Supporting Information

Materials. Chitosan (CS, M_W=5.6×10^5 Da, degree of deacetylation 91.13 %) was purchased from the Zhejiang Yuhuan Biotechnology Co., Ltd. (China). Mel was purchased from Suzhou Lide Chemistry Co., Ltd. (China). 4-Pyridinecarboxaldehyde was purchased from Aladdin Reagen Co., Ltd. (China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma-Aldrich Chemical Co., Ltd. (St. Louis, MO). SKBR3 cells were purchased from ATCC (USA) and grown in base medium (ATCC-formulated McCoy's 5a Medium Modified, Catalog No. 30-2007) containing 10 % fetal bovine serum.

Synthesis of carboxymethyl chitosan (CMCS). CMCS was prepared according to the literature.[S1] Chitosan (1.0 g) was stirred with 30 mL isopropanol in flask for 6 h and then filtered. The filter residue was stirred with NaOH solution (50 %, w/w) thoroughly and then frozen in the refrigerator overnight. Chloroacetic acid (1.5 g) dissolved in 10 mL isopropanol was dropped onto the frozen chitosan mixture. The mixture was stirred for 20 h at the temperature of below 10℃. The reaction mixture was then filtered and the residue was washed with absolute ethanol. The crude product was dialyzed against deionized water and then freeze-dried to obtain CMCS (1.15 g). The CMCS was characterized by FT-IR spectroscopy (BREKER TENSOR 27, GER). The viscosity-average molecular weight of CMCS measured by an Ubbelohde viscometer is 5.0×10^5 Da. The degree of substitution of carboxymethyl groups was estimated by an elemental analysis (Elementar vario EL III, GRE). N,O-substitution degree of CMCS is about 0.74, and the degree of N-substitution is about 0.12.

Preparation of Pyridine-carboxymethyl chitosan (Py-CMCS). Py-CMCS was synthesized through Schiff's reaction. 4-pyridinecarboxaldehyde (4-Py) dissolved in ethanol was added dropwise into CMCS water solution. The mixture was stirred for 24 h at room temperature. The solution was dialyzed against deionized water for 24 h, and then freeze-dried to obtain Py-CMCS.

The degree of substitution of Py to C-2 amino groups of Py-CMCS was estimated by UV (PerkinElmer Lambda 750 S, USA). Py-CMCS (0.01 g) was dialyzed against 100 ml 1 M HCl for 24 h. The dialyzed solution was collected and the concentration of 4-Py was determined by UV at 257 nm.

The FT-IR spectra of CMCS (curve a) and Py-CMCS (curve b) are shown in Figure S1. Compared with curve a, a new band at 1645 cm^{-1} in curve b is attributed to the -C=N- group of Schiff base[S2] and other three new bands at 1560 cm^{-1}, 1388 cm^{-1}

and 966 cm^{-1} are attributed to the pyridine ring.[S3] These results indicate successful conjugation of 4-Py to C-2 amino groups of CMCS via Schiff base bonds.

The structure of Py-CMCS is also characterized by ^1H NMR spectroscopy (Varian NMR System 600, USA) (see Figure S2). The assignments of the ^1H NMR signals of Py-CMCS are given as follow. ^1H NMR (600 MHz, D_2O, δ): 8.45 (Py Ha), 7.58 (Py Hb), 4.79 (H-l), 4.19 (H-5), 4.08 (CH_2COOH), 3.6-3.9 (H-3, H-4, H-6), 3.28 (H'-2), 3.03 (H-2), 1.95 ($COCH_3$).

The pH stability of Py-CMCS is characterized by UV (see Figure S3). Py-CMCS (0.01 g) were dialyzed against 100 ml water solutions in the pH range of 1.0-9.0 for 24 h. The dialyzed solutions were collected and the concentrations of 4-Py were determined by UV at 257 nm. Then, the remaining Py on Py-CMCS was calculated. The Py-CMCS is stable in the pH range of 5.0 to 9.0.

Preparation of Py-CMCS·Mel micelles. Py-CMCS (100 mg) was dissolved in 100 mL deionized water in a sealed flask. The Mel solution, which was prepared by dissolution of Mel (100 mg) in ethanol (20 ml) under protection of nitrogen, was added dropwise into the flask and stirred at pH 7.4 for 10 h. The whole solution was dialyzed against 500 mL deionized water for 12 h. Water was changed once every 2 h after 4 h. (All of the steps were carried out under protection of nitrogen below 15℃.) The dialyzed solution was collected for determination of drug-loading. After dialysis, the solution was divided into two parts. One part was directly used for pH-sensitivity studies and morphological observation; the other was freeze-dried for FT-IR characterization and analysis of drug release. The micelle samples prepared with different degrees of substitution of Py were named Py-CMCS·Mel micelles-1, Py-CMCS·Mel micelles-2 and Py-CMCS·Mel micelles-3, respectively.

Drug loading determination of Py-CMCS·Mel micelles. The concentration of Mel in the dialyzed aqueous solution was determined by HPLC (Agilent 1100, USA) coupled to UV detection at 261 nm. (Three independent determinations were made, and the results were averaged.) Then the weight of Mel in the micelles was calculated. Drug loading rate was expressed by equation 1.

$$\text{Drug loading (\%)} = \left(\frac{W}{W_0} \right) \times 100\% \quad (1)$$

where W is the weight of drug loaded in micelles and W_0 is the weight of micelles.

The drug loading (%) of Py-CMCS·Mel micelles-1, Py-CMCS·Mel micelles-2 and Py-CMCS·Mel micelles-3 was 15.25, 24.53 and 10.82, respectively.

pH-sensitivity studies and ζ-potentials of Py-CMCS·Mel micelles. The dialysed solution of micelles was divided into six parts and the pH of each part was adjusted by 0.1 M HCl and 0.1 M NaOH. The pH-sensitivities of Py-CMCS·Mel micelles were studied by measurement of the mean particle diameter of the micelles at 25℃ in the pH range of 5.0-9.0. The ζ-potentials were determined by Malvern Nano ZS3600 nano zeta sizer (Malvern Co. UK) in the pH range of 4.0-9.0. Sodium chloride was added to keep the ionic strengths of the media constant at 0.1 M.

In vitro drug release of Py-CMCS·Mel micelles. The freeze-dried Py-CMCS·Mel micelles-2 was chosen for the in vitro drug release. The micelles were dissolved in three phosphate-citrate PBS buffers of pH 5.0, pH 7.0 and pH 7.4 to obtain a concentration of 1.0 mg/mL. The solutions were injected into dialysis bags, and then immersed into the release media (40 ml) with different pHs. The release studies were carried out at an oscillation condition of 37℃, 50 r/min. 2 μl of the solutions were withdrawn and analyzed with HPLC at different time intervals. Before and after each analysis, nitrogen was purged into the release media for 30 s. Cumulative drug release was expressed by equation 2.

$$\text{Cumulative drug release (\%)} = \left(\frac{M_t}{M_0}\right) \times 100\% \qquad (2)$$

where M_t is the amount of drug released at time t and M_0 is total amount of drug in micelles. The release data were averaged based on three independent measurements.

In vitro cytotoxicity. Evaluation of cytotoxicity of the micelles was performed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method. SKBR3 cells were seeded into 96-well microtiter plates at a density of 1×10^4 cells/well in RPMI 1640 medium and incubated for 24 h. The cells were treated with 20 μL of several dilutions of the micelles, equivalent concentration of pure melphalan or with the polymeric carriers alone. A 20 μL buffer solution was used as control. After incubation 48 h, 10μL MTT (5 mg/mL) solution in PBS (pH 7.4) was added to each well and further incubated in 5% CO_2 incubator at 37℃ for 1 h. After removal of the unreacted dye and medium, 200 μL DMSO was added to dissolve the formazan crystals formed in live cells. Finally, the optical densities (OD) were measured at 590 nm using a microplate reader (BIO-RAD, Model550, USA). The relative cell inhibition ratio (%) was calculated by (OD control-OD sample)/OD control×100. The data were all corrected by the blank group with media in the absence of SKBR3 cells.

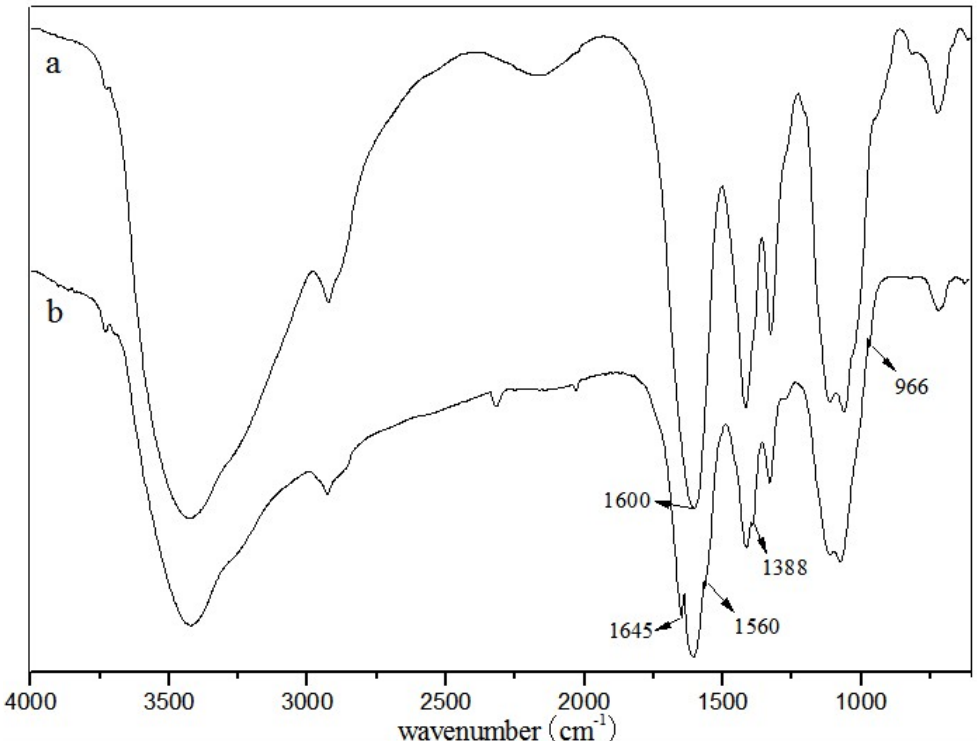

Fig.S1 FT-IR spectra of: CMCS (a) and Py-CMCS (Py_2) (b)

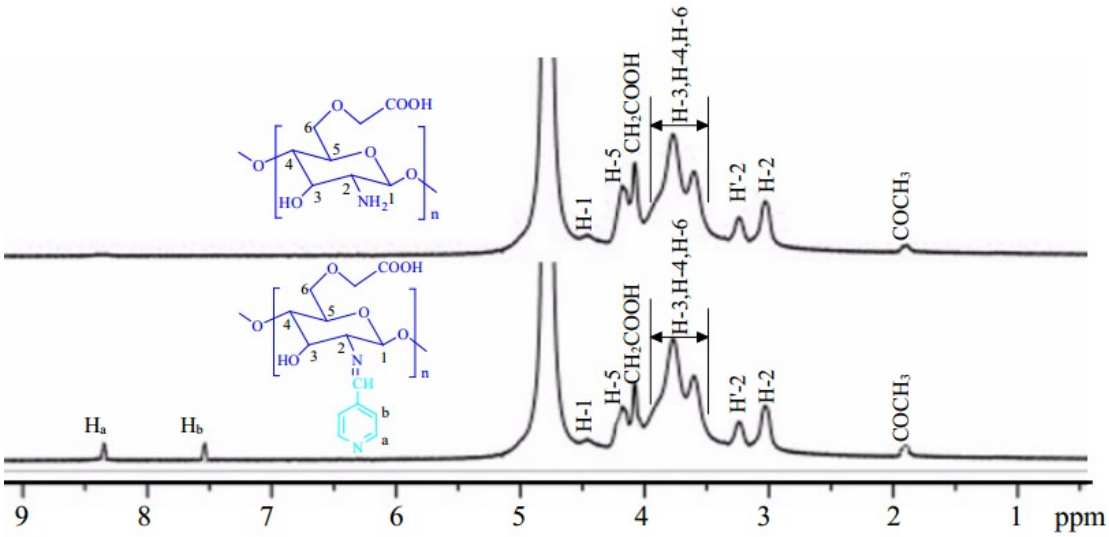

Fig.S2 ^1H NMR spectra in D_2O of: CMCS (a) and Py-CMCS (Py_2) (b)

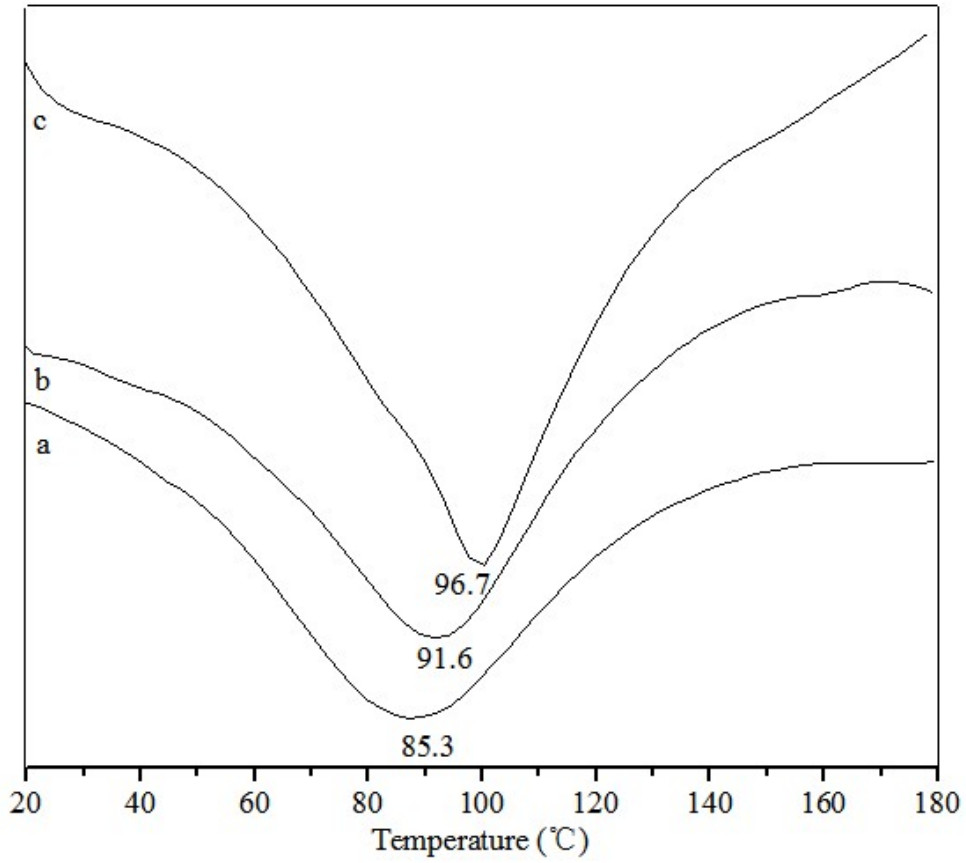

Fig.S3 DSC thermogram of Py-CMCS/Mel (1:1) mixture (a), Py-CMCS·Mel micelles-2 (0.6:1) (b) and Py-CMCS·Mel micelles-2 (1:1) (c).

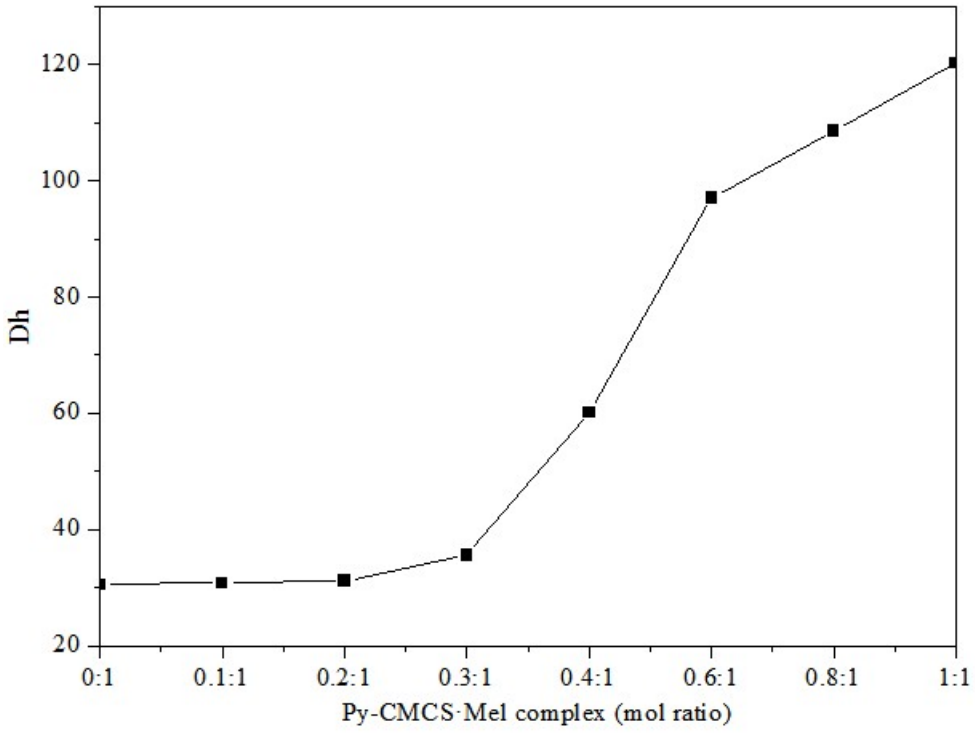

Fig.S4 Effect of addition of Mel on the particle size of Py-CMCS (Py_2) solution

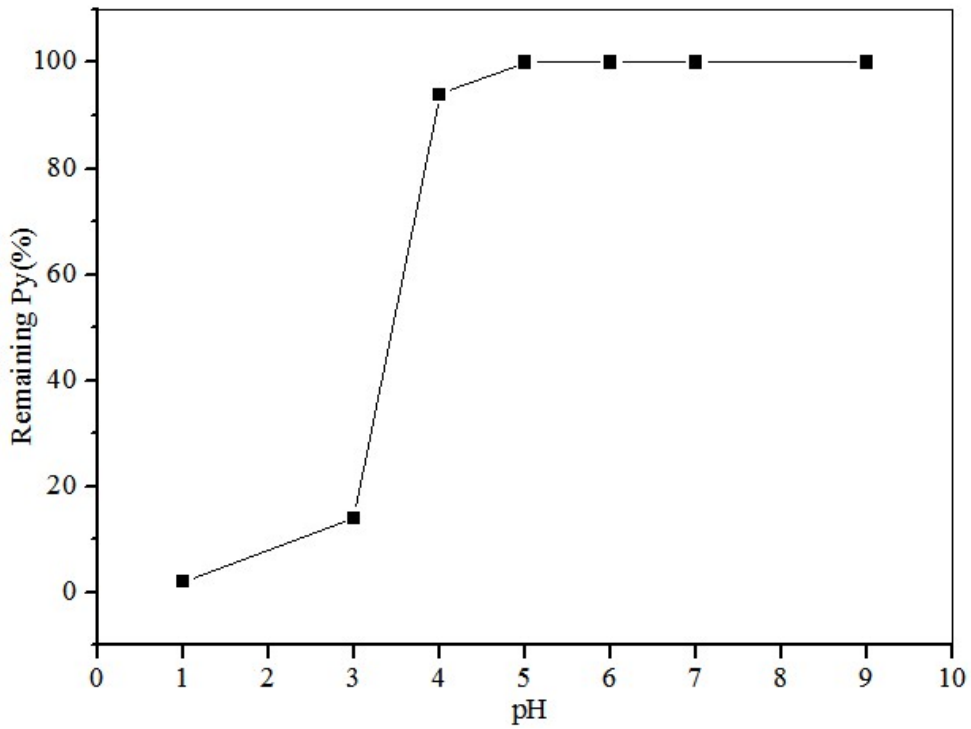

Figure S5. pH stability of Py-CMCS (Py_2)

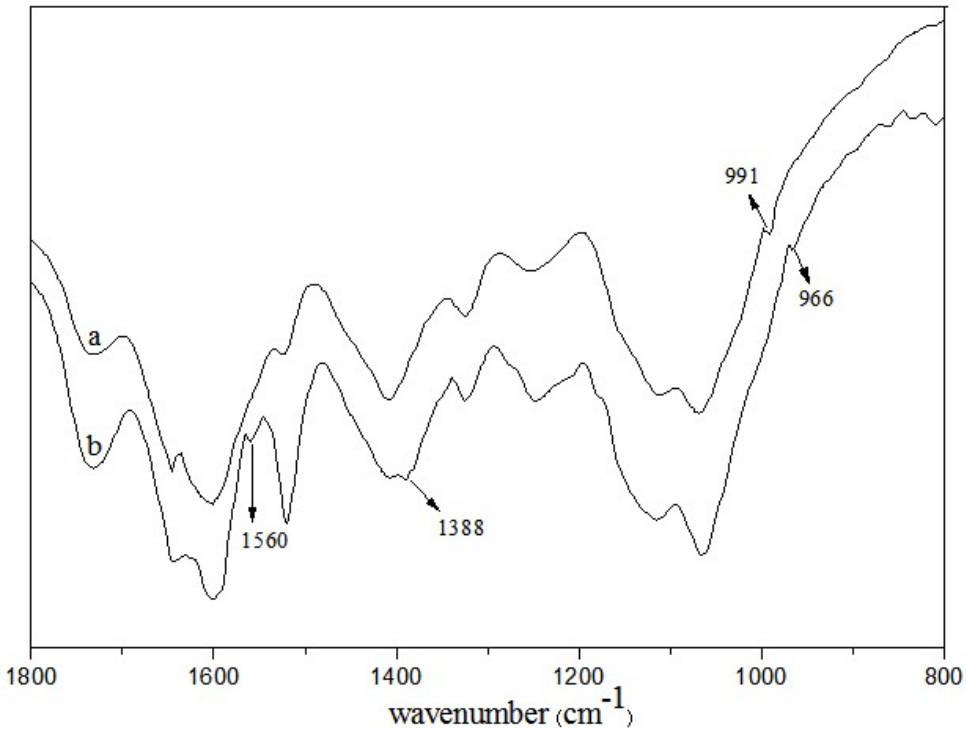

Figure S6. FT-IR spectra of Py-CMCS·Mel micelles-2(1.12:1) at pH 7.4 (a) and pH 5.0 (b)

Table 1 Effect of the composition of Py-CMCS·Mel complex on the polydispersity index (PDI) values of the micelles

Py-CMCS·Mel complex (mol ratio)	PDI		
	Py-CMCS·Mel micelles-1	Py-CMCS·Mel micelles-2	Py-CMCS·Mel micelles-3
0.4:1	0.053±0.011	0.063±0.014	0.054±0.018
0.6:1	0.069±0.015	0.078±0.012	0.071±0.016
0.8:1	0.073±0.014	0.069±0.016	↓
1:1	0.064±0.018	0.085±0.013	↓
1.12:1	0.082±0.018	0.077±0.017	↓

↓sediment

[S1] H. W. Tu, Y. Qu, X. H. Hu, Y. H. Yin, H. Zheng, P. H. Xu, F. L. Xiong. *Carbohydrate Polymers*. 2010, *82*, 440-445.

[S2] Y. H. Yin, S. Xu, D. Chang, H. Zheng, J. L. Li, X. P. Liu, P. H. Xu and F. L. Xiong. *Chem. Commun*. 2010, *46*, 8222-8224.

[S3] A. T. Rodriguez, M. Chen, Z. Chen, C. Jeffrey Brinker, and H. Y. Fan. *J. AM. CHEM. SOC*. 2006,*128*, 9276-9277.