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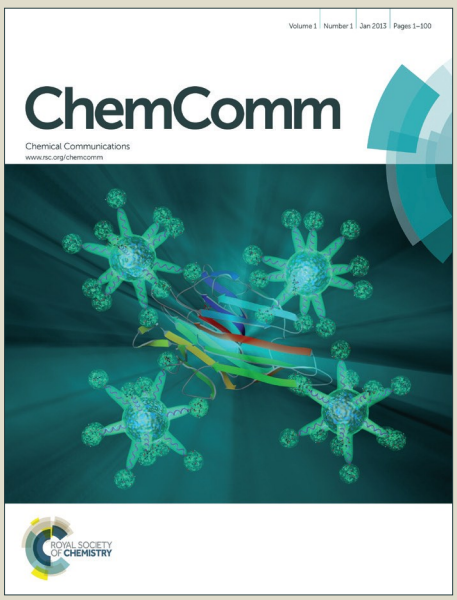

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In vivo observation of the pH alternation in mitochondria upon various external stimuli

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The pH of mitochondria (pH_m) has a close relationship with many biological processes. Here we developed a new indicator Mito-pH-1 for ratiometric fluorescent detection of mitochondria pH value, which has excellent tolerance to environment change. And Mito-pH-1 has been firstly used to monitor the change of pH_m under temperature and H_2O_2 stimuli in living cells.

Mitochondria are found in almost all eukaryotic cells.[1] The alkaline pH level (~8.0) in mitochondria (pH_m) is widely believed to be required for normal physiological function, and disruptive variations in pH_m can lead to dysfunction of the organelle.[2, 3] pH_m plays a central role in formation of proton gradient and electrochemical potential that drive the adenosine 5′-triphosphate (ATP) synthesis.[4] It is also involved directly or indirectly in several metabolic processes happened in mitochondria, such as the generation of reactive oxygen species (ROS),[5] the homeostasis of calcium ions,[6] as well as the trigger of cellular apoptosis and degeneration[7]. In addition, recent studies reveal that pH_m can be influenced by several bioactive species and environmental parameters.[8] Unfortunately, it has been difficult to precisely understand how pH_m is regulated in the cell and consequently regulates metabolic processes, because the conventional methods are based on a rapid cell extract analysis rather than in vivo studies.[9]

Fluorescent indicator has been widely considered to be a powerful imaging technique because of its high spatial resolution and direct observation capability.[10,11] Despite the important physiological effects of pH_m, indicators quantifying the in vivo variation of pH_m are extremely scarce. Only several mutants of fluorescent proteins (FPs)[12-14] have been developed as pioneering examples of the pH_m indicator. However, the expensive purification and tedious transfection processes limit broad applications of these genetically encoding proteins. Last year, two small molecules, a member of cyanine family (Spring Red) and a naphthalimide derivative, were created for in vivo pH_m imaging.[15-18] Although both pH_m indicators have advantages of readily processing, synthetic convenience, and relatively low cost, their specific pH dependent fluorescence intensity is significantly interfered by the excitation power and microenvironment, making the observation of pH_m levels less accurate.

Recently, a compound using covalently-linked two fluorophores has been developed as a sole ratiometric pH_m indicator.[19] Although this indicator exhibits a good sensitivity to pH_m, constant temperature is a prerequisite for accurate measurements. Body temperature is generally regulated at a constant setpoint, but many prevalent diseases can cause slight or mortal temperature changes.[20] The temperature fluctuation reduces the metric accuracy significantly, whether intensity-based indicators or ratio-based ones with two different fluorophores, because the thermal activation of radiationless processes make the luminescence efficiency temperature-dependent for almost all organic luminophores.[21]. Therefore, it is of great challenge to develop in vivo fluorescent pH_m indicators with stable fluorescence signals over a wide temperature range.

Table 1. Quantum yields of Mito-pH-1 at various pH values.

pH	4.0	5.0	6.0	6.5	7.0	7.5	8.0	8.5	9.0	10.0	11.
Φ	0.76	0.74	0.73	0.73	0.72	0.72	0.71	0.69	0.68	0.66	0.6

Radiative and nonradiative decays are intrinsic characteristics that completely determined by fluorophore structure. Fluorescent dyes with similar conjugation systems always tend to show closer fluorescent behaviors. Following the idea, we designed and synthesized a novel ratiometric indicator Mito-pH-1 on the basis of a hydroxypyrene derivative (Scheme S1). The easy-synthesized indicator operates through protonation and deprotonation of phenolic hydroxyl, giving a neutral form and anionic one, respectively (Figure 1a). The anionic and neutral forms of the Mito-pH-1 luminogen have similar electronic structures in either ground state (S_0) or excited state (S_1) (Figure S1 and Table S1). The c

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transition dipole moments from S_1 to S_0 in the anionic and neutral forms also suggest the similar emission possibility.[22] It is rational to expect that both forms show similar response to temperature. Furthermore, the extended conjugate-structure and the ketone substituent of the luminogen endow it an excited state with ππ*/nπ* mixture characteristic, promising a high luminescent efficiency even in an aqueous environment (Table 1). The specific anchor group positively charged triphenylphosphonium (TPP) facilitates entrance and retainment of Mito-pH-1 in mitochondria.[23] By using Mito-pH-1 as the indicator, we have successfully observed the dynamics of pH_m in several situations, including cytosolic acidification /alkalization, cool/overheat and H_2O_2 stimulations. The results provided here show an interesting difference of pH_m response between tumor and normal cells, demonstrating the highly practical efficiency of Mito-pH-1 in monitoring pH_m.

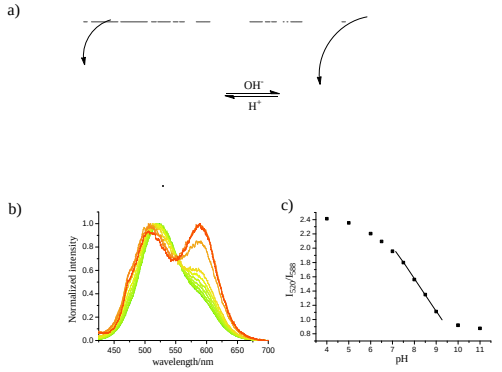

Figure 1. a) Mechanism of the Mito-pH-1 emission changing with the pH value. b) Normalized fluorescence spectra of Mito-pH-1 (1.0 μM) recorded at different pH values (4.0-11.0). c) Plot of Mito-pH-1 fluorescence intensity ratio (I_{520}/I_{588}) versus pH. λ_{ex}=405 nm.

The in vitro fluorescent characteristics and performances of Mito-pH-1 were firstly examined in water/1,4-dioxane mixed solvent, which provides a similar microenvironment to the inner space of mitochondria.[24] Mito-pH-1 displays a sensitive spectroscopic response to variant pH levels (Figure 1b). With increasing the pH value from 4.0 to 11.0, the absorption band shifts gradually from 410 (neutral phenolic form) to 500 nm (anionic form) with a distinct isosbestic point at 450 nm (Figure S2). The emission intensity at 588 nm shows a relative increase compared with that at 520 nm, giving a measurable ratio (I_{520}/I_{588}) of the fluorescence intensity related to the variation of pH levels. The ratio shows a perfect reversibility between the pH values 4.0 and 9.0 (Figure S3), and a linear relationship between I_{520}/I_{588} and the pH value is observed in the alkaline region (pH = 7.0–9.0, Figure 1c). The pKa value of Mito-pH-1 is 7.33±0.04, which was calculated with the Henderson-Hasselbach-type mass action equation that log[(Imax-I)/(I-Imin)]=pH-pKa,[25] where Imax, Imin, and I represent the maximum, minimum, and observed fluorescence intensity at a given pH value, respectively. These results suggest Mito-pH-1 as a good candidate for the pH_m indicator.

As noted above, the inert response to temperature is an important criterion for a proper in vivo pH_m indicator. The fluorescence of Mito-pH-1 at different temperature was further evaluated with the pH value of 8. Although the fluorescence intensity for both neutral and anionic forms decreases slightly (~5%) with increasing temperature from 25 to 45 °C, the intensity ratio (I_{520}/I_{588}) does not show any variations (Figure 2a). The superior insensitivity of ratiometric fluorescence to temperature probably originates from the approximate decay processes of the two forms. In addition to temperature, an inert response to bio-active matters is also essential for being a specific indicator, because the matrix environment of mitochondria is an aqueous solution of various bio-active molecules alongside ions.[26] To determine the possible interference, the intensity ratio of Mito-pH-1 was assessed in the absence and presence of essential metal ions, redox chemicals and chemical transmitter, which are relatively significant abundance in mitochondria. No notable response change was observed when Mito-pH-1 was exposed to the additaments, indicating that Mito-pH-1 has no reaction activity with most of the environmental substances. This excellent tolerance of Mito-pH-1 to bio-active substances is in keeping with our molecular design. Mito-pH-1 does not have any apparent reaction active sites. The sole possibility of reduction reaction of Mito-pH-1 with ROS is also significantly diminished by the intramolecular charge transfer.[27]

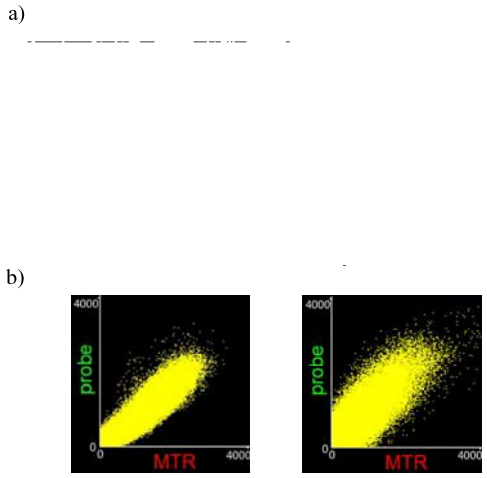

Figure 2. a) Fluorescence responses of Mito-pH-1 in PBS (pH = 8.0) to diverse substances. b) The correlation of Mito-pH-1 and MTR intensities in HeLa cells (left) and NIH/3T3 Fibroblasts (right). λ_{ex}=405 nm.

The attractive in vitro performance of Mito-pH-1 urged us to further investigate its ability to localize and stain mitochondria in living cells. HeLa cells and NIH/3T3 cells were first stained with Mito-pH-1 (1.0 μM) for 40 minutes and then with Mito-Tracker Red (MTR, 1.0 μM), a commercial mitochondrial specific fluorescent dye, for another 20 minutes. The fluorescence images of the neutral and anionic forms of Mito-pH-1 collected from two different channels are perfectly overlapped with that from MTR (Figure 2b), suggesting a good cell-membrane permeability of Mito-pH-1. The high Pearson correlation coefficients of 0.970 and 0.960 in tumor and normal cells demonstrate a specific targeting on mitochondria of Mito-pH-1 (Figure 2b). The standard 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay also confirms the good biocompatibility of Mito-pH-1 for HeLa cells and NIH/3T3 cells (Figure S5). Moreover, to ensure the accuracy of in vivo monitoring pH_m, the approach that the H^+/ K^+ ionophore nigericin induced pH

homogenization was utilized to execute calibrations in HeLa and NIH/3T3 cells.[28] The in vivo images of red and green channels show clearly a sensitive fluorescent response to the pH change in the range of 7.0 - 9.0 (Figure S6). This relationship between the fluorescence ratio from the two channels and the pH value can be described by linear equations: ratio = 4.66 − 0.39pH (correlation coefficient R = 0.992) in HeLa cells, and ratio = 4.69 − 0.39 pH (R = 0.991) in NIH/3T3 cells, respectively (Figure S7). The excellent performance of Mito-pH-1 in cells makes the following in vivo mapping of pH_m operable and reliable.

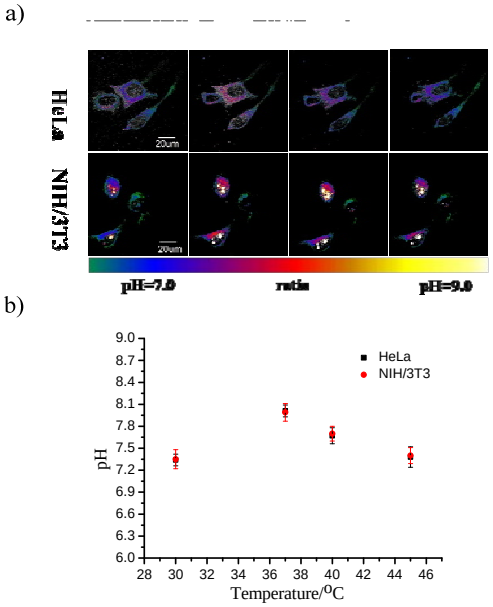

Figure 3. a) Ratiometric images of HeLa and NIH/3T3 Fibroblasts cells treated with Mito-pH-1 (1.0 μM) at different temperatures. b) The plot of pH alteration of HeLa (mean of 18 cells) and NIH/3T3 Fibroblasts cells (mean of 16 cells) versus temperature. The color strip represents the pseudocolor change with pH. λ_{ex}=405 nm.

The disruptive variation in ambient temperature can influence the performance of several intracellular functional molecules and structural components.[29] The resulted cold or heat stress further causes more or less damages on homeostasis in cells.[30] To date, most studies on the relationship between temperature and the pH value focus on the extracellular or cytoplasmic circumstances, and rare attention has been paid to the influence of temperature on pH_m.[31] To assess the impact of the variation of ambient temperatures on pH_m, the confocal microscopy images of HeLa and NIH/3T3 cells stained with Mito-pH-1 upon in-situ cooling or heating were taken, and their ratiometric images at different temperatures were obtained. The calibrated ratios show gradual changes with increasing temperature from 30 to 45 °C, suggesting a regular alternation of pH_m levels in HeLa cells and NIH/3T3 cells (Figure 3).

The measured pH_m values of HeLa and NIH/3T3 cells at 37 °C are 8.01 ± 0.08 and 7.99 ± 0.12, respectively, which are in good agreement with the general reports.[32] The pH_m levels decrease to 7.34 ± 0.08 and 7.35 ± 0.13 at 30 °C in the tumor and normal cells, respectively. The acidification originates probably from proton leak or mitochondrial uncoupling that result in a potential energy of the proton gradient being released as heat, which is the self-protecti behavior of living cells to minimize the cold damage.[33] The sam acidification tendency is observed with increasing temperature. A 40 °C, the pH_m values in the tumor and normal cells increase to 7.6 ± 0.11 and 7.70 ± 0.10, respectively. One possible reason for th phenomena is the increased permeability of the mitochondrial inner membrane during heating.[34] When the temperature reach 45 °C, the pH_m levels decrease again to 7.38 ± 0.14 and 7.40 ± 0.11 in HeLa and NIH/3T3 cells, respectively. The disappearance of th proton gradient means the halt of the ATP production.[35] The above results clearly manifest that whether cold stress or heat stress can lead to a dysfunction of mitochondria. The visible responses of pH_m to temperature in both tumor and normal cells demonstrate th reality of Mito-pH-1 being an in vivo pH_m fluorescent indicator.

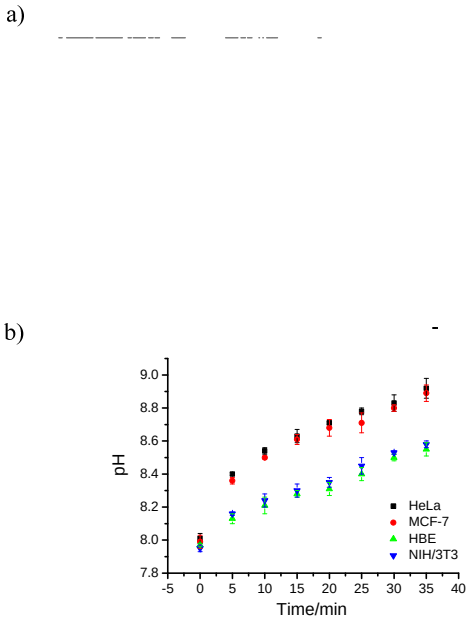

Figure 4. a) Ratiometric images of HeLa, NIH/3T3 Fibroblasts, MCF-7 and HBE cells loaded Mito-pH-1 (1.0 μM) treated with H_2O_2 (0 μM) for 0 minute and 35 minutes. b). The plot of pH alteration of HeLa (mean of 17 cells), NIH/3T3 Fibroblasts (mean of 20 cells), MCF-7 (mean of 15 cells) and HBE (mean of 22 cells)) versu challenge time of H_2O_2. The color strip represents the pseudocol change with pH. λ_{ex}=405 nm.

ROS are constantly generated and eliminated in mitochondria an play important roles in the modulation of signal transductio cascades and transcription factors[36]. In contrast to temperature hydrogen peroxide (H_2O_2) as the major ROS production is direct involved in bio-energy metabolism,[37] which is probably more sensitive to pH_m fluctuations. To obtain the relationship betw en pH_m and H_2O_2, HeLa and NIH/3T3 cells stained with Mito-pH-1 w challenged with H_2O_2 (100 μM), respectively. The real tim dynamics of the ratio responses demonstrate that the pH_m leve increases mono-directionally upon stimulation of H_2O_2 in bot tumor and normal cells (Figure 4 and Figure S8). However, the important result is that the increasing rates are remarkab different in tumor cells and normal cells. The discrepancy of this pH_m response inspired us to further examine the effects of ROS o other cells. The in vivo ratiometric images of another common

tumor and normal cells, MCF-7 and HBE cells, stained with Mito-pH-1 upon stimulation of H_2O_2 (100 μm) were obtained, and similar discrepancy of the pH_m response was observed. The calculated mean difference is only ~0.4% between two tumor or normal cells but ~3.6% for interclass, demonstrating that the discrepancy between tumor and normal cells are reliable.

The biological mechanism of the cell-related increase rate of pH_m is not very clear, but several studies have demonstrated that pH_m controls the rate of oxidative phosphorylation.[38] The higher pH_m level ensures the sustained uptake of pyruvate and other essential substances for oxidative metabolism and elevates ATP synthetic rates in mitochondria.[39] On the other hand, the oxidative stress induced by H_2O_2 activates the nuclear enzyme poly(ADP-ribose) polymerase. This activation dramatically depletes nicotinamide adenine dinucleotide in cytosol, thus, inhibits the glycolysis process.[40] As well known, the aerobic glycolysis in cytosol rather than efficient oxidative phosphorylation in mitochondria is the predominant energy metabolism way for tumor cells (Warburg effect). We assume therefore that the halt of glycolysis impels the higher rate of oxidative phosphorylation in tumor cells to maintain their advantageous survival ability. Moreover, the matrix alkalization is an indication of early apoptosis in mammalian cells. It is reasonable that tumor cells are more susceptible to H_2O_2-induced apoptosis than normal cells because of their generally elevated ROS levels.[41] The results shown here provide a potential strategy for discriminating tumor cells from normal cells.

In conclusion, a targetable and low-cost indicator Mito-pH-1, which shows a desirable ratiometric fluorescent response to pH variations, has been developed. The advantages of high specificity to mitochondria, low cytotoxicity, and excellent tolerance to environment change make Mito-pH-1 a well-suited indicator for in vivo continuously monitoring pH_m under stimuli. Quantitative determination of pH_m for several cells has been successfully performed by using Mito-pH-1 as the indicator. For the first time, our results show that pH_m varies with both temperature and ROS stimulations. It is very interesting that tumor and normal cells present responses to H_2O_2 stimulus in different extents, which is possibly originated from the difference between the bio-energy metabolisms of two classes of cells. We expect that the detection of pH_m can be promoted to be a novel method for discriminating tumor cells from normal cells in near future.

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