Synergistic Lysosomal Activatable Polymeric Nanoprobe Encapsulating pH Sensitive Imidazole Derivative for Tumor Diagnosis

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Developing optical tumor imaging probes with minimal background noise is very important for its early detection of small lesions and accurate diagnosis of cancer. To overcome the bottleneck of low signal to noise ratio and sensitivity, it needs further improvement in fluorescent probe design and understanding of tumor development process. Recent reports reveal that lysosome’s acidity in cancer cells can be below 4.5 with high Na⁺/H⁺ exchange activity, which makes it an ideal target intracellular organelle for cancer diagnosis based on the variation of pH. Herein, a boron 2-(2'-pyridyl) imidazole complex derivative (BOPIM-N) is developed, with the ability to show a pH-activatable “OFF–ON” fluorescent switch by inhibiting twisted intramolecular charge transfer upon protonation at pH 3.8–4.5, which is studied for its selective viable cancer cell imaging ability in both in vitro and in vivo experiments. Interestingly, BOPIM-N can specifically emit green fluorescence in lysosomes of cancer cells, indicating its promising cancer cell specific imaging ability. More importantly, nanoformulated BOPIM-N probes can be specifically light-ON in tumor bearing site of nude mice with resolution up to cellular level, indicating its potential application in tumor diagnosis and precision medicine.

Selecting imaging of cancer cells and tumors has great importance in medical diagnosis or elucidating cancer-associated process, but it remains a challenge to differentiate the viable cancer cells of various types and normal ones with high selectivity.[1] Besides to antibody recognition,[1c,2] tumor-associated enzymes and the pH of tumor/cell microenvironment are two important factors for tumor selective imaging.[3] Tumor-related enzymes (i.e., matrix metalloproteinases or β-galactosidase) can also occur in healthy tissues which produce undesirable background signals, but these enzymes based probes are limited by tumor types.[4] By contrast, anaerobic glycolysis (or Warburg effect) has been recognized as a solid cancer hallmark regardless of its phenotypes, where the production of lactic acid (pKₐ = 3.7) causes acidic tumor microenvironment (pH of around 6.5).[5] Accordingly, various pH-sensitive fluorescent probes have recently emerged, such as fluorescent gold nanoparticles (AuNPs) with deposition of cationic conjugated polyelectrolyte[6] and fluorescent dye encapsulated cationic micelles, for diagnostics of cancer cells.[7] However, this strategy remains a concern as pH of the acidic intracellular vesicles or lysosomes in normal cells can range from 4.5 to 6.5,[8] which might overlap with the extracellular acidity of cancer cells.[9] This disadvantage might induce undesired background noises and impair the small population living cancer cell detection.

To effectively avoid such pH range overlap, an alternative hallmark of cancer cells could be exploited is the lysosomal acidity of cancer cells, which is greatly differential from that of normal cells. The lysosome acidity in malignant tumor can be below 4.5, in contrast to lysosomes in normal cells that maintain the acidic environment of 4.5–6.5.[10] This difference raises a specific circumstance in highly metabolic cancer cells (typical pH in lysosome ranging from 3.8 to 4.7) that provides an attractive opportunity of precise diagnosis. The cause for this low pH in malignant tumor is enzymatic activity maximizing as the Na⁺/H⁺ exchange activity is significantly enhanced.[11] Naturally, exploiting the greatly differential lysosomal acidity of
normal and cancer cells might serve as a wide-spectrum target of intracellular organelle for label-free selective imaging of cancer cells with low signal interferences. Nonetheless, recently developed lysosome-tracker dyes based on rhodamine,[12] BODIPY,[13] naphthalimide,[14] or cyanine[15] could only achieve specific tumor imaging by costly conjugation to tumor receptor specific antibodies, rather than considering this special pH difference between lysosome in tumor tissues and that in normal ones.[16] Therefore, developing a biocompatible pH sensor with the ability to control its fluorescence OFF–ON switch at pH 3.8–4.5 might significantly minimize the background signals originating from noncancer cells or stromal tissues,[17] and serve as a novel and simple strategy for selective viable cancer cell imaging.

To fulfill improving the signal-to-noise ratio, one more critical step involved is to efficiently transport the pH sensor in vivo with tumor site accumulation ability. Among currently feasible candidates for transportation including molecular conjugates or inclusion complex with biocompatible macromolecules, nanoparticle carriers, with enhanced permeation retention (EPR) effect for excellent tumor site accumulation and pump resistance combating ability, are attractive as small molecule delivery system for improved solubility, bioavailability, and retention time.[18] Compared with inorganic nanoparticles, organic polymeric nanocarriers are widely studied as they can be nontoxic, biocompatible, and biodegradable.[19] Specially, poly(polyethylene glycol (PEG)/polypropylene glycol (PPG)/polylactide (PLA) urethane)s copolymer, made of PLA, PEG, and PPG, have been used as carriers for sustained small molecular delivery or on-demand release to tumor site with minimum toxicity and side effects,[20] although polymeric nanoparticles have not been evaluated for the delivery of pH sensitive probe for tumor diagnosis.

In order to address the above issues, herein, we designed a biofriendly nanoprobe targeting to lysosome’s acid environment in tumor cells, based on a boron 2-(2′-pyridyl) imidazole complex derivative (BOPIM-N) with specific fluorescence light-ON ability at pH 3.8–4.5, being encapsulated in biodegradable and biocompatible copolymer poly(PEG/PPG/PLA urethane)s nanoparticles for passive accumulation in tumor thanks to nanoparticle’s EPR effect (as shown in Scheme 1). On one hand, the fluorescence activation at pH 3.8–4.5 could effectively minimize the background noise from undesired lysosome uptake of probe by normal tissues. On the other hand, the EPR effect of poly(PEG/PPG/PLA urethane)s nanoparticles could significantly increase the passive accumulation of probe in tumor of in vivo study and hence further minimize the background noise, while reducing cancer cell’s undesired pump resistance. Together, such synergistic strategy for minimizing background noise can be important for precise tumor diagnosis. Furthermore, the design of polymeric nanoparticle based on copolymer poly(PEG/PPG/PLA urethane)s could increase the hydrolytic degradability of nanoparticle, as well as the biocompatibility of BOPIM-N for safe in vivo study. To the best of our knowledge, this is a pioneer report on the biological application of BOPIM-N for cancer cell diagnosis as well as on using biodegradable and biocompatible poly(PEG/PPG/PLA urethane)s as nanoparticle carrier for probe encapsulation, which might be beneficial.

Scheme 1. a) Schematic illustration of the BOPIM-N structure with reversible and acidic pH-induced fluorescence activation. N,N-dimethylanilinophenyl group as electron donor with free intramolecular rotation ability causes fluorescence quenching, but its twisted molecular confirmation could be stabilized upon protonation to induce increased fluorescence emission. b) Schematic illustration of design of biocompatible polymeric BOPIM-N nanoprobe, based on pH-sensitive imidazole derivative BOPIM-N with cancer cell’s low pH lysosome light-ON ability encapsulated in biocompatible and biodegradable poly(PEG/PPG/PLA urethane)s, for potential tumor diagnosis.
for solving safety concerns after diagnosis. In short, our lysosomal activatable polymeric nanoprobe, on the basis of synergistic effect of pH sensitive imidazole derivative and biodegradable nanocarriers, would be promising for selective imaging with minimal background noise and wide-spectrum tumor diagnosis with high precision.

BOPIM complex could show intense emissions with large Stokes shift, which favored their applications as a robust fluorescent probe. In this report, we found that BOPIM-N, a BOPIM derivative bearing substituted dimethylamino moieties with a pKₐ of 3.12, could turn on its fluorescence upon protonation of its dimethylamino groups in water with pH below 4.5 (Figure 1a–b; Scheme S1, Supporting Information), whose detailed protonation-induced emission mechanism was revealed by molecular calculation, based on a density function theory (as shown in Figure 1c). Substituted dimethylamine group of BOPIM-N showed highest occupied molecular orbital (HOMO) character to induce charge transfer to BOPIM main body as lowest unoccupied molecular orbital (LUMO), which was characterized as twisted intramolecular charge transfer. Thus, the fluorescence of BOPIM-N was greatly inhibited. Interestingly, the protonated dimethylamino groups had LUMO character while BOPIM main body acted HOMO, which greatly impaired electron transfer and led to fluorescence recovery. Hence, its fluorescence signals could be modulated at pH 3.8–4.5, which could be easily observed by naked eye (as shown in Figure 1a, left panel). More importantly, this fluorescence change was reversible, which favored their bio-sensing applications. Meanwhile, control BOPIM-c without dimethylamino moieties was not able to

![Figure 1. Fluorescence properties and in vitro imaging of different cancer cell lines and non-cancer cells after the addition of BOPIM-N or BOPIM-c. a) Fluorescence images of BOPIM-N or BOPIM-c probes with response to different pH environments. b) pH-dependent fluorescence intensity changes of acidic pH-activatable BOPIM-N (20 µm) are presented. c) Frontier molecular orbitals optimized at the B3LYP functional with 6–31G (d, p) basis set, to further clarify the mechanism of fluorescence enhancement. HOMO and LUMO of BOPIM-N and BOPIM-N+2H⁺, and electrostatic potential surface maps over an electronic isodensity of 0.000400e Å⁻³ at B3LYP/6-31G* level were simulated to show well charge separation state in BOPIM-N but not in that upon protonation. Positive charge-rich region is dark blue. d) Confocal microscope images of HepG2, A549, HeLa, and LO2 cells obtained after the addition of the BOPIM-N for 3 h. e) Confocal microscope images of HepG2, A549, HeLa, and LO2 cells were obtained after the addition of the BOPIM-N for 3 h.](image-url)
show pH-dependent fluorescence change (Figure 1a, right panel). To be highlighted, with solvent pH reduction from 4.5 to 3.8, more than 500 times of maximum emission intensity increase at 520 nm was observed (Figure 1b; Figure S1, Supporting Information), indicating its promising application as specific light ON probe in the lysosome of cancer cells with pH below 4.5. Furthermore, BOPIM-N displayed a reversible and fast responsive fluorescence emission between pH 3.8 and 7.4, and satisfactory fluorescence stability in physiological intracellular species, such as ions, saccharides or proteins (as shown in Figure S2 in the Supporting Information). In short, BOPIM-N might act as a fluorescent pH sensor with precise control of the fluorescence OFF–ON switch at pH 3.8–4.5, which favored its lysosome imaging applications for specific cancer cell imaging.

Next, the selective response of BOPIM-N in living cancer cell was demonstrated in in vitro study with cellular level precision before in vivo tumor diagnosis application (Figure 1d,e). BOPIM-N, with specific pH-activatable “OFF–ON” fluorescence switch ability upon protonation of dimethylaminophenyl groups at pH below 4.5, could specifically emit green fluorescence in a number of cancer cell lines from different organs (e.g., HepG2 liver cancer, A549 lung cancer, or HeLa cervical cancer cells), but not in normal cells (e.g., LO2 liver cells). Fluorescent images of cells, with incubation of BOPIM-N or BOPIM-c for 3 h, were obtained. BOPIM-N gave strong green emission in HepG2, A549, and HeLa cancer cells, but no signal was observed in LO2 normal cells (as shown in Figure 1d), indicating that BOPIM-N could act as a specific tracker in living cancer cells but not in normal ones. Significantly different from pH-activatable probe, BOPIM-c could even show strong fluorescence in both cancer cells and normal cells (as shown in Figure 1e), indicating that BOPIM-c was not suitable for specific tumor imaging.

To further investigate the subcellular locations of pH-activatable probe BOPIM-N in cancer cells, we imaged HepG2 liver cancer cells with addition of 20 μm BOPIM-N using confocal microscope. BOPIM-N emitted spot-like green fluorescence in HepG2 cancer cells (as shown in Figure 2a, left panel) and matched well with the commercial LysoTracker with an overlap coefficient averaging 0.88 ± 0.02 (0 represents no colocalization and 1 represents a perfect colocalization, as shown in Figure S3a in the Supporting Information), while almost no background emission was observed in LO2 normal cells, indicating that BOPIM-N could not act as a lysosome tracker in normal cells. In contrast, always-on probe BOPIM-c emitted green fluorescence in both HepG2 cancer and LO2 normal cells, regardless of its cellular locations (as shown in Figure 2a, right panel). To confirm the probe fluorescence modulation by pH, we further incubated HepG2 cells with acetic acid supplemented medium, as it was a cell-permeable weak acid. Interestingly, pH-activatable BOPIM-N probe emitted green fluorescent spot with good colocalization with red fluorescent lysosome, before treatment of acetic acid (as shown in Figure S3b in the Supporting Information, left panel). With time going, the green fluorescence signals expanded greatly (Figure S3b in the Supporting Information, right panel), clearly demonstrating the acidification effect and the pH-dependent fluorescence of BOPIM-N. More interestingly, the lysosome pH value could be estimated to be 3.8 in HepG2 cancer cells by calculating the fluorescence intensity of BOPIM-N before and after acetic acid treatment, as shown in the Supporting Information. To confirm that the fluorescence signals only from living cells, HepG2 cancer cells were incubated with either control or pH-activatable reagents and then treated with 30% ethanol. The ethanol treatment led to fast diminishment of BOPIM-N fluorescence signals, where the signals of control BOPIM-c remained unchanged (as shown in Figure S3c in the Supporting Information). Considering the fast cell death due to treatment of ethanol, these results indicated that the signals of pH-activatable BOPIM-N reflected cell viability, which was important for normal function of lysosome. In short, these results showed that pH-dependent activation of BOPIM-N was due to its lysosome accumulation (proposed mechanism as shown in Figure 2b) in cancer cells, and only the viable cancer cells uptaking BOPIM-N in their lysosomes exhibited fluorescence signals, which might be beneficial to cancer diagnosis with minimal background fluorescence.

Subsequently, to examine the potential of BOPIM-N in in vivo tumor diagnosis, we conducted fluorescence imaging of nude mice bearing tumors developed from HepG2 cancer cells with treatments of an always-on BOPIM-c control or a pH-activatable BOPIM-N molecule. In order to increase the tumor uptake of fluorescence probe, polymeric nanoparticles were employed to encapsulate BOPIM molecules due to nanoparticle’s EPR effect for passive accumulation in tumor.[21] In details, poly(PEG/PPG/PLA urethane)s were synthesized according to previous reports[20,22] and utilized to encapsulate BOPIM probes by nanoemulsion technique to form nanoformulated BOPIM-c nanoprobes or BOPIM-N nanoprobes with size of 110–180 nm (as shown in Figure S4 in the Supporting Information), as these nanofomulations are nontoxic, biodegradable, and stable in serum medium, and the loading efficiency was calculated to be 97.7% by characterizing the amount of leaked BOPIM-N after dialysis. Similar to BOPIM-N, nanoformulated BOPIM-N nanoprobes also showed pH-responsive fluorescence emission (as shown in Figure S5 in the Supporting Information), indicating their pH activatable fluorescence light-ON ability without diffusing out BOPIM-N molecules from polymeric nanoparticle cores, which was beneficial to in vivo diagnosis. It was noteworthy that the nanoformulated BOPIM-N nanoprobe exhibited (as shown in Figure S5d in the Supporting Information) a fluorescent emission red shift when increasing the pH value from 2 to 8, in contrast to the fluorescent emission of BOPIM-N without polymeric nanoparticle protection (as shown in Figure S1a in the Supporting Information).

Furthermore, the encapsulation of BOPIM-N in polymeric nanoparticles was found to increase their cellular uptake (as shown in Figure S6 in the Supporting Information). In order to investigate the possible cellular uptake pathway of nanoformulated BOPIM-N nanoprobes, we conducted a study of cellular uptake of nanoformulated BOPIM-N nanoprobes with coloading of Rhodamine B in the polymeric nanoparticles at 37 °C, 4 °C, and with sodium azide (NaN₃) as adenosine triphosphate (ATP) inhibitor. As shown in Figure S7 in the Supporting Information, inhibition of protein or enzyme activity at 4 °C or inhibition of ATP synthesis by NaN₃ could significantly reduce the active uptake of BOPIM-N nanoprobes, indicating that BOPIM-N nanoprobes were taken up into HepG2 cancer cells via an energy-dependent
endocytosis, which could benefit their accumulation in lysosome.\textsuperscript{[20]} In addition, flow cytometry was also conducted to characterize the number of LO2 and HepG2 cells with uptake of fluorescent BOPIM-c and BOPIM-N nanoprobes (Figure S8, Supporting Information). The experimental results showed that LO2 cells could uptake lots of always-ON BOPIM-c nanoprobes, indicating that the nanoprobes could be internalized by LO2 cells (more than 97% cells with fluorescence signals from BOPIM-c nanoprobes). By contrast, the ratio of LO2 cells with fluorescence signals from BOPIM-N nanoprobes (similar polymeric nanoparticles with encapsulation of BOPIM-N) was only less than 3%, indicating that the specific light-ON of BOPIM-N nanoprobe was not due to the endocytosis amount difference.

By taking the above advantages as well as nanoformulation's possible EPR effect, these nanoprobes were vein tail injected into nude mice bearing solid tumors, to investigate their cancer diagnosis ability. Interestingly, the control BOPIM-c nanoprobes exhibited undesired signals from normal tissues, while BOPIM-N nanoprobes showed specific signals in HepG2 tumors (Figure 3a). Furthermore, we found that the fluorescence signal of BOPIM-N nanoprobes from tumors of nude mice increased with increasing injection time, indicating the increasing uptake of NPs through blood vessel up to 5 h post-injection (as shown in Figure 3a; Figure S9 in the Supporting Information).

The ex vivo fluorescence images of various organs and tissues from HepG2 tumor-bearing mice intravenously injected

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**Figure 2.** The proposed mechanism of acidic lysosome activatable probe in cancer cell. a) Fluorescence confocal images of HepG2 cells with probes BOPIM-N or BOPIM-c (green channel), costained with commercial LysoTracker-Red RB-EDA to visualize the location of lysosomes. b) A schematic illustration of BOPIM-N specific light-ON in cancer cells. Nonfluorescent probes were uptaken by cancer cells with accumulation in lysosomes, which provided unique acidic environment for specific probe light-ON.
with BOPIM-N nanoprobes were taken at 5 h postinjection (Figure 3b). The main tissues including heart, liver, spleen, lung, kidney, and tumor were isolated to study the fluorescence signal distribution of the BOPIM-N nanoprobes. Obviously, fluorescence signal was only observed in solid tumor tissues, while there was almost no detectable fluorescence from other tissues. More importantly, hematoxylin & eosin (HE) staining and confocal images of tumor tissue sectioning from mice treated with BOPIM-N nanoprobes showed strong fluorescence signals from cytoplasm of viable cancer cells (Figure 3c), indicating a similar light-ON mechanism as that in in vitro study (Figure 2) and highlighting the detection of tumor at cellular level by using this novel nanoprobe. These results suggested that BOPIM-N remained its OFF or quenched state at normal tissues, while its fluorescence could be restored only with uptake of acidic lysosome in tumors with cellular precision, by combining the advantages of nanoparticles' enhanced accumulation in tumor as well as BOPIM-N's specific pH activatable light-ON ability.

As biocompatibility of probe was important for its safe biomedical application,[19,23] the cytotoxicity of BOPIM derivatives as well as their nanofomulations was assessed by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (as shown in Figure S4 in the Supporting Information). The viability of HEK293 normal cells or HepG2 liver cancer cells remained around 90% even treated with 100 µm BOPIM-N.
or BOPIM-N nanoprobes for 24 h, demonstrated its good biocompatibility of BOPIM probe in vitro. This result revealed that BOPIM-N showed significantly low cytotoxicity to the normal cells, thus holding great promise to serve as a safe and efficient fluorescent probe for in vivo applications.

In conclusion, a novel pH-activatable BOPIM-N probe has been rationally designed to control its fluorescence switch OFF-ON pH value by carefully modulating its electron donor groups. It was demonstrated that such small molecular probe BOPIM-N could efficiently differentiate various types of cancer cells from normal cells, by specific fluorescence ON in response to the abnormal lysosomal acidity of cancer cells. More importantly, nanoformulated BOPIM-N nanoprobes with size of 110–180 nm in biocompatible and biodegradable poly(PEG/PPG/PLA urethane)s polymers could synergistically improve signal-to-noise ratio and efficiently identify tumor tissues in vivo with resolution up to cellular level, thereby promising new opportunities for non-invasive in vivo tumor detection, as well as contributing to accurate tumor diagnosis for precision medicine and a better understanding of tumor progression. Furthermore, BOPIM derivatives hold the potential for deep tumor imaging after further modification of electron acceptor core to be near-infrared probe.

**Experimental Section**

**Synthesis and Characterization of BOPIM-N:** The corresponding fabrication of BOPIM-N was by using the interaction between 4-dimethylaminobenzaldehyde and 2-cyano-pyridine, as well as its further modification by adding Et3N and BF3∙OEt2 in anhydrous CH2Cl2 and purification by a silica gel column. And the stock solution of BOPIM-N according to literature procedure.[24] In details, 10 mmol ligand was evaporated, before silica column purification (yield 45%). 1H NMR, 13C NMR, and mass spectrum characterization of product could be found in the Supporting Information.

**Preparation of BOPIM-N Nanoprobe:** Biodegradable and biocompatible poly(PEG/PPG/PLA urethane)s were obtained as described.[17c,23a] In details, 0.3 g PLA-diol, 1.8 g PEG, and 0.9 g PPG were conjugated with assistance of hexamethylene disocyanate and trace amount of dibutyltin dilaurate in 1 mL 1,2-dichloroethane for 48 h. The resultant crude product was purified by precipitation in mixture of methanol and diethyl ether (yield, 80%). 1H NMR and 13C NMR of products could be found in the Supporting Information.

Furthermore, BOPIM-N (1 mg) was dissolved in CHCl3 (0.5 mL), mixed with poly(PEG/PPG/PLA urethane)s (4 mg). The resulting solution was added dropwise to a 10 mL Pluronic F127 aqueous solution under vigorous shaking for 12 h to evaporate organic solvent. After that, the BOPIM-N-loaded nanoparticles (named BOPIM-N nanoprobes) were filtered and the amount of BOPIM-N (yield, 90%) was determined by UV absorption. The particle size was analyzed by Hitachi SU-70 scanning electron microscope and Malvern Nano-Sizer.

**Confocal Imaging:** Imaging of internalization of BOPIM-N and BOPIM-c into HepG2, A549, Hela, and LO2 cells was taken by confocal microscope. A solution of BOPIM-N or BOPIM-c (green channel) with concentration of 20 μM in phosphate-buffered saline (PBS) was added into culture medium for 3 h before imaging. Furthermore, LysoTracker-Red RB-EDA (red channel) was added in the medium with a dilution ratio of 1000:1 for 1 h. The treated cells were washed, before further visualization by using a Zeiss LSM5 EXCITER Confocal Laser Scanning Microscope equipped with a Plan Apo ×40/1.3 objective lens (Zeiss).

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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**Conflict of Interest**

The authors declare no conflict of interest.

**Keywords**

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