Talanta 131 (2015) 666–671

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

A profluorescent ratiometric probe for intracellular pH imaging

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ARTICLE INFO

Article history:
Received 10 May 2014
Received in revised form
11 August 2014
Accepted 15 August 2014
Available online 23 August 2014

Keywords:
Intracellular pH
Iridium(III) complex
Near-infrared
Ratiometric
Fluorescein
Profluorescent

ABSTRACT

A ratiometric pH probe composed of a fluorescein moiety and an ionic near-infrared-emitting phosphorescent cyclometalated iridium(III) complex bis(6-(benzo[b]thien-2-yl)phenanthridinato)(4-(3-carboxypropyl)-4′-methyl-2,2′-bipyridine)iridium(III) was synthesized. With good cell permeability, the probe demonstrated a linear ratiometric response to the pH variation in the physiological range in HeLa cell assay.

1. Introduction

Intracellular pH (pHi) has strong impact on cellular proliferation, endocytosis, modulation, and apoptosis [1–3]. Abnormality of pHi is associated with several diseases such as carcinoma. Hypoxia surrounding rapidly proliferating and metabolic cancer cells accompanies pH change inside and outside cancer cells [4–8]. On the other hand, different compartments and organelles in normal cell varied in pH values [9], for instance, endosomes [10] and lysosomes [11] have an acidic intracompartmental pH value 4–6. Thus the precise measurement of intracellular pH and its spatial–temporal distribution is of great importance to advance our knowledge of cell behavior and function as well as to understand disease mechanism and to develop diagnostic methods. Efforts in designing and developing new pHi probes [12–15] and sensors [16–18] have been made to achieve this goal.

With subtle molecular designs, ratiometric fluorescent probing [19–22] is promising because its signal detection modality avoids unfavorable influence of concentration variation and fluorescence fluctuation in different targets and time range. The ratiometric fluorescent probing is thus receiving increasing attention in acquiring quantitative information of intracellular pH.

Fluorescein derivatives [23] are the most common in vitro and in vivo pH indicators. Their fluorescent intensity changes with the pH value of surrounding environment due to the pH-dependent dissociation equilibria wherein only monoanion and dianion emit fluorescence [24,25]. Fluorescein derivatives have been conjugated with other partnering luminescent substances (small molecules, nanoparticles and fluorescent proteins) to form ratiometric probes with dual emissions. These luminescent substances as fluorescein's partners have been extensively discussed in a recent review [20]. An unaddressed group of luminescent molecules is metal complexes, e.g., phosphorescent cyclometalated iridium(III) complexes, which, however, were thoroughly reviewed as promising bioimaging reagents in a more recent review paper [26]. Meanwhile, the anticancer activity of iridium(III) complexes [27–29] attracts increasing attention as their potential of inducing apoptosis [30,31] and antiangiogenesis [32]. The general chemical stability and wide tunability of photophysical properties of iridium(III) complexes offers possibility of designing ratiometric probes with unlimited combinations of luminophores meeting the requirements [14,20] for the pH probes. When choosing fluorescein as the pH-sensitive moiety, which emits light at 520–530 nm, an ideal pH-insensitive phosphorescent moiety as fluorescein's partners would be the one that, under the same excitation wavelength, emits phosphorescence with wavelength well separated from that of fluorescein.

The NIR emissive iridium(III) complexes (BTPH and its acid form BTPHSA) reported by Zhang et al. [33] were chosen by us to be our first demonstration of such fluorescein/iridium(III) complex

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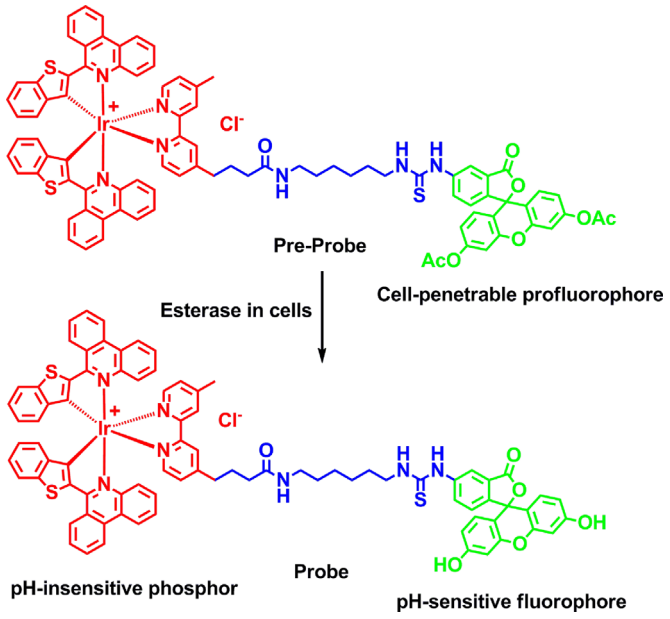
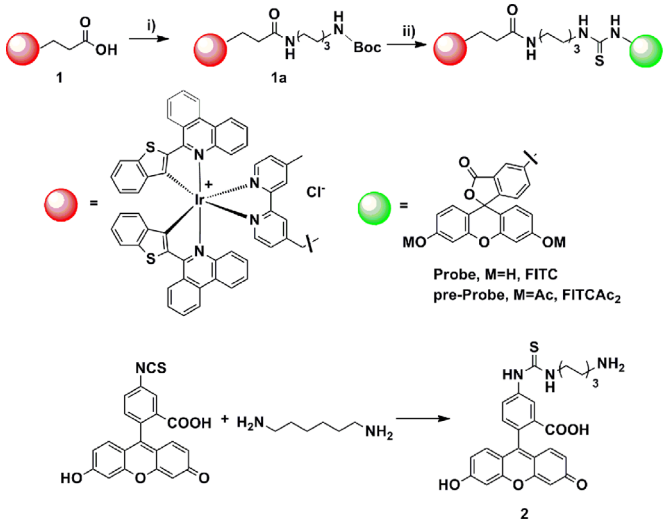
http://dx.doi.org/10.1016/j.talanta.2014.08.039
0039-9140/

couple. The phosphorescent moiety demonstrated in Scheme 1 is a variation of BTPHSA with a 2,2′-bipyridine derivative, instead of acetylacetonato, as the ancillary ligand. It was synthesized as an acid form (**1** in Scheme 2) capable of coupling to the fluorescein moiety to yield the dual-emitting probe under a single wavelength excitation. Complex **1** possesses a larger Stokes' shift and thus is able to discriminate itself from fluorescein moiety in emission spectroscopy. The good separation of the emission wavelengths from these two luminophores also prevents the interference of each other in the confocal microscopy. It is well known that fluorescein refuses to enter the cell because of its negative charge under the physiological conditions [34]. Although the positive charge of iridium(III) moiety favors the cell penetration [26], it was found that the **Probe** was unable to penetrate into cells. As usual,



Scheme 1. Molecular design of the ratiometric pH probe based on a pH-sensitive fluorescein moiety and a pH-insensitive phosphorescent iridium(III) complex.



Scheme 2. The structures and syntheses of **1**, **Probe** and **pre-Probe**. (i) HDABoc, DCC/HOBt/DMF, 24 h; (ii) 20% TFA/CH_2Cl_2, 1 h; **Probe**, M=H, FITC, 1% TEA/CH_2Cl_2; **pre-Probe**, M=Ac, $FITCAc_2$, 1% TEA/CH_2Cl_2. Red and green balls indicate near-infrared phosphorescent iridium(III) complex and green-emitting fluorescein emitter, respectively. (For interpretation of the references to color in this scheme legend, the reader is referred to the web version of this article.)

we synthesized a **pre-Probe** with the iridium(III) moiety coupled to the fluorescein diacetate moiety, which is non-fluorescent, but turns into fluorescent fluorescein through esterase hydrolysis in the cell [35].

2. Materials and methods

2.1. Chemicals and instruments

Bis(6-(benzo[b]thien-2-yl)phenanthridinato)(4-(3-carboxypropyl))-4′-methyl-2,2′-bipyridine)iridium(III) chloride (**1**) was synthesized in our lab. FITC isomer I, di-tert-butyl dicarbonate (Boc_2O) and 1,6-hexylenediamine were purchased from Aladdin, N,N′-dicyclohexyl carbodiimide (DCC) from Sigma-Aldrich, and hydroxybenzotriazole (HOBt) from GL Biochem. Nigericin and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich. Other chemicals and solvents were commercially available in analytical grade and used without further purification except otherwise specified.

GC–MS was determined on the Agilent 7890A GC system equipped with 5975C inert triple-axel MS detector. ESI-TOF-MS was measured on the Agilent 1200/6200 TOF-MS system. ^1H and ^{13}C NMR spectra were acquired from Varian 400 MHz NMR spectrometer. UV–visible absorption and excition/emission spectra were measured on a Lamda 25 UV/Vis spectrometer (PerkinElmer) and F4600 fluorescence spectrophotometer (Hitachi), respectively. Deionized water (18 MΩ cm, Milli-Q prepared) and UPLC grade organic solvents were used for spectroscopic measurements. The pH values were determined with a PB-10 pH meter (Sartorius), calibrated with a triple-point method before use.

2.2. Cell culture

HeLa cells (American Type Culture Collection) were cultured in Dulbecco's modified Eagle medium (DMEM, Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich) and 1% penicillin–streptomycin (Sigma-Aldrich) and grew to exponential phase prior to use. HeLa cells were grown at 37 °C under 5% CO_2 atmosphere.

2.3. Synthesis and characterization

2.3.1. *Bis(6-(benzo[b]thien-2-yl)phenanthridinato)(4-(3-carboxypropyl))-4′-methyl-2,2′-bipyridine)iridium(III) chloride (1)*

ESI-TOF-MS: *m/z* ($[M-Cl]^+$) calcd 1069.2222, found 1069.2227. ^1H NMR (400 MHz, $CDCl_3$) δ 9.37 (t, *J*=8.6 Hz, 2H), 8.56 (s, 2H), 8.35–8.22 (m, 5H), 8.18 (s, 1H), 7.94 (s, 4H), 7.84–7.78 (m, 2H), 7.30–7.04 (m, 8H), 6.80–6.56 (m, 6H), 2.62 (s, 2H), 2.34 (s, 3H), 2.23 (d, *J*=6.0 Hz, 2H), 1.75 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 175.18, 167.88, 167.80, 159.45, 159.24, 156.29, 155.11, 155.01, 152.78, 145.61, 145.58, 143.72, 143.59, 143.54, 138.55, 138.42, 133.71, 133.56, 133.32, 128.86, 128.70, 128.41, 128.10, 127.92, 127.86, 127.77, 127.29, 127.00, 126.89, 126.74, 125.48, 125.40, 124.77, 124.73, 124.04, 124.02, 122.85, 122.79, 122.76, 122.70, 122.66, 122.55, 122.05, 122.00, 34.14, 34.00, 25.54, 21.11.

2.3.2. *tert-Butyl 6-aminohexylcarbamate (HDABoc)*

HDABoc was synthesized according to Dardonville et al. [36]. To CH_2Cl_2 solution of 1,6-hexylenediamine (20.06 g, 172.6 mmol) was added dropwise di-tert-butyl dicarbonate (7.6 g, 34.8 mmol) dissolved in CH_2Cl_2. After 20 h of stirring at room temperature, the mixture was filtered. The filtrate was concentrated and redissolved in ethyl acetate, washed with water. Removal of solvent yielded a milky liquid (5.36 g, 71.2%). GC–MS: *m/z* ($[M-But]^+$)