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Phosphatidylserine-functionalized liposomes-in-microgels for delivering genistein to effectively treat ulcerative colitis

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Materials and methods.

Cell culture for *in vitro* anti-inflammation assay

The MTT method was also used to investigate the inhibitory effects of Gen or Gen@Li NPs on LPS-induced cellular inflammatory responses. RAW 264.7 macrophages were grown in 96-well plates and cultured for 24 hours. The medium was changed to serum-free medium containing Gen or Gen@Li NPs. After incubation for 24 hours, the media were discarded, and macrophages were exposed to LPS (1 μg/mL) for 24 hours. Then, the macrophages were incubated with 100 μL of MTT for 4 hours. After incubation with MTT, the medium was removed, and 50 μL dimethyl sulfoxide (DMSO) was added. Finally, the absorbance of each well was measured at 490 nm. LPS-stimulated macrophages were used for the positive control, and untreated LPS macrophages were used for the negative control.

Determination of inflammatory cytokine content and oxidative stress

RAW 264.7 macrophages were grown in 6-well plates and incubated at 37 °C for 24 hours. Then macrophages were treated with Gen and Gen@Li NPs for 24 hours. Subsequently, macrophages were treated with LPS (1μg/mL) for 24 hours. The cell supernatant was gathered and some inflammatory factors containing IL-6, IL-1β, and TNF-α were measured by ELISA kits.

The levels of reactive oxygen species (ROS), superoxide dismutase (SOD), and glutathione (GSH) in the cell were evaluated by ROS assay kit (E004-1-1, Nanjing Jiancheng Bioengineering Institute, Nanjing), SOD assay kit (WST-1 method, A001-3-2, Nanjing Jiancheng Bioengineering Institute, Nanjing), GSH assay kit (A006-2-1,

Nanjing Jiancheng Bioengineering Institute, Nanjing).

Determination of intracellular ROS level and cell apoptosis

RAW 264.7 macrophages were grown in 6-well plates and incubated at 37 °C for 24 hours. Macrophages were then exposed to Gen or Gen@Li NPs for 24 hours. Subsequently, macrophages were treated with LPS (1μg/mL). The levels of reactive oxygen species (ROS) and apoptosis in the cells were evaluated by ROS assay kit (E004-1-1, Nanjing Jiancheng Bioengineering Institute, Nanjing) and Hoechst 33342 assay kit (C1018, Beyotime Biotechnology, Shanghai).

Cellular uptake

RAW 264.7 macrophages were seeded in 6-well plates and cultured for 24 hours. After exposure to rhodamine b (Rho-B)-Gen or Gen@Li NPs for 2 hours, macrophages were thoroughly rinsed with PBS and fixed in paraformaldehyde for 20 min. Then, the nucleus of the cells was stained with DAPI for 10 min. Untreated cells were used as a negative control. Images were acquired on a fluorescent inverted microscope using the rhodamine b channel and the DAPI channel. Thereafter, we performed a fluorescence quantification experiment. Macrophages were seeded in 6-well plates at a density of 1×10^5 cells/well. Then, the complete media were replaced with Rho-B-Gen or Gen@Li NPs contained medium. At time intervals (1, 2 and 4 h), cells were rinsed thoroughly with PBS and then observed with a fluorescent inverted microscope. Relative fluorescence quantification was analyzed using Image J software, and the relative fluorescence intensity was the rhodamine B fluorescence intensity/number of cells.

Cytotoxicity of Gen or Gen@Li NPs

The cytotoxicity of Gen or Gen@Li NPs to RAW 264.7 macrophages was determined. Macrophages were cultured at density of 10^5 μL/well in 96-well plates in a 37 °C incubator for 24 h. Then, macrophages were exposed to different concentrations of Gen and Gen@Li suspensions for 24 hours. Next, macrophages were incubated with 100 μL of MTT in a 37 °C incubator for 4 hours. Subsequently, the media were discarded and 50 μL DMSO was added to each well and then measured at 490 nm.

Haemolysis analysis

Mouse blood samples were added to NaCl solution and red blood cells (RBCs) were obtained by centrifugation at 1000 rpm for 5 min. After washing with NaCl solution, RBCs were diluted with PBS solution (pH 7.4). Samples were divided into the following groups: (1) Positive control group: purified water; (2) Negative control group: PBS; (3) Gen group; (4) Gen@Li NPs group and (5) Gen@Li microgels group. The diluted RBC suspension was mixed uniformly with the above samples, and incubated at 37 °C for 3 h. After centrifugation at 1000 rpm for 5 min, optical images were taken and 100 μL of each supernatant was added into a 96-well plate. Finally, the absorbance was measured at 540 nm.

Figures captions:

Figure S1. EDS of Gen@Li NPs (i), Alg microgels (ii) and Gen@Li microgels (iii). Sample sizes are three (n=3).

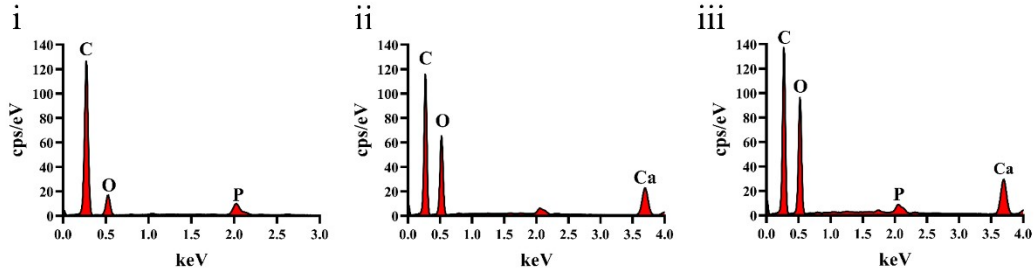
Figure S2. Determination of anti-inflammatory activities and oxidative stress level *in vitro*. Changes of (A) IL-1β, (B) IL-6, (C) TNF-α levels in RAW264.7 cells treated with Gen and Gen@Li NPs. Determination of (E) NO, (F) SOD and (G) GSH. Data were expressed as mean ± SD. Sample sizes are three (n=3).

Figure S3. Biocompatibility. (A) Cytotoxicity of Gen or Gen@Li NPs to RAW264.7 cells. (B) Hemolysis analysis. Data were expressed as mean ± SD. Sample sizes are three (n=3).

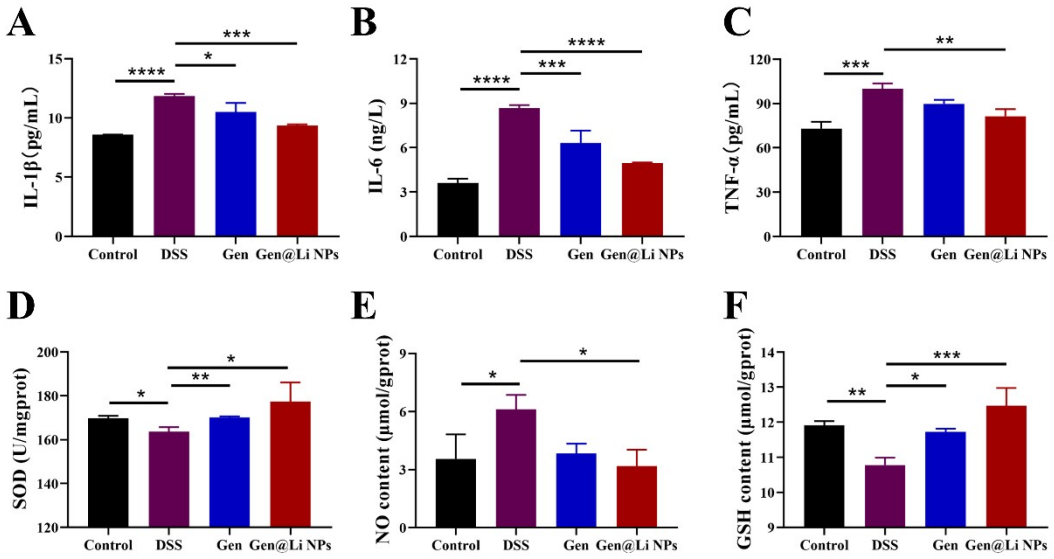
Figure S4. Intestinal permeability indicated by serum FITC-Dextran concentration. Data were expressed as mean ± SD. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Sample sizes are three (n=3).

Table S1. Characteristics of Li NPs and Gen@Li NPs. The values are mean ± SD (n=3).

Figure S1



94 **Figure S2**



95

Figure S3

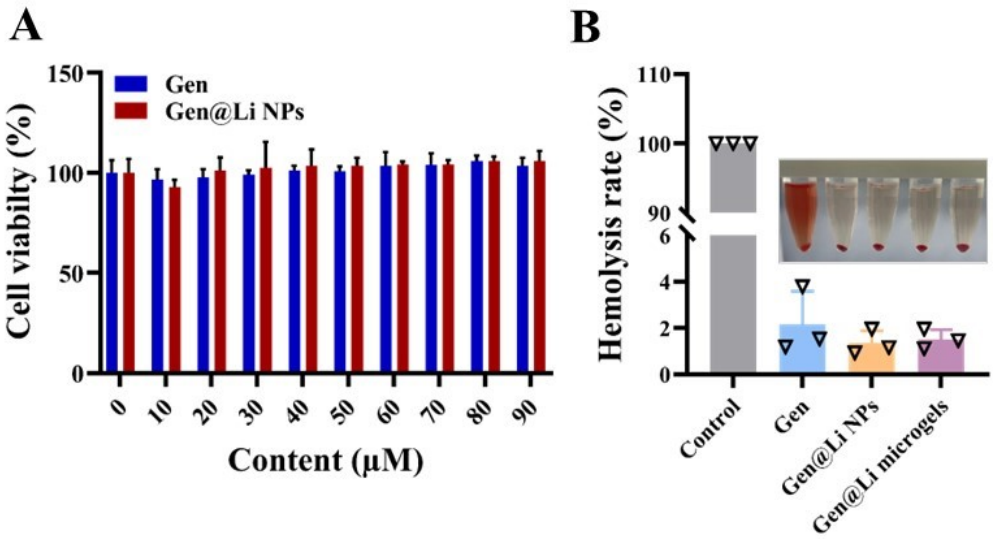
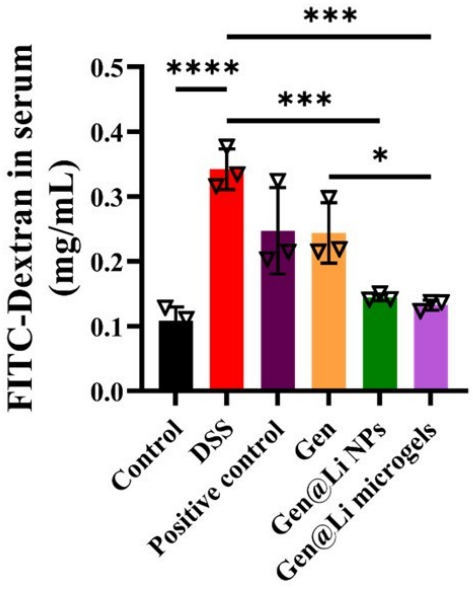

A
Gen
Gen@Li NPs
Cell viabilty (%)
150
100
50
0
0
10
20
30
40
50
60
70
80
90
Content (μM)
B
Hemolysis rate (%)
110
100
90
6
4
2
0
Control
Gen
Gen@Li NPs
Gen@Li microgels

Figure S4


FITC-Dextran in serum (mg/mL)
0.5
0.4
0.3
0.2
0.1
0.0
****
***
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*
Control
DSS
Positive control
Gen
Gen@Li NPs
Gen@Li microgels

Table S1

	Particle Size	PDI	Zeta-potential(mv)
Li NPs	159.80±1.56	0.32±0.0087	-16.33±1.16
Gen@Li NPs	245.90±9.61	0.32±0.06	-28.10±1.93