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ARTICLE TYPE

Easily fixed simple small ESIPT molecule with aggregation induced emission for fast and photostable "turn-on" bioimaging

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An excited state intramolecular proton transfer (ESIPT) fluorogen, due to its easy hydrogen bonding and AIE characteristics, has been demonstrated a fast and selective light-up of the cytoplasm of HeLa cells within 20 seconds. Besides, the low-cost material preparation, fair cytocompatibility, good photostablilty and wide pH application range all indicate its great potentials for commercialization.

The development of chromophore dyes with high sensitivity, noninvasiveness and biocompatibility is of great scientific value and has important practical implications in biomedical imaging and biotechnology in that it offers a direct visualization tool for detecting biological molecules and monitoring biological events in living cells.[1-4]

It is well known that fluorescent dyes such as inorganic quantum dots (QDs),[5,6] fluorescent proteins,[7]and upconversion nanophosphors (UCNP)[8] have been widely investigated as fluorescent probes for fluorescence bioimaging. However, inorganic quantum dots (QDs) are highly cytotoxic in the oxidative environment because of their formation of heavy metals and chalcogens, which greatly limits them in vivo applications.[9-11] Fluorescent proteins such as green fluorescent protein (GFP) and its variants suffer from inherent susceptibility to proteolytic enzymes, severe spectral overlap with biosubstrate autofluorescence, and poor photostability, resulting in the instability in biological environment.[12] For the UCNP, extremely low upconversion efficiency and complicated processing technique hinder their developments and applications.[8] In comparison, small-molecule fluorescent and phosphorescent organic dyes[13] offer a unique approach due to their low toxicity, high sensitivity, fast response time and technical simplicity characteristics.[14] As we know, phosphorescent materials have the shortcomings of large molecular volume, cytotoxicity, rare source and high cost.[15-17] Meanwhile, most nomadic organic fluorophores suffer from severe luminescence quenching when accumulating in living cells,[18,19] intense photobleaching, and easily exuding from cells.[20-22] It is thus envisioned to develop organic dyes exhibiting intense luminescence even when accumulated, high photostability, high selectivity and in-cell fixity.

In recent decades, a novel series of bioimaging and biosensing dyes exhibiting unique characteristics of aggregation-induced emission (AIE) have been discovered.[23-25] These AIE dyes are non-emissive in molecularly-dispersed solution, and become highly luminescent in aggregated state (for example, nanoparticles, powders and crystals). The AIE mechanism at the single molecular level can be attributed to the restriction of the intramolecular rotations (RIR),[26-28] restriction of intramolecular charge transfer (ICT)[29] or twisted intramolecular charge transfer (TICT),[30,31] and restriction of cis-trans isomerization.[32] At supramolecular level, the AIE characteristics can be related to some specific molecular packing types, such as dimer/excimer stacking,[33,34]J-aggregation,[35] herringbone stacking,[36] or even the weakly coupled H-aggregation.[37] Particularly, the AIE phenomenon has been demonstrated to be useful in monitoring activities of various biomolecule detection.[38-40] These assays rely on previously "dark" AIE molecules combining with bio-molecules through noncovalent interactions (such as hydrophobic or electrostatic interaction) to yield highly emissive aggregated complexes.

As we know, for the purpose of biomedical imaging detection, the internalization of cell dye should be as quick as possible. However, to the best of our knowledge, the cells generally internalized the majority of fluorescent bioimaging dyes within 10 min to 2 h,[41-47] which means a relatively long detection. This is possibly because it is not easy for relatively large molecules or particles to penetrate membrane through the 0.4~1nm pores.[48] Many efforts have been made to develop more sensitive chromophores for cellular quick-imaging studies.[49,50] Very few fluorescent dyes have been reported to be internalized by the cells within 1~2 min,[51] indicating a rapid uptake. Only some algal cells were reported to internalize fluorescent dyes within several seconds.[52]

In our previous work, a series of AIE-active excited state intramolecular proton transfer (ESIPT) compounds have been developed with in-depth investigation of their AIE mechanisms.[53-55] ESIPT molecules are normally more stable as enol forms in the ground state and more stable as keto forms in the excited state. In the ESIPT process, an extremely fast four-level photophysical cycle (E-E*-K*-KE), mediated by intramolecular H-bonds, occurs immediately after photoexcitation.[56] Therefore, an abnormally large Stokes shift without self-absorption is detected, which is beneficial to AIE characteristics. Here, we have developed an easily synthesized organic small-molecule ESIPT probe with strong luminescence, which displays AIE characteristic and bears hydrogen bonding group to realize fast, selective and photostable "turn-on"

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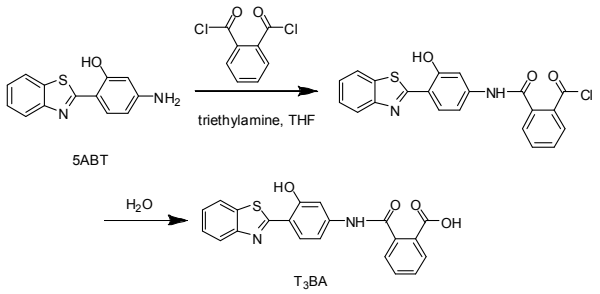
bioimaging. Featured by short response time of 20 seconds *via* quick penetration, boosting fluorescence when fixed and concentrated in cytoplasm *via* hydrogen bonding, and photostable emission under intense illumination, the probe performed as an excellent fluorescent bio imaging dye, which rapidly and selectively stained cytoplasm of living HeLa cells without interfering with their proliferation processes.



Scheme 1.Synthetic route of T3BA

The **T3BA** compound has been synthesized by single-armed acylation of phthaloyl dichloride with 5-amino-2-(benzo[d]thiazol-2-yl)phenol (5ABT),[53] due to decreased reaction activity of further acylation (Scheme 1). **T3BA** is soluble in THF, DMF and DMSO, but exhibits poor solubility in water. The AIE phenomenon can be easily observed by the naked eyes. The THF solution was almost non-emissive, whereas its powder or nanoparticles dispersed in water exhibited a significantly enhanced emission (Fig. 1 inset). The fluorescence quantum efficiency of its THF solution and powder were measured as 0.08 % and 4 %, respectively.

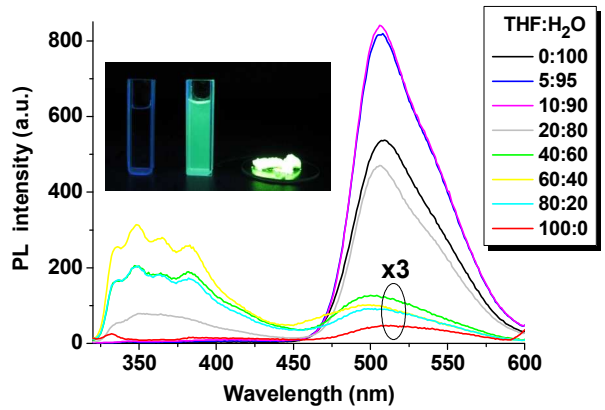


Fig. 1 Fluorescence spectra of 10^{-5} M T3BA in the THF/H_2O mixture at different water fractions. Inset: photographs of 10^{-5} M T3BA under UV illumination at 365 nm in THF solution, water, and powder from left to right, respectively.

In THF solution, **T3BA** mainly exhibited two emissions around 320~420 nm and 470~600 nm, respectively. The emission around 320~420 nm corresponds to the enol emission, whereas the low-energy emission peaked at 506~513 nm corresponds to the keto emission.[53] When the water fraction increased gradually from 0% to 60%, the fluorescence intensity only slightly increased. Further increasing the water fraction to 80%, the emission enhancement suddenly became evident, indicating formation of nanoparticles which exhibited prominent AIE characteristics. Meanwhile, the absorption spectrum exhibited a sudden decrease of intensity and change of spectrum profile with increase of the relative absorption around 300 nm (Fig. S3). The maximum fluorescence efficiency of the nanoaggregates achieved 90% water content, which was enhanced by 52 times higher than that of the molecularly dispersed THF solution. In the nanoparticles, the enol emission around 320~420 nm gradually disappeared, while the keto emission around 470~600 nm became the dominant emission.

Cytotoxicity assay with the cultural concentrations of **T3BA** at 5, 10, 25, 50, 75, 100 μM in DMSO buffer (Fig. S4) demonstrated that more than 93% HeLa cells survive with the cultural concentration of **T3BA** lower than 50μM, and more than 85% still survive with higher cultural concentration of 100 μM. Repeated experiments indicated that, with a low cultural concentration of 5μM **T3BA**, the dye shows an unexpected result with the viabilities of HeLa cells more than 105 %, probably due to the growth promotion effect of low concentration of **T3BA**, which needs further investigation. These results implied that **T3BA** displays excellent biocompatibility and non-toxicity, and can be utilized in living cell as an excellent imaging agent.

As anticipated, these small **T3BA** molecules can quickly penetrate cell membranes and lead to fast fluorescence staining, as demonstrated by confocal fluorescence microscopy experiments (Fig. 2). The luminescence boosting in the cytoplasm of cells can be rapidly detected within 20 s (Fig. 3), indicating great potentials for instant clinical diagnosis. The overlay of luminescent images and bright-field images confirmed that **T3BA** were located mainly in the cytoplasm of cells but not in the membrane and nucleus. This is possibly because the caboxyl group of **T3BA** can easily form hydrogen bond (such as C=O···OH, (C=O)OH···O=C, and (C=O)OH···O-H) with the active constituents in cytoplasm (such as amino acid, glycogen, polyhydroxybutyrate, lipid), and thus fixed and concentrated in cytoplasm with boosting emission immediately after these small **T3BA** molecules penetrate the membrane. Therefore, due to the AIE characteristics, the rapid and selective light-up of the cytoplasm of HeLa cells can be observed (Scheme 2). Consistently, quantification by line plots revealed that the fluorescence intensity of the cytoplasm (region 3 and 1) is much higher than that of the nucleus (region 2), indicating selective cytoplasm uptake of **T3BA** (Fig. S5). This **T3BA** uptake process is revealed as energy-independent (Fig. S6), by investigating the location of **T3BA** when active cellular uptake was blocked by incubation at 4 °C or pretreatment with the metabolic inhibitors 2-deoxy-D-glucose and oligomycin.[57,58]

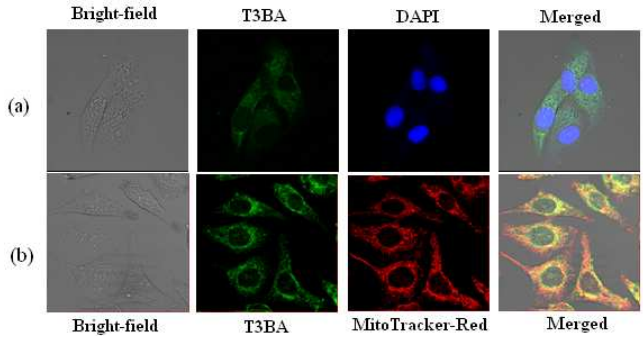


Fig. 2 Confocal luminescence images of living HeLa cells incubated with 20μM of T3BA for 10 min at 37 °C and co-stained with DAPI (a) and MitoTracker Red (b).

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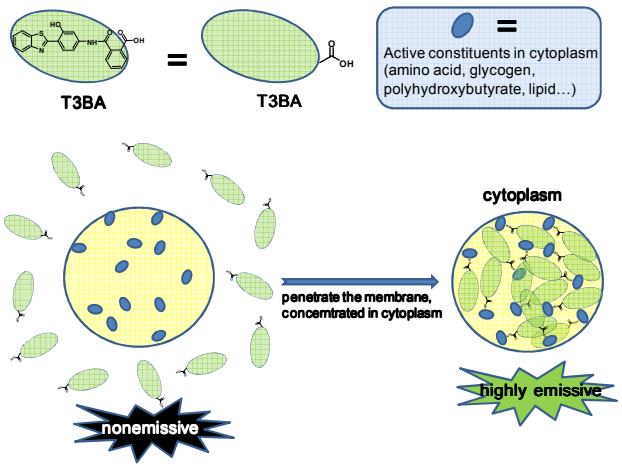

Scheme 2.The imaging mechanism of T3BA in cytoplasm

Under continuous laser scanning upon excitation at 405 nm, the fluorescence intensity decreased less than 10% after 10 minutes continuous laser illumination (Fig. S7), suggesting its good photostability. This is beneficial for bioimaging, especially in long-term studies.[59] The constancy of the fluorescence in living HeLa cells stained with **T3BA** was investigated *via* long time observation for 24 h (Fig. 3). Luminescence in the cytoplasm of cells can be rapidly detected within 20 s. The fluorescence intensity continuously increased over time and reached maximum within 3 min. This indicated that, even without a molecular transporter, small **T3BA** molecules are rapidly internalized by the cells and exhibit selective cytoplasm staining. Even after 24 h incubation, the fluorescence intensity of **T3BA** maintained high and the cells did not divide. The PI staining showed that the **T3BA**-treated cells were still viable. All these results indicated the good constancy of **T3BA** in cell imaging. Moreover, the fluorescence intensity of T3BA in living HeLa cells was barely affected when the pH varied from 4 to 10 (Fig. S8), indicating a good suitability in various cell culture mediums and fluctuating biological environment.

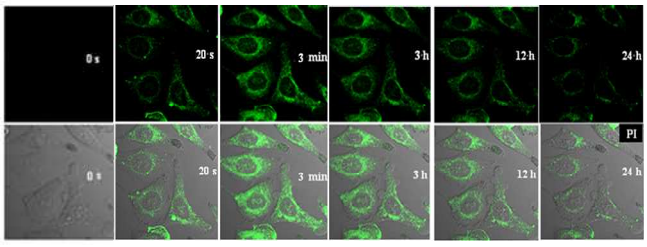

Fig. 3 Time-lapse imaging in situ of HeLa cells at 0 s, 20 s, 3 min, 3 h, 12 h, and 24 h, respectively. Inset: PI staining is included to indicate cell viability.

The signal to noise (S/N) ratio of T3BA is comparable to the commonly commercialized dyes of MitoTracker Red and LysoTracker Red (see Fig. S9). Considering its much simplified molecular structure (compared with MitoTracker Red and LysoTracker Red, see Fig. S10), facile and low-cost synthetic route, as well as fair cytocompatibility, good photostablilty and wide PH application range, T3BA is demonstrated as an attractive candidate as commercialized bioimaging dye.

Conclusions

In this work, a new AIE-active and hydroxyl-bearing small-molecular compound, 5-Amino-2-(benzo[d]thiazol-2-yl)-phenol, has been developed to act as a desirable cell-staining probe. Thanks to the fast responsibility and enhanced emission when concentrated in cell culture media, the probe outperforms as an excellent fluorogen for quick and selective imaging of cytoplasm of living cells without influencing cell proliferation. This indicates that T3BA acts as a promising candidate material for specific staining and targeting of cellular compartments by further modification and improvement.

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Notes and references

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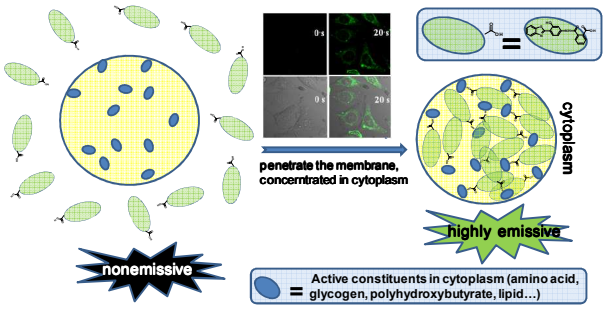

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