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A BODIPY-derived fluorescent probe for cellular pH measurements

Fangfei Han [1], Yanmei Xu [1], Dechen Jiang *, Yu Qin *, Hongyuan Chen

State Key Laboratory of Analytical Chemistry for Life Science, School of Chemistry and Chemical Engineering, Nanjing University, Nanjing 210093, China

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ABSTRACT

In this study, BODIPY-appended calix[4]arene was chosen as a fluorescent probe for intracellular pH. The compound with cell permeability can monitor the minor pH change near neutrality inside the cell and is the first BODIPY-derived probe reported for cytosolic pH. Owing to a high level of cell retention and minor cytotoxicity of the probe, stable fluorescence is provided in the cells for 24 h, facilitating the precise observation of intracellular pH. A model of cell apoptosis was designed by exposure of the cells to a low concentration of hydrogen peroxide. An increase in the fluorescence of the cells confirmed that BODIPY-appended calix[4]arene sensed the fluctuation of the cellular pH during early cell apoptosis. The developed fluorescent pH probe will be useful for the study of cell apoptosis.

The maintenance of normal intracellular pH is important for cellular activities such as cell growth and apoptosis, ion transport, and endocytosis [1–5]. The study of the connection between intracellular pH and cellular activities needs the precise measurement of intracellular pH. Different methods have been developed to monitor pH value in the cells, including electrochemistry [6], nuclear magnetic resonance (NMR)[2] [7], absorbance spectroscopy [8], and fluorescence probe analysis [9]. Among all of these methods, fluorescent probe analysis has the advantages of high spatial and temporal resolution on cell observation. In addition, fluorescent techniques have only minor interruptions of the cellular activity that helps the collection of cellular information.

To fulfill the requirement for cell observation, the fluorescent probe needs high selectivity, brightness, water solubility, cell permeability, and cell retention as well as minimal toxicity. Up to now, no fluorescent probe has been developed to perfectly satisfy all of these criteria at once. The most successful fluorescent probe is BCECF [2′,7′-bis-(2-carboxyethyl)-5-(and-6-)carboxyfluorescein] [10] (see Fig. 1), which has been widely applied in cell biology. The commercially available acetoxymethyl ester of BCECF can diffuse into the cell and be hydrolyzed by endogenous cellular esterase into the charged fluorescent dye. The four or five negative charges on the dye at physiological pH result in cell retention of the dye. In addition, BCECF has a pK_a value of 7.0, which permits the probe to sense near neutral cytosolic pHs (6.8–7.4) [11]. The main problem with BCECF is significant leakage from the cells [10]. In addition, BCECF is not ideal for tracking the acidic compartment pH (4.5–5.5). To overcome these problems, HPTS (8-hydroxypyrene-1,3,6-trisulfonic acid) was synthesized with three sulfonate groups [12,13]. The tri- or tetraanionic dye at physiological condition exhibits cell retention, which has been applied to measure cytoplasmic pH or acidic organelle pH in many cell types [14,15]. The limitation of HPTS is a lack of cell permeability. Microinjection, electroporation, and scrape loading are normally used to load HPTS into the cells, which interrupt the cellular activities [16].

BODIPY (4,4-difluoro-4-bora-3*a*,4*a*-diaza-*s*-indacene)-derived probes are the other family of fluorescent dye that has attracted attention since the 1990s [17,18]. BODIPY has several features, including robustness against light, high fluorescence quantum, and fine tunable spectroscopic properties. Some BODIPY-derived compounds have been applied to monitor intracellular pH and have weak fluorescence in nonprotonated form at the cytosol and become highly fluorescent when the functional group is protonated at the acidic compartments [19]. The dependence of fluorescence on pH is explained by different mechanisms such as photo-induced electron transfer (PET) [20], photo-induced intramolecular charge transfer (ICT) [21], and resonance energy transfer (RET) [22]. Although the number of articles on BODIPY-derived probes has increased greatly, to the best of our knowledge, no BODIPY-derived fluorescent probe has been reported to monitor

* Corresponding authors. Fax: +86 25 83594846 (D. Jiang), fax: +86 25 83592562 (Y. Qin).
E-mail addresses: dechenjiang@nju.edu.cn (D. Jiang), qinyu75@nju.edu.cn (Y. Qin).

[1] These authors contributed equally to this work.

[2] *Abbreviations used:* NMR, nuclear magnetic resonance; BCECF, 2′,7′-bis-(2-carboxyethyl)-5-(and-6-)carboxyfluorescein; HPTS, 8-hydroxypyrene-1,3,6-trisulfonic acid; BODIPY, 4,4-difluoro-4-bora-3*a*,4*a*-diaza-*s*-indacene; AM, acetoxymethyl ester; CFDA SE, carboxyfluorescein diacetate, succinimidyl ester; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; CMFDA, 8-chloromethyl-4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3*a*,4*a*-diaza-*s*-indacene.

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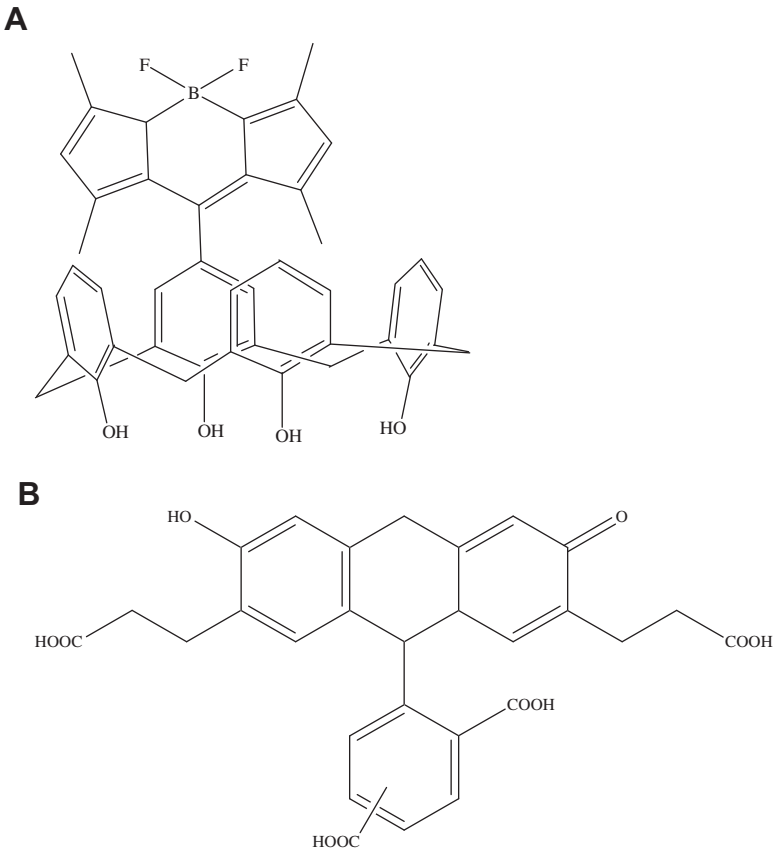

Fig.1. Chemical structures of BODIPY-appended calix[4]arene (A) and BCECF acetoxymethyl ester (B).

near neutral cytosolic pH, which is the most important part for the measurement of intracellular pH.

To apply the BODIPY-derived dye as cytosolic pH probe, a series of candidates were investigated in our group. Because pH sensors with 1:1 binding stoichiometry have a usable quantitative pH response in an approximate pK_a range from −1 to +1, the pK_a value of the candidate should be close to 7.0 [18]. BODIPY-appended calix[4]arene is a fluorescent dye with four phenolic groups synthesized in 2001 (shown in Fig. 1) that has a fluorescent response within a pH range of 11.53 to 2.53 with a pK_a value of 6.5 [23]. Thus, in this study, BODIPY-appended calix[4]arene was chosen to monitor the near neutral cytosolic and acidic pH inside the cell. In addition, cell retention and cytotoxicity of the probe in the cells were investigated. The results suggested that the probe can be used to study early cell apoptosis.

Materials and methods

Materials

2′,7′-Bis-(2-carboxyethyl)-5-(and 6-) carboxyfluorescein, acetoxymethyl ester (BCECF AM), LysoTracker Red DN-99, carboxyfluorescein diacetate, succinimidyl ester (CFDA SE), and Hoechst 33342 were purchased from the Beyotime Institute of Biotechnology (Nantong, China). 4-*tert*-Butylcalix[4]arene was obtained from J&K Scientific (Beijing, China). All other chemicals were obtained from Sigma–Aldrich unless indicated otherwise. HeLa cells were purchased from the Institute of Biochemistry and Cell Biology, Shanghai Institute for Biological Sciences, Chinese Academy of Science (Shanghai, China). Ultrapure water was used throughout. Buffer solutions were sterilized.

Synthesis of BODIPY-appended calix[4]arene

BODIPY-appended calyx[4]arene was synthesized in our group following the procedure reported in the literature [23]. 4-*tert*-Butylcalix[4]arene (13.3 g, 20 mmol), phenol (9.02 g, 96 mmol), and aluminum chloride anhydrous (14 g, 105 mmol) were added to toluene in N_2. The solution was stirred at room temperature for 1 h and added into 0.2 M HCl solution. The organic layer was separated, and the rest of the toluene was removed under reduced pressure. The product (calix[4]arene, 4.5 g, 51%) was recrystallized by $CH_3OH/CHCl_3$. Then, calix[4]arene (1 g, 2.3 mmol) was dissolved in dry chloroform and the solution was cooled to −15 °C. After 1,1-dichlorodimethyl ether (0.35 g, 2.8 mmol) and $SnCl_4$ (3.15 g, 12 mmol) were rapidly added, the reaction mixture was stirred at room temperature for 1 h and quenched with water. The solvent was removed under reduced pressure, and the residue was purified by silica gel column chromatography (petroleum ether/CH_2Cl_2, 8:7), which was confirmed to be 5-formylcalix[4]arene. The yield was 0.18 g (15%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 10.23 (br, 3H, OH), 7.61 (s, 2H, ArH), 7.10 (td, 4H, J = 7.1 Hz, 1.6 Hz, ArH), 7.06 (d, 2H, J = 7.8 Hz, ArH), 6.77 (t, 2H, J = 7.5 Hz, ArH), 6.73 (t, H, J = 7.5 Hz, ArH), 4.29 (br, 4H, $ArCH_2Ar$), 3.60 (br, 4H, $ArCH_2Ar$).

To synthesize BODIPY-appended calix[4]arene, trifluoroacetic acid was added into a solution of 2,4-dimethylpyrrole and 5-formylcalix[4]arene in N_2-saturated dichloromethane. The clear yellow solution was stirred for 3 h at room temperature under N_2, and then a solution of *p*-chloranil in dichloromethane was added. When the reaction mixture turned dark red, the stirring was continued for another 30 min. A bright green fluorescence was observed after Et_3N and $BF_3{\cdot}Et_2O$ were added. After 10 h, the desired product was purified by silica gel column chromatography