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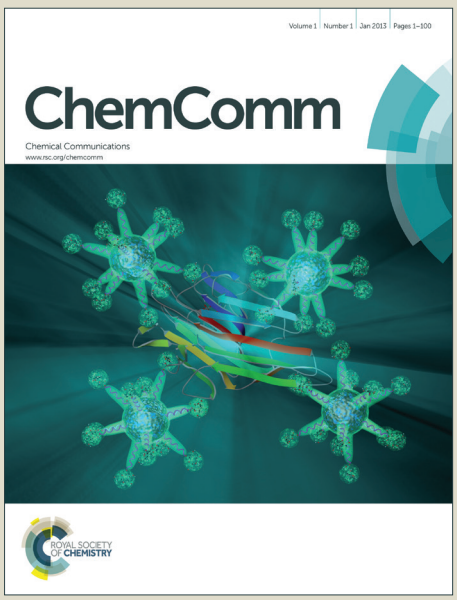

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A Near-Infrared Multifunctional Fluorescent Probe with Inherent Tumor-targeting Property for Bioimaging

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A mitochondria-targeting probe by conjugating a quaternary ammonium cation with glucosamine modified pH-activated cyanine was designed and synthesized. This probe has excellent selectivity and sensitivity toward pH, stability, cellular membrane permeability and low cytotoxicity. Owing the acidic feature of tumor and the more negative mitochondrial membrane potential of tumor cells than that of normal cells, this probe can selectively accumulate in tumor cells and light up its fluorescence. It has been successfully applied for *in vivo* tumor imaging with high signal-to-noise ratio. Moreover, this multifunctional switchable sensor was also employed for fluorescent imaging of the fluctuation of intracellular pH in HeLa cells.

Tumor is one of diseases seriously threatening human life and health. It has been demonstrated that early diagnosis is crucial to improve the survival rates of cancer patients.[1] Thus, the development of effective methodologies with sufficient specificity and sensitivity for visualization of tumor in their early stages is highly desirable but remains a considerable challenge.

Optical imaging has emerged as a potential tool for *in vivo* tumor imaging by virtues of noninvasive, nonradioactive, real-time imaging, sensitive, convenient and visualized.[2] However, the current strategy for constructing active tumor-targeting fluorescent probes requires chemical or biological conjugation of probes with appropriate tumor-targeting ligands including peptides, proteins, antibodies, aptamers and so on.[3] For these approaches, these sensors could be targeted only to specific cancer cell types and the conjugation may also alter the specificity, affinity and the distribution of these agents.[4] Hence, developing innovative strategies to overcome these limitations is urgent. Recently, some near-infrared (NIR) heptamethine indocyanine dyes (lipophilic cations) have been identified with preferential mitochondria-targeting accumulation property of tumor cells owing to the higher magnitude of mitochondrial membrane potential in tumor cells than that of normal cells.[5] This finding will broaden the current approach for the construction of tumor-targeting contrast agents. Unfortunately, these reported dyes are "always on" type agents, their high background autofluorescence signals would result in a low signal-to-noise ratio (SNR) and even the "false positive" results when circulating in the blood stream.[6] Therefore, it remains a challenge to acquire excellent probes with sufficient specificity and sensitivity for satisfactory tumor imaging.

Compared to the "always on" probes, pH-responsive fluorescent probes which were designed according to the acidic feature of tumor (universal phenomena of all solid tumors[7]) can avoid the "false positive" results and offer a high level of selectivity, has recently received considerable attention.[8] Based on this, we present herein a novel, biocompatible, inherent tumor-targeting and specific NIR pH-activated probe (**10**) for *in vitro* and *in vivo* tumor imaging. This probe was constructed by employing a non-*N*-alkylated NIR indolium cyanine as the selective indicator for tumor with a dramatic off-on fluorescence response signal and a quaternary ammonium cation as the biomarker for mitochondria of tumor as well as the hydrophilic group (as illustrated schematically in scheme1). The cationic group was expected to facilitate the accumulation of probe in mitochondria of tumor to achieve the tumor-targeting due to the higher mitochondria membrane potential in tumor cells. For further improving probe's biocompatibility and aqueous solubility, glucosamine functional group was also introduced. In this study, an analogue of **10** (compound **7**, shown in scheme1), lacking the lipophilic cations was also designed and synthesized as a control to investigate the effect of cationic group of probe **10** on cellular distribution.

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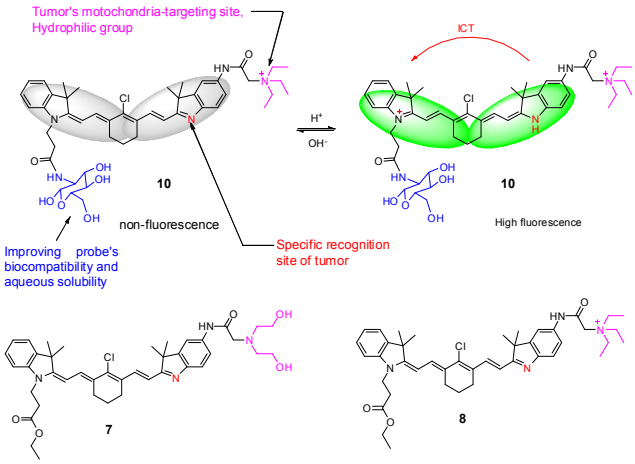

Scheme1 Proposed mechanism of probe **10** for tumor-targeting and specific imaging, and the structures of the control systems (analogue **7** and intermediate **8**).

Probe **10** and **7** were prepared via the synthetic routes outlined in scheme S1 (ESI†). Briefly, compounds **1** and **2** were prepared according to our previously reported method,[9] and then these compounds were treated with **3** in ethanol to yield compound **4**. Reduction of **4** with tin(II) chloride dihydrate offered compound **5**. Acylation of **5** with chloroacetyl chloride afforded **6** in a satisfied yield. Diethanolamine or TEA was subsequently employed in the nucleophilic substitution of compound **6** to present compound **7** or compound **8**. After the hydrolyzation of compound **8** to acid **9**, amide **10** was obtained from **9** and D-glucosamine hydrochloride through the formation **9**-NHS active ester in the presence of *N,N*-diisopropylethylamine. The structures of target compound and intermediates were confirmed by NMR and HRMS.

To demonstrate our rationale, the optical responses of probe **10** and the control probes (compound **7** and **8**) upon pH were firstly evaluated in its ethanol-water (2/8, V/V) solution. As shown in Fig.1, for the compound **10**, under alkaline medium, only an absorption maximum at 525 nm was observed, while as the pH decreased, a new absorption peak appeared at 790 nm and progressively increased with a distinct isosbestic point being observed at 608 nm. Meanwhile, the color of the solution changed from pink to pale green, which indicated that this probe could serve as a visual indicator for medium pH. The fluorescence spectral of the probe upon pH values were depicted in Fig.2. Upon excitation at 760 nm, an emission band at 814 nm appeared and underwent a concomitant monotonic increase with the decrease of medium pH from 11.70 to 2.53. The same trends were observed in compounds 7 and **8** (as shown in Fig.S1 and Fig.S2, ESI†). The pH-dependent spectral response arises from the protonation and deproronation of the indole nitrogen atoms. The ICT process was enhanced upon *N* atoms protonation, which results in the bathochromic shift of the absorption maxima and the remarkable enhancement of fluorescence emission. Notably, a sharp approximate linear fluorescence intensity increase (about 4-fold enhancement) occurred within the narrow range of pH 7.48～6.02 (Fig.2 (B)), which cover both physiological conditions of tumor and normal tissue, making this pH-sensitive probe a promising fluorescence indicator for monitoring slight pH fluctuation in physiological environments and imaging of tumor.

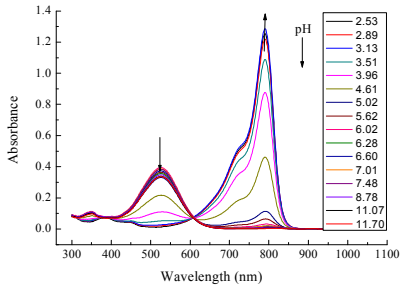

Fig.1 UV-Vis spectra of compound **10** (1×10^{-5} M) upon pH in ethanol-water (2/8, V/V) solution

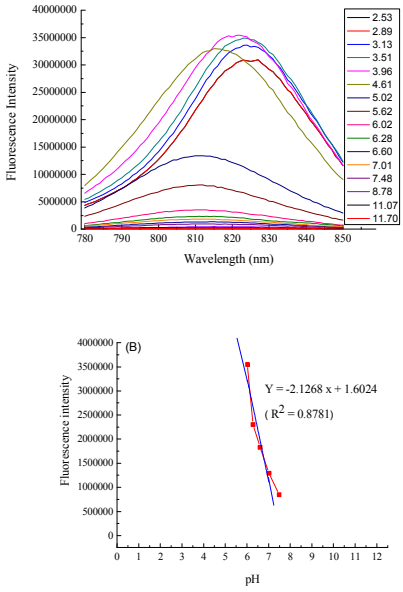

Fig.2 (A) Fluorescence spectra of compound **10** (1×10^{-5} M) at different pH values. (B) Changes in the fluorescence intensity at 814 nm upon pH (6.02 to 7.48).

The photostability of **10** (1×10^{-5} M) was then measured by using a 254 nm UV lamp as an excitation source. Its time-dependent fluorescence spectra at pH = 4.01 were recorded during 60 min. As shown in Fig.3, almost no changes of fluorescence intensity were observed within the scanning period, confirming that this probe was stable to medium, light and air. Moreover, it displayed a strong reversibility when the solution was circularly adjusted forth and back between 3.0 and 9.0 (Fig.4). From these results, we could conclude that this probe exhibits reversible response to H^+ and exports a steady singal, indicating that this probe could serve as a switch on sensor for practical application. Furthermore, the photophysical properties of compound 10 at varying pH, including absolute fluorescence quantum yield (ϕ_f) and fluorescence lifetime (FLT) were measured (as shown in Tab.1 and Fig.S3, ESI†).

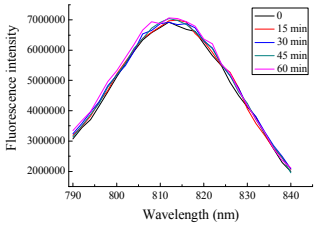

Fig.3 Time-depnedent changes of fluorescence spectra of **10** (1×10^{-5} M) at pH=4.01 in ethanol-water (2/8, V/V) solution during 60 min

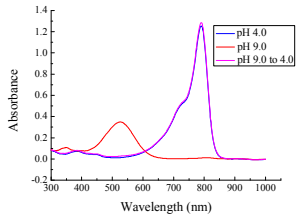

Fig.4 Reversible changes of the absorption spectra of **10** (1×10^{-5} M) between pH 3.0 and 9.0 in ethanol-water (2/8, V/V) solution

Tab.1 Fluorescence quantum yield and fluorescence lifetime of **10** in in ethanol-water (2/8, V/V) solution at varying pH

Entry	pH=3	pH=4	pH=5	pH=6	pH=7
ϕ_f	0.0264	0.0221	0.0193	0.0159	-
FLT/(ns)	0.6797	0.6601	0.6436	0.5909	-

- Fluorescence intensity was too low for fluorescence lifetime and fluorescence quantum yield meacurement.

Subsequently, the selectivity of probe **10** (1×10^{-5} M) was evaluated at pH = 4.01 with a wide array of the possible competitive species, including metal ions (Na^+, K^+, Mg^{2+}, Zn^{2+} and Ca^{2+}) made from their chloride salts, H_2O_2 as well as metabolic thiols GSH, Lys, Hcy (1×10^{-5} M, respectively). As expected (Fig.S4, ESI†), no remarkable changes were observed upon addition of these potential interferents, enabling itself to be a promising pH-sensitive probe for practical application in a complex environment. Moreover, it was worth noting that this probe's excitation and emission wavelengths all located in the region of "biological window",[10] which render it preferable for *in vivo* imaging by the virtues of deep tissue penetration and low background autofluorescence.[11]

Encouraged by the above results, the capacity of this probe for monitoring the fluctuation of intracelllar pH in living cells was explored. And its cytotoxicity towards HeLa cells were firstly evaluated using standard cell viability protocols (MTT assay).[12] HeLa cells were incubated for 24 h and then treated with **10** for 24 h with various concentrations (0.5, 1, 5 and 10 *equiv.* of testing concentration (1×10^{-5} M)). As shown in Fig.S5 (ESI†), upon incubation with a significantly high concentration of probe (5 *equiv.* of testing concentration, 5×10^{-5} M), the cell viability was still well and the survival rate is higher than 70%, indicating that this probe was suitable for living cell imaging. And then HeLa cells were incubated with **10** in a 6-well plate and the intracellular pH of the cells were adjusted by HCl from 7.4 to 4.0. The confocal fluorescence imaging was carried out subsequently. Interestingly, as can be seen from Fig.6, almost non-fluorescence was observed in the HeLa cells in normal culture medium (pH 7.4), while the intracellular fluorescent signal increased greatly as the cellular pH decreased from 7.4 to 4.0. The fluorescence switch on manner and the fast response strongly suggested that this probe could serve as a switchable sensor for real-time monitoring of the pH changes in living cells and has a potential for studying pH-related biological processes. Moreover, since the physiological conditions of tumor and normal tissues just fall into the pH region of 6.0 to 7.4,[12(a),13] the results also indicated that this probe was a promising switchable indicator for tumor specific imaging.

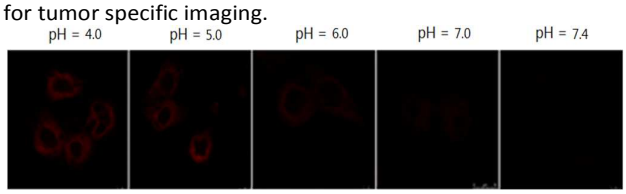

Fig.6 Confocal microscopy images of probe **10** in HeLa cells at pH 4.0, 5.0, 6.0, 7.0 and 7.4, respectively

To further prove our concept, the localization experiment of **10** in HeLa cells was performed with a membrance potential dependent mitochondrial tacker, rhodamine 123 (Rh123).[14] As can be seen from Fig.7, the fluorescence image region produced by **10** overlaps well with that obtained by Rh123 (upper row in Fig.7). And as expected, similar results were obtained with the intermediate (compound **8**). In contrast, with compound **7**, a control system lacking the cation unit, a poor overlap was observed (under row, Fig.7). Besides, the fluorescence residences of **10** and **7** in HeLa cells were also investigated. In this experiment, HeLa cells were separately preincubated with media containing **10** or **7** (1×10^{-5} M) for 2 h at 37 ℃, then the media was replaced by PBS buffer and incubated for additional 1 h and 5 h in fresh culture media at 37 ℃, respectively. The results show that fluorescent signal produced by **10** was retained even after 5 h, while the fluorescent signal of **7** faded with time. From these remarkable differences, we can conclude that probe **10** could selectively accumulate in the mitochondria of tumor cells, is likely attributed to the higher mitochondrial membrane potential in tumor cells. The electrostatic interaction between the cationic probe and the negatively charged inner surface of mitochondria may extend the residence of this probe. The above results further demonstrated that the chemistry strategy for fabrication of the inherent tumor-targeting probe is feasible, and this design strategy could broaden the current approach of the construction of tumor specific contrast agents.

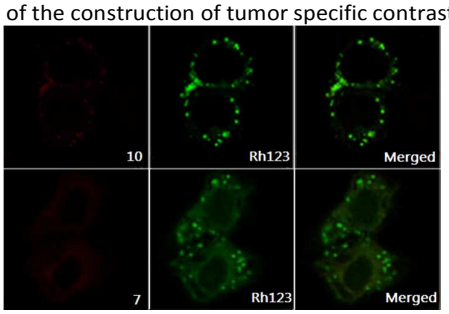

Fig.7 Cofocal microscopy images of **10** with Rh123 (upper row) and 7 with Rh123 (under row) in HeLa cells

Having achieved successful cell imaging, a further effort was made to determine whether this probe could be competent for *in vivo* tumor imaging. Probe **10** (1×10^{-5} M in PBS containing 1% DMSO, 200μL) was injected *in situ* into the tumor-bearing nude mice, followed by real-time monitoring by a Bethold Night OWL LB 983 *in vivo* Imaging System.[15] For control, **10** of the same amount was subcutaneously injected into the homologous normal tissues (left hind limb). Interestingly, as shown in Fig.8, a remarkable NIR fluorescent signal focusing on the tumor was immediately detected upon injection this probe into the tumor-bearing nude mice. And the fluorescent signal in tumor was detectable for up to 24 h. While almost no signal was detected in the control tissues during the whole process. The above results demonstrated that this probe can be fast and specifically activated by tumor cells with excellent optical properties, indicating a fairly bright application prospect in *in vivo* tumor imaging. The aforementioned results confirmed that this probe hold great potential as a versatile molecular probe platform for *in vivo* tumor imaging, and the further *in vivo* studies are currently underway.

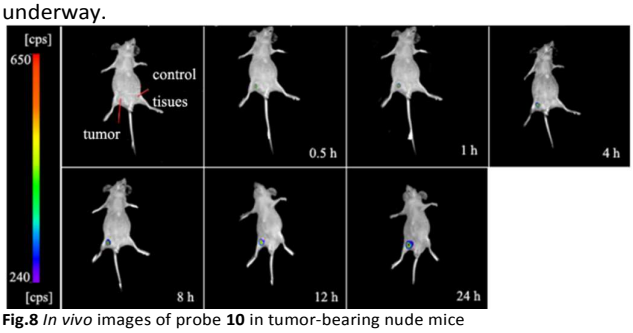

Fig.8 *In vivo* images of probe **10** in tumor-bearing nude mice

In summary, we have developed a novel multifunctional NIR pH-activated fluorescent probe by a simple and flexible strategy for bioimaging of cells and tumor-bearing mice. This probe has such outstanding properties as excellent selectivity and sensitivity toward pH, stability, rapid response time, cellular membrane permeability and low cytotoxicity, and as a result, it was successfully employed to monitor the fluctuation of intracellular pH in living cells with fluorescence switch on manner. Furthermore, this probe can preferentially accumulate in the mitochondria of tumor cell and it was also successfully applied to *in vivo* tumor-targeting imaging with superior signal-to-noise ratio. The results of this effort strongly suggest that the probe constructed by introduction the quaternary ammonium cation to the backbone of a pH-activated NIR probe can serve as an effective tool for *in vivo* imaging of tumor. Moreover, the design of this strategy could broaden the current approach of the construction of tumor specific contrast agents.

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