Pluronic Nanoparticle-Modified Modular Bacterial Robots for Therapy of Tumors and Inflammatory Bowel Disease

Jinghao Fan, Lin Huang, Mingkang Liu, Hongmei Liu*, and Decheng Wu*

Guangdong Provincial Key Laboratory of Advanced Biomaterials, Department of Biomedical Engineering, Southern University of Science and Technology, Shenzhen, 518055, People's Republic of China.

E-mail: liuhm@sustech.edu.cn; wudc@sustech.edu.cn

1.1. Materials

Pluronic F127 (PF127) and pyrene were purchased from Sigma-Aldrich (USA). Dess–Martin periodinane, deuterated chloroform ($CDCl_3$), chloroform, puerarin, doxorubicin hydrochloride (Dox), Cy5.5, and rhodamine B were obtained from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Dextran sulfate sodium salt (DSS, molecular weight 36,000–50,000 Da) was purchased from MP Biomedicals (USA).

1.2. Cell Lines and Bacterial Strains

4T1 cell line was cultured in RPMI 1640 containing 5% fetal bovine serum and 1% penicillin/streptomycin. The EcN was a gift from Prof. Yilan Liu (University of Waterloo). *Bifi.* was purchased from China Center of Industrial Culture Collection (Beijing, China). EcN was cultured in LB medium, while *Bifi.* was cultured anaerobically in TPY broth.

1.3. Synthesis and Characterization of PF127-CHO

PF127-CHO was synthesized in one step from PF127. PEO-PPO-PEO triblock copolymer PF127 (0.0390 mmol) was dissolved in dichloromethane, and Dess-Martin periodinane (0.0390 mmol) was added. The reaction was carried out at room temperature under nitrogen and in the dark for 24 h. After the reaction was completed, the mixture was cooled to room temperature and filtered 3-4 times to remove the excess Dess-Martin periodinane. Then, the reaction solution was concentrated under reduced pressure to a viscous state. The viscous solution was dropped into stirring n-hexane at room temperature and stirred. The mixture was centrifuged 3-5 times until the supernatant was clear. The precipitate was collected and dried in a vacuum drying oven overnight to obtain the aldehyde-modified PEO-PPO-PEO triblock copolymer, which was in the form of a white powder.

The infrared spectra of PF127 and PF127-CHO were tested using an iS20 Fourier Transform Infrared Spectrometer (Thermo Fisher, USA). Additionally, the samples were dissolved in deuterated chloroform (final concentration 10 mg/mL) and the hydrogen nuclear magnetic resonance spectra (^{1}H NMR) of the samples were tested using a 400 MHz nuclear magnetic resonance spectrometer (AVANCE Digital 400, Bruker, Germany).

1.4. Synthesis and Characterization of PF127-CHO micelles

PF127-CHO micelles were prepared by the thin-film hydration method. Specifically, PF127-CHO was dissolved in 5 mL of chloroform to form a homogeneous solution. The solution was then subjected to rotary evaporation under reduced pressure to obtain a thin film. Subsequently, 5 mL of phosphate-buffered saline (PBS) was added to hydrate the film at room temperature. When drug encapsulation was required, it was

performed according to the hydrophilicity or lipophilicity of the drug during the corresponding preparation steps. The preparation of PF127 micelles followed a similar procedure as that of PF127-CHO micelles.

The prepared PF127 and PF127-CHO micelles were purified by dialysis in PBS. The particle size and polydispersity index (PDI) of the micelles were measured using DLS at 25 °C with Zetasizer Nano ZS (Nano ZS90, Malvern) under the condition. The morphology of PF127-CHO micelles was observed using transmission electron microscopy (TEM). The critical micelle concentration (CMC) of micelle was experimentally determined using the pyrene fluorescence probe method.

1.5. Drug Loading and Release Experiments

PF127-CHO micelles containing 2 mg of puerarin were dissolved in 10 mL of PBS solution and transferred to a shaker for incubation at 37 °C and 90 rpm. At predetermined time points, 1 mL of supernatant was withdrawn for analysis, and an equal volume of fresh aqueous was added back to the original solution. The absorbance of the samples was measured at 250 nm using a UV spectrophotometer. The puerarin concentration and cumulative release amount were calculated, and the release profile was plotted. The release experiment for doxorubicin at different pH was performed following the same procedure, with the absorbance measured at 480 nm, corresponding to the maximum absorption of Dox.

1.6. Construction of Pluronic Nanoparticles-Modified Modular Bacterial Robots

The aqueous solution of PF127-CHO micelles was added to the *E. coli* suspension (OD600 = 1), and the mixture was shaken and incubated at 37 °C (200 rpm) for 2 h. Subsequently, the suspension was centrifuged at 5000 rpm for 3 min, and the precipitate was collected and gently resuspended in cold sterile PBS. This washing step was repeated 2–3 times to remove unbound drug components. The final bacterial robots were resuspended in PBS and used for subsequent experiments.

1.7. Efficiency Experiment of Bacterial Loading PF127-CHO Micelles

In this experiment, PF127-CHO micelles loaded with Cy5 fluorescent dye were incubated with Gram-negative bacteria *E. coli* to study their binding conditions. The experiment was analyzed using confocal microscopy (CLSM), ultraviolet spectrophotometer, and flow cytometer.

CLSM imaging: The constructed bacterial robots were obtained, and 10 mL of bacterial liquid was aspirated into the confocal microplate. Finally, the Cy5 fluorescence on the surface of the bacteria was observed through CLSM (EXmax = 646 nm, EMmax = 664 nm).

Ultraviolet spectrum analysis: The constructed bacterial robots were obtained with

incubation times of 1, 2, and 3 h. Then, 700 - 1000 mL of bacterial liquid was aspirated into the cuvette, and the ultraviolet absorption of the bacterial robots under different incubation times was detected using an ultraviolet spectrophotometer.

Flow cytometer analysis: The constructed bacterial robots were obtained with incubation times of 1, 2, and 3 h. After collecting the bacteria, the binding efficiency of PF127-CHO micelles to *E. coli* was analyzed using a flow cytometer.

1.8. The Universal Experiment of Pluronic Nanoparticles-Modified Modular Bacterial Robots

In this part of the research, the types of bacteria and encapsulated drugs were expanded to investigate the universality of pluronic nanoparticle-modified modular bacterial robots. In addition to Gram-negative bacteria, the capability of Gram-positive bacteria to load PF127-CHO micelles was also examined. Furthermore, hydrophobic rhodamine B was encapsulated within PF127-CHO micelles. The specific experimental procedures are detailed in the section titled "**Construction of pluronic nanoparticle-modified modular bacterial robots**".

1.9. Activity Detection of Pluronic Nanoparticles-Modified Modular Bacterial Robots

The aqueous solution of PF127-CHO micelles was added to the *E. coli* suspension (OD600 = 1) and the mixture was shaken and incubated at 37 °C (200 rpm). After incubation, the bacterial suspension was diluted at a volume ratio of 1:100 (v/v) into fresh LB medium, and the OD600 values were recorded every 10 min using a microplate reader. To determine the number of bacteria, the suspension was further diluted and inoculated onto LB agar plates, which were incubated at 37 °C overnight. Colony-forming units (CFU) were counted the following day.

1.10. In Vivo Anti-Tumor Treatment Experiment

To evaluate the in vivo anti-tumor efficacy of pluronic nanoparticles-modified modular bacterial robots, a 4T1 breast cancer model of BALB/c mice was used in this experiment. The evaluation of anti-tumor efficacy will be conducted through the following indicators: changes in tumor size and weight, as well as histological analysis.

The 4T1 tumor-bearing BALB/c mice (with tumor volume ranging from 50 to 100 mm^3) were randomly divided into four groups, with three mice in each group. Mice were intratumorally injected with Dox, EcN, or EcN loaded with Dox drugs (EcN + PF127-CHO@Dox). The injections were given once every other day for a total of three times. During the treatment process, the tumor volume (tumor volume was calculated using the formula $V = (\text{length} \times \text{width}^2)/2$) was measured to detect the growth of the tumor. Additionally, the weight of the mice was recorded every other day to assess the

safety and side effects of the treatment. After the 14th day of treatment, the mice were euthanized, and the tumors were dissected and collected. The tumor size and weight were measured. The effects of different formulations on tumor growth were analyzed and compared. Finally, the collected tumor tissues were used for histological analysis.

1.11. In Vitro Deep Penetration of Bacterial Robots in Tumor Spheroids

The tumor spheroids were incubated with the medium containing free Dox, Dox@PF127-CHO, and bacterial robots at a Dox concentration of 100 μM for 8 h, respectively. After washing with PBS, the tumor spheroids were placed in cavity microscope slides. The images of the tumor spheroids were captured by LSM980 (Zeiss) using Z-stack imaging with 25 μm intervals from the top of the spheroid to the middle by CLSM.

1.12. Biosafety and Toxicity Profile Evaluation of Pluronic Nanoparticles-Modified Modular Bacterial Robots

After treatment with PBS and pluronic nanoparticles-modified modular bacterial robots, the major organs (heart, liver, spleen, lung, and kidney) of BALB/c mice were collected at day 14 and preserved for histological assay. Meanwhile, the whole blood and serum were also collected for the blood biochemistry analysis and the blood routine analysis.

1.13. Bacterial Viability of Bacterial Robots

Equal amounts of EcN (1×10^8 CFU) and bacterial robots (containing 1×10^8 CFU BL and 5 mg PF127-CHO) were incubated in 1 mL medium supplemented with either SGF (pH 1.2), SIF (pH 6.8) or SICF (pH 7.8) and incubated at 37 °C with gentle shaking to investigate their stabilities in the GI environment. At predetermined time points, bacterial viability of both was measured. For the determination of bacterial viability, 50 μL of each diluted sample was taken and spread on LB plates. The colonies were counted after 24 h incubation at 37 °C.

1.14. DSS-induced IBD of Mice

Female Balb/C mice aged 6−8 weeks were randomly divided into five groups: the healthy group, the DSS group, the puerarin group, the *Bifi.* group, and the *Bifi.* + puerarin@PF127-CHO group after one week acclimatization. Mice received 3% DSS supplemented in drinking water for 7 days to induce the IBD model, followed by normal water. Healthy mice were provided with normal water only. And then, mice were orally continuously administered with puerarin (25 mg/kg), *Bifi.* (1×10^7 CFU) and *Bifi.* + puerarin@PF127-CHO groups (equivalent dose of puerarin and *Bifi.*) of the respective bacteria for 4 days. During the experiment, the body weight of each mouse was measured and recorded daily. On day 12, all mice were euthanized, and their colons

were collected for measurement and histological analysis. Histopathological evaluation of colon tissue based on neutrophil and monocyte infiltration (0−3), epithelial structural damage (0−3), muscle thickening (0−3), goblet cell loss (0−1), and crypt structure loss (0−1).

1.15. Gut Microbiota 16S Sequencing Assay

To investigate the changes in gut microbiome, fecal samples from different treatment groups were collected on the final day of the experiment. These samples were immediately frozen in liquid nitrogen and subsequently stored until sequencing. DNA was extracted from the fecal samples using the E.Z.N.A.™ Mag-Bind Soil DNA Kit (OMEGA). The bacterial 16S rRNA gene (V3-V4 region) was amplified using the primers pairs 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3') with an ETC 811 PCR thermocycler (EASTWIN). The microbial composition was then determined on the Illumina MiSeq platform. Operational taxonomic units (OTUs) were clustered using UPARSE (version 7.1), and taxonomic classification of the representative OTU sequences was performed using the RDP Classifier.

1.16. Statistics Analysis

Statistical analysis was performed using GraphPad Prism 9.0. All values and error bars were calculated by expressing the mean ± S.D.. One-way analysis of variance (ANOVA) or unpaired two-tailed t-tests were used to analyze data. $P < 0.05$ was considered significant (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

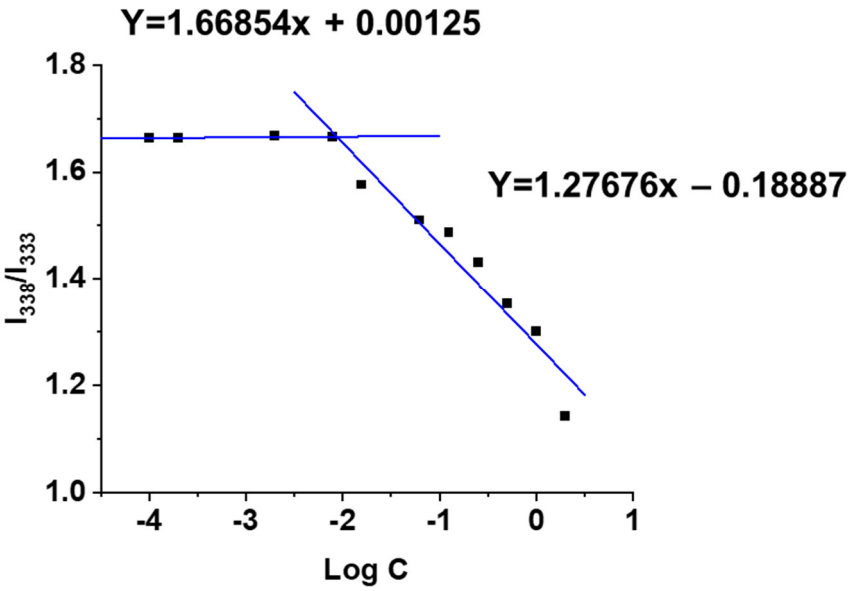

Figure S1. CMC of PF127-CHO.

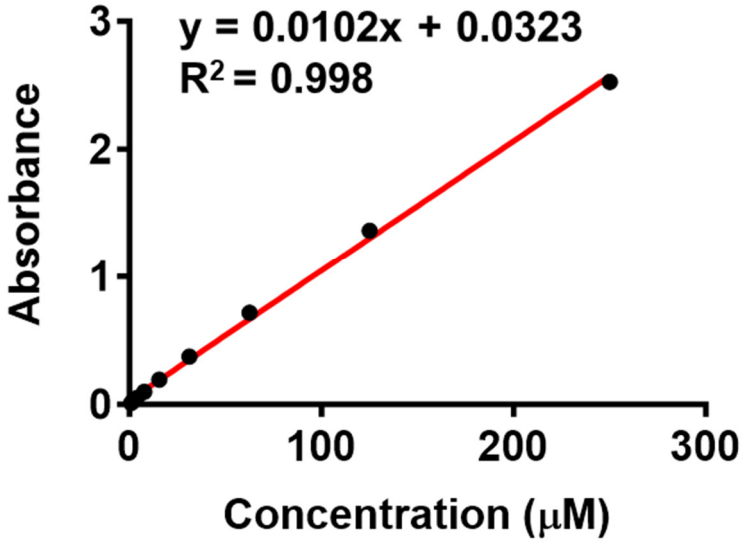

Figure S2. The standard curve of Dox.

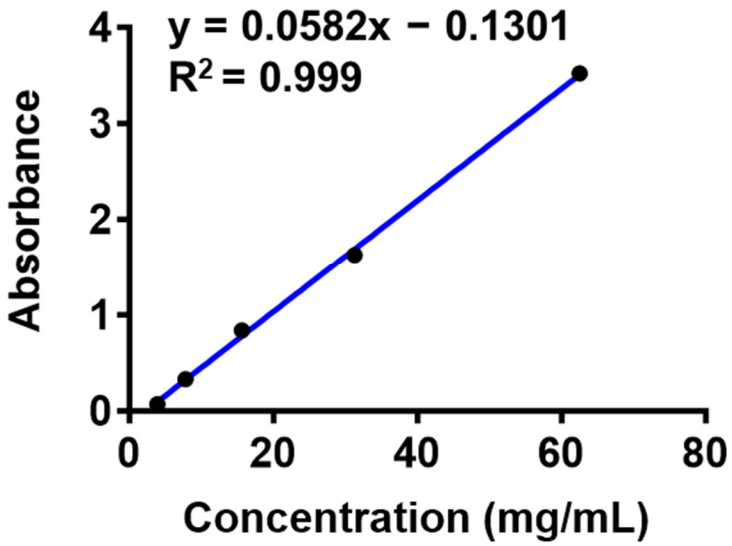

Figure S3. The standard curve of puerarin.

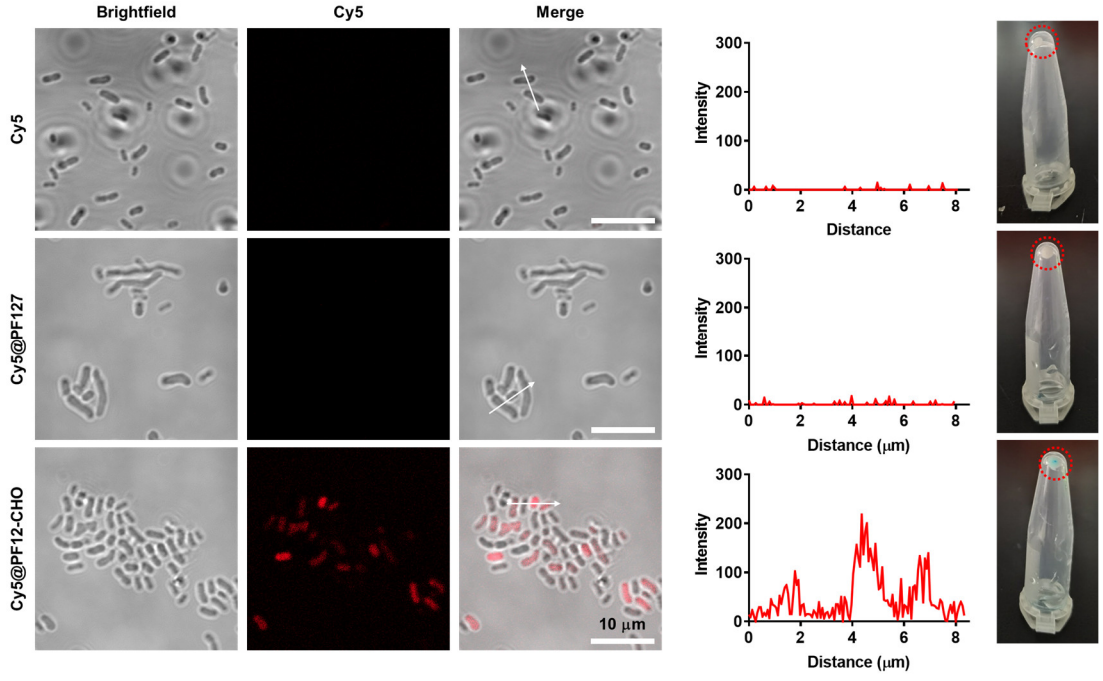

Figure S4. Representative confocal images and the corresponding intensity profile analysis of *E.coli* with different treatments. The white arrows show the corresponding intensity profile analysis. The red channels indicate Cy5. The inserted image showed the color change of the bacterial precipitates after different treatments. Scale bar, 10 μm.

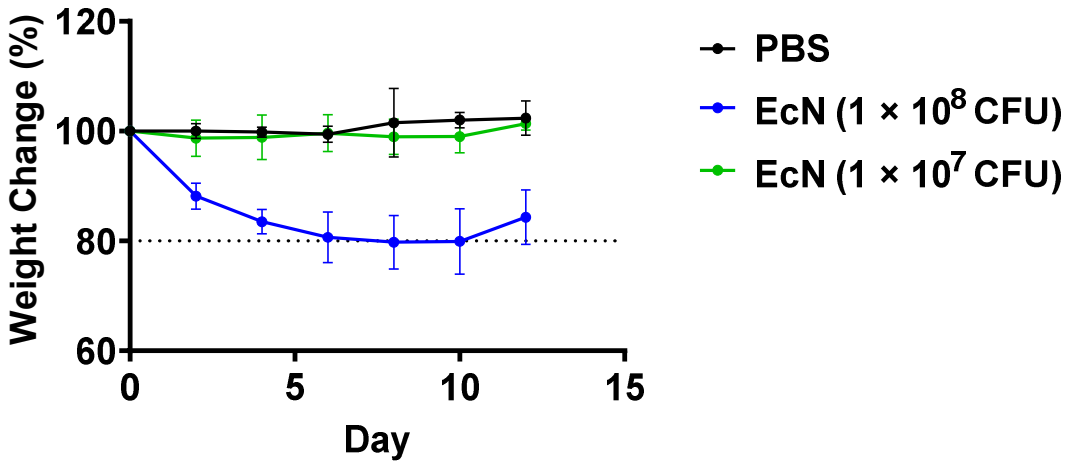

Figure S5. Body weight change of mice during treatment with various doses of EcN. The data represent as mean ± S.D. (n = 5).

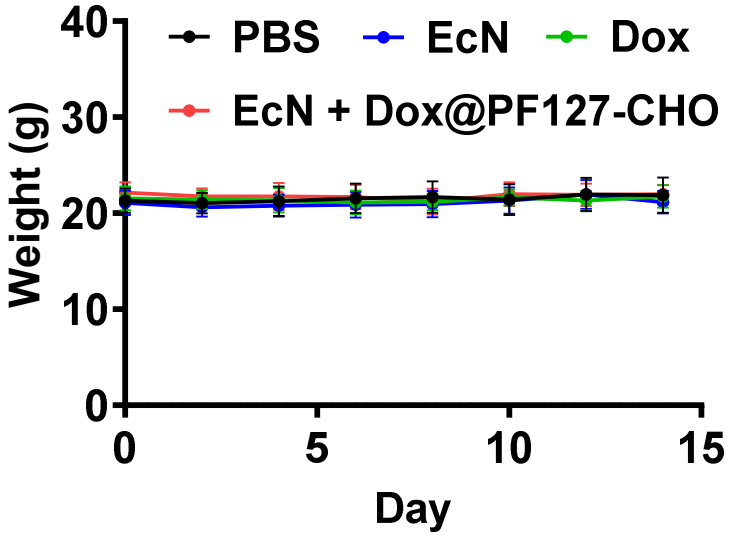

Figure S6. Body weight after different treatments during 14 days. The data represent as mean ± S.D. (n = 5).

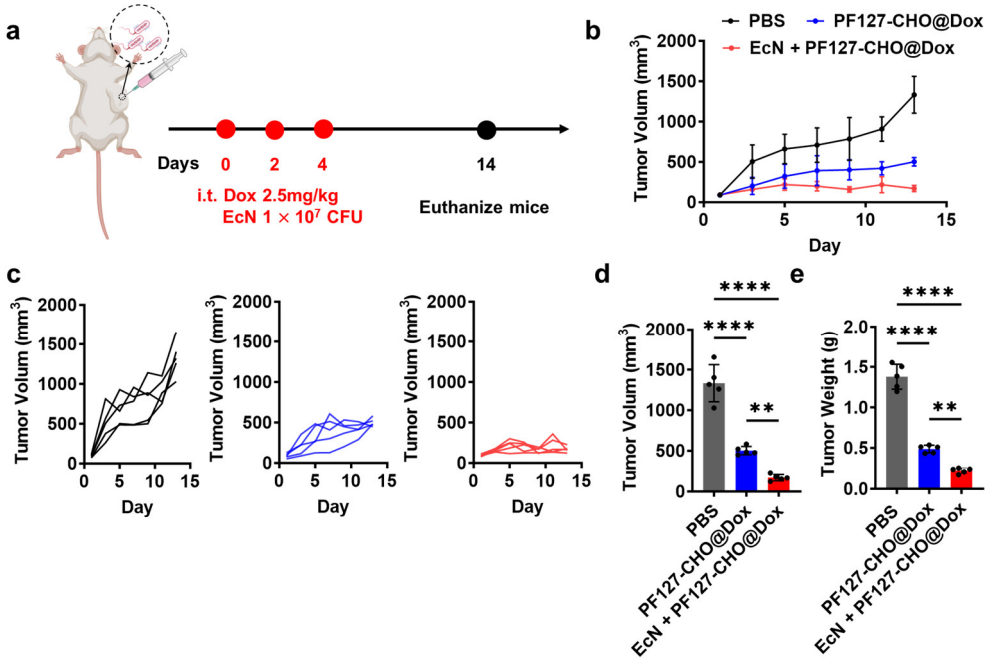

Figure S7. (a) Schematic illustration of the treatment schedule. (b) Summary of tumor growth curves in each group (n = 5). (c) Growth curves of tumors of individual mice (n = 5). Statistical graph of (d) tumor volume and (e) tumor weight at day 14 (n = 5). The data represent as mean ± S.D.. Statistical significance was determined by ordinary one-way ANOVA with Bonferroni multiple comparisons test. (**$p < 0.01$; **** $p < 0.0001$).

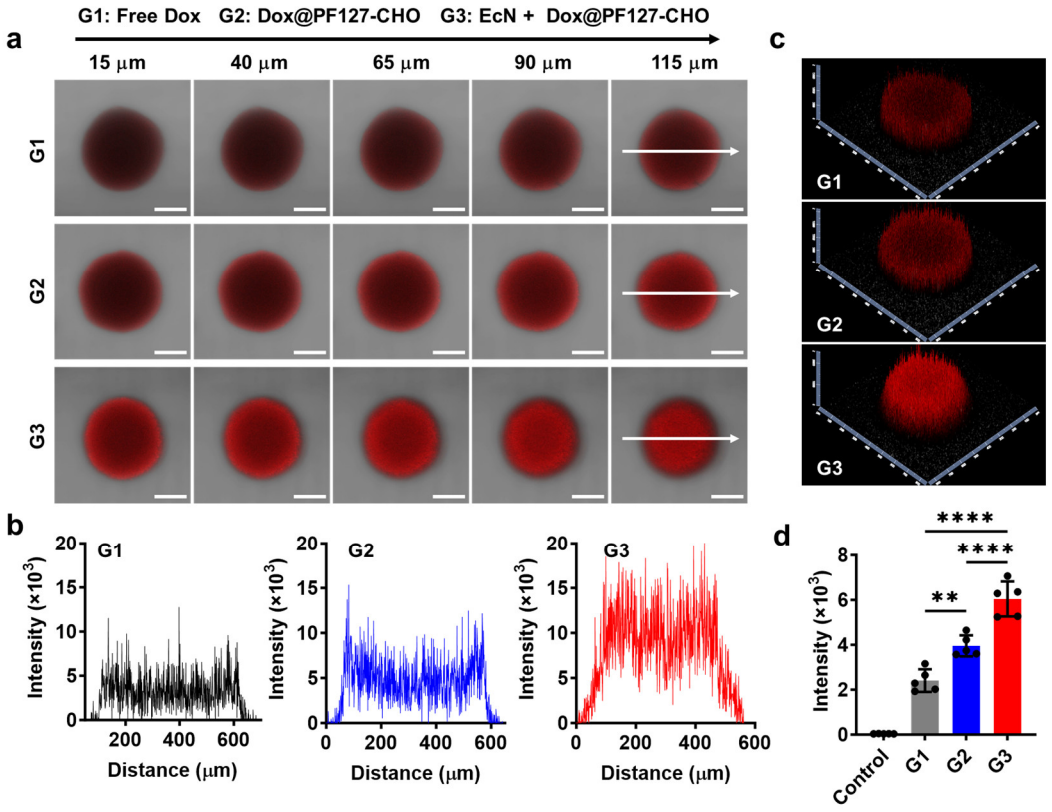

Figure S8. (a) The confocal images of 4T1 tumor spheroids with a multilevel scan started at the top of tumor spheroid in 25 mm intervals. Scale bar = 200 mm. (b) The corresponding intensity profile analysis of 4T1 spheroids. The white arrows show the corresponding intensity profile analysis. (c) Surface plot images of 4T1 spheroids with different treatments. (d) The Dox accumulation level of the tumor spheroids was quantified by the mean fluorescence intensity of Dox (n = 5). The data represent as mean ± S.D.. Statistical significance was determined by ordinary one-way ANOVA with Bonferroni multiple comparisons test. (**$p < 0.01$; **** $p < 0.0001$).

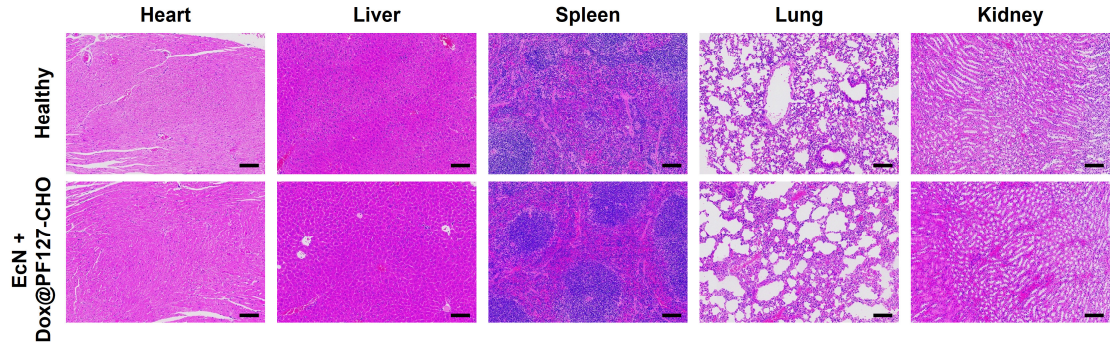

Figure S9. H&E staining of the main organs (heart, liver, spleen, lung, and kidneys) after different treatments in day 14. Scale bar, 100 μm.

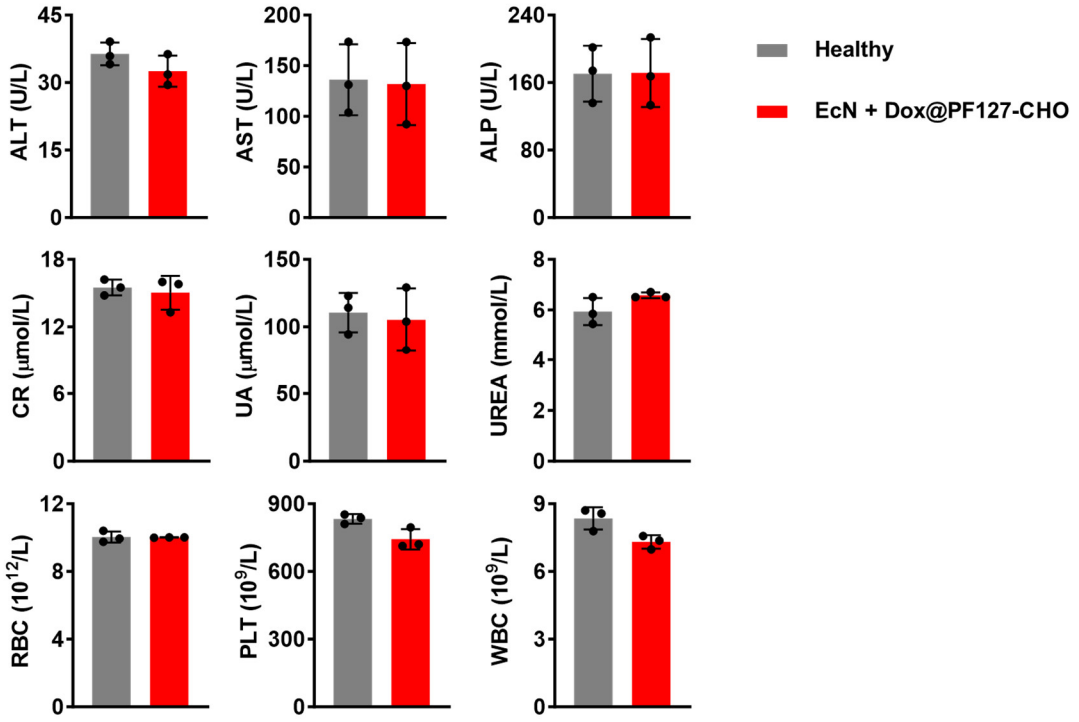

Figure S10. Blood biochemistry and routine tests after different treatments in day 14. The data represent as mean ± S.D. (n = 3).

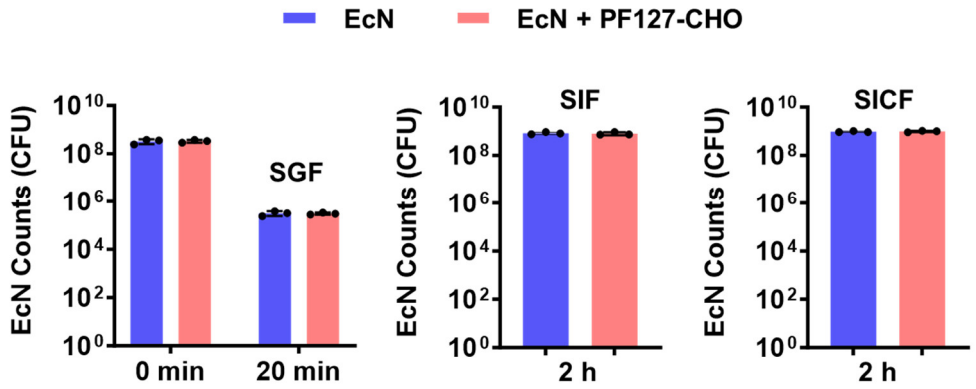

Figure S11. Corresponding viability of EcN in SGF, SIF and SICF. The data represent as mean ± S.D. (n = 3).

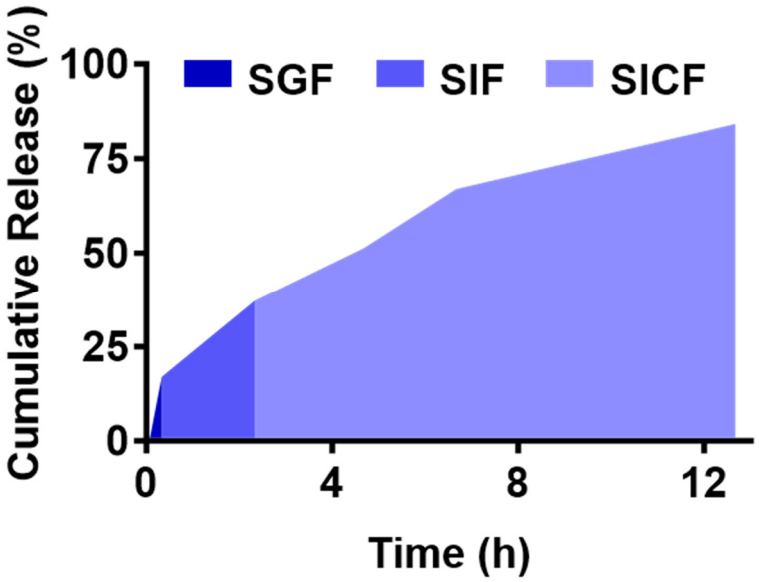

Figure S12. The release behavior of Puerarin@PF127-CHO in SGF, SIF, and SICF at 37 °C. The data represent as mean ± S.D. (n = 3).

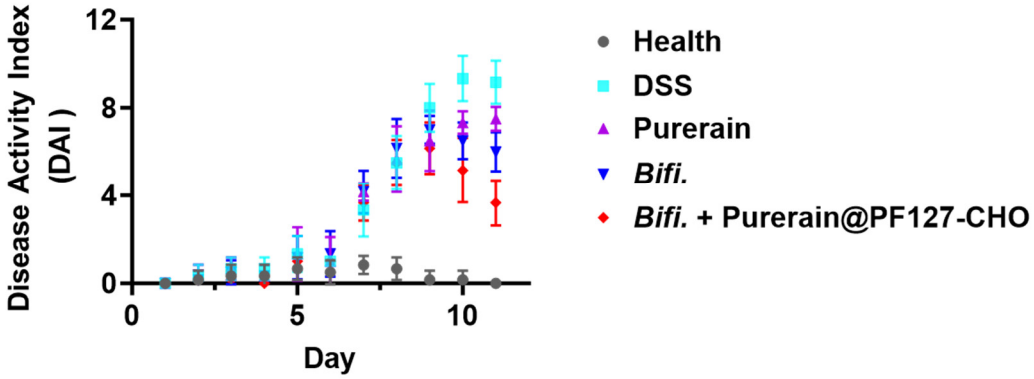

Figure S13. Changes in DAI for 11 days, which is the summation of the stool consistency index (0–3), faecal bleeding index (0–3) and weight loss index (0–4). The data represent as mean ± S.D. (n = 6).

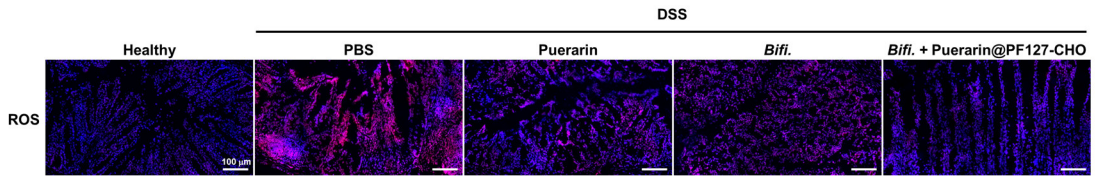

Figure S14. Representative ROS staining of colonic sections of colons of mice on day 12 after the indicated treatments. Scale bar: 100 μm.

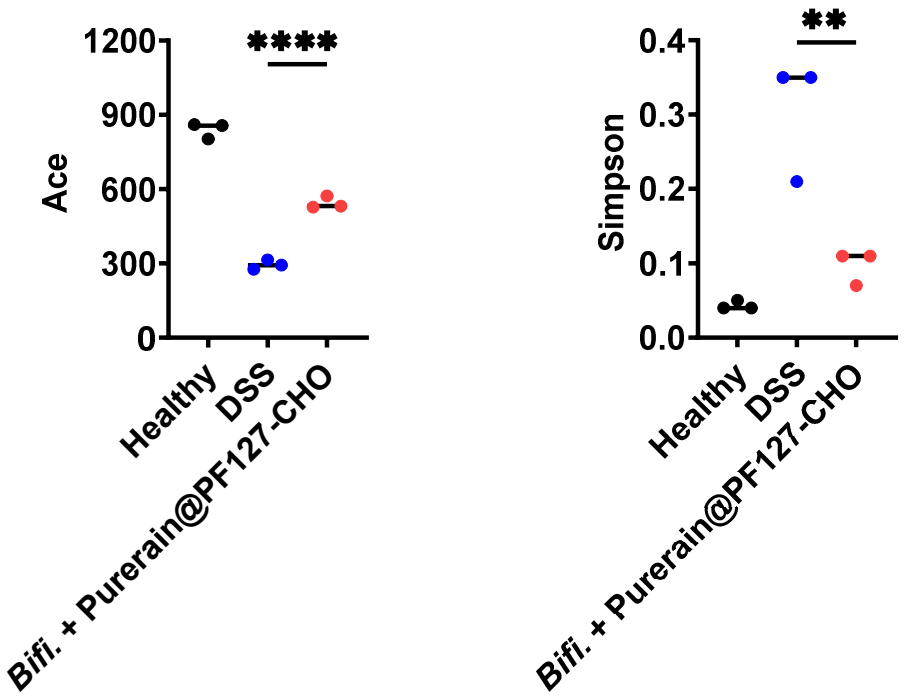

Figure S15. Alpha diversity displayed by Ace and Simpson index. The data represent as mean ± S.D. (n = 3).