Supporting Information

Silencing of Intestinal Glycoprotein CD98 by Orally Targeted Nanoparticles Enhances Chemosensitization of Colon Cancer

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Materials

PLGA (molecular weight: 38-54 kg/mol), sodium nitrite, chitosan, PVA (86-89% hydrolyzed, low molecular weight), CPT, CM, AOM and Triton X-100 were supplied by Sigma-Aldrich (St. Louis, MO). Chitosan was depolymerized using sodium nitrite. Viscosity-average molecular weight of depolymerized chitosan was measured as 1.8×10^4 using an Ubbelohde viscometer. Mouse CD98 antibody for western blot and mouse siCD98 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Purified anti-mouse CD98 antibody for NPs conjugation was from Biolegend (San Diego, CA). $F(ab')_2$ Preparation Kits, 2-mercaptoethanol and Zeba Desalting Columns were obatined from Pierce Thermo Scientific (Rockford, IL). DC Protein Assay Kits was supplied from Bio-Rad Laboratories (Hercule, CA). Heterobifunctional PEG derivative (MAL-PEG-NHS, molecular weight: 2000) were from Jenkem Technology (Beijing, P. R. China). Paraformaldehyde (16%) was obtained from Electron Microscopy Science (Hatfield, PA). Vybrant® MTT cell proliferation assay kit and Annexin V-FITC/PI Apoptosis Detection Kit were supplied by Molecular Probes (Eugene, OR). Hematoxylin and eosin were purchased from Richard-Allan Scientific (Kalamazoo, MI). DSS (molecular weight: 36-50 kDa) was from MP Biomedicals (Solon, OH). 10% buffered formalin was obtained from EMD Millipore (Billerica, MA).

Induction of Colon Cancer Mouse Model and Examination of CD98 Expression

C57BL/6 female mice (10-week old, The Jackson Laboratory) were used in the animal experiments. The experimental protocols were approved by the Institutional Animal Care and Use Committee of Georgia State University. Colon cancer in the mouse model was induced following methods described in our previous study.[1] Total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA). The complementary DNA (cDNA) was synthesized using

the Maxima First Strand cDNA Synthesis Kit (Fermentas, Hanover, MD). Thereafter, mRNA expression levels were analyzed by qRT-PCR using Maxima SYBR Green/ROX qPCR Master Mix (Fermentas, Hanover, MD). The data were normalized in relation to the internal control: 36B4. Relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. Primer sequences for qRT-PCR were listed in Table S1.

Approximately 5–10 mm pieces of tissue samples were homogenized in a radioimmunoprecipitation assay buffer supplemented with protease inhibitors (Roche Diagnostics, Indianapolis, IN) on ice. The homogenates were centrifuged at 12,000 rpm for 10 min at 4 ^{o}C. Total cell lysates (50 mg per lane) were resolved on 4–15% gradient polyacrylamide gels (Bio-Rad Laboratories) and transferred to nitrocellulose membranes (Bio-Rad Laboratories). Membranes were then probed with primary antibodies: CD98 and GAPDH. After washes, membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (GE Healthcare, Piscataway, NJ), and blots were detected using the enhanced chemiluminescence detection kit (GE Healthcare).

Physicochemical Characterization of NPs

Particle size, monodispersity and zeta potential of NPs were determined in deionized water by DLS using a multi-angle particle sizing method (90 Plus/BI-MAS) or DLS under an electric field (ZetaPlus analyzer, Brookhaven Instruments). The particle size, PDI and zeta potential of NPs were based on the average of repeated measurements. For stability test, NPs were suspended in simulated colonic fluid (pH 6.8), and incubated for 48 h at 37 ^{o}C. At appropriate time points, the particle sizes and zeta potentials were measured.

The morphology of PEG-siCD98/CPT-NPs and Fab'-siCD98/CPT-NPs was viewed using TEM (LEO 906E, Zeiss, Germany) at an 80 kV accelerating voltage. NP suspensions were dropped

onto 400-mesh carbon-coated copper grids and dried prior to analysis.

To determine loading amount of siRNA in NPs, NPs (3 mg) were dissolved in 0.5 mL of dichloromethane. The released siRNA was extracted from the organic phase using 0.8 mL Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, pH = 8.0). TE buffer was added to the organic solution, and the resultant mixture was vortexed vigorously for 5 min and then centrifuged at 13,400 g for 5 min at 4 ^{o}C. The supernatant was collected and analyzed for double-stranded RNA content using a Quant-IT^{TM} PicoGreen^{TM} assay according to the procedures recommended by the manufacturer (Invitrogen).

Loading amount and encapsulation efficiency of CPT in NPs were measured based on CPT fluorescence using a plate reader (Perkin Elmer, Boston, MA). Briefly, NPs (3 mg) were dissolved in an acidic dimethyl sulfoxide solution (0.1% HCl, v/v), and the resulting solutions were transferred to a black 96-well plate. The fluorescence intensity was detected at emission wavelength of 430 nm (excitation at 360 nm). The drug loading and encapsulation efficiency were defined as follows:

$$\text{Drug loading} = \frac{\text{Weight of drug in NPs}}{\text{Weight of NPs}} \times 100\% \quad (1)$$

$$\text{Encapsulation efficiency} = \frac{\text{Weight of drug in NPs}}{\text{Weight of drug feeded}} \times 100\% \quad (2)$$

Morphology of hydrogel loaded with NPs was examined using SEM (LEO 1450VP, Zeiss, Germany). The NP-loaded hydrogel was freeze-dried, mounted on a carbon adhesive tape, and sputter-coated with a mixture of gold and palladium (60:40) in an argon atmosphere under low pressure.

Release Profiles of siCD98 and CPT from Fab'-siCD98/CPT-NPs

NPs (3 mg) were suspended in PBS (1.0 mL) and incubated at 37 ^{o}C with gentle shaking (120

rpm). The siRNA amount released from NPs was measured at various time intervals over 72 h. For each measurement, NP suspension was centrifuged at 13,400 g for 5 min, and the collected supernatant was used to determine siRNA content using the same method as that described above. The precipitated NPs were re-suspended in an equal volume of fresh PBS solution and incubated under the same conditions for continuous monitoring of siRNA release.

CPT amount released from NPs was assessed using a dialysis method. Briefly, NPs were dispersed in PBS to form a suspension (equal to 250 μg of drug). The suspension was transferred into a regenerated cellulose dialysis tube (molecular weight cut-off = 10 kDa) and the sample-filled tube was closed tightly at both ends to keep each tube surface area equivalent. The closed bag was subsequently immersed in 20 mL PBS release medium containing 0.1% Tween-80. Tween-80 was employed in PBS to maintain the solubility of CPT in aqueous phase. The tube was put in a water bath shaking at 120 rpm at 37 ^{o}C. At appropriate time points, outer solution was taken for measurement and fresh release medium was added. The amount of CPT in the outer solution was measured using the same method as described above.

The *in vitro* release behaviors of drugs from NP-embedded hydrogel were examined in gradually pH changing buffers: hydrochloricacid/potassium chloride buffer (pH 1.2), phosphate buffered saline buffer (pH 7.2) and acetic acid/sodium acetate buffer (pH 6.8), which were selected based on the normal variations of the pH along the GIT from the stomach, the proximal small intestine to the ileocecal region. The Fab'-CPT-NP (II)-embedded hydrogel was transferred into a regenerated Cellulose Dialysis tube (molecular weight cut-off = 10 kDa). The closed bag was subsequently immersed in 20 mL release medium (pH 1.2, 7.2 and 6.8). The tube was put in a water bath shaking at 120 rpm at 37 ^{o}C. At appropriate time points, outer solution was taken for measurement and fresh release medium was added. The amount of CPT in the

outer solution was measured using the same method as described above. TEM was used to observe the morphology of NPs released from hydrogel in acetic acid/sodium acetate buffer (pH 6.8) after 24 h of incubation.

Induced Cell Apoptosis

Colon-26 cells were cultured in 6-well plates overnight before NPs (CPT: 10 μM) were added in the medium. After further incubation for 4, 8 and 16 h, cells were rinsed with PBS thrice. Cells were then collected using accutase and analyzed by apoptosis assay using an Annexin V-FITC/PI Vybrant Kit. The emitted green fluorescence of Annexin V and red fluorescence of PI were recorded using 525 and 575 nm band pass filters, respectively (excitation at 488 nm). The flow analysis was based on 5000 gated cell events using FCM Canto^{TM} (BD Biosciences, San Jose, CA).

Wound Healing Assay

Scratch and Boyden chamber assays were performed to evaluate the functional roles of NPs in colonic epithelial cell migration. *In vitro* scratch wounds were generated by scraping the cell monolayer with a sterile p10 pipet tip. After the injury was made to the monolayer, the cells were gently washed, and the wounds were imaged separately prior to/after the addition of various NPs (CPT: 10 μM) using a microscope. The relative migration rate of control cells was designated as 100% and healing rate of other plates were compared with control cells.

Cell Migration Assay

Migration assay was done using cell culture inserts. Cell culture inserts with pores (8 μm) were placed in the 12-well companion plate containing 100 μL of RMPI 1640 medium. In the upper half of the insert, 1.5×10^5 cells were placed inside the chamber and incubated overnight. The cells were treated with serum-free medium and various NP suspensions. And the serum

containing medium was added to the lower chamber of the 12-well plate. After 24 h incubation, the cells on the upper surface of membrane were scrubbed off with humid cotton buds, while cells on the bottom surface were immobilized with cold 70% ethanol, stained with 0.1% crystal violet for 30 min. Cells presented in the lower part of the inserts were determined by counting cells in five microscopic fields per well, and the extend of migration was expressed as an average number of cells per microscopic field.

***Ex Vivo* Imaging**

To track the Fab'-functionalized NPs in GIT after oral administration, near infrared dye DiR was employed as a fluorescence probe. The fabrication process of Fab'-DiR-NPs was exactly the same as that for Fab'-CPT-NPs, except that CPT was replaced by DiR. The amount of DiR in Fab'-DiR-NPs was examined by a UV-Vis spectra method. Fab'-DiR-NP-embedded hydrogel was orally administered to the mice with colon cancer at an equivalent DiR concentration (0.5 mg DiR/kg per mouse). After oral administration for respective 8 or 24 h, the mice were sacrificed to obtain GIT. The images were captured using an IVIS spectrum imaging system (PerkinElmer/Caliper LifeSciences, Hopkinton, MA).

***In Vivo* Tissue Uptake of NPs**

To track the distribution of NPs in colon tumor tissues, CM was employed as a fluorescence probe. The fabrication processes of PEG-CM-NPs and Fab'-CM-NPs were exactly the same as that for PEG-CPT-NPs and Fab'-CPT-NPs, except that CPT was replaced by CM. Mice with colon cancer were orally administered with PEG-CPT-NPs or Fab'-CPT-NP-embedded hydrogel (1.5 mg CM/kg mouse). After 12 h oral administration, mice were sacrificed by CO_2 euthanasia. Colon tissues were extracted, rinsed with PBS and then embedded in Optimal Cutting Temperature compound. Eventually, tissue sections (5 μm) were stained with DAPI and Alexa

Fluor 568 phalloidin. An Olympus fluorescence microscope with a Hamamatsu Digital Camera ORCA-03G was used to record the images.

Statistical Analysis

Statistical analysis was conducted using ANOVA test followed by a Bonferroni post-hoc test (GraphPad Prism) or Student's *t*-test. Data were presented as mean ± standard error of mean (S.E.M.). Statistical significance was confirmed when $*P<0.05$ and $**P<0.01$.

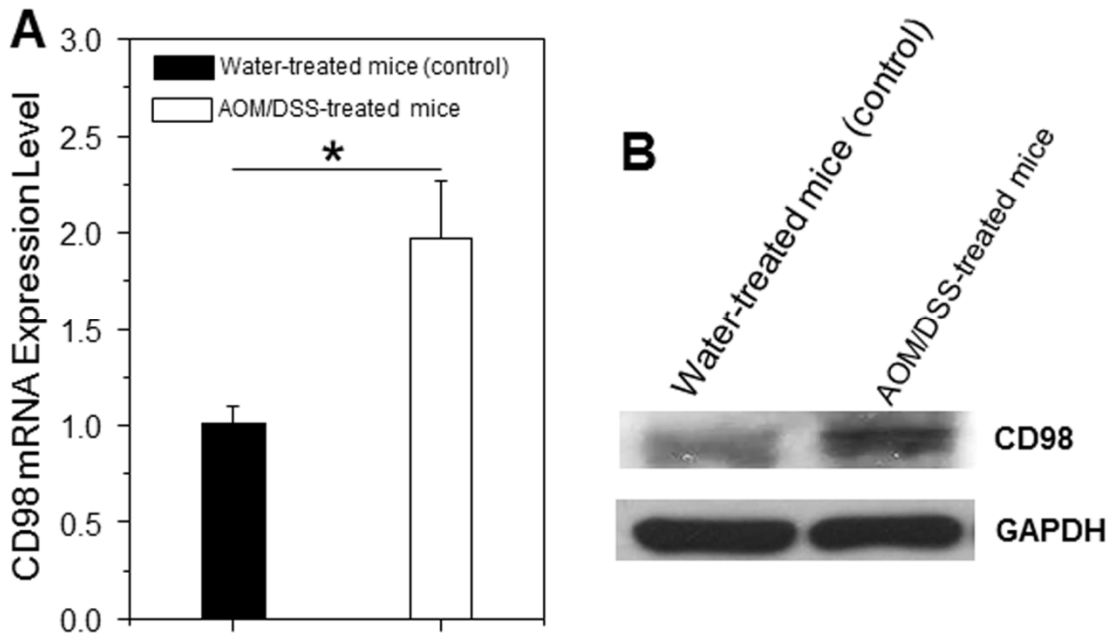

Figure S1. Upregulation of colonic CD98 expression in mice treated with AOM/DSS. (A) The colonic mRNA levels of CD98 were quantified by quantitative real-time reverse-transcription polymerase chain reaction and normalized to mRNA levels of the ribosomal protein, 36B4. Statistical significance was assessed using Student's *t* test (*$p < 0.05$ and **$p < 0.01$). Each point represents the mean ± S.E.M. (n = 5). (B) The protein levels of CD98 in the colons of healthy control mice and mice treated with AOM/DSS were analyzed by Western Blot.

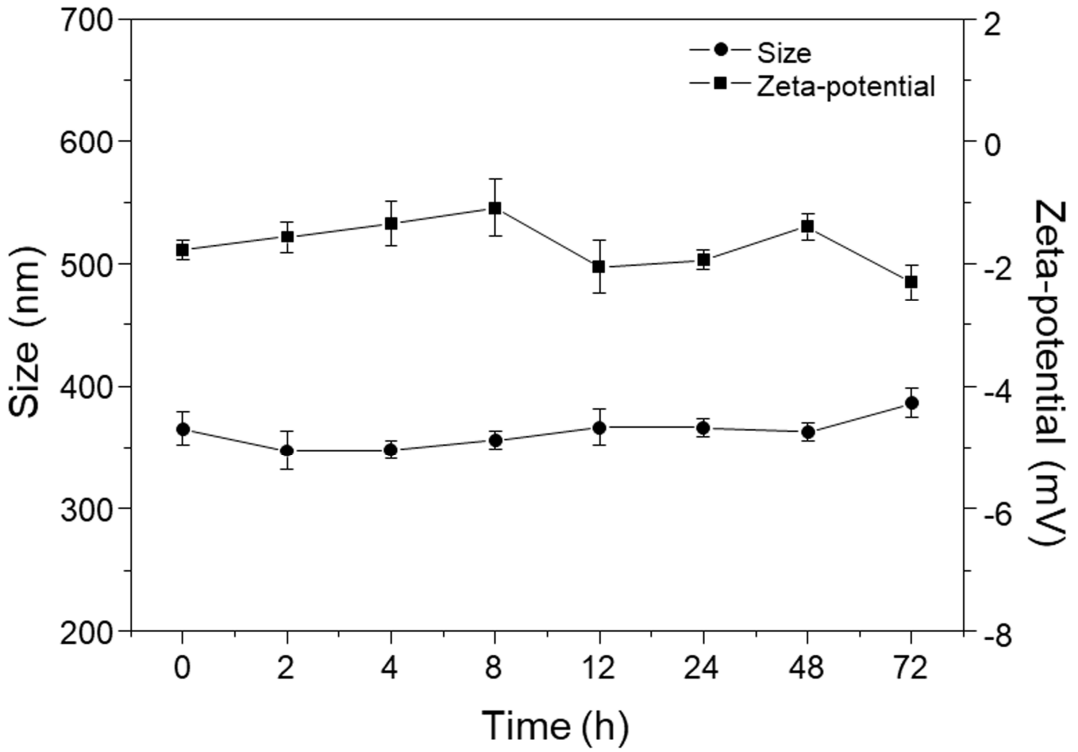

Figure S2. Changes of particle sizes and zeta potentials of Fab'-siCD98/CPT-NPs (II) in stimulated colonic fluid as a function of time.

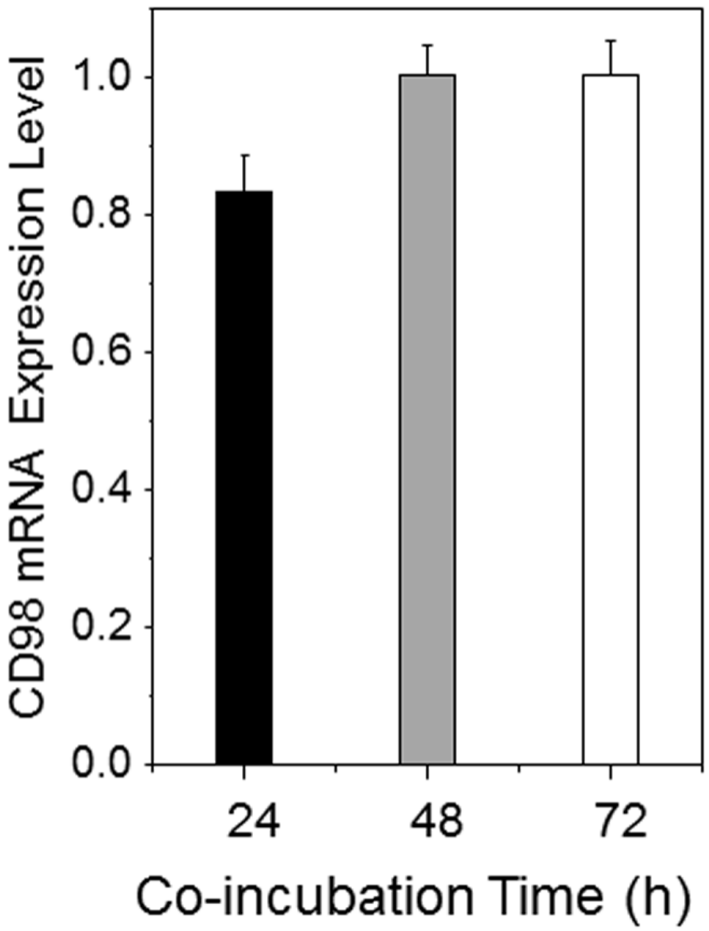

Figure S3. CD98 mRNA expression levels of Colon-26 cells treated with blank Fab'-NPs. CD98 mRNA expression levels of Colon-26 cells exposed to blank Fab'-NPs at the NP concentration of 25 μg/mL for 24, 48 and 72 h, respectively. Each point represents the mean ± S.E.M. (n = 3).

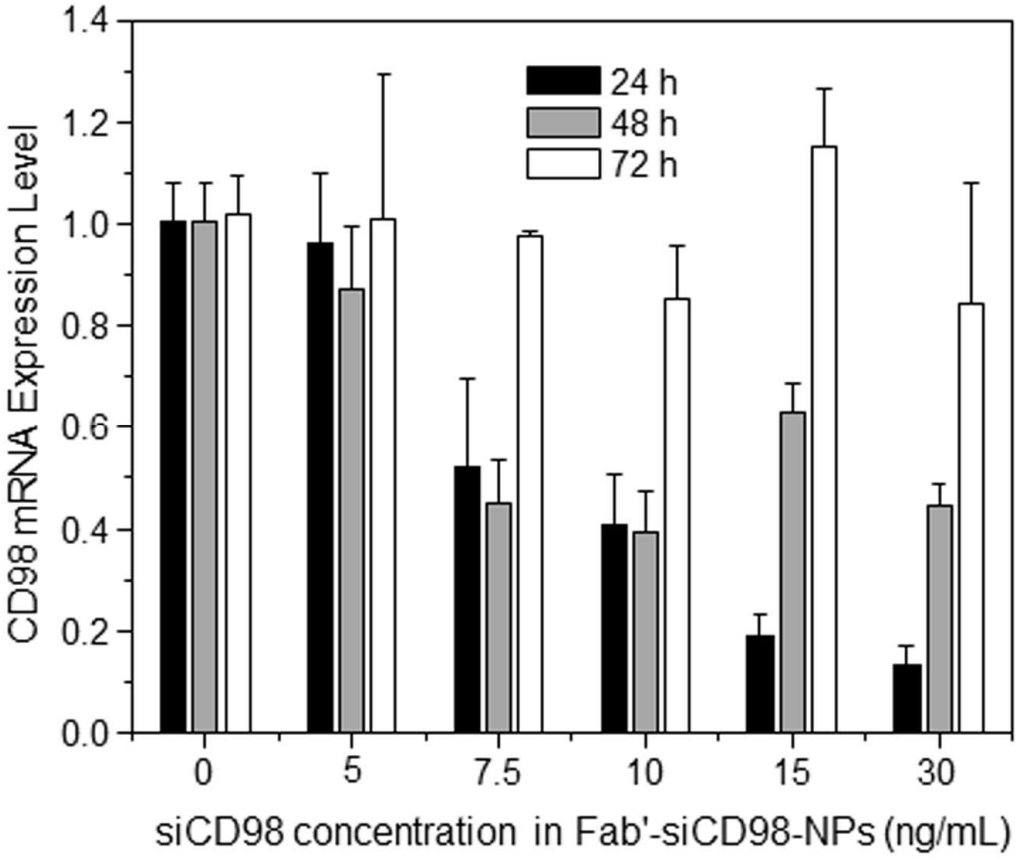

Figure S4. CD98 mRNA expression levels of Colon-26 cells exposed to Fab'-siCD98-NPs at different concentrations (5, 7.5, 10, 15 and 30 ng/mL) of siCD98 for 24, 48 and 72 h, respectively.

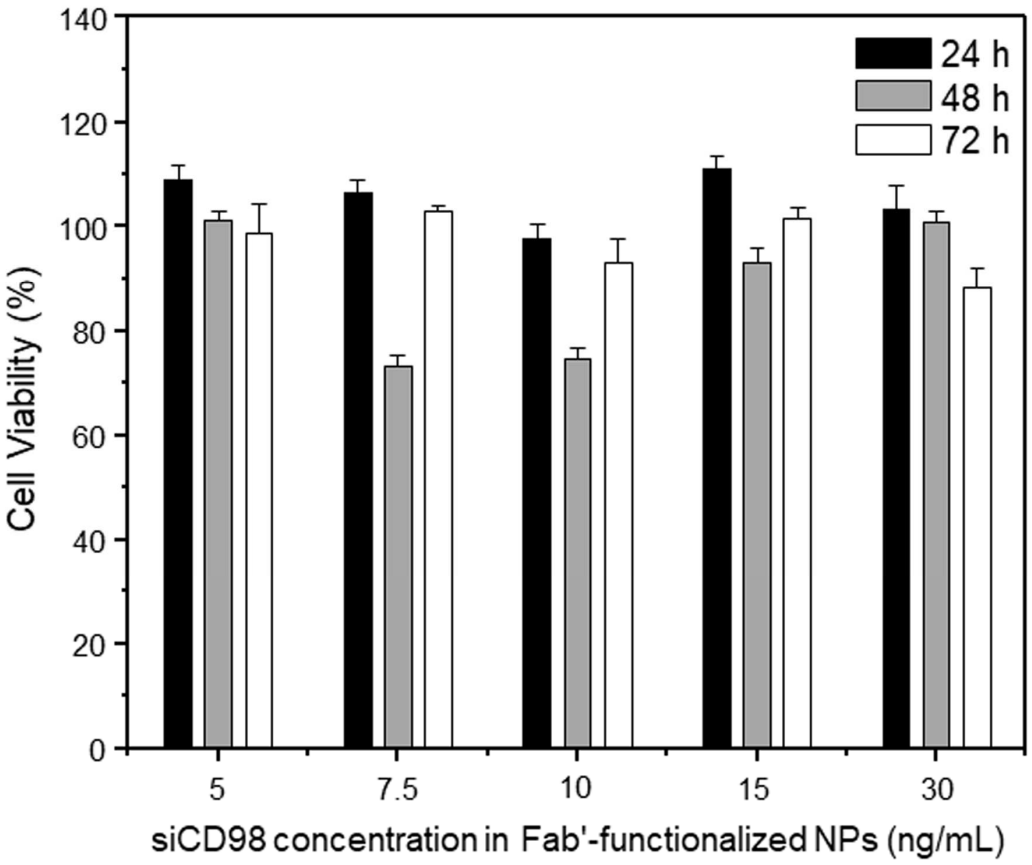

Figure S5. *In vitro* cytotoxicity of Fab'-siCD98-NPs in Colon-26 cells. The cell viability of Colon-26 cells exposed to Fab'-siCD98-NPs at different concentrations of siCD98 (5, 7.5, 10, 15 and 30 ng/mL) for 24, 48 and 72 h, respectively. Each point represents the mean ± S.E.M. (n = 5).

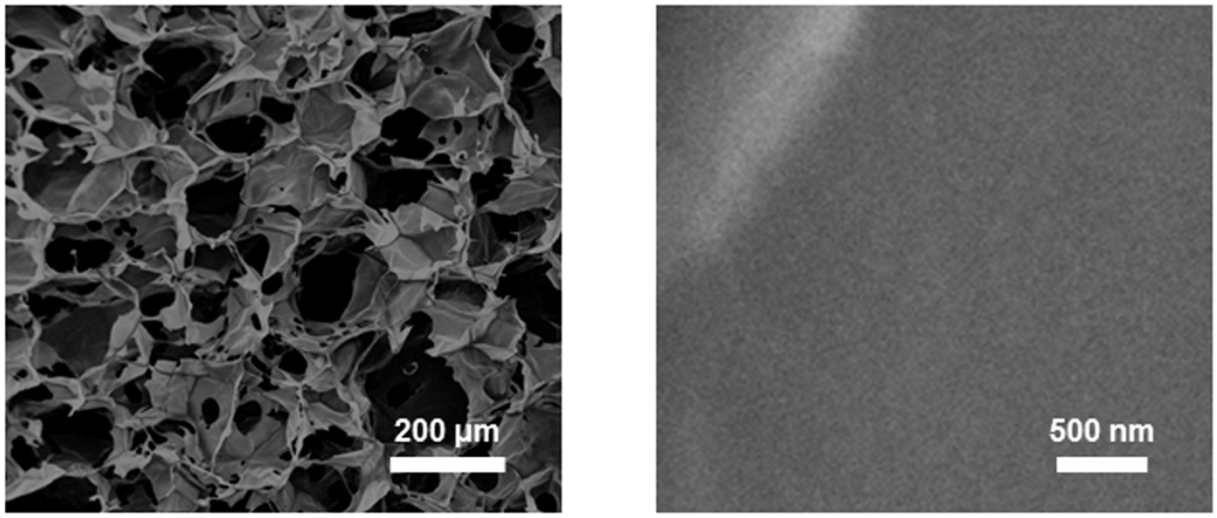

Figure S6. Representative scanning electron microscope images of the resulting hydrogel (chitosan and alginate) without NPs.

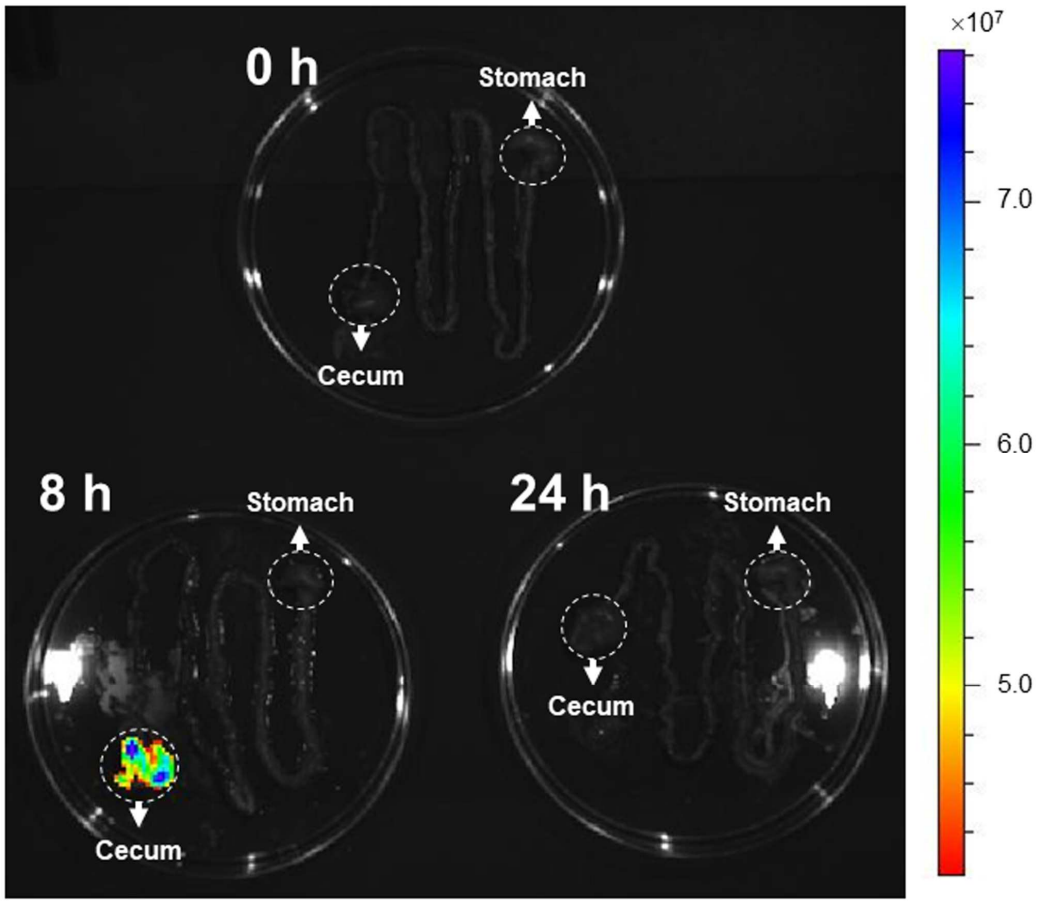

Figure S7. Typical images of stomach, small intestine and caecum imaging showing the distribution of orally Fab'-DiR-NPs embedded in hydrogel at three different time points (0, 8 and 24 h).

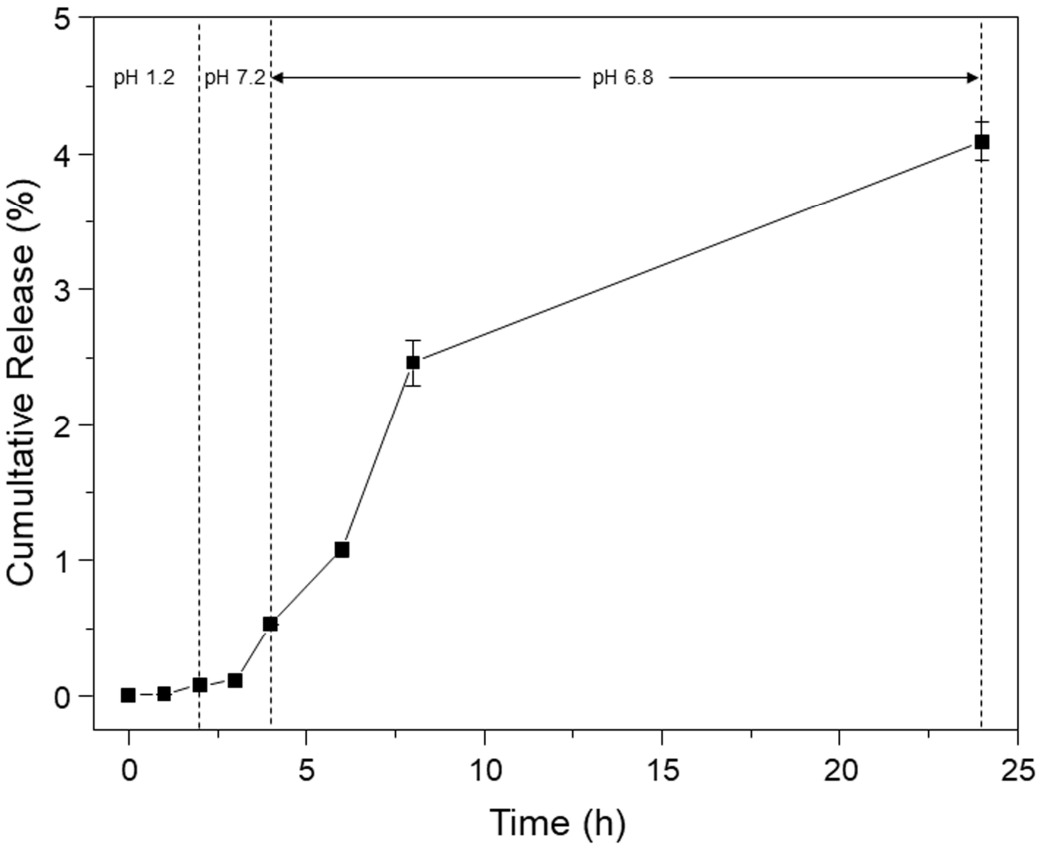

Figure S8. Release profiles of CPT from Fab'-CPT-NP (II)-embedded hydrogel incubated in a buffer that gradually changed pH over 24 h (n = 3).

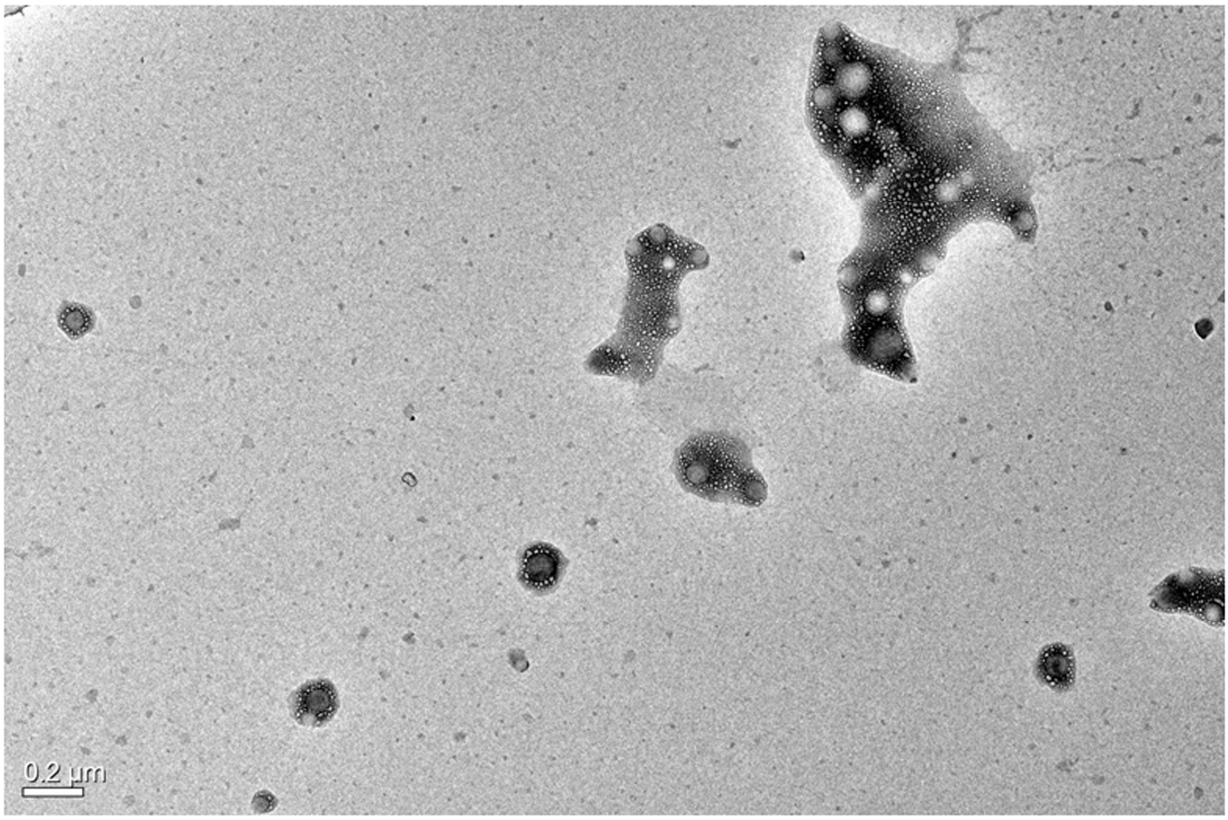

Figure S9. TEM image of Fab'-siCD98/CPT-NP (II) released from hydrogel.

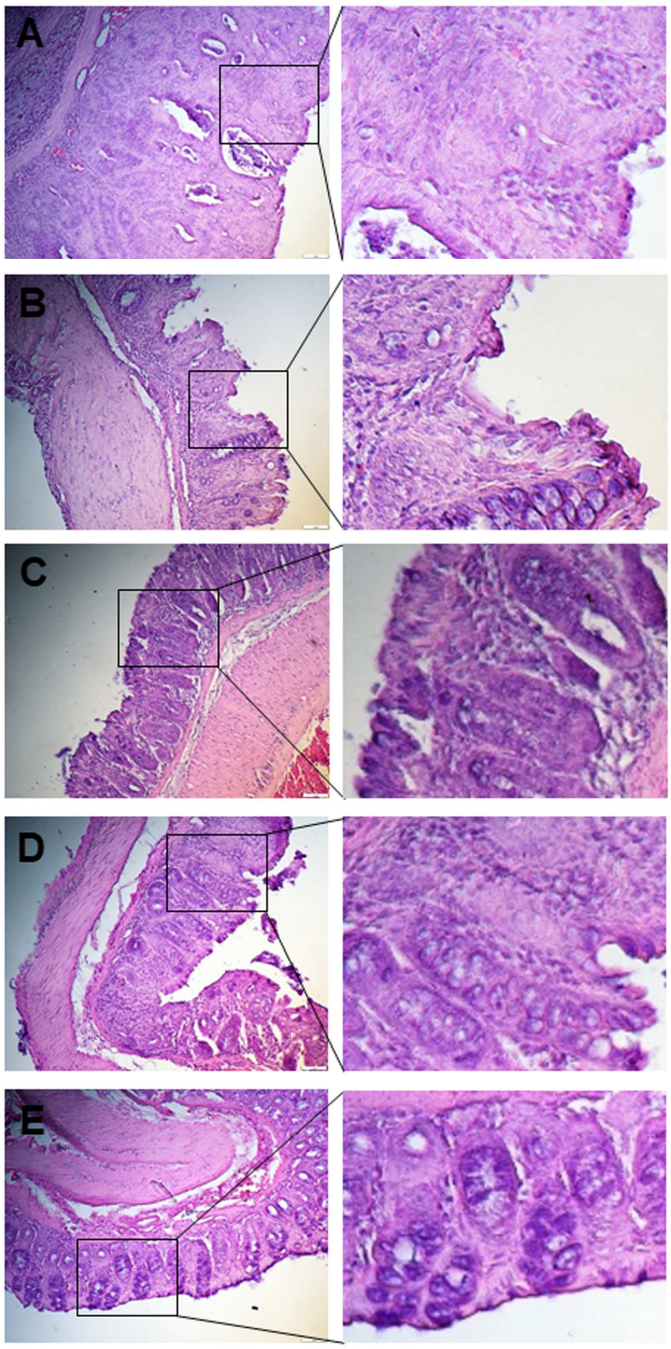

Figure S10. Representative H&E-stained colon sections from AOM/DSS-treated mice administered with blank NP, Fab′-siCD98-NP, Fab′-CPT-NP, siCD98/CPT-NP or Fab′-siCD98/CPT-NP-embedded hydrogel. Scale bar represents 100 μm.

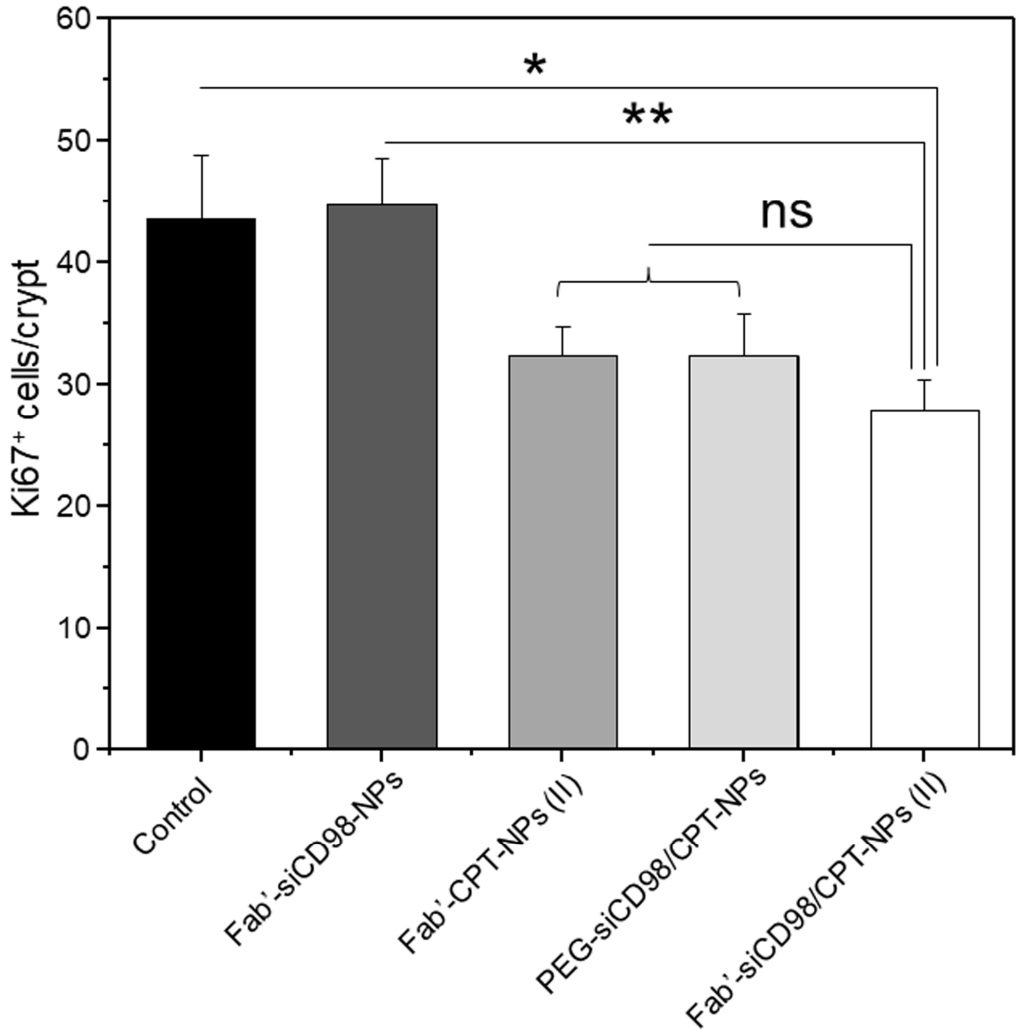

Figure S11. Ki67^{+} cells were counted and averaged per crypt. Statistical significance was assessed using ANOVA followed by a Bonferroni post-test (*$p < 0.05$ and **$p < 0.01$). Data are expressed as means ± S.E.M. (n = 6-10 per group).

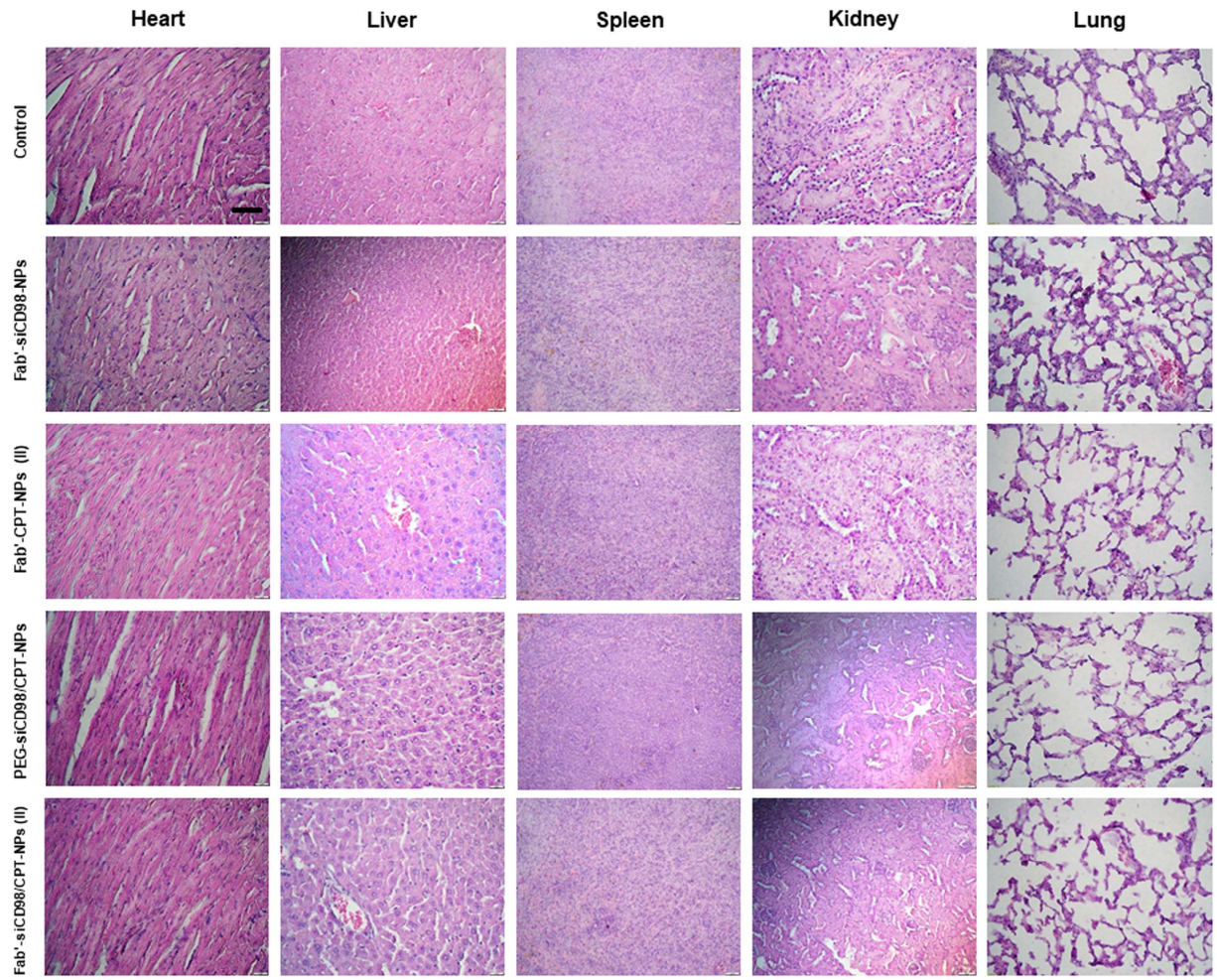

Figure S12. H&E staining of sections of major organ tissues obtained from AOM/DSS-treated mice after treatment with blank NP, Fab′-siCD98-NP, Fab′-CPT-NP, siCD98/CPT-NP or Fab′-siCD98/CPT-NP-embedded hydrogel. Scale bar represents 20 μm.

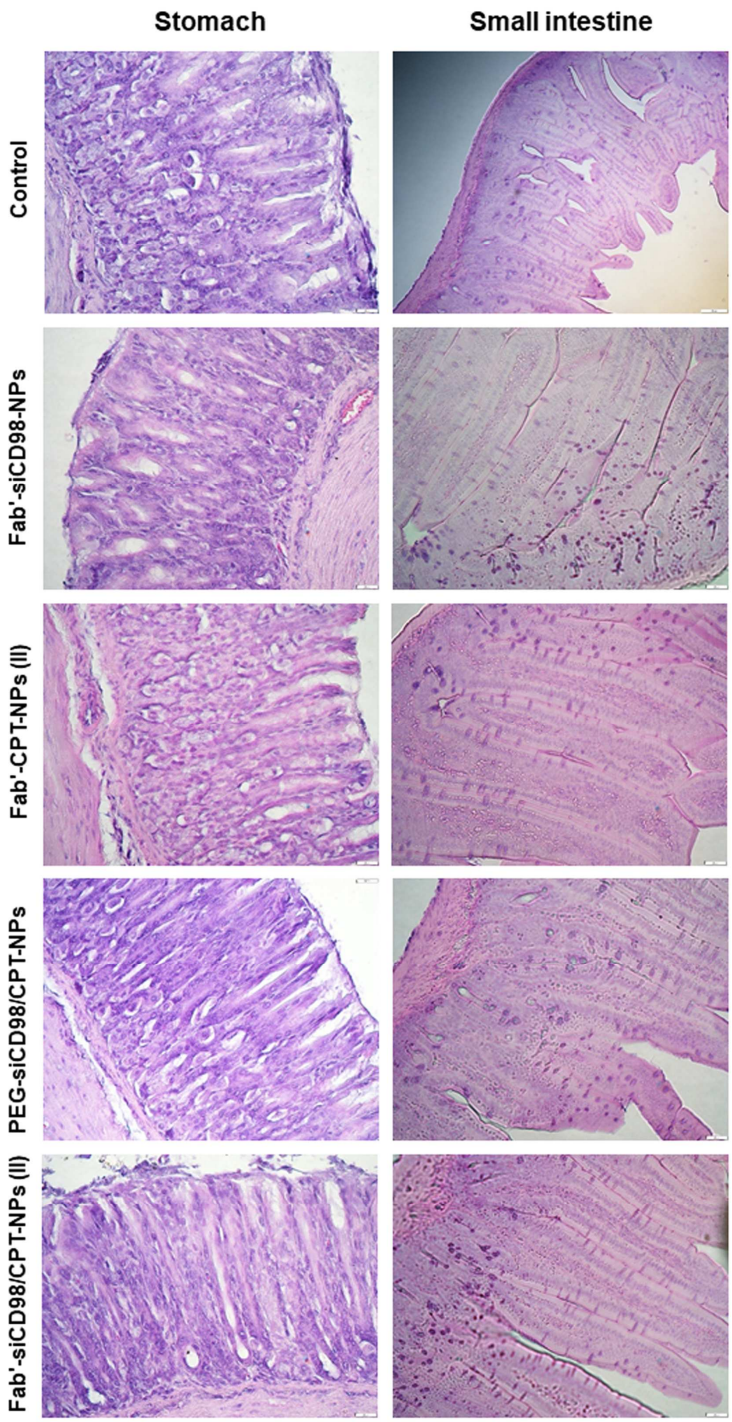

Figure S13. H&E staining of sections of stomach and small intestine obtained from AOM/DSS-treated mice after treatment with blank NP, Fab′-siCD98-NP, Fab′-CPT-NP, siCD98/CPT-NP or Fab′-siCD98/CPT-NP-embedded hydrogel. Scale bar represents 20 μm.

Table S1. qRT-PCR primers used in this study.

Primer name	Sequence	Description
CD98-F	5’-GAGGACAGGCTTTTGATTGC-3’	CD98 gene RT-PCR forward primer
CD98-R	5’-ATTCAGTACGCTCCCCAGTG-3’	CD98 gene RT-PCR reverse primer
TNF-α-F	5’-AGGCTGCCCCGACTACGT-3’	TNF-α gene RT-PCR forward primer
TNF-α-R	5’-GACTTTCTCCTGGTATGAGATAGCAAA-3’	TNF-α gene RT-PCR reverse primer
IL-6-F	5’-ACAAGTCGGAGGCTTAATTACACAT-3’	IL-6 gene RT-PCR forward primer
IL-6-R	5’-TTGCCATTGCACAACTCTTTTC-3’	IL-6 gene RT-PCR reverse primer
IL-12-F	5’-GCCAGTACACCTGCCACAAA-3’	IL-12 gene RT-PCR forward primer
IL-12-R	5’-TGTGGAGCAGCAGATGTGAGT-3’	IL-12 gene RT-PCR reverse primer
IL-10-F	5’-ATGCAGGACTTTAAGGGTTACTTGGGTT-3’	IL-10 gene RT-PCR forward primer
IL-10-R	5’-ATTTCGGAGAGAGGTACAAACGAGGTTT-3’	IL-10 gene RT-PCR reverse primer
Bcl-2-F	5’-GTACCTGAACCGGCATCTG-3’	Bcl-2 gene RT-PCR forward primer
Bcl-2-R	5’-GGGGCCATATAGTTCCACAA-3’	Bcl-2 gene RT-PCR reverse primer
36B4-F	5’-TCCAGGCTTTGGGCATCA-3’	36B4 gene RT-PCR forward primer
36B4-R	5’-CTTTATCAGCTGCACATCACTCAGA-3’	36B4 gene RT-PCR reverse primer

Reference

1. Viennois, E.; Xiao, B.; Ayyadurai, S.; Wang, L. X.; Wang, P. Q.; Zhang, Q.; Chen, Y.; Merlin, D. Micheliolide, a New Sesquiterpene Lactone that Inhibits Intestinal Inflammation and Colitis-Associated Cancer. *Lab. Investig.* **2014**, *94,* 950–965.