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Dopamine-Modified Cationic Conjugated Polymer as a New Platform for pH Sensing and Autophagy Imaging

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A dopamine-modified conjugated polymer PFPDA is synthesized and characterized. At low pH, dopamine exists in its hydroquinone form and lacks the ability to quench fluorescence. At high pH, the proportion of the quinone form of dopamine increases due to its autooxidation, and efficient intramolecular electron transfer from the polymer main chain to quinone occurs, resulting in the quenching of the fluorescence of PFPDA. Thus, PFPDA exhibits a fluorescence "turn-on" response at low pH. PFPDA possesses excellent photostability and exhibits no cytotoxicity, which makes it a good fluorescent material for pH sensing and cell imaging. A light-induced hydroxyl anion emitter, MGCB, is also used to change the pH of the solution and thus regulate the fluorescence of PFPDA via remote control under light irradiation. Because the cytoplasm becomes acidic when cell autophagy occurs, PFPDA can also be used for autophagy imaging of HeLa cells with good selectivity.

1. Introduction

In recent decades, pH sensing in aqueous media has attracted a great deal of interest in many fields such as analytical chemistry, cellular biology, medicine and environmental protection.[1,2] The acid-response pH sensing of cellular environment is quite crucial for many reasons. For example, the decrease of intracellular pH can cause apoptosis,[3] and acidic organelles such as lysosomes and endosomes also functionalize in endocytic and digestive processes.[4] Fluorescence technology is one of the important methods to achieve rapid and sensitive pH sensing due to the advantage of high sensitivity and low background noise, thus it is widely applied in pH sensing.[5] Fluorescence pH sensing can be achieved by the use of many kinds of substances, such as quantum dots (QDs), fluorescent proteins and other small organic dyes, via intramolecular or intermolecular charge/energy transfer mechanisms.[6–8] However, each kind of method has its own limitations despite of its specific advantage. For example, small organic dyes usually suffer from low water solubility and comparatively poor photostability, which limit their use in biological research.[9] QDs possess advantages of narrow emission, and high brightness and photostability, but inherent cytotoxicity limits their further application in cell biology.[10,11] Therefore, it is of great significance to develop other kinds of fluorescence systems for pH sensing.

Conjugated polymers (CPs), coordinating the action of many repeated units and possessing strong light-harvesting and significant optical signal amplification properties, serve as a platform for highly sensitive biological and chemical detections.[12,17] Several conjugated polymers containing various cationic or anionic moieties have been reported to establish fluorescence pH-sensing systems on the basis of the different aggregation forms of CPs in different pH environments.[1,18–21] However, for most of these systems, the fluorescence is "turned-off" at low pH (acidic condition). In the meantime, the pH-response range of these CPs is often not located around physiological pH due to the intrinsic p*K*a values of the cationic or anionic moieties. Thus, application of these CPs in cell biology is limited. In this paper we report a "turn-on" pH sensing system at low pH by linking a dopamine moiety to a conjugated polymer to afford dopamine-modified polyfluorene derivative (PFPDA). This system is not based on an aggregation effect but the redox property of dopamine. The oxidized dopamine form (quinone) dominates in aqueous media with high pH and thus the quenching effect toward the conjugated polymer can be regulated by the pH change.[22] The pH-depentent redox property of dopamine and the signal amplification characteristics of CPs are combined to realize rapid, sensitive, and acid-responsive pH sensing. An organic dye, malachite green carbinol base, is reported to have the ability of releasing hydroxyl anions and thus altering the pH of solution via ultraviolet irradiation,[23,24] therefore it can be utilized in regulation of the fluorescence of PFPDA via remote control under light irradiation. Finally, this pH sensing system is also able to be applied in the autophagy imaging of HeLa cells (human cervical carcinoma cell line) because a portion of cytoplasm becomes acidic when autophagy occurs.[25,26]

2. Results and Discussion

Our new CP-based pH assay is illustrated in **Scheme 1**. The dopamine is covalently linked to the side chain of a water-soluble conjugated polymer (PFPDA). Dopamine can exhibit different redox states and change between hydroquinone (reduced state) and quinone (oxidized state) reversibly in aqueous media with a

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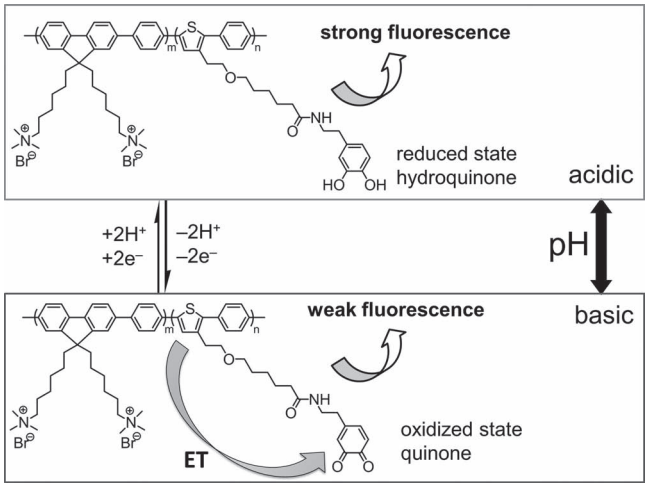


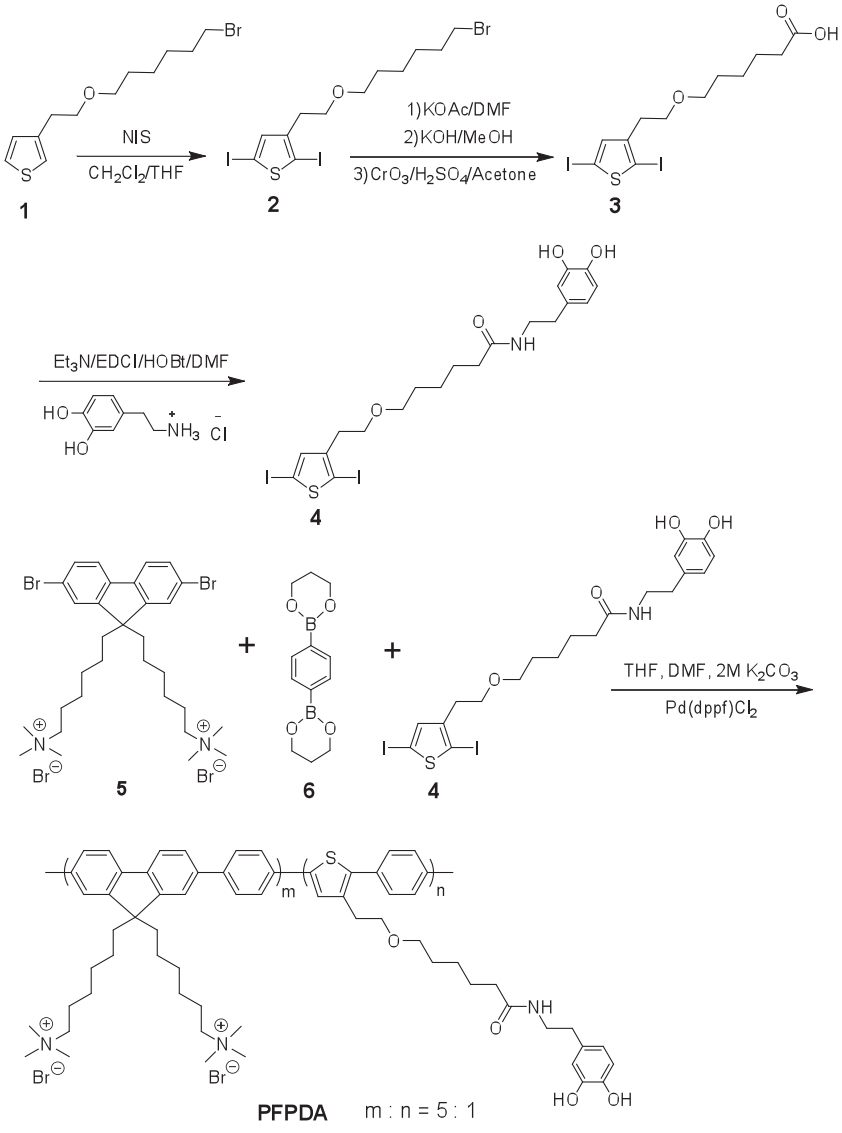
Scheme 1. Schematic representation of pH sensing on the basis of PFPDA.

change in pH.[22] At low pH, dopamine exists as its hydroquinone form and lacks the ability to quench fluorescence. The electron transfer (ET) from the polymer main chain (donor) to hydroquinone (acceptor) is absent and strong emission is observed upon excitation of the polymer. As pH is increased, the proportion of quinone form of dopamine increases due to its autooxidation. In this case, efficient intramolecular ET from polymer main chain to quinone occurs and the fluorescence of PFPDA is quenched. Thus, a different fluorescent response of the PFPDA is expected as the environmental pH changes.

The synthetic routes for monomers and the polymer PFPDA are outlined in **Scheme 2**. 3-(2-((6-bromohexyl)oxy)ethyl)thiophene (**1**) was treated with N-iodosuccinimide (NIS) to afford compound **2**. Via a multistep reaction, the compound **1** was converted to compound **3** followed by conjugation with dopamine to give monomer **4**. Coupling monomers **4**, **5**, and **6** via a Suzuki reaction in the presence of catalyst $Pd(dppf)Cl_2$ gave polymer PFPDA with 47% yield. The ratio of the two copolymerized units was calculated from ^1H NMR to be 5/1. The PFPDA was dissolved in water containing 5% DMSO (v/v) at room temperature to be used for further experiments.

The photophysical properties of PFPDA were investigated in phosphate buffered saline (PBS). As shown in **Figure 1**a, the UV-vis absorption spectrum of PFPDA shows a maximum peak at 380 nm, while the emission spectrum exhibits a maximum peak at 455 nm and a shoulder peak at 425 nm upon excitation at 390 nm. We studied the fluorescence intensity of PFPDA under light irradiation. Figure 1b shows that PFPDA maintained its original fluorescence intensity upon continuous irradiation by a mercury lamp (100 W) at 380 nm for 60 s. Thus, PFPDA exhibits excellent photostability, which is significant for further sensing and imaging investigation. The quantitative cytotoxicity of PFPDA was measured using an MTT method. As shown in Figure 1c, the cell viability of HeLa cells did not decrease after 24 h incubation with varying PFPDA concentration (0–100 μM). The low toxicity of PFPDA is critical for its application in cell imaging.

The pH response of PFPDA was investigated in PBS with different pH. As shown in **Figure 2**a, the fluorescence intensity of PFPDA increased as the pH changed from high to low. The maximum emission intensity at 455 nm decreases linearly with increasing pH value in the pH range of 5.0–9.0 (Figure 2b). For this system, there is a good response around neutral pH (6.0–8.0), facilitating utilization in a biological environment. At low pH, dopamine exists as its hydroquinone form and lacks the ability to quench the fluorescence of PFPDA. At higher pH, the proportion of quinone of dopamine increases due to its autooxidation. This results in electron transfer from PFPDA to



Scheme 2. The synthetic route of PFPDA.

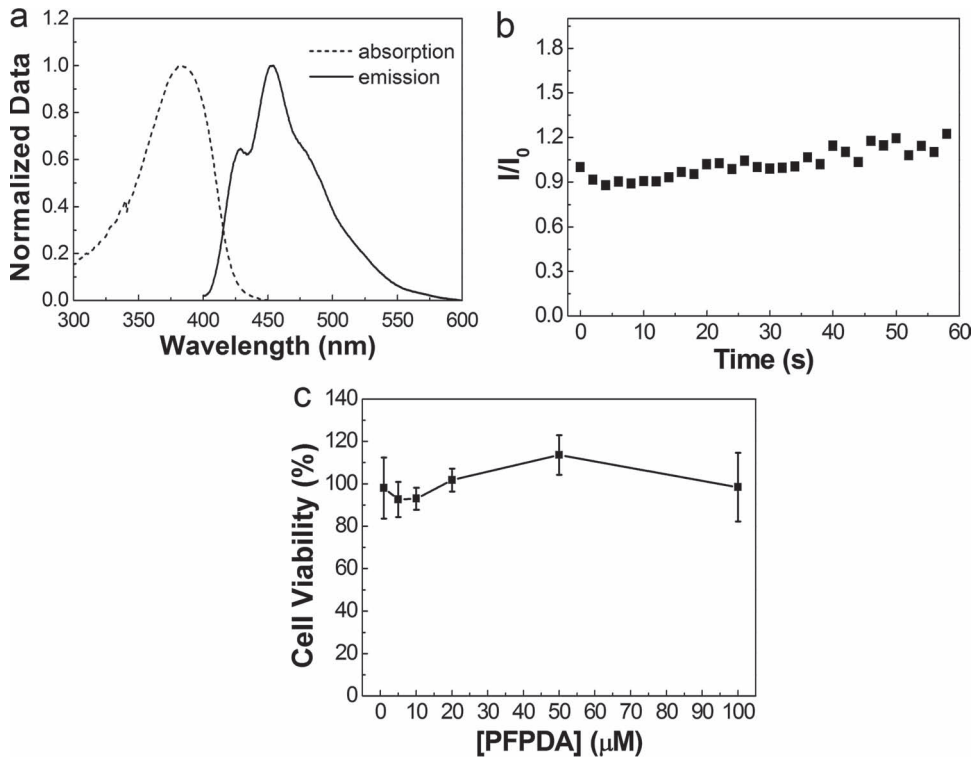


Figure 1. a) Normalized UV-vis absorption and emission spectra of PFPDA in PBS (pH = 7.0). The excitation wavelength is 390 nm. b) Photostability of PFPDA upon irradiation at 380 nm under a mercury lamp (100 W). c) Cell viability of HeLa cells incubated with various concentrations of PFPDA. Standard deviations calculated from six replicate samples.

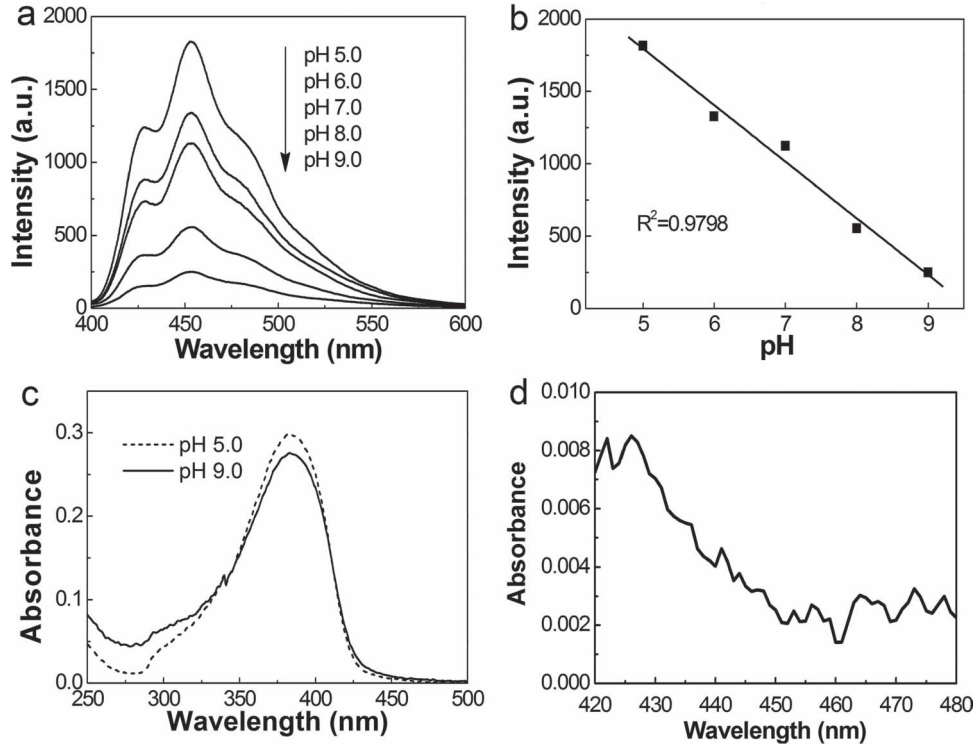


Figure 2. a) Fluorescence spectra of PFPDA in PBS at different pH values (5.0, 6.0, 7.0, 8.0, and 9.0). b) The maximum fluorescence intensity of PFPDA at 455 nm as a function of pH value; [PFPDA] = 1 μM in RUs. The excitation wavelength is 390 nm. c) Absorption spectra of PFPDA in PBS at pH 5.0 and 9.0. Measurements were performed in phosphate buffered saline solution; [PFPDA] = 10 μM. d) Absorption spectra of PFPDA at pH 9.0 between 420 and 480 nm with subtraction of the residual absorbance of PFPDA at pH 5.0.

quinone and the fluorescence of PFPDA is quenched. The formation of quinone from dopamine was verified by UV-vis spectroscopy. Figure 2c compares the absorption spectra of PFPDA in PBS at pH 5.0 and 9.0; an intensity increase at 440 nm is observed for pH 9.0 (Figure 2d), which provides the evidence of the autooxidation of dopamine from its hydroquinone to quinone form.[27] The fluorescence quantum yields of PFPDA in aqueous solution were also measured with the value of 2% at pH 9.0 and 11% at pH 5.0.

Organic dye, malachite green carbinolbase (MGCB), was utilized to achieve the regulation of fluorescence of PFPDA. It is reported that MGCB can release hydroxide anions in the presence of UV light and recombine with the OH^- after the UV light is stopped,[23–24] thus the solvent pH can be changed by the hydroxide anions released by MGCB. In order to improve the solubility of MGCB in aqueous media, the surfactant cetyltrimethylammonium bromide (CTAB) was used in our experiments. The working system was established by adding MGCB and PFPDA to the aqueous solution containing CTAB (50 mM) and NaCl (200 mM). The UV-vis spectra of the solution were recorded under 254 nm UV light. As shown in **Figure 3**a, the absorbance at 621 nm increases as the solution was irradiated under the 254 nm UV light, elucidating the generation of hydroxyl anion. It is noted that the peak at 621 nm reaches a maximum after irradiation for about 15 min, which shows that MGCB is completely converted to the malachite green cation. In this case, the solution exhibited a deep green color due to the formation of the malachite green cation. The fluorescence spectra of PFPDA in this working system were then measured upon irradiation under 254 nm UV light. The fluorescence spectra were recorded every 3 min for 15 min. As shown in Figure 3b, the maximum emission intensity of PFPDA at 425 nm decreased by a half after 15 min irradiation. The control experiment showed that the maximum emission intensity of PFPDA did not change in the absence of MGCB. These experiments showed that fluorescence regulation of PFPDA was achieved by the MGCB-induced pH change via UV irradiation. It is noted that the fluorescence of PFPDA can recover to its original intensity after 30 min with the UV light switched off (Figure 3c). The cycling of the fluorescence intensity of PFPDA at 425 nm in the presence of MGCB under excitation at 390 nm was realized by turning the UV light on and off alternately (each cycle consisted of 20 min with light and 40 min without light). From the six cycles shown in Figure 3d, we can see that the intensity of each cycle only slightly decreased. In general, this system showed comparatively excellent cycling properties.

Finally, PFPDA was applied to autophagy imaging of HeLa cells. The HeLa cells were treated with rapamycin (25 μM) for 8 h to induce autophagy followed by staining with PFPDA.

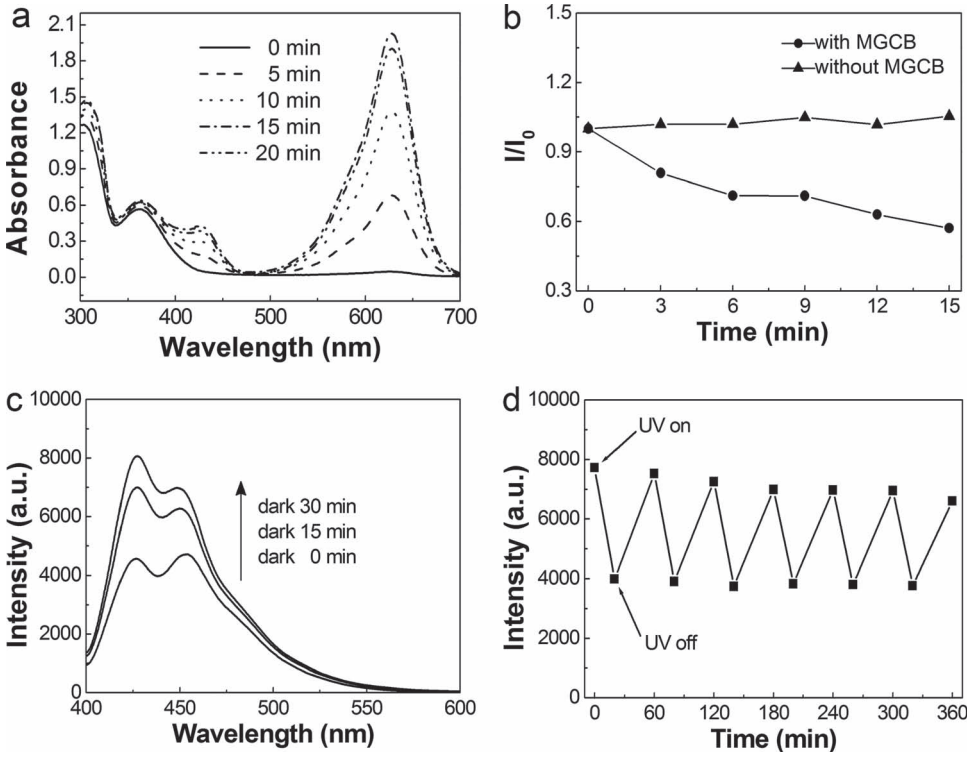


Figure 3. a) UV-vis absorption spectra of PFPDA and MGCB under irradiation at 254 nm UV light for 0–20 min; [PFPDA] = 2.5 μM, [MGCB] = 100 μM. b) Maximum fluorescence intensity of PFPDA at 425 nm with and without the addition of MGCB under irradiation at 254 nm for 15 min. The excitation wavelength is 390 nm; [PFPDA] = 1 μM, [MGCB] = 100 μM. c) Fluorescence spectra of PFPDA in the presence of MGCB after the sample was irradiated at 254 nm for 15 min and kept in the dark for another 15 min and 30 min. The excitation wavelength is 390 nm; [PFPDA] = 2 μM, [MGCB] = 100 μM. d) Cycling of maximum fluorescence intensity of PFPDA at 425 nm in the presence of MGCB with the UV light on and off. The excitation wavelength is 390 nm; [PFPDA] = 2 μM, [MGCB] = 100 μM.

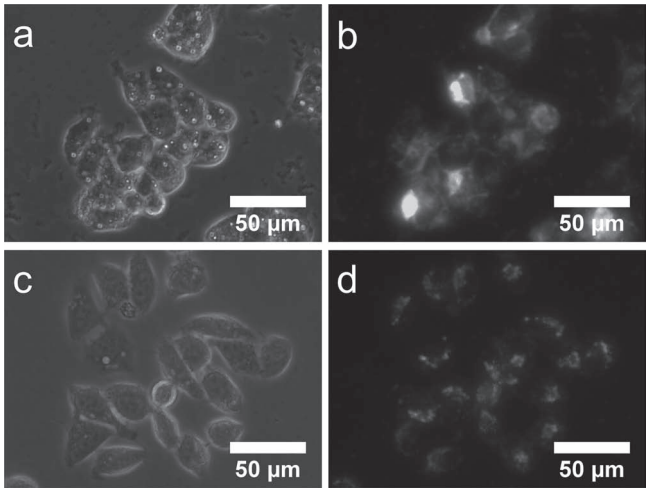


Figure 4. Phase contrast image (a) and fluorescence image (b) of rapamycin-treated HeLa cells stained with PFPDA. Phase contrast image (c) and fluorescence image (d) of HeLa cells stained with PFPDA without treatment with rapamycin. [PFPDA] = 10 μM, [rapamycin] = 25 μM.

The pH of lysosomal internal environment is around 5.0 and thus is acidic compared with the slightly basic cytosol with pH 7.2. In the process of autophagy, a portion of the cytoplasm is sequestered in autophagosome and eventually fused with lysosomes, leading to a pH decrease of the cytoplasm and thus the increase of fluorescence intensity from the PFPDA in the cells. As shown in **Figure 4**a,b, the HeLa cells stained with PFPDA exhibit brighter emission intensity with the addition of rapamycin compared with the control experiment (without treatment with rapamycin) (Figure 4c,d). The brightness intensity of the fluorescence of PFPDA in HeLa cells treated with rapamycin is five times higher than that of cells without treatment with rapamycin. It is noted that no difference was observed when we used the commercially available dye (monodansylcadaverine (MDC)) to image autophagy of HeLa cells with and without treatment of rapamycin. Thus, our new pH sensing system can be applied in the autophagy imaging of cells with good selectivity.

3. Conclusions

A dopamine-modified conjugated polymer PFPDA was synthesized and characterized, exhibiting a fluorescence "turn-on" response at low pH values. PFPDA possesses excellent photostability and exhibits no cytotoxicity, which makes it a good fluorescent material for pH sensing and cell imaging. We utilized the redox properties of dopamine to realize a reversible pH response of the fluorescence intensity of PFPDA. A light-induced hydroxyl anion emitter MGCB was also used to change the pH of the solution and thus regulate the fluorescence of PFPDA via remote control under light. PFPDA was also used for turn-on autophagy imaging of HeLa cells because a portion of the cytoplasm becomes acidic when autophagy occurs.

4. Experimental Section

Materials: Solvents were obtained from Beijing Chemical Co. and used without further purification. THF was dried under sodium. DMF was distilled under P_2O_5. 2,7-Dibromofluorene, $Pd(dppf)Cl_2$ (98%) was obtained from Pacific ChemSource, Inc. (China), malachite green carbinol base and rapamycin were purchased from Sigma-Aldrich. Dopamine hydrochloride and cetyltrimethylammonium bromide (CTAB) were purchased from Alfa-Aesar.

Instruments: ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AV400 instrument. Mass spectra were measured on a SHIMADZU LCMS-2010 spectrometer for ESI, a Bruker Biflex spectrometer for MALDI-TOF, and a Waters GCT spectrometer for high resolution mass spectra (HRMS). Elemental analysis was performed on a Flash EA1112 instrument. UV-vis absorption spectra were performed using a JASCO V-550 spectrophotometer. Florescence spectra were recorded on a Hitachi F-4500 fluorometer equipped with a xenon lamp as excitation source at room temperature, and the PMT voltage was 700 V. The pH value was measured on a Mettler Toledo FE20 pH indicator, calibrated with standard buffers of pH = 4.00, 7.00, and 10.00 at room temperature. Phase contrast bright-field and fluorescence images were taken with fluorescence microscopy (Olympus 1 × 71) with a mercury lamp (100 W) as the excitation source. MGCB was irradiated in a WD-9403F UV viewing cabinet.

*Synthesis of 3-(2-((6-Bromohexyl)oxy)ethyl)-2,5-diiodothiophene (**2**)*: To a stirred solution of 3-(2-((6-bromohexyl)oxy)ethyl)thiophene (**1**) (1.0 g, 3.4 mM) in a 1:1 (*v*/*v*) solvent mixture of dichloromethane and acetic acid (40 mL) was slowly added N-iodosuccinimide (1.93 g, 8.6 mM) at 0°C in the dark. The mixture was stirred at room temperature overnight and then was washed with 10% sodium thiosulfate, saturated aqueous $NaHCO_3$ and brine. The organic phase was dried over Mg_2SO_4, filtered and the solvent was removed under vacuum. Column purification was performed on silica gel with petroleum ether/ethyl acetate (40:1) as the eluent solvents to afford the pure compound as a white powder (1.55 g, 94%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.98 (s, 1H), 3.54 (t, 2H), 3.42 (dd, 4H), 2.80 (t, 2H), 1.91–1.84 (m, 2H), 1.61–1.54 (m, 2H), 1.49–1.34 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 146.13, 138.33, 77.99, 75.83, 70.77, 69.71, 33.93, 32.76, 32.44, 29.48, 27.96, 25.40. MS (MALDI-TOF): *m*/*z* 540.6 ($M–H^+$); $C_{12}H_{17}BrI_2OS$: calculated for C 26.54, H, 3.16; found C 26.93, H 3.26.

*Synthesis of 6-(2-(2,5-Diiodothiophen-3-yl)ethoxy)hexanoic acid (**3**)*: To a solution of compound **2** (1.0 g, 1.8 mM) in 15mL DMF was added KOAc (1.8 g, 18 mM). The mixture was vigorously stirred at 60 °C for 2 h under nitrogen atmosphere. After cooling to room temperature the solvent was removed under reduced pressure. The residue was treated with KOH (1.24 g, 22 mM) in methanol (15 mL) at 60 °C for 2 h. The mixture was concentrated and extracted with dichloromethane, followed by washing by water and drying over Mg_2SO_4. After evaporation of the solvent, the compound acquired was dissolved in acetone and the solution was cooled to 0 °C. Jones' reagent was added dropwise until the orange color persisted and the mixture was stirred at room temperature overnight. After isopropanol was added dropwise to quench the reaction, the solvent was removed under vacuum. The solid was dissolved in 1 M HCl and extracted with CH_2Cl_2. The combined organic layers were extracted with 1 M NaOH, and the aqueous layer was then acidified followed by extracting with CH_2Cl_2. The combined organic layers were dried over anhydrous Mg_2SO_4, filtered and concentrated under reduced pressure to give a yellow solid (628 mg, 69%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.29 (s, 1H), 6.97 (s, 1H), 3.54 (t, 2H), 3.43 (t, 2H), 2.79 (t, 2H), 2.37 (t, 2H), 1.69–1.55 (m, 4H), 1.43–1.36 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 179.69, 146.04, 138.29, 78.00, 75.85, 70.65, 69.71, 33.96, 32.38, 29.27, 25.69, 24.45. HRMS (ESI) calculated for $C_{12}H_{16}I_2O_3S$ [$(M+H)^+$]: 494.8983, found: 494.8963.

*Synthesis of N-(3,4-Dihydroxyphenethyl)-6-(2-(2,5-diiodothiophen-3-yl)ethoxy)hexanamide (**4**)*: To a solution of compound **3** (200 mg, 0.4 mM) in DMF (6 mL) at 0 °C was sequentially added NEt_3 (340 μL, 2.4 mM), HOBt (110 mg, 0.8 mM), EDCI (170 mg, 0.9 mM), and dopamine hydrochloride (230 mg, 1.2 mM). The reaction mixture stirred at 0 °C

for 1 h and at room temperature overnight. After DMF was removed under reduced pressure, 10 mL water was added and the aqueous layer was extracted with chloroform. The collected organic layer was washed with 1 M HCl. The organic layer was dried over Mg_2SO_4, filtered and concentrated under vacuum. Column purification was performed on silica gel with petroleum ether/ethyl acetate (1:2) as eluent solvents to afford a colorless liquid (167 mg, 65.5%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.88 (s, 1H), 6.94 (s, 1H), 6.81 (d, 1H), 6.69 (s, 1H), 6.57 (d, 1H), 6.19 (s, 1H), 5.66 (s, 1H), 3.57 (t, 2H), 3.50–3.44 (m, 4H), 2.80 (t, 2H), 2.69 (t, 2H), 2.16 (t, 2H), 1.63–1.51 (m, 4H), 1.37–1.29 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.25, 145.80, 144.32, 143.12, 138.20, 130.44, 120.36, 115.64, 115.37, 78.17, 75.97, 70.64, 69.59, 41.02, 36.59, 34.77, 32.22, 29.10, 25.73, 25.43. HRMS (ESI) calculated for $C_{12}H_{16}I_2O_3S$ [M+H]: 629.9669, found: 629.9653.

Synthesis of PFPDA: To a solution of monomer **5** (154 mg, 0.2 mM), monomer **4** (31 mg, 0.05 mM) and monomer **6** (62 mg, 0.25 mM) in 8 mL THF and 2 mL DMF was added 2 mL of aqueous potassium carbonate (2.0 M). The resulting mixture was degassed and then $Pd(dppf)Cl_2$ (15 mg, dppf = diphenylphosphinoferrene) was added under a nitrogen steam. The mixture was vigorously stirred at 80 °C for 2 days and then cooled down to room temperature. The solvents were removed under vacuum and the solid was dissolved in methanol and precipitated by acetone. The precipitate was collected and dialyzed in water using a dialysis membrane with a cut-off of 3500 g mol^{-1} for two days to yield a yellow solid (115 mg, 47%). ^{1}H NMR (400 MHz, DMSO) δ 8.58, 7.98–7.80, 7.57–7.36, 7.16, 6.62, 3.34, 3.17, 2.95, 2.08, 1.46, 1.14–1.09, 0.66.

Assay for Photostability of PFPDA: The solution containing PFPDA was dropped on a glass plate and covered with a coverslip. The sample was continuously irradiated by a mercury lamp (100 W) with a 380/30 nm excitation filter. Fluorescence emission intensities of the sample was recorded with fluorescence microscopy (Olympus 1 × 71).

In Vitro Cell Viability Assay: HeLa cells were seeded in 96-well tissue culture plates at a density of 8000 cells per well and maintained overnight in a DMEM medium. Cells were then treated with various concentrations of PFPDA (1, 5, 10, 20, 50, and 100 μM) respectively, followed by incubation at 37 °C for 24 h. After discarding the culture medium, the cells were then treated with 100 mL of MTT (1 mg/mL in PBS) followed by incubation at 37 °C for 4 h. After the supernatant was removed, the cells were lysed by adding 100 μL DMSO per well, and the absorbance of the purple formazan was recorded at 520 nm using a Spectra MAX 340PC plate reader.

Regulation of Fluorescence of PFPDA with MGCB: Malachite green carbinolbase (MGCB) was dissolved in DMSO. A surfactant cetyltrimethylammonium bromide (CTAB) (50 mM) was dissolved in 200 mM NaCl aqueous solution and the whole solution served as the working system with a slightly acidic pH value. To a fluorimeter cuvette with 1 mL CTAB (50 mM) in NaCl (200 mM) was added MGCB (100 μM) and PFPDA (2 μM). The whole system was irradiated under 254 nm UV light. The fluorescence of PFPDA was recorded under excitation at 390 nm.

Fluorescence Imaging of Autophagy in HeLa Cells: 20 μL of 1.0 mM PFPDA and 13 μL rapamycin (3.8 mM in DMSO) was added into 2 mL of serum-free DMEM medium containing HeLa cells in 35 mm × 35 mm plate (the final concentration: [PFPDA] = 10 μM, [rapamycin] = 25 μM). The plate was incubated at 37 °C for 8 h, then the medium was removed and the cells were washed with phosphate buffered saline (PBS, pH 7.4) twice. The fluorescent images were recorded on fluorescence microscopy using a 380/30 nm excitation filter with 100 ms exposure time. Another plate of HeLa cells as a control was treated in the same way except for the addition of rapamycin.

Acknowledgements

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