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Two-Photon Fluorescence Vascular Bioimaging with New Bioconjugate Probes Selective Towards the Vascular Endothelial Growth Factor Receptor 2

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Abstract

We report the synthesis and characterization of two amine reactive fluorescent dyes with efficient two-photon absorption (2PA) properties and high fluorescence quantum yields. Bioconjugation of these dyes with the DC-101 antibody proved to be useful for selectively imaging the vascular endothelial growth factor receptor 2 (VEGFR-2) in cells expressing this receptor *in vitro* and in “whole” mounted excised tumors (*ex vivo*) by two-photon fluorescence microscopy (2PFM). The penetration depths reached within the tumors by 2PFM was over 800 μm . In addition, the concentration of dye required for incubation of these bioconjugates was in the picomolar domain, the probes possessed very good photostability, and the 2PFM setup did not require any additional means of increasing the collection efficiencies of fluorescent photons to achieve the relatively deep tissue imaging that was realized, due, in large part, to the favorable photophysical properties of the new probes.

Keywords

Angiogenesis; VEGFR-2; bioconjugates; two-photon absorption; fluorescent dyes; two-photon fluorescence microscopy; tumor imaging

Introduction

The development of methodologies and materials to image cancer tumors is an area of increasing interest in the chemical and biomedical communities. The conjugation of fluorescent dyes with antibodies and peptides, used as targeting vectors, is a common strategy to selectively image tumors. In the interest of following tumor growth and drug efficacy, researchers have targeted the vasculature rather than the tumor itself to determine

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Supporting Information Available: ¹H NMR and ¹³C NMR data. Two-photon fluorescence micrographs of excised whole mount organs and glands of the mice that had been injected with bioconjugate **B2** (negative control) and 2PFM of other negative controls are shown. A picture of the scanning process while taking a 2PFM micrograph is shown as is verification of the quadratic dependence of the microscope system for two-photon excitation, and spectral scan vs. solution emission spectrum of model probe **11**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

tumor size, morphology, and characteristics by different technologies (immunohistochemistry, fluorescence microscopy, photoacoustic microscopy, etc).¹⁻⁵

Angiogenesis is the process of forming new blood vessels to support tissue growth. This fundamental physiological process promotes embryonic development, tissue repair, and fertility, although it also promotes chronic inflammation, tumor growth, and tumor metastasis.⁶ Tumors in particular rely on angiogenesis for their continued growth. It has been demonstrated that solid tumors will not grow larger than 2–3 mm in diameter in the absence of new blood vessels and require angiogenesis to form metastases.⁷ Vascular endothelial growth factor (VEGF) is a protein that stimulates vascular endothelial cell growth, survival, and proliferation. Thus, VEGF facilitates survival of existing vessels, contributes to vascular abnormalities that may impede effective delivery of antitumor compounds, and stimulates new vessel growth.⁸⁻¹⁰

The VEGF family consists of five members (VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor), which show different affinities for one of the three VEGF tyrosine kinase receptors (VEGFR-1, VEGFR-2 and VEGFR-3). VEGFR-1 and VEGFR-2 are expressed in vascular endothelial cells. Moreover, there is evidence that VEGFR-2 is the major mediator of VEGF-driven responses in endothelial cells and it has been considered to be crucial in angiogenic processes.¹⁰

In 2005, Backer and coworkers reported the use of a boronated polyamidoamine dendrimer able to target vasculature by means of a VEGF-containing bioconjugate that directed the dendrimer to a specific receptor (VEGFR). The incorporation of a near infrared (NIR) dye (Cy5) was employed to track the bioconjugate. The targeting of tumor vasculature endothelial cells, rather than the tumor itself, was achieved since endothelial cells in tumor neurovasculature express a significantly greater number of VEGFR-2 when compared to quiescent endothelial cells.¹¹ More recent work has demonstrated the use of VEGF bioconjugates containing NIR fluorescent dyes to study the possible expression of VEGFRs in bladder inflammation.¹²

The use of 2PA absorbing fluorophores offers several advantages over their one-photon NIR counterparts. In the nonlinear absorption process the fluorescence emission is proportional to the square of the intensity of the incident light providing the system with numerous intrinsic advantages, e.g., deeper penetration, more confined excitation areas, less scattering, and minimal photodamage outside of the focal volume.¹³

The scientific community has access to a wealth of efficient 1PA fluorescent dyes with emissions that range from the UV to the IR and that are commercially available. However, there is a need to develop new dyes with high fluorescence quantum yields and 2PA cross sections that meet the high photostability demands that two-photon fluorescence microscopy (2PFM) imposes for repeated three-dimensional (3D) imaging. We previously described a strategy to create 2PA amine reactive probes that can be conjugated to proteins or antibodies.^{14, 15} In this work, we introduce the synthesis and characterization of two new fluorescent amine reactive dyes based on a fluorenyl core. The fluorophore design was based on a small series of efficient 2PA dyes that were recently reported.¹⁶ The architecture of the fluorophores involved a fluorenyl core flanked by a triple bond- thiophene bridge (π) that led to an electron withdrawing group on one side (A). On the other, the fluorenyl core was tethered to the anti-VEGFR-2 that served as a donor (D). This resulted in systems that have a D- π -A dipolar structure.

These probes were used to prepare VEGFR-2 antibody bioconjugates (anti-VEGFR-2) that selectively bound to the VEGFR-2 in vascular endothelial cells that overexpress this specific receptor in *in vitro* experiments. Furthermore, the conjugation of these new 2PA dyes to the

VEGFR-2 antibody was used to image tumor vasculature of whole-mounted excised tumors by 2PFM (Figure 1). In vitro, and, more significantly, *ex vivo*, thick tumor 2PFM imaging was accomplished, revealing the 3D tumor microvasculature morphology.

Experimental Section

Materials and methods

Key intermediates **1**, **2**, and **7** were prepared as described in the literature.^{15, 16} All reactions were carried out under N₂. Microwave-assisted procedures were carried out in a CEM Discover unit microwave in 10 mL closed vessels; temperature, pressure and power were programmed at a maximum value (as indicated), depending on the reaction. All other reagents and solvents were used as received from commercial suppliers. ¹H and ¹³C NMR spectra were recorded in CDCl₃ on a Varian NMR spectrometer at 500 and 125 MHz, respectively. Mass analyses (APCI, atmospheric pressure chemical ionization) were performed at University of Florida. The analyses were carried out on a Finnigan LCQ - Quadrupole Ion Trap (Thermo Finnigan). Monoclonal anti-human VEGFR-2/KDR-Fluorescein isothiocyanate bioconjugate was obtained from R&D Systems and was used as a positive control without further treatment.

Synthetic procedures and characterization

Synthesis of 2-(5-(9,9-bis(2-(2-ethoxyethoxy)ethyl)-7-nitro-9H-fluoren-2-yl)thiophen-2-yl)benzothiazole (3)—In a 10 mL vessel, under nitrogen, 9,9-bis(2-(2-ethoxyethoxy)ethyl)-2-iodo-7-nitro-9H-fluorene **1** (200 mg, 0.335 mmol), 2-(5-(tributylstannyl)thiophen-2-yl)benzothiazole **2** (207 mg, 0.408 mmol) and Pd(PPh₃)₂Cl₂ (6 mg, 0.008 mmol) were dissolved in toluene (2 mL). The mixture was heated in the microwave for 25 min, at 130 °C, 150 psi and 150 W, filtered through a celite plug, and purified by column chromatography using as a solvent a mixture of hexane:ethyl acetate (2:1) to yield 216 mg (98%) of a light orange solid. m.p. 75.7-76.5 °C. ¹H NMR (500 MHz, CDCl₃) δ 8.26-8.34 (m, 2H, Ph-H), 8.06 (d, *J*=8.06 Hz, 1H, Ph-H), 7.89 (d, *J*=8.06 Hz, 1H, Ph-H), 7.73-7.84 (m, 4H, Ph-H), 7.69 (d, *J*=3.93 Hz, 1H, Thy-H), 7.49-7.52 (m, 1H, Ph-H), 7.48 (d, *J*=3.93 Hz, 1H, Thy-H), 7.38-7.42 (m, 1H, Ph-H), 3.34-3.40 (m, 4H, CH₂), 3.27-3.31 (m, 4H, CH₂), 3.15-3.22 (m, 4H, CH₂), 2.79-2.93 (m, 4H, CH₂), 2.45-2.56 (m, 4H, CH₂), 1.10 (t, *J*=6.98 Hz, 6H, CH₃). ¹³C NMR (125 MHz, CDCl₃) δ 161.1, 153.9, 152.1, 151.2, 147.7, 147.5, 146.3, 138.6, 137.0, 135.0, 134.8, 129.7, 126.8, 126.0, 125.6, 124.8, 123.9, 123.2, 122.2, 121.7, 121.0, 120.2, 119.3, 70.4, 69.8, 67.1, 66.8, 52.5, 39.6, 15.2. MS (APCI) *m/z* calcd [M+H]⁺ 659.2244, found 659.2256.

Synthesis of 7-(5-(benzothiazol-2-yl)thiophen-2-yl)-9,9-bis(2-(2-ethoxyethoxy)ethyl)-9H-fluoren-2-amine (4)—In a 10 mL vessel, under nitrogen, 2-(5-(9,9-bis(2-(2-ethoxyethoxy)ethyl)-7-nitro-9H-fluoren-2-yl)thiophen-2-yl)benzothiazole **3** (300 mg, 0.45 mmol) and 10% Pd/C (30 mg) were dissolved in a 1:1 mixture of THF:EtOH (4 mL). NH₂NH₂·2H₂O (300 mg, 6.0 mmol) was added to the mixture dropwise at room temperature, and then heated in the microwave to 110 °C, 100 W and 100 psi for 30 min. The mixture was filtered through a silica plug with ethyl acetate, and after removing the solvent under reduced pressure, the material was purified by column chromatography using as a solvent ethyl acetate to yield 280 mg (97%) of a dark yellow solid. m.p. 120.8-121.4 °C. ¹H NMR (500 MHz, CDCl₃) δ 8.04 (d, *J*=7.88 Hz, 1H, Ph-H), 7.86 (d, *J*=7.88 Hz, 1H, Ph-H), 7.59-7.67 (m, 3H, Ph-H Thy-H), 7.55 (d, *J*=7.69 Hz, 1H, Ph-H), 7.45-7.50 (m, 2H, Ph-H), 7.34-7.40 (m, 2H, Ph-H, Thy-H), 6.75 (d, *J*=7.99 Hz, 1H, Ph-H), 6.68 (dd, *J*=7.99 Hz, *J*=1.92 Hz, 1H, Ph-H), 3.38-3.44 (m, 4H, CH₂), 3.33-3.37 (m, 4H, CH₂), 3.21-3.26 (m, 4H, CH₂), 2.77-2.86 (m, 4H, CH₂), 2.31-2.46 (m, 4H, CH₂), 1.13 (t, *J*=7.05 Hz, 6H, CH₃). ¹³C NMR (125 MHz, CDCl₃) δ 161.3, 153.8, 151.1, 149.0, 148.8, 146.7, 141.6, 135.3, 134.6,

130.8, 130.7, 129.6, 129.6, 125.4, 125.1, 123.3, 122.8, 121.46, 121.1, 120.1, 119.0, 114.5, 109.8, 70.1, 69.7, 67.0, 66.6, 50.9, 39.9, 15.1. MS (APCI) m/z calcd $[M+H]^+$ 629.2502, found 629.2508.

Synthesis of 2-(5-(9,9-bis(2-(2-ethoxyethoxy)ethyl)-7-isothiocyanato-9H-fluoren-2-yl)thiophen-2-yl)benzothiazole (5)—In a two-neck round bottom flask and under nitrogen, a solution of 7-(5-(benzothiazol-2-yl)thiophen-2-yl)-9,9-bis(2-(2-ethoxyethoxy)ethyl)-9H-fluoren-2-amine **4** (250 mg, 0.398 mmol) in chloroform (2 mL) was combined with an aqueous solution of CaCO_3 (103 mg, 1.03 mmol) in an ice bath. Thiophosgene (0.042 mL, 0.44 mmol) was added to the vial dropwise while stirring the mixture and the reaction proceeded for 4 h. After completion, a 10% HCl solution was added until the gas formation ceased. The mixture was poured into water, extracted with methylene chloride, dried over magnesium sulfate and purified by column chromatography using as a solvent a mixture of hexane:ethyl acetate (2:1) to yield 200 mg (74%) of a light orange solid. m.p. 103.2–104.0 °C. ^1H NMR (500 MHz, CDCl_3) δ 8.04 (d, $J=8.10$ Hz, 1H, Ph-H), 7.86 (d, $J=8.10$ Hz, 1H, Ph-H), 7.70–7.73 (m, 1H, Ph-H), 7.61–7.68 (m, 4H, Ph-H, Thy-H), 7.46–7.50 (m, 1H, Ph-H), 7.41 (d, 1H, $J=4.10$ Hz, Thy-H), 7.34–7.39 (m, 1H, Ph-H), 7.32 (d, $J=1.68$ Hz, 1H, Ph-H), 7.21 (dd, $J=7.97$ Hz, $J=1.68$ Hz, 1H, Ph-H), 3.36–3.42 (m, 4H, CH_2), 3.30–3.35 (m, 4H, CH_2), 3.18–3.23 (m, 4H, CH_2), 2.78–2.91 (m, 4H, CH_2), 2.38–2.47 (m, 4H, CH_2), 1.12 (t, $J=7.11$ Hz, 6H, CH_3). ^{13}C NMR (125 MHz, CDCl_3) δ 161.0, 153.7, 151.1, 150.3, 148.0, 139.6, 139.0, 136.2, 135.4, 134.7, 133.1, 130.2, 129.6, 129.5, 124.2, 122.9, 121.5, 121.4, 121.1, 120.92, 120.85, 120.7, 120.6, 120.4, 70.1, 69.6, 66.9, 66.6, 51.8, 39.6, 15.1. MS (APCI) m/z calcd $[M+H]^+$ 671.2066, found 671.2068. UV-vis (DMSO) λ_{max} 397 nm.

Synthesis of 1-(7-(5-(benzothiazol-2-yl)thiophen-2-yl)-9,9-bis(2-(2-ethoxyethoxy)ethyl)-9H-fluoren-2-yl)-3-butylthiourea (6)—In a small vial under nitrogen, a mixture of 2-(5-(9,9-bis(2-(2-ethoxyethoxy)ethyl)-7-isothiocyanato-9H-fluoren-2-yl)thiophen-2-yl)benzothiazole **5** (56 mg, 0.083 mmol), and *n*-butylamine (0.074 mL, 1.0 mmol) at room temperature was stirred for 1 h. The remaining *n*-butylamine was removed under vacuum and the residue was purified by column chromatography with hexane:ethyl acetate (1:1) to yield 50 mg (80%) of a bright yellow solid. m.p. 67.0–68.0 °C. ^1H NMR (500 MHz, CDCl_3) δ 8.04 (d, $J=8.13$ Hz, 1H, Ph-H), 7.84–7.94 (m, 2H, Ph-H, N-H), 7.67–7.73 (m, 4H, Ph-H), 7.66 (d, $J=4.01$, 1H, Thy-H), 7.47–7.52 (m, 1H, Ph-H), 7.42 (d, 1H, $J=4.01$ Hz, Thy-H), 7.35–7.40 (m, 1H, Ph-H), 7.32 (d, $J=1.46$ Hz, 1H, Ph-H), 7.21 (d, $J=7.74$ Hz, 1H, Ph-H), 6.34–6.39 (m, 1H, N-H), 3.62–3.69 (m, 2H, CH_2), 3.32–3.41 (m, 4H, CH_2), 3.24–3.30 (m, 4H, CH_2), 3.12–3.21 (m, 4H, CH_2), 2.78–2.98 (m, 4H, CH_2), 2.36–2.44 (m, 4H, CH_2), 1.57–1.65 (m, 2H, CH_2), 1.35–1.43 (m, 2H, CH_2), 1.10 (t, $J=6.96$ Hz, 6H, CH_3), 0.95 (t, $J=7.55$ Hz, 3H, CH_3). ^{13}C NMR (125 MHz, CDCl_3) δ 180.6, 161.1, 153.72, 153.71, 150.1, 148.1, 140.1, 138.3, 136.1, 134.68, 134.67, 132.8, 129.5, 123.8, 122.9, 121.5, 121.4, 120.63, 120.61, 120.60, 120.48, 120.47, 120.0, 70.0, 69.6, 67.1, 66.5, 51.9, 39.6, 31.0, 20.2, 15.1, 15.0, 13.9. MS (APCI) m/z calcd $[M+H]^+$ 744.2958, found 744.2952. UV-vis (DMSO) λ_{max} 400 nm.

Preparation of bioconjugate B1—A mixture containing 1000 μL of monoclonal DC-101 antibody (4 mg/mL) and 1000 μL of NaHCO_3 (1 M) was combined with a fresh solution of isothiocyanate **5** in DMSO in a 1:6 molar protein:dye proportion and incubated for 1 h in the dark at room temperature. A mixture containing 100 μL of polyclonal VEGFR-2 antibody (1 mg/mL) and 100 μL of NaHCO_3 (1 M) was combined with a fresh solution of isothiocyanate **5** in DMSO in a 1:10 molar protein:dye proportion and incubated for 2 h in the dark at room temperature. The biconjugate was purified by using disposable PD-10 desalting columns from GE equilibrated and eluted with PBS buffer 7.4. Fractions

containing the bioconjugate were identified using a spectrophotometer by monitoring bands at 280 nm and 400 nm. The degree of labeling was determined to be 3.

Synthesis of 2-(5-((9,9-bis(2-(2-ethoxyethoxy)ethyl)-7-nitro-9H-fluoren-2-yl)ethynyl)thiophen-2-yl)benzothiazole (8)—In a 10 mL vessel, under nitrogen, 9,9-bis(2-(2-ethoxyethoxy)ethyl)-2-iodo-7-nitro-9H-fluorene **1** (413 mg, 0.725 mmol), 2-(5-ethynylthiophen-2-yl)benzothiazole **7** (175 mg, 0.725 mmol), Pd(PPh₃)₂Cl₂ (21 mg, 0.03 mmol), CuI (5.7 mg, 0.03 mmol), were dissolved in a 1:4 mixture of Et₃N:toluene (3 mL). The mixture was heated in the microwave for 1 h, at 130 °C, 150 psi and 150 W, filtered through a celite plug, and purified by column chromatography using as a solvent a mixture of hexane:ethyl acetate (2:1) to yield 505 mg (74%) of a light orange solid. m.p. 105.0-106.5 °C. ¹H NMR (500 MHz, CDCl₃) δ 8.33 (d, *J*=2.00 Hz, 1H, Ph-H), 8.26 (dd, *J*=8.36 Hz, *J*=2.00 Hz, 1H, Ph-H), 8.02 (d, *J*=7.95 Hz, 1H, Ph-H), 7.85 (d, *J*=7.95 Hz, 1H, Ph-H), 7.73-7.79 (m, 2H, Ph-H, Thy-H), 7.66-7.68 (m, 1H, Ph-H), 7.54-7.60 (m, 2H, Ph-H, Thy-H), 7.46-7.50 (m, 1H, Ph-H), 7.36-7.40 (m, 1H, Ph-H), 7.31 (d, *J*=3.92 Hz, 1H, Thy-H), 3.35-7.42 (m, 4H, CH₂), 3.27-3.31 (m, 4H, CH₂), 3.15-3.21 (m, 4H, CH₂), 2.80-2.93 (m, 4H, CH₂), 2.43-2.54 (m, 4H, CH₂), 1.12 (t, *J*=6.85 Hz, 6H, CH₃). ¹³C NMR (125 MHz, CDCl₃) δ 160.5, 153.8, 151.1, 147.5, 146.0, 138.8, 138.6, 134.9, 133.3, 133.1, 128.6, 128.5, 126.9, 126.7, 126.6, 123.4, 123.3, 121.7, 121.4, 119.2, 119.1, 96.0, 84.2, 70.3, 69.7, 66.9, 66.7, 52.3, 39.4, 15.2. MS (APCI) *m/z* calcd [M+H]⁺ 683.2244, found 683.2231.

Synthesis of 7-((5-(benzothiazol-2-yl)thiophen-2-yl)ethynyl)-9,9-bis(2-(2-ethoxyethoxy)ethyl)-9H-fluoren-2-amine (9)—In a 10 mL vessel, under nitrogen, 2-(5-((9,9-bis(2-(2-ethoxyethoxy)ethyl)-7-nitro-9H-fluoren-2-yl)ethynyl)thiophen-2-yl)benzothiazole **8** (250 mg, 0.366 mmol) and 10% Pd/C (25 mg) were dissolved in a 1:1 mixture of THF:EtOH (4 mL). NH₂NH₂·2H₂O (220 mg, 4.4 mmol) was added to the mixture dropwise at room temperature, and then heated in the microwave to 110 °C, 100 W and 100 psi for 30 min. The mixture was filtered through a silica plug with ethyl acetate, and after removing the solvent under reduced pressure, the material was purified by column chromatography using as a solvent a mixture of hexane:ethyl acetate (1:3) to yield 200 mg (84%) of a light orange solid. m.p. 110-111 °C. ¹H NMR (500 MHz, CDCl₃) δ 8.03 (d, *J*=8.09 Hz, 1H, Ph-H), 7.84 (d, *J*=8.09 Hz, 1H, Ph-H), 7.52-7.57 (m, 2H, Ph-H, Thy-H), 7.43-7.52 (m, 4H, Ph-H), 7.35-7.39 (m, 1H, Ph-H), 7.28 (d, *J*=4.09 Hz, 1H, Thy-H), 6.71-6.74 (m, 1H, Ph-H), 6.64-6.68 (m, 1H, Ph-H), 3.79 (s, 2H, NH₂), 3.39-3.46 (m, 4H, CH₂), 3.32-3.39 (m, 4H, CH₂), 3.20-3.26 (m, 4H, CH₂), 2.74-2.85 (m, 4H, CH₂), 2.29-2.44 (m, 4H, CH₂), 1.15 (t, *J*=7.02 Hz, 6H, CH₃). ¹³C NMR (125 MHz, CDCl₃) δ 160.6, 153.7, 151.3, 148.1, 147.1, 142.0, 137.7, 134.7, 133.0, 131.8, 130.6, 128.1, 127.4, 125.6, 125.5, 122.9, 121.7, 120.8, 118.9, 118.0, 115.1, 113.9, 110.2, 97.2, 82.2, 70.1, 69.7, 67.0, 66.7, 50.9, 39.9, 15.4. MS (APCI) *m/z* calcd [M+H]⁺ 653.2502, found 653.2510.

Synthesis of 2-(5-((9,9-bis(2-(2-ethoxyethoxy)ethyl)-7-isothiocyanato-9H-fluoren-2-yl)ethynyl)thiophen-2-yl)benzothiazole (10)—In a two-neck round bottom flask and under nitrogen, a solution of 7-((5-(benzothiazol-2-yl)thiophen-2-yl)ethynyl)-9,9-bis(2-(2-ethoxyethoxy)ethyl)-9H-fluoren-2-amine **9** (178 mg, 0.273 mmol) in chloroform (2 mL) was combined with an aqueous solution of CaCO₃ (80 mg, 0.80 mmol) in an ice bath. Thiophosgene (0.026 mL, 0.34 mmol) was added to the vial dropwise while stirring the mixture and the reaction proceeded for 4 h. After completion, a 10% HCl solution was added until the gas formation ceased. The mixture was poured into water, extracted with methylene chloride, dried over magnesium sulfate, and purified by column chromatography using as a solvent a mixture of hexane:ethyl acetate (2:1) to yield 170 mg (90%) of a bright yellow solid. m.p. 86-88 °C. ¹H NMR (500 MHz, CDCl₃) δ 8.05 (d, *J*=7.97 Hz, 1H, Ph-H), 7.87 (d, *J*=7.97 Hz, 1H, Ph-H), 7.63-7.67 (m, 2H, Ph-H), 7.53-7.61 (m, 3H, Ph-H Thy-H),

7.47-7.52 (m, 1H, Ph-H), 7.37-7.42 (m, 1H, Ph-H), 7.30-7.33 (m, 2H, Ph-H), 7.21-7.24 (m, 1H, Ph-H), 3.39-3.45 (m, 4H, CH₂), 3.30-3.35 (m, 4H, CH₂), 3.18-3.23 (m, 4H, CH₂), 2.75-2.85 (m, 4H, CH₂), 2.34-2.45 (m, 4H, CH₂), 1.15 (t, $J=7.01$ Hz, 6H, CH₃). ¹³C NMR (125 MHz, CDCl₃) δ 160.4, 153.7, 151.2, 149.4, 139.8, 138.8, 138.1, 135.5, 134.7, 132.3, 130.5, 126.8, 126.5, 126.2, 124.9, 123.1, 123.0, 121.60, 121.58, 121.4, 121.2, 120.9, 120.6, 120.0, 96.4, 83.3, 70.1, 69.6, 67.3, 66.6, 51.7, 39.5, 15.1. MS (APCI) m/z calcd [M+H]⁺ 695.2067, found 695.2076. UV-vis (DMSO) λ_{max} 391 nm.

Synthesis of 1-(7-((5-(benzothiazol-2-yl)thiophen-2-yl)ethynyl)-9,9-bis(2-(2-ethoxyethoxy)ethyl)-9H-fluoren-2-yl)-3-butylthiourea (11)—In a small vial under nitrogen, a mixture of 2-(5-((9,9-bis(2-(2-ethoxyethoxy)ethyl)-7-isothiocyanato-9H-fluoren-2-yl)ethynyl)thiophen-2-yl)benzothiazole **10** (58 mg, 0.083 mmol), and *n*-butylamine (0.074 mL, 1.0 mmol) at room temperature was stirred for 1 h. The remaining amine was removed under vacuum and the residue was recrystallized from hexane:ethyl acetate (9:1) to yield 48 mg (75%) of a light yellow solid. m.p. 118.5-119.5 °C. ¹H NMR (500 MHz, CDCl₃) δ 8.05 (d, $J=8.07$ Hz, 1H, Ph-H), 7.88 (d, $J=8.07$ Hz, 1H, Ph-H), 7.78 (s, 1H, N-H), 7.66-7.73 (m, 2H, Ph-H), 7.54-7.62 (m, 3H, Ph-H Thy-H), 7.48-7.53 (m, 1H, Ph-H), 7.37-7.43 (m, 1H, Ph-H), 7.29-7.33 (m, 2H, Ph-H, Thy-H), 7.15-7.22 (m, 1H, Ph-H), 6.36 (s, 1H, N-H), 3.62-3.71 (m, 2H, CH₂), 3.34-3.43 (m, 4H, CH₂), 3.22-3.32 (m, 4H, CH₂), 3.10-3.22 (m, 4H, CH₂), 2.74-2.97 (m, 4H, CH₂), 2.34-2.45 (m, 4H, CH₂), 1.61 (quintuplet, $J=7.05$ Hz, 2H, CH₂), 1.40 (sextuplet, $J=7.05$ Hz, 2H, CH₂), 1.13 (t, $J=7.05$ Hz, 6H, CH₃), 0.96 (t, $J=7.83$ Hz, 6H, CH₃). ¹³C NMR (125 MHz, CDCl₃) δ 180.6, 168.1, 160.4, 153.7, 149.2, 140.4, 138.2, 138.1, 134.7, 132.9, 132.7, 128.5, 128.4, 126.9, 126.5, 126.3, 123.1, 121.54, 121.5, 121.4, 121.3, 120.2, 120.1, 96.4, 83.1, 70.0, 69.6, 67.1, 66.5, 51.9, 45.5, 39.6, 30.9, 20.2, 15.1, 13.8. MS (APCI) m/z calcd [M+H]⁺ 768.2958, found 768.3010. UV-vis (DMSO) λ_{max} 391 nm.

Preparation of bioconjugate B2—A mixture containing 1000 μL of monoclonal DC-101 antibody (4 mg/mL) and 1000 μL of NaHCO₃ (1 M) was combined with a fresh solution of isothiocyanate **10** in DMSO in a 1:6 molar protein:dye proportion and incubated for 1 h in the dark at room temperature. The bioconjugate was purified by using disposable PD-10 desalting columns from GE equilibrated and eluted with PBS buffer 7.4. Fractions containing the bioconjugate were identified using a spectrophotometer by monitoring bands at 280 nm and 391 nm. The degree of labeling was determined to be 3.

Preparation of IgG containing bioconjugate—A mixture containing 190 μL of IgG antibody (21.16 mg/mL) in 890 μL of PBS buffer pH=7.4 and 1000 μL of NaHCO₃ (1 M) was combined with a fresh solution of isothiocyanate **10** in DMSO in a 1:6 molar protein:dye proportion and incubated for 1 h in the dark at room temperature. The bioconjugate was purified by using disposable PD-10 desalting columns from GE equilibrated and eluted with PBS buffer 7.4. Fractions containing the bioconjugate were identified using a spectrophotometer by monitoring bands at 280 nm and 391 nm. The degree of labeling was determined to be 3.

Measurements

Spectra of amine reactive probes and model compounds were acquired in DMSO solutions with concentrations on the order of 10^{-6} M at room temperature using 1 cm quartz cuvettes. Absorption spectra were recorded with an Agilent 8453 UV-visible spectrophotometer. Steady-state fluorescence spectra were measured with a PTI Quantamaster spectrofluorimeter in the photon counting regime of the PMT using an L-format configuration. The fluorescence spectra were corrected for the spectral dependence of the PMT. Fluorescence quantum yields were determined relative to 9, 10-diphenylanthracene in

cyclohexane. Absorption spectra of bioconjugates **B1** and **B2** were measured in PBS buffer using 1 mm quartz cuvettes.

Two-photon absorption spectra were measured by the two-photon fluorescence (2PF) method relative to Rhodamine B in methanol,¹⁷ using solutions with concentrations of approximately 10^{-4} M in spectroscopic grade DMSO following previously reported methodology.¹⁶

Cell culture and incubation

PAE (Porcine aortic endothelial) and PAE/KDR (commercially available Porcine aortic endothelial expressing 2×10^5 VEGFR-2 per cell) were cultured in F-12 Nutrient mixture (Ham's medium, Invitrogen) containing 10% fetal bovine serum (FBS) (Atlanta Biologicals) and 1% penicillin/streptomycin, at 37 °C, under a 5% CO₂ environment. Cells were cultured on N° 1 round 12 mm coverslips that were previously treated with poly-D-lysine. The treated cover slips were placed in 24-well plates and 60,000 cells/well were seeded and incubated at the same conditions as indicated above until 75-85% confluency was reached on the coverslips. Cells were then fixed with 3.7% solution of paraformaldehyde in pH=7.4 PBS buffer for 10 min. The fixing agent was extracted and washed (2×) with PBS. To reduce autofluorescence, a fresh solution of NaBH₄ (1 mg/mL) in pH=8 PBS buffer was used to treat the fixed cells (2×). Subsequently, cells were permeabilized with a 0.1% saponin solution in PBS containing 5% BSA. After permeabilization, PAE-KDR cells were incubated for 1 h at room temperature in a solution containing 0.4 μM solution of bioconjugate **B2** and the monoclonal anti-human VEGFR-2/KDR-Fluorescein isothiocyanate bioconjugate (25 μg/mL) in culture media. PAE (negative control) and PAE-KDR cells were incubated for 2 h in a 0.4 μM solution of bioconjugate **B2** in PBS. The dye solutions were extracted by suction and the coverslipped cells were washed abundantly with PBS (4×). The coverslipped cells were then washed with buffer PBS (2×) and mounted on microscope slides using Prolong Gold (Invitrogen) as a mounting media.

Tissue immunofluorescence staining

For the imaging of tumor endothelial cells, 10 μm thick sections were prepared from OCT-embedded frozen blocks. Tumor sections were incubated with rat anti-mouse CD31 antibody (BD Bioscience, San Diego, CA) and then Alexa Fluor® 594-conjugated anti-rat Ig (Invitrogen).

Mouse models and tumor excision

Lewis lung carcinoma cells were purchased from ATCC (Manassas, VA) and cultured in DMEM (Invitrogen) supplemented with 10% FBS. Experiments involving mice were done in accordance with protocols approved by the Sanford-Burnham Medical Research Institute Internal Animal Care and Use Committee. Seven-week-old male C57BL/6 mice were purchased from Harlan Laboratories (Indianapolis, IN) and acclimated for 1 week upon delivery. Each mouse was injected subcutaneously into the right flank with 3×10^6 of Lewis lung carcinoma cells suspended in 0.1 mL of PBS. DC-101 antibody or mouse control IgG conjugated with 2PA probe (bioconjugate **B3**) was injected at a dose of 13.4 nmol (600 μg as a protein) per mouse via the tail vein 10 days after the inoculation of tumor cells. Two hours after the injection of the bioconjugate probe, mice were perfused with PBS and then 10% neutral-buffered formalin via left ventricle under the deep anesthesia with isoflurane. Tumors were excised and fixed with 10% neutral-buffered formalin for 18 hours at 4 °C. After fixation, tumors were rinsed twice with PBS and cut into two pieces at the center of the tumors; one was used for 3-D two-photon fluorescence imaging, and the other was embedded in OCT compound (Sakura Finetek, Torrance, CA) for the immunohistochemistry analysis.

One-photon fluorescence imaging

Cell imaging—One-photon confocal fluorescence microscopy was performed on a Leica SP5 II equipped with a spectral scanner. Micrographs of the fixed cells were taken under the following conditions: excitation of **B2** was done at 405 nm, excitation the FITC anti-VEGFR-2 conjugate was done at 476 nm; fluorescence collection of both channels was done by setting the spectral window range from 490-620 nm. A water immersion, 63 ×, 1.3 N. A. objective was used for cell imaging.

Tissue sections—One-photon fluorescence microscopy images were recorded on an Olympus IX-81 DSU microscope equipped with a Hamamatsu EM-CCD C9100 digital camera. One-photon fluorescence images were taken using a custom made filter cube (Ex: 377/50; DM: 409; Em:525/40) for bioconjugates **B2**, a FITC filter cube (Ex:477/50; DM: 507; Em:536/40) for FITC anti-VEGFR-2, and a TexRed filter cube (Ex:477/50; DM: 507; Em:536/40) for the Alexa Fluor® 594-CD31 antibody bioconjugate.

Two-photon fluorescence imaging

Two-photon fluorescence microscopy (2PFM) imaging was performed on a modified Olympus Fluoview FV300 laser scanning microscopy system equipped with a broadband, tunable Coherent Mira Ti:sapphire laser (200 fs pulse width, 76 MHz repetition rate), pumped by a 10 W Coherent Verdi frequency doubled Nd:YAG laser. The Ti:sapphire laser, tuned and modelocked to 700 nm (for cell imaging) or to 820 nm (for “whole” tumor for imaging), was used as the two-photon excitation source. Two-photon induced fluorescence was collected by a 60× (UPLANSAP0, N.A. = 1.35 Olympus) or, for ex vivo tumor imaging, with a 10× (UPlanFL, N. A. = 0.30) microscope objective. A high transmittance (>95%) short-pass filter (cutoff 685 nm, Semrock) was placed in front of the PMT detector within the FV300 scanhead in order to filter off background radiation from the laser source (700 nm or 820 nm). The optical nonlinearity (quadratic dependence) for two-photon excitation was verified with dye **11**-doped poly(methyl methacrylate) films (see Figure S4 in Supporting Information). The average laser power used for excitation of the tumors from mice injected with **B2** was 22 mW and 90 mW for all the control samples.

Results and Discussion

Synthesis of new fluorescent probes and preparation of bioconjugates

The synthesis of the 2PA amine reactive probes **5** and **10** was achieved in short reaction times and high yields by using microwave-assisted synthesis, as illustrated in Schemes 1 and 2, respectively. Briefly, fluorene derivative **1** was coupled to intermediates **2** and **7** either by Stille or Sonogashira coupling to yield nitro derivatives **3** and **8**, respectively. These nitro derivatives were reduced to the corresponding amine intermediates **4** and **9**.¹⁸ Transformation of these amines into the corresponding isothiocyanates **5** and **10** was performed by a previously reported method.^{14, 15, 19}

In order to better understand the scope and properties of these probes, conjugation with *n*-butylamine was performed to generate model compounds **6** and **11** that would closely resemble the optical properties of the bioconjugates. Once obtained, amine reactive probes and models were structurally and photophysically characterized.

The amine-reactive probes, isothiocyanates **5** and **10**, were conjugated to anti-VEGFR-2 for 2 h at room temperature in a 10:1 molar ration of dye:protein. Purification was achieved by using a commercially available, Sephadex packed, GPC column. The antibody of choice for this conjugation was DC-101, mainly because the epitope for this antibody is extracellular. In these studies, where mice were going to be injected with the antibody conjugate, it was

clear that the location of the antigenic determinant had to be on the surface of the endothelial cells of the tumor vessels in order to maximize the labeling efficiency of the bioconjugate. Furthermore, DC-101 (Bevacizumab) has been FDA approved for the treatment of non-small cell lung cancer, colon cancer and, and breast cancer; and is currently being studied for the treatment of ovarian, prostate, renal, pancreatic and liver cancers.

Photophysical properties

Photophysical characterization was performed for amine-reactive probes **5** and **10** and model compounds **6** and **11** (Figure 2). Table 1 reveals that there are intense absorption maxima for amine-reactive probes and model compounds located between 390-400 nm, whereas there was a red shift in the emission maxima when the dyes were conjugated to *n*-butylamine due to the dipolar nature of the model adducts. In addition, a larger Stokes shift was observed for the more conjugated model compound **11**.

In our previous studies, we observed that the presence of isothiocyanate groups in the fluorene system plays an important role by quenching the fluorescence of the dye, possibly by inducing intersystem crossing and allowing the molecule to relax by nonradiative pathways but the fluorescence seems to be restored (switched on) upon conjugation.^{14, 15} Interestingly, in the case of these new dyes, this effect was not observed. For the probe **5**, the quantum yield increased upon conjugation to the amine, as was observed previously for a similar molecule. However, for probe (**10**), there was a significant decrease in the fluorescence quantum yield after conjugation with *n*-butylamine. (Table 1). This important result suggests that luminescent properties cannot be predicted based only on similarities between structures.

In 2PFM applications where the upconverted fluorescence of the probe is directly interrogated the efficiency of the probe depends on basically three parameters, namely the two-photon absorption cross section, the fluorescence quantum yield, and the quantum yield of photodecomposition. The first two parameters are of utmost importance and reflect the probability the probe has to absorb two-photons at a specific wavelength and the ratio of photons the molecule emits by means of fluorescence in relation to the photons it has absorbed, respectively. Combined, the two-photon absorption cross section and the fluorescence quantum yield conform a parameter referred to as action cross section which is the product of these values ($\delta \cdot \Phi$). The parameter that is frequently overlooked is the photodecomposition quantum yield (η) that is a good indicator of how quickly a fluorophore photobleaches per photons absorbed. The figure of merit, F_M , is the action cross section normalized by the photodecomposition quantum yield ($F_M = \delta \cdot \Phi / \eta$) and is a more comprehensive parameter for evaluating the overall efficiency of a fluorescent probe. In the particular case of compounds **6** and **11** (Table 1) the efficiencies were quite favorable when compared to fluorescein ($F_M = 6 \times 10^6 \text{ GM}$).

Lifetimes for these series of compounds were also measured in DMSO at concentrations of approximately 10^{-6} M , as shown in Table 1. A difference of 1.2 ns was found between the fluorescence lifetime of models **6** and **11**. Even though they have similar fluorescence maxima, with this difference in lifetime it may be possible to distinguish between bioconjugates containing these dyes and different antibodies or vectors by fluorescence lifetime imaging microscopy (FLIM).

Anisotropy of samples were measured under one-photon excitation in poly-THF at concentrations of approximately 10^{-6} M ; while two-photon absorption (2PA) cross sections of model compounds were determined by the fluorescence method using DMSO solutions in concentrations of ca. 10^{-5} M . Rhodamine B in methanol was employed as a standard¹⁷ (Figure 3). Both compounds have a significant 2PA cross section value within the measured

range (700-940 nm). Anisotropy spectra exhibited a constant value at wavelengths where the maximum of linear absorption were located, corresponding to the first electronic transition $S_0 \rightarrow S_1$ (one-photon allowed); in this region, there is a decrease in the 2PA cross section value. The anisotropy began to decrease below 350 nm, reaching a minimum at around 325 nm leading to a second “plateau”; this may correspond to a higher electronic transition $S_0 \rightarrow S_n$, a two-photon allowed transition, resulting in a high 2PA cross section at this excitation wavelength.

A maximum in the 2PA spectra within the frame of the main linear absorption band was observed at around 820 nm for both model compounds. This transition correlated nicely to that responsible for the shoulder located at around 425 nm in the linear absorption spectrum. These dyes can be excited by 2PA at wavelengths as long as 850-900 nm and the fluorescence emission ranging from 400 - 800 nm. These probes have great versatility because they can be efficiently excited over a range of wavelengths and their emission is in the visible to NIR range, required for deep tissue imaging.

After bioconjugation of the isothiocyanates with the selected antibody and purification by gel permeation chromatography, fractions containing the bioconjugates were analyzed by UV-vis spectrometry. Figure 4 shows the absorption spectrum of isothiocyanate **10** (red) and biconjugate **B2** (green). The concentration of the protein in the bioconjugate was determined to be ca. 50 μM and the degree of labeling (DOL) was found to be 3. The concentration of the dye in the purified solution was estimated to be ca. 0.4 μM by using the extinction coefficient of the model compound. Preparation of bioconjugates following this method was performed more than once to evaluate the reproducibility of the method; repeated preparation of **B1** and **B2** generated these same results consistently.

Cell Imaging

To study the scope of the new bioconjugates, probes **B1** and **B2** were incubated with porcine aortic endothelial (PAE-KDR) cells that express VEGFR-2, as it is known that this receptor is found in higher concentrations when angiogenesis is occurring.¹⁰ Preliminary studies demonstrated that fluorescence images obtained with **B2** were superior to those obtained with **B1** (not shown). Thus, bioconjugate **B2** was used for the subsequent experiments. PAE-KDR cells were coincubated with a commercially available fluorescent bioconjugate containing fluorescein isothiocyanate and a monoclonal VEGFR-2 antibody (DC101), as a positive control, and our DC101 bioconjugate **B2**. After 1 h of incubation, epifluorescence micrographs of the incubated cells revealed a high degree of colocalization between the two probes (Figures 5A-G). Figure 5C shows very good colocalization of the commercial FITC antibody and bioconjugate **B2**. To examine the selectivity of bioconjugate **B2** towards the VEGFR-2, porcine aortic endothelial (PAE) cells that do not overexpress the receptor were used as a negative control. There is more evident autofluorescence in panel F, these are separate images. In Panel B the field of view was reduced as a result of digitally zooming in that particular area, subsequently the histograms of each micrograph are significantly different. This was particularly evident after no-neighbor deconvolution was performed on each micrograph. Micrographs of cells PAE cells showed little to no specific fluorescence when incubated with B2 (Figure 5I) or the VEGFR-2 antibody FITC conjugate (Figure 5H).

In the interest of comparing the difference resulting from exciting by one- versus two-photon absorption, PAE-KDR cells that had been previously incubated with **B2** were imaged by exciting at 405 nm and 810 nm, respectively. Collection of the one-photon fluorescence was confocal, as it was passed through a 120 μm pinhole (Figure 6 B). On the other hand, the upconverted fluorescence on the 2PFM was collected keeping the microscope pinhole completely open (Figure 6C). Fluorescence appeared to be much more localized in the 2PFM (Figure 6C) than in its one-photon counterpart. This is likely due to the fact that in the

confocal image a significant proportion of the fluorescence being collected is autofluorescence since the molecule is being excited at 405 nm. On the other hand, in 2PFM autofluorescence is not as much of a concern with excitation at 815 nm, i.e., most of what is collected is fluorescence from bioconjugate **B2**.

Whole Tumor Ex Vivo Imaging

Microscopy of biological specimens deep within the tissue is severely limited by scattering of visible light. However, many relevant physiological events occur within tissue of living organisms. Imaging features deeper within tissue at micrometer resolutions has been possible by 2PFM because scattering decreases with increasing wavelengths. Within biological tissue only unscattered (ballistic) photons are effective in exciting molecules by the absorption of two-photons.^{20, 21} Efficient 2PA fluorophores with high two-photon absorption cross sections at near IR wavelengths have been previously reported.^{16, 22} Thus, 2PFM benefits from the use of near IR light that is less likely to be scattered in tissue. The maximum imaging penetration depth within biological tissue (z_{max}) has been estimated to be linearly dependant on the scattering length for excitation light in tissue, $l_s^{(ex)}$, but logarithmically dependant on the collection efficiency of the system (fraction of fluorescent photons detected), two-photon action cross-section ($\delta\Phi$)^{21, 23} Limitations in most Ti:sapphire laser sources (1 W, 100 fs, 80 MHz, 680-1000 nm) restrict what can be done with these sources to increase imaging depth. These new, more efficient probes aim to increase the two-photon action cross section of the probe to enable imaging at greater depths in tissue.

Most efforts in improving the image quality and penetration depth of 2PFM have been oriented towards increasing collection efficiencies of the emitted fluorescence photon,²⁴ microscope objective,²¹ or repetition rates of the excitation source.²⁵ Herein, the focus has been to optimize the two-photon action cross section of fluorophores **6** and **11** in order to maximize the 2PFM imaging penetration within tumor tissue.¹⁶ The optimal dye (**10**) was conjugated to the DC-101 antibody to facilitate observation of the formation of new vessels that overexpress VEGFR-2 receptor deep within the tumor.

A solid tumor is comprised of cancer cells and host stromal cells alike.^{26, 27} Neoplastic and host cells rely on blood vasculature to supply themselves with nutrients and get rid of toxins. All of these cells are exposed to hypoxic and acidic microenvironment that induces the production of positive or negative regulators of angiogenesis. In the past 20 years more than 20 angiogenic stimulators have been discovered, VEGF being one of the most important factors. Vascular function is key in determining the tumor microenvironment. Vasculature in healthy tissue is very well structured; in contrast, tumor vessels have very poor morphology and are conformed by immature cells in a mesh-like architecture that confers a leaky property to the vessel. Because of its deep penetration, 2PFM offers an excellent means of monitoring tumor microenvironment, angiogenesis regulation and normalization of tumor vasculature by anti-angiogenic agents.^{28, 29}

The initial efficiency and selectivity of bioconjugate **B2** was evaluated in C57BL/6 male mice. Lewis Lung Carcinoma cells (3×10^6 cell/site) were implanted subcutaneously into the mice flanks. Eleven days postransplantation the tumor diameter had reached approximately 10 mm, the mice were tail vein injected with 400 μ g of the DC-101 bioconjugate **B2**. After 2 h the mice were perfused with PBS and sacrificed by formalin injection to the heart. Tumors were excised and fixed, and half was kept "whole" for tumor *ex vivo* microscopy and the other half for immunofluorescent histological experiments. The "whole" tumors were mounted on a glass cover slip and immobilized for imaging. 2PFM was conducted using both low (10 $\times$) and high (60 $\times$, oil immersion) N.A. objectives. The unconverted fluorescence was filtered prior to reaching the PMT (long pass 690 nm).

The whole tumor *ex vivo* 2PFM imaging experiments showed excellent results allowing the visualization of vasculature over 800 μm deep in the tumor tissue (Figure 7). In Figure 7, the orange boxes (A-F) show layers corresponding to optical sections along Z-axis (blue). The optical plane X-Y sections (X-axis in red, Y-axis in green) sections (Figures 7B, D, and F) at 738, 533, and 82 μm , respectively, show the fluorescence contribution of these optical layers along the Z-axis. Fluorescence from two-photon excitation of the probe was recorded up 830 μm into the tumor. The relative position of these planes along the Z-axis can be seen in the micrographs shown in Figures 7A, C, and E, where the corresponding optical plane (orange square) is shown in the respective projection of the X-Z plane along the Y-axis. Projection of X-Y planes along the Z-axis by the reconstruction of all of the optical planes (H) shows image of chaotically intertwined vessels within tumor where VEGFR-2 was overexpressed. The micrographs reveal how sensitive the 2PA phenomenon is to movements in the objective along the Z-axis and illustrates how useful this dye can be to reveal the formation of vasculature by 2PFM. Furthermore, the penetration that was achieved through a media with such wide distribution of refractive indices illustrates the potential of these efficient 2PA probes in monitoring the formation and development of vasculature in tumors by 2PFM imaging.

The selectivity of probe **B2** towards the tumor is evidenced by the excellent contrast observed in the micrographs in Figure 7, i.e., there was very little, nonspecific fluorescence within the tumors mainly attributable to autofluorescence. The bioconjugate was also specific to tumors. This observation was verified by the 2PFM images of key organs of the mice that had been injected with **B2** (Supporting Information Figure S1). The organs and glands observed were kidneys, liver, heart, brain, lung, and spleen. Primarily only autofluorescence from native structures within each organ (e.g., elastin in muscle and actin in the heart) or residual dye that was being excreted (kidney) was observed.

Tissue sections (400 μm thick) of the tumors of the mice that had been injected with **B2** were costained with an Alexa Fluor® 594-CD31 antibody to confirm, by fluorescence colocalization, that probe **B2** was, indeed, targeting vasculature (Figure 8). The tissue section was fixed and permeabilized for incubation with the Alexa Fluor® 594-CD31 conjugate for 1 h at room temperature. Figure 8 B shows the epifluorescence (1PA) corresponding to the sites where the DC-101 bioconjugate **B2** had accumulated 2 h following the tail vein injection. The vasculature was imaged by fluorescence of the Alexa Fluor® 594 dye (Figure 8C). The overlay of micrographs B and C is shown in Figure 8D where a high degree of colocalization can be observed for many vessels. As expected, not all vessels colocalized presumably because some expressed higher levels of the VEGFR-2 than others.

The selectivity of the **B2** conjugate to the VEGFR-2 was also investigated by making an IgG conjugate with isothiocyanate **10**, which was injected in the same dose and conditions in control mice. Model compound **11** was also injected into different control mice under the same conditions to demonstrate the specificity of the monoclonal antibody **B2**. Only nonspecific fluorescence was observed from imaging these tumors by 2PFM (Supporting Information Figure S2).

Conclusions

We developed and synthesized new two-photon absorbing fluorescent amine reactive probes and bioconjugates. Photophysical characterization was performed for amine reactive probes, model compounds, and bioconjugates. The new probes demonstrated a number of desirable characteristics and optical properties, including high photostability and target selectivity. Successful imaging by one and two-photon excitation with the bioconjugate **B2** in cells was

performed, demonstrating that the new bioconjugates selectively bind VEGFR-2 in cell culture, tissue sections, and whole tumors. The DC-101 bioconjugate **B2** is a powerful new probe for selectively imaging the vascular endothelial growth factor receptor 2 (VEGFR-2) in cells expressing this receptor *in vitro* and in “whole” mounted excised tumors (*ex vivo*) by two-photon fluorescence microscopy (2PFM) imaging. This selectivity imparted excellent contrast in 2PFM micrographs that captured tumor vasculature features up to 800 μm within the tumor, despite the refractive index heterogeneity that is characteristic of this type of tissue. These images were recorded without having to increase the collection efficiency of fluorescent photons with additional artifacts and with a simple, relatively low numerical aperture objective fully exploiting the working distance of this objective. Since the tumors were “whole” mounted without prior treatment, the conditions are close to that of the *in vivo* environment. Hence, the new bioconjugate should be an candidate to continue with *in vivo* imaging of tumors models through, e.g., a dorsal skinfold chamber where the first layers of tissue are significantly easier to access.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Depiction of a VEGFR-2 expressing tumor. Tumor cells induce the formation of new vasculature by expressing a higher concentration of VEGFR-2 which enables tumor growth. After treated with a 2PA, fluorescent antibody-bioconjugate the tumor vessels can be imaged by 2PFM.

Figure 2. Normalized absorption (left) and fluorescence (right) spectra of compounds **5**, **6**, **10**, and **11** in DMSO.

Figure 3. Normalized absorption (dash lines), two-photon absorption cross section (symbols and lines), and anisotropy ($2\times$, solid lines) of model compounds **6** (A) and **11** (B).

Figure 4. Normalized absorption of isothiocyanate **10** (red, solid line) and bioconjugate **B2** (green, dashed line).

Figure 5.

One-photon confocal micrographs of PAE-KDR and PAE cells. Cells were incubated with VEGFR-2 antibody-FITC conjugate (red) and DC101 bioconjugate (**B2**, green). A, B, and C show magnification of area enclosed by the blue box in D, F, and G. Colocalization corroborates VEGFR-2 selectivity (C and G) of **B2**. Fluorescence in negative control PAE cells (H-I) is much lower in intensity and less specific than in PAE-KDR (A-G). Scale bars: 5 μm in A and 20 μm in G.

Figure 6.

One- and two-photon fluorescence micrographs of PAE-KDR (porcine aortic endothelial cells expressing VEGFR-2) incubated with bioconjugate **B2** (15 μ M, 45 min, A-C) and PAE-KDR, PAE cells without **B2** incubation (D-I). A, D, G are DIC images, B, E, H are X-Y projections of one-photon confocal fluorescence (Excitation: 405 nm; Emission: spectral scanner window 480-620 nm). C, F, I are X-Y projections along Z-axis of two-photon fluorescence micrographs (Excitation: 810 nm; Emission: spectral scanner window 480-620 nm). 20 μ m scale bar in A applies for B and C. 20 μ m scale bar in I applies for D-I.

Figure 7.

Whole mount tumor 2PFM micrographs of excised Lewis Lung Carcinoma excised tumors. Mouse was tail vein injected 400 ug of DC-101 bioconjugate **B2** (cartoon in G). Orange boxes (A, C, E) or squares (B, D, F) show 40 μm optical sections along Z-axis. B, D and F are X-Y (red-green axes) plane projections along the Z-axis (blue) of optical sections centered at 740 μm , 540 μm , and 40 μm , respectively; A, C, and E show relative position of corresponding optical section section in the Z-axis. Projection of X-Y planes along the Z-axis (H) shows image of chaotically intertwined vessels within tumor. Micrographs were collected at 820 μm , 10 $\times$. Tissue penetration exceeded 830 μm . Scale bars F: 20 μm ; H: 10 μm .

Figure 8. Micrographs of tissue sections (400 μm thick) of tumors excised from a mouse injected with **B3**. Bright field image is shown in A and one-photon epifluorescence micrographs are shown B and C. Overlay of DC-101 bioconjugate **B2** (B) and Alexa Fluor® 594-CD31 antibody conjugate (C) is shown in D, colocalization confirms that probe **B2** targeted vasculature.

Scheme 1. Synthesis of B1

Scheme 2. Synthesis of B2

Table 1

Linear absorption maximum ($\lambda_{\text{max}}^{\text{abs}}$), emission maximum ($\lambda_{\text{max}}^{\text{em}}$), fluorescence quantum yield (Φ), molar absorptivity coefficient (ϵ^{max}), fluorescence lifetime (τ), photodecomposition quantum yield (η), and figure of merit (F_M) of compounds **5**, **6**, **10**, and **11** in DMSO.

Compound	$\lambda_{\text{max}}^{\text{abs}}$, nm	$\lambda_{\text{max}}^{\text{em}}$, nm	Φ	$\epsilon^{\text{max}} 10^{-3}, \text{M}^{-1}\text{cm}^{-1}$	τ , ns	$\eta 10^6$	$F_M 10^{-6}$, GM
5	397 $\pm$ 1	469 $\pm$ 1	0.80 $\pm$ 0.05	61	0.95 $\pm$ 0.08	--	--
6	400 $\pm$ 1	583 $\pm$ 1	1.00 $\pm$ 0.05	53	2.00 $\pm$ 0.08	4.23 $\pm$ 0.04	13 $\pm$ 2
10	391 $\pm$ 1	466 $\pm$ 1	0.70 $\pm$ 0.05	60	0.85 $\pm$ 0.08	--	--
11	391 $\pm$ 1	580 $\pm$ 1	0.20 $\pm$ 0.05	54	0.79 $\pm$ 0.08	1.60 $\pm$ 0.02	23 $\pm$ 3

¹ Fluorescence quantum yield measured relative to 9,10-diphenylanthracene in cyclohexane.