Supplementary Materials

1 Materials and methods

1.1 MTT assay

Typically, exponentially growing MCF-7 and L929 cells were seeded in 96-well plates at 1×10^4 cells/well and pre-incubated for 24 hours. Then, the cells were incubated with Rg5, Rg5-BSA NPs and FA-Rg5-BSA NPs at the Rg5-equivalent doses of 6.25, 12.5, 25, 50, and 100 μM for 24 and 48 hours. Subsequently, 50 μL of 0.5% MTT was added to each well and the cells were incubated for 4 hours at 37 °C. Next, the medium was replaced with 150 μL of DMSO. The optical density of the wells was measured at 490 nm using a microplate reader (Power Wave XS2, BioTek Instruments Inc., USA).

1.2 Staining assay

Briefly, the MCF-7 cells were cultured overnight in 6-well plates at a density of 2×10^5 cells/well in 2 mL medium. Then, Rg5, Rg5-BSA NPs and FA-Rg5-BSA NPs at 50 μM were added to the cells separately for 24 hours. Cells treated with culture medium served as control. After the supernatant was discarded and the cells were rinsed twice with PBS, the cells were stained with Hoechst 33342 solution at 10 μg/mL for 15 minutes in the dark, or with AO/EB staining solution at 2 μg/mL for 5 minutes, then washed twice again with ice cold PBS. The stained cells were observed using an inverted fluorescence microscope (Nikon, Tokyo, Japan).

1.3 Confocal laser scanning microscopy

The MCF-7 cells were seeded at a density of 4×10^4 cells/dish into a glass-bottom cell culture dish (NEST, Ø90 mm×20 mm, China) supplemented with folic acid-free medium or medium containing 20 mM FA. Then, the medium was replaced with fresh medium containing FITC-labeled Rg5-BSA NPs, FA-Rg5-BSA NPs, and FA-Rg5-BSA NPs plus 20 mM free FA (note: the cells were pretreated with FA for one hour before adding FA-Rg5-BSA NPs to investigate if FA specifically mediated cellular uptake) at the same concentration of 250 μg/mL for 3 hours at 37 °C, respectively. After that, the culture medium was aspirated, and the cells were fixed and stained with DAPI at 1 μg/mL. Then, the cells were washed and imaged by confocal laser scanning microscopy (CLSM, Olympus Fluoview FV-1000, Tokyo, Japan).

1.4 Flow cytometry

Typically, the MCF-7 cells were cultured in 6-well plates, grown overnight and then incubated with free FITC, FITC-labeled Rg5-BSA NPs, FA-Rg5-BSA NPs, and FA-Rg5-BSA NPs plus 20 mM free FA with an equivalent FITC concentration of 250 μg/mL for 3 hours at 37 °C. The untreated cells served as control. After incubation, the suspension was removed and the wells were washed with PBS to remove the NPs outside the cells. Subsequently, the samples were trypsinized and harvested to obtain a cell suspension, and then resuspended in 200 μL PBS for flow cytometry analysis.

1.5 Human breast cancer xenograft mouse model

Five-week-old female BALB/c nude mice (16±2 g) were purchased from Hunan SJA Lab Animal

Co., Ltd. (Hunan, China). They were humanely cared for with free water and food. Procedures involving animals and their care were conducted in accordance with the National Institutes of Health (NIH) guidelines and all animal experiments were performed in compliance with the Animal Ethics Procedures and Guidelines of the People's Republic of China and approved by the Northwest University Animal Ethics Committee.

To examine the in vivo anti-tumor effects of Rg5, Rg5-BSA NPs, and FA-Rg5-BSA NPs, subcutaneous tumor xenograft models were established in the right limb armpit regions of nude mice by injecting 1×10^7 MCF-7 cells/mouse/100 μL after one week of acclimation. When the tumors reached a volume of approximately 200 mm^3, these mice were randomly divided into four groups (n=6) (day 0), and injected intraperitoneally (i.p.) with the control, free Rg5, Rg5-BSA NPs, and FA-Rg5-BSA NPs (15 mg/kg Rg5 equivalents) every day for three consecutive weeks. During the treatment, the tumor volume was measured using a digital caliper, and the body weights were monitored every 3 days. The tumor volume was calculated using the formula: tumor volume (mm^3) = (length) × (width) ^{2} × 0.5. After 21 days of treatment, the mice were humanely sacrificed by cervical dislocation, dissected to obtain the tumors, then weighed and imaged.

2 Results and discussion

2.1 The stability analysis

The stability of nanoparticles in plasma determines the successful delivery of drug as it prevents particle aggregation or embolism from occurring in the systemic circulation. As shown

in Figure S1, the Rg5-loaded nanoparticles in FBS were completely stable at 4 °C, as no significant changes were observed in the particle size of Rg5-BSA NPs and FA-Rg5-BSA NPs for 5 days. Because the negative zeta potential of the nanoparticles interacts with the negatively charged components of the FBS to create a mutually exclusive force, the stability of nanoparticles is maintained in FBS. Thus, this suggested that Rg5-loaded nanoparticles are kinetically stable and propitious for further use as a drug carrier.

2.2 TG analysis

The thermal properties of BSA, Rg5, Rg5-BSA NPs, and FA-Rg5-BSA NPs were analyzed by TG analysis. The TG data showed the stability of the nanoparticles and the degradation of the materials with respect to increasing temperature. Figure S1 clearly showed that the Rg5-BSA NPs began to lose weight at 100 °C, which was due to water loss, and started to degrade at 200 °C. A sudden drop in weight was found beyond 250 °C, due to the loss of small molecules, ammonia and CO_2. As a result of FA, the FA-Rg5-BSA NPs underwent an abrupt decrease in weight loss at the beginning. There were great differences in weight loss approximately 300 °C for FA-Rg5-BSA NPs (28.94%), Rg5-BSA NPs (23.42%), Rg5 (10.91%), and BSA (24.95%). Beyond 500 °C, a fast degradation ratio for Rg5-BSA NPs was observed compared to that for BSA, which is attributed to the crystalline nature of Rg5 entrapped in Rg5-BSA NPs. However, a relatively slower rate of degradation was observed in FA-Rg5-BSA NPs in the presence of FA. In addition, beyond 600 °C, no obvious change was observed in BSA and Rg5-BSA NPs due to char formation in the nitrogen atmosphere. The total reduction rate of weight loss was approximately 41.14% for FA-Rg5-BSA NPs, 79.16% for Rg5-BSA NPs and 82.54% for Rg5.The

slow degradation rate of FA-Rg5-BSA NPs compared to that of Rg5, indicated the stability of the FA-Rg5-BSA NPs.

2.3 DSC analysis

DSC thermograms could provide thermal behavior information, including new peaks, endothermic peaks, peak shape, peak temperature, and enthalpy changes. DSC was utilized to confirm the existing form of the Rg5 in the formulations and to investigate the interaction between Rg5 and nanoparticles. A drug has better dissolution, absorption and bioavailability when the drug within nanoparticles is in an amorphous state. Figure S2 showed the DSC thermograms of BSA, Rg5, Rg5-BSA NPs, and FA-Rg5-BSA NPs. The Rg5 powders exhibited an endothermic melting peak at 369.84 °C, which implied the crystalline nature of Rg5. However, the characteristic peak of Rg5 disappeared in the thermogram of Rg5-BSA NPs and FA-Rg5-BSA NPs, indicating that Rg5 was in an amorphous state after encapsulation into the nanoparticles. While the DSC thermogram of FA-Rg5-BSA NPs showed a single peak at 137.38 °C, there was no peak near the Rg5-BSA NPs melting point, which confirmed the formation of a nanodrug delivery system. This result indicated the stability of the obtained product.

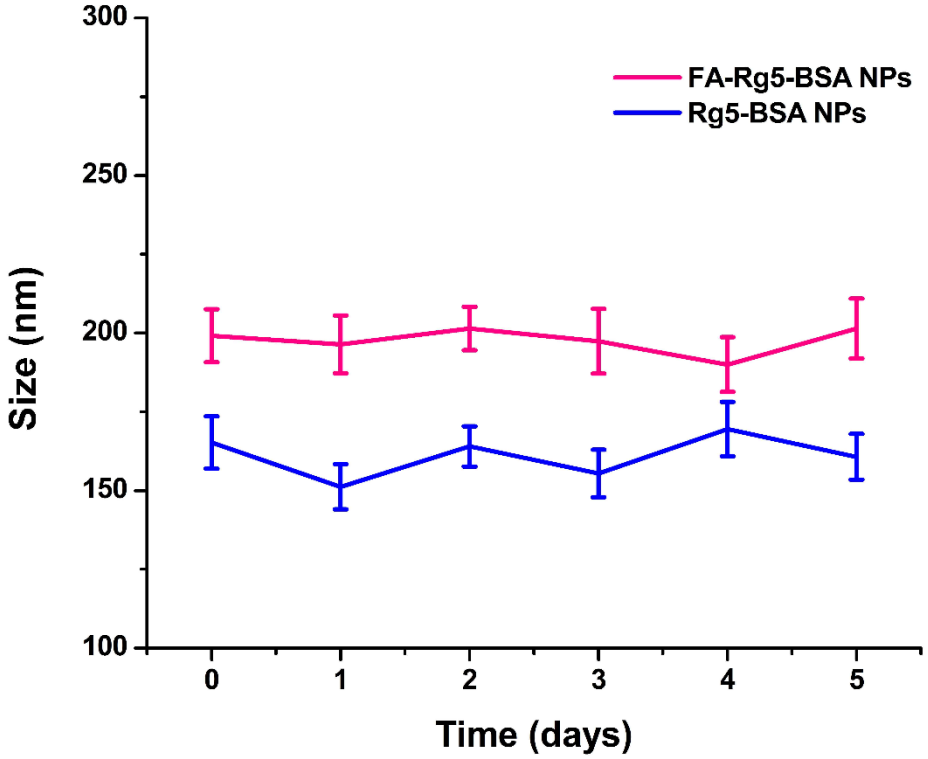

Figure S1 The stability of Rg5-BSA NPs and FA-Rg5-BSA NPs in FBS. Data are represented as mean ± SD, n = 3.

Abbreviations: BSA, bovine serum albumin; Rg5, ginsenoside Rg5; FA, folic acid; NPs, nanoparticles.

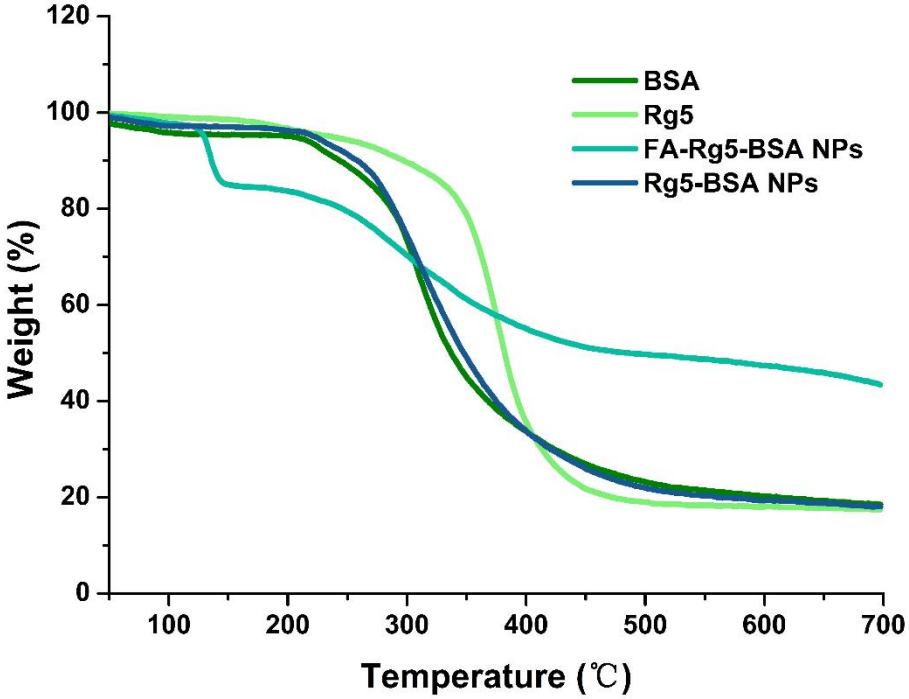

Figure S2 TG analysis curves of BSA, Rg5, FA-Rg5-BSA NPs and Rg5-BSA NPs.

Abbreviations: BSA, bovine serum albumin; Rg5, ginsenoside Rg5; FA, folic acid; NPs, nanoparticles; TG, thermo gravimetric.

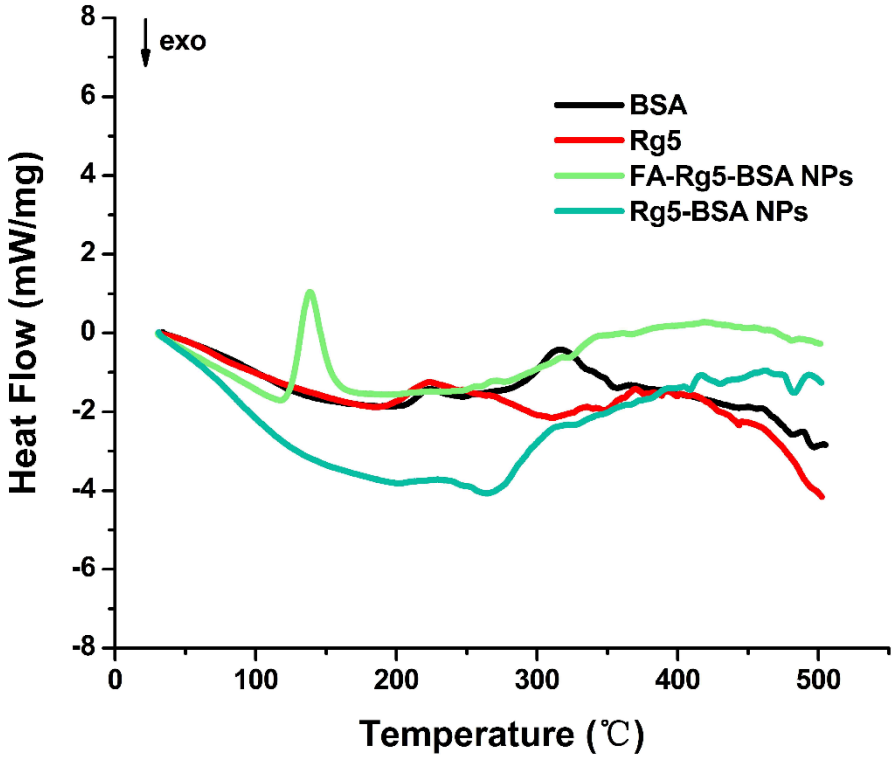

Figure S3 DSC analysis curves of BSA, Rg5, FA-Rg5-BSA NPs and Rg5-BSA NPs.

Abbreviations: BSA, bovine serum albumin; Rg5, ginsenoside Rg5; FA, folic acid; NPs, nanoparticles; DSC, differential scanning calorimetry.

Table S1 The effect of aqueous solution concentration of BSA on Rg5-BSA NPs

BSA concentration（mg/mL）	Particle size (nm)	PDI	Entrapment efficiency (%)
5	262.5±5.1	0.235±0.124	56.38±4.96
10	258.8±4.7	0.158±0.145	71.22±4.64
15	**201.0±5.7**	**0.109±0.094**	**66.36±5.58**
20	233.0±3.9	0.172±0.155	62.33±4.32

Table S2 The effect of pH value of BSA solution on Rg5-BSA NPs

pH	Particle size (nm)	PDI	Entrapment efficiency (%)
7	301.4±6.1	0.154±0.143	53.21±3.77
8	277.6±5.6	0.097±0.069	69.37±5.19
9	**223.5±4.4**	**0.119±0.106**	**75.36±4.44**
10	321.4±5.3	0.236±0.213	81.56±5.67

Table S3 The effect of volume ratio of ethanol to water on Rg5-BSA NPs

Volume ratio of ethanol to water	Particle size (nm)	PDI	Entrapment efficiency (%)
1	229.6±3.4	0.235±0.133	68.86±4.74
2	203.8±4.9	0.147±0.126	73.81±4.98
3	**158.7±5.6**	**0.088±0.074**	**79.28±3.36**
4	154.4±2.4	0.097±0.082	54.44±3.76

Table S4 The effect of molar ratio of Rg5 to BSA on Rg5-BSA NPs

Molar ratio of Rg5 to BSA	Particle size (nm)	PDI	Entrapment efficiency (%)
5	243.8±3.5	0.115±0.084	59.28±2.95
10	233.6±5.2	0.212±0.146	66.44±3.88
15	199.7±4.1	0.083±0.062	69.54±2.79
20	**174.8±3.0**	**0.072±0.053**	**87.29±2.55**
25	209.6±4.7	0.229±0.185	77.63±3.50

Table S5 The effect of the rate of adding ethanol on Rg5-BSA NPs

The rate of adding ethanol (mL/min)	Particle size (nm)	PDI	Entrapment efficiency (%)
0.1-1.0	229.6±3.4	0.117±0.096	60.35±2.95
1.0-1.5	203.8±4.9	0.214±0.185	77.38±3.28
1.5-2.0	**160.7±3.1**	**0.073±0.064**	**75.43±2.84**

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121 **Table S6** The effect of stirring speed on Rg5-BSA NPs

Stirring speed (r/min)	Particle size (nm)	PDI	Entrapment efficiency (%)
300	234.7±5.8	0.198±0.164	78.48±3.48
600	**194.8±3.6**	**0.186±0.155**	**83.47±4.29**
900	215.9±4.2	0.093±0.085	80.63±3.34

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123 **Table S7** The effect of stirring time on Rg5-BSA NPs

Stirring time (h)	Particle size (nm)	PDI	Entrapment efficiency (%)
6	255.7±5.3	0.081±0.066	59.29±4.17
12	208.3±2.6	0.131±0.104	63.28±3.74
24	**198.6±3.3**	**0.076±0.062**	**75.65±4.11**

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125 **Table S8** The effect of the molar ratio of folic acid to BSA on FA-Rg5-BSA NPs

Molar ratio of FA to BSA	Particle size (nm)	PDI	The amount of FA (μg/mg BSA NPs)
5	312.1±3.6	0.293±0.056	73.2±18.55
10	307.6±3.7	0.219±0.089	67.5±16.36

15	**233.6±4.2**	**0.153±0.045**	**71.4±19.48**
20	240.8±2.9	0.185±0.048	78.85±24.52

Table S9 The effect of reaction time after adding folic acid on FA-Rg5-BSA NPs

Reaction time after adding FA (h)	Particle size (nm)	PDI	The amount of FA (μg/mg BSA NPs)
1	307.4±3.9	0.156±0.052	50.5±13.57
3	288.6±3.7	0.257±0.064	54.3±19.65
6	**246.8±2.5**	**0.268±0.057**	**60.5±23.44**
12	322.9±5.4	0.215±0.086	65.7±29.53

Table S10 The effect of centrifugal speed on FA-Rg5-BSA NPs

Centrifugal speed (r/min)	Particle size (nm)	PDI	The amount of FA (μg/mg BSA NPs)
7000	267.4±3.5	0.179±0.096	63.42±23.83
10000	**230.7±4.7**	**0.181±0.058**	**88.74±19.38**
12000	209.5±4.6	0.278±0.045	53.79±27.46

Notes: Data are represented as mean ± SD, n = 3.

Abbreviations: BSA, bovine serum albumin; Rg5, ginsenoside Rg5; FA, folic acid; NPs, nanoparticles.