Supporting Information

A Superstable Sandwich-type Composite of a Single-benzene-based Fluorophore and Chitosan as a Fluorescent Authentication Barcode

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1. Experimental section

1.1. General information

Chitosan (low molecular weight) was purchased from Sigma-Aldrich. Dimethyl 1,4-cyclohexanedione-2,5-dicarboxylate (DCD) was purchased from TCI (Tokyo, Japan). Ethanol, acetic acid, and buffer solutions were purchased from Samchun Chemicals (Rep. of Korea). Dimethyl sulfoxide (DMSO, Product No. 1.02952.1000) was purchased from Merck (Darmstadt, Germany). Commercially available reagents and anhydrous solvents were used without further purification. Chemical reactions were performed under open-air reflux conditions. Excitation and emission spectra of the powder sample were measured using a fluorescence spectrophotometer (Hitachi, F-7100). UV/vis absorption and emission spectra were measured using an Agilent Cary 8454 spectrophotometer (Agilent Technologies, California, USA) and an RF-6000 spectro-fluorophotometer (Shimadzu Corp., Kyoto, Japan). The fluorescence intensity of the materials was measured with a fluorescence imaging system (FTIS, VISQUE® InVivo Elite, Vieworks Co., Ltd., Rep. of Korea). The surface element of the particles was analyzed using energy-dispersive X-ray spectroscopy (EDS, E-max Evolution EX-370 Analyzer) at the Korea Basic Science Center (Korea University, Seoul, Rep. of Korea). Attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy was performed using a Thermo Scientific Nicolet™ iS™ 5 FT-IR spectrometer instrument (16 scans, Waltham, MA, USA). Time-resolved fluorescence (TRF) signals of c-SSH were measured in the solid state. Experiments were carried out using a time-correlated single-photon counting (TCSPC) method.

1.2. Synthesis of SBBF-Chitosan

Dimethyl 1,4-cyclohexanedione-2,5-dicarboxylate (0.438 mmol) and chitosan (1 mmol) were mixed in 10 ml of EtOH and 500 µL of AcOH and reacted at 80°C for 18 h. After the reaction, the precipitate was filtered and washed with ethyl acetate and water and then dried under vacuum conditions. The product obtained after drying was approximately 170 mg, with a yield of approximately 97%. The melting point of **SC** was measured using a thermal analysis system (TAS, melting point: 222.4 ± 2.0°C.).

1.3. Characterization of SC

To identify the structural changes of SC, the infrared absorption spectrum of SC was analyzed using Attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy. Time-resolved fluorescence (TRF) signals of SC was measured in solid-state. The sample was excited using a 388 nm pulse (LDH-P-C-520, PicoQuant) and the sample TRF signals were collected at 610 nm. In order to observe the structural formulation, image-based analysis was

conducted by scanning electron microscope (SEM, SU8220, Hitachi, Japan) imaging at the Korea Basic Science Center (Korea University, Seoul, Rep. of Korea). The surface element of particles was analyzed using energy-dispersive X-ray spectroscopy (EDS, E-max Evolution EX-370 Analyzer) at the Korea Basic Science Center (Korea University, Seoul, Rep. of Korea).

1.4. Stability analysis of SC

UV/vis absorption spectra were measured using a spectrophotometer (Agilent Technologies Cary 8454, Santa Clara, CA, USA), and fluorescence spectra were recorded on a spectro-fluorophotometer (SHIMADZU CORP. RF-6000, Kyoto, Japan), with a 1 cm standard quartz cell (internal volume of 1 mL, Hellma Analytics, Germany). All absorption and emission spectra were obtained at room temperature (25 °C).

1.5. Tablet preparation

Both **SC**/Eudragit formulation and tablets were made using a tablet press, and the external drug was also formed using Eudragit EPO.

1.6. Animals

Five-week-old male Balb/c nu/nu mice were obtained from DBL Co., Ltd. (Incheon, Korea). The mice were housed 5 per cage (27×22×14 cm) with free access to food and water in the animal room and were kept under a 12 h light/dark cycle (lights on from 07:30 to19:30) at a constant temperature (23±1 °C) and with relative humidity (60±10%). The mice were kept in the animal room for a week before all experiments. Animal treatment and maintenance were performed according to the Animal Care and Use Guidelines by Kyung Hee University. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Kyung Hee University (Approval No.: KHSASP-22-024).

1.7. FTIS mouse tissue imaging

After the **SC** (5 mg/mL, p.o.) administration, the main organs (brain, heart, lung, intestine, spleen, kidney, and liver) of the mouse were dissected to perform further ex vivo analysis. The fluorescence tissue imaging system (FTIS, VISQUE InVivo Elite, Vieworks Co. Ltd., Rep. of Korea) was used for the ex vivo tissue fluorescence imaging. The imaging experiment was carried out in a dark room. The data were acquired by tracking the signals of **SC** (PE Channel: 390–490 nm excitation, 575–640 nm detection channel).

1.8. Hemolysis assay

The blood was obtained from the hearts of the mice anesthetized with isoflurane. The red blood cells (RBCs) were extracted by centrifugation at 4 °C (3,000 rpm, 3 min) and washed in 1× cold PBS (2 times). The concentrations of **SC** for the test were 2.5, 5, and 10 mg/mL. **SC** was

treated to the purified RBCs (8% working concentration, v/v, in cold 1× PBS). [Positive control: 0.1% (working concentration) Triton X-100]. The mixture was incubated in a shaking incubator (200 rpm, 37 °C) for 1 h and then was centrifuged at 3000 rpm at 4 °C. The supernatant was measured under absorption at 450 nm.

1.9. Statistical analysis

The results were analyzed by ANOVA with the Newman–Keuls multiple comparison test. All statistical results were analyzed using Prism 8.0 software (GraphPad, La Jolla, California, USA).

2. Supporting Figures

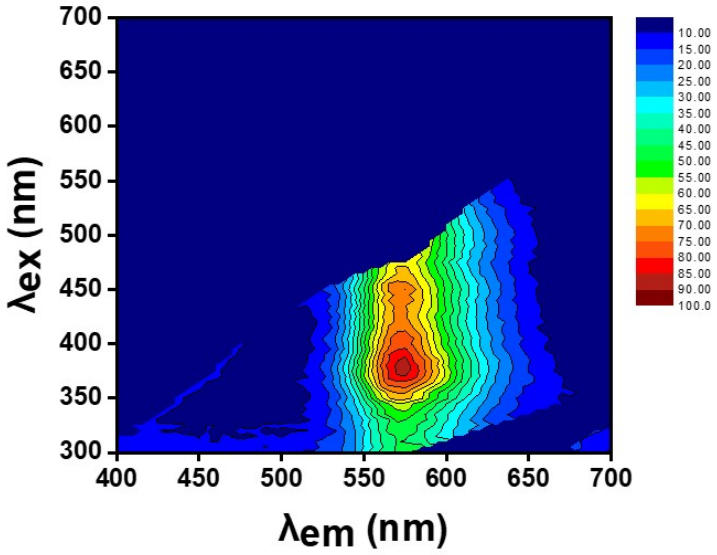

Fig. S1. 2D photoluminescence excitation (PLE) spectrum (λ_{ex}: 400–700 nm) of **SC**. X axis: emission, Y axis: excitation. Intensity: red (strong), blue (weak).

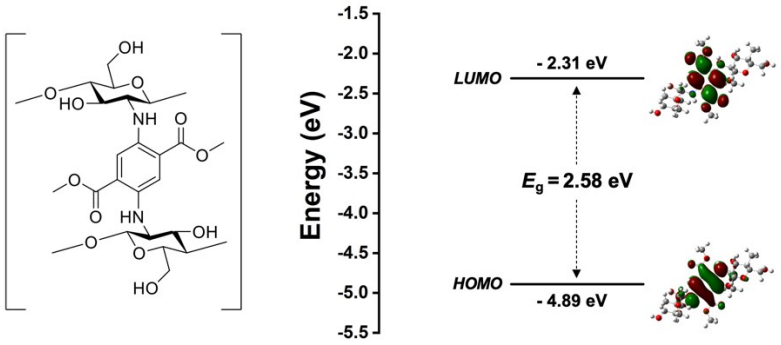

Fig. S2. DFT calculation (B3LYP/6-31+G(3d)) results for the most stable conformational structure (HOMO-LUMO) of the monomeric structure.

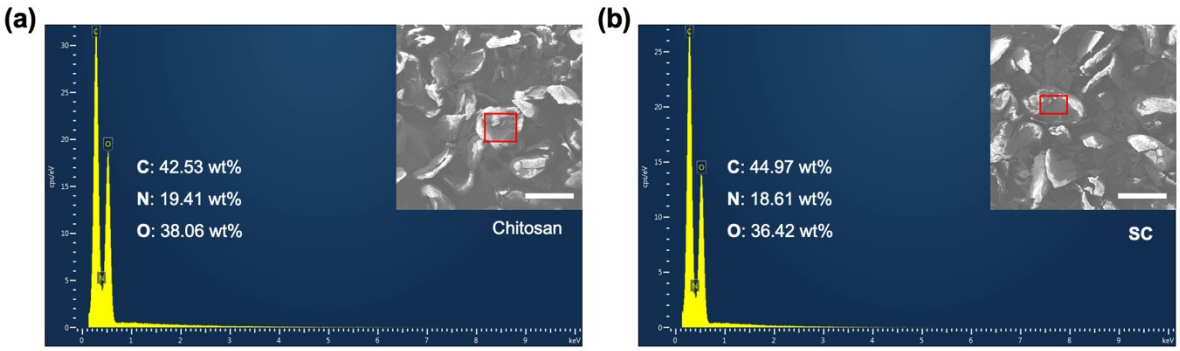

Fig. S3. EDS mapping spectrum of (a) chitosan and (b) **SC**. Inset: SEM images of chitosan and **SC**. Scale bar: 200 μm.

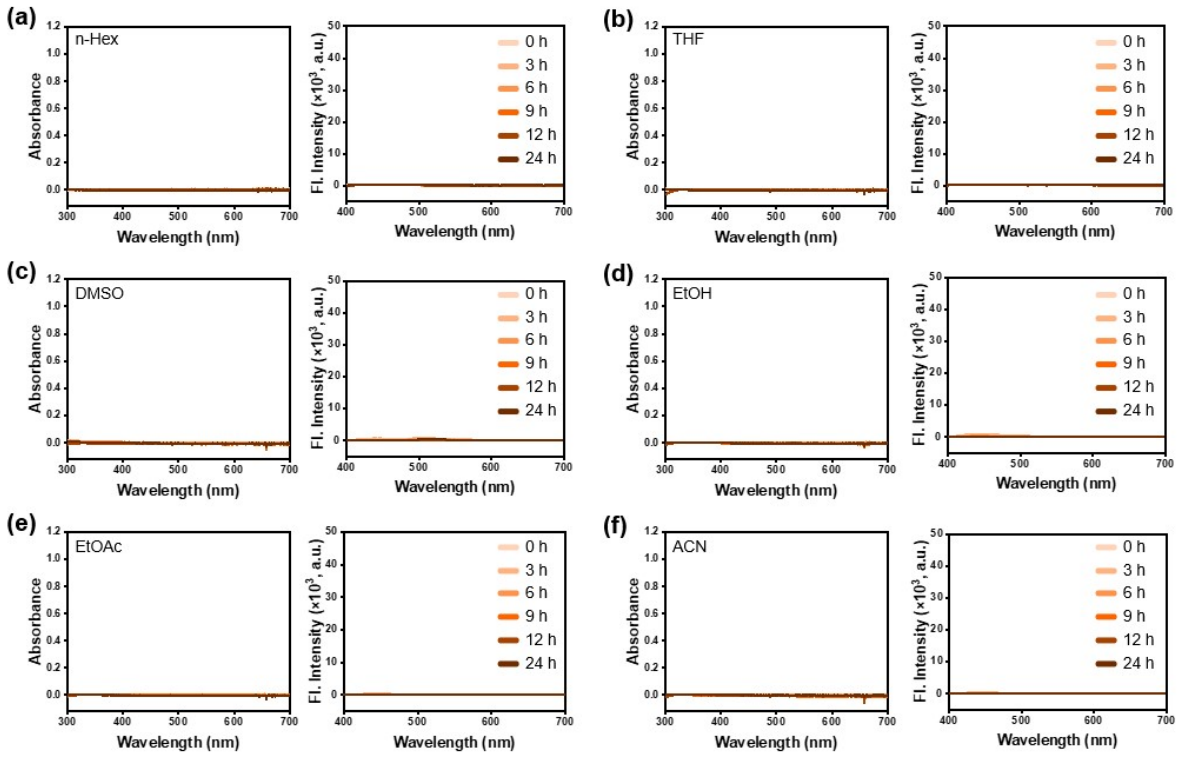

Fig. S4. The absorption and emission spectrum of **SC** suspension (2 mg/mL) supernatant in (a) n-hexane, (b) tetrahydrofuran, (c) dimethyl sulfoxide, (d) ethanol, (e) ethyl acetate, and (f) acetonitrile. Inset time: incubation time at 25 °C.

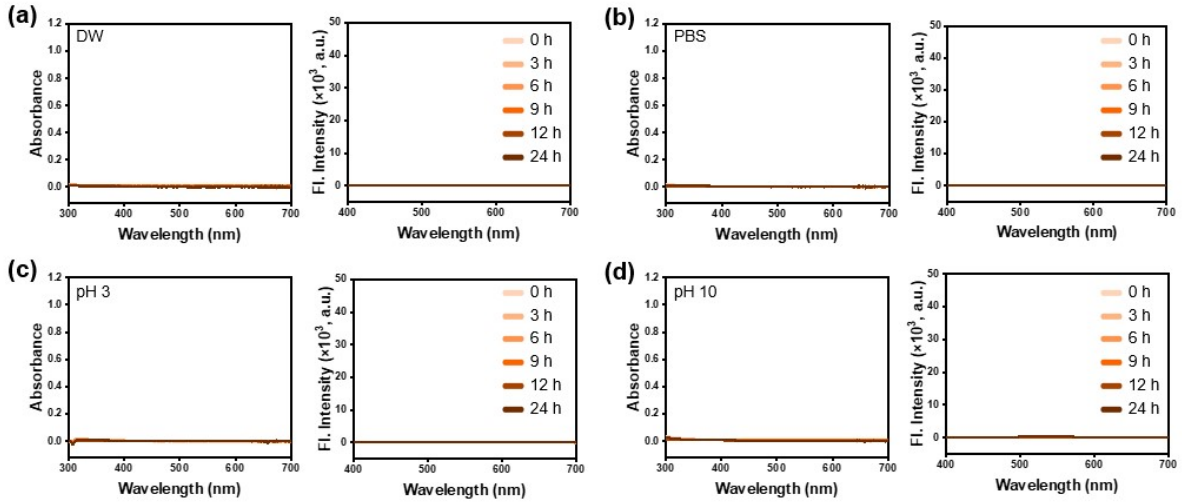

Fig. S5. The absorption and emission spectrum of **SC** suspension (2 mg/mL) supernatant in (a) deionized water, (b) PBS (pH 7.4), (c) pH 3 buffer solution, and (d) pH 10 buffer solution. Inset time: incubation time at 25 °C.

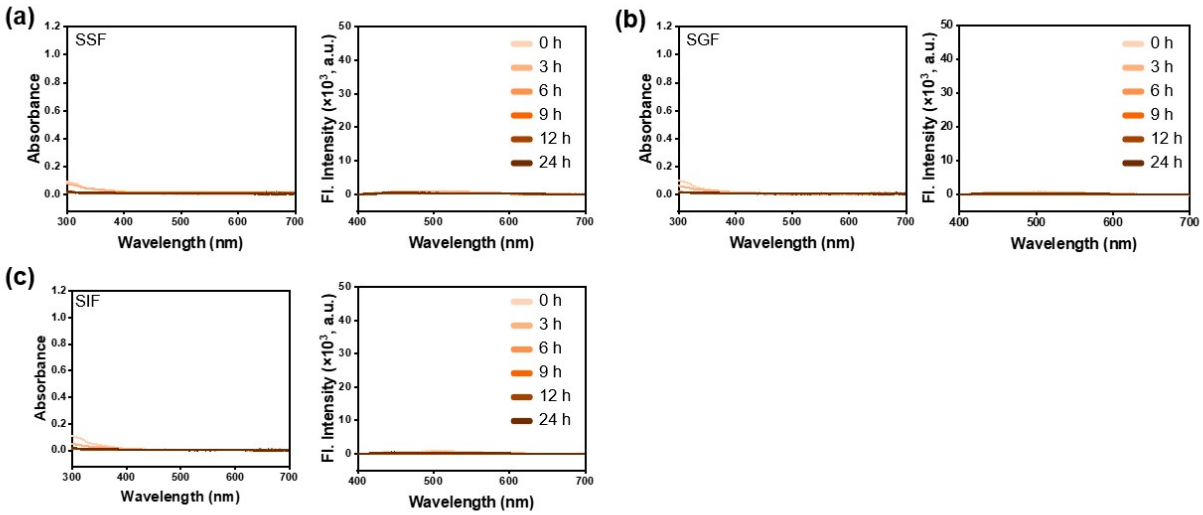

Fig. S6. The absorption and emission spectrum of **SC** suspension (2 mg/mL) supernatant in (a) simulated salivary fluids, (b) simulated gastric fluids, and (c) simulated intestinal fluids. Inset time: incubation time at 25 °C.

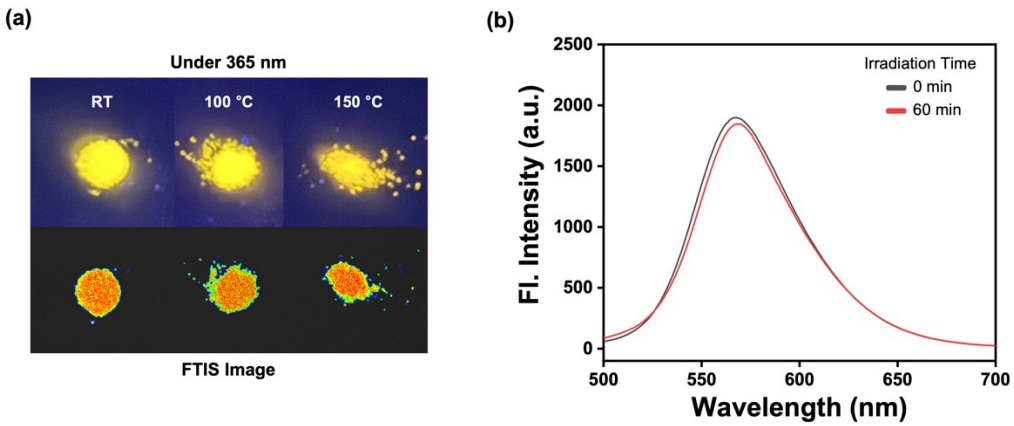

Fig. S7. (a) Fluorescence images of **SC** with temperature change. Incubation time: 1 h. Top: image at 365 nm; Bottom: FTIS image. The fluorescent imaging was conducted at the PE detection channel (390–490 nm excitation, 575–640 nm detection). (b) Emission spectra of **SC** under laser irradiation (50 mW/cm^2, 365 nm).

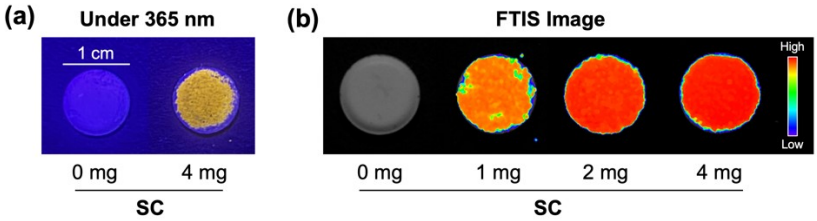

Fig. S8. (a) Image of a tablet with **SC** attached to the surface under 365 nm. (b) Fluorescence images of a tablet with **SC** attached to the surface. The fluorescent imaging was conducted at the PE detection channel (390–490 nm excitation, 575–640 nm detection).

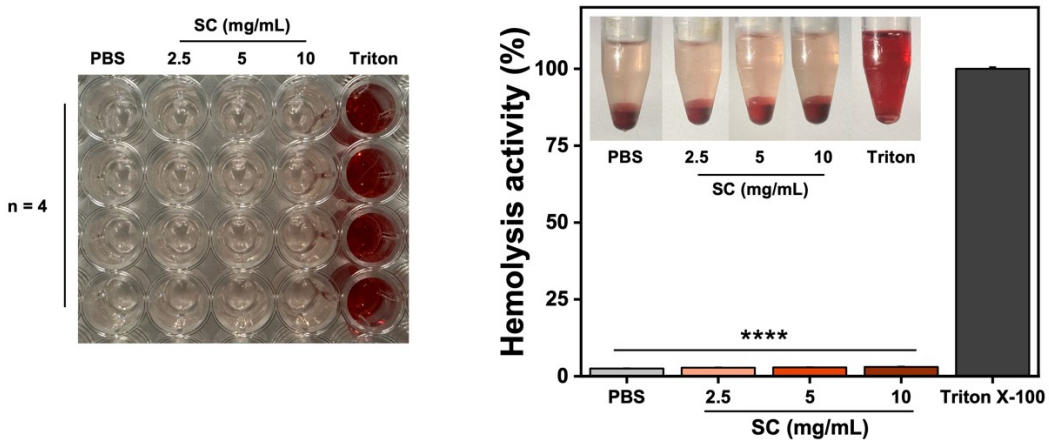

Fig. S9. Hemolysis analysis results of **SC**. The data are shown as the means ± S.E.M. (n = 4). ****<0.001 compared to the Triton X-100 group.

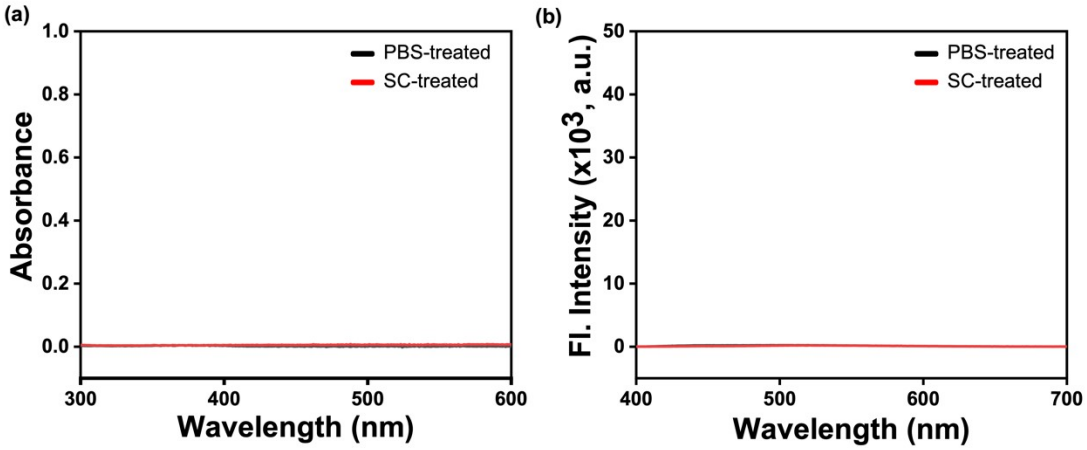

Fig. S10. (a) UV/vis and (b) emission spectra of urine from mice. Urine was collected 2 to 4 hours after PBS and **SC** administration.

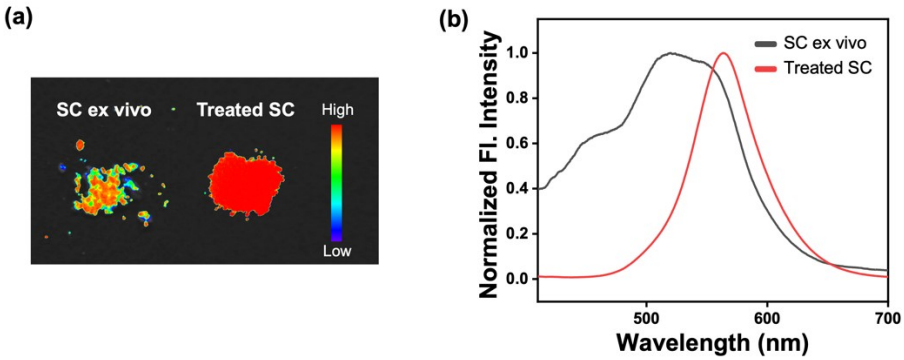

Fig. S11. (a) Fluorescence images of **SC** metabolites obtained from the sigmoid colon of the mice and treated **SC**. The fluorescent imaging was conducted at the PE detection channel (390–490 nm excitation, 575–640 nm detection). (b) Emission spectra of **SC** metabolites obtained from the sigmoid colon of the mice and treated **SC**.

3. Supporting Tables

Table S1. Simulated digestion medium composition.

(unit: mM)

Constituent	Simulated Salivary Fluid	Simulated Gastric Fluid	Simulated Intestinal Fluid
KCl	15.1	6.9	6.8
KH_2PO_4	3.7	0.9	0.8
$NaHCO_3$	13.6	25.0	85.0
NaCl	-	47.2	38.4
$MgCl_2(H_2O)_6$	0.15	0.12	0.33
$(NH_4)_2CO_3$	0.06	0.5	-
HCl	1.1	15.6	8.4