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Construction of ratiometric fluorescent probe based on inverse electron-demand Diels–Alder reaction for pH measurement in living cells

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ABSTRACT

To endow fluorescent functions to the inverse electron demand Diels–Alder (IEDDA) reaction products, introduction of extraneous fluorophores is usually inevitable. However, this strategy is blamed for complex construction and background fluorescence. It is desirable to construct IEDDA fluorescent products in a more convenient manner. In this work, we reported the *in-situ* generated green fluorescence in IEDDA reaction. The fluorescence intensity of IEDDA products RA-TZ-1 and RA-TZ-2 decreased in reduced pH values (from pH 7.0–3.0). Based on this novel property, we developed a ratiometric fluorescent probe RB–RA-TZ-2 by conjugation with another probe RB-NH_2 (Em = 584 nm) with inverse response toward pH values. By single excitation, the fluorescence intensity ratio (I_{584}/I_{488}) showed a linear response (R = 0.9797) to H^+ in pH range of 3.5–5.0. Colocalization imaging with LysoTracker Green indicated the capacity of RB–RA-TZ-2 to detect lysosomal pH (Pearson's coefficient 0.81). Confocal microscopy was applied to measure and image intracellular pH in living cells.

1. Introduction

Bioorthogonal techniques such as copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC), [1,2] strain-promoted alkyne-azide cycloadditions (SPAAC) [3], photoclick reactions [4] and inverse electron-demand Diels–Alder reaction (IEDDA) [5,6] have revolutionized conjugation strategies in chemical biology. Among these techniques, IEDDA is highlighted for its excellent specificity, fast reaction rate, catalyst free and biocompatibility features [7], which has been applied in applications such as genetic code expansion [8,9], protein modification [10,11],nuclear medicine [12], and fluorescent imaging [13,14].

Usually, neither the IEDDA bioorthogonal pairs (dienophile and diene) nor the IEDDA products are fluorescent. To endow fluorescent function to IEDDA products, introduction of extraneous fluorophores is inevitable [15,16]. One classical fluorescent labeling strategy begins with decoration of a biochemical reporter with a dienophile group, and decoration of an extraneous fluorophore with 1,2,4,5-tetrazine diene group [17,18]. By means of IEDDA reaction, the biochemical reporter group successfully conjugates with the fluorophore, followed by the recovery of fluorescence results from the breakdown of 1,2,4,5-tetrazine structure. This strategy is ingenious, however has deficiencies such as complex construction and background fluorescence. Simple fluorescent conjugation strategy without extraneous fluorophores is ideal but challenging.

Recently, several strategies are reported to meet this demand. Shang's group endowed proteins with green fluorescence through IEDDA reaction between non-fluorescent styrene and 1,2,4,5-tetrazines [19]. Vazquez's group reported that by combination of trans-cyclooctenes (TCOs) with 1,2,4,5-tetrazines, IEDDA products with various fluorescence were developed [20].

In this study, we began with the confirmation of *in situ* fluorescence from the IEDDA reaction by simply mixing Reppe anhydride with 1,2,4,5-tetrazines (Scheme 1a). This "click-fluorescence" conjugation strategy showed a promising application prospect in construction of fluorescent functional products (Scheme 1b).

Moreover, IEDDA products RA-TZ-1 and RA-TZ-2 exhibited gradually decreased emission in reduced pH solutions (from pH 7.0–3.0). On the other hand, rhodamine amide derivatives were widely applied in pH sensing studies for their sensitivity to acid environment [21–23]. Importantly, it responded to reduced pH solutions (from pH 7.0–3.0) with a gradually increased fluorescence emission, which just contrasted with that of RA-TZ-1 and RA-TZ-2. It was also well known that probes with single fluorescence emission suffered low measurement accuracy

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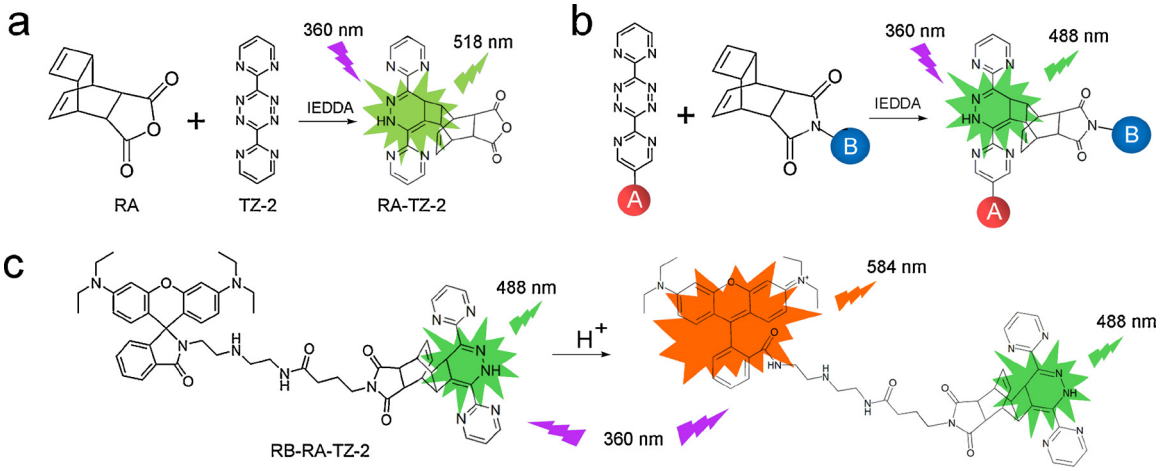

Scheme 1. (a) *in situ* fluorescence from the IEDDA reaction between Reppe anhydride and 1,2,4,5-tetrazines; (b) Application prospect of "click-fluorescence" conjugation strategy; (c) pH sensing mechanism of probe R B-R A-TZ-2.

because of instrumental or operating error. However, this defect could be improved by ratiometric fluorescence strategy, whose measurement accuracy could be largely improved by means of self-calibration of two different emission bands in one individual probe [24–27]. Therefore to demonstrate the potentials of this novel "click-fluorescence" conjugation strategy in a universal manner based on the IEDDA fluorescence and rhodamine fluorescence, the construction of a concise ratiometric fluorescent pH probe was carried out.

Herein, a reported rhodamine based probe RB-NH_2 [28] was transformed into probe R B-R A with the ability to trigger IEDDA reaction with 1,2,4,5-tetrazines. By the IEDDA reaction between R B-R A and tetrazine TZ-2, a ratiometric fluorescent pH probe R B-R A-TZ-2 was developed. The special pH sensing mechanism of R B-R A-TZ-2 derived from the fluorescence intensity ratio between orange fluorescence (584 nm) and green fluorescence (488 nm) from rhodamine part and RA-TZ part respectively (Scheme 1c). By single excitation at 360 nm, the fluorescent intensity ratio (I_{584}/I_{488}) changed correspondingly in pH values. In addition, confocal microscopy was conducted to measure and image intracellular pH in living RAW 264.7 cells.

2. Experimental section

2.1. Materials and instruments

All commercial chemicals were purchased from commercial suppliers and used without further purification. Twice distilled water was used throughout all experiments. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded by Bruker AV 400 MHz spectrometers. High-resolution mass spectra (HRMS) was recorded by the Waters Xevo G2-XS Q-Tof matrix-assisted time of flight mass spectrometer using high performance liquid chromatography (HPLC) and electro-spray ionization (ESI) technique. UV–vis absorption spectra were recorded by SHIMADZU UV-1800 spectrophotometer. Fluorescence spectra was recorded by HORIBA Fluorolog-3 modular spectrometer with a Xe lamp as excitation source. Absolute fluorescence quantum yield measurement was conducted by integrating sphere on HORIBA Fluorolog-3 modular spectrofluorometer [29]. Fluorescence lifetime was detected by DeltaTime TCSPC on HORIBA Fluorolog@-3 spectrometer equipped with a HORIBA NanoLED source (N-455 nm). Carl Zeiss LSM710 confocal microscope was applied for living cells fluorescent imaging.

Synthesis of tetrazine TZ-1, Reppe anhydride (RA) and RA-NHS were according to published procedures [30–32].

2.2. Synthesis of tetrazine TZ-2

2-pyrimidinecarbonitrile (1.05 g, 10 mmol, 1.0 eq) was suspended in dry EtOH (20 mL). With the dropwise addition of hydrazine hydrate (1.56 mL, 25 mmol, 2.5 eq), the orange mixture was stirred at 85 °C with reflux under nitrogen protection for 16 h. The orange precipitate was collected by vacuum filtration and washed by EtOH. The intermediate product was suspended in acetic acid (10 mL). In fume hood, isopentyl nitrite (1 mL, 7.15 mmol) was added dropwise with stirring. The orange mixture turned purple with release of toxic gas nitrogen dioxide. After 8 h oxidation, the purple precipitate was collected and washed by EtOH. Crude purple product went for a flash chromatography in silica gel column (DCM, followed by DCM/MeOH 10:1). The purple filtrate was collected and dried to obtain TZ-2 as purple solid. (700 mg, 2.94 mmol, 70%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.23 (d, J = 4.9 Hz, 4 H), 7.87 (t, J = 4.9 Hz, 2 H). ^{13}C NMR (101 MHz, DMSO-d_6): 163.45, 159.41, 159.07, 123.67.

2.3. Synthesis of RA-TZ-1

To a solution of RA (22.2 mg, 0.11 mmol, 1.1 eq) in 30 mL DMSO, TZ-1 (28.0 mg, 0.1 mmol, 1.0 eq) was added. During the 18 h stirring at room temperature, the solution turned yellow from purple. Green fluorescence was witnessed using hand-held 365 nm UV lamp. Fresh DD water was added followed by DCM extraction to remove DMSO, the yellow crude product in DCM was dried by MgSO4. Column chromatography in silica gel column (DCM/MeOH 10:1) was conducted to afford the IEDDA product RA-TZ-1 as light-yellow solid (31.8 mg, 0.07 mmol, 70%). ^{1}H NMR (400 MHz, DMSO-d6) δ 9.12 (d, J = 4.9 Hz, 2 H), 8.33 (d, J = 8.2 Hz, 2 H), 8.19 (d, J = 8.0 Hz, 2 H), 7.66 (t, J = 5.1 Hz, 1 H), 5.89 (q, J = 6.9, 6.2 Hz, 2 H), 4.35 (t, J = 3.9 Hz, 1 H), 4.20 (t, J = 4.1 Hz, 1 H), 3.84 (d, J = 4.9 Hz, 1 H), 3.71 (s, 1 H), 3.68 – 3.60 (m, 2 H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 173.84, 173.67, 167.39, 158.77, 149.03, 145.43, 138.19, 132.92, 131.09, 130.74, 130.31, 127.96, 45.98, 45.65, 44.27, 43.76, 35.72, 35.29. ESI-MS calcd for $C_{25}H_{18}N_4O_5$, $[M+H]^+$ 455.1355, found 455.1385.

2.4. Synthesis of RA-TZ-2

To the solution of RA (22.2 mg, 0.11 mmol, 1.1 eq) in 10 mL acetonitrile, TZ-2 (23.8 mg, 0.1 mmol, 1.0 eq) was added. During the 18 h stirring at room temperature, the solution turned yellow from purple. Green fluorescence was witnessed using hand-held 365 nm UV lamp. After removal of the solvent, the crude product was purified by column chromatography in silica gel column (DCM/MeOH 20:1) to afford the