Electronic Supplementary Material (ESI) for Nanoscale.

Supporting Information

Biomimetic Nanocomposite Made of Ginger-derived Exosome and Inorganic-framework for high-performance Delivery of Oral Antibody

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Materials and methods

Materials and reagents

Tetraethyl orthosilicate (TEOS), Fluorescein iso-thiocyanate (FITC), Tetramethylrhodamine (TRITC), Sulfo-Cyanine7 (Cy7) and L-lysine and 3-amino-propyltriethoxysilane (APTES) was purchased from Aladdin (Shanghai, China). DMEM (High Glucose, Gibco; Thermo Fisher Scientific, Inc.). FBS (Bioind Fetal Bovine Serum). INF was purchased from BioChemPartner (Shanghai, China). TNF-a ELISA kit was purchased Adanti Biotechnology Co., Ltd. All transport inhibitors were purchased from Dalian Meilun Biotechnology Co., LTD. All organelle probes were purchased from Beyotime Biotechnology Co., LTD. Transwell (12 mm diameter, 0.4 μm pore size, Costar®, Corning Costar Co., Cambridge, MA, USA). All chemicals for HPLC assay were of analytical grade and purchased from Shandong Yuwang industrial limited. Other chemicals were all analytical as required.

Isolation and purification of GEs

Gingers were washed three times, and after final washing, the gingers with skin removed were juiced in a cold room, and the collected juice was diluted with PBS, and then differentially centrifuged (3000 g for 30 min, 13000 g for 30 min, 17000 g for 60 min) at 4°C. For purification of GEs, a discontinuous sucrose gradient (8%, 30% and 60% [g/v]) was loaded to make a cushion. The suspension was transferred to the topside of sucrose cushion carefully without disturbing the interface and ultracentrifuged at 100,000 × g for 1.5 h, 4°C.The band between 8/30% layers was harvested. and then the precipitate was suspended with PBS to achieve GEs. The concentration of GEs was determined by detecting protein concentration using BCA protein quantification assay kit. The verification of GEs was confirmed by SDS-Page. The morphology of GEs was evaluated using a Hitachi HC-1 electron microscope through a conventional procedure[3]. The GEs were stored at -20°C for later use.

Synthesis and characterization of LMSN

Pre-LMSN was synthesized according to the previous method [1]. In a typical procedure, 0.5 g of CTAB was dissolved in an emulsion system composed of 70 mL of H_2O, 0.8 mL of aqueous ammonia (NH_4OH, 30%), 20 mL of ethyl ether and 10 mL of ethanol. After the mixture was vigorously stirred with a magnetic stirring rate of 1000 rpm for 0.5 h at 4°C, 2.5 mL of TEOS was quickly poured into the abovementioned mixture. The resulting mixture was vigorously stirred with a magnetic stirring rate of 1000 rpm at 4 °C for 4 h. After that, stop the reaction and place the erlenmeyer flask at room temperature for 24 hours. A white precipitate was obtained by centrifugation at 11000 rpm for 5 min, which was washed with ethanol and pure water three times. And a subsequent CTAB template extraction was performed by treating the product in ethanolic HCl (15 mL of HCl (37%) in 120 mL of ethanol) by stirring at 70 °C for 36 h. The prepared pre-LMSNs were functionalized with aminopropyl groups by APTES and the obtained samples were named LMSNs.

Preparation of INF/LMSN

INF was used as model protein drugs. For drug loading, a protein-to-carrier ratio of 2:1 (w/w) was used to prepare protein-loaded LMSN nanoparticles. Drug loading was performed by dispersing 5-mg LMSNs in protein solution (10-mg protein in 20-mL phosphate buffer solution (PBS), pH6.8) and stirring gently at 4 ℃ for 4 h. At suitable time intervals, samples of the suspension were centrifuged at 4500g for 5 min. The amount of protein was determined by measuring the amount of supernatant solution removed from the sample [2]. INF content of the supernatant was calculated using a standard calibration curve. And the drug loading efficiency (%) was calculated by the equation as below:

$$loading\ efficiency\ (\%) = \frac{INF_{total} - INF_{free}}{LMSN_{total} + INF_{total} - INF_{free}}$$

Preparation and characterization of INF/LMSN@GE

INF/LMSN@GE was synthesized according to the previous method [3]. The isolated GEs and INF/LMSN were mixed uniformly in a volume ratio of 1:1~3 (w/w). The mixture was extruded through a Micro-extruder (Avanti, 610007-1Ea PC Membranes 0.4μm) at least 11 times. After that, the mixture was centrifugated (12000 g for 10 min) at 4°C to remove the empty GEs. The sediments were washed in a cold PBS and centrifuged once more. The pellets (INF/LMSN@GEs) were resuspended in PBS and stored at -80°C for use. The zeta potential and particle size of LMSN@GE were measured by by a Particle Size Analyzer Nicomp 380 (Particle sizing systems, USA), and the morphology of LMSN@GE was observed by TEM. The verification of LMSN@GE was confirmed by SDS-Page (supported by Servicebio Biotechnology Co., Ltd.)

Fluorescent labeling of INF, LMSN and GEs

TRITC-labeled INF (T-INF) was first synthesized based on the reaction between the primary amino groups of INF and the isothiocyanate groups of TRITC as previously reported [4]. Cy7 labeled INF was prepared with the same method using TRITC-labeled INF.

5 μg of FITC were suspended in 3 mL of ethanol. After addition of 20 mg of LMSNs, the suspension was stirred for 24 h at 25°C. And FITC-labeled LMSNs (FITC-L MSNs) were collected by centrifugation at 11000 rpm for 5 min, which was washed with ethanol and pure water three times to remove the residual FITC molecules. Other fluorescent dyes (Dir, Did) were diluted in 1mL PBS, then the fluorescent-labeled GEs were prepared with the same method using FITC-labeled LMSN.

Fluorescence resonance energy transfer (FRET) analysis

To further investigate the interaction between LMSN and GE, FRET analysis was performed. FITC and TRITC were used as FRET pairs. TRITC labeled GE (T-GE) and FITC labeled LMSN (F-LMSN) were prepared using the procedure described above.

The fluorescence intensity was measured through a fluorescence spectrophotometer (Shimadzu RF-5301, Japan) with an excitation wavelength of 440 nm and the emission spectrum was recorded from 500 to 600 nm. FRET efficiency (E) and the distance between the donor and acceptor (R) were calculated as following equations:

$$E = 1 - \frac{F_{DA}}{F_D}$$

$$R = R_0 - \sqrt[6]{\frac{1}{E} - 1}$$

where F_{DA} is the intensity in the presence of the acceptor, F_D is the intensity in the absence of the acceptor, and R_0 is the FOrster distance at 50% transfer efficiency. For FITC-TRITC, R_0 is 55 nm [5].

Stability of nanoparticles

The *in vitro* stability of GEs in simulated gastric fluid (SGF, pH 1.2) and simulated intestinal fluid (SIF, pH 7.4) was evaluated. SGF was prepared 2 with pepsin (1%), 2.0g/L NaCl, and 7ml/L concentrated HCl at a pH of 1.2 to simulate the stomach conditions. SIF was prepared with pancreatin (10%) pH7.2, which consist of 0.1mol/L NaH2PO4 50ml and 0.1mol/L NaOH 42.5ml for 1L of solution to simulate intestine conditions. SGF and SIF stock solution was filtrated through a 0.22μm Millipore filtration membrane before use. To perform the stability study, GEs were transferred into proper medium and incubated at 37°C. At selected time points, the stability of GEs was evaluated by testing the particle size distribution, zeta potential and TEM image.

In vitro drug release

The release profile of INF from LMSN and LMSN@GE was performed in SGF and SIF. At selected time points, samples were centrifuged (10000 rpm for 1 min) at 4°C，the supernatant was taken out for ELISA analysis and replenished with an equal volume of fresh buffer. INF concentrations were calculated according to standard curves at the corresponding buffer solutions.

Conformational stability of INF

Circular dichroism (CD) spectroscopy (Bio-Logic MOS 450, France) was used to measure the secondary conformational changes of the INF loaded in, and released from LMSNs with respect to the native ones. Samples were diluted so that they contained 0.2 mg/mL protein with PBS (pH = 6.8) prior to testing. Spectra were collected from 190 to 260 nm with a resolution of 1 nm at 25°C. A baseline correction with the PBS used was conducted.

Cytotoxicity assay

HT29 cells were obtained from the Cell Bank in Shanghai Institute of Cell Biology, China. The cells were cultured at 37 °C under a humidified 5% CO_2 in Dulbecco's

Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% antibiotic solution (Gibco, Invitrogen, USA). The cytotoxicity of GE, LMSN and LMSN@GE was analyzed on HT29 cells by MTT assay. The cells were seeded in 96-well plate with a density of 10^4 cells per well and incubated overnight at 37 °C. NPs with different concentrations were added into the wells and incubated with the cells for another 24 hours. After that, the cell viability was tested by MTT assay according to the protocol.

Cellular uptake

HT29 cells and 3T3 cells were seeded in six-well plates (3×10^5 cells in 2 mL medium per well) and incubated for 24 h. Then FITC-LMSN and FITC-LMSN@GE (with FITC-LMSN concentration 200 $ng \cdot mL^{-1}$) were added to the well and incubated for 4 h at 37°C, respectively. Consequently, the cells were trypsinized, washed three times with cold PBS, and resuspended in 500 μL PBS and then the FITC signal was detected by using flow cytometry (Becton Dickinson, USA). The autofluorescence of untreated cells was used as the control. The results were analyzed using FlowJo Version 7.6 software (Flowjo, USA). All the experiments were performed in triplicate and the result displayed a representative of three independent experiments.

2×10^4 HT29 cells were seeded in 24-well plates with cell microscope slide for 24 h. FITC-LMSN@Dir-GE (with FITC-LMSN concentration 200 $ng \cdot mL^{-1}$) were added to cell microscope slides and incubated with HT29 cells for 4 h at 37°C. After washing with PBS, the cells were fixed with 4% paraformaldehyde for 10 min, followed by staining with Hoechst 33342. The distribution of FITC-LMSN@Dir-GE in HT29 cells was visualized using Olympus FV1000S-SIM/IX81 confocal laser scanning microscope (CLSM, Olympus, Japan).

TEM of LMSN@GE in intestinal epithelial cells

The sections of colonic tissues from colitis mice treated with LMLN@GE were imaged on a Hitachi H7650 microscope. Sections were processed as follows: firstly, fixed in 3% glutaraldehyde and 1% osmium tetroxide, then dehydrated in increasing ethanol concentrations and subsequently embedded in epoxy resin. Ultra-thin slices were cut from polymerized epoxy resin blocs, followed by 1% uranyl acetate and Reynold's lead citrate staining of the sections. The observation was supported by Servicebio Biotechnology Co., Ltd.

Colitis models

Colitis models were set up according to the previous method. All animal experiments were conducted following the National Institutes of Health guidelines for the care and use of laboratory animals and approved by the Institutional Animal Care and Ethics Committee of Shenyang Pharmaceutical University. Colitis was induced by feeding 2% DSS to female C57BL/6 mice (Liaoning Changsheng Biotechnology co., Ltd., China, 6 week, 18-22 g) for 6 d.

Biodistribution and inflamed colon targeting

The colitis mice were randomly divided into three groups and administrated with Cy7-INF (i.v.) or Cy7-INF/LMSN (p.o.) and Cy7-INF/LMSN@GE (p.o.). The dose of INF was kept the same in all groups which are 10 mg/kg. The mice were sacrificed 24 h post-administration. The tissues and organs were collected and observed under the IVIS imaging system (PerkinElmer). The images were analyzed by Living Image® software.

Colitis treatment

As shown in Figure S1, After DSS solution feeding finished, the colitis mice were randomly divided into six groups. The dose of INF was kept the same in all groups which are 10 mg/kg each day. Group (I): Healthy mice without treatment, Group (II): The DSS induced colitis mice treated with saline, Group (III): The DSS induced colitis mice treated with INF solution (i.v.), Group (IV): The DSS induced colitis mice treated with GE (p.o.), Group (V): The DSS induced colitis mice treated with INF/LMSN (p.o.), Group (VI): The DSS induced colitis mice treated with INF/LMSN@GE (p.o.), (n=7).

The body weight was recorded every day and the severity of colitis in the mice was assessed on day 16. The severity was evaluated by the DAI (0-16), including loss of body weight (1, 1-5%; 2, 5-10%; 3, 10-15%; 4, 15-20%), rectal bleeding (0, normal; 1, semi-normal; 2, positive hemoccult; 3, blood traces in stool visible; 4, gross rectal bleeding) and stool consistency (0, normal; 1, semi-normal; 2, loose stool; 3, loose stool that adhered to the anus; 4, liquid stools that adhered to the anus). Colons were collected after the sacrifice of mice and the length was measured.

H&E stain and IHC

Once the tissues were harvested from mice post-treatment, the tissues were immediately fixed in formalin for 48 h in 4 °C. Servicebio Biotechnology Co., Ltd provided the testing services.

TNF-α detection by ELISA method

RAW264.7 cells were obtained from the Cell Bank in Shanghai Institute of Cell Biology, China. The cells were cultured at 37 °C under a humidified 5% CO2 in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% antibiotic solution (Gibco, Invitrogen, USA). The cells were seeded in 24-well plate with a density of 5×10^4 cells per well and incubated overnight at 37 °C. 100 ng/mL LPS were added into the cells to activate the macrophage cells. GE, Free INF, INF/LMSN, INF/LMSN@GE and INF from SIG treated INF/LMSN and INF/LMSN @GE were used to inhibit the LPS induced TNF-α production. The INF concentration is 20 μg/mL in all groups. The supernatant of cell culture was collected and centrifuged at 5000 r/min at 4°C. The supernatants were collected and stored at -80 °C. The TNF-α was detected by ELISA kits (Adanti Biotechnology Co., Ltd.) according to its protocols.

Colons from treated mice were weighed and homogenized in cold PBS (w/v = 1:10)

with protease inhibitor cocktail for 20 min. Homogenates were centrifuged at 5000 r/min at 4°C. The supernatants were collected and stored at -80 °C. The TNF-α was detected by ELISA kits (Adanti Biotechnology Co., Ltd.) according to its protocols.

Statistical analysis

All values were presented as mean ± SD. Statistical differences were determined using unpaired Student's t test with a two-tailed P value when comparing between 2 independent groups, and one-way ANOVA analysis with Student-Newman-Keuls using SPSS program. Differences were considered significance at $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

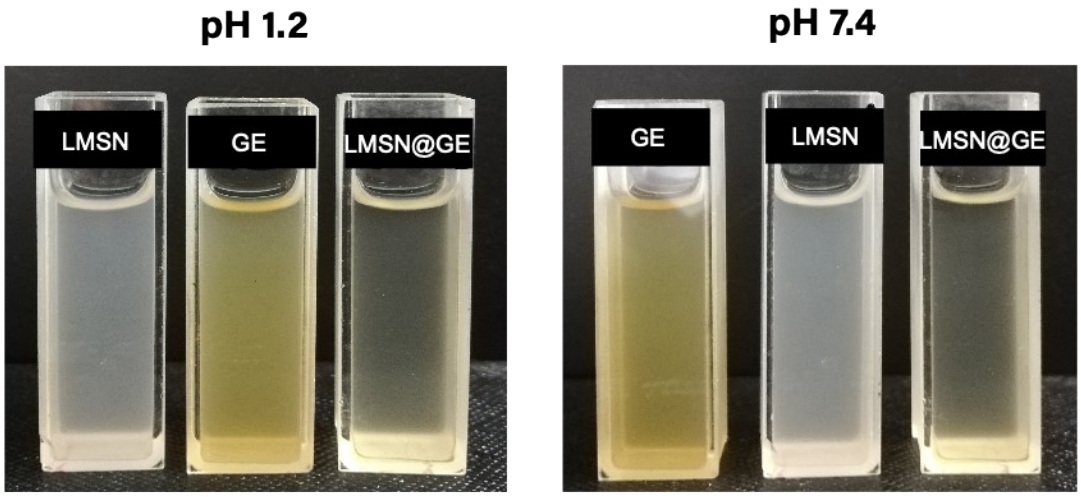

Fig. S1 The stability of NPs in SGF for 2 h and in SIF for 6 h.

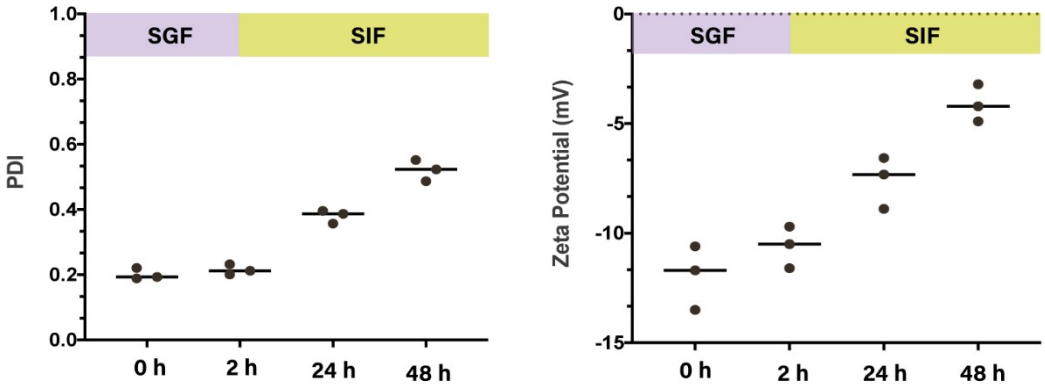

Fig. S2 The PDI and zeta potential change of GE in SGF and SIF for 48 h.

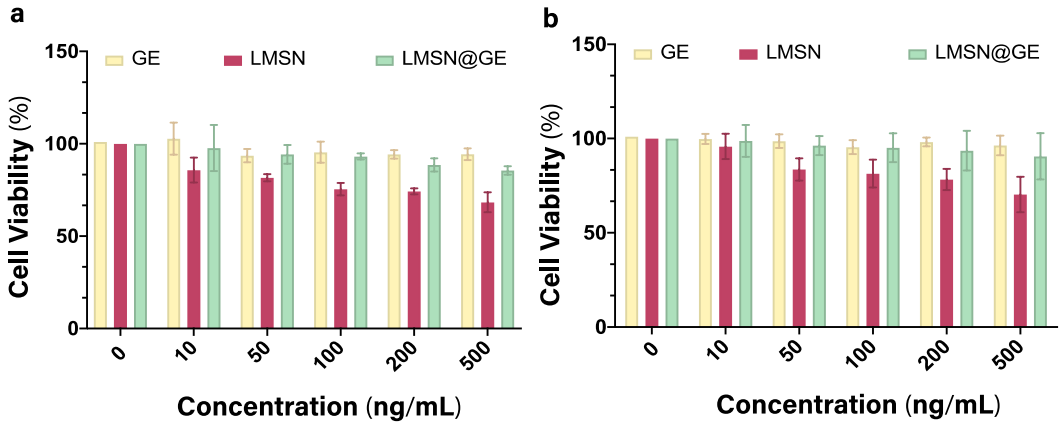

Fig. S3 Cell viability of HT29 cells (a) and Raw 264.7 cells (b) incubated with GE, LMSN and LMSN@GE for 48 h. (n=5).

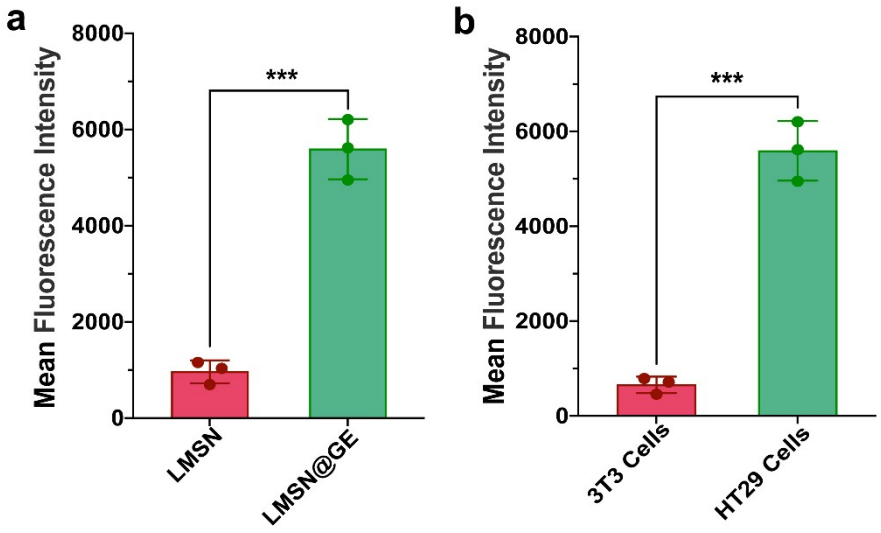

Fig. S4 (**a**) Flow cytometry analysis of the distinct cellular uptake of LMSN and LMSN@GE by HT-29 cells, LMSN was labeled by FITC. (**b**) Flow cytometry analysis of the specific uptake of LMSN@GE by HT-29 cells compared to 3T3 cells, LMSN was labeled by FITC. (n=3).

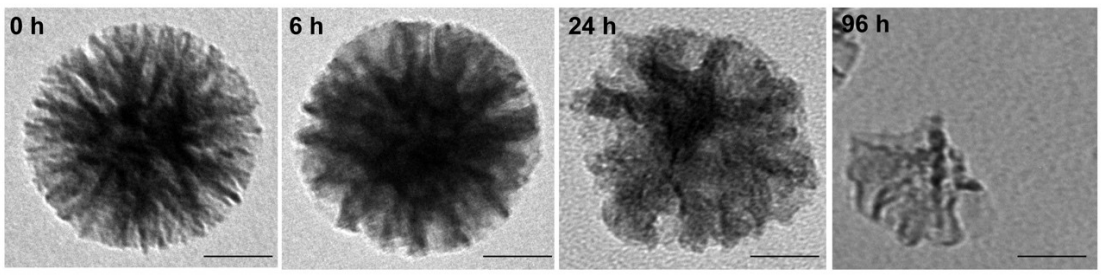

Fig. S5 The degradation rate of the LMSN by the simulated body fluid. Scale bars, 50 nm.

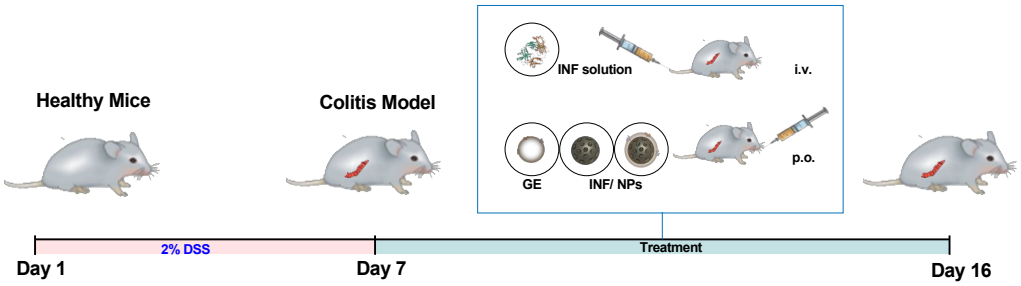

Fig. S6 Therapeutic schedule of colitis treatment.

Table S1 Structural parameters of the LMSN and the LMSN@GE.

Sample	W_{BJH}(nm)	S_{BET}(m^2/g)	V_p(cm^3/g)
LMSN	19.58	399.6	2.082
LMSN@GE	-	48.15	0.6591

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