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Biotinylated Bioluminescent Probe for Long Lasting Targeted *in Vivo* Imaging of Xenografted Brain Tumors in Mice

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Abstract

Bioluminescence is a useful tool for imaging of cancer in *in vivo* animal models that endogenously express luciferase, an enzyme that requires a substrate for visual readout. Current bioluminescence imaging, using commonly available luciferin substrates, only lasts a short time (15–20 min). To avoid repeated administration of luciferase substrate during cancer detection and surgery, a long lasting bioluminescence imaging substrate or system is needed. A novel water-soluble biotinylated luciferase probe, B-YL (1), was synthesized. A receptor-targeted complex of B-YL with streptavidin (SA) together with a biotinylated epidermal growth factor short peptide (B-EGF) (SA/B-YL/B-EGF = 1:3:1, molar ratio) was then prepared to demonstrate selective targeting. The complex was incubated with brain cancer cell lines overexpressing the EGF receptor (EGFR) and transfected with the luciferase gene. Results show that the complex specifically detects cancer cells by bioluminescence. The complex was further used to image xenograft brain tumors transfected with a luciferase gene in mice. The complex detects the tumor immediately, and bioluminescence lasts for 5 days. Thus, the complex generates a long lasting bioluminescence for cancer detection in mice. The complex with selective targeting may be used in noninvasive cancer diagnosis and accurate surgery in cancer treatment in clinics in the future.

Graphical abstract

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Glioblastoma; bioluminescence; in vivo imaging; epidermal growth factor

INTRODUCTION

Imaging has become one of most important techniques in identifying and monitoring cancer in noninvasive diagnosis as well as in accurate surgical resection during treatment.^{1–6} First, it is crucial to detect tumor cells at an early stage of development.⁷ The most commonly used methods for detecting cancer in patients include magnetic resonance imaging (MRI), computerized tomography (CT), and positron-emission tomography (PET). Second, cancer treatments can include surgical resection, chemotherapy, and radiation.^{8,9} Surgery is one of the most effective ways to remove tumors and avoid metastatic disease spread. Surgery cures approximately 45% of all cancer patients with solid tumors. To be considered successful, a surgeon must remove the entire tumor and any lymph nodes or satellite nodules containing tumor cells. Partial removal of the solid tumor and incomplete removal of all the tumor cells decrease a patient's survival rate by 5-fold. Therefore, it is important to map the solid tumor accurately using imaging before surgery and double check for any residual tumor cells during and after surgery.

Recently, fluorescence imaging has been suggested for use in both cancer detection and resection; however, it is a light-dependent method.⁵ This strategy uses an external light source to excite exogenously added fluorescence agents and is not very versatile because many biological molecules present in the body have significant absorption of wavelengths at both visible and infrared regions of the light spectrum. Further, many wavelengths generated from the external excitation light source cannot penetrate tissues to reach the imaging fluorescent molecules, especially when they are in solid tumors. When the fluorescent molecules are not excited, no light is emitted for detection of the cancer. To overcome this problem, luminescence imaging is being developed to emit light from within the solid tumor. The process involves an enzyme, such as luciferase, which catalyzes the oxidation of a substrate, luciferin, to generate a bioluminescent signal, which is measurable by photon emission.¹⁰ Light is generated without application of an external excitation light source. The enzymatically generated photons are able to travel through solid tumors during the detection process.⁷

Although humans and animal models of cancer do not have naturally occurring bioluminescent genes, such as luciferase, the genes or proteins can be introduced for

imaging purposes. For instance, bacteria encoded with a luciferase gene can accumulate in C6 glioma tumors in a mouse model. The expressed luciferase is then used for cancer detection *in vivo*.¹¹ Furthermore, mammalian cells genetically modified with a luciferase gene can be delivered directly into tumors in live animals for tumor detection using bioluminescence.^{7,12,13}

In addition, tumor bioluminescence can be a useful tool during surgery as shown in an animal model.⁸ Bioluminescence was able to precisely detect tumors both preoperatively and intraoperatively. For the enzyme to produce bioluminescence within the tumor, D-luciferin or other small molecule substrates, such as coelenterazine, vargulin, and 6-aminoluciferin derivatives, must be administered.^{10,14–22} However, bioluminescence generated from these substrates lasts for a very short time, only 15 to 20 min per administration.¹⁰ This is not practical in a clinical setting as the surgery to remove a brain tumor takes 3–5 h. Checking and rechecking for tumor remnants by bioluminescence would require multiple new administrations of substrate. The additional application steps would lengthen the surgical time and potentially complicate the surgery and reduce the success rate of the surgery. Therefore, a new substrate with longer lasting bioluminescence signal is needed.

To overcome current limitations in bioluminescence imaging of cancers for detection and surgery, here, we report the development of a novel platform system for detection of brain tumors in mice *in vivo* (Scheme 1). First, we synthesized a novel biotin containing bioluminescent probe, B-YL (**1**), which acts as a substrate for luciferase. The probe possesses an aminoluciferin unit as a bioluminescent reporter, a poly-(ethylene glycol) (PEG-1000) link for improving cell penetrating ability, and a biotin tail for binding to streptavidin.^{19,23,24} Then, we constructed a complex, which contains streptavidin (SA), the bioluminescent probe B-YL, and a biotinylated epidermal growth factor short peptide (B-EGF) (SA/B-YL/B-EGF = 1/3/1, molar ratio), to target the complex. The EGF peptide binds to the EGF receptor, a biomarker overexpressed in 30–50% of high-grade gliomas. We then applied the complex to detect implanted brain tumor cells encoded with the luciferase gene by bioluminescence *in vitro* and *in vivo*.²³

RESULTS AND DISCUSSION

The synthesis of substrate B-YL is outlined in Scheme 2. Synthesis began with compound **2**.¹⁵ Compound **2** was amidated with propargylamine (**3**) in the presence of 1-ethyl-3-(3-(dimethylamino)propyl)-carbodiimide (EDC) and 1-hydroxy-benzotriazole (HOBt), resulting in compound **4** (88% yield). The latter was reacted with biotinylated azidoPEG (**5**) by click chemistry in the presence of Cu(CH₃CN)₂PF₆ and tris-[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA), affording compound **6** (74% yield).²⁵ Finally, compound **6** was reacted with D-cysteine in the presence of K₂CO₃ at pH 8.5 to produce probe B-YL (**1**) in 67% yield. The details of the synthetic procedures for the B-YL are described in the Methods.

The azidoPEG (**5**) is also a new and useful synthetic intermediate. The synthesis of compound **5** is also shown in Scheme 3.

After synthesis, the biotinylated probe, B-YL, was evaluated *in vitro* with a commercially available firefly luciferase. B-YL displayed bioluminescence with a maximum emission at 590 nm and was oxidized by commercial luciferase to emit bioluminescence photons (Figure 1).

The mechanism for generating bioluminescence is shown in Scheme 4. Luciferase, for example, from a firefly, generates light from a luciferin-based substrate in a multistep process. First, the substrate is adenylated by Mg-ATP to form luciferyl adenylate and pyrophosphate. Luciferyl adenylate is then oxidized by oxygen to form a dioxetanone ring. Decarboxylation forms an excited state oxyluciferin, which tautomerizes between the keto–enol forms. The reaction emits light as the oxyluciferin returns to the ground state.¹⁰

The B-YL substrate was then incubated with the brain cancer cell line U87-luc, which was derived from the parental brain cancer cell line U87 after stable transfection with a luciferase gene. As shown in Figure 2a, B-YL applied to cancer cells without luciferase, parental U87 cells, did not reveal bioluminescence activity regardless of the number of cells. However, as shown in Figure 2b, when there were as few as 7500 U87-luc cells or more in a well, bioluminescence signal was detected by using B-YL. B-YL clearly adsorbed across the cell membrane and was oxidized by luciferase. Therefore, B-YL can be used for the detection of the cancer cells by bioluminescence.

Using classical avidin–biotin complex (ABC) formation, complexes were made with a streptavidin core, which lacks any carbohydrate modification and has a near-neutral pH, and biotinylated ligands in the presence or absence of free biotin (B). The complex EGF-B/SA/B-YL (SA/B-YL/B-EGF = 1/3/1, molar ratio) is a targeting complex, which can be used as an active imaging agent for the detection of brain cancer cells.¹⁸ Complex EGF-B/SA/B-YL possesses targeting functionality because it has an EGF short peptide (12 amino acids). The complex B/SA/B-YL (SA/B-YL/B = 1/3/1, molar ratio) is a control complex with no targeting ability (no conjugated EGF short peptide).

The EGF-B/SA/B-YL complex was then tested for the ability to target and image brain cancer cell lines that overexpress the biomarker EGFR using bioluminescence *in vitro*. U87 (gray hatch bars) and U87-luc (black bars) cells were treated with free B-YL (150 µg/mL), untargeted B/SA/B-YL complex (150 µg/mL), or EGFR-targeted EGF-B/SA/B-YL complex (50–150 µg/mL). Bioluminescence was detected immediately. As shown in Figure 3, increasing concentrations of EGF-B/SA/B-YL complex actively accumulated into both U87 and U87-luc cells due to the presence of the EGF short peptide. However, only cells with luciferase were able to convert the EGF-B/SA/B-YL complex into detectable photons. Bioluminescence intensity depends directly on the amount of administered complex. Signal intensity increased linearly with increasing concentrations of the EGF-B/SA/B-YL complex. Cells without luciferase (U87) did not oxidize the EGF-B/SA/B-YL complex and release photons.

Untargeted B/SA/B-YL passively accumulated within the brain cancer cells. Luciferase activity for untargeted B/SA/B-YL was comparable to that of adsorbed free B-YL at the same concentration in U87-luc cells. The bioluminescence signal intensity from the targeted

EGF-B/SA/B-YL complex is 2.5-fold higher than that of the untargeted B/SA/B-YL complex, suggesting that the complex EGF-B/SA/B-YL could image cancer cells U87-luc much better than complexed B/SA/B-YL. Neither of the complexes (B/SA/B-YL or EGF-B/SA/B-YL) nor free B-YL were converted to bioluminescence photons by U87 cancer cells because they do not have luciferase activity.

The targeted EGF-B/SA/B-YL complex or untargeted B/SA/B-YL complex was then administered to mice ($N = 5$ per group) with subcutaneously implanted xenograft brain tumors derived from U87-luc cancer cells (right flank) or U87 cancer cells (left flank) and evaluated for bioluminescence activity.²³ In Figure 4a, targeted EGF-B/SA/B-YL revealed increasing bioluminescence in the right flank tumor region (U87-luc) of a mouse from 0.3 to 6 h. The signal was strong 6 h after injection of the complex. In contrast, administration of the untargeted B/SA/B-YL resulted in a weak bioluminescence signal after 0.3 h. The signal was completely gone after 4 h. The results suggest that the EGF-B/SA/B-YL complex is a suitable imaging agent for detection of luciferase activity within tumors.

Furthermore, in Figure 4b, longitudinal imaging of mice injected with either targeted EGF-B/SA/B-YL complex (solid line) or untargeted B/SA/B-YL complex (dashed line), revealed that the signal from untargeted B/SA/B-YL complex peaked at 0.02 h after injection, suggesting that the B/SA/B-YL complex had an even shorter retention time than that reported for D-luciferin in mice (20–30 min). Both the B/SA/B-YL complex and D-luciferin do not have targeting function. Bioluminescence signal intensities were then normalized across the treatment groups ($N = 5$ for each group) at 0, 0.02, 6, and 24 h (Figure 4c).

When the EGF-B/SA/B-YL complex was used, the maximum bioluminescence intensity was achieved at 24 h, suggesting the retention time for the EGF-B/SA/B-YL complex was very long. When the B/SA/B-YL complex was used, the maximum bioluminescence intensity was achieved at 0.02 h (12 min); there is 1200-fold increase in terms of retention time of bioluminescence from EGF-B/SA/B-YL. Furthermore, it took approximately 3 h to accumulate EGF-B/SA/B-YL within the tumor, EGF-B/SA/B-YL can be retained, and bioluminescence will last 24 h. The signal of bioluminescence finally disappeared after the sixth day, suggesting that the bioluminescence from EGF-B/SA/B-YL complex had very long retention time. It could be used to image cancer cells long-term *in vivo*. Current studies are underway to evaluate the ability of the biotinylated bioluminescent probe to cross the BBB in orthotopic brain tumors in mice and spontaneously formed gliomas in dogs.

CONCLUSIONS

In conclusion, a novel biotinylated probe, B-YL, has been synthesized. B-YL possesses a biotin unit for binding to streptavidin, a PEG linker for improving cell penetration, and a luciferin unit for bioluminescence triggered by luciferase. After synthesis, the B-YL was tested and found to be a substrate of firefly luciferase. B-YL was then used to form complexes with streptavidin together with biotinylated EGF short peptide to create targeted and untargeted complexes. The EGF-B/SA/B-YL complex produced stronger bioluminescent signal in brain cancer cell lines with an encoded luciferase gene (U87-luc). By using the EGF-B/SA/B-YL complex, bioluminescence signal in xenograft brain tumors

in mice was also detected. More importantly, the bioluminescence in the xenografted mouse lasted several days, suggesting that the targeted complex could be a long lasting bioluminescent agent for brain cancer imaging *in vivo*. To the best of our knowledge, this is the first long lasting bioluminescent imaging agent, which can last as long as 5 days *in vivo*. This imaging agent could find applications in cancer detection and surgery in the brain using bioluminescence, when tumors are removed and the surgery lasts several hours.

METHODS

General Methods

All chemicals used were purchased from commercial sources and used without further purification. Firefly luciferase was purchased (Sigma, MO). Flash chromatography was performed with SiliaFlash P60 silica (70–230 mesh; SiliCycle, Canada) and monitored by thin layer chromatography (TLC) with silica gel matrix plates (pore size 60 Å; Sigma-Aldrich, MO). The ^1H spectra were recorded on a 400 MHz Bruker Nanobay-400 (9.4 T) instrument (Bruker, MA) with methanol- d_4 , DMSO- d_6 , and CDCl_3 . The chemical shifts of protons are given in ppm relative to the signal of tetramethylsilane (TMS) as the internal standard. The purification of compound **1** was carried out using a high performance liquid chromatography (HPLC) instrument (Dionex Ultimate 3000; Thermo Scientific, MA) as described in more detail below in the preparation and purification of probe **1**. Luminescence spectroscopy measurement was performed using a SynergyMx (BioTek, VT). High-resolution mass spectrometry (HRMS) spectra were performed in the Mass Spectrometry Service Center of UC Riverside, CA. A Waters GCT Premier mass spectrometer (Waters, MA) was utilized with electron ionization (EI) and chemical ionization (CI) capabilities. Positive or negative ion mode was performed. Accurate mass measurements for these ionization modes were carried out as outlined.²⁶

Poly(ethylene glycol)₂₃-diazide (8)—PEG-1000 (20 g, 0.02 mol) was dehydrated by refluxing with toluene (100 mL), which was distilled out after reflux. After cooling, the PEG-1000 was dissolved in THF (150 mL). To the solution were added mesyl chloride (4.88 mL) in one portion and triethylamine (9.16 mL) in tetrahydrofuran (THF) (80 mL) dropwise over 30 min at 0 °C. The mixture was stirred for 1 h at 0 °C and 3 h at room temperature, affording PEG dimesylate solution in THF.²⁷ A small amount of solution was extracted with dichloromethane, washed with water and dried over Na_2SO_4 . After concentration, the residue was used for characterization. ^1H NMR (400 MHz, CDCl_3 , ppm) δ 3.11 (s, J = 5.2 Hz, 4 H), 3.65 (s, 85 H).

To the solution of PEG dimesylate in THF were added sodium bicarbonate (5%, 44 mL) and sodium azide (5.2 g). The resultant mixture was concentrated to 250 mL, stirred at room temperature overnight, and refluxed for 5 h. After cooling, the mixture was extracted with dichloromethane (3×100 mL). The resultant organic layer was dried over MgSO_4 and concentrated to give PEG diazide (20.7 g, 98%). The product was used for the next step without further purification. ^1H NMR (400 MHz, CDCl_3 , ppm) δ 3.40 (t, 6 H), 3.67 (s, 81 H), 3.78–3.80 (m, 4 H), 4.40–4.41 (m, 4 H). Mass calculated for $\text{C}_{44}\text{H}_{88}\text{N}_6\text{O}_{21}^+$ ($\text{M} + \text{H}^+$) 1037, found 1032.

Amino-poly(ethylene glycol)₂₃-azide (9)—To the PEG diazide (**8**, 10.7 g, 10.2 mmol) solution in ethyl acetate (EtOAc, 60 mL) were added HCl (1 N, 12 mL) and triphenylphosphine (2.67 g, 10.2 mmol). The mixture was stirred for 12 h at room temperature. Water (12 mL) was added into the reaction mixture, and the mixture was extracted with EtOAc (2 × 50 mL) to remove unreacted PEG diazide. Solid potassium hydroxide (9.28 g) was added at 0 °C. The mixture was then extracted with CH₂Cl₂ (3 × 50 mL). The organic layer was washed with brine, dried over Na₂SO₄, and concentrated to 9.8 g of amino containing PEG compounds (azido PEG monoamine and PEG diamine) in 94% yield.² ¹H NMR (400 MHz, CD₃OD, ppm) δ 2.80–2.82 (m, 2 H), 3.39–3.42 (m, 2 H), 3.55 (m, 2 H), 3.63 (s, 86 H). Mass calculated for C₄₄H₉₀N₄O₂₁⁺ (M + H⁺) 1011, found 1012.

N-(Azido-poly(ethylene glycol)₂₃)-hexahydro-2-oxo-(3a*S*,4*S*,6a*R*)-1*H*-thieno[3,4-*d*]imidazole-4-pentanamide (5)—To the above amino containing PEG compounds **9** (9.8 g) in DMF (70 mL) and NEt₃ (2.76 mL) was added biotin NHS (3.93 g, 11.48 mmol). After stirring overnight at room temperature, the reaction mixture was concentrated, dissolved in CH₂Cl₂, centrifuged to a clear solution, and purified with column chromatography (silica gel, dichloromethane–dichloromethane/ acetone/methanol (2:7:1)–dichloromethane/methanol (9:1)) to yield product **5** (67%).²⁸ ¹H NMR (400 MHz, CDCl₃, ppm) δ 1.45–1.49 (m, 2 H), 1.70–1.75 (m, 4 H), 2.28 (t, *J* = 7.9 Hz, 2 H), 2.72–2.79 (m, 1 H), 2.92 (dd, *J* = 12.8, 4.8 Hz, 1 H), 3.17–3.18 (m, 1 H), 3.41 (t, *J* = 5.0 Hz, 2 H), 3.59 (t, *J* = 5.0 Hz, 2 H), 3.65–3.75 (m, 75 H), 4.34–4.37 (m, 1 H), 4.53–4.56 (m, 1 H), 6.21 (s, 1 H), 6.76 (s, 1 H). Mass calculated for C₅₄H₁₀₄N₆O₂₃SN⁺ (*n* = 22, M + Na⁺) 1259.677, found 1259.681.

N'-Propargyl N''-(2-Cyano-6-benzothiazolyl)-glycinamide (4)—To a solution of *N*-(2-cyano-6-benzothiazolyl)-glycine (**2**, 0.57 g, 2.6 mol) in tetrahydrofuran (100 mL) and acetonitrile (70 mL) were added propargyl amine (**3**, 0.33 mL, 5.2 mmol), 1-ethyl-3-(dimethylamino)propylcarbodiimide (EDC, 0.996 g, 5.2 mmol), and 1-hydroxybenzotriazole (HOBt, 0.8 g). The mixture was stirred for overnight, concentrated in vacuo, dissolved in dichloromethane (50 mL), washed with saturated sodium bicarbonate (50 mL), and dried over magnesium sulfate. After concentration, residue was purified by column chromatography (silica gel, hexane, ethyl acetate, then acetone) to yield 0.59 g (88%) of product **4**.³ ¹H NMR (400 MHz, CD₃COCD₃, ppm) δ 2.65 (t, *J* = 2.4 Hz, 1 H), 3.97 (d, *J* = 5.6 Hz, 2 H), 4.05 (dd, *J* = 5.6, 2.5 Hz, 2 H), 6.30 (s, 1 H), 7.17 (m, 1 H), 7.19 (s, 1 H), 7.79 (s, 1 H), 7.96 (d, *J* = 8.6 Hz, 1 H). Mass calculated for C₁₃H₁₁N₄OS⁺ (M + H⁺) 271.065, found 271.064.

N-(5-(N''-(2-Cyano-6-benzothiazolyl)-glycinamido-methyl)-1*H*-1,2,3-triazolyl)-poly(ethylene glycol)₂₃-hexahydro-2-oxo- (3a*S*,4*S*,6a*R*)-1*H*-thieno[3,4-*d*]imidazole-4-pentanamide (6)—To a solution of **4** (0.185 g, 0.72 mmol) and *N*-(azido-poly(ethylene glycol)₂₃)-hexahydro-2-oxo-(3a*S*,4*S*,6a*R*)-1*H*-thieno [3,4-*d*]imidazole-4-pentanamide (**5**, 0.45 g, 0.36 mmol) in DMF (4.0 mL) were added tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA, 3.3 mg) and Cu(CH₃CN)₂PF₆ (29.8 mg) under N₂ stream at room temperature. The resultant mixture was stirred at 40 °C overnight. After evaporation of solvent in high vacuum, the residue was purified with column chromatography (silica gel,

dichloromethane–dichloromethane/ methanol (88/12 (v/v))) to yield 0.402 g (74%) of product **6**.²⁵ ¹H NMR (400 MHz, CD₃OD, ppm) δ 1.47–1.48 (m, 2 H), 1.63–1.74 (m, 4 H), 2.27 (t, *J* = 7.5 Hz, 2 H), 2.75 (d, *J* = 12.8 Hz, 1 H), 2.98 (dd, *J* = 12.8, 2.5 Hz, 1 H), 3.22–3.24 (m, 1 H), 3.37–3.39 (m, 2 H), 3.60–3.65 (m, 82 H), 3.86 (d, *J* = 5.0 Hz, 2 H), 3.96 (s, 2 H), 4.31–4.35 (m, 1 H), 4.51–4.60 (m, 5 H), 7.08–7.10 (m, 2 H), 7.92–7.94 (m, 2 H), 8.02 (s, 1 H). Mass calculated for C₆₃H₁₀₇N₁₀O₂₂S₂ (*n* = 18, M + H⁺) 1419.700, found 1419.700.

6-(3-N-{N-[5-(Hexahydro-2-oxo-1H-thieno[3,4-d]imidazol-4-yl)-1-oxopentyl]-, [3aS-(3aα,4β,6aα)]}-aminoethyl-poly(ethylene glycol)₂₁-oxyethyl-1H-1,2,3-triazol-5-yl-methylamino-oxomethylamino)-2-benzothiazolyl]-4,5-dihydro-, (4S)-4-thiazole-carboxylic Acid (1)—To a solution of compound **6** (15.07 mg, 0.9 mmol) in methanol (6 mL), was added a solution of D-cysteine (9.31 mg) in water (1.8 mL). The mixture was titrated with potassium carbonate (0.05 M) to pH 8. The mixture was then stirred for 10 min at room temperature. After concentration with N₂ stream, the residue was purified with high performance liquid chromatography (HPLC) using a reverse phase column (Luna 5 μ C18 column, 4.6 μ, 250 mm; Phenomenex, CA)²⁹ with a 20–100% acetonitrile gradient at a flow rate of 5 mL/min and the detector set at 260 nm, affording probe **1** (10.1 mg, 67% yield).¹⁵ ¹H NMR (400 MHz, CD₃OD, ppm) δ 1.46–1.47 (m, 2 H), 1.65–1.69 (m, 4 H), 2.24 (m, 2 H), 2.74 (d, *J* = 12.4 Hz, 1 H), 2.93–2.97 (m, 1 H), 3.22 (m, 1 H), 3.33–3.66 (m, 84 H), 3.82 (s, 2 H), 3.91 (s, 2 H), 4.10–4.12 (m, 1 H), 4.33 (s, 1 H), 4.52 (s, 4 H), 6.90–6.99 (m, 2 H), 7.76–7.60 (m, 2 H). Ultraviolet maximum absorption was at 365 nm and maximum fluorescence emission was at 515 nm in Leibovitz's L-15 medium (pH 7.8). Mass calculated for C₆₆H₁₁₂N₁₁O₂₄S₃ (*n* = 18, M + 1H⁺) 1538.704, found 1538.706.

Measurement of Bioluminescence Spectrum of Probe B-YL (1)

In a well of 96 quartz plate (500 μL), HEPES buffer (30 mM) solution (400 μL, pH = 7.7) containing MgSO₄ (5.0 mM), ATP (2.6 mM), DTT (3.5 mM), CoA (1.5 mM), and commercially available firefly luciferase (40 μg/mL) were mixed. The probe B-YL was then added and mixed to a final concentration (32 μM). Bioluminescence spectrum of B-YL was then taken immediately with a Synergy Mx (BioTek, VT) over the wavelength range 520–650 nm.

Formation of B-YL Complexes

Preparation of a Complex of Streptavidin with B-YL and Free Biotin (B/SA/B-YL, 1:3:1 Molar Ratio)—Streptavidin (189 μM, stock solution A) was prepared by dissolving streptavidin solid (10.0 mg) in PBS buffer (1.0 mL, pH 7.4). Biotin was prepared in dimethyl sulfoxide (DMSO) (1.0 mM). Biotin (1.0 mM, 0.189 mL) and B-YL (6.5 mM, 0.0852 mL) were added to DMSO (0.20 mL), resulting in solution B after mixing. Solution A was then mixed with solution B evenly. The resulting mixture was incubated at room temperature for 30 min. The mixture was then transferred into a membrane filter cassette (20 kDa cutoff) and dialyzed in PBS buffer (1000 mL) overnight. The B/SA/B-YL solution was removed from the cassette. Concentration was measured by UV spectroscopy (76.4 μM, 353 μg/mL).

Preparation of a Complex of Streptavidin with B-YL and EGF Peptide (EGF-B/SA/B-YL, 1:3:1 Molar Ratio)—Biotinylated EGF short peptide (B-Tyr-His-Trp-Tyr-Gly-Tyr-Thr-Pro-Gln-Asn-Val-Ile-NH₂) was prepared in dimethyl sulfoxide (DMSO) (1.0 mM). Biotinylated EGF short peptide (1.0 mM, 0.189 mL) and B-YL (6.5 mM, 0.0852 mL) were added to DMSO (0.20 mL), resulting in a solution B after mixing evenly. Solution A was then mixed with B evenly. The resulting mixture was incubated at room temperature for 30 min. The mixture was then transferred into a membrane filter (cassette, 20 kDa cutoff) and dialyzed in PBS buffer (1000 mL) overnight. The EGF-B/SA/BYL solution was drawn out of the membrane filter. Concentration was measured by UV spectroscopy (111 μ M, 512 μ g/mL).

***In Vitro* Bioluminescence Imaging**

For the *in vitro* luciferase assay, cells were plated in triplicate on black walled 24-well plates with increasing cell numbers (0–60000 cells) of either U87 not expressing luciferase or with luciferase. Cells were grown overnight with regular growth medium. After 24 h, the regular medium was replaced with 100 μ g/mL B-YL Leibovitz's L-15 medium with MgCl₂ (5 mM). Bioluminescence images were taken immediately after adding the substrate into the cells using an IVIS 200 In vivo Imaging System (PerkinElmer, MA).

***In Vivo* Bioluminescence Imaging**

U87 cells were implanted subcutaneously on the left (without luciferase) and right flanks (with luciferase) of athymic nude mice as per IUCAC approved protocols. Prior to *in vivo* imaging, the mice were anesthetized with isoflurane. Targeted EGF-B/SA/B-YL ($N = 5$) or untargeted B/SA/B-YL ($N = 5$) solution was then injected intraperitoneally (150 μ g/kg body weight). The mice were imaged using an IVIS Spectrum. Mice were imaged every hour for 8 h and then every 24 h thereafter. Relative luminescence was quantitated by creating a region of interest (ROI) over the tumor and expressed as photons/(s·cm²). To standardize the data, a ROI was drawn around a white light image of each tumor. Light emission was quantified from the same surface area (ROI) for each tumor. Corresponding gray scale photographs and color luminescence images were superimposed and analyzed using Living Image analysis software version 3.1 (Caliper Life Sciences, CA).³⁰

Statistical Analysis

All data is expressed as mean $\pm$ SD. All data analysis was performed using GraphPad Prism software version 6 (La Jolla, CA) unless specified. Multiple variables were analyzed via analysis of variance techniques; a p value <0.05 was considered statistically significant.

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ABBREVIATIONS

B	biotin
BBB	blood–brain barrier
CT	computerized tomography
EDC	1-ethyl-3-(3-(dimethylamino)propyl)-carbodiimide
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EtOAc	ethyl acetate
HPLC	high performance liquid chromatography
HRMS	high-resolution mass spectrometry
Mg-ATP	magnesium-adenosine triphosphate
MRI	magnetic resonance imaging
NMR	nuclear magnetic resonance spectroscopy
PEG	poly(ethylene glycol)
PET	positron-emission tomography
SA	streptavidin
THF	tetrahydrofuran
TLC	thin layer chromatography
UV–vis	ultraviolet–visible spectroscopy

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Figure 1.
Luminescence spectrum of probe **1** after treatment with commercially available firefly luciferase.

Figure 2.

B-YL substrate was used to identify enzymatic activity in brain tumor cell lines transfected with luciferase. Triplicate wells were plated with increasing numbers of U87 cells either without luciferase (a) or with luciferase (b). In each plate, row 1, wells 1–3, no cells; wells 4–6, 1875 cells per well; row 2, wells 1–3, 3750 cells per well; wells 4–6, 7500 cells per well; row 3, wells 1–3, 15000 cells per well; wells 4–6, 30000 cells per well; row 4, wells 1–3, 60000 cells per well. Incubation buffer, Leibovitz's L-15 medium with MgCl_2 (5 mM). Each well was incubated with B-YL (100 $\mu\text{g/mL}$). Plates were imaged using an IVIS 200 In

vivo Imaging System. Escalating luminescence was observed only in wells containing luciferase-expressing cells (b). No luminescence was observed in cells lacking luciferase (a).

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Figure 3.

Relative bioluminescence intensities of imaging of brain cancer cells U87 (gray hatch bars) and brain tumor cell lines transfected with luciferase U87-luc (black bars) after treating with free B-YL substrate (150 µg/mL), B-SA-B-YL (complex of B-YL substrate with streptavidin and free biotin, 150 µg/mL, untargeted), and EGF-B-SA-B-YL (complex of B-YL substrate with streptavidin and biotinylated EGF short peptide, 50, 75, and 150 µg/mL, targeted).

Figure 4.

In vivo imaging of xenograft U87-luc brain tumors in mice using targeted EGF-B-SA-B-YL or untargeted B-SA-B-YL complexes (150 μ g/kg body weight). (a) Bioluminescence images from two representative mice, one from each treatment group, using complex EGF-B-SA-B-YL (top) and B-SA-B-YL (bottom). (b) Longitudinal bioluminescence signal intensities (0–144 h) of representative mice using complexes EGF-B-SA-B-YL (solid line) and B-SA-B-YL (broken line). (c) Bioluminescence signal intensities were normalized for each treatment group ($N=5$) at 0, 0.02, 6, and 24 h ($p < 0.001$).

Scheme 1.

Schematic of a Novel Platform Involving a Probe, B-YL, for Targeted Imaging of Brain Cancer Cells by Long Lasting Bioluminescence

Scheme 2.
Synthesis of Probe B-YL (1)

Scheme 4.
Production of Bioluminescence from Probe B-YL (1)