Supplementary

Coordination-driven Assembly of Catechol-modified Chitosan for Kidney-specific Delivery of Salvianolic Acid B to Treat Renal Fibrosis

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Methods

Materials

The near-infrared dyemono-NHS ester Cy7-SE and fluorescein isothiocyanate (FITC) were obtained from Nanjing Wobio Science and Technology Co., Ltd. (Nanjing, China). Lyso-Tracker red and DAPI were purchased from Beyotime Institute of Biotechnology (Nantong, China). DMEM/F12 culture medium was purchased from Nanjing Kaiji Biotechnology (Nanjing, China). Hyp assay kit purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China)，Lipid Perooxidation MDA assay purchased from Beyotome Biotechnology (Nantong, China).

Synthesis of catechol-conjugated chitosan (HCA-Chi)

0.5 g LMWC (M_n 5000) was dissolved in 150 ml deionized water (pH 5.0, adjusted with 1 M HCl). 0.6 g of HCA and 1.3 g of EDC were dissolved in 5 ml of water and stirred for 10 min. The solutions were mixed by magnetic stirring for 12h and the product was then purified by successive dialysis (MWCO 1000) against deionized water for 36 h. The final product was lyophilized and kept in a desiccator.

The ^{1}H NMR spectrum was measured by a Bruker Avance spectrometer (Billerica, MA, USA), operating at 500 MHz with D_2O as solvent and tetramethylsilane (TMS) as internal standard. The degree of HCA conjugation was calculated by comparing the relative peak area of a catechol group (3H, aromatic ring proton, δ 6.76, D_2O) and an acetyl group (3H, COCH3, δ 1.95, D_2O) on a chitosan backbone. UV-Vis spectroscopy was also used to determine the catechol content, as a result of HCA conjugation, based on the absorbance of catechol at 280 nm.

Cell culture, cytotoxicity and cellular uptake

HK-2 cells (American Type Culture Collection, ATCC) were cultured in DMEM/F12 (Hyclone) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (100 IU/ml) under standard conditions (37 °C, 5% CO_2). The viability of HK-2 cells treated with HCA-Chi and HChi-Ca-Sal B was assessed by MTT assay. For cellular uptake, FITC-labeled HCA-Chi was synthesized. FITC-nanocomplex dispersed in serum-free DMEM/F12 was added to HK-2 cells seeded in 35 mm glass bottomed dishes and co-cultured at 37^{o}C. After incubation for 2 h, the medium was aspirated and rinsed twice with ice-cold buffer, and the cells were observed by confocal laser scanning microscopy (CLSM, Leica TCS SP5, Germany).

Two-color co-localization experiment was performed to investigate the intracellular fate of the HChi-Ca-Sal B nanocomplex in HK-2 cells using CLSM. Lyso-Tracker™ Red, which stains acidic compartments, was diluted with PBS, and incubated with HK-2 cells at 37 ℃. After 45min of incubation, the medium was aspirated and the cells were rinsed three times with ice-cold buffer, and then observed by CLSM**.**

Western Blotting

HK-2 cells were homogenized in lysis buffer for 30min on ice. The supernatants were collected after centrifugation at 10,000 rpm at 4°C for 15 min. Protein concentration was determined using a BCA protein assay kit. Equal amounts of protein were separated by 10% SDS gel electrophoresis (SDS-PAGE) and then transferred to a nitrocellulose membrane. After blocking with 5% nonfat milk in TBST at room temperature for 1 h, the membrane was incubated with primary antibody at 4°C overnight. After washing in TBST, the blots were incubated with

horseradish-coupled secondary antibody for 2h at room temperature. The signals were visualized using an enhanced chemical luminescent (ECL) system. Quantification was performed by measuring the intensity of the signals with an NIH image software package.

Results

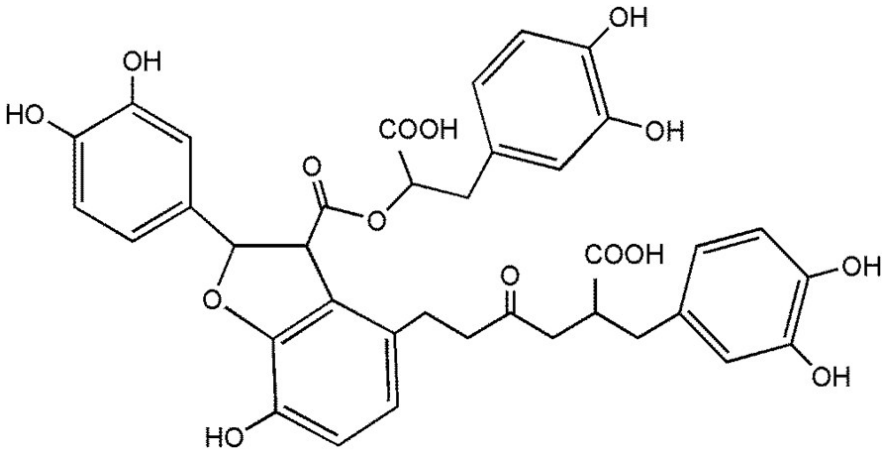

Figure S1. The chemical structure of salvianolic acid B

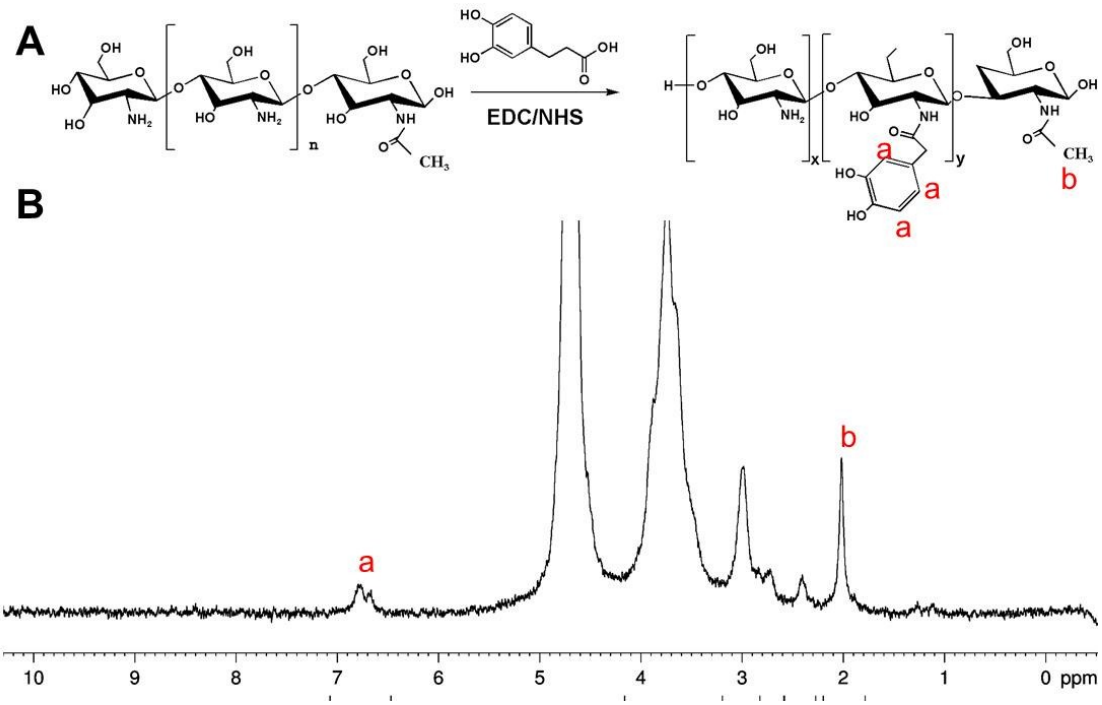

Figure S2. (A) Synthesis of catechol-modified chitosan (HCA-Chi); (B) ^{1}H NMR spectrum of HCA-Chi

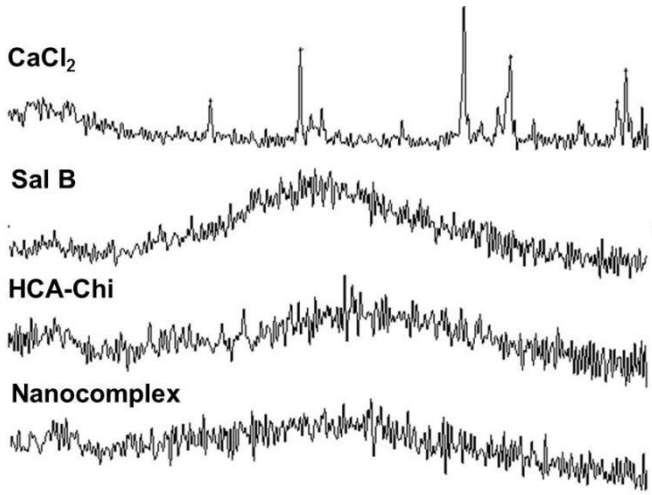

Figure S3. Power X-ray diffraction patterns: Calcium chloride, Sal B, HCA-Chi and HChi-Ca-Sal B nanocomplex.

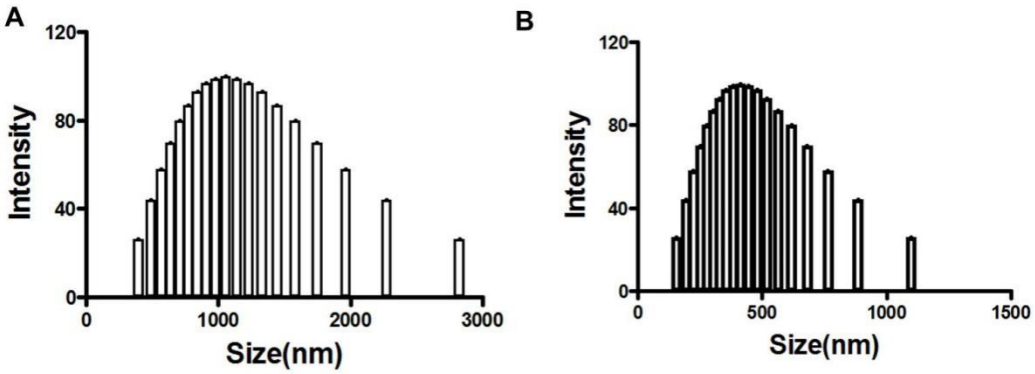

Figure S4. Size distribution of HCA-Chi solution (A) and acidized (pH 5.0) HCA-Chi-Ca-Sal B nanocomplex (B)

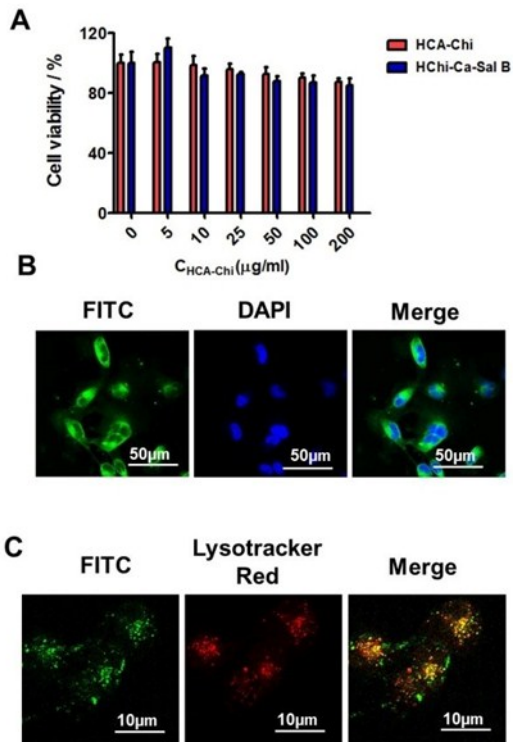

Figure S5. **(A)** *In vitro* cytotoxicity of HCA-Chi and HChi-Ca-Sal B against HK-2 cells after 24 h incubation (n =3). **(B)** CLSM images showing uptakeof HChi-Ca-Sal B (at a Sal B dose of 20μg/ml) after incubation with HK-2 cells for 2h. **(C)** Two-color colocalization images, taken by CLSM, of HK-2 cells incubated with FITC-labeled HChi-Ca-Sal B (at a Sal B dose of 20μg/ml) for 4h.

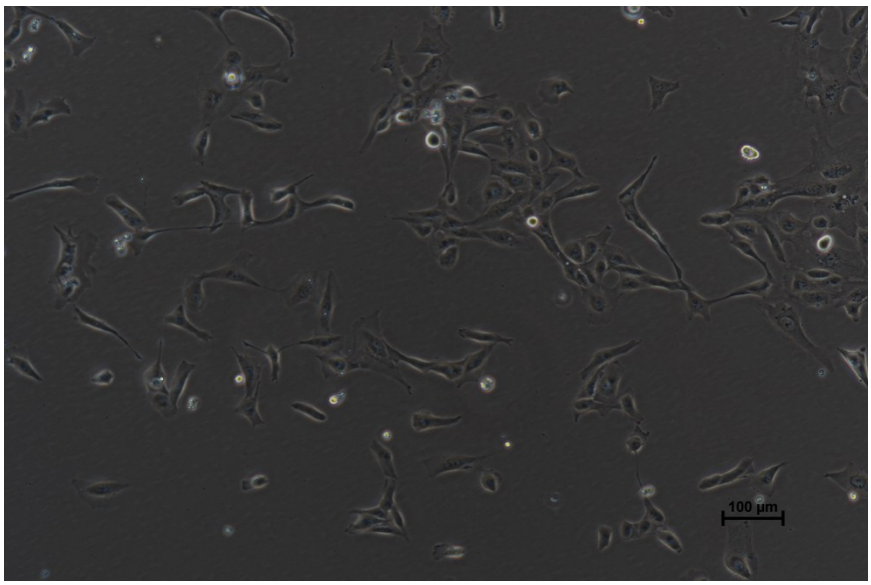

Figure S6 Image showing the morphology of HK-2 cells treated with TGF-β1 (10 ng/ml) for 72 h.

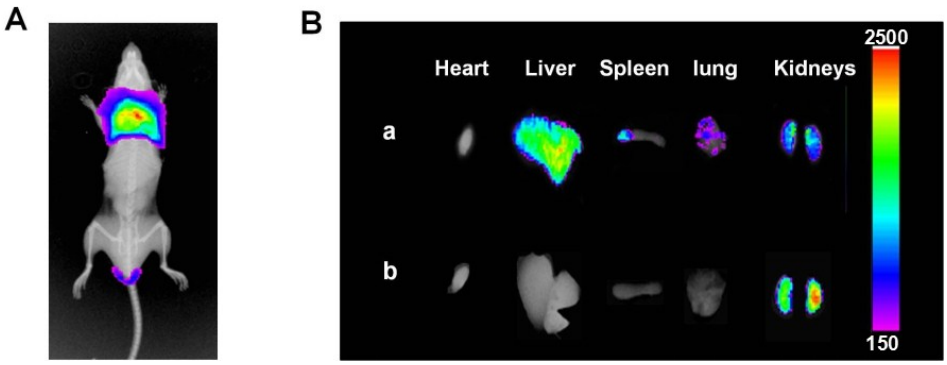

Figure S7 (A) NIRF images of ICR mice following IV injection of free Cy7 at 8 h; (B) NIRF images of lung, heart, liver, spleen, intestine and kidney isolated from mice 8 h post-injection of free Cy7 (a) and the Cy7-SE-labeled nanocomplex (b).

Table S1 Effect of HCA-Chi-Ca-Sal B and free Sal B on TGF-β1-induced EMT of HK-2 cells (length/width ratio). Values are expressed as mean ± S.D

	Cell length/Width ratio
Control	2.27±0.40
TGF-β1 (10 ng/ml)	7.15±1.92
100 μmol/l free Sal B + TGF-β1 (10 ng/ml)	2.18±0.67
10 μmol/l free Sal B + TGF-β1 (10 ng/ml)	2.08±0.57
1 μmol/l free Sal B + TGF-β1 (10 ng/ml)	4.05±0.94
0.1 μmol/l free Sal B + TGF-β1 (10 ng/ml)	4.53±1.00
100 μmol/l HCA-Chi-Ca-Sal B + TGF-β1 (10 ng/ml)	2.14±0.58
10 μmol/l HCA-Chi-Ca-Sal B + TGF-β1 (10 ng/ml)	2.22±0.58
1 μmol/l HCA-Chi-Ca-Sal B + TGF-β1 (10 ng/ml)	2.76±1.31
0.1 μmol/l HCA-Chi-Ca-Sal B + TGF-β1 (10 ng/ml)	3.73±1.28