Electronic Supplementary Information

The crosslink enhanced emission (CEE) in non-conjugated polymer dots: from photoluminescence mechanism to cellular uptake mechanism and internalization

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1. Experimental Section

Materials: The polyethyleneimine (PEI, Mw=600, 1800, 10000) was obtained from Aladdin reagent. The carbon tetrachloride (CTC) was purchased from Beijing Chemical Works.

Preparation of PDs-1. The PEI (1.0 g) and CTC (3.0 g) were added to a round-bottom flask. Then, the system was stirred at 90 ºC for 6 hours. When the reaction system was cooled to room temperature, 50 mL of deionized water was added to dissolve the products (the excess CTC was removed). At last, the products were subjected to dialysis (Mw=3500) to obtain the PDs-1 (inner products of the dialysis bag).

Preparation of PDs-2. The PDs-2 was prepared by the previous method.[1] The PEI (1.0 g) was dissolved in 50 mL deionized water. Then the solution was transferred to a poly (tetrafluoroethylene) (Teflon)-lined autoclave (100 mL) and heated at 200 ºC for 5 h. After the reaction, the reactors were cooled to room temperature by water or naturally. The product, which was brown-yellow and transparent, was subjected to dialysis (Mw=3500) to obtain the PDs-2 (inner products of the dialysis bag).

Preparation of PDs-3. 1.0 g citric acid was dissolved in the 10 mL deionized water, and the reaction system was put in the domestic microwave (700 W) to react for 5 min. The obtained yellow viscous product was dissolved in the 10 mL deionized water, and PEI (1.0 g) was added in the round-bottom flask. Then, the system was stirred at 90 ºC for 6 hours. At last, the products were subjected to dialysis (Mw=3500) to obtain the PDs-3 (inner products of the dialysis bag).

Preparation of PDs-4. The electrochemical-synthesized C-dots were prepared as follows:[2] The electrolyte of the electrochemical process was prepared by mixing ethanol/H_2O (100 mL; volume ratio=99.5:0.5) with 0.2 g NaOH. The graphite rods (diameter=0.5 cm) were adopted as an anode and cathode, as well as the carbon source in a typical electrochemical synthesis. The C-dots were synthesized with a current intensity in the range of 100 $mA \cdot cm^{-2}$. The as-prepared C-dots were subjected to dialysis to remove the excess ions. Then, the obtained yellow viscous product (100 mg) was dissolved in the 10 mL deionized water, and PEI (1.0 g)

was added to the round-bottom flask. Then, the system was stirred at 90 ºC for 6 hours. At last, the products were subjected to dialysis (Mw=3500) to obtain the PDs-4 (inner products of the dialysis bag).

Characterization: High-resolution transmission electron microscope (HTEM) was recorded on FEI Tecnai F20. Fluorescence spectroscopy was performed on a Shimadzu RF-5301 PC spectrophotometer. UV-vis absorption spectra were obtained using a Shimadzu 3100 UV-vis spectrophotometer. IR spectra were taken on a Nicolet AVATAR 360 FT-IR spectrophotometer. The confocal microscopy images were taken at Olympus Fluoview FV1000. Binding energy calibration was based on C1s at 284.6 eV. AFM images were recorded in the tapping mode with a Nanoscope IIIa scanning probe microscope from Digital Instruments under ambient conditions. Matrix-assisted laser desorption/ionization reflect time-of-flight (MALDI-TOF) technique was recorded on Bruker autoflex speed TOF with tetracyanoquinodimethane (TCNQ) as the matrix. Zeta potential and DLS measurements were performed using a Zetasizer Nano-ZS (Malvern Instruments). Each sample was measured 5 times and the average data was presented.

Time-resolved photoluminescence. Nanosecond fluorescence lifetime experiments were performed by the time-correlated single-photon counting (TCSPC) system under right-angle sample geometry. A 379/405 nm picosecond diode laser (Edinburgh Instruments EPL375, repetition rate 2 MHz) was used to excite the samples. The fluorescence was collected by a photomultiplier tube (Hamamatsu H5783p) that was connected to a TCSPC board (Becker&Hickel SPC-130). The time constant of the instrument response function (IRF) was about 300 ps.

Femtosecond transient absorption setup. The TA setup consisted of 400 nm pump pulses and doubled from 800 nm laser pulses (~100 fs duration, 250 Hz repetition rate) generated from a mode-locked Ti: sapphire laser/amplifier system (Solstice, Spectra-Physics) and broadband white-light probe pulses generated from 2-mm-thick water. The TA data were collected by a fiber-coupled spectrometer connected to a computer. The group velocity dispersion of the transient spectra was compensated by a chirp program. All of the measurements were performed at room temperature.

The cellular (PC12 cells) uptake mechanism and internalization of PD.

1. Materials and methods

1.1 Materials

Fetal bovine serum (FBS), phosphate buffer solution (PBS), Dulbecco's Modified Eagle Medium (DMEM), penicillin/streptomycin, trypsin/EDTA, anhydrous dimethyl sulfoxide,

Propidium Iodide (PI), MitoTracker Red, ER-Tracker Red, Lysosome-Tracker were obtained from Life Technologies (Grand Island, NY, USA). Sodium azide (SA), genistein, 2-deoxy-D-glucose (2-DG), chlorpromazine, tetrazolium salt (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Paraformaldehyde was purchased from Sinopharm Chemical Reagent Co (Shanghai, China). Cytochalasin D (CytoD) was obtained from Bioaustralis (Smithfield, NSW, Australia).

1.2 Cell culture

The differentiated rat adrenal pheochromocytoma cells (PC12) were purchased from the cell bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere with 5% CO_2.

1.3 Cell viability assay of PDs

The cytotoxic effect of PDs was evaluated using the MTT cell viability assay, in which MTT is reduced in metabolically active cells to form insoluble purple formazan crystals, which are solubilized by the addition of DMSO. Briefly, PC12 cells were seeded in 96-well plate at 1×10^4 cells/well in 200 μL culture medium under 37 °C and 5% CO_2. After 24 h incubation, different concentrations of PDs (2, 10, 30, 40, 50 μg/mL) were added and incubated for another 24 h or 4 h at 37 °C. Then, nanoparticle dispersion in each well was replaced by 180 μL serum-free medium (SFM) and 20 μL MTT (5 mg/mL). Cells were further incubated for 3 h; the supernatant was removed by inverting the plates to decant the liquid; 200 μL/well DMSO was added to dissolve the crystals remaining at the bottom of the plate. The absorbance was measured at 490 nm using a Synergy HT microplate reader (Bio-Tek, Winooski, VT). Cell viability was expressed as a percentage relative to the untreated cells, which served as the control.

1.4 Uptake mechanism of PDs

1.4.1 Time-dependent cellular uptake

Time dependence of cellular uptake was determined using both confocal laser scanning microscope (CLSM) and fluorescence-activated cell sorter (FACS). For CLSM, PC12 cells (5×10^4 cells/dish) were seeded in a 35 mm glass-bottom dish and incubated for 24 h. PDs (20 μg/mL) was added and incubated for different time (15, 30, 60, 120, 360 min). After that, the cells were rinsed 3 times with PBS and added 1 mL PBS for scanning. Live cell imaging was applied here to avoid the autofluorescence of the fixative paraformaldehyde and scanning was performed at the excitation (Ex) wavelength of 405 nm and emission (Em) wavelength of 430-470 nm with a FV1000Olympus IX81 CLSM (Osaka, Japan) using a 40× objective lens.

Three different visual fields of each time point containing 30-40 cells were analyzed and triplicate experiments were done at three independent time points. The mean fluorescence intensity (MFI) of 100-120 cells in all 9 images were analyzed by Image J software and the images shown here were representative of the original data.

As for the FACS analysis, PC12 cells (5×10^5 cells/well) were seeded in 6-well plates and incubated for 24 h. PDs (20 μg/mL) was added and incubated for different time (15, 30, 60, 120, 360 min). Subsequently, the cells were washed 3 times with PBS, trypsinized, harvested, centrifuged, and resuspended in 200 μL PBS. Finally, the MFI of 20000-30000 cells at each time point was measured using a FACSAria II flow cytometer with a 405 nm laser.

1.5 Energy-dependent cellular uptake

The energy-dependent cellular uptake was performed with low-temperature incubation and ATP (adenosine triphosphate) depletion treatments. PC12 cells (8×10^5 cells/well) were seeded in 6-well plates and incubated for 24 h. The cells were pre-incubated at 4°C or in SFM supplemented with 10 mM SA and 50 mM 2-DG at 37 °C for 45 min, respectively. PDs (20 μg/mL) was added and co-incubated for an additional 2 h, and cells were then washed three times with PBS, trypsinized, harvested, centrifuged, and resuspended in 200 μL PBS. The MFI of cellular uptake was measured using a FACSAria II flow cytometer with a 405 nm laser.

1.6 Cellular uptake pathways of PDs

1.6.1 The cytotoxicity of cellular uptake inhibitors

The cytotoxicity of cellular uptake inhibitors against PC12 cells was determined using the MTT assay. Briefly, PC12 cells (1×10^4 cells/well) were seeded in 96-well plates and incubated for 24 h. Genistein (50 μg/mL), chlorpromazine (10 μg/mL), and CytoD (5 μM) were separately added and incubated for 4 h. Then, the culture medium was replaced with 180 μL SFM and 20 μL MTT (5 mg/mL) and the sample was incubated for an additional 3 h. Subsequently, the supernatant was removed by inverting the plates to decant the liquid; 200 μL/well DMSO was added to dissolve the crystals remaining at the bottom of the plate. The absorbance was measured at 490 nm using a microplate reader.

1.6.2 The effect of uptake inhibitors on endocytotic pathways of PDs

The endocytotic pathways of PDs were investigated by the addition of specific pharmacological inhibitors and detected by both FACS and CLSM. PC12 cells (5×10^5 cells/well) were seeded in 6-well plates and incubated for 24 h. Genistein (50 μg/mL), chlorpromazine (10 μg/mL) and CytoD (5 μM) were separately added into SFM and pre-incubated at 37 °C for 45 min. PDs (20 μg/mL) was then added and incubated for an

additional 1 h. Subsequently, cells were then washed three times with PBS, trypsinized, harvested, centrifuged, and resuspended in 200 μL of PBS. The cellular uptake efficiency was determined using a FACSAria II flow cytometer with the 405 nm laser.

1.7 Intracellular distribution of PDs

1.7.1 Cellular organelles specific staining

To observe co-localization, mitochondria, endoplasmic reticulum, lysosomes of cells were labeled with MitoTracker Red, ER-Tracker Red, and LysoTracker Red respectively, under the manufacturer's instructions. CLSM observation was performed on the FV1000 IX81 microscope with a 60× oil immersion objective. The excitation/emission wavelengths of PDs, MitoTracker Red, ER-Tracker Red, LysoTracker Red, and Golgi-RFP were 405/430-470 nm, 559/580-625 nm, 559/600-630 nm, 559/575-640 nm, and 559/565-620 nm, respectively.

1.7.2 Transmission electronic microscopy (TEM)

PC12 cells (1×10^6 cells/well) were seeded in 6-well plates and incubated for 24 h. PDs (20 μg/mL) was added and incubated for different time (15, 30, 120 min). Subsequently, the cells were washed 3 times with PBS, trypsinized, harvested, centrifuged. Samples of fresh cells were fixed overnight at room temperature with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer at a pH of 7.4. Samples were then rinsed three times of 10 min in 0.1 M sodium cacodylate buffer and post-fixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 h. After washing steps, the samples were dehydrated in a graded series of ethanol ending with propylene oxide. Samples were then embedded in EPON. About 70 nm thin sections were prepared with a Reichert Ultracut E ultramicrotome. The sections were observed using a JEM-2100F high-resolution transmission electron microscope (JEOL, Japan) operating at 120 kV accelerating voltage.

1.8 The rat Schwann cells (RSC96) were cultured and analyzed by similar methods as PC12 cells.

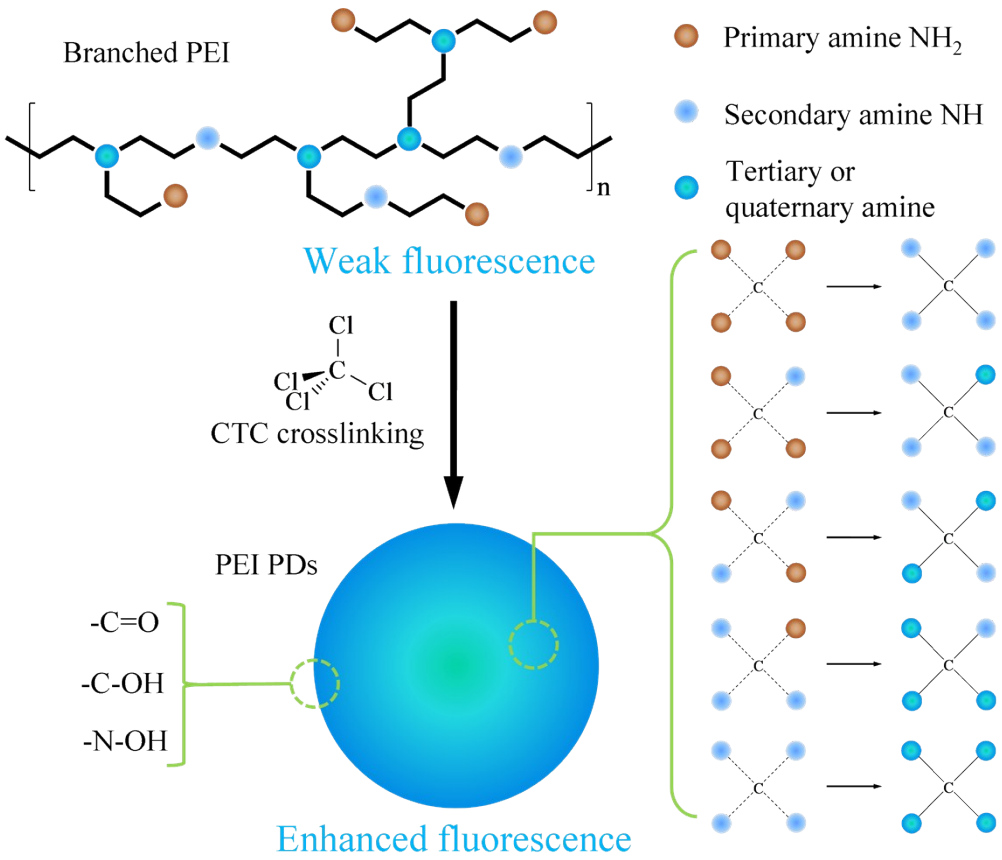

Figure S1. The scheme of crosslinking the branched PEI by CTC. The used PEI possesses 25% primary amine, 50% secondary amine and 25% tertiary amine. **The PDs in S1 to S9 were corresponding to PD 1.**

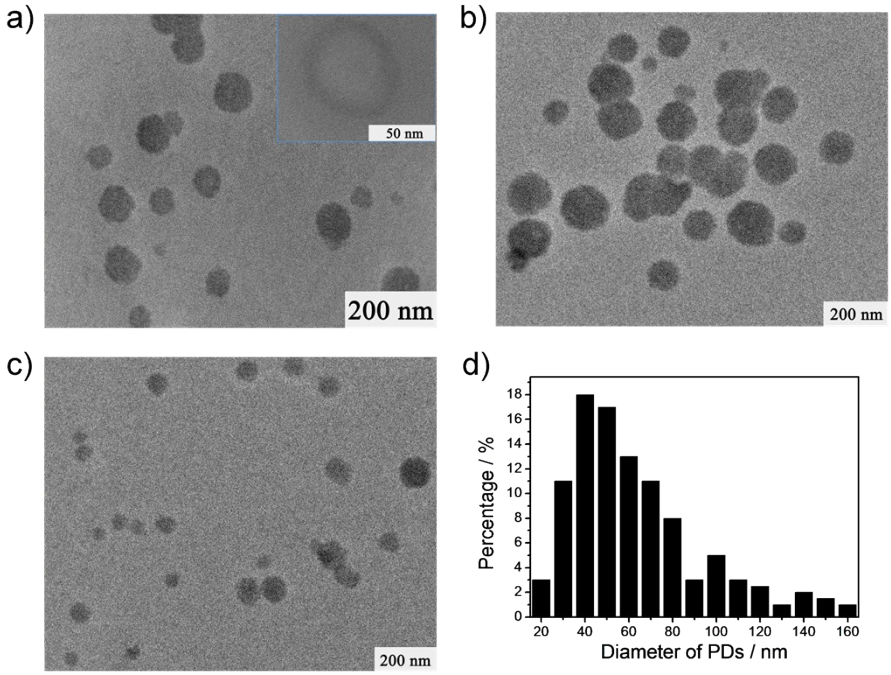

Figure S2. TEM images of the PDs. The PDs possess a wide size distribution, when we subjected the PDs to TEM imaging, different sizes were found in the TEM image, as a result,

we have to provide several typical images (a, b and c), showing the wide size distribution of PDs. The inset of a) was the enlarged image, showing the amorphous form of PDs. The average diameter of PDs was ca. 63 nm.

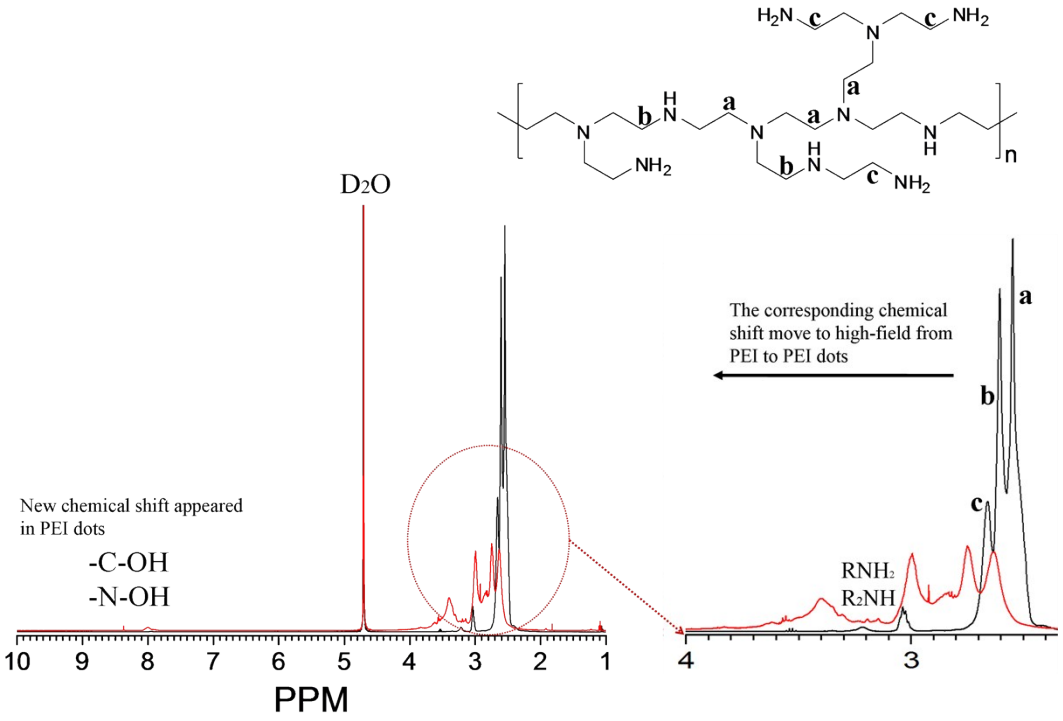

Figure S3. The 1H NMR spectra of bare PEI (red line) and PDs (black line). The corresponding chemical shift moved to high-field from PEI to PEI PDs, and new chemical shifts appeared in PEI PDs.

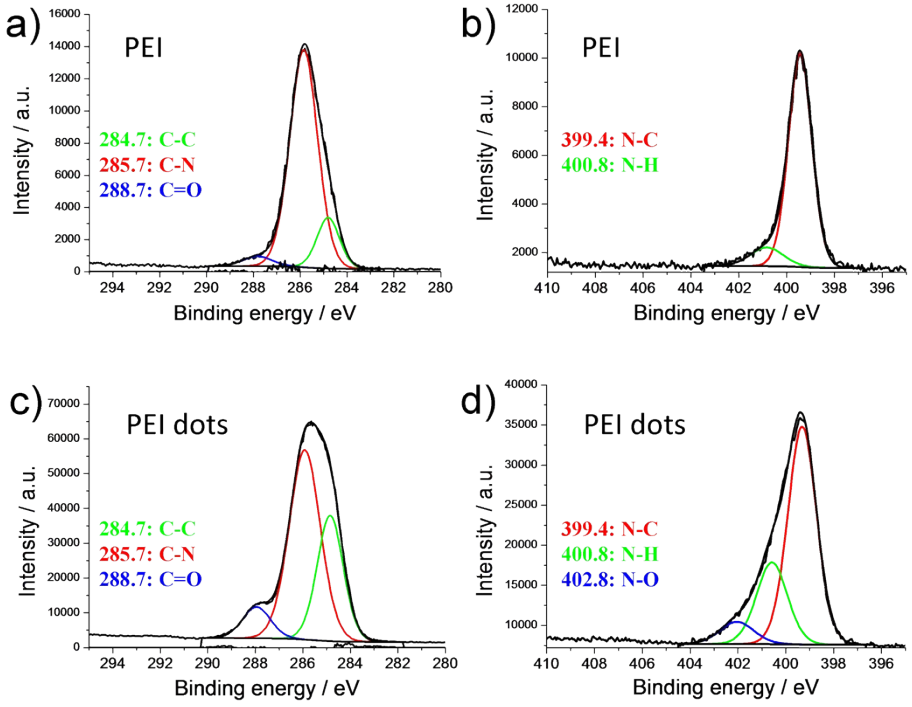

Figure S4. XPS analysis of bare PEI and PDs, respectively.

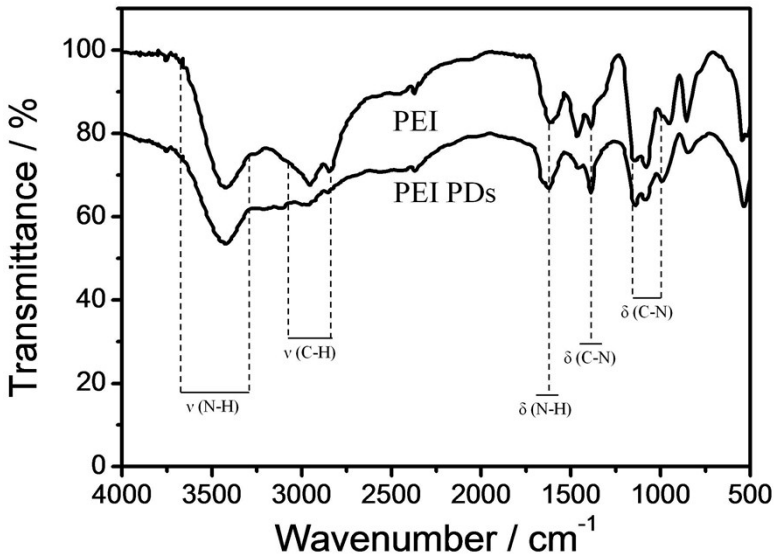

Figure S5. FT-IR spectra of the bare PEI and PDs, respectively.

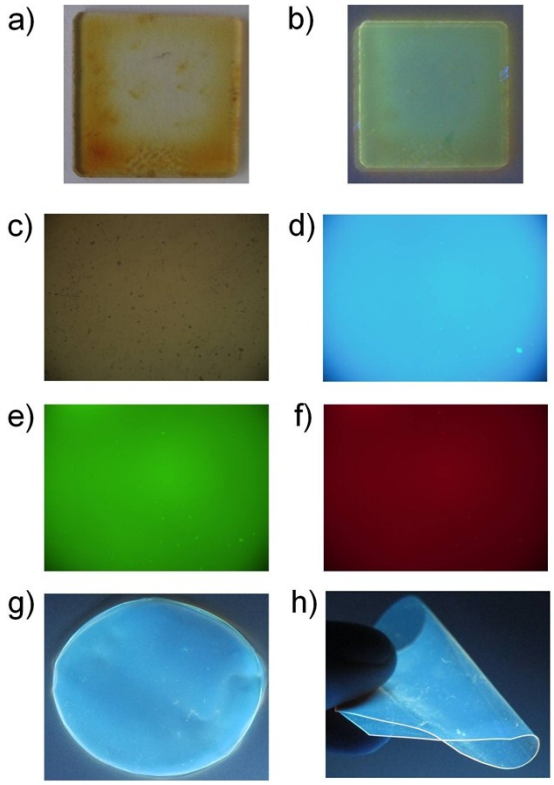

Figure S6. The dried film on the glass based on PDs aqueous solution. a-b) The photo of the PDs film under the white light and UV excitation, respectively.c-f) The microscope images of PDs film under natural light (c), UV (d), blue light (e) and green light (f) excitations. g-h) The bulk nanocomposite was prepared by PDs and PVA.

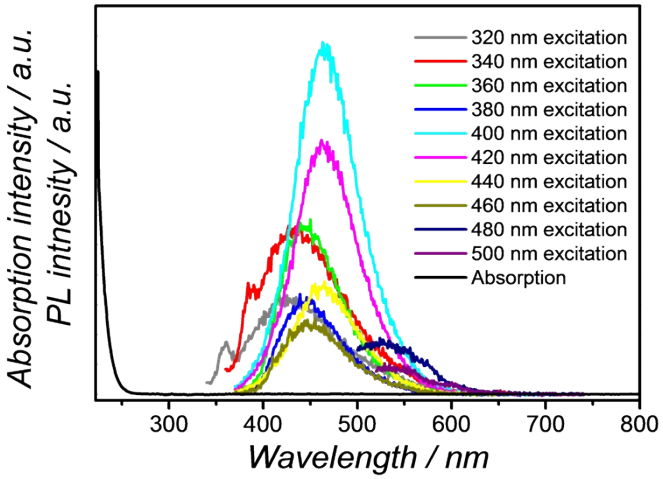

Figure S7. The absorption and fluorescence spectra of the bare PEI.

Table S1. Quantum yields (QY) measurements.

Quinine sulfate (0.1M H_2SO_4 as the solvent; QY=0.54) was chosen as the standard. The QYs of PDs (in water) were determined by the point-reference method.

$$\phi_x = \phi_{st}(I_x / I_{st})(\eta_x^2 / \eta_{st}^2)(A_{st} / A_x)$$

Where φ is the QY, I is the measured integrated emission intensity, η is the refractive index of the solvent, and A is the optical density. The subscript *"st"* refers to the standard with known QY and *"x"* refers to the sample. To minimize re-absorption effects, absorption in the 10 mm fluorescence cuvette was kept below 0.10 at the excitation wavelength (360 nm).

Series	ABS	Peak area	QY / %
Quinine sulfate	0.089	54596.4	54
Bare PEI	Hardly to be determined		
PD 1	0.098	3018.8	2.7
PD 2	0.091	3308.0	3.2
PD 3	0.08	8724.5	9.6
PD 4	0.085	482.8	0.5

Figure S8. The different graphic patterns were obtained by PDs ink.

Table S2. The lifetime of the PDs at 375 nm excitation and probed at different wavelength sections. The following table is the related values.

Wavelength (nm)	τ_1 (ns)	Percent (%)	τ_2 (ns)	Percent (%)	$\tau_{average}$ (ns)
440	0.93	49.38	5.71	50.62	3.349636
460	1.07	46.04	5.98	53.96	3.719436
480	1.17	44.68	6.19	55.32	3.947064
500	1.19	43.75	6.17	56.25	3.99125
520	1.23	44.46	6.3	55.54	4.045878
540	1.24	44.83	6.25	55.17	4.004017
560	1.13	44.26	6.08	55.74	3.88913
580	1.18	47.44	6.15	52.56	3.792232

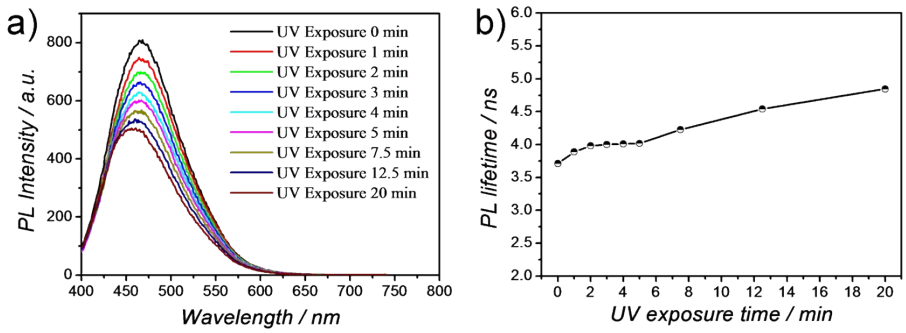

Figure S9. The PL property and lifetime of PDs after 2000 W UV exposure with different time. It was interesting that the steady PL decreased during the high power UV exposure, while the PL lifetime increased. The precise mechanism was unclear, but it was possible that the UV exposure can induce the degradation of crosslinked polymer chains in PDs, and the radiative process decreased while the nonradiative process increased.

Table S3. The QYs of PDs prepared by different degrees of crosslinking.

The quality ratio of PEI/CTC	Quantum yield / %
3:1	2.7
5:1	1.8
10:1	1.7
20:1	0.8

Data note: the quantum yield was determined by quinine sulfate as the standard.

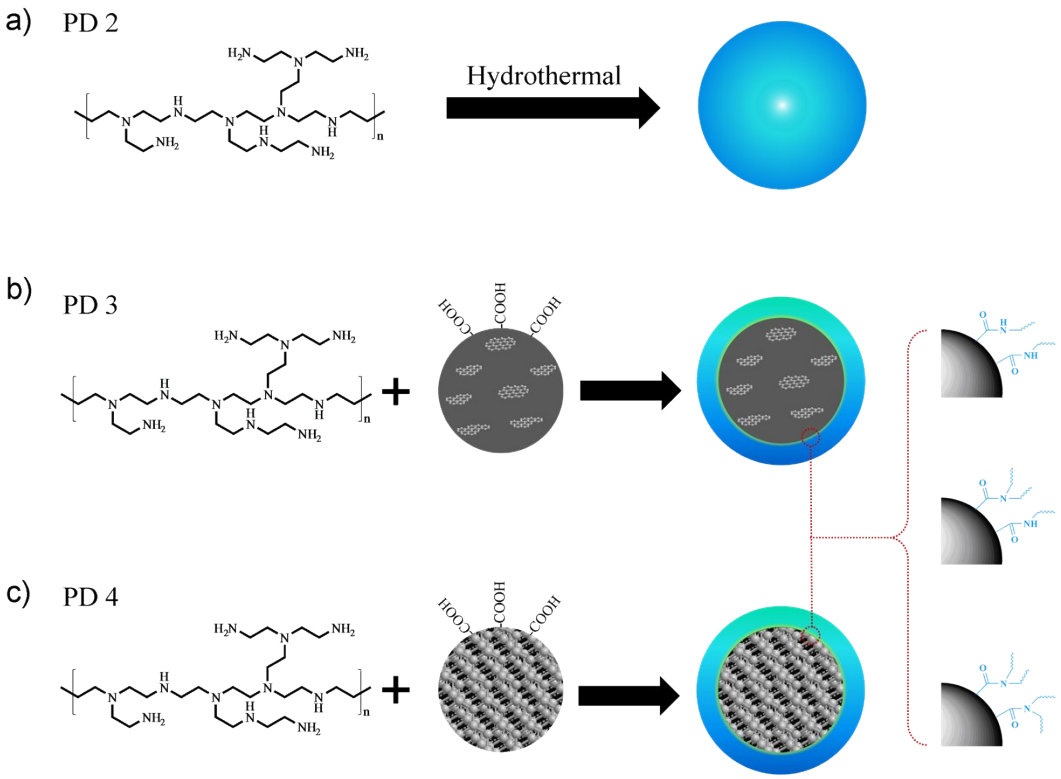

Figure S10. a-c) The scheme of PD 2-4. The PD 2 was prepared by the hydrothermal route, in which the PEI was formed to a sphere aggregation during the hydrothermal process. PD 3 and 4 were prepared by coating the PEI on two different carbon cores (the amorphous carbon core and the carbon core with perfect carbon lattice).

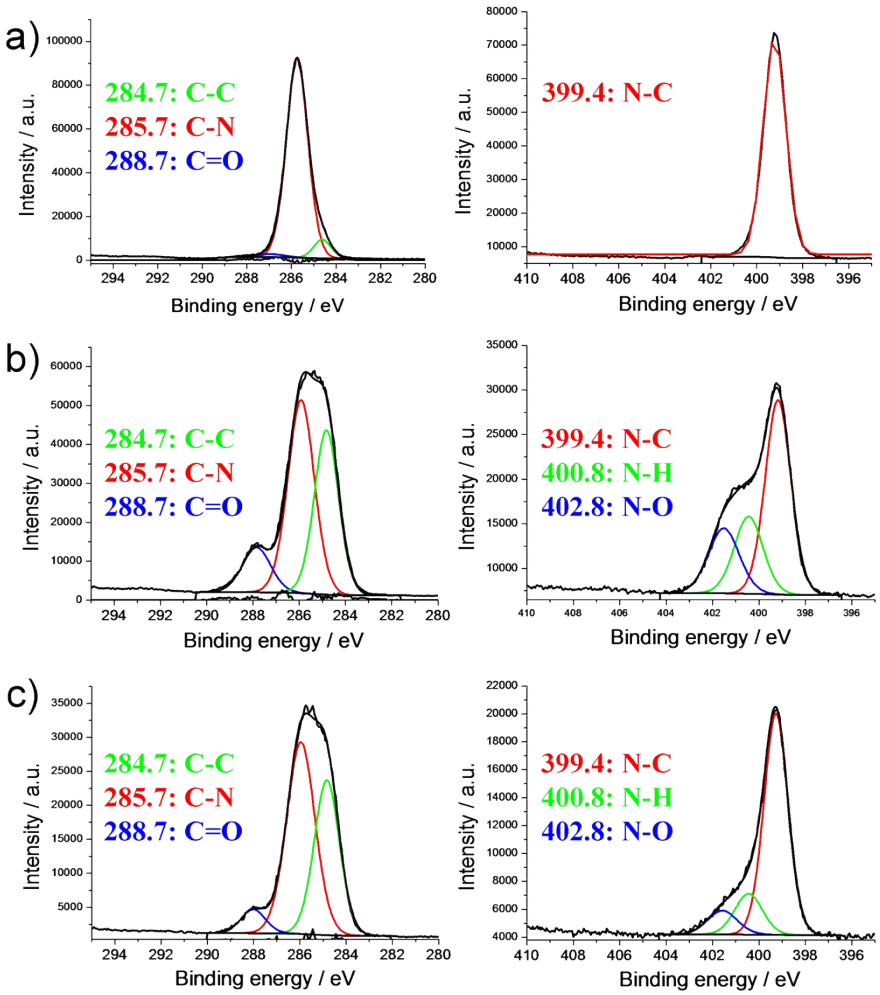

Figure S11. a-c) The XPS analysis of PD 2-4, respectively.

Table S4. The Zeta potential and particle size of PD 1-4.

Serials	Zeta potential / mV	Particle size by DLS / nm
PD 1	9.80	179.8
PD 2	-4.59	163.6
PD 3	0.0446 (-17.3 for carbon core)	117.5 (34.3 for carbon core)
PD 4	1.94 (-28.1 for carbon core)	143.6 (48.1 for carbon core)

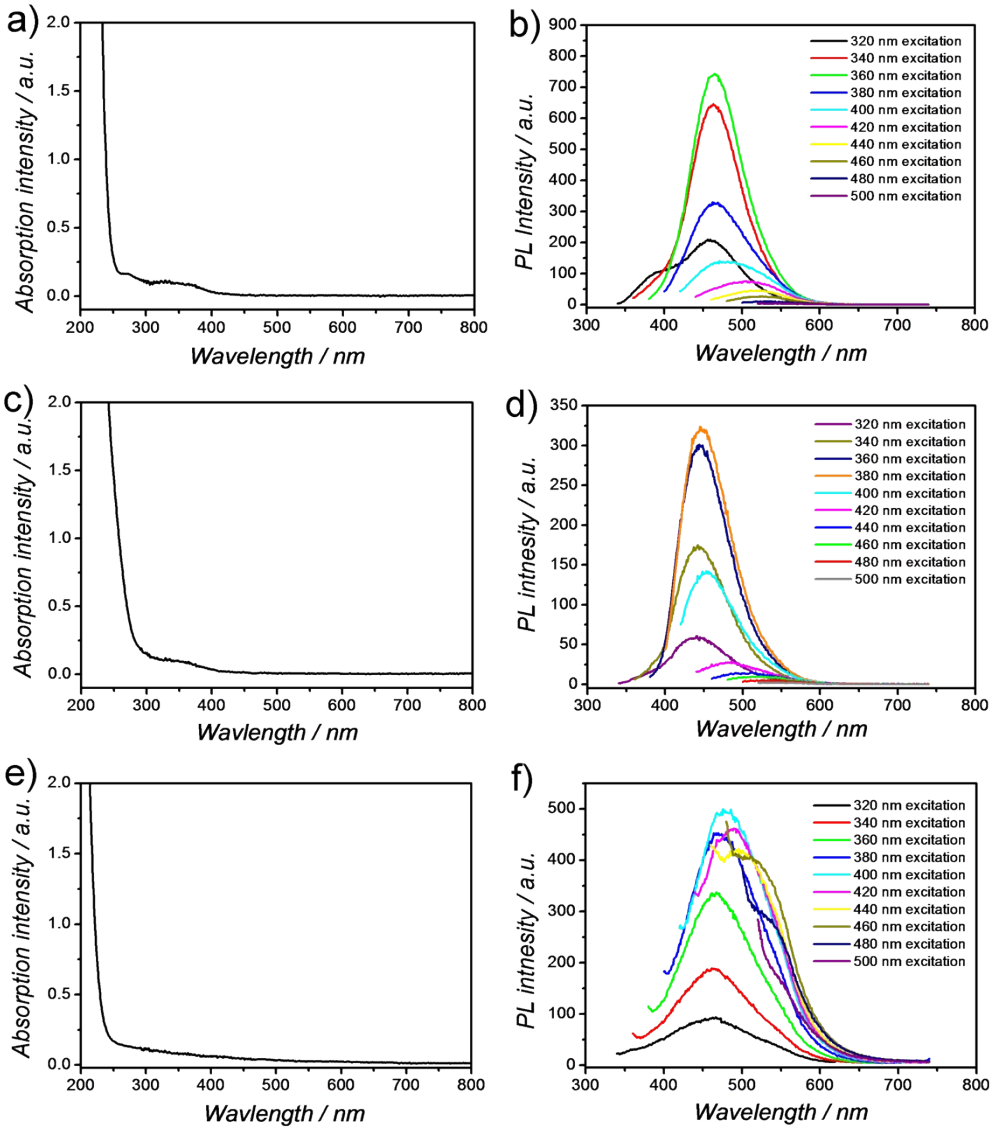

Figure S12. The optical properties of the PD 2-4. a, b) The absorption and fluorescence spectra of PD 2. c, d) The absorption and fluorescence spectra of PD 3. e, f) The absorption and fluorescence spectra of PD 4.

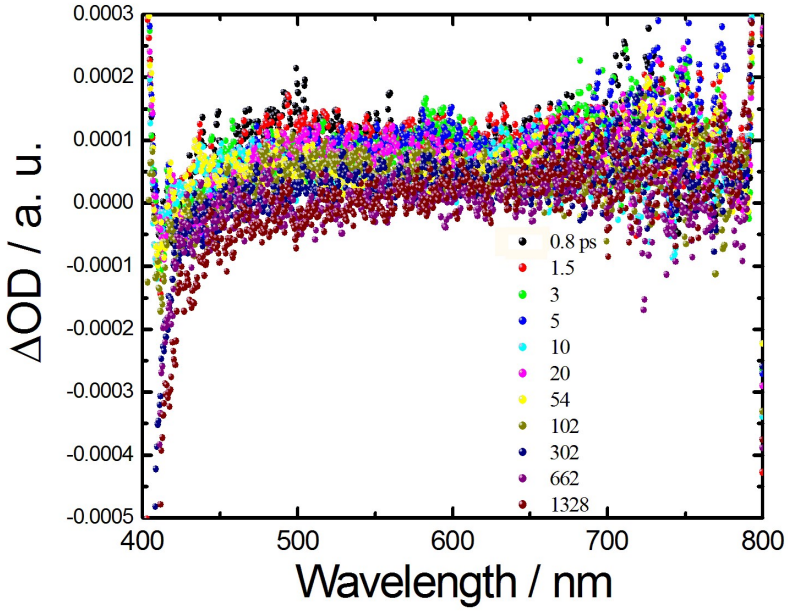

Figure S13. The transient spectra of bare PEI.

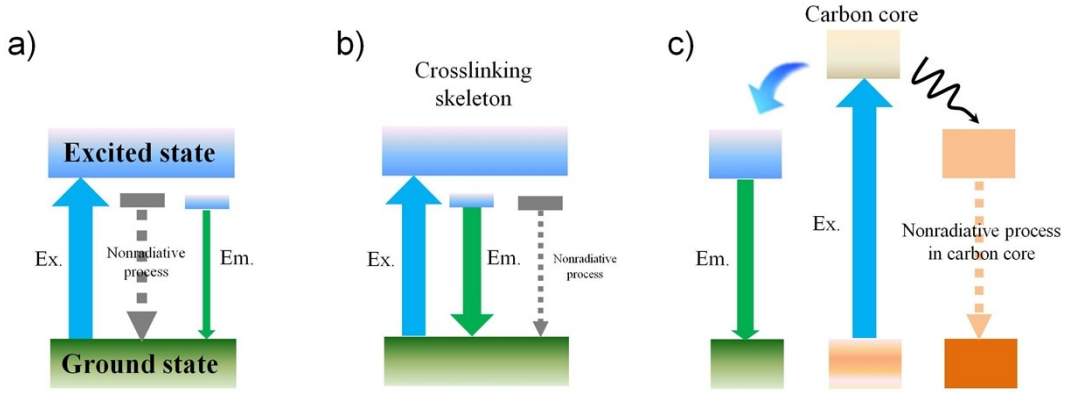

Figure S14. a) The suggested electronic state of bare PEI. b) The suggested electronic state of PD 1 and 2. c) The suggested electronic state of PD 3 and 4.

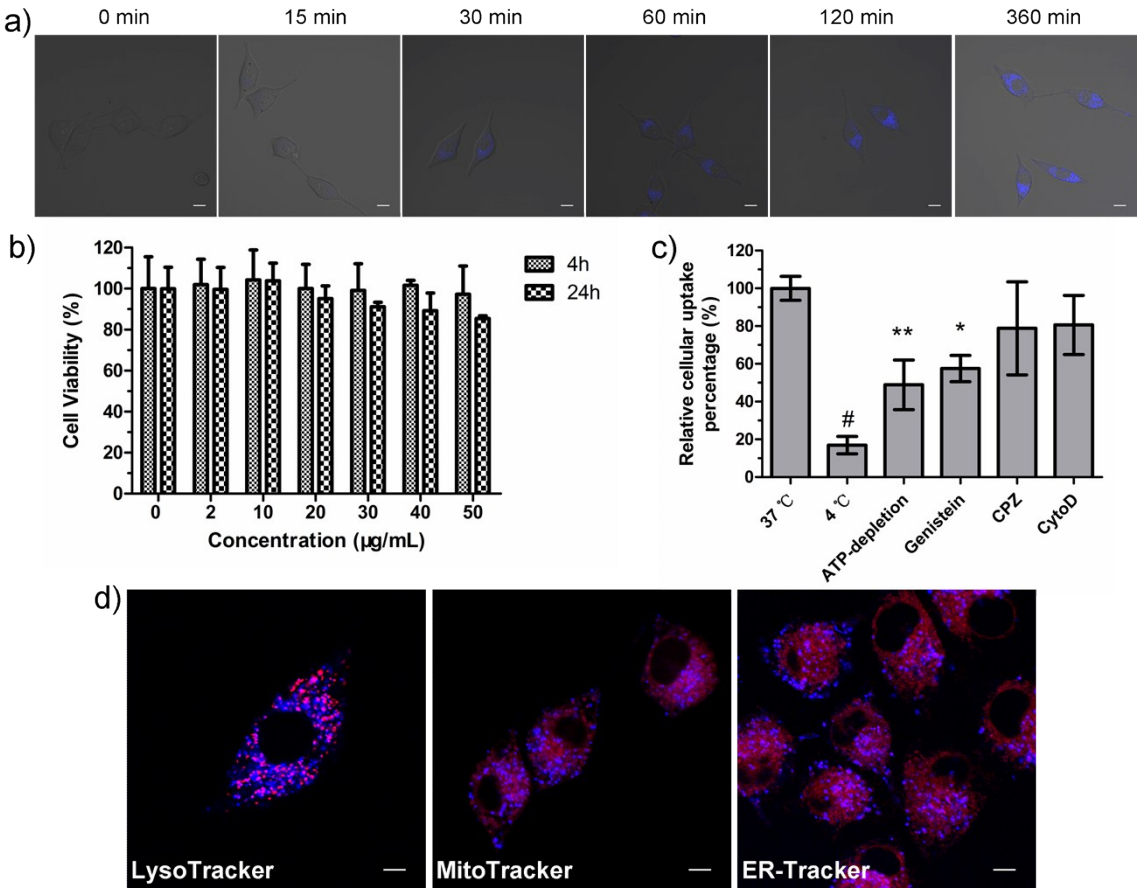

Figure S15. The cellular (RSC96) uptake mechanism and internalization of PDs. a) The time-dependent cellular uptake of passivated PDs determined by CLSM (The scale bar is 10 μm). b) Cytotoxicity of PDs. c) The FACS quantitative data for the uptake of PDs by RSC96 cells incubated at 37 °C, 4 °C, ATP-depletion, inhibitors Genistein, chlorpromazine, and cyto D treatments, respectively. d) PDs co-localized with cellular organelles specific dyes in RSC96 cells. LysoTracker red, ER-Tracker red and MitoTracker red were used to label, lysosomes, endoplasmic reticulum and mitochondria, respectively (The data on PDs co-localized with PI was omitted, because the PDs can not enter the nucleus just like PC12 cells). The scale bar is 5 μm.

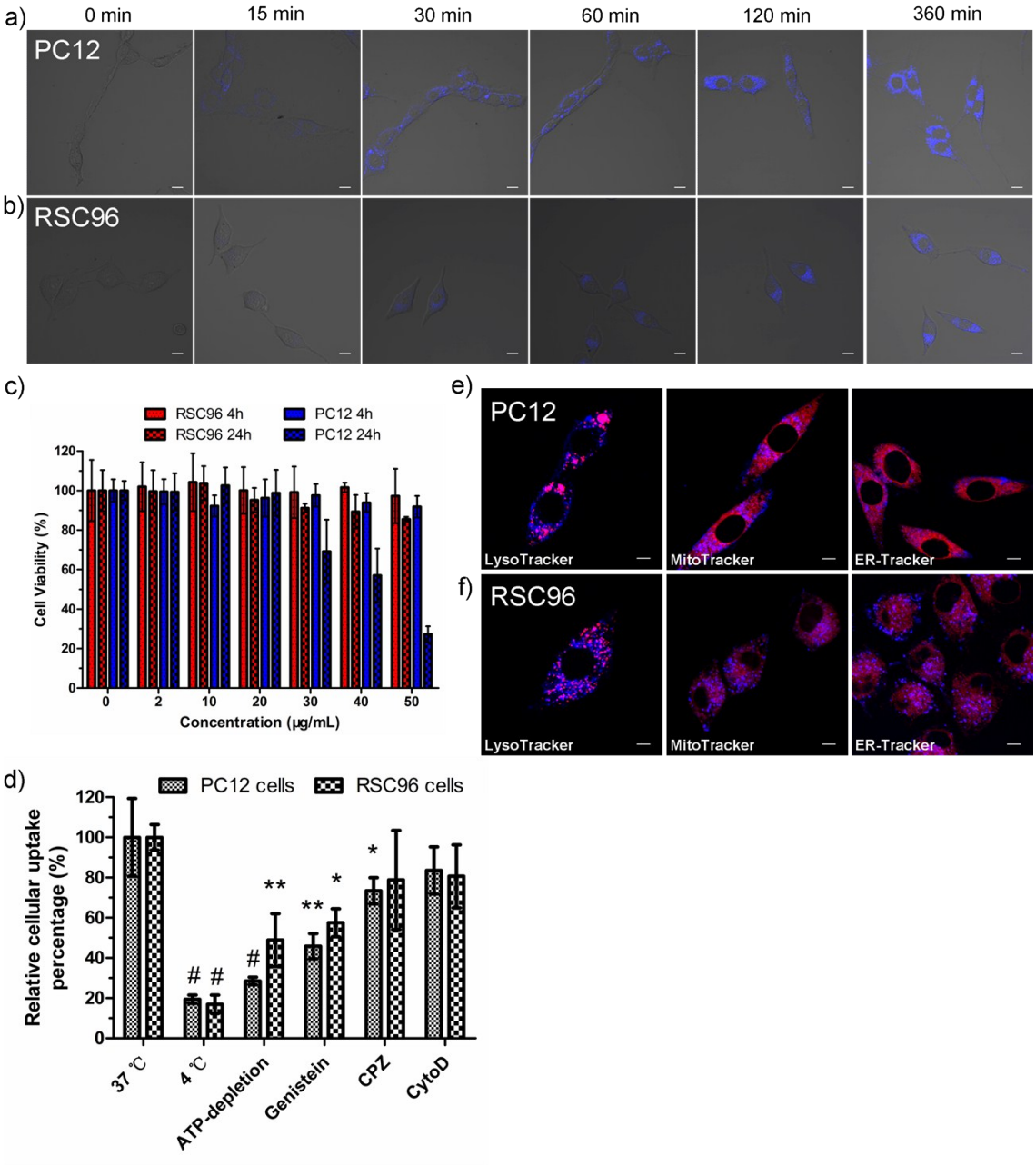

Figure 16. The PDs possessed similar cellular uptake mechanism and internalization for two different cell lines, the image was the mixed result by Figure 4 and S15. a-b) The time-dependent cellular uptake of passivated PDs determined by CLSM (The scale bar is 10 μm). c) Cytotoxicity of PDs. d) The FACS quantitative data for the uptake of PDs by PC12 and RSC96 cells incubated at 37 °C, 4 °C, ATP-depletion, inhibitors Genistein, chlorpromazine and, cyto D treatments, respectively. e-f) PDs co-localized with cellular organelles specific dyes in PC12 and RSC96 cells. LysoTracker red, ER-Tracker red, and MitoTracker red were

used to label lysosomes, endoplasmic reticulum and mitochondria, respectively. The scale bar is 5 μm.

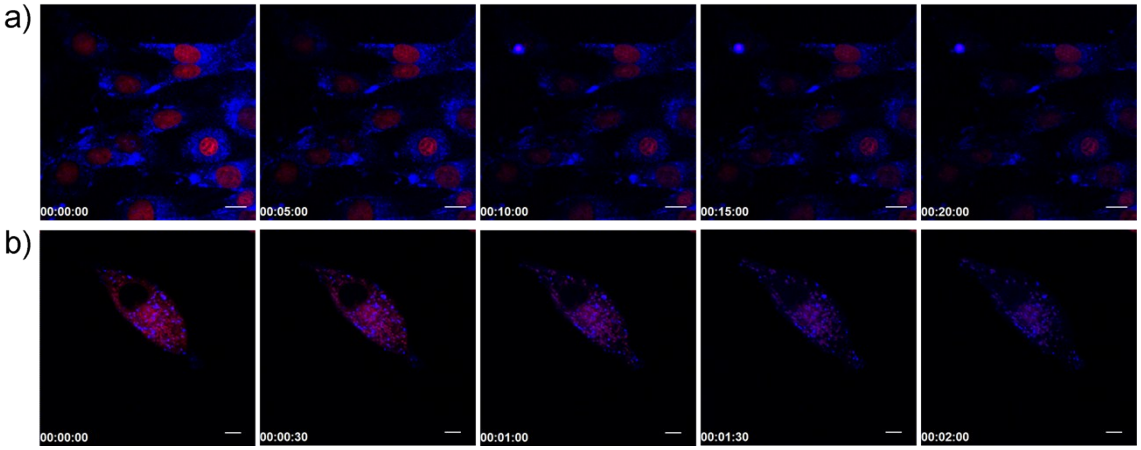

Figure S17. The photo-stability of PDs in cells compared with commercial dyes. a) The PC 12 cells were labelled by PDs and PI, and submitted to the CLSM exposure for 20 min. b) The RSC96 cells were labelled by PDs and Mitotracker, and submitted to the CLSM for 2 min. It was found that the PL of Mitotracker nearly disappeared for only 2 min exposure while the PDs kept the original PL intensity. The scale bar was 10 μm for a) and 5 μm for b).

ESI references

[1] S. Zhu, J. Zhang, L. Wang, Y. Song, G. Zhang, H. Wang, B. Yang, *Chemical Communications* **2012**, *48*, 10889.

[2] H. Li, X. He, Z. Kang, H. Huang, Y. Liu, J. Liu, S. Lian, C. H. Tsang, X. Yang, S. T. Lee, *Angewandte Chemie* **2010**, *49*, 4430.