A charge transfer type pH responsive fluorescent probe and its intracellular application†

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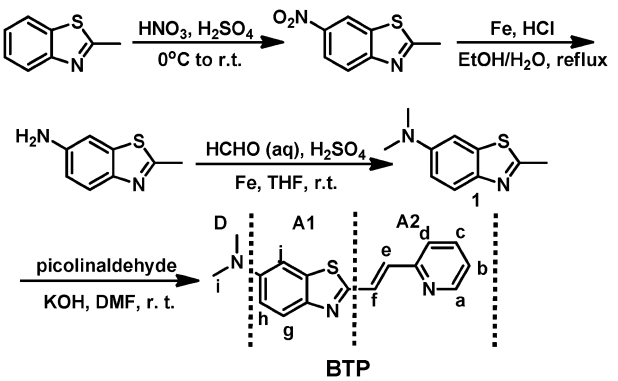
A new charge transfer pH fluorescent probe **BTP** has been prepared by the ethylene bridging of benzothiazole and pyridine. The probe exhibits a specific fluorescent response to pH with a large Stokes shift, and the pH-induced emission enhancement factor (EEF) is about 22-fold when the pH is increased from 3.2 to 5.2. The presence of metal cations such as Na^+, K^+, Ca^{2+}, Mg^{2+} and other transition metal cations does not interfere with its fluorescent pH response. In addition, the intracellular pH fluorescent imaging ability of the probe has been confirmed on macrophage cells using a confocal microscope.

Introduction

Intracellular pH plays a central role in many cellular events, such as cell growth, endocytosis, cell adhesion and other cellular processes.[1–4] Determination of pH by fluorescent methods is attracting much more attention in pH imaging and sensing due to the advantages over other techniques including rapid response time, high signal-to-noise ratio, non-invasiveness, and excellent pH sensitivity.[5,6] In general, there are two types of synthetic pH probes, one type for cytosol that work at a pH of 6.8–7.4,[5,7–12] and another type for the acidic organelles (for example, lysosomes) functioning in the pH range 4.5 to 6.0.[13–16] Although lots of the former type of probe have been reported, little attention has been paid to the latter. In fact, the abnormal pH values in acidic organelles have been reported to be closely associated with some cellular dysfunction. The defective pH regulation of acidic compartments in human breast cancer cells and the elevated lysosome pH in neural ceroid lipofuscinoses are the well known examples.[17] On the other hand, many fluorescent pH probes still suffer from the severe excitation interference caused by a shorter Stokes shift.[14,15] Therefore, the development of specific pH fluorescent probes of large Stokes shift for the acidic pH range is appealing to the fields of chemistry, cytology and pathology.

Benzothiazole derivatives with a D (donor)–π–A (accepter) structure type are frequently adopted as the fluorophore to construct intramolecular charge transfer (ICT) fluorescent probes displaying a large Stokes shift.[18] Rurack and coworkers have reported an ICT sensor for $H_2PO_4^-$ *via* ethylene bridging of the bisamidopyridine receptor and benzothiazole. Moreover, ICT sensors for cations have also been constructed by bridging azocrown ether and benzothiazole. All these sensors display a large Stokes shift (more than 100 nm).[18a,b] In this work, a new fluorescent pH probe for acidic micro-environments, **BTP**, was constructed by ethylene bridging of benzothiazole and pyridine to form a D–π–A fluorophore. Herein, pyridine was introduced as the H^+ binding moiety since the low pK_a value (5.3 at 20 °C) of the pyridine N favors H^+ binding in acidic conditions.[19] In addition, its benzothiazole motif, which has a low affinity for H^+ and metal cations, is of benefit to the specific pH response of probe.[8] The ethylene bridge between benzothiazole and pyridine makes **BTP** an D–π–A type fluorophore with the dimethylamino group and benzothiazole/pyridine acting respectively as D and A, favoring a large Stokes shift and reducing the excitation interference (Scheme 1).



Scheme 1 Synthesis of **BTP**.

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Results and discussion

Synthesis of BTP

BTP was prepared simply in a good yield (78%) by stirring the mixture of picolinaldehyde and 6-(*N*,*N*-dimethyl)amino-2-methylbenzothiazole (**1**) in the presence of KOH at room temperature for 24 h. Compound **1** was synthesized from 2-methylbenzothiazole following a reported procedure undergoing nitration, reduction and methylation in sequence.[20]

Spectroscopic properties and optical responses to pH

The fluorescence and UV–vis pH titration of **BTP** were performed in aqueous media (containing 1% methanol) over a pH range from 3 to 12. Free **BTP** in aqueous solution (pH = 6.02) exhibits an intense emission band centered at 596 nm (λ_{ex} = 397 nm) with a large Stokes shift of 199 nm in neutral conditions (Fig. S7 of the ESI†). Its quantum yield in neutral conditions was determined as 0.22 with quinine sulfate in 0.5 M H_2SO_4 as per ref. 21. The large Stokes shift should help to reduce the excitation interference. In fact, **BTP** displays stable emission when the pH value is higher than 5.2 (Fig. S8 and S9 of the ESI†). However, **BTP** shows very weak fluorescence when the pH value is lower than 3.1. Distinct emission enhancement can be observed without emission shift when the pH is raised from 3.2 to 5.2, and the emission enhancement factor is around 22 [Fig. 1(a)]. Fig. 1(b) illustrates the fluorescent pH titration profile according to the emission intensity at 597 nm. Fitting the profile with the Henderson–Hasselbach equation gives a pK_a of 4.22 for **BTP**.[22] This pK_a value almost matches the typical pH values of acidic organelles such as lysosomes, suggesting **BTP** might be suitable for detecting the altered pH of lysosomes.

The addition of transition metal cations such as Hg^{2+}, Cd^{2+}, Pd^{2+}, Cu^{2+}, Ni^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+} and Ag^{+} to **BTP** solution does not lead to any notable emission change (Fig. 2). The results confirm the specific fluorescent response of **BTP** to H^{+}. In addition, the presence of Na^{+}, K^{+}, Ca^{2+} and Mg^{2+}, which are abundant in cells, at a concentration of 1000 times as that of other metal cations does not interfere with the pH sensing behaviour of **BTP** in acidic environments. All these results suggest that **BTP** might be a suitable candidate as a staining agent for fluorescent intracelluar pH imaging.

^{1}H NMR spectra of **BTP** at different pH values have been determined in CD_3OD/D_2O (10 : 1, v/v) (Figs. 3 and 4). The results disclose that the chemical shifts of **BTP** protons remain almost constant when the pH is higher than 4.35 (pD values have been converted into pH values).[23] When decreasing the pH from 4.35 to 3.72, one can see the clear down-field shift for the signals of the pyridine protons H_d, H_b and H_c. The H_d signal shifts from 7.36 to 7.38, H_b from 7.67 to 7.70 and H_c from 7.86 to 7.89. These results suggest that the protonation of pyridine N (**N2**) may occur in this process, although the down-field shift of H_a is very minor. The constant chemical shift of the dimethylamino group protons (H_i) in this process implies that the amino N (**N1**) is still not protonated even at pH 3.72. The distinct down-shift of all **BTP** protons can only be observed when the pH becomes lower than 2.99. The ^{1}H NMR titration implies that the emisson enhancement of

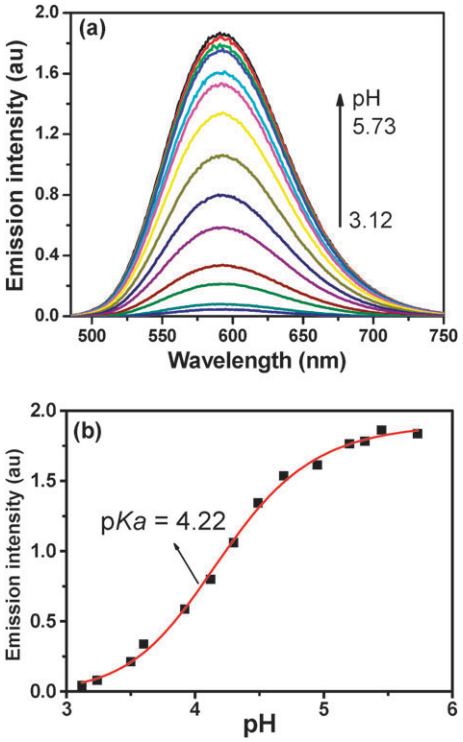

Fig. 1 (a) Emission spectra of **BTP** (5×10^{-6} M) in aqueous solutions (MeOH–water = 1 : 99, v/v) at different pH, (b) emission intensity at 597 nm *versus* pH in acidic conditions according to the fluorescent pH titration. λ_{ex} = 397 nm.

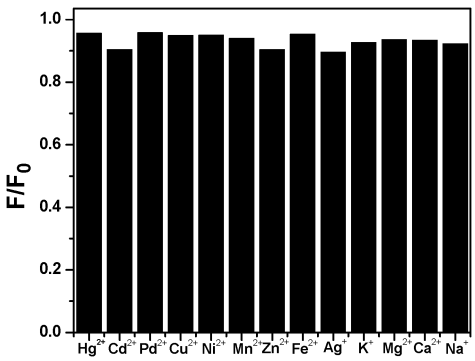

Fig. 2 Emission change at 597 nm of **BTP** (5×10^{-6} M) in aqueous solution induced by different metal cations (1 : 99, MeOH–water, v/v; 50 mM HEPES, 100 mM KNO_3; pH 7.40), λ_{ex} = 397 nm. The final concentration of Hg^{2+}, Cd^{2+}, Pb^{2+}, Cu^{2+}, Ni^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+}, Hg^{2+} and Ag^{2+} is 5 μM, respectively, while that of Na^{+}, K^{+}, Ca^{2+} and Mg^{2+} is 5 mM, respectively.

BTP from pH 3.2 to 5.2 should be ascribed to the deprotonation of pyridininum. In fact, the pyridine N protonation-induced emission quenching has also been reported by other scientists.[24] The protonation of the dimethylamino group at pH <3.0 may destroy the ICT effect of the **BTP** molecule, which makes **BTP** become non-fluorescent. The N atom (**N3**) in the benzothiazole ring is difficult to protonate due to its lone electron pair being involved in the aromatic conjugation system.[25,26]

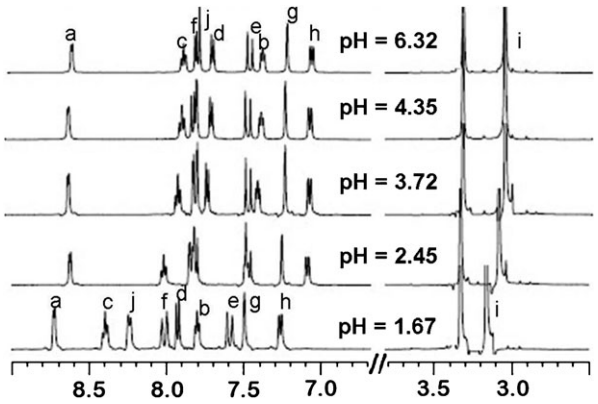

Fig. 3 ^{1}H NMR spectra of **BTP** in CD_3OD/D_2O (10 : 1, v/v) at different pH values.

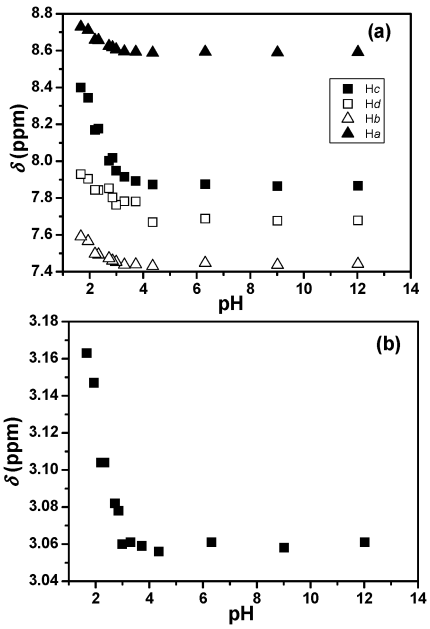

Fig. 4 pH Titration profiles of **BTP** according to the chemical shifts of the pyridine protons (a) and *N*,*N*-dimethylamine protons (b) at different pH values (pH values were converted from pD by pH = pD–0.4).[23]

The UV–vis titration of **BTP** from pH 3.86 to 5.0 exhibits an increase in the π–π* transition band at 390 nm which is accompanied by a decrease in the broad ICT band (at 417 nm) and in a minor band at 341 nm (Fig. 5). The clear isobestic points at 441 nm and 350 nm imply that only one reaction occurred from pH 3.86 to 5.0, which can be ascribed to the conversion of free **BTP** to the protonated [**BTP** + H^+] according to the ^{1}H NMR titration data. On the other hand, the UV spectra of **BTP** exhibit a stable maximal absorption band centered at 390 nm (ε = 2.23 × 10^4 M^{-1} cm^{-1}) at pH 5–12 (Fig. S10 of the ESI†) similar to the stable emission band above pH 5.

Intracellular pH imaging with BTP as the imaging agent

The intracellular pH imaging ability of **BTP** has been investigated on macrophage cells by laser scanning confocal

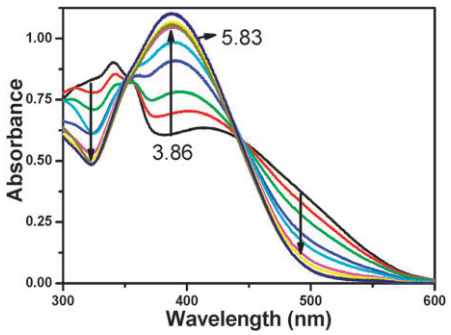

Fig. 5 Absorption spectra of **BTP** (5 × 10^{-6} M) in aqueous solutions (MeOH–water, 1 : 99, v/v) at different pH values. The arrows display the variation of absorbance from pH 3.86 to 5.83.

microscopy. The macrophage cells after 10 min of incubation with **BTP** (10 μM, in PBS) for 10 min at 37 °C show punctuated fluorescence inside the cells especially near the nucleus and cell membrane, displaying the preferential affinity of **BTP** for lysosomes upon excitation at 405 nm (Fig. 6b and Fig. S11). The subsequent single rinse with PBS (pH 7.4) induces only a very minor decrease of fluorescence in the macrophage cells (Fig. 6c), suggesting **BTP** is difficult to exclude from macrophage cells by rinsing. However, the fluorescence in macrophage cells can be distinctly depressed (Fig. 6d) by incubating the cells with ATP (1 mM) for 3 min. The distinct emission decrease is consistent with the fact that the extracellular ATP stimulation normally induces the pH decrease of lysosomes.[27] The fine cell permeability and effective imaging ability for the pH variation around pH 4 indicate that **BTP** is an effective intracellular pH imaging agent.

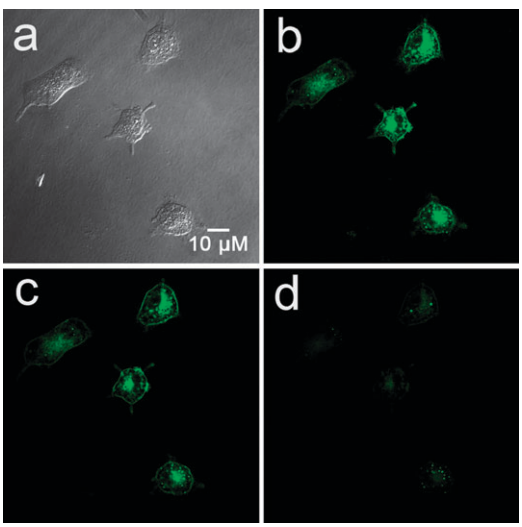

Fig. 6 Confocal fluorescence imaging of macrophage cells. (a) Bright-field transmission image of the cells stained with **BTP** (10 μM, PBS solution) at 37 °C for 10 min; (b) fluorescence image of (a); (c) fluorescence image of cells in (b) after rinsing once with PBS solution for 3 min (200 μL); (d) fluorescence image of macrophage cells in (c) followed by further treatment with 1 mM ATP solution for 3 min. λ_{ex}, = 405 nm; band path for detection = 550–650 nm.

Conclusions

In summary, a D–π–A type fluorescent pH probe **BTP** has been synthesized by linking the 2-pyridine motif to benzothiazole. This probe has a pK_a of 4.22 and a remarkable emission enhancement is observed when increasing the pH from 3.2 to 5.2. The large Stokes shift, fine cell permeability and specific pH fluorescent response make **BTP** an effective intracellular pH imaging agent for acidic microenvironments. The current study demonstrates that the conjugation of the fluorophore with pyridine to form an ICT fluorophore might be an effective strategy to construct new fluorescent pH probes of large Stokes shift for acidic microenvironments.

Experimental sections

Materials and general methods

All reagents were of analytic grade. The stock solutions of metal ions for fluorescence discrimination were prepared respectively by dissolving $MnCl_2$, $PbCl_2$, $FeCl_2$, $Zn(NO_3)_2 \cdot 7H_2O$, $CaCl_2$, NaCl, $CuSO_4$, $NiCl_2 \cdot 6H_2O$, KCl, $CdCl_2 \cdot 2.5H_2O$, $HgCl_2$, $MgCl_2 \cdot 6H_2O$, $AgNO_3$ in doubly distilled water. The ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker DRX-300 spectrometer with TMS as the internal standard in $CDCl_3$ or CD_3OD/D_2O. Mass spectrometric data were determined with a LCQ (ESI-MS, Thermo Finnigan) mass spectrometer. Elemental analyses for C, H and N were performed on a Perkin-Elmer 240 C analyzer. Fluorescence measurements were performed on an AMINCO Bowman series 2 with 3 nm slit for both excitation and emission. The fluorescence quantum yield was determined using quinine sulfate solution (Φ = 0.546 in 0.5 M H_2SO_4) as per ref. 21. Absorption spectra were measured on a Shimadzu UV-3100 or an UV–vis–NIR spectrophotometer. All pH and pD measurements were determined using a Model PHS-3C meter.

Sample preparation for UV–vis and fluorescence determination

A stock solution of **BTP** (0.5 mM) was prepared in MeOH. The **BTP** solution for spectroscopic determination was obtained by diluting the stock solution to 5 μM with deionized water. In the titration experiments, 3 mL of **BTP** solution (5 mM, 0.5 mM) was poured into a quartz optical cell of 1 cm optical path length each time and the slight pH variations of the solution were achieved by adding the minimum volumes of NaOH or HCl. Spectral data were recorded immediately after each addition. To determine the fluorescence response of **BTP** to different metal cations, the stock solution of **BTP** was diluted to 5 μM with HEPES solution (50 mM HEPES, 100 mM KNO_3; pH 7.40).

UV–vis and fluorescence pH titration

The UV titration experiments on **BTP** were carried out by adding the diluted HCl or NaOH aqueous solution to 3 mL of **BTP** solution (5 μM, 1 : 99, MeOH–water v/v) in a cuvette. The spectra and pH values were recorded after the solution was completely mixed. The fluorescence titration experiment was investigated in a similar procedure and the excitation wavelength was 397 nm.

^{1}H NMR titration

The ^{1}H NMR study of the pH titration were carried out on Bruker DRX-500 (500 MHz) by adding DCl or NaOD in D_2O to the solutions of **BTP** (8×10^{-3} M) in CD_3OD: D_2O (10 : 1, v/v) to modulate the different pH values. Chemical shift was referenced to an external sample of TMS (δ = 0.00 ppm). The experiments were carried out at 298 K.

Selective fluorescence response of BTP to different metal cations

Stock solutions of the metal ions (1.2 mM) were prepared in deionized water. The fluorescence response of **BTP** to different metal cations was determined in 5 μM **BTP** buffered solution (1 : 99, MeOH–water, v/v; 50 mM HEPES, 100 mM KNO_3; pH 7.40). Metal cation solution (12.5 μL, 1.2 mM) was added to 3 mL of this solution, and the fluorescence spectra were determined after complete mixing. The excitation wavelength was 397 nm. The final concentration of alkaline or alkaline earth metal cation was 1000 times as high as that of other cations.

Cell culture and intracellualr pH fluorescence imaging

Murine peritoneal macrophages were isolated from 6- to 12-week-old C57BL/6J mice by using a reported protocol.[28] After culturing on coverslips for 2 h, the floating cells were removed by extensive washing, and the attached cells were maintained in RPMI 1640 medium (Invitrogen) containing 10% fetal bovine serum, penicillin (100 units/mL), streptomycin (100 mg mL^{-1}) and 5% CO_2 at 37 °C. After removing the incubation media and rinsing with PBS three times, the cells were stained by incubating the cells in 10 μM **BTP** solution for 10 min at room temperature. Then the cells were imaged with an Olympus FV1000 microscope equipped with a 60 × 1.40 NA oil-immersion objective. After removing the **BTP** media, the cells were treated with PBS for 3 min followed by imaging. Then the PBS solution was removed and the cells were cultured with **BTP** for 10 min followed by rinsing with 1 mM ATP solution for 3 min. Then the cells were imaged again. For all imaging, the samples were excited at 405 nm, and the band path was 550–650 nm.

Synthesis

A mixture of 6-(*N*,*N*-dimethyl)amino-2-methylbenzothiazole (0.70 g, 3.65 mmol), picolinaldehyde (0.50 g, 3.65 mmol) and powdered KOH (0.93 g, 16.6 mmol) in 3 mL DMF was stirred at 25 °C for 24 h. Then, the mixture was partitioned between H_2O (5 mL) and CH_2Cl_2 (10 mL). The aqueous portion was extracted with CH_2Cl_2 (3 × 10 mL). Then, the combined organic extracts were dried over anhydride $MgSO_4$ followed by removing the solvent *via* evaporation *in vacuo*. The crude product was purified on silica gel (EtOAc: CH_2Cl_2 = 1 : 10) to afford **BTP** as red powder. Yield 0.80 g (78%); m.p. 148–149 °C. Elemental analysis, found: C, 68.23; H, 5.48; N, 15.12; calc. for $C_{16}H_{15}N_3S$: C, 68.30; H, 5.37; N, 14.93. ^{1}H NMR ($CDCl_3$, 300 MHz), δ (ppm): 3.06 (s, 6H, BT–N(CH_3)$_2$), 6.94–6.98 (dd, 1 H, BT–H), 7.09 (d, 1H, J = 5.1 Hz BT–H), 7.20–7.24 (m, 1H, Py-H), 7.48–7.51 (d, 1H, J = 7.8 Hz, BT–H), 7.69–7.75 (m, 1 H, Py–H), 7.44–7.49 and 7.79–7.85

(2 × d, 2H, J = 15.2 Hz, –CH=CH–), 7.88 (d, 2 H, J = 9.3 Hz, Py–H), 8.66 (d, 1H, J = 4.8 Hz, Py–H). ^{13}C NMR ($CDCl_3$, 75 MHz), δ (ppm): 39.62, 102.28, 113.52, 122.40, 122.96, 123.31, 125.67, 133.95, 136.62, 137.34, 149.26, 149.69, 153.87, 161.49. ESI-MS (positive mode, m/z): Calcd. 282.11, found: 282.19 for $[M+H]^+$.

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