Electronic Supplementary Information (ESI)

Oral nanotherapeutics with enhanced mucus penetration and ROS-responsive drug release capacities for delivery of curcumin to colitis tissues

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Supplementary methods

1. Physicochemical characterizations of NPs

The average hydrodynamic particle sizes (nm), PDI values, and zeta potentials (mV) of various NPs were measured using a DLS instrument (Malvern Zetasizer Nano S90, Worcestershire, UK). The average values of these parameters were based on the measurements of 3 batches of these NPs.

The morphologies of various NPs were observed using a JEOL JSM-6510LV SEM (Tokyo, Japan). NPs were suspended in water, and a drop of the NP suspension was added to the surface of silicon chips. The air-dried NPs were coated with platinum under vacuum before SEM examinations.

The loading amount and encapsulation efficiency of CUR in various NPs were determined by measuring the intrinsic fluorescence intensity of CUR. NPs were dissolved in DMSO. Thereafter, the solution (150 μL) was transferred to a black 96-well plate, and the fluorescence intensity of CUR was measured at 528 nm emission wavelength (485 nm excitation wavelength) using a microplate reader (Biotek Instruments, USA).

The loading amount and of encapsulation efficiency CAT in P-CUR/CAT-NPs was determined as described below. NPs (3 mg) were vortexed in 0.5 mL of dichloromethane. The released BSA/CAT was extracted from the organic phase using 0.8 mL PBS. PBS was added to the organic solution, and the resultant mixture was vortexed vigorously for 5 min and then centrifuged at 12 000 rpm for 5 min at 4°C. The BSA/CAT content in the supernatant was analyzed by the Bio-Rad protein assay. The encapsulation rate was applied to determine the CAT loading, assuming that BSA and CAT are loaded in the same way in a homogeneous aqueous phase.
XRD spectra of pristine CUR, pristine CAT, pristine PLGA, pristine PF127, and various NPs were examined on an XRD-7000 instrument (Shimadzu, Japan) by scanning at a speed of 2°/min from 10° to 50° (2θ) under conditions of 40 kV and 30 mA.

2. Drug release profiles of NPs

The dialysis method was used to determine the drug release profiles of various NPs in different buffers (pH 7.4 or 5.5) with or without H$_2$O$_2$ (10 mM). Various CPT-loaded NPs were dispersed in buffers, and the CPT amount in NPs was set as 200 μg. NP suspensions were transferred into cellulose dialysis bags (molecular weight cut-off = 10 kDa), and these bags were sealed tightly at both ends. Subsequently, the closed bags were put into 50 mL centrifuge tubes, which were filled with 20 mL of releasing buffers containing Tween-80 (1%, w/v). Tween-80 was used to keep the solubility of CPT in buffers. Thereafter, the tubes were kept in a rotary shaker at 120 rpm at 37°C. At pre-determined time points, the outer releasing buffer was taken for measurements, and the equal volume of fresh buffer was added. The CPT concentrations in the outer releasing buffers were determined using a microplate reader (Biotek Instruments, USA).

3. In vitro biocompatibility of NPs

Raw 264.7 macrophages were seeded in 96-well plates at a density of $2.0 \times 10^4$ cells per well and incubated in complete culture medium overnight. Various NPs were suspended in serum-free medium to obtain NP suspensions containing various CUR concentrations (0 – 25 μM), and the resulting suspensions (100 μL) were added to the wells. After 5 h co-incubation, complete culture medium (100 μL) was added to each well and further incubated for 19 h. Subsequently, cells were incubated with MTT (0.5 mg/mL) for 3 h. The media were discarded, and DMSO (50 μL) was added to each well before spectrophotometric measurements at 570 nm. Untreated cells
were used as a negative control, whereas cells with the treatment of Triton X-100 solution (1%, w/v) were treated as a positive control.

4. **In vitro cell internalization profiles of NPs**

Raw 264.7 macrophages were seeded in cell culture dishes (2 cm in diameter) at a density of $4 \times 10^4$ cells per dish and incubated in complete culture medium overnight. Various NPs were suspended in a serum-free medium to form NP suspensions at a CUR concentration of 20 $\mu$M, and the resulting suspensions (1 mL) were added into dishes. After co-incubation for 5 h, cells were washed with PBS containing calcium and magnesium for 3 times, and fixed in paraformaldehyde solution (4%, v/v) for 20 min. Subsequently, the cytoskeleton and the nucleus were stained with AF633-labeled phalloid and DAPI, respectively. The fluorescence images were taken with a confocal laser scanning microscope (Zeiss-800, Germany).

5. **Quantification of cell internalization efficiencies of NPs**

Raw 264.7 macrophages were seeded in 6-well plates at a density of $3 \times 10^5$ cells per well and incubated in complete culture medium overnight. Various NPs were suspended in a serum-free medium to form NP suspensions at a CUR concentration of 20 $\mu$M, and the NP suspension (2 mL) was added into each well. After co-incubation for respective 1, 3, and 5 h, cells were washed with PBS containing calcium and magnesium for 3 times. Cells were collected using trypsin and evaluated using flow cytometry (FCM, ACEA Novocyte™, USA).

6. **In vivo bio-distribution profiles of NPs**

UC mouse model was established by replacing the drinking water with DSS solution (3.5%, w/v) for 5 days. Hydrogel-embedding P-CAT/Cy7-NPs (Cy7, 5mg/kg) were orally administered to mice with UC. At pre-determined time points (12, 24, and 48 h), GIT and the major organs were
excised and imaged by using an IVIS spectrum imaging system (FX Pro90200, Care stream, USA).

In addition, the stomach, duodenum, jejunum, ileum, cecum, and colon from the P-CAT/CUR-NP-treated mice were excised at the time point of 24 h, rinsed with PBS, and embedded in Optimal Cutting Temperature (OCT) compound. These tissues were sectioned into slices (5 µm) and stained with DAPI. The fluorescence images were taken with a confocal laser scanning microscope (Zeiss-800, Germany).

7. Ly6G staining

Paraffin-embedded tissue sections were deparaffinized in xylene and rehydrated using an ethanol gradient. Tissue sections were incubated with hydrogen peroxide (3%, v/v) in PBS for 30 min at room temperature. Epitope retrieval was performed by treating the tissues with 10 mM sodium citrate buffer (pH 6.0) with 0.05% Tween-20 at 100°C for 10 min in a pressure cooker. Ly6G staining sections were blocked with 10% normal goat serum with BSA (1%, w/v) in TBS for 2 h at room temperature followed by incubation with rat monoclonal anti-Ly6G antibody (1:500 dilution) (Abcam, Cambridge, MA, USA) in TBS with BSA (1%, w/v) at 4°C overnight. Tissue sections were treated with their respective biotinylated secondary antibodies for 45 min at room temperature (Vector laboratories PK-6101 and BA-9400). Color was developed using the Vectastain ABC kit (Vector Laboratories) followed by DAB reaction. Sections were then counterstained with hematoxylin, dehydrated in an ethanol and xylene. Images were acquired at 20× magnification using an Olympus microscope equipped with a D-26 color camera.

8. Statistical analysis
Statistical analysis was conducted using an ANOVA test followed by a Bonferroni post-hoc test (Graph Pad Prism). Data were shown as mean ± standard error of the mean (S.E.M.). Statistical significance was represented by *$p<0.05$ and **$p<0.01$. 
Fig. S1 XRD patterns of (a) pristine CUR, (b) pristine CAT, (c) pristine PLGA, (d) pristine PF127, (e) CUR-NPs, (f) P-CUR-NPs, and (g) P-CAT/CUR-NPs.
**Fig. S2** *In vitro* cytotoxicity of CUR-NPs, P-CUR-NPs, and P-CAT/CUR-NPs against Raw 264.7 macrophages after co-incubation for 24 h based on MTT assays. Triton X-100 (0.1%) was used as a positive control to produce a maximum cell death rate (100%); cell culture medium was used as a negative control (death rate defined as 0%). Each point represents the mean ± S.E.M. (n=5).
Fig. S3 Colon images of mice from different groups at day 11.
Fig. S4 Representative Ly6G immunostaining images of colons from (i) the healthy control group, (ii) the DSS control group, (iii) the CUR-NP-treated DSS group, (iv) the P-CUR-NP-treated DSS group, and (v) the P-CAT/CUR-NP-treated DSS group, respectively. Arrows indicate the infiltration of monocytes, granulocytes, and neutrophils (hematoxylin counterstaining was used to visualize histological damage in the mouse colon). Scale bars: 50 μm.
Fig. S5 Representative H&E staining images of heart, liver, spleen, lung, and kidney obtained from various mouse groups on Day 11. Scale bar = 200 µm.