Simultaneously and Selectively Imaging a Cytoplasm Membrane and Mitochondria Using a Dual-Colored Aggregation-Induced Emission Probe

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ABSTRACT: Analysis of subcellular organelles (e.g., a cytoplasm membrane and mitochondria) during cellular processes can provide particularly useful information for our understanding of cell chemistry and biology. For this purpose, fluorescent probes capable of dynamically imaging multiple organelles in a simultaneous and selective manner are highly demanded, yet such probes are scarcely reported due to the challenges in molecular design. In this study, we developed a dual-colored aggregation-induced emission (AIE) probe TPNPDA-C12 with twisted intramolecular charge transfer (TICT) to visualize the membrane and mitochondria of the same cells through distinct fluorescence channels simultaneously. We also successfully used the probe to monitor and distinguish the dynamic changes of the organelles during cell apoptosis and necrosis induced by reactive oxygen species (ROS) and cytotoxins.
tracking cellular processes. A handful of dual-colored fluorescent probes have been developed for the nucleus and mitochondria through a difference in charge interactions, as well as lysosomes and lipid droplets via their distinct physiological environment. Nevertheless, no dual-colored fluorescent probe for simultaneously imaging the cell membrane and mitochondria is available so far.

To address the challenges, in this work, we designed and synthesized an amphiphilic and positively charged AIE probe, TPNPDA-C12 (Figure 1a), to image the cytoplasm membrane and mitochondria simultaneously and selectively. Our design of a TPNPDA-C12 molecule was based on three desired properties: (1) amphiphilicity with a critical micelle concentration (CMC) suitable for cell membrane binding along with positives for mitochondria targeting; (2) a combined AIE and twisted intramolecular charge transfer (TICT) mechanism to allow fluorescence color changes in response to different environments in the two organelles upon stimuli; (3) biocompatibility and photostability to monitor the cell processes.

**EXPERIMENTAL SECTION**

**Analytical Procedures.** The stock solution (10 mM) of TPNPDA-C1, TPNPDA-C12, TPNPDA-C15, and TPNPDA-C18 were prepared in ethanol. In a typical detection, 30 μL of stock solutions was added into a test tube, which was diluted to 2.97 mL of ethanol/ethyl acetate (or glycerin or 1× Dulbecco’s phosphate buffered-saline (DPBS) buffer) to get final solutions with different ethyl acetate (or glycerin) fractions from 0 to 99% and the fluorescence spectra were recorded. For TPNPDA-C12, the photograph, UV absorption, and fluorescence spectra of the solutions in different solvents at 100 μM were recorded.

**Living Cell Staining and Imaging.** Cells were grown in a 35 mm Petri dish with a coverslip at 37 °C. Modified staining procedures of TPNPDA-C1, TPNPDA-C12, TPNPDA-C15, and TPNPDA-C18 for most living cell imaging experiments are as follows. Stock solutions (1 mM) of the probes in ethanol were prepared. After that, 10 μL of stock solution was added in 1 mL cultured adherent cells (HeLa, HepG2, or MCF-7) to get a final staining dye concentration at 10 μM. The cells were incubated with the probe at 37 °C. After 10 min, cells were washed three times by 1× DPBS buffer, then 1 mL of a culture medium (Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS)) was added again for living cell imaging. Fluorescent images of the yellow channel were excited by 405 nm laser and collected at 490–560 nm, and the red channel was excited by 488 nm laser and collected at 600–700 nm.

**Colocalization Experiments.** Hela cells were stained by TPNPDA-C12 with a modified staining method and commercial subcellular organelle probes with a standard staining manual. The detailed experimental procedure is provided in the Supporting Information (SI).

**Dynamic Imaging for Apoptosis and Necrosis Induced by ROS (H₂O₂) or Cytotoxin (STS).** HeLa cells were first stained by TPNPDA-C12 with a modified staining method mentioned above. Five microliters of 3% H₂O₂ or 10 μL of 1 mg/mL STS-DMSO stock solution was added and used with a CLSM’s time mode for taking pictures every 2 min for a total of 30 min.

Other experimental details, including materials and instrumentation, synthesis method, calculation, cell culture, cytotoxicity, fluorescence lifetime imaging, and detailed colocalization experiments, are provided in the SI.

**RESULTS AND DISCUSSION**

**Dual-Color Photoluminescence Property Study.** As we expected, TPNPDA-C12 exhibited clear AIE characteristics in a cosolvent solution composed of ethyl acetate (poor solvent) and ethanol (good solvent), emitting fluorescence only in the presence of ≥60% ethyl acetate (Figures 1b,c, and S1). Interestingly, unlike traditional AIE fluorophores, TPNPDA-C12 had dual-colored fluorescence that changed from red to yellow (λ_{max} = 567 nm, quantum yield (QY) = 2.2%) when the ethyl acetate fraction increased from 60 to 99%. The red fluorescence (Figure 1d,e) was also observed in a glycerin solution with minimal ethanol (λ_{max} = 650 nm, QY = 3.2%), as

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**Figure 1.** (a) Chemical structure and the design principle of TPNPDA-C12. (b) Fluorescence intensity of 100 μM TPNPDA-C12 at 567 nm with different ethyl acetate/ethanol volume fractions. λ_{ex} = 373 nm. (c) Photographs of 100 μM TPNPDA-C12 solution with different ethyl acetate/ethanol volume fractions under room light (top) or UV (bottom). (d) Fluorescence intensity of 100 μM TPNPDA-C12 at 650 nm with different glycerin/ethanol volume fractions. λ_{ex} = 500 nm. (e) Photographs of 100 μM TPNPDA-C12 solution with different glycerin/ethanol volume fractions under room light (top) or UV (bottom). (f) Photographs of 100 μM TPNPDA-C12 solution with 99% ethyl acetate or glycerin under room light (left) or UV (right). Fluorescence pictures were taken under a 365 nm UV lamp.
well as an ethanol solution under a low temperature 77 K in liquid nitrogen (Figure S2). Dynamic light scattering (DLS) analysis of the solutions (Figure 2a–c) indicated the formation of molecular aggregates in 99% ethyl acetate (also found as ∼100 nm spherical particles under transmission electron microscopy (TEM) after staining) but neither in ethanol nor in 99% glycerin (<6 nm as a solvent background). These results suggested that the emissions in yellow and red were ascribed to the molecular aggregates and restricted monomers of TPNPDA-C12, respectively. The yellow fluorescence has a longer lifetime (2.3 ns) than that of the red (0.11 ns, Figure 2d), in accordance with the general lifetimes for aggregates. The UV absorption spectra of TPNPDA-C12 in different solvents underwent a red shift with increasing solvent polarity, supporting the role of TICT in the fluorescence color change (Figures S3 and S4). Therefore, we concluded the dual-colored fluorescence of TPNPDA-C12 as a result of both AIE and TICT effects.

Figure 2. (a) Dynamic light scattering (DLS) analysis for 100 μM TPNPDA-C12 in (a) ethanol, (b) 99 vol % glycerin, and (c) 99 vol % ethyl acetate. Inset: the particle size of 100 μM TPNPDA-C12 in 99 vol % ethyl acetate measured by TEM. (d) Fluorescence decay curve and lifetime of 100 μM TPNPDA-C12 in 99 vol % ethyl acetate (pink), 99 vol % PBS buffer (blue), and 99 vol % glycerin (red). (e) Top view and the edge view of the optimized molecular geometry at an S0 state. R represents the C12 alkyl chain. (f) Emission wavelength with different C1−N2−C3−C4 dihedral angles (the red star represents the fully relaxed S1 geometry). Inset: the first pair of natural transition orbitals (NTO) at a D−A perpendicular geometry (left), optimized S1 geometry (middle), and S0 geometry (right). The NTO weights are all larger than 96%.

Figure 3. (a) Confocal fluorescence images of HeLa cells stained with TPNPDA-C12. Y: yellow channel (excited by 405 nm laser and collected at 490−560 nm), R: red channel (excited by 488 nm laser and collected at 600−700 nm), Mer: merged channel, TD: bright field. Scale bar is 20 μm. (b) Schematic of TPNPDA-C12 for simultaneously dual-color imaging the cell membrane and mitochondria. (c) Confocal fluorescence images of HeLa cells for colocalization of TPNPDA-C12 with DiO, MTD, LyDR, and Hoechst. Scale bar is 10 μm.
The microscopic mechanism of the dual-colored fluorescence is investigated with ab initio quantum chemistry calculations (see S1 for the computational details). As the key dihedral angle $\varphi_{C1-N2-C3-C4}$ (Figure 2e) between the donor and the acceptor changes from a perpendicular geometry through a fully relaxed twisted $S_0$ geometry to a planar geometry (close to the $S_0$ state), the emission wavelength is blue-shifted monotonically, which is mainly due to the change of the excitation character from a pure intramolecular charge transfer state to a mixed local excitation/charge transfer state and finally a local excitation dominant state, as shown in Figure 2f. Therefore, because of the well-known aggregation-induced restriction of an intramolecular rotation effect, the D–A dihedral angle in the aggregate is close to that of the near planar $S_0$ geometry, giving a blue shift emission. In addition to the difference in excitation characters of the aggregate as described above, a previous computational work first coined “aggregation-induced blue-shifted emission” due to the reduced relaxation energy of the excited state in the aggregate, which was found to be general for AIE chromophores and not specifically for the D–A molecule in this work. We believe these two effects both contributed to the blue-shifted emission of TPNPDA-C12 in its molecular aggregates.

For the amphiphility of TPNPDA-C12, we measured its critical micelle concentration (CMC) in an aqueous PBS buffer for its potential in staining the cytoplasm membrane. The CMC was calculated based on the curve of the fluorescence intensity depending on the concentration of TPNPDA-C12 in PBS (Figure S5a). The intersection of the two tangent lines gave a CMC value of about 45 $\mu$M, which was also confirmed by DLS analysis of the samples (Figure S5b), showing the presence of particles with the average diameter of 80.5 nm only at concentrations above 45 $\mu$M TPNPDA-C12. The CMC value and micelle size of TPNPDA-C12 in PBS were indeed appropriate for cellular studies. Besides, the fluorescence of TPNPDA-C12 remained unchanged under a pH range of 4.45–9.07 (Figure S5c), indicating its stability in live-cell imaging. On the other hand, we found low cytotoxicity of TPNPDA-C12 even at concentrations as high as 20 $\mu$M for various cell lines (Figures S8 and S9).

Simultaneous and Selective Live-Cell Imaging. Under a confocal laser scanning microscope (CLSM), we were very excited to observe a yellow fluorescence in the cytoplasm (490–560 nm emission channel excited by 405 nm laser) and a red fluorescence in the cell membrane (600–700 nm emission channel by 488 nm laser) in HeLa cells simultaneously using TPNPDA-C12 as the sole probe at 10 $\mu$M (Figure 3a). Based on the dual-colored fluorescence of TPNPDA-C12 showing yellow for molecular aggregates and red for restricted monomers (Figures 1f and S10), we assumed TPNPDA-C12 fused into the phospholipid bilayer of the cell membrane as monomers because of its much higher CMC over phospholipids. The red fluorescence in the cell membrane could also be explained by the high viscosity of the phospholipid bilayer, similar to glycerin (Figure 3b). In contrast, to our surprise, aggregation of TPNPDA-C12 occurred in the cytoplasm according to the yellow fluorescence inside the cells, though the concentration of TPNPDA-C12 used in cell culture was much lower than its CMC. Fluorescence lifetime imaging microscope (FLIM) measurement confirmed the presence of restricted monomers in the cell membrane and molecular aggregates in the cytoplasm by the characteristic lifetimes (Figure S11). Therefore, we believe there must be an intracellular microenvironment accumulating the probe to high local concentrations to form the TPNPDA-C12 aggregates. To pinpoint the exact location of TPNPDA-C12 in subcellular organelles, we performed colocalization experiments using commercially available probes known for specific subcellular organelles. As shown in Figures 3c and S14, Pearson’s coefficient of each commercial dye was calculated by the colocalization scatter plots. The red fluorescence of TPNPDA-C12 was found nicely colocalized with the cytoplasm membrane dye DiO (coefficient as high as 0.85). The yellow fluorescence of TPNPDA-C12, on the other hand, overlapped finely with the mitochondria dye MitoTracker Deep Red (coefficient as high as 0.86), and almost without apparent correlation with the lysosome dye LysoTracker (coefficient as only 0.44, similar to MTG and LysoTracker as 0.35, Figure S15). Besides, for the endoplasmic reticulum (ER) and the Golgi apparatus, the yellow fluorescence also exhibits little correlation (Figure S16, coefficient as only 0.51 and 0.54, respectively), similar to the coefficient of commercial mitotracker green (MTG) with the ER and the Golgi apparatus (0.48 and 0.46, respectively, Figure S17). These results indicated that TPNPDA-C12 was simultaneously distributed in the cell membrane and mitochondria inside live HeLa cells, with a selective dual-colored fluorescence in red and yellow, respectively. Besides HeLa cells, we also studied the behaviors of TPNPDA-C12 in HepG2 and MCF-7 cells and found their localization of red and yellow fluorescence was the same, suggesting its general use regardless of cell lines (Figure S18).

Effect of the Alkyl Chain Length on Subcellular Localization. The length of an alkyl chain usually affects the CMC and solubility of amphiphilic molecules. In addition to TPNPDA-C12, we synthesized its analogues with alkyl chains in the length of 1, 15, and 18 carbons (named as TPNPDA-C1, TPNPDA-C15, and TPNPDA-C18, respectively). They had almost the same fluorescence properties in poor solvents as well as in glycerin compared with TPNPDA-C12 (Figures S19–21), since they shared the same AIE and TICT characteristics of the identical core fluorophore. However, when applied for living cell imaging on HeLa cells, TPNPDA-C1, TPNPDA-C12, TPNPDA-C15, and TPNPDA-C18 displayed a dramatic difference in intracellular localizations. As shown in Figure 4, TPNPDA-C15 with a longer alkyl chain than TPNPDA-C12 could only stain the cell membrane in red while giving no yellow fluorescence in the cytoplasm, probably due to its stronger amphiphility blocking its entry into an intracellular environment. Colocalization experiments show that the colocalization coefficient of TPNPDA-C15 with DiO reached a high value of 0.92 (Figure S23). TPNPDA-C18 was unable to stain the cells at all, likely because it had an ultralow CMC and formed large aggregates in a medium with little cellular uptake. When the alkyl chain was reduced to only one carbon, TPNPDA-C1 initially showed a similar pattern in imaging HeLa cells as TPNPDA-C12, but the red fluorescence in the cell membrane was not stable for TPNPDA-C1 and gradually diffused over time, indicating the critical role of sufficient amphiphility in probe design (Figure S22).
TPNPDA-C12 to monitor the cytoplasm membrane and mitochondria during apoptosis and necrosis, the important ways of cell death. 25,47,48,49 Many research studies have been reported with a variety of fluorescent probes for ratiometrically26,47 or dynamically25,48,49 imaging apoptotic and necrotic processes. 3,4,5 However, no probe is reported yet to study the roles of multiple subcellular organelles in apoptosis and necrosis. We introduced high concentration of hydrogen peroxide (H$_2$O$_2$) as ROS stimulation to induce rapid cell apoptosis and necrosis. ROS-induced apoptosis and necrosis destroyed the integrity of the cell membrane, and dead cells could be identified by staining with propidium iodide (PI) that only entered the cells after the rupture of the cell membrane. 12,13 As shown in Figure 5a and Movie S1, after adding 5 mM H$_2$O$_2$ in HeLa cells stained by TPNPDA-C12, the yellow fluorescence in mitochondria almost remained constant, while the red fluorescence in the cell membrane gradually weakened over time and disappeared within 30 min.

This result indicated that ROS-induced apoptosis and necrosis started with relatively fast destruction of the cell membrane when mitochondria in the cytoplasm were not damaged yet. On the other hand, we used staurosporine (STS), a commonly used chemical drug for cytotoxin-induced apoptosis and necrosis, to see its difference from ROS-induced cell death. Interestingly, in 30 min after addition of 10 μM STS, the yellow fluorescence enhanced significantly and the red fluorescence remained almost unchanged (Figure 5b and Movie S3). Since it was difficult to intuitively determine through the images whether the cells were already in the apoptotic phase after 30 min incubation with STS, we used flow cytometry to quantitatively characterize the cell viability. HeLa cells were preincubated with 10 μM STS or equal DMSO as a control and then treated by the apoptosis assay kit of annexin V—allophycocyanin (APC)/PI double staining. As shown in Figure S24a, the fluorescence of neither APC nor PI was detected in HeLa cells incubated without STS (UL = 0.51%, UR = 2.93%) and live cell remained 95.72% (LL). For the sample after STS treatment for 30 min, 56.95% of the cells were stained by annexin V—APC (UL), indicating these cells were in an apoptotic phase (Figure S24b). It was also found that 39.62% of the cells were stained by both annexin V—APC and PI (UR) that had already died of necrosis. Based on these results, we speculated that STS as a competitive inhibitor of protein kinases through the prevention of ATP binding to the kinase 51 acted on mitochondria and inactivated protein kinases there. The damage caused in mitochondria might increase mitochondria membrane permeability and allow dispersed TPNPDA-C12 molecules in the cytoplasm to accumulate into mitochondria and form aggregates to enhance the yellow fluorescence. During this process, the integrity of the cell membrane was not compromised, so the red fluorescence was not affected.

**CONCLUSIONS**

In conclusion, we designed and synthesized an amphiphilic AIE probe TPNPDA-C12, which could simultaneously and selectively image a cell membrane and mitochondria in a dual-colored mode. Through charge interaction, TPNPDA-C12 accumulated in mitochondria to form molecular aggregates displaying a yellow fluorescence. On the other hand, TPNPDA-C12 fused into the cytoplasm membrane by amphiphilic interaction and the restricted monomers there showed a red fluorescence. The alkyl chain length of TPNPDA analogues determined their distribution in the subcellular organelles. We also successfully used TPNPDA-C12 to dynamically visualize the cell membrane and mitochondria during distinct ROS- and cytotoxin-induced cell apoptosis and necrosis. Because of its advantages in dual-colored fluorescence, low cytotoxicity, and simultaneous and selective imaging of the cytoplasm membrane and the mitochondria, we believe TPNPDA-C12 is a promising probe to study live cell processes for cell biology and disease mechanisms.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.0c02596. Materials and instruments, the synthesis method and experimental procedures, UV and FL spectra, confocal fluorescent images, flow cytometric analysis, and
characterizations of the synthesized compounds (NMR, ESI-MS) (PDF)
TPNPDA-C12-stained HeLa cells after adding 5 μL of a 3 wt % H₂O₂ solution in water recording for 30 min (AVI)
TPNPDA-C12-stained HeLa cells after adding 5 μL of water recording for 30 min (AVI)
TPNPDA-C12-stained HeLa cells after adding 10 μL of a 1 mg/mL STS solution in DMSO recording for 30 min (AVI)
TPNPDA-C12-stained HeLa cells after adding 10 μL of DMSO recording for 30 min (AVI)

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Author Contributions
Y.Z. and Y.D. contributed equally in probe design and synthesis, characterization, and live-cell imaging. All authors have given approval to the final version of the paper. Dr. J.R. and Prof. Z.S. contributed in theoretical calculation and analysis. Associate Prof. Y.X. contributed to paper writing instruction and revision. Prof. A.T. contributed to all of the works except for theoretical calculation and analysis.

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Notes
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■ ABBREVIATIONS USED

AIE aggregation-induced emission
ACQ aggregation-caused quenching
TICT twisted intramolecular charge transfer
ROS reactive oxygen species
MMP mitochondria membrane potential
CMC critical micelle concentration
NTO natural transition orbitals
LE local excitation
CT charge transfer
CLSM confocal laser scanning microscope
FLIM fluorescence lifetime imaging microscope
DLS dynamic light scattering
TEM transmission electron microscopy
STS staurosporine

■ REFERENCES
