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AIE/FRET-Based Versatile Nanoparticles PEG-Pep-TPE/DOX for Cancer Therapy and Real-Time Drug Release Monitoring

*Tian-Tian Wang,[‡1] Qi-Chun Wei,[‡2] Zhen-Tao Zhang,[1] Meng-Ting Lin,[1] Jie-Jian Chen,[1] Yi Zhou,[1] Ning-Ning Guo,[1] Xin-Cheng Zhong,[1] Wen-Hong Xu,[2] Zhan-Xiang Liu,[1] Min Han,[*1] Jian-Qing Gao[*1]*

‡: These authors contributed equally

1. Experimental section

1.1 Synthesis of PEG-Pep-TPE

1.1.1 Synthesis of tetraphenylethylene carboxaldehyde (TPE-CHO)

TPE-CHO was synthesized referring to a published article.[1] 1-Bromo-4-[(1E)-3,3-diphenylprop-1-en-1-yl] benzene (6 mmol) and 4-Formylphenylboronic acid (9 mmol) were dissolved in toluene (40 mL), then tetrabuylammonium bromide (TBAB, 0.6 mmol) and 1.2 M potassium carbonate aqueous solution were added to the above solution. The mixture was stirred at room temperature for 0.5 h under N_2 gas protection. After that, $Pd(pph_3)_4$ (5.3×10^{-3} mmol) as a catalyst was added to the solution, which was heated up to 90 °C and refluxed for 24 h while TLC was used to monitor the progress of the reaction. After the completion of the reaction, the mixed solution was cooled to room temperature, and then poured into water extracted with ethyl acetate for three times; the organic layer was dried over anhydrous magnesium sulfate and then evaporated to yield crude TPE-CHO. The crude compound was purified by silica gel column chromatography (CH_2Cl_2: Hexane=1:2).

1.1.2 Synthesis of Peptide-ss- (Peptide: Phe-Phe-Lys-Tyr)

Thiodiglycolic acid (20 mg), HOBT(8 mg), TBTU(100 mg) and DIEA(20 μL) were disoloved in DMF(5 mL), then stirred for 2 h. Besides, 5 mL DMF consist of peptide (20 mg) was prepared and injected into the above solution drop by drop. After 12 h, the reaction solution was purified by dialysis (molecular weight cutoff 1000) in distilled water for 24 h, and then the mixture inside the dialysis tube was lyophilized to obtain Peptide-ss-.

1.1.3 Synthesis of PEG-Pep

The synthesis method was the same as that of Peptide-ss. Peptide-ss-(50 mg), HOBT (8 mg), TBTU (50 mg) and DIEA (20 μL) were dissolved in 5 ml of DMF for 2 h, then mixed with PEG-NH_2 2000 (150 mg) and stirred for 12 h. After that, the reaction solution was purified by dialysis (molecular weight cutoff 3500) in distilled water for 24 h, and then lyophilized to obtain PEG-Pep.

1.1.4 Synthesis of PEG-Pep-TPE

PEG-Pep and TPE-CHO (PEG-Pep: TPE-CHO=1:5) were dissolved in 10 ml THF with 2 drops of glacial acetic acid as a catalyst, and reacted for 12 h. After the reaction solution was evaporated under reduced pressure and reconstituted with water, the supernatant by ultracentrifugation at 3000 rpm was lyophilized to obtain PEG-Pep-TPE in the final.

1.1.5 Synthesis of PEG-Pep-TPE(C-N)

The compounds PEG-Pep-TPE (11 mg), $NaBH_3CN$ (0.7 mg) and bromocresol green (3 mg) was dissolved in a 1:1 mixture of methanol and THF under anhydrous and anaerobic conditions, after which a 1:1mixture of methanol and acetic acid were added dropwise until the reaction mixture was remained yellow and then stirred for 3 h at 0 °C. After that, saturated $NaHCO_3$ solution was added continually until the solution turned near colorless and no more bubble appeared. The resulting solution was dialyzed against distilled water for 2 days before being lyophilized.

1.2 Preparation of DOX-Loaded PEG-Pep-TPE nanoparticles

DOX-loaded PEG-Pep-TPE nanoparticles were prepared by thin film dispersion method: 10 mg PEG-Pep-TPE was dissolved in 30 mL methanol, followed by adding DOX (10 mg), then evaporated slowly for 3 h. And after that, it was reconstituted with deionized water to obtain the DOX-load nanoparticles. Besides, the drug loading efficiency (LE) of the DOX-loaded

nanoparticles was calculated according to the following equation: LE (wt %) = (weight of loaded drug/ (weight of polymer) ×100%

1.3 GSH/pH-responsive behaviors of blank nanoparticles in physical stimulation

The environment of acidic and high GSH in tumor cells was established as follows: a certain amount of GSH was added into 0.5 mL of the blank nanoparticle solution, and the final GSH concentration was 10 mM. Besides, 1 M HCl solution was used to adjust the pH to 5.0. After that, the mixture was incubated in a constant temperature shaking chamber for 12 h, 24 h respectively and then observed the change of their particle size and morphology.

1.4 Cell lines

The cancer cell lines A549 [Institute of Biochemistry and Cell Biology of Chinese Academy of Science (Shanghai, China)] were cultured in complete growth media (RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin) at 37°C in a humid atmosphere maintained of 5% CO_2.

1.5 GSH/pH-responsiveness of blank nanoparticles in cells

The logarithmic growth phase A549 cells were cultured in 50 mL cell culture flasks at 37 °C, 5% CO_2 overnight, and then treated with PEG-Pep-TPE nanoparticles preparation (100 μg/ml) for 12 h. After digestion by the enzyme, the mixture was centrifuged at 1200 rpm for 5 min and the precipitates were washed by pH 7.4 PBS twice. Then the cell pellet after centrifugation was fixed in 2.5% glutaraldehyde PBS overnight and frozen in 4 °C for sample preparation. In the final, the morphology of preparation in cells was observed under the transmission electron microscope.

1.6 FRET Analysis in vitro

PEG-Pep-TPE nanoparticles and PEG-Pep-TPE/DOX nanoparticles were dissolved in the mixed

solution of THF and water (V_{water}=99%) respectively until the final concentration of PEG-Pep-TPE was 1mg/mL. After the fluorescence intensity was assessed, a certain amount of GSH solid was added into the group of DOX-loaded nanoparticles, and the final GSH concentration was 10 mM. Meanwhile,1 M HCl solution was used to adjust the pH to 5.0. At selected time intervals for 0, 0.5, 1, 1.5, 2, 4 h, the fluorescence intensity was measured in the wavelength range of 400-650 nm.

1.7 *In vitro* release assay

Respectively, the 2 mL free DOX solution, PEG-Pep-TPE NPs +DOX, PEG-Pep-AIE/DOX NPs+ GSH-OEt and PEG-Pep-AIE/DOX NPs was transferred into a dialysis tube (MWCO = 8000-14000). It was noted that PEG-Pep-AIE/DOX NPs+ GSH-OEt composed of DOX-loaded nanoparticles and 2 μM GSH-OEt, and GSH-OEt was a GSH-responsive promotor which could increase the GSH level after being hydrolyzed by intracellular lactase.[2] Then, they were incubated in 30 mL PBS (pH 7.4) at 37 °C (n=3). The 2 mL PBS outside tube was collected and replaced with the same volume of fresh PBS at predetermined time points. The cumulative release percentage of DOX was detected by a UV/NIR spectrophotometer in the final.

1.8 Cytotoxicity Assessment

The *in vitro* cytotoxicity was assessed by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. A549 cells were seeded in 96-well plates at a density of 3500 cells per well and cultured in 5% CO_2 at 37 °C overnight. The medium was replaced by 150 μL fresh culture medium containing free DOX, PEG-Pep-TPE nanoparticles and PEG-Pep-TPE/DOX nanoparticles at the final drug concentrations of 2、1、0.5、0.25、0.125、0.063、0.031、0.016 μg/mL for 72 h followed by MTT assay (n=6). Furtherly, as the above method, A549 cells were

respectively incubated with free DOX solution, PEG-Pep-TPE NPs+ DOX, PEG-Pep-AIE/DOX NPs+ GSH-OEt and PEG-Pep-AIE/DOX NPs at the final drug concentrations of 1、0.5、0.25、0.125、0.063、0.031 μg/mL for 48 h followed by MTT assay (n=6).

1.9 Cellular uptake and release assay

For the cellular uptake and release assay, A549 cells were seeded in Glass Chamber at a density of 2.5×10^5 cells per well and cultured in 5% CO_2 at 37 °C overnight (n=5). 1) Acceptor photobleaching and spectral imaging: The medium was then removed and incubated with DOX-loaded nanoparticles for 3 h while the final concentration of DOX was 10 μg/mL. To study the release of DOX-loaded nanoparticles in cells over the time, A549 cells were treated with fresh medium for another 0, 1, 2, 3, 4 h after the drug-containing medium was removed. 2) Sensitized emission: The medium was then removed and incubated with PEG-Pep-TPE NPs, free DOX and DOX-loaded nanoparticles for 3 h while the final concentration of DOX was 10 μg/mL. After that, the drug-containing medium was removed and A549 cells were treated with fresh medium for another 0, 1, 2, 3, 4 h. Then the culture medium was removed, and cells were fixed with the 0.4% formaldehyde for 30 minutes after the dishes were rinsed with PBS (pH = 7.4). Finally, A549 cells were observed under a confocal laser scanning microscope (CLSM) with excitation at 405 nm for TPE-CHO and 488 nm for DOX by the typical acceptor photobleaching method.[3]

1.10 F-actin assay for synergism mechanism investigation

A549 cells were seeded in Glass Chamber at 15×10^4cells per well and incubated at 37 °C, 5% CO_2 overnight. Then the preparation of free DOX, PEG-Pep-TPE nanoparticles and PEG-Pep-TPE/DOX nanoparticles were dissolved in the fresh medium until the final concentration of DOX was 2 μg/mL. The mixture was added into each glass dishes with the medium removed.

Meanwhile, the cells were not treated with drugs and just incubated with fresh medium as the control group. After incubated for 12 h, the dishes were rinsed with PBS (pH = 7.4) with the medium removed and the F-actin was stained with Alexa Fluor™ 488 Phalloidin. After the staining solution was removed, cells were rinsed three times with PBS and the morphology of actin was observed under Nikon A1R confocal microscope.

1.11 Dynamical behaviors of PEG-Pep-TPE(C-N) nanoparticles in cells

A549 cells were seeded in Glass Chamber at 15×10^4 cells per well and incubated at 37 °C, 5% CO_2 overnight. Then the medium was then removed and A549 cells were incubated with PEG-Pep-TPE(C-N) nanoparticles at the final polymer concentrations of 500 μg/mL for 1.5 h. Subsequently, A549 cells were treated with fresh medium and were observed dynamically under OLYMPUS SPIN SR10 confocal microscope for another 9 h.

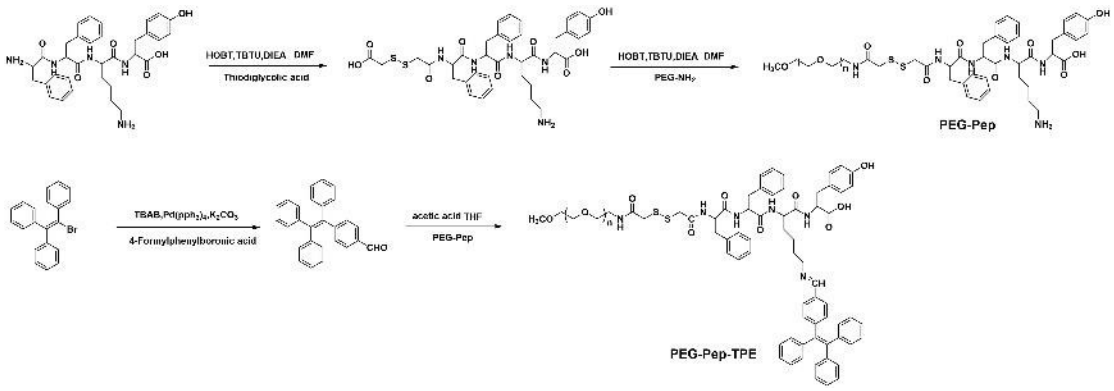


Figure S1. Synthetic route for PEG-Pep-TPE polymer.

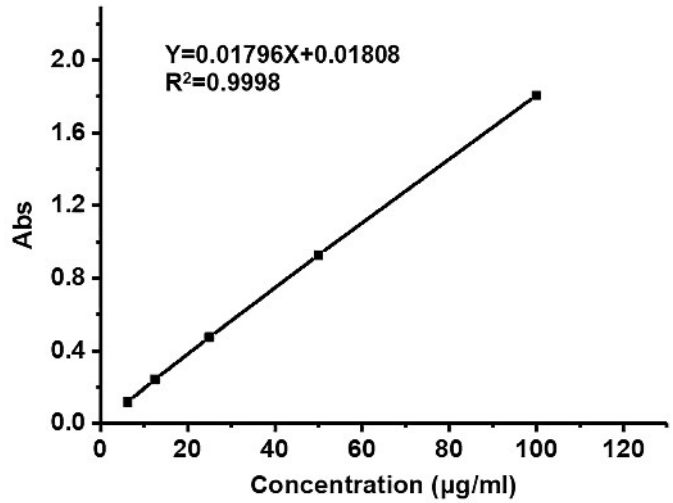


Figure S2. The standard curve of adsorption and mass concentration of DOX in water was made out by UV spectrophotometer under 488 nm laser.

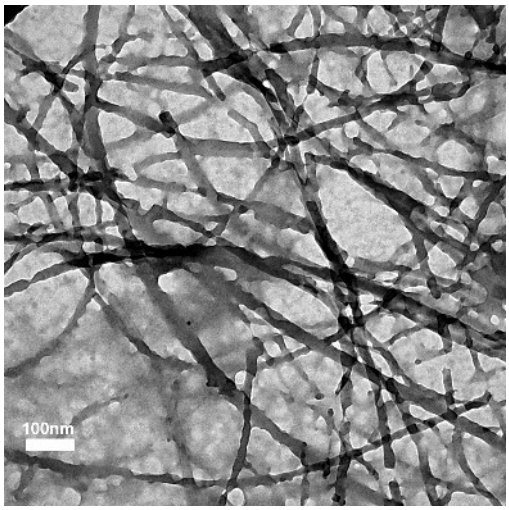


Figure S3. TEM image of PEG-Pep-TPE NPs pretreated with pH 5.0 and 10 mM GSH for 24 h.

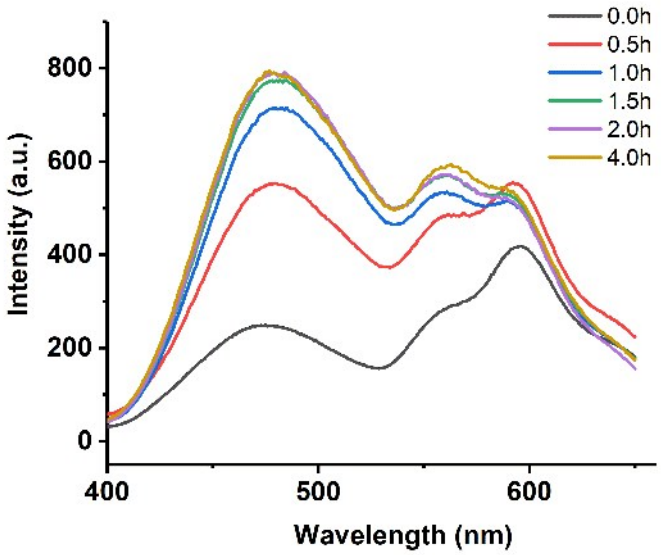
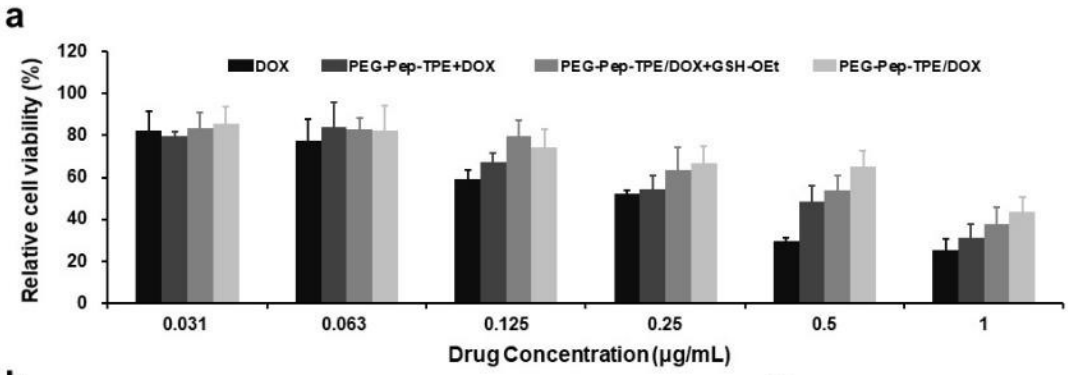


Figure S4. Fluorescence spectra of PEG-Pep-TPE/DOX NPs in the presence of pH 5.0 and 10 mM GSH at selected time intervals.



b

Samples	IC50(μg/mL)	In vitro release(%)
Free DOX	0.2273	94.49
PEG-Pep-TPE+DOX	0.3735	93.59
PEG-Pep-TPE/DOX+GSH-OEt	0.6448	77.16
PEG-Pep-TPE/DOX	0.7566	34.08

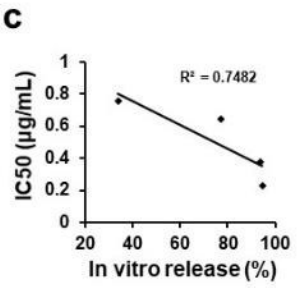


Figure S5. a) in vitro cytotoxicity of free DOX solution, PEG-Pep-TPE NPs+DOX, PEG-Pep-TPE/DOX NPs+2μM GSH-OEt and PEG-Pep-TPE/DOX NPs against the A549 cells after 48h incubation were determined by MTT assay (n=6), LE(%) = 3%; b) the IC50 values at 48h and in vitro DOX release kinetics in PBS at 6h of each drug groups were listed in table, respectively; c) the correlation between IC50 values and in vitro release was acquired from table b.

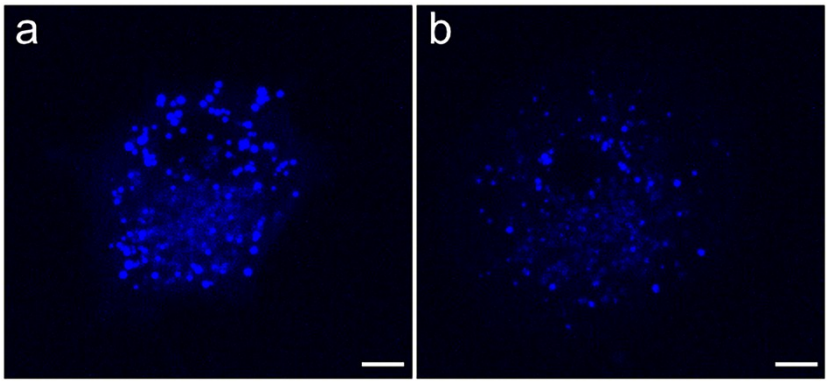


Figure S6. CLSM images of A549 cells at 1.5 h (a)and 10.5 h (b) after incubation with the PEG-Pep-TPE(C-N) NPs for 1.5 h at a dose of 500 μg/mL (PEG-Pep-TPE(C-N) polymer), scale bars represent 5 μm in all images.

Notes and references

[1] X. Wang, Y. Yang, Y. Zhuang, P. Gao, F. Yang, H. Shen, H. Guo, D.C. Wu, Fabrication of pH-Responsive Nanoparticles with an AIE Feature for Imaging Intracellular Drug Delivery, Biomacromolecules 17(9) (2016) 2920.

[2] B.Y. Chung, S.R. Choi, I.J. Moon, C.W. Park, Y.H. Kim, S.E. Chang, The Glutathione Derivative, GSH Monoethyl Ester, May Effectively Whiten Skin but GSH Does Not, Int J Mol Sci 17(5) (2016).

[3] H.C. Ishikawa-Ankerhold, R. Ankerhold, G.P. Drummen, Advanced fluorescence microscopy techniques--FRAP, FLIP, FLAP, FRET and FLIM, Molecules 17(4) (2012) 4047-132.