Intracellular pH-Induced Fluorescence Evoking to Track Nanoparticles in Cells

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Experimental Section

Materials

1'-(2-Methacryloxyethyl)-3',3'-dimethyl-6-nitrospiro-(2H-1-benzopyran-2,2'-indoline) (SP) and L-Malic acid was purchased from Aladdin (China). mPEG (Mw = 2000 g/mol), N,N-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) were purchased from Sigma-Aldrich and used as received. Cinnamyl alcohol (CIN) was purchased from TCI (Japan). Paclitaxel (PTX) was purchased from Meilun Biology Technology Company (Dalian, China). Tetrahydrofuran (THF) was dried in sodium (Chengdu Kelong Chemicals Ltd, China) and distilled before used. All the other reagents and solvents were analytical grade and used as received. Roswell park memorial institute (RPMI-1640), 100 × mycillin, fetal bovine serum and 4', 6-diamidino-2-phenylindole (DAPI) were purchased from Life Technologies Co, (Carlsbad, CA) and used for cells test. Cell Counting Kit-8 assay (CCK-8) was purchased from Dojindo Laboratorise (Kumamoto, Japan). Mouse breast cancer cell (4T1) and human hepatoma cell (HepG2) were obtained from the Chinese Academy of Science Cell Bank for Type Culture Collection (Shanghai, China).

Characterizations

The ^{1}H NMR spectra were performed on a Bruker DRX-300 spectrometer operating with 400 MHz. The gel permeation chromatography (GPC) was performed on an Agilent 1100 Series, and analyzed with GPC-SEC data analysis software. FTIR spectra were recorded on a Thermo Scientific Nicolet iS10 spectrophotometer over the wavenumber range of 4000–400 cm^{-1}. Particle size was performed on Zetasizer Nan-ZS (Malvern Instrument Ltd., Malvern, UK) equipped with a He-Ne laser beam at 633 nm (scattering angle: 90 °). Atomic force microscopy (AFM) was employed with a Nanoscope III from Digital Instruments (MFP-3D-BIO) operated in the tapping mode in air using microfabricated Si (type NCH) cantilevers. The transmission electron microscopy (TEM) measurements were performed on a Hitachi model H-600-4 transmission electron microscope with an accelerating voltage of 80 kV. The molecular simulation of PMA was performed on Materials Studio 5.0.

Synthesis of PMA-mPEG-CIN-SP polymer

L-Malic acid (20.0 g, 0.149 mmol) was added to a 100 mL bottom-round flask with a magnetic stirrer and then heated to 100℃ in an oil bath under reduced pressure for 72 h. The received PMA was dissolved in anhydrous

THF and precipitated in large amount of cold ethyl ether, and then the precipitate was vacuum-dried at room temperature.

PMA (1.00 g, 8.62 mmol), DMAP (0.1 g, 0.82mmol), cinnamyl alcohol (0.46 g, 3.45mmol) and mPEG (3.45 g, 1.73mmol) were dissolved in 20 mL of anhydrous THF in an ice bath under nitrogen atmospheres. A solution of DCC (1.78 g, 8.64mmol) in THF (20 mL) was added dropwise into the mixture solution and stirred at room temperature for 48 h. The white precipitate was removed by filtration, followed by concentrated and precipitated in diethyl ether. The white precipitate was vacuum-dried. The synthesis of PMA-mPEG-CIN-SP polymer was the same as the above mentioned synthesis of PMA-mPEG-CIN polymer. The molar ratio of SP molecules to carboxyl groups in PMA was designed to be 0.5.

Preparations of blank and PTX loaded nanoparticles

Blank NPs were prepared by solvent evaporation method. Briefly, 2 mL of PMA-mPEG-CIN-SP polymer (20 mg) solution in tetrahydrofuran (THF) was added dropwise (1 drop every 20s) to 10 mL of deionized water. After 24 h stirring, the resulting solution was centrifugalized at 3000 r/min for 10 min and passed through a 0.45 μm pore-size syringe filter (Milipore, Garrigtwohill, Co. Cork, Ireland).

Paclitaxel-loaded polymeric NPs were prepared as follows: 1 mL of drug stock solution (1mg/mL) in THF was respectively added to the vial containing 10 mg of PMA-mPEG-CIN-SP and PMA-mPEG-CIN polymer. After 24 h, the solution was centrifugalized at 3000 r/min for 10 min and passed through a 0.45 μm pore-sized syringe filter (Millipore, Carrigtwohill, Co. Cork, Ireland) to remove any insoluble drug. The amount of drug loaded in nanoparticles was determined by high performance liquid chromatography (HPLC) using an Agilent HPLC system consisting of a 1260-pump, and a 1260-ultraviolet detector (Agilent, USA). An Agilent ODS_3 column (250×4.6 mm, 5 μm, Agilent, USA) was used for chromatographic separation and the detector wavelength was 227 nm. Acetonitrile and water with the ratio of 1:1 were used as the mobile phase.The flow rate was 1.5 mL/min.The drug loading content (DLC) was calculated using the following equation:

$$DLC\,(\%) = \frac{Mass\ of\ drug \text{ in } the\ nanoparticels}{Mass\ of\ drug\ loaded\ nanoparticles} \times 100\% \qquad (1)$$

Acidochromic properties of PMA-mPEG-CIN-SP nanoparticles

Polymeric micellar solutions with different pHs (5.0, 5.5, 6.8, 7.4) were obtained by adding acetate buffer solutions with pHs 7.4, 6.8, 5.5 and 5.0 respectively into cuvette containing polymeric NPs solution. At definite time intervals, the cuvette containing polymeric NPs was immediately measured by UV-Vis absorption spectra using a Hitachi U-3900 spectrophotometer at the wavelength in the range of 420~650 nm and fluorescence spectrophotometer with a FLS 920 spectro photometer (Edinburg) at a wavelength scan rate of 1200 r/min. The excitation wavelength is 420 nm, and the emission spectrum was recorded at the wavelength ranging from 450 to 750 nm. The operating environment was in dark.

Cell culture

Mouse breast cancer cell (4T1) and human hepatoma cell (HepG2) were cultured in HEPES-buffered RPMI-1640 (pH 7.2) and Dulbecco's modified eagle medium (DMEM) (pH 7.2) respectively, supplemented with 10 v/v % heat-inactivated fetal bovine serum (FBS), 100 IU/mL penicillin and 100 μg/mL streptomycin (all ingredients obtained from Gibco, USA) at 37 °C in a humidified atmosphere with 5% CO_2.

Cytotoxicity assay

3T3 fibroblasts with the density of 5×10^3 cells per well were seeded in 96-well plates and cultured overnight for 70%-80% cell confluence. The medium was replaced with 100 μL of fresh complete medium, and the nanoparticles with different concentrations (1, 0.5, 0.2, 0.1, 0.05, 0.01 mg/mL) were added. The cells were incubated with nanoparticles for another 24 h, followed by removed the culture media, and then the cells were rinsed with PBS (pH 7.4). 100 μL of culture media containing 10 % Cell Counting Kit-8 (CCK8, v/v) was added to each well. After the cells were incubated for 2 h, the 96-well plates were detected at 450 nm using microplate reader (Model 550, Bio-Rad, USA). The cell viability was calculated according to the following formula:

$$Cell\ viability\,(\%)=\frac{(OD\ value\ of\ test\ group-OD\ value\ of\ blank\ group)}{(OD\ value\ of\ control\ group-OD\ value\ of\ blank\ group)}\times100\% \qquad (2)$$

Intracellular tracking nanoparticles

4T1 cells were seeded in 35 mm diameter glass dishes (Invitrogen, USA) at a cell density of 5×10^4 cells/mL and cultured with RPMI-1640 free of fetal bovine serum (FBS). After reaching 60% confluence, the cells were treated with PMA-mPEG-CIN-SP polymeric nanoparticles ([C] = 2 mg/mL). After 2, 6, 12 and 24 h incubation, the cells were rapidly washed with PBS, and the live cells were observed by confocal laser scanning microscopy (CLSM, Leica TCP SP5) with the excitation wavelengths of 535 nm and emission wavelength of 610 nm. For the cells incubated with 24 h, it was washed three times with PBS and fixed with 4% paraformaldehyde for 10 min. The nuclei were counterstained by DAPI for 5 min. The culture media were replaced with PBS.

Cellular uptake of polymeric NPs

Monolayers of 4T1 cells were cultured with PMA-mPEG-CIN-SP polymeric NPs (2 mg/mL) in RPMI-1640 culture medium for 12 h. After the treatment, cells were washed three times with cold PBS, and then were harvested in a 2 mL EP tube by trypsinization. The sample for TEM observation was prepared by a conventional way [1].

In vitro anticancer activity

In a 96-well culture plate, 4T1 and HepG2 cells (2×10^4 cells/mL) were seeded with 200 μL of RPMI-1640 and DMEM media for 24 h. PTX loaded nanoparticles were added to the media-removed 96-well plates with different PTX concentrations (from 10^{-4} to 10 μg/mL). After incubated for 48 h, the culture media were removed and the cells were rinsed with PBS (pH 7.4) for three times. 100 μL of culture media containing 10 %

CCK8 (v/v) was added to each well. The 96-well plates were detected at 450 nm using the microplate reader (Thermo Fisher, US) after 2 h incubation. The cell viability was calculated according to the formula (1).

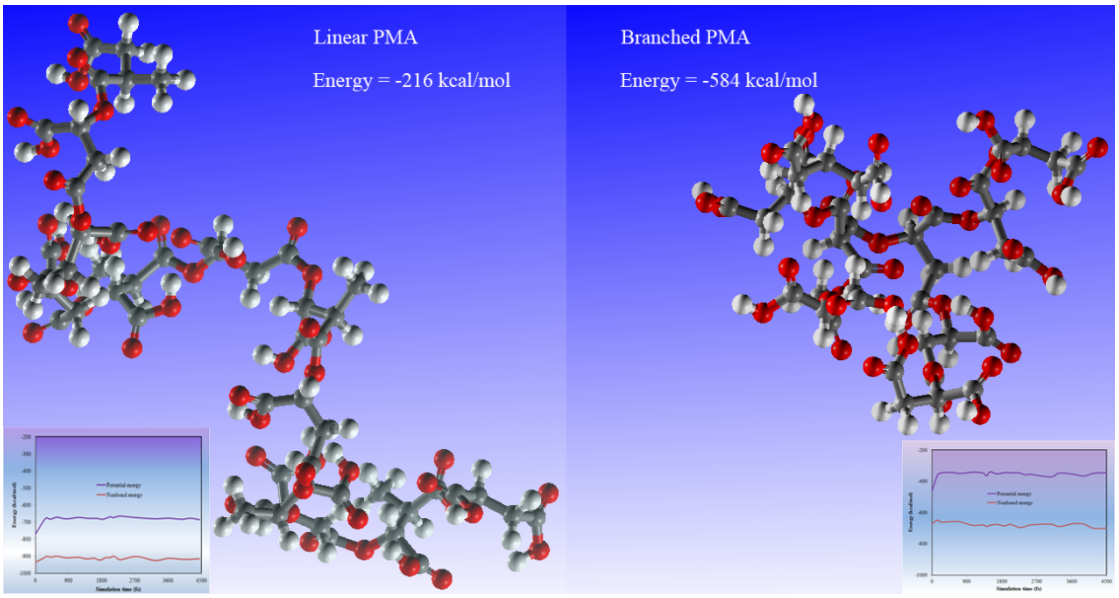

Figure S1. The molecular simulation of the structure of poly(α,β-malic acid), the energy of hyperbranched structure (-584.09 kcal/mol) was much lower than that of linear structure (-216.04 kcal/mol).

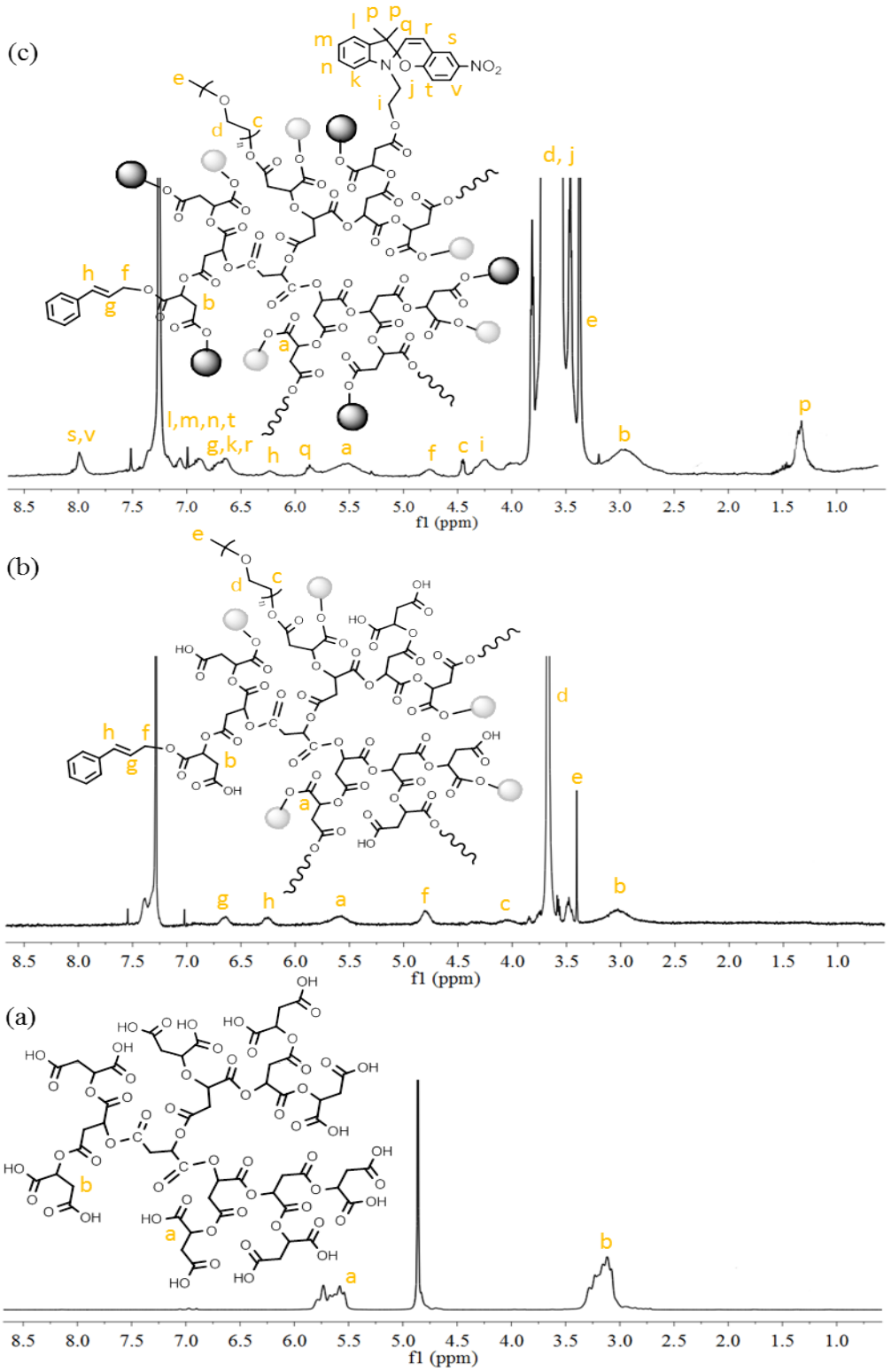

Figure S2. The ^{1}H NMR spectra of PMA (a), PMA-mPEG-CIN (b) and PMA-mPEG-CIN-SP (c).

Table S1. The molecular weight of PMA-mPEG-CIN-SP polymer

Polymer	Mn (g/mol)			Mw	PDI
	^{1}H NMR	GPC	MS		
PMA	1500	1750	1456	2590	1.48
PMA-mPEG-CIN	3800	3890	3684	5174	1.33
PMA-mPEG-CIN-SP	4500	4158	4135	5655	1.36

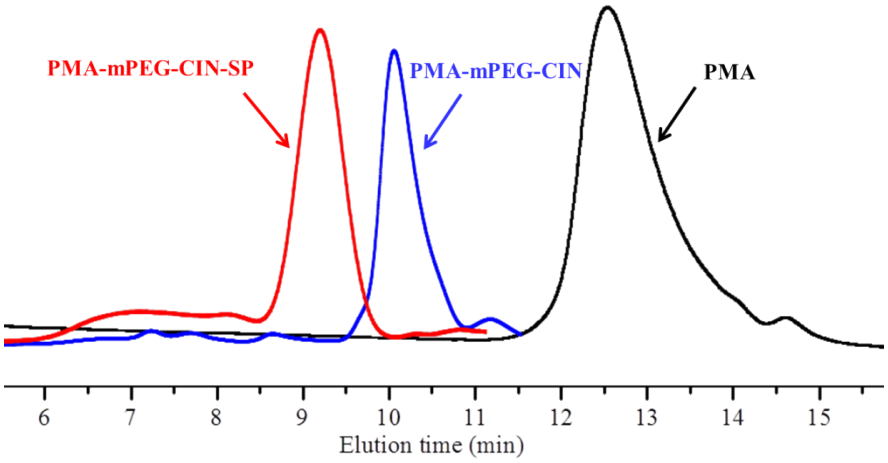

Figure S3. The GPC traces of PMA, PMA-mPEG-CIN and PMA-mPEG-CIN-SP.

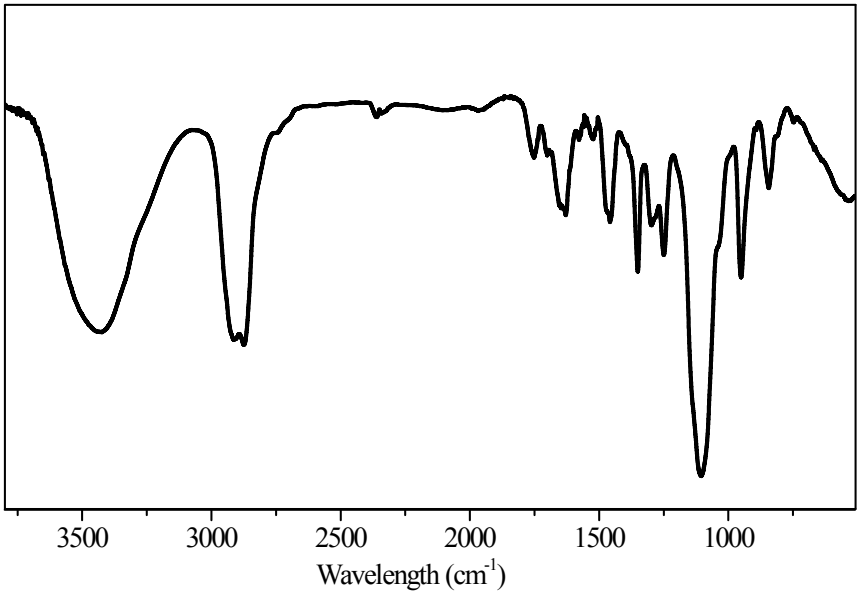

Figure S4. FTIR spectrum of PMA-mPEG-CIN-SP polymer.

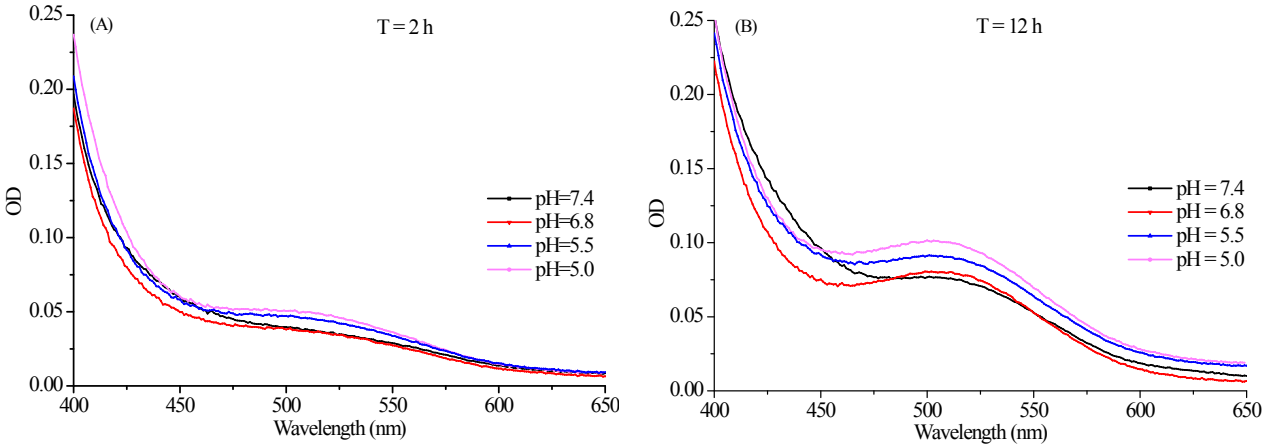

Figure S5. UV/vis absorption spectra of PMA-mPEG-CIN-SP nanoparticles in aqueous media with different pH values for 2 h (A) and 12 h (B).

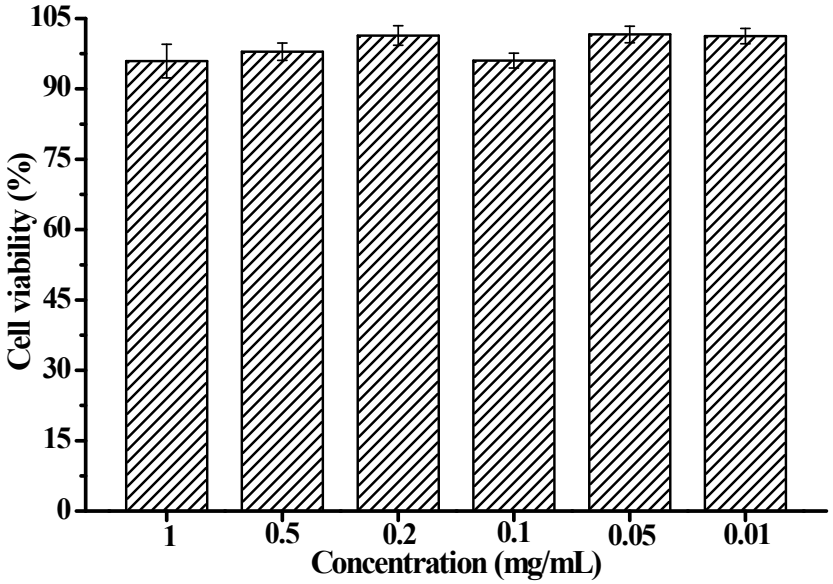

Figure S6. The cytotoxicity of PMA-mPEG-CIN-SP nanoparticles incubated with 3T3 fibroblasts for 24 h.

REFERENCES

1. S. Bontha, A. V. Kabanov and T. K. Bronich, *Journal of Controlled Release*, 2006, **114**, 163-174.