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Two novel two-photon excited fluorescent pH probes based on the A-π-D-π-A system for intracellular pH mapping

Shuyi Chen [a], Meng Zhao [a], Jian Su [a], Qiong Zhang [a], Xiaohe Tian [b, *], Shengli Li [a], Hongping Zhou [a], Jieying Wu [a, **], Yupeng Tian [a, c]

[a] Department of Chemistry, Key Laboratory of Functional Inorganic Materials Chemistry of Anhui Province, Anhui University, Hefei 230601, PR China
[b] School of Life Science, Anhui University, Hefei 230039, PR China
[c] State Key Laboratory of Coordination Chemistry, Nanjing University, Nanjing 210093, PR China

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ABSTRACT

The pH value plays a significant role in lots of systems such as chemical reactions and biological processes. Herein, two novel two-photon excited fluorescent (2PEF) pH probes abbreviated as **L1** and **L2**, based on the A-π-D-π-A system were facilely synthesized by one-step solid-state reaction at room temperature. Probes **L1** and **L2** exhibited linear response over the weakly acidic pH range (4.33–6.73 for **L1**, 2.99–5.28 for **L2**) and remarkable emission ratio enhancement with the pK_a of 5.12 and 3.85, respectively. Interestingly, the position of the nitrogen atom had influence on the pH-dependent single-photon excited fluorescence, two-photon absorption (2 PA) across-section and linear region of pH response in probes **L1** and **L2**. Furthermore, the probes exhibited favorable two-photon absorption, high sensitivity, low cytotoxicity, and capability of mapping intracellular pH distribution. Therefore, probes **L1** and **L2** could act as practical tool for the sensitively detection of pH in weakly acidic conditions and tracking pH difference in living cells *via* two-photon fluorescence imaging.

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1. Introduction

Dynamic monitoring the pH fluctuations is critical for many industrial and biological processes; hence many researchers devoted themselves to develop novel sensors for monitoring pH variations [1–3]. Most of the intracellular metabolic pathways, such as proliferation, apoptosis, endocytic process and defense, are mainly managed by pH values in the weakly acidic pH environment. Lysosomes are membraneous acidic organelles (pH 4.0–5.5), which include plenty of proteins and enzymes playing vital roles in biological activities [4–9]. Thus, the lysosomal targeting and imaging are crucial in the fundamental research and clinical applications. The dyes for lysosomal localization were first served as an essential tool to study biological image by fluorescence microscopy in 1964 [10]. More recently, well-designed fluorescent dyes as probes have been employed to dynamic monitoring the pH fluctuations and have lysosomal localization capability simultaneously [11–13]. Although those probes were partially addressed by using a combination of a lysosome-specific dye and pH-sensitive fluorescent probe, the situation may be very complicated due to spectral cross talks and strong intrinsic autofluorescence background [14].

Fluorescent probes have become an indispensable tool in the study of pH fluctuations on account of its high sensitivity and specificity [15,16]. Two kinds of pH-sensitive fluorescent probes have been developed. One is the turn-on probe based on a photo-induced electron transfer system [17]. The other is the ratiometric probe based on a spectral shift in the absorption and/or emission during proton binding [18,19]. Of these two kinds of probes, the ratiometric probes have been widely used for quantitative analysis owing to the ability to reduce the environmental impacts. To monitor acidic pH, small molecules have been widely used as ratiometric one-photon probes, however, there are only few reports on two-photon excited fluorescence (2PEF) pH probes in recent years [20,21]. The major obstacle encountered with one-photon fluorescence probes is that it requires a rather short excitation wavelength, which limits their applications in live cell imaging. Consequently, probes with large two-photon absorption (2 PA) cross-section have become the technique of choice for non-invasive biological imaging in living cells and tissues [22–25]. Since 2 PA

* Corresponding author.
** Corresponding author.
E-mail addresses: t.xiaohe731@gmail.com, xiaohe.t@ahu.edu.cn (X. Tian), jywu1957@163.com (J. Wu).

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probes tackle cellular autofluorescence, photodamage, photobleaching and shallow penetration depth, which are capable of significantly enhancing the accuracy of measurement under intracellular conditions [26–29].

The aforementioned concerns encourage us to design two-photon excitable probes for dynamic monitoring the pH fluctuations and specially targeting lysosomes. Toward this end, we report two novel lysosome-targeted ratiometric 2PEF probes (**L1** and **L2,** Scheme 1) to monitor acidic pH. The probes were prepared through one-step solid-state reactions and gave quantitative yields. Such A-π-D-π-A system is extensively adopted as template to construct intramolecular charge transfer (ICT) fluorescent probes displaying desirable two-photon photophysics [30–34]. Meanwhile, we equipped the system with two pyridine moieties (A) of the end of the designed small molecule to obtain favorable pH sensitivity and two ethoxyl moieties (D) to acquire excellent cell membrane permeability and supply moderate electron donors for enhancing the basicity of the pyridine moieties. As expected, **L1** and **L2** exhibited pronounced pH-dependent red-shift properties both in the absorption and emission spectra, high quantum yield, large 2 PA cross-section to overcome autofluorescence and capability of mapping intracellular pH distribution. The influence of nitrogen atom position on pH-dependent optical properties and specific features in living cells *via* two-photon fluorescence imaging also have been investigated.

2. Experimental section

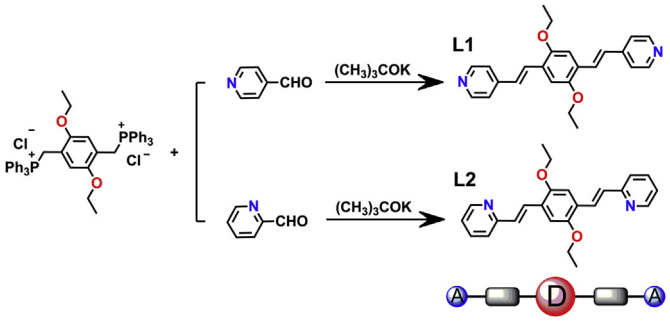
2.1. Materials and apparatus

All chemicals were purchased as reagent grade and used without further purification. The solvents were dried and distilled according to standard procedures. IR spectra (4000 − 400 cm^{-1}), as KBr pellets, were recorded on a Nicolet FT-IR 870 SX spectrophotometer. ^1H NMR spectra were performed on a Bruker 400 Hz Ultra shield spectrometer and were reported as parts per million (ppm) from TMS (δ) and ^{13}C NMR spectra were obtained on a Bruker Advance 100 MHz NMR spectrometer.

2.2. Optical measurements

2.2.1. The linear spectra measurement

All the solvents used for absorption and fluorescence measurements were HPLC grade. For dilute solutions of $c = 1.0 \times 10^{-5}$ mol/L, in quartz cuvettes of 1 cm path length, one-photon absorption (OPA) spectra were recorded on SHIMADZU UV-3600 spectrophotometer. One-photon excited fluorescence (OPEF) spectra were recorded on a Hitachi F-7000 fluorescence spectrophotometer. The concentrations are 1.0×10^{-5} mol/L. The fluorescent quantum yields were measured with integrating sphere.



Scheme 1. Synthetic routes of **L1** and **L2**.

2.2.2. Fluorescence lifetime

For time-resolved fluorescence measurements, the fluorescence signals were collimated and focused onto the entrance slit of a monochromator with the output plane equipped with a photomultiplier tube (HORIBA HuoroMax-4P). The decays were analyzed by 'least-squares'.

2.2.3. 2PA cross-sections

The 2PEF spectra were measured at femtosecond laser pulse and Ti: sapphire system (680–1080 nm, 80 MHz, 140 fs) as the light source. The δ values of samples were determined by the following equation [35]:

$$\delta_s = \delta_r \frac{\Phi_r}{\Phi_s} \frac{c_r}{c_s} \frac{n_r}{n_s} \frac{F_s}{F_r}$$

where the subscripts "s" and "r" represent sample and reference (here, fluorescein in ethanol solution at a concentration of 5.0×10^{-4} mol/L was used as reference), respectively. F is the overall fluorescence collection efficiency intensity of the fluorescence signal collected by the fiber spectra meter. Φ, *n* and *c* are the quantum yield of the fluorescence, the refractive index of solvent, and the concentration of the solution, respectively.

2.3. Synthesis

2.3.1. Synthesis of 4,4′-((1E,1′E)-(2,5-diethoxy-1,4-phenylene) bis(ethene-2,1-diyl)) dipyridine (*L1*)

2,5-Diethoxy-1,4-bis(triphenylphosphonium)benzene dichloride (4.0 g, 5.0 mmol) [30] and 4-pyridinecarboxaldehyde (1.3 g, 12 mmol), fresh t-BuOK (2.3 g, 20 mmol) were crashed together with a pestle and mortar at room temperature for 0.5 h and the resultant yellow powder was obtained. Yield 1.6 g (Scheme 1, 87%). Melting point: 226 °C. ^1H NMR (DMSO-d_6, 400 MHz) δ 8.56 (d, $J = 5.6$ Hz, 4 H), 7.68 (d, $J = 16.6$ Hz, 2 H), 7.54 (d, $J = 5.7$ Hz, 4 H), 7.39 (t, $J = 8.3$ Hz, 4 H), 4.17 (q, $J = 6.8$ Hz, 4 H), 1.43 (t, $J = 6.9$ Hz, 6 H). ^{13}C NMR (DMSO-d_6, 100 MHz) δ 14.71, 64.52, 111.36, 120.74, 126.09, 126.96, 127.20, 144.57, 150.02, 150.72. Elemental analysis for **$C_{24}H_{24}N_2O_2$**, Calcd (%): C 77.39, H 6.49, N 7.52, found: C 77.46, H 6.56, N 7.41. MS (ESI-MS): calc: 372.1838 (M), found: 373.1924 ($[M+H]^+$). IR (KBr, cm^{-1}) selected bands: 3429 (s), 2977 (m), 2931 (w), 2879 (w), 1628 (m), 1594 (s), 1472 (m), 1425 (m), 1388 (m), 1328 (m), 1263 (m), 1211 (s), 1113 (m), 1050 (s), 990 (w), 966 (s), 851 (m), 800 (m).

2.3.2. Synthesis of 2,2′-((1E,1′E)-(2,5-diethoxy-1,4-phenylene) bis(ethene-2,1-diyl)) dipyridine (*L2*)

2,5-Diethoxy-1,4-bis(triphenylphosphonium)benzene dichloride (5.9 g, 7.5 mmol) [30] and picolinaldehyde (1.9 g, 18 mmol), fresh t-BuOK (3.6 g, 30 mmol) were crashed together with a pestle and mortar at room temperature for 0.5 h and the resultant yellow powder was obtained. Yield 2.2 g (Scheme 1, 79%). Melting point: 198 °C. ^1H NMR (DMSO-d_6, 400 MHz) δ 8.59 (d, $J = 4.1$ Hz, 2H), 7.95 (d, $J = 16.2$ Hz, 2H), 7.79 (td, $J = 7.7, 1.7$ Hz, 2H), 7.49 (d, $J = 7.8$ Hz, 2H), 7.42 (d, $J = 16.2$ Hz, 2H), 7.38 (s, 2H), 7.25 (dd, $J = 6.8, 4.9$ Hz, 2H), 4.18 (q, $J = 6.9$ Hz, 4H), 1.42 (t, $J = 6.9$ Hz, 6H). ^{13}C NMR (DMSO-d_6, 100 MHz) δ 14.81, 64.37, 111.07, 122.21, 122.61, 126.16, 126.42, 128.62, 136.83, 149.52, 150.76, 155.26. Elemental analysis for **$C_{24}H_{24}N_2O_2$**, Calcd (%): C 77.39, H 6.49, N 7.52, found: C 77.31, H 6.42, N 7.63. MS (ESI-MS): calc: 372.1838 (M), found: 373.1924 ($[M+H]^+$). IR (KBr, cm^{-1}) selected bands: 3453 (m), 3017 (w), 2928 (w), 1625 (m), 1582 (vs), 1561 (m), 1500 (s), 1465 (vs), 1418 (vs), 1337 (s), 1208 (vs), 1150 (m), 1109 (m), 1048 (vs), 977 (m), 821 (w), 770

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