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Strained alkyne substituted near infrared BF2 azadipyrrromethene fluorochrome

Dan Wu  
*Royal College of Surgeons in Ireland*, danwu@rcsi.ie

Shane Cheung  
*Royal College of Surgeons in Ireland*, shanecheung@rcsi.ie

Corry James O'Sullivan  
*Royal College of Surgeons in Ireland*, corryjosullivan@rcsi.ie

Yinghua Gao  
*Donghua University*

Zhi-Long Chen  
*Donghua University*

*See next page for additional authors*

Citation

Introduction

Fluorescence imaging offers exciting possibilities for the real-time recording of complicated biological processes and the visualization of specific tissue regions within whole organisms. Specifically, the near-infrared (NIR) spectral region (700–900 nm) provides optimal imaging spectral wavelengths to reduced light toxicity and avoidance of competing endogenous chromophore absorbance. This is of specific advantage for use in prolonged live cellular and in vivo imaging thereby bridging the divide of cellular and animal research while also providing a translational route to clinical use for fluorescence-guided surgery.

In conjunction with optimal wavelengths the covalent attachment of a targeting moiety to help address a region of interest at a subcellular or whole organism level is typically required. Lysine-activated ester and cystine-maleimide remain the most commonly used transformations for bio-conjugation with more recently the non-natural amino acid residue based azide-alkyne addition reaction growing in popularity. The first of such reactions were Cu(I) catalysed though more recently the limitations of using a toxic metal catalyst has led to the increasing adoption of strain-promoted azide alkyne cycloadditions (SPAAC) which are rapidly grown in importance. Exploitation of the intrinsic reactivity of substituted cyclooctynes with organic azides has been successfully used in SPAACs for bio-conjugations in aqueous media, cell membrane labelling and other applications. Several cyclooctyne derivatives have been specifically developed such as mono- and di-fluorinated cyclooctyne (MOFO and DIFO), dibenzocyclooctyne (DBO), dibenzoazacyclocyclynone (DBAC) and bicyclononyne (BCN) in combination with a variety of fluorophores for these purposes.

Currently, the number of NIR fluorophores with the capability for SPAACs is relatively limited with the DIBAC substituted cyanine dye ADIBO-Cy5.5 and the MOFO substituted Atto 700 dye (dye structure not disclosed) being only recently described (Fig. 1). Clearly a distinct need exists for new classes of NIR emitting fluorochromes with SPAAC capability, and thus, intensive efforts are currently being put into their development.

![Fig. 1 Structures of ADIBO-Cy5.5 and MOFO-Atto 700 fluorochromes.](image_url)
The excellent photophysical and stability properties of the BF₂-azapyromethene (NIR-AZA) fluorophore class has attracted the attention of many research teams for use in numerous material and biologically related applications. For example, the absorption of fluorophore 3a is 709 nm with emission at 740 nm in formulated aqueous solution (Fig. 2, ESI Fig. S1). Fluorochromes 3b-f which are based on derivatization of this structure have been recently reported containing activated ester, maleimide and terminal alkynes conjugating groups (Fig. 2). However, a strained alkyne fluorochrome has yet to be developed which is the focus of this work. Numerous strained alkynes have been developed for the specific purpose of azide bioconjugation reactions though our attention was drawn to the bicyclo[6.1.0]non-4-ynyl (BCN) derivative. Positive features of BCN is its ease of preparation, good bench stability, lower lipophilicity and excellent SPAAC reaction kinetics. It has been successfully employed for both bioconjugation and bioorthogonal labelling.

As such, the target selected was NIR-AZA 4 which synthetically would require the sequential functionalization of 3a with both a strain-promoting BCN conjugation group and an aqueous solubilizing alkyl-sulfonate group (Fig. 3). Following synthesis BCN-NIR-AZA 4 was assessed for its ability to bio-conjugate in mild aqueous conditions and the in vitro and in vivo imaging capability of its conjugates determined.

**Results and Discussion**

The synthetic route commenced with the mono alkylation of bisphenol azadipyrromethene 3a with N-Boc-2-bromoethylamine and cesium carbonate in dry DMSO at 80 °C to produce 5 in a 70% yield. Subsequent Boc-deprotection of 5 was completed with TFA (10%) in CH₂Cl₂ for 2 h to give 6 following which introduction of the BCN group was achieved by coupling with (1R,8S,9s)-bicyclo[6.1.0]non-4-yn-9-yl methyl (4-nitrophenyl) carbonate 7 in dry DMF, providing 8 in 65% yield following purification. The final synthetic step functionalized 8 with the water solubilizing alkyl sulfonate group by reflux with 1,3-propanesultone in dry THF in presence with cesium carbonate for 1 h. During the reaction the NIR-fluorochrome product precipitated and was isolated in a 75% yield. NMR and MS analysis was consistent with the expected product structure (Scheme 1).

The spectroscopic properties of key intermediates 5, 6, 8 and fluorochrome 4 were examined in organic and aqueous solvents (Table 1). Organic-soluble derivatives 5, 6, 8 all showed similar sharp absorption and emission band qualities in chloroform with absorption and emission maxima at 684±1 nm and 710 nm respectively. Extinction coefficients (ε) and fluorescence quantum yields (Φ_flu) ranged from 80-83,000 and 0.30-0.32, respectively (entries 1-3). Encouragingly, the spectra properties of 4 in water maintained these desirable photophysical properties. For example, λₘₐₓ = 690 nm (ε = 72,000) and λₑₓᵣ = 722 nm (Φ_flu = 0.18) were recorded in aqueous buffer for 4 (Table 1, entry 4).
**Table 1. UV-Vis and Fluorescence Properties of 4, 5, 6 and 8**

<table>
<thead>
<tr>
<th>Entry</th>
<th>Comp.</th>
<th>Solvent</th>
<th>λ\textsubscript{abs} (nm)</th>
<th>Ext. coeff.</th>
<th>λ\textsubscript{flu} (nm)</th>
<th>Φ\textsubscript{flu}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>CHCl(_3)</td>
<td>685</td>
<td>83,000</td>
<td>710</td>
<td>0.32</td>
</tr>
<tr>
<td>2</td>
<td>6(^{[b]})</td>
<td>CHCl(_3)</td>
<td>684</td>
<td>80,000</td>
<td>710</td>
<td>0.32</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>CHCl(_3)</td>
<td>684</td>
<td>82,000</td>
<td>710</td>
<td>0.30</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>H(_2)O/1% SDS</td>
<td>690</td>
<td>72,000</td>
<td>722</td>
<td>0.18</td>
</tr>
</tbody>
</table>

\(^{[a]}\) Compound 3a (Φ\textsubscript{flu}=0.30) was used as a standard. \(^{[b]}\) Measured as free amine. \(^{[c]}\) H\(_2\)O/1% SDS.

*N*-Boc-4-azido-L-homoalanine was selected as a test azide for reaction with 4 to provide the triazole amino acid substituted product 9 (Scheme 2). To explore the conjugation conditions, the model reaction was carried out in PBS with different conditions and reaction conversions monitored over time by reverse phase HPLC (Fig. 4).

Encouragingly, as can be seen from the conversion plots, the reaction of BCN-NIR-AZA 4 fluorophore with 5 equiv. of N\textsubscript{Boc}-4-azido-L-homoalanine was completed within 30 mins at both rt and 37 °C (Fig. 4, green and blue traces). Upon reduction of the azide equivalence to 1.1 the reaction was complete in 2 h at 37 °C (red trace).

Next, the azido functionalized cyclic RGD peptide [cyclo[Arg-Gly-Asp-D-Phe-Lys(azide)]] was chosen for its excellent tumour targeting ability via the \(\alpha\_5\beta\_3\) integrin heterodimeric cell surface receptors overexpressed in tumour cells.\(^{[15]}\) The SPAAC reaction of 1.1 equiv. of azido functionalized cyclic RGD peptide with 4 in PBS at 37 °C was judged to have reached completion by HPLC in 2 h (Scheme 2). The cRGD-conjugate 10 was purified by reverse phase prepartive HPLC and characterised with HRMS and MALDI-TOF mass spectrometry (ESI, Fig. S5).

The photophysical properties of conjugates 9 (black trace) and 10 (red trace) are shown in Table 2 and were found to maintain the excellent absorption and fluorescence properties of fluorochrome 4. Comparison of the data for conjugates 9 and 10 revealed only minor differences in the maxima values of \(\lambda\textsubscript{abs} (\pm 1 \text{ nm})\) and \(\lambda\textsubscript{flu} (\pm 1 \text{ nm})\) in aqueous solution (Table 2).

**Table 2. UV-Vis and Fluorescence Properties of 9 and 10**

<table>
<thead>
<tr>
<th>Comp.</th>
<th>(\lambda\textsubscript{abs}) (nm)</th>
<th>Fwhm(^{[c]}) (nm)</th>
<th>Ext. coeff.</th>
<th>(\lambda\textsubscript{flu}) (nm)</th>
<th>Φ\textsubscript{flu}</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>695</td>
<td>54</td>
<td>69,000</td>
<td>720</td>
<td>0.18</td>
</tr>
<tr>
<td>10</td>
<td>694</td>
<td>60</td>
<td>67,000</td>
<td>721</td>
<td>0.17</td>
</tr>
</tbody>
</table>

\(^{[a]}\) H\(_2\)O/1% SDS. \(^{[b]}\) Full width at half maximum height. \(^{[c]}\) Compound 3a (Φ\textsubscript{flu}=0.30) used as a standard.

To explore the ability of conjugated NIR-AZAs to be internalized in cells, HeLa Kyoto cells were first incubated with 9 and 10 for 2 h, fixed with formaldehyde, followed by nuclei staining with Hoechst 33342 and imaged. These preliminary results showed that an effective internalization of the fluorescent conjugates had occurred with localization throughout the cytosol though more concentrated in the perinuclear region (Fig. 5, panels A, B). In order to visualise the process in real time live cell imaging was carried out with 10 which revealed that the initial cell membrane interaction was rapid with fluorescence observed within 2 min and increasing steadily to 10 min (Fig. 5, panels C-F). For movie of this process see SI Fig. S4.1 Further studies were conducted in which cells were pre-incubated with 10 for 10, 30, 60 and 120 min and...
subsequently imaged with representative fields of view shown in Fig. 5 (panels G-J). At 10 and 30 min the membrane is clearly distinguishable with localization of 10 predominantly at the cell membrane (panels G, H; see SI Fig. S4.2 for Z-stacks). At 60 and 120 minutes internal trafficking vesicles could be observed within the cytosol as internalization had occurred and the outline of the nucleus can be discerned (panels I, J; see SI Fig. S4.2 for Z-stack). Analysis of live cell images following a 120 min incubation with 10 showed distinct point like organelle staining indicative of internalization and trafficking within the cells (See SI Fig. S4.3).

As the photophysical properties of the NIR-AZA fluorophore class are in vivo compatible the cRGD conjugate 10 could be also tested in vivo. For this purpose, subcutaneous tumours from the human esophageal cancer cell line Eca-109 were grown in mice and the ability of 10 to tumour localize was investigated. Following an intravenously tail vein injection of 10 (2 mg/kg), NIR fluorescence images (using emmission filter 700(20) nm) were recorded at regular intervals for 48 h (see ESI, Fig. S5) A high emmission intensity from organs such as heart, liver and other tissues including tumour was evident 2 h post administration. Tumour versus liver selectivity was best after 6 h post administration. The optimal tumor to background differential occurred between at 6 and 10 h post injection, with almost complete diminishment of fluorescence after 24 h.

Fig. 5. A composite fluorescence image of fixed HeLa-Kyoto cells following 2 h incubation with conjugates 9(A) and 10 (B) at 5 µM cell nuclei stained blue with Hoechst 33342). Sum montage of live HeLa-Kyoto cell imaging at 0 min (C), 2 min (D), 6 min (E), 10 min (F) following introduction of 10. Live HeLa-Kyoto cell imaging after pre-incubation with 10 for 10 min (G), 30 min (H), 60 min (I), 120 min (J). Scale bars = 10 µm.

Fig. 6. In vivo imaging of 10 using Eca-109 subcutaneous tumour model in mice. (A) Photo image (B) NIR fluorescence imaging 10 h post i.v. administration of 10 (excit. 640(30) nm, emiss, 700(20) nm) (C) NIR fluorescence imaging 10 h post i.v. administration of 10 with intensity scale adjusted. (D) Profile of tumour NIR fluorescence (red solid line) and liver (red dashed line) over time following i.v. tail injection of 10. Background area (grey solid line). Values determined from the same sized ROI from background area and tumour averaged for n=3.

Conclusions
In summary, the first BCN strained alkyne derived BF₂ azadipyrrromethene has been synthesised and shows excellent NIR photophysical properties. Reaction with azido amino acid and RGD peptide sequences were efficient in PBS at rt or 37 °C without the need for any additional reagents or catalysts. The cRGD peptide conjugated NIR-AZA showed impressive real-time cellular imaging and the ability to tumour localise in vivo mouse efficient uptake and good localisation at the tumour site.
Experimental procedures

Chemicals. All reagents were purchased from Sigma-Aldrich and used without further purification unless otherwise noted. All reactions involving air-sensitive reagents were performed under nitrogen in oven-dried glassware using syringe-septum cap technique. All solvents were purified and degassed before use. The peptide cyclo[Arg-Gly-Asp-D-Phe-Lys(Azide)] was purchased from Peptides International and used as supplied.

Instrumentation. 1H and 13C nuclear magnetic resonance spectra were recorded on a 400 MHz spectrometer. Electrospray mass spectrometry was carried out using an Advion Expression CMS. MALDI MS data was acquired on a Waters MALDI Q-TOF Premier MS. Chromatographic separation was carried out under pressure on Merck silica gel 60 using flash-column techniques. Reactions were monitored by thin-layer chromatography (TLC) carried out on 0.25 mm silica gel coated aluminum plates (60 Merck F254) using UV light (254 nm) as visualizing agent. High performance liquid chromatography (HPLC) was performed with a Shimadzu instrument equipped with a Shimadzu SPD-20AV prominence UV/Vis Detector. Separations employed YMC RP Triart Phenyl 5 μm HPLC column (150 × 6 mm I.D.), with a water:CH3CN solvent gradient. Fluorescence emission spectra measurements were conducted with a Varian Cary Eclipse fluorescence spectrophotometer with the excited and emissive slit of 5 nm. UV-Vis spectra were recorded using a Varian Cary 50 Scan UV-Visible spectrophotometer using 1 cm path length quartz cuvettes.

Synthetic Procedures.

Synthesis of fluorophore 5. Compound 3a 9 (200 mg, 0.38 mmol) and Cs2CO3 (246 mg, 0.76 mmol) were dissolved in dry DMSO (6 ml) and stirred at rt under N2 atmosphere for 10 min. 2-(Boc-amino)ethyl bromide (94 mg, 0.42 mmol) was added and the solution stirred at 80 °C for 60 min. The mixture was cooled to rt and partitioned between AcOEt (100 ml) and PBS buffer at pH 7 (100 ml). The organic phase was washed with water (3×100 ml), brine (50 ml), dried over Na2SO4, filtered and evaporated to dryness. The crude product was purified by alumina column chromatography eluting with CH2Cl2 (100%). Further purification with silica gel chromatography eluting with CH2Cl2:ethyl acetate 90:10 gave the product as the dark blue solid 5 (178 mg, 70 %), mp 158-159 °C. 1H NMR (400 MHz, DMSO-d6): δ 8.24-8.08 (m, 8H), 7.64 (s, 1H), 7.60-7.38 (m, 7H), 7.14 (d, J = 8.9 Hz, 2H), 7.08 (t, J = 5.6 Hz, 1H, NH), 6.96 (d, J = 8.9 Hz, 1H), 4.11 (t, J = 5.6 Hz, 2H), 3.35 (m, 2H), 1.41 (s, 9H) ppm. (OH not observed). 13C NMR (100 MHz, DMSO-d6): δ 161.4, 160.9, 158.5, 156.0, 155.7, 144.8, 143.9, 142.3, 141.1, 132.4, 132.0, 131.7, 131.5, 129.6, 129.3, 129.1, 128.7, 128.6, 123.5, 121.4, 120.1, 119.1, 116.0, 114.8, 77.8, 66.7, 39.4, 28.2 ppm. Ammonium carbonate (CH3COONH4) 688 nm, λmax em (CHCl3): 710 nm. MS ESI [M-H-]: 671.26, C25H34N4O2BF2 requires 671.51. HRMS (MALDI-TOF) for C25H34N4O2BF2 calc.: 727.2719, found: 727.2747.

Synthesis of fluorophore 6. A solution of compound 5 (100 mg, 0.15 mmol) in dry CH2Cl2 (18 ml) was cooled to 0 °C and treated dropwise with TFA (2 ml). Reaction mixture was warmed to rt and stirred under N2 for 2 h. Solvent was removed under vacuo and the residual TFA was removed azeotropically with serial additions of CH2Cl2 and subsequent removal under vacuo. The solid was suspended in CH2Cl2 (10 ml), filtered and washed with CH2Cl2 (50 ml) to yield the product 6 as a dark purple solid (76 mg, 74%), mp 239-240 °C. For NMR analysis conversion to free amine could be achieved by dissolving in AcOEt and washed by NaHCO3, H2O and brine sequentially. The solvent was dried and the solid was passed through silica chromatography by using DCM:MeOH (10:1) to give free amine format fluorophore. 1H NMR (400 MHz, DMSO-d6): δ 8.54 (br.s, 2H, NH2), 8.25-8.10 (m, 8H), 7.68 (s, 1H), 7.56-7.40 (m, 7H), 7.18 (d, J = 8.9 Hz, 2H), 7.03 (d, J = 8.9 Hz, 2H), 4.36 (t, J = 5.0 Hz, 2H), 3.23 (t, J = 5.0 Hz, 2H) ppm. (OH not observed). 13C NMR (100 MHz, DMSO-d6): δ 161.9, 160.1, 159.1, 155.4, 145.1, 143.7, 142.6, 140.8, 132.5, 132.1, 131.7, 131.4, 129.6, 129.2, 129.1, 129.0, 128.7, 128.6, 121.4, 121.1, 120.4, 118.7, 116.1, 115.0, 64.5, 38.1 ppm. λmax abs (CHC13): 684 nm, λmax em (CHCl3): 710 nm. MS ESI [M-H-]: 571.21, C32H32N5O2BF2 requires 571.41. HRMS (MALDI-TOF) for C32H32N5O2BF2 calc.: 727.2195, found: 727.2181.

Synthesis of fluorophore 8. A solution of 6 (70 mg, 0.12 mmol) in dry DMSO (8 ml) was treated with ((1R,8S,9s)-bicyclo[6.1.0]non-4-yn-9-yl)methyl (4-nitrophenoxy)carbonyl 713a (58 mg, 0.18 mmol) and TEA (0.02 mL, 0.12 mmol), and stirred at rt under N2 for 2 h. The mixture was partitioned between EtOAc (30 ml) and 0.1 M HCl solution (30 ml), the organic phase was washed twice with the H2O (2 × 30 ml) and brine (30 ml), dried over anhydrous Na2SO4, filtered and evaporated to dryness. Purification by column chromatography, packed and loaded with CH2Cl2:ethyl acetate 70:30 gave the product as a red metallic solid 8 (60 mg, 65%), mp 150-151 °C. 1H NMR (400 MHz, CDCl3): δ 8.07-7.99 (m, 8H, 7.47-7.36 (m, 6H), 7.04 (s, 1H), 6.99-6.92 (m, 5H), 5.23 (t, J = 5.9 Hz, 1H, NH), 4.19 (d, J = 8.1 Hz, 2H), 4.08 (t, J = 5.0 Hz, 2H), 3.63-3.56 (m, 6H), 2.33-2.15 (m, 6H), 1.64-1.52(m, 2H), 1.41-1.32 (m, 1H), 0.94 (t, J = 9.7 Hz, 2H) ppm. 13C NMR (100 MHz, CDCl3): δ 160.7, 158.8, 158.6, 157.7, 157.0, 145.5, 145.2, 143.5, 143.1, 133.5, 132.4, 131.9, 131.6, 129.4, 129.3, 129.2, 128.9, 128.5, 124.7, 124.0, 118.8, 118.5, 115.9, 114.6, 98.9, 67.1, 63.3, 40.5, 29.1, 21.4, 20.2, 17.7 ppm. λmax abs (CHCl3): 685 nm, λmax em (CHCl3): 710 nm. MS ESI [M-H-]: 747.28, C32H32BF2N4O4 requires 747.29. HRMS (MALDI-TOF) for C32H32BF2N4O4 calc.: 748.3032, found: 748.3022.

Synthesis of fluorophore 4. A solution of 8 (30 mg, 0.04 mmol) in dry THF (10 ml) was treated with 1,3-propane sulfone (10 mg, 0.08 mmol) and cesium carbonate (26 mg, 0.08 mmol) and the mixture stirred at reflux for 1 h. The reaction mixture was cooled to rt, the precipitate filtered and washed with dry DCM (3 × 25 ml). Precipitate was purified by Sephadex G-25 desalting column eluting with CH3CN:H2O (1:9) to give the product as a dark green solid 4 (26 mg, 75%), mp 215-216 °C. For NMR and HRMS analysis, 4 was transformed into its
sulfonic acid by dissolving in 0.5 M HCl / CH3CN (2mL, v/v=1:1), and passing through a Sephadex G-25 column with product fractions freeze-dried. 1H NMR (400 MHz, MeOD-d4): δ: 8.14-8.00 (m, 8H), 7.44-7.34 (m, 6H), 7.18 (d, J = 10.5 Hz, 2H), 7.10-6.89 (m, 4H), 4.66 (brs, 1H, NH), 4.15 (t, J = 6.8 Hz, 2H), 4.07 (t, J = 5.4 Hz, 2H), 3.97 (d, J = 6.8 Hz, 2H), 3.49 (t, J = 5.4 Hz, 2H), 3.05-2.94 (m, 2H), 2.44-2.14 (m, 6H), 2.11-1.94 (m, 2H), 1.42-1.22 (m, 3H), 0.81-0.61 (m, 3H). 13C NMR (100 MHz, MeOD-d4): δ: 164.7, 164.4, 161.3, 161.1, 160.7, 158.8, 148.7, 148.2, 147.9, 146.7, 146.2, 146.1, 145.9, 145.6, 135.4, 134.7, 132.1, 132.0, 131.3, 127.2, 126.9, 121.7, 121.5, 117.4, 101.2, 81.3, 81.0, 80.7, 71.9, 69.8, 69.7, 43.0, 36.1, 28.0, 26.8, 25.9, 23.6. λmax (MeOH): 685 nm, λmax (EtOH): 711 nm. MS ESI [M-H]: 869.43, C49H42BF4N2O4S requires 869.75. HRMS (MALDI-TOF) for C49H42BF4N2O4S, calcd.: 869.2992, found: 869.2975.

Preparation of Azido-L-homoalanine conjugate 9. N-Boc-4-azido-L-homoalanine (1.54 mg, 6.3 µmol) was dissolved in PBS (2 mL, pH 7.2), purged with N2 for 30 min in a 20 mL glass vial equipped with a magnetic stir bar. A solution of 4 (5 mg, 5.75 µmol) in N2 purged PBS (2.0 mL) was added dropwise, and the reaction stirred at 37 °C for 2 h. The reaction mixture was purified through a Sephadex G-25 and then a Sep Pak C18 reverse-phase cartridge. The pure fractions were combined and water was removed by freeze drying to give conjugate 9 (6 mg, 95%). MS ESI (M-H): 1114.80, C55H62BF3N2O4S requires 1114.00. HRMS (MALDI-TOF) for C55H62BF3N2O4S, calcd.: 1113.4163, found: 1113.4164.

Preparation of cyclic RGD peptide conjugate 10. cRGD peptide cyclo[Arg-Gly-Asp-d-Phe-Lys(Azide)] (2.39 mg, 3.79 µmol) was dissolved in N2 purged PBS (1.2 mL, pH 7.2) in a 5 mL glass vial. A solution of 4 (3 mg, 3.45 µmol) in PBS (1.2 mL, pH 7.2) was added dropwise and the reaction stirred at 37 °C for 2 h. The reaction mixture was purified by RP preparative HPLC (CH3CN:H2O=70:30). The pure fractions were combined and water was removed by freeze drying to give conjugate 10 (3.1 mg, 60%). MS MALDI-QTOF (M+Na)+: 1522.6064, C57H64BF4N2O4SNa requires 1522.6002. HRMS (MALDI-TOF) for C57H64BF4N2O4SNa, calcd.: 1498.6026, found 1498.6026.

Cell Culture

HeLa-Kyoto cells were cultured in Dulbecco’s modified Eagle’s medium (1000mg/L-glucose, 110mg/mL sodium pyruvate supplemented with 10% foetal bovine serum, 1% v/v L-glutamine and 1% v/v penicillin-streptomycin). Cells were imaged on an inverted Olympus IX73 widefield fluorescence microscope maintained at 37 °C with a 60X, N.A. 1.42 oil immersion objective. Z-scans were acquired with a z-step 0.5µm over 12µm. A z-scan was acquired every 30 sec. Exposure = 100ms, obj. = 60x, NA = 1.42, filter = 703/73nm BP.

In vivo fluorescence imaging studies. All in vivo experiments were conducted in Fudan University, Shanghai, China in compliance with relevant laws, and their institutional guidelines for animal protocols and the guidelines issued by the Ethical Committee of Fudan University which approved the experiments. Mouse model were established by subcutaneous injection of ~5×10⁶ human esophageal cancer (Eca-109) cells in 200 µL PBS into the right forelimb of five-week-old male Balb C nu/nu mice. When implanted tumor sizes were more than 10 mm in diameter, tumors were excised and small pieces of the tumor (approximately 2 mm square pieces) were implanted subcutaneously into the right forelimb of Balb C nu/nu mice (5 weeks old). When the tumors reached a size of 5-10 mm in diameter after implantation (7-10 days), the mice were used for tumor targeting studies. This method was found to more effective at successfully growing tumor than injection of Eca-109 cells.

Tumor targeting with cRGD Conjugate 10. For tumor targeting studies, conjugate 10 (2 mg/kg, dissolved in PBS) was intravenous tail injected in nude mice bearing Eca-109 tumors. The fluorescence images of the whole mice were obtained using the in vivo Imaging System (IVIS Spectrum CT, PerkinElmer) with an excitation and emission filters 640(30) and 700(20) nm at 0, 20, 30, 40, 50 min, and 1, 2, 4, 6, 8, 10, 12, 24 48 h after introduction.

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