Near-infrared AIEgen-functionalized and Diselenide-linked Oligo-ethylenimine with Self-sufficing ROS to Exert Spatiotemporal Responsibility for Promoted Gene Delivery

Yongkang Huang,[a#] Qixian Chen,[b#] Hongguang Lu,[a] Jinxia An,[a] Huajie Zhu,[a] Xiangjie Yan,[a] Wei Li,[a] Hui Gao[a*]

[a]School of Chemistry and Chemical Engineering, Tianjin Key Laboratory of Organic Solar Cells and Photochemical Conversion, Tianjin University of Technology, Tianjin 300384 (P. R. China).

[b]School of Life Science and Biotechnology, Dalian University of Technology, No. 2 Linggong Road, Dalian 116024 (P. R. China).

E-mail: hgao@tjut.edu.cn (H. Gao).

Electronic Supplementary Information (ESI) available. See DOI: 10.1039/x0xx00000x

Author Contributions

#(Y. H., Q.C.) These authors contributed equally.

1. Experimental section

1.1 Reagents and chemicals

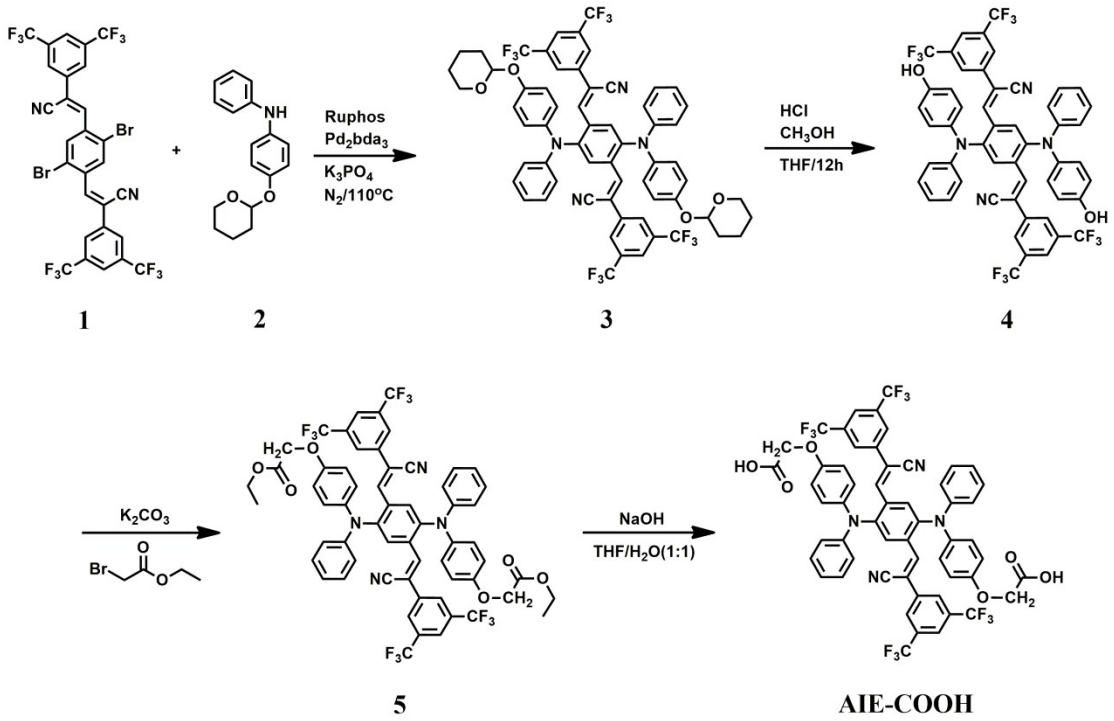
Selenium powder, sodium borohydride ($NaBH_4$), 3-chloropropionic acid chrormic trioxide, 3,5-bis(trifluoromethyl)phenylacetonitrile, bis(dibenzylideneacetone)palladium(0) [$Pd_2(dba)_3$] dichlorofluorescein diacetate (DCF-DA), 2-dicyclohexylphosphino-2',6'-diisopropoxybiphenyl (Ruphos) and dithiothreitol were purchased from Energy Chemical Company (Shanghai, China). 1,4-dibromo-2,5-dimethylbenzene, ethyl bromoacetate, N-pheny-4-(tetrahydro-2H-pyran-2-yi)oxy)aniline, 4-hydroxydiphenylamine, pyridinium p-toluenesulfonate, N-hydroxysuccinimide (NHS) and 1-ethyl-3-[3-(dimethylamino)- propyl] carbodiimide (EDC) were purchased from Heowns (Tianjin, China). Oligoethylenimine (OEI_{600}) was obtained from Aladdin (Shanghai, China). 9,10-Anthracenediylbis(methylene)dimalonic acid (ABDA) and polyethylenimine [M_w: 25 kDa, branched] were purchased from Sigma-Aldrich. 3-(4,5-Dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was used in the cell cytotoxicity assay (Dojindo, Japan). Ethidium bromide (EtBr) was obtained from Macklin (Shanghai, China). GSH and GSSG Assay Kit assay kit was obtained from Beyotime Biotechnology Company (Shanghai, China). All other reagents were obtained from Tianjin Chemical Reagent Co. (Tianjin, China).

^{1}H-NMR spectra of AIEgen derivatives and the polymers were recorded on a 400 MHz Bruker Avance-400 spectrometer (400 MHz, Bruker, Freemont, CA). The chemical shifts were referred to the solvent peaks, δ = 2.50 ppm for DMSO. UV-Vis spectra were recorded on a Nanophotometer NP80 Touch spectrophotometer. Fluorescence spectra were carried out with a Hitachi F4500 spectrofluorophotometer and Maya2000Pro optical fiber spectrophotometer. The molecular weight was evaluated by gel permeation chromatography (GPC), using the ViscoGEL C-Series columns (C-MBLMW-3078) from Malvern and 5% aqueous acetic acid medium as a mobile phase at a flow rate of 0.7 mL min^{-1} at 35 °C. Molecular weights were calibrated with PolyCALTM Dextran Std-T68K (Worcestershire ER14 1XZ, UK) as standards.

Dynamic light scattering (DLS) measurements were performed using a Malvern Nano-ZS instrument at 25 °C. The morphology of AIE nanoparticles (AIE NPs) was observed using a JEOL JSM-6700F type field emission scanning electron microscope (SEM).

1.2 Synthesis of 2,2'-((((2-((E)-2-(3,5-bis(trifluoromethyl)phenyl)-2-cyanovinyl)-5-((Z)-2-(3,5-bis (trifluoromethyl)phenyl)-2-cyanovinyl)-1,4-phenylene)bis(phenylazanediyl))bis(4, 1-phenylene))bis(oxy))diacetic acid (AIE-COOH)

Organic monomer with aggregation-induced emission (AIEgen) was synthesized as previously described using a Knoevenagel reaction of 1 and 2 to obtain compound 3. Compound 3 was deprotected to yield compound 4.[1] Compound 4 (1.0 mmol) and K_2CO_3 (3.0 mmol) was dissolved in 2 mL THF followed by dropwise addition of bromoacetate (2.0 mmol) under nitrogen with magnetic stirring. The reaction mixture was allowed to proceed overnight at 70 °C for 48 h. The crude product was purified by silica gel chromatography (petroleum ether : ethyl acetate = 5 : 1), and then evaporated to dryness under reduced pressure to give 5. Compound 5 was dissolved in 4 mL THF/H_2O (1 : 1) and hydrolyzed by NaOH at 65 °C for 4 h to obtain the mixture solution. The solvent was removed via rotary evaporation. The solid crude product was dissolved in water and acidified with hydrogen chloride until pH value reach 2.0. The pure AIE-COOH was obtained by filtration. ^{1}H NMR (400 MHz, DMSO) δ 12.97 (s, 2H), 8.12 (d, J = 5.8 Hz, 4H), 7.84 (s, 4H), 7.59 (s, 2H), 7.22 (t, J = 7.8 Hz, 5H), 7.07 (d, J = 8.9 Hz, 4H), 6.96 (d, J = 7.9 Hz, 5H), 6.88 (t, J = 7.4 Hz, 3H), 6.83 (d, J = 9.0 Hz, 4H), 4.53 (s, 3H). Yield: 89%.



Scheme S1 Synthetic scheme of the AIE-COOH.

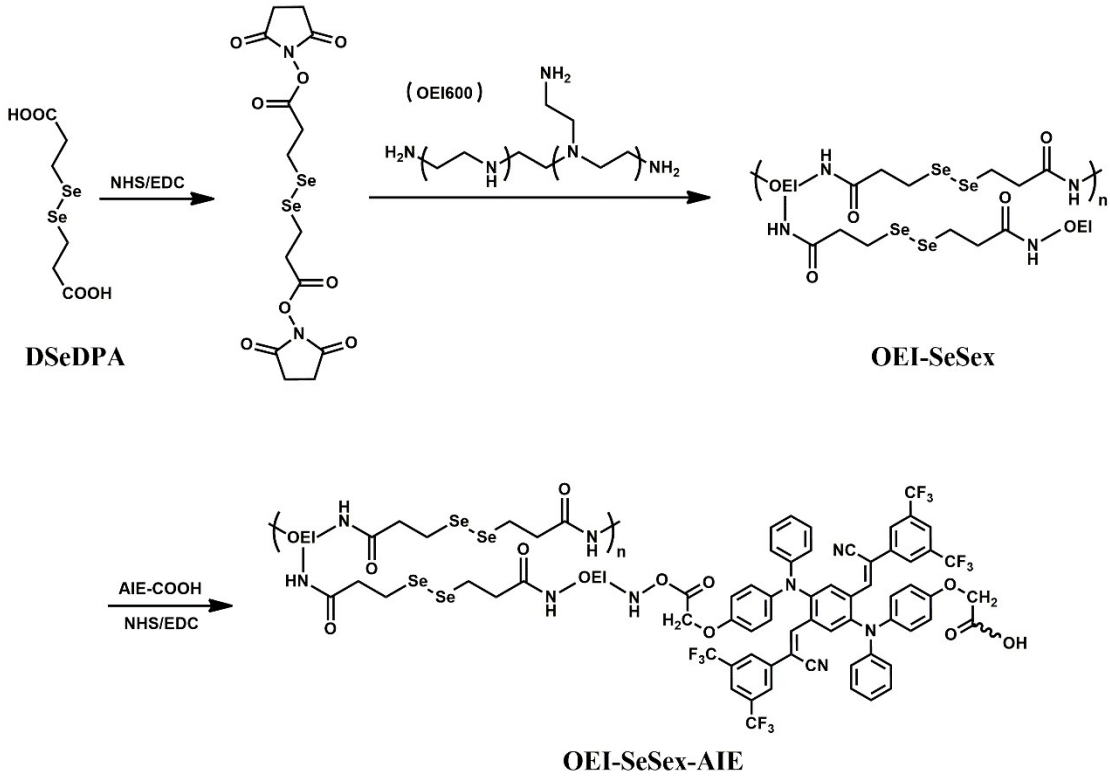
1.3 Aggregation-induced emission characters of AIE-COOH

AIE-COOH powder was dissolved in anhydrous THF solution (15 mM) as stock solution. UV-vis spectra of AIE-COOH were recorded on a Nanophotometer NP80 Touch spectrophotometer. The THF/water mixtures with different water fractions were prepared by slowly adding distilled water into THF under sonication at room temperature. The concentration of AIE-COOH was maintained at 10 μM. The emission measurement of the resultant mixtures was performed immediately in the wavelength range of 600-800 nm.

1.4 Synthesis of OEI-SeSex-AIE polymer

OEI-SeSex were synthesized according to previous reports by cross-linking OEI_{600} with 3,3'-diselanediyldipropanoic acid (DSeDPA).[2] OEI-SeSex-AIE copolymer was then obtained by activating AIE-COOH with EDC and NHS followed by reacting with OEI-SeSex. Briefly, AIE-COOH (0.17 mmol) and EDC (16.8 mmol) were dissolved in 3.0 mL anhydrous DMSO in a flask with magnetic stirring for 15 min. NHS (4.2 mmol) was dissolved in 1 mL anhydrous DMSO and added into the mixture with magnetic stirring for 3 h to activate the carboxyl. OEI-SeSex (1.7 mmol) was dissolved in 1 mL

of anhydrous DMSO and then added into the activated AIE-COOH with magnetic stirring for 48 h to obtain crude product. The pure products of OEI-SeSex-AIE were obtained after dialysis (Spectra/Por RC, MWCO 8-14 kDa) against deionized water for 48 h and lyophilization. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.13 (s, 18H), 7.86 (s, 20H), 7.58 (s, 15H), 7.22 (s, 31H), 7.04 (s, 29H), 6.96 (s, 32H), 6.88 (s, 60H), 4.89 (s, 29H), 3.51 – 3.19 (m, 138H), 2.67 (s, 8H), 2.55 – 2.46 (m, 780H), 2.33 (s, 15H), 2.18 (d, J = 11.9 Hz, 34H), 2.18 (d, J = 11.9 Hz, 41H), 1.65 (s, 1H), 1.24 (s, 119H), 1.07 (d, J = 7.0 Hz, 33H). Yield: 81.6%.



Scheme S2 Synthetic scheme of the polymer OEI-SeSex-AIE.

1.5 AIE NPs assembly

Either OEI-SeSex-AIE (0.5g) or AIE-COOH (0.5g) was dissolved in the mixed solution of 3.0 mL H_2O and 3.0 mL anhydrous DMSO, respectively, and stirred for 24 h and then dialysis (Spectra/Por RC, MWCO 8-14 kDa) and lyophilization to obtain OEI-SeSex-AIE/AIE. AIE NPs were formed by adding the OEI-SeSex-AIE/AIE solutions of prescribed concentrations to an equal volume of pDNA solution (50 μg/mL) to obtain the catiomer/pDNA weight ratios (c/p) of 5. The two solutions were mixed and vortexed

for 30 s, followed by incubation at room temperature for 30 min to obtain the AIE NPs.

1.6 Particle size measurements of AIE NPs

The size of AIE NPs were determined by Zetasizer Nano ZS90 instrument (Malvern Instruments, Southborough, MA) at 25 °C. All AIE NPs samples were prepared in water as mentioned above.

1.7 Morphology of AIE NPs

The morphologies of AIE NPs were investigated by scanning electron microscope (SEM) performed on a JSM-6700F (JEOL Ltd., Japan) instrument at an acceleration voltage of 10 kV. A drop of AIE NPs solution was deposited onto a double-polished SiO_2 slice, followed by air-drying for 48 h. The specimens were manufactured by coating the above samples with a thin gold layer.

1.8 ROS production upon daylight

To study the ability of the AIE-COOH and AIE NPs to generate ROS, dichlorofluorescein diacetate (DCF-DA) was used to detect the ROS generation upon daylight irradiation. To convert DCF-DA to dichlorofluorescein (DCF), 0.25 mL of 1 mM DCF-DA in ethanol was added to 1 mL of 0.01 N NaOH and allowed to stir for half an hour at room temperature. The hydrolysate was then neutralized with 5 mL of 1 × PBS at pH 7.4, and stored in ice for further use. 20 μL of the above solution was mixed with 1 mL of AIE-COOH (0.15 mM) or AIE NPs (125 μg mL^{-1}) and exposed to daylight irradiation for different time intervals at a power density of 10 mW cm^{-2} (All the light irradiation was in the same condition). The fluorescence change in the solution was measured with excitation at 488 nm and the emission was collected at 525 nm.

1.9 ROS quantum yield

The ROS quantum yield of AIE NPs in water (Φ) upon daylight irradiation (10 mW cm^{-2}) was determined using ABDA as an indicator and using Rose Bengal (RB) as the standard reference. ABDA solid (200 μM) was dissolved in DI water. The AIE NPs (100 μg mL^{-1}) or RB (25 μg mL^{-1}) was then added in aqueous solution. The absorbance decrease of ABDA

at 400 nm was recorded for different durations of light irradiation to obtain the decay rate of the photosensitizing process. And the ROS yield is calculated using the following equation:

$$\Phi_{\text{AIE NPs}} = \Phi_{\text{RB}} (K_{\text{AIE NPs}} \bullet A_{\text{RB}}) / K_{\text{RB}} \bullet A_{\text{AIE NPs}}$$

Where $K_{\text{AIE NPs}}$ and K_{RB} are the decomposition rate constants of the photosensitizing process determined by the plot ln (A_0/A) versus irradiation time. A_0 is the initial absorbance of ABDA while A is the ABDA absorbance after different irradiation times. $A_{\text{AIE NPs}}$ and A_{RB} represent the light absorbed by AIE NPs and RB, which are determined by integration of the absorption bands in the wavelength range of 400-800 nm. Φ_{RB} is the ROS quantum yield of RB, which is 75% in water. The decomposition rate constants of AIE NPs ($K_{\text{AIE NPs}}$) and RB (K_{RB}) were calculated as 0.0005 and 0.0011, respectively. The integrations of the optical absorption in the wavelength range of 400-800 nm for RB (A_{RB}) and OEI-Se-AIE ($A_{\text{AIE NPs}}$) were 212.58 and 87.12, respectively.

1.10 Agarose gel electrophoresis

Gel retardation assay was conducted to estimate the encapsulation efficiency of the polymers to pDNA. 5 μL of AIE NPs solution in H_2O at different c/p was mixed with 2 μL 40% glycerol and 1 μL loading-buffer. The mixture was dripped into the channel of agarose gel containing 0.8%wt EtBr, which immersed in TBE buffer. Electrophoresis was performed under 80 V for 90 min. DNA was visualized by UV illuminator (Gel Documentation Systems, Bio-Rad, Hercules, CA).

1.11 Assessment of micellar stability

AIE NPs (12.5 μg ml^{-1}) were used to detect the release of pDNA after 10 minutes of daylight (10 mW cm^{-2}), and the control group was not illuminated. Heparin is a competitive anion. EtBr (1 eq per base pair) was added to the mixed system for an additional 30 min. Increasing amounts of heparin was added to the mixed system. After 75 minutes, the fluorescence of free EtBr (F_i), pDNA/EtBr (F_0), and EtBr/complex were recorded after addition of heparin (F_h) on a Safire plate reader (Tecan, Medford, MA; λ_{ex} = 523 nm, λ_{em} = 587 nm). Relative fluorescence intensities were calculated from the following equation:

$$\text{Relative fluorescence} = \frac{(F_h - F_i)}{(F_0 - F_i)} \times 100$$

An increase in relative fluorescence is indicative of micelle destabilization.

1.12 Cell culture

Hela cell lines were incubated in RPIM 1640 media (Thermo Fisher Scientific from Shanghai, China) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics in humidified environment containing 5% CO_2 at 37 °C.

1.13 *In Vitro* transfection

Transfection assays were performed using a plasmid CAG-Luc as the reporter gene in HeLa cell lines. Briefly, cells were seeded on 24-well plates at a density of 5×10^4 cells in 400 μL of medium/well for 24 h. Then, the medium in each well was replaced with a fresh one. After being fed by AIE NPs (12.5 μg ml^{-1}) for four hours, the cells treated with AIE NPs are exposed to daylight for different times from 0-3 minutes. Followed by another 44 h of incubation, the cultured cells were washed with PBS twice and lysed in 100 μL of the cell culture lysis reagent. Luciferase gene expression was quantified by a commercial kit (Promega Co. Cergy Pontoise, France) and a luminometer (FLX800, BioTek, Gene company Limited). The protein concentration of each well was analyzed using a protein assay kit (Pierce). Gene expression results were expressed as relative light units (RLUs) per milligram of cell protein (RLU/mg protein). The results were presented as a mean and standard deviation obtained from four samples. Following a similar protocol, the transfection assays of Hela cells incubated with AIE NPs at varied c/p ratios were detected, and the PEI/pDNA complex (concentration of pDNA is 2.5 μg ml^{-1}) at N/P ratio of 10 was used as a control group.

1.14 Intracellular ROS assays.

The intracellular generation of ROS was determined by the fluorescence by confocal laser scanning microscopy (CLSM) (Zeiss LSM510, Oberkochen, Germany). HeLa cells were treated with AIE NPs (12.5 μg ml^{-1}) for 4 h. Then, the medium was replaced with

fresh one, and the cells were irradiated with daylight for different time intervals. DCF-DA (final concentration 10 mM) was addend the cells were incubated for 20 min. Later, the DCF-DA solution was removed and then repeatedly washed 3 times with PBS. Subsequently, another 1 mL PBS was added and the cells were incubated for additional 20 min. Finally, the cells were observed via CLSM (λ_{ex} = 488 nm, λ_{em} = 525 nm).

1.15 Intracellular distribution

CLSM was performed to explore insight into the internalization and subcellular localization of AIE NPs. HeLa cells were plated onto a 35 mm glass bottom dish at a density of 10^5 cells, incubated in 400 μL RPIM 1640 medium supplemented with 10% FBS for 24 h. The medium was exchanged with 1 mL of free-serum medium at pH 6.8, followed by addition of 100 μL AIE NPs (12.5 μg ml^{-1}) (labeled with Cy5). After 4 h of culture in humidified atmosphere of 5% CO_2 at 37 °C, the cells were washed with PBS, the cells in the light treatment group were exposed to daylight for 3 minutes at this time, followed by 20 h post incubation in 1 mL of fresh media. Then, cells were washed with PBS and treated with Lyso Tracker Green DND-26 (YEASEN, Shanghai, China) to stain lysosome. The cells were subjected to fixation with 75% alcohol prior to CLSM observation. The observation was carried out by a LSM510 (Nikon 108, Japan). For Cy5-pDNA: λ_{ex} = 633-635 nm, λ_{em} = 662-737 nm. For DAPI: λ_{ex} = 402 nm, λ_{em} = 461 nm. For Lyso Tracker Green: λ_{ex} = 504 nm, λ_{em} = 511 nm. For AIEgen: λ_{ex} = 493 nm, λ_{em} = 662-737 nm. The colocalization degree of late endosome/lysosome and Cy5-pDNA was calculated according to the formula: Colocalization degree (100%) = the number of yellow pixels/the total number of yellow and red pixels × 100%.

1.16 Intracellular glutathione assay

Hela cell lines were cultured in 6-well plates at a density of 3 × 10^5 cells for 24 h. The cells were seeded with AIE NPs (12.5 μg ml^{-1}) and treated in presence and absence of daylight irradiation after replacing the fresh medium. The glutathione (GSH) content was measured using the GSH and GSSG Assay Kit assay kit (Beyotime Biotechnology Company) according to the manufacturer's instructions.

1.17 Cytotoxicity assays

To determine the cytotoxicity, a MTT-based cell viability test was performed in 96-well plates. HeLa cells were seeded at a density of 5000 cells per well respectively. After overnight culturing, the cells were treated with AIE NPs (12.5 μg ml^{-1}) at c/p ratio of 5 or PEI/pDNA (concentration of pDNA is 2.5 μg ml^{-1}) at N/P ratio of 10 for 4 h. For daylight-treated experimental groups, the wells with AIE NPs upon 4 h incubation were exposed to daylight irradiation (10 mW cm^{-2}) for 0-3 minutes. The cell culture medium was then replaced by fresh cell culture medium and further cultured for 44 h. Then, the medium in the wells were removed and the cells were washed with PBS buffer and then incubated in fresh medium (100 μL) and 10 μL of MTT solution (5 mg mL^{-1}) for 3 h. After removing MTT solution, 100 μL of filtered DMSO was added into each well to dissolve all the crystals formed. The cell viability was accessed by means of MTT absorbance at 570 nm recorded using a microplate reader (Epoch, BioTek, Gene company Limited). The cell viability in each well was calculated from the obtained values as a percentage of control wells. The results were presented as a mean and standard deviation obtained from six samples. Following a similar protocol, the cell viability of Hela cells incubated with AIE NPs at varied c/p ratios were detected, and the PEI/pDNA complex (concentration of pDNA is 2.5 μg ml^{-1}) at N/P ratio of 10 was used as a control group.

1.18 Statistics analysis: Significant differences in cell viability, transfection efficiency and intracellular GSH content between any two groups were evaluated using Student's *t* test.

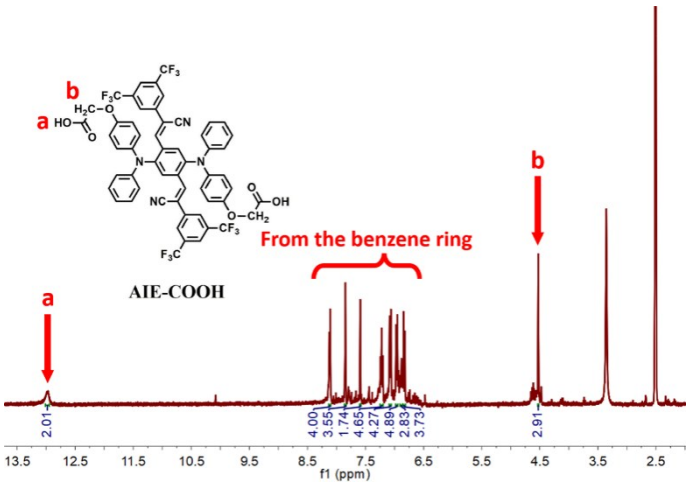


Fig. S1 ^{1}H NMR spectrum of AIE-COOH in DMSO-d_6.

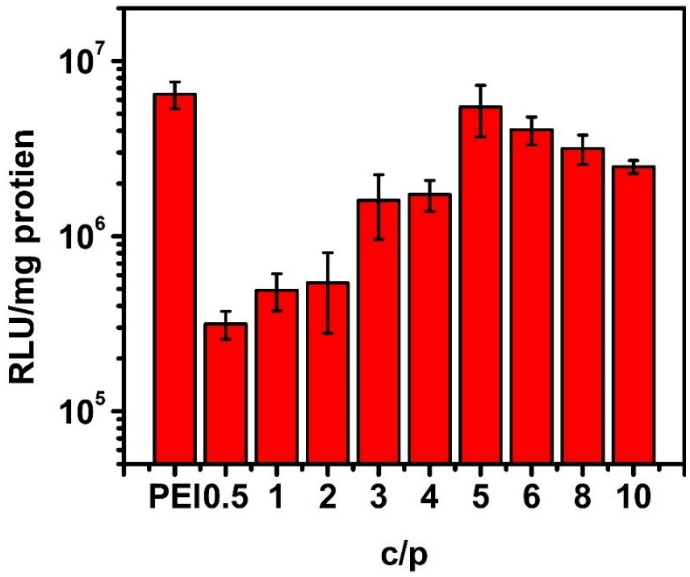


Fig. S2 Transfection efficiency of AIE NPs in Hela cells at varied c/p ratios. PEI/pDNA complex at N/P ratio of 10 was used as a control group. The results were presented as a mean and standard deviation obtained from four samples.

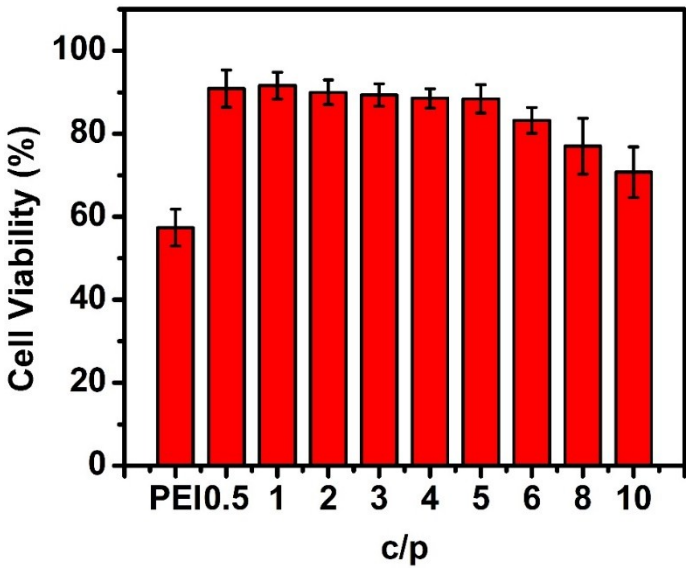


Fig. S3 Cell viability of Hela cells in presence of AIE NPs at varied c/p ratios. PEI/pDNA complex at N/P ratio of 10 was used as a control group. The results were presented as a mean and standard deviation obtained from six samples.

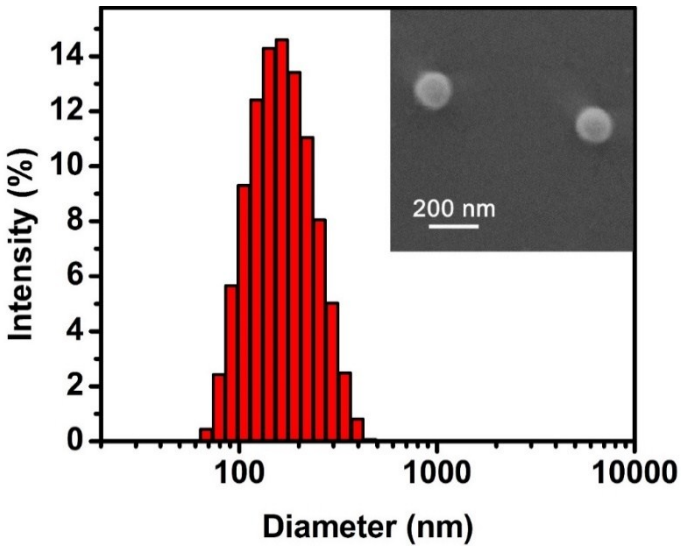


Fig. S4 Formation of OEI-SeSex-AIE/AIE nanoparticles characterized by DLS measurement and SEM observation.

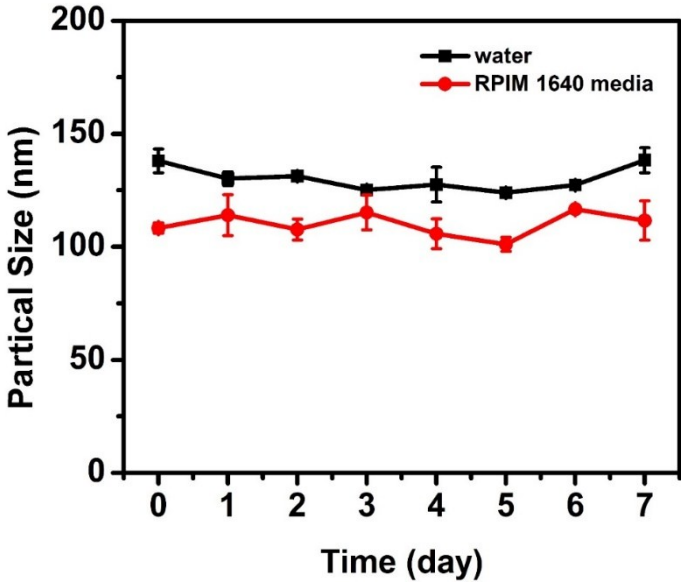


Fig. S5 Average hydrodynamic diameter changes of AIE NPs (12.5 μg mL^{-1}) upon incubation in water and RPIM 1640 media at 37 °C for 7 days.

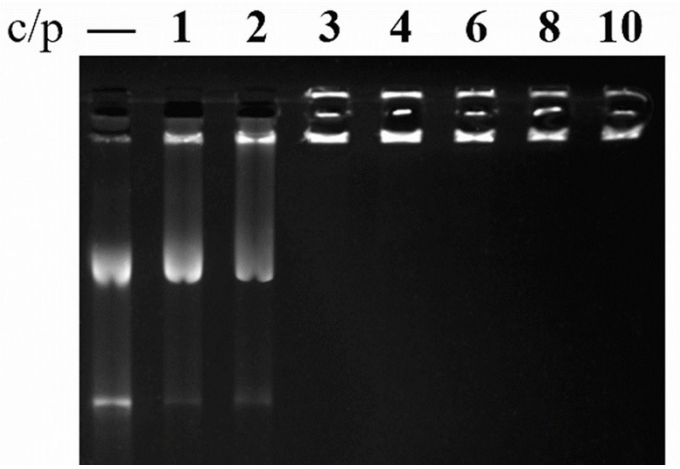


Fig. S6 Gel retardation assay of AIE NPs at varied c/p ratios.

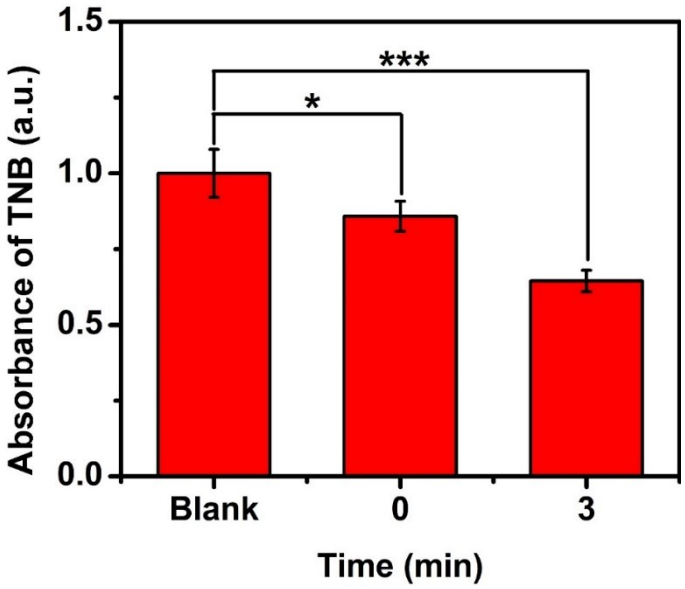


Fig. S7 Concentration of GSH in cells tested by the absorbance of TNB. Hela cells were treated with AIE NPs (12.5 μg mL^{-1}) for 4 h in all of the tests. **P*< 0.05. ****P*< 0.001.

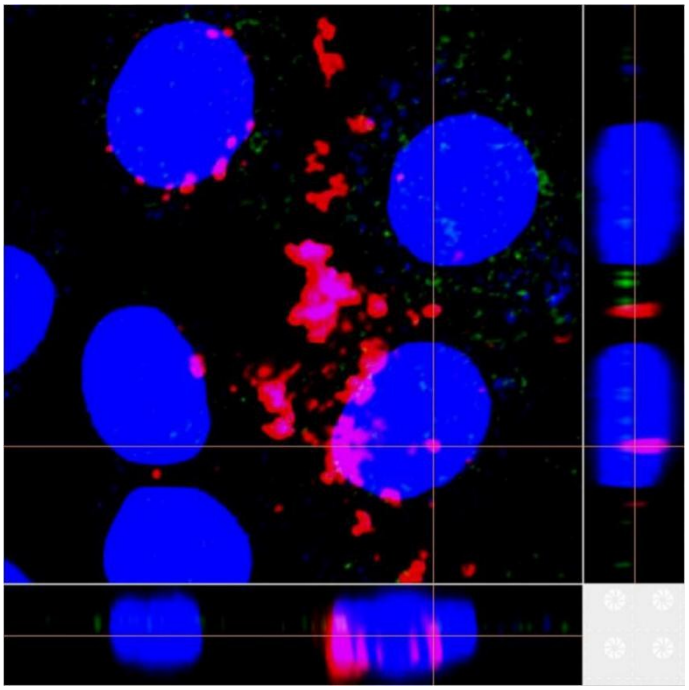


Fig. S8 CLSM images of Hela cells fed with AIE NPs (12.5 μg ml^{-1}). The cells were co-stained with DAPI. Representative slices of views from different directions of cell. For Cy5-pDNA, λ_{ex} = 633-635 nm, λ_{em} = 662-737 nm. For DAPI, λ_{ex} = 402 nm, λ_{em} = 461 nm.

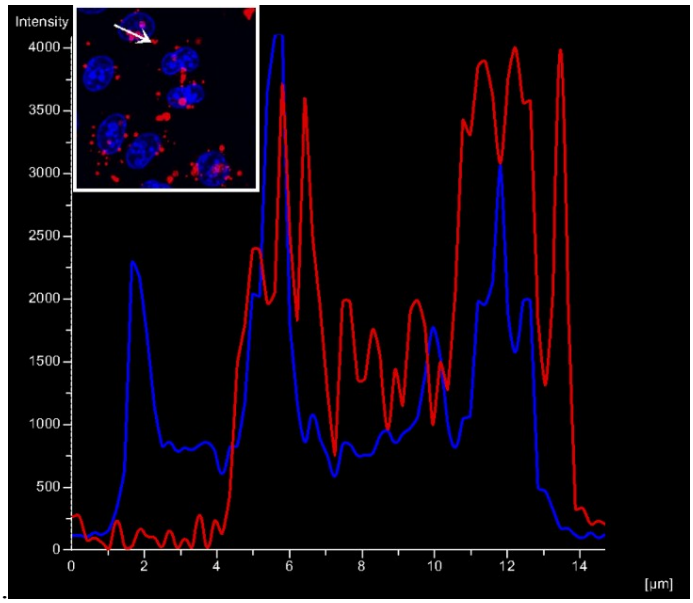


Fig. S9 The corresponding fluorescence intensity profiles of the areas marked by white arrow in the confocal images. HeLa cells were exposed to daylight for 3 minutes after feeding with AIE NPs (12.5 μg mL^{-1}) for 4 hours and then incubated for another 20 hours. The red line represents the Cy5-labeled pDNA and the blue line represents the nucleus.

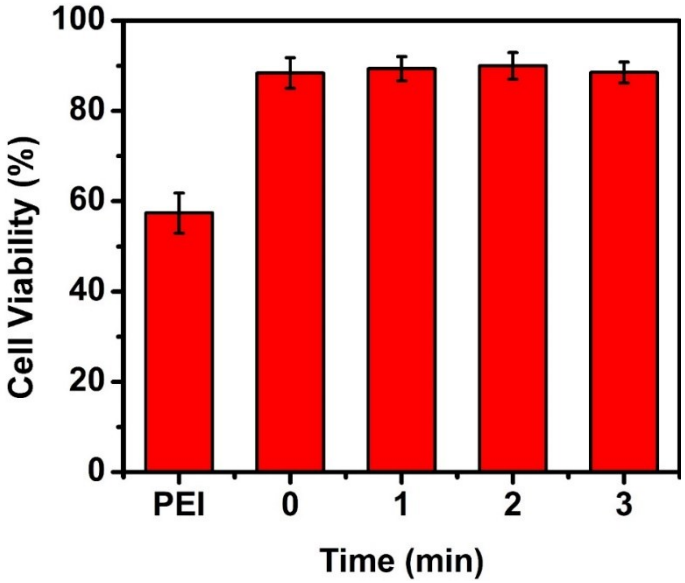


Fig. S10 Cell viability of Hela cells fed with AIE NPs (12.5 μg mL^{-1}) in presence and absence of daylight, as compared to PEI.

2. Reference:

(1) Y. Guan, H. Lu, W. Li, Y. Zheng, Z. Jiang, J. Zou and H. Gao, *ACS Appl. Mater. Interfaces*, 2017, **9**, 26731.

(2) D. Yue, G. Cheng, Y. He, Y. Nie, Q. Jiang, X. Cai and Z. Gu, *J. Mater. Chem. B*, 2014, **2**, 7210.