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A super-sensitive ratiometric fluorescent probe for monitoring intracellular subtle pH fluctuation

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ABSTRACT

A new type of xanthene-based fluorescent probe capable of intracellular subtle pH fluctuation sensing was developed. The probe underwent reversible deprotonation-protontation processes and therefore generated two states with distinct contrast in fluorescence emission features. In the physiological range (pH 7.0–8.0), the probe markedly presented 31.2% fluorescence signal change upon pH fluctuation of 0.1 pH unit, enabling sensitivity infinitely superior to previous fluorescent pH probes. The preliminary biological experiments revealed the large dynamic range of the probe for intracellular pH sensing within the range of pH 6.0–8.0. Owing to its sensitive responses to pH fluctuation in the physiological pH range, the probe definitely demonstrated its ability for "distinguishing" tumor from normal cells based on the difference in microenvironment pH level between the former and the latter. The figure of merits of the probe, including p*K*a (7.45) in the physiological pH range, excellent photostability, extraordinary large dynamic range for pH sensing, as well as the preliminary bioimaging results regarding differentiating tumor from normal cells are indicative of its potential for providing the microenvironment information regarding the interfacial features at the perimeter of tumor tissues by presenting high-contrast fluorescence images.

1. Introduction

Among a broad variety of environmental conditions within specific microenvironments in living systems such as intracellular pH and redox homeostasis, intracellular pH unequivocally plays crucial roles in cellular behaviors and many important physiological and pathological processes [1–4]. For instance, it has been demonstrated that most proteins are highly dependent on local pH within specific microenvironment for maintaining their structures and functions and subtle fluctuation in pH can exert a drastic effect on their structures and activities [5]. As another example, the intravesicular pH within single synaptic vesicles was demonstrated directly associated with the loading of neurotransmitters into these functional nanostructures for propagating nerve impulses [6]. It is beyond doubt that the maintenance of an appropriate pH within specific microenvironments in cells is of great significance to their normal physiology [7–9]. Specifically, alterations in the cytosolic pH have been proposed to closely related to cellular proliferation, cell migration and apoptosis [10,11]. Additionally, the imbalance of mitochondrial pH homeostasis has been linked with a significant early sign in mitochondrial-dependent apoptosis [4]. Furthermore, increasing evidence definitely demonstrated the significant roles of acidic extracellular microenvironment in the growth and metastasis of tumors [12,13]. It deserves mentioning that the intracellular pH condition in living cells is not homogeneous throughout the whole cell and it virtually varies greatly among different organelles [4]. Under physiological conditions, the extracellular pH is slightly alkaline with pH ranging among ~7.35–7.45. Even when bathed in large volumes of such slightly alkaline medium, the lysosome compartments can reach pH as low as 4.5–4.7 while the mitochondrial matrix is markedly alkaline with pH of ~8.0 [14,15]. Taking the wide distribution of pH condition in living systems and the crucial roles of intracellular and extracellular pH microenvironment on physiological processes, accurately determining the pH level in complex biological systems is of paramount importance and represents a challenge to biology, physiology, and medicine.

Owing to their salient advantages in terms of high sensitivity, excellent spatiotemporal resolution, real-time and nondestructive detection, simplicity of manipulation and applicability to intracellular

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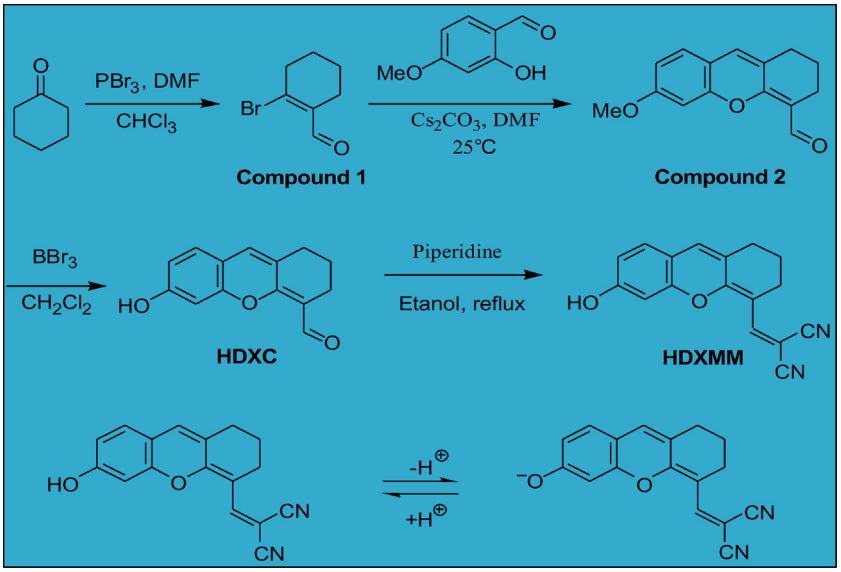

Scheme 1. Synthetic route to the target pH probe HDXMM and the proposed response mechanism of the probe to pH change.

detection over other strategies such as acid-base indicator, potentiometric titration, and nuclear magnetic resonance (NMR) spectroscopy [16–19], fluorescence-based approaches have played active roles at the forefront of biological pH sensing in recent years [20–26]. Specifically, various pH-sensitive fluorophores [21,27–32], fluorescent nanoprobes with different formats [32–35] and pH-sensitive green fluorescent protein (GFP) variant [36], have come to represent the method of choice for the biological pH sensing. Despite considerable advancements, development of fluorescent probes with ideal dynamic range for monitoring subtle physiological pH fluctuation still remains a challenge-most probes developed to date do not present enough contrast in beaconing fluorescence signal upon variation of pH value [36–43]. For instance, the pH-sensitive mutant of GFP pHluorin merely presented ~10% change in fluorescence brightness per pH unit, which definitely impedes its practical applications in sensing tiny pH fluctuation [36].

In this work, a new type of ratiometric pH probe (called HDXMM hereafter) for pH sensing was developed by conjugating an electron-withdrawing group, malononitrile, with a xanthene derivative carrying a phenol moiety (Scheme 1). It is noted that another type of fluorescent probe featured with a xanthene derivative in conjugation with an indolium cation moiety have been developed for lysosomal pH sensing [31]. Specifically, such probe displayed a p*K*a of 5.0 due to the acidic nature of indolium cation moiety [44] and xanthene derivative [45], which makes the probe suitable for mapping the pH levels of lysosomes with physiological pH range of 3.8–5.0 [31]. It is known that the electronic characteristics of the substituted moiety generally exert significant influence on the p*K*a of chromophores [46]. Taking this, along with its relatively high p*K*a value (11.2) [47], malononitrile moiety was used as the electron-withdrawing group in this work for constructing the target pH probe with increased p*K*a, within or close to the physiological range of extracellular pH conditions, and thus suitable for detecting the pH level in the range from near-neutral to weakly basic conditions. It was expected that the xanthene derivative carrying a phenol moiety in conjugation with a malononitrile group may afford a fluorophore with higher p*K*a value as compared to the counterpart one with the involvement of indolium cation moiety. The HDXMM probe underwent reversible deprotonation-protontation processes and therefore generated two interconvertible states with distinct fluorescence emission features [31,32,48,49]. The p*Ka* of HDXMM was determined as ~7.45, indicating the suitability of such probe for measurement of pH in the physiological pH range. More importantly, as compared to the fluorescent pH probes reported to date, the HDXMM probe is capable of presenting huge pH-induced contrast in beaconing fluorescence signal in the physiological pH range. Specifically, upon changing the pH value from 6.0 to 8.0, the ratiometric beaconing fluorescence signal of the HDXMM probe displayed contrast with magnitude more than 20-fold. Additionally, the excellent photostability, prompt response, outstanding selectivity, and low cytotoxicity of HDXMM probe were also confirmed. The preliminary fluorescence imaging experiment results have revealed the response ability of HDXMM probe to pH fluctuation of cell culture media in the physiological pH range. Moreover, the practicability of HDXMM probe for discriminating tumor cells from normal cells based on the difference in pH level of the cytoplasm between cancer cells and normal cells have also been validated. Such feature-packed probe is expected to further advance pH sensing capabilities in practical biological systems such as identifying the boundary between tumor and surrounding normal tissue based on the difference in pH between them.

2. Experimental

2.1. Chemicals

All chemicals and solvents were purchased from commercial suppliers and used without further purification unless otherwise stated. Silica gel (300–400 mesh) for column chromatography was purchased from Qingdao Ocean Chemicals Inc. Ultrapure water (over 18kΩ) was prepared via a Milli-Q water purification system (Millipore).

2.2. Apparatus

^{1}H NMR spectra and ^{13}C NMR were recorded in $CDCl_3$ at ambient temperature on a Shimadzu (Japan) 400 Hz Spectrometer. Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectra were recorded with a Bruker BIFLEX-III mass spectrometer. UV–vis absorption spectra were acquired on a UV-2550 spectrophotometer (Shimadzu, Japan). Steady-state fluorescence spectra were recorded on FluoroMax-4 spectrofluorometer (Horiba Jobin Yvon, NJ, USA). The pH values were measured with a Sartorius (PB-10) digital pH meter with a combined glass-calomel electrode.

2.3. Synthesis and characterization of fluorescent probe

For synthesizing the target HDXMM probe, a *de novo* strategy without the involvement of cyanine dyes and with high yield was applied in the present work (Scheme 1) [50]. The molecular structures of