The Development of a Highly Photostable and Chemically Stable Zwitterionic Near-Infrared Dye for Imaging Applications

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**Experimental Procedures**

**Material and Method**

All reactions were performed in oven-dried glassware under a positive pressure of nitrogen. Unless otherwise noted, starting materials and solvents were purchased from Aldrich and Acros organics and used without further purification. c(RGDyK) was purchased from ChinaPeptides Co., Ltd. NMR spectra were recorded on a Bruker AMX500 (500 MHz) NMR spectrometer. Chemical shifts are reported as δ in units of parts per million (ppm) and coupling constants are reported as a $J$ value in Hertz (Hz). Mass of all the compounds was determined by LC-MS of Agilent Technologies with an electrospray ionization source.

Spectroscopic and quantum yield data were measured on a SpectraMax M2 spectrophotometer (Molecular Devices). Data analysis was performed using Graph Prism 5.0.

**Quantum Yield Measurements**

Quantum yields for all the fluorescent compounds were measured by dividing the integrated emission area of their fluorescent spectrum against the area of ICG in DMSO excited at 730 nm ($\Phi_{ICG} = 0.13$). Quantum yields were then calculated using equation (1), where $F$ represents the integrated emission area of fluorescent spectrum, $\eta$ represents the refractive index of the solvent, and $Abs$ represents absorbance at excitation wavelength selected for standards and samples. Emission was integrated from 750 nm to 850 nm.

$$\Phi_{\text{sample}} = \Phi_{\text{reference}} \left( \frac{F_{\text{sample}}}{F_{\text{reference}}} \right) \left( \frac{\eta_{\text{sample}}}{\eta_{\text{reference}}} \right) \left( \frac{Abs_{\text{reference}}}{Abs_{\text{sample}}} \right)$$

(1)

**Photostability Experiment**

Strong UV Lamp Procedure: 1 nmol compounds in 96-well black plate was added 1 µL DMSO and followed by 99 µL PBS buffer (pH 7.4). The final concentrations of compounds were 10 µM. Fluorescence intensity measurements were carried out in each 10 min interval for a total period of 1 h ($\lambda_{\text{ex}} = 760$ nm, $\lambda_{\text{em}} = 800$ nm). UV lamp: UVP Blak-Ray1B-100AP high intensity mercury lamp, 100 W, 365 nm).
Cell Culture and Imaging Experiments

U87MG and MCF7 cells were cultured in high-glucose (4500 mg/L) containing-Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. 24-36 h prior to imaging, cells were seeded in clear bottom, 96-well plate. ZWCC compounds were added to cultured cells to reach final concentration of 5 µM and incubated for 4-6 h at 37 ºC. Cells were washed with PBS buffer twice before imaging. To confirm that ZWCC-linker-cRGD binds specifically to integrin αvβ3, U87MG cells were incubated with 10 μM RGD before ZWCC-linker-cRGD were added. Live cells images were acquired on an inverted Ti-E microscope (Nikon Instruments Inc), equipped with a customised Ex 750 nm/ Em 800 nm filter for NIR fluorescence acquisition, as well as DAPI for Hoechst33342 fluorescence acquisition. Images were analysed using NIS Elements 3.10 software.

In Vivo Bio-distribution of ZWCC dyes in BALB\c nude mice

BALB/c nude mice (18 - 20 g) were obtained from the Biological Resource Centre (Biomedical Sciences Institutes) and anesthetized by intraperitoneal injection of ketamine (150 mg/kg)/xylazine (10 mg/kg) at 8 weeks of age. The mice were placed under the microscope before the survival surgery. An anesthetic gas (Isofluorane) was maintained during bio-distribution study for 1 h. The ZWCC (500 µM x 100 µL in saline containing 1% of PEG, 0.1% of tween20, 10 µL/g, and 1% DMSO) were injected intravenously (i.v.) after capturing the first images as a control. Time dependent fluorescence images were acquired with a Leica M205 FA Fluorescent Stereo Microscope using a 3.95X objective lens with a NIR camera, measured and processed with NIS-Elements 3.10.

Chemical Synthesis

Synthesis of ZWCC: Precursor chloro dye was synthesized as reported.² Precursor chloro dye (60 mg, 0.074 mmol) and 4-carboxyphenylboric acid (24.5 mg, 0.147 mmol) in H₂O were reacted in microwave reactor in the presence of a catalytic amount of Pd(PPh₃)₄ and K₂CO₃ for 1 h. After reaction, the mixture was washed with anhydrous ethyl acetate, and the water phase was separated and dried under reduced pressure. The crude product was further purified by HPLC (yield: 45%).
$^1$H NMR (500 MHz, CH$_3$CN+D$_2$O): $\delta$ 8.89 (s, 1H), 8.69 (d, $J$ = 7.8 Hz, 2H), 8.22 (d, $J$ = 8.3 Hz, 2H), 8.19 (s, 1H), 7.65 (s, 1H), 7.62 (d, $J$ = 7.3 Hz, 2H), 7.51 (d, $J$ = 8.3 Hz, 2H), 6.65 (d, $J$ = 13.9 Hz, 2H), 6.01 (s, 1H), 4.35 (t, $J$ = 6.8 Hz, 4H), 3.97 – 3.87 (m, 4H), 3.58 (s 18H), 3.26 (m, 4H), 2.67 – 2.42 (m, 6H), 1.54 (s, 12H).

$^{13}$C NMR (126 MHz, CH$_3$CN+D$_2$O): $\delta$ 172.79, 171.29, 163.43, 149.42, 143.47, 142.24, 141.37, 141.10, 135.08, 134.40, 133.72, 130.32, 129.53, 129.09, 127.43, 120.26, 110.91, 101.26, 63.45, 53.53, 48.91, 40.94, 27.38, 24.81, 21.22.

HRMS $m/z$ (C$_{49}$H$_{62}$N$_4$O$_8$S$_2$) calculated: 898.4009, found: (M+Na)$^+$ 921.3874.
Scheme S1. General synthetic scheme of ZWCC-linker-cRGD.

ZWCC \xrightarrow{EDC, DMSO} \text{ZWCC-NHS} \xrightarrow{DMSO, TEA} \text{ZWCC-C6-COOH}

ZWCC-NHS \xrightarrow{cRGD, DMSO} \text{ZWCC-C6-cRGD}

ZWCC \xrightarrow{EDC, DMSO} \text{ZWCC-NHS} \xrightarrow{DMSO, TEA} \text{ZWCC-PEG4-COOH}

ZWCC-PEG4-NHS \xrightarrow{cRGD, DMSO} \text{ZWCC-PEG4-cRGD}
Synthesis of **ZWCC-NHS, ZWCC-C6-NHS and ZWCC-PEG4-NHS**: Each of relevant acid (1 equiv) was mixed with N-Hydroxysuccinimide (3 equiv), EDC (3 equiv) in DMSO. The mixture was stirred at room temperature and the reaction was monitored using LCMS. After reaction, collect the solid after adding enough anhydrous ethyl acetate to the reaction mixture. The product was used for the next step without further purification. All compounds were confirmed by HPLC-MS.

Synthesis of **ZWCC-C6-COOH** and **ZWCC-PEG4-COOH**: ZWCC-NHS (1 equiv) was mixed with each of linker (2 equiv), TEA (2 equiv) in DMSO. The mixture was stirred at room temperature for 2 h. After reaction, collect the solid after adding enough anhydrous ethyl acetate to the reaction mixture. The crude product was purified by HPLC to afford the corresponding compounds as green solid. All compounds were confirmed by HPLC-MS.

Synthesis of **ZWCC-C6-cRGD** and **ZWCC-PEG4-cRGD**: Each of NHS ester (1 equiv) was mixed with cRGD (2 equiv), TEA (2 equiv) in DMSO. The mixture was stirred at room temperature for 2 h. After reaction, collect the solid after adding enough anhydrous ethyl acetate to the reaction mixture. The crude product was purified by HPLC to afford the corresponding compounds as green solid. All compounds were confirmed by HPLC-MS.
Figure S1. Absorption and emission spectra of ICG (a and b) and ZWCC (c and d) in different solvent systems at a concentration of 5 µM (λ<sub>ex</sub> = 730 nm).
Table S1. Photophysical data of ICG and ZWCC in various aqueous buffers and organic solvents.

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Figure S2. Concentration-weighted absorption spectra of ZWCC measured at different dye concentrations between 0.5 – 40 µM in H₂O.
Figure S3. Normalized Excitation and emission spectra of ZWCC (5 µM) in 4 different solvent system ($\lambda_{ex} = 730$ nm) (a) DMSO, (b) PBS, (c) H$_2$O, (d) MeOH.
Figure S4. Chemical stability of ZWCC under pH 5 (a) and pH 10 (b), UV detection: 700 nm.
Figure S5. Chemical reaction activity of ZW800-1 and ZWCC with primary amine. Reaction condition: DMF:DMSO=4:1, rt, 4 h.
(a) LCMS data for reaction of ZWCC and tert-butyl 2-aminoethylcarbamate.

(b) LCMS data for reaction of ZW800-1 and tert-butyl 2-aminoethylcarbamate.

**Figure S6.** Reverse-phase HPLC monitoring of reaction after 4 h. (UV detection: 650 nm, MS detection: ESI-MS).
Figure S7. Biodistribution and clearance of ZWCC in BALB/c nude mouse in vivo. Bright field and fluorescence images (NIR camera) were captured after and before the injection of ZWCC dye through tail vain. a) & e) before the injection of dyes. b) & f) 1 min after the injection c) & g) after 30 min and d) & h) after 60 min of post injection respectively. 100 μL of 500 μM ZWCC dye in PBS buffer containing 1% of PEG, 0.1% of tween20, 10 μL/g, and 1% DMSO was injected.
Figure S8. Fluorescence staining of ZWCC-linker-cRGD in integrin αvβ3 positive cells. Lower panels show blocking by free cRGD before staining by ZWCC-linker-cRGD. Red colour is from ZWCC compounds (middle panels) and blue colour is from Hoechst stain (left panels) for nuclei visualization. Merged images of the two are shown in the right panels. Images were captured on a 20x objective. Scale bar is 100 μm.
HRMS m/z (C_{82}H_{114}N_{14}O_{16}S_{2}^{2+}) calculated: 807.3984, found: 807.3954.
HRMS m/z (C_{87}H_{124}N_{14}O_{20}S_{2}) calculated: 874.4274, found: 874.4243.
References
