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An Efficient Biomimetic Nano-Regulator Inducing Simultaneous Calcium Ion/Nitric Oxide/Energy Metabolism Triple Homeostasis Disruption for Synergetic Cancer Therapy

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Materials:

Calcium chloride anhydrous ($CaCl_2$), L-Arginine (L-Arg), Ammonium bicarbonate (NH_4HCO_3), Tetraethyl orthosilicate (TEOS) were purchased from China National Pharmaceutical Group Corporation (Shanghai, China). Glucose Oxidase from Aspergillus niger (GOx), N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-(dimethylamino)propyl) carbodiimide hydrochloride (EDC), polyvinylpyrrolidone (molar Mt 10,000, PVP-10) were purchased from Sigma-Aldrich. Annexin V-FITC/PI Apoptosis detection Kit was purchased from Meilunbio Biotechnology Co., LTD (Dalian, China). Calcein-AM/PI Double staining Kit, Mitochondrial Membrane Potential Detection Kit (JC-1) were purchased from G-Clone Biotechnology Co., LTD (Beijing China). Enhanced ATP Assay Kit were purchased from Beyotime Biotechnology (Shanghai, China). The water used was Mill-Q secondary ultrapure water (18.2MΩ/cm). MCF-7 cells and 4T1 cells were purchased from Procell Life Science&Technology Co.,Ltd. (Wuhan, China), and HEB cells were obtained from Zhimin Lu lab in Zhejinag University Cancer

Center(Hangzhou, Zhejiang 310029, China)The chemical reagents used in the experiment were of analytical grade and used without purification.

Synthesis of $CaCO_3$/L-Arg (CL) nanoparticles: $CaCl_2$ (166.0 mg) and L-Arg (0.4 mL, 10 mg/mL, aqueous solution) were dissolved in absolute ethanol (100 mL) in a 500 mL glass beaker, which was covered by a tinfoil with several holes. Then the beaker was placed together with 8.0 g NH_4HCO_3 in a vacuum drying oven at 30 °C and the reaction was allowed for 48 h. The white product was centrifuged at 8,000 rpm and redispersed in absolute ethanol. As for bare $CaCO_3$ nanoparticles, L-Arg was not added to the reaction system.

Synthesis of $CaCO_3$/L-Arg @SiO_2 nanoparticles (CLS): Silica shell was synthesized according to previous literature with some modifications. $CaCO_3$/L-Arg (4.0 mg, ethanol solution) and PVP-10 (0.35 mL, 1 mg/mL, ethanol solution) were dispersed in absolute ethanol (20 mL) under stirring for 30 min. Then, TEOS (0.2 mL) was dropwise added, and the mixture was continuously stirred for 2 h. After that, ammonium hydroxide (0.5 mL, 25 %, v/v) was added, and the mixture was continuously stirred for 12 h. The mixture was centrifuged (8000 rpm, 10 min) and washed with absolute ethanol for three times. Then the products were redispersed in absolute ethanol (20 mL) containing PVP-10 (0.3 mL, 1 mg/mL, ethanol solution) and the mixture was stirred for 30 min. After that, TEOS (0.15 mL) was added and the mixture was stirred for another 2 h. Finally, ammonium hydroxide (0.8 mL) was added, and the mixture was continuously stirred overnight. The product was centrifuged and washed with absolute ethanol at 8000 rpm for five times. As for the

synthesis of $CaCO_3$ @SiO_2 nanoparticles (CS), $CaCO_3$/L-Arg was replaced by bare $CaCO_3$ with the same amount and the procedure was the same.

Synthesis of SiO_2 /L-Arg nanoparticles (SL): 0.25g CTAB was dissolved in 120 mL H_2O, and NaOH (2 M, 0.75mL) were added. The mixture was reacted at 80℃ for 30 min under stirring. Then TEOS (1.25 mL) and APTES (0.25 mL) were added dropwise to the solution and the mixture was reacted for 2 h. The white product was centrifuged and washed with methanol at 8000 rpm. The product was redispersed in 50 mL methanol containing 3 mL HCl, the mixture was refluxed for 24 h. The product was centrifuged and washed twice with methanol and ethanol respectively. The above product and L-Arg were added to 20mL H_2O, and the mixture was stirred for 48 h, the product was centrifuged and washed with ethanol washed with absolute ethanol at 8000 rpm.

Synthesis of $CaCO_3$/L-Arg @SiO_2-GOx nanoparticles (CLSG): Amino modified CLS was firstly prepared. 8 mg $CaCO_3$/L-Arg@SiO_2 and 16 μL APTES were added to 40 mL absolute ethanol, the mixture was allowed reflux for 4 h. The product was centrifuged at 8000 rpm and washed with ethanol to obtain CLS-NH_2. As for GOx modification, 8 mg GOx, 190.0 mg EDC and 114.0 mg NHS were dissolved in 2 mL H_2O and the mixture was sit in the dark for 30 min to activate the carboxyl group of GOx. Then, the activated GOx were added to CLS-NH_2 in 20 mL H_2O and reacted for 1 h.

Preparation of Cell Membrane Fragments: Cell membrane fragments were prepared according to previous literature with some modifications. U87 MG cells were cultured

and collected by centrifugation, then dispersed in Tris buffer (1 mL containing 10 μL EDTA protease inhibitor), which was placed in 4 ºC for 1 h. Then the cells were treated with ultrasound, centrifuged at 4 °C (3000 rpm, 10 min), and the supernatant was collected. Finally, the supernatant was centrifuged at 4 °C (14 000 rpm, 30 min) and collect sediment for standby.

Synthesis of CLSGM nanoparticles: The prepared CLSG was redispersed in 10 mL H_2O, and the above cell membrane fragments were added, the mixture was stirred for 1 h. The product was centrifuged and washed with H_2O.

Cell culture: The U87 MG cells were obtained from Procell Life Science & Technology Co., Ltd. The U87 MG cells were cultured in DMEM Medium supplemented with FBS (10 vol%), penicillin (100 U/mL) and streptomycin (100 μg/mL) in an atmosphere of CO_2 (5 vol%) and air (95 vol%) at 37°C. The cells were passaged every two days with 0.25% Trypsin EDTA.

Detection of pH decrease by GOx in vitro: 2 mg CLSG was dispersed in 10 mL H_2O at 37 ºC in the presence or absence of glucose (1 mg/mL) respectively. The pH value of the solution was recorded every 3 min.

Detection of H_2O_2 in vitro: 50 μL of TMB ($1x10^{-4}$ M, DMSO) and 20 μL of HRP were added into CLSG solution (1 mg/mL, 2 mL) with or without glucose (1 mg/mL), respectively. UV-Vis spectra was measured after the mixture was incubated at 37 ºC for 30 min.

As for fluorescence analyse, two groups of CLSG in PBS buffer solution (1 mg/mL, 2 mL) with or without glucose (1 mg/mL), respectively, were incubated with 20 μL of

H_2O_2 probe Cy-O-Eb with a final concentration of 10 μM in the darkness at 37 ºC for 1 h. Then the fluorescence intensity was detected by using FLS-980 Edinburgh Fluorescence Spectrometer. Ex=750 nm, Em=785-850 nm.

Detection of NO in vitro: Three groups of CLSG with different concentrations (0.1, 0.5, and 1.0 mg/mL，repectively) in PBS buffer solution containing NO probe (RhBS, 10 μM) were incubated with glucose (0, 1, 2 and 4 mg/mL, respectively) in the darkness at 37 ºC for 1 h. Then the fluorescence intensity was detected by using FLS-980 Edinburgh Fluorescence Spectrometer. Ex=540 nm, Em=575-650 nm.

Cell targeting: U87 MG cells, MCF-7 cells, 4T1 cells, and HEB cells were seeded on glass bottom dishes and incubated in a carbon dioxide incubator overnight to make the cell stick to the wall. Then, complete medium and CLSRM with a concentration of 200 μg/mL was added and the cells were incubated for another 12 h. The cells were washed with PBS to remove free CLSRM before confocal imaging. As for flow cytometry analyse, U87 MG cells, MCF-7 cells, 4T1 cells, and HEB cells were cultured in cell-culture dishes and incubated with CLSRM (200 μg/mL) for 12 h. Then the cells were collected by trypsin digestion and washed with PBS before analyzed using flow cytometry.

MTT assay: U87 MG cells were inoculated on 96 well plates and incubated in a carbon dioxide incubator overnight. After removing the medium, CLSGM with different concentrations or different nanoparticles: CSM, CLSM, CSGM, SLGM, CLSGM with a concentration of 20 μg/mL medium (Glucose free) were added to each well respectively, the cells treated with the medium only were set as the negative

control group. After 12 h of incubation, the medium was changed to complete medium, and the cells were continue incubating for 12 h, then the medium was replaced by MTT solution (150 μL). After 4 h of incubation, the solution was discarded and the crystal was dissolved by DMSO. The absorbance at 495 nm was measured by using a microplate reader.

Intracellular ATP Measurement: Intracellular ATP was measured using an ATP detection kit. Briefly, U87 MG cells were firstly inoculated on 6 well plates and incubated in carbon dioxide incubator overnight. After removing the medium, glucose free medium containing different nanoparticles (CSM, CLSM, CSGM, SLGM, CLSGM) with a concentration of 50 μg/mL were added, and one group without any nanoparticle was set as the blank control. After incubation for 6 h, the medium was replaced with complete medium and the cells were incubated for another 4 h and then the intracellular ATP level was detected.

JC-1 Staining to Measure Mitochondrial Membrane Potential: The cells were inoculated into glass bottom dishes and incubated in a carbon dioxide incubator overnight. After removing the medium, glucose free medium and different probes: CSM, CLSM, CSGM, SLGM, CLSGM with a concentration of 50 μg/mL were added, and the group without any probe was set as the blank control. After incubation for 2 h, the medium was replaced with complete medium and the cells were incubated for another for 2 h. Then the cells were stained with the JC-1 probe and the changes of mitochondrial membrane potential in cells were detected by the laser confocal fluorescence imager.

Intracellular NO detection: The cells were inoculated into glass bottom dishes and incubated in a carbon dioxide incubator overnight. After removing the medium, glucose free medium and different probes: CSM, SLGM, CLSGM with a concentration of 50 μg/mL were added, at the same time, the group without any probe was set as the blank control. After incubation for 2 h, the medium was replaced with complete medium and the cells were incubated for another 2 h. When changing the medium, 20 μL RhBS and 20 μL Hochest33342 were added to each glass dish. Intracellular NO fluorescence imaging was conducted by laser confocal imager.

Intracellular H_2O_2 detection: The cells were inoculated into glass bottom dishes and incubated in a carbon dioxide incubator overnight. After removing the medium, glucose free medium and different probes: CSM, CSGM with a concentration of 50 μg/mL were added, at the same time, the group without any probe was set as the blank control. After incubation for 2 h, the medium was replaced with complete medium and the cells were incubated for another 2 h. When changing the medium, 20 μL Cy-O-Eb and 20 μL Hochest33342 were added to each glass dish respectively. Intracellular H_2O_2 fluorescence imaging was conducted by laser confocal imager.

Intracellular Ca^{2+} Detection: The cells were inoculated into glass bottom dishes and incubated in a carbon dioxide incubator overnight. After removing the medium, glucose free medium and different probes: CSM, CLSGM with a concentration of 50 μg/mL were added, at the same time, the group without any probe was set as the blank control. After incubation for 2 h, the medium was replaced with complete medium and the cells were incubated for another 2 h. When changing the medium, 20 μL Ca^{2+}

probe (Fluo-4, AM) and 20 μL Hochest33342 was added to each glass dish respectively. Intracellular NO fluorescence imaging was conducted by laser confocal imager. As for ICP-MS analyse, U87 MG cells were firstly incubated in cell-culture dishes with CLSRM (50 ug/mL) in glucose free medium. After incubation for 2 h, the medium was replaced with complete medium and the cells were incubated for another 2 h. Then the cells were collected by trypsin digestion and washed with PBS twice. Subsequently, 5.0×10^7 cells were collected through cell counting and treated with aqua regia for 4 h. The solution was finally diluted to 10 mL for ICP-MS analyse.

Intracellular ROS detection: The cells were inoculated into glass bottom dishes and incubated in a carbon dioxide incubator overnight. After removing the medium, glucose free medium and different probes: CLSM, CLSGM with a concentration of 50 μg/mL were added, at the same time, the group without any probe was set as the blank control. After incubation for 2 h, the medium was replaced with complete medium and the cells were incubated for another 2 h. Before confocal imaging, 20 μL ROS probe (DCFH-DA) and 20 μL Hochest33342 were added to each glass dish. Intracellular ROS fluorescence imaging was detected and photographed by laser confocal imager.

Intracellular lysosomal co-localization assay: GOx in CLSM synthesis step was replaced with Rhodamine B. The cells were inoculated into glass bottom dishes and incubated in a carbon dioxide incubator overnight. Then complete medium and CLSRM with a concentration of 200 μg/mL was added and the cells were incubated

for another 1 h, 2 h, 4 h and 6 h, respectively. 20 μL LysoTracker Green DND-26 and 20 μL Hochest33342 were added 1 h before confocal experiments.

Live/Dead Cell Staining Assays: The cells were inoculated into glass bottom dishes and incubated in a carbon dioxide incubator overnight. After removing the medium, glucose free medium and different probes: CSM, CLSM, CSGM, SLGM, CLSGM with a concentration of 50 μg/mL were added, at the same time, the group without any probe was set as the blank control. After incubation for 2 h, the medium was replaced with complete medium and the cells were incubated for 4 h. According to the instructions of the kit, after the cells were washed with buffer for 3 times, Calcein-AM and PI were added and the cells incubated at 37 ^{o}C for 15 min. The fluorescence images were captured by laser confocal imager.

Apoptosis detection:

(1) The cells were inoculated into glass bottom dishes and incubated in a carbon dioxide incubator 12 h. After removing the medium, glucose free medium and different probes: CSM, CLSM, CSGM, SLGM, CLSGM with a concentration of 50 μg/mL were added, at the same time, the group without any probe was set as the blank control. After incubation for 2 h, the medium was replaced with complete medium and the cells were incubated for 2 h. After washing three times with PBS, cells were fixed with 4% paraformaldehyde at room temperature for 15-30 min. PBS was used to wash the cells three times. The cells were then permeabilized with 0.1% Triton X-100 PBS at room temperature for 10 minutes, followed by three washes with PBS-T. The cells were blocked with 3% BSA PBS-T solution for 10-30 minutes, then washed

three times with PBS-T. Cleaved-caspase3 primary antibody, diluted in 1% BSA PBS-T, was added in appropriate amounts and incubated overnight at 4℃. The cells were washed four times with PBS-T. Fluorescent secondary antibody, diluted in 1% BSA PBS-T, was added in appropriate amounts and incubated at room temperature in the dark for 30 minutes. The cells were washed five times with PBS-T, and then stained with Hochest33342 dye (5 μg/mL) at room temperature for 3-5 minutes. The cells were washed twice with PBS-T, and then mounted with 80% glycerol for imaging using confocal microscopy.

(2) The cells were inoculated on 6 well plates and incubated in a carbon dioxide incubator overnight. After removing the medium, glucose free medium and different probes: CSM, CLSM, CSGM, SLGM, CLSGM with a concentration of 50 μg/mL were added, and the group without any probe was set as the blank control. After incubation for 2 h, the medium was replaced with complete medium and the cells were incubated for another 4 h. Then the cells were digested with trypsin without EDTA, and collected by centrifugation and washed twice with PBS. Finally, the cells were redispersed in buffer and Annexin-FITC/PI was added. After 15 min incubation in darkness at room temperature, the apoptosis was detected by flow cytometry analysis.

U87 MG Tumor Model: All procedures of animal study were approved by Ethics Committee of Medical College of Qingdao University (QDU-AEC-2022270). BALB/c mice (approximately 4-5weeks old, ~18 g) were housed under normal conditions with 12 h light and dark cycles and given access to food and water ad

libitum. The U87 MG xenograft tumor model was used as an example to evaluate the therapeutic effect. To this end, $5x10^6$ U87 MG cells in 60 μL of serum-free RPMI 1640 medium were injected subcutaneously into the right axillary region of BALB/c mice. After the tumor size had reached approximately 120 mm^3, the mice were used in subsequent experiments (tumor volume = $W^2 \times L/2$, W = width, L = length)

In vivo Fluorescence Imaging: Tumor bearing BALB / c mice were injected with CLSRM (50 mg kg^{-1}) *via* tail vein, and fluorescence imaging was measured at different time points (2, 4, 6, 8, 10 and 12 h).

In vivo Verification of Antitumor Effects: The U87 MG tumor-bearing BALB/c mice were randomly assigned to 6 groups (more than 5 mice in each group). And mice in each group received different treatments: (I) Control, (II) CSM, (III) CLSM, (IV) CSGM, (V) SLGM, (VI) CLSGM *via* intravenous injection with the dose of 50 $mg \cdot kg^{-1}$ and the mice in control group were injected with PBS. The tumor sizes and body weights were measured every other day for 14 days. The survival rate of mice in each treatment group within 50 days was recorded. The mouse is considered dead either when it has truly died or when its tumor volume reaches 1 cm^3. The H&E staining of the five major organs (heart, liver, spleen, lung, and kidney) were tested at 7 days post injection. H&E staining, TUNEL staining and Ki67 staining of tumor were detected at 1 day post injection.

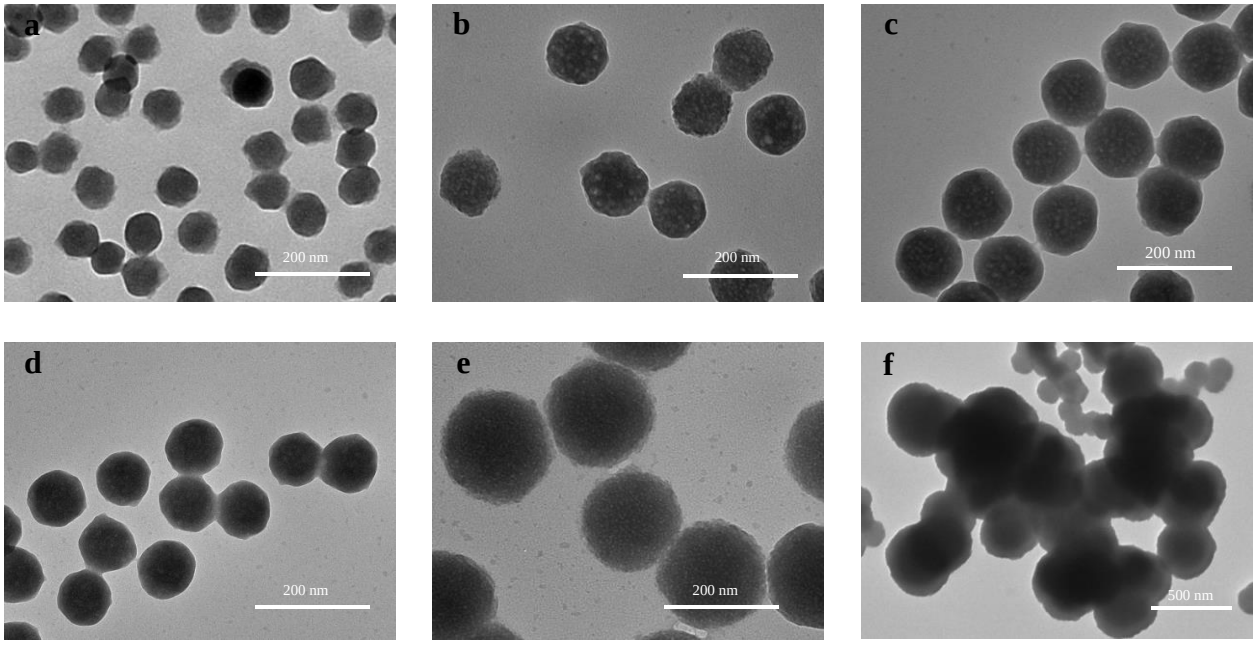

Fig. S1 (a to f) TEM images of $CaCO_3$ nanoparticles with different water percentage in alcohol of 0 %, 0.2 %, 0.4 %, 0.5 %, 1.0 % and 2 % (v %) in the absence of L-Arg during the preparation, respectively.

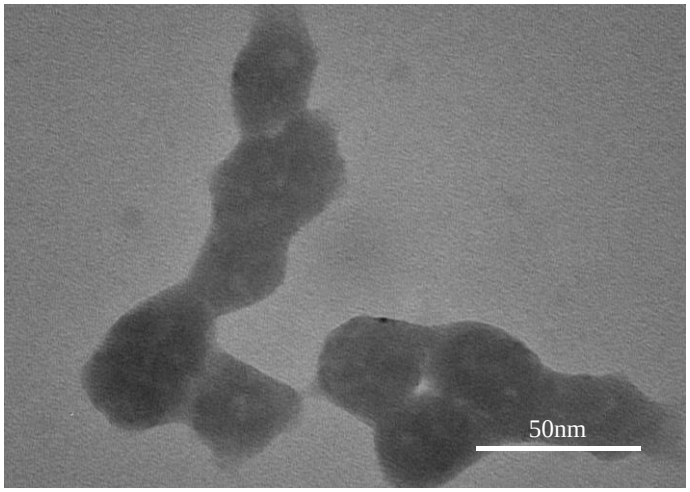

Fig. S2 TEM image of $CaCO_3$ nanoparticles with L-Arg in the absence of water during the preparation.

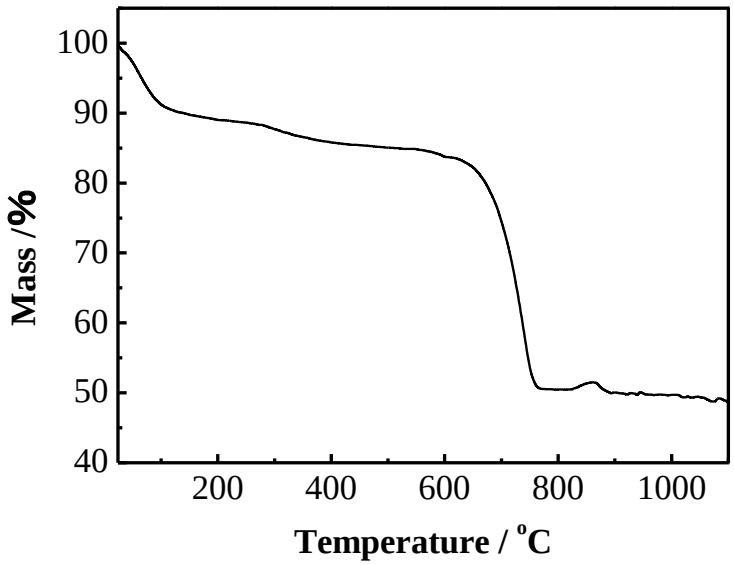

Figure S3. TGA analyse of CL.

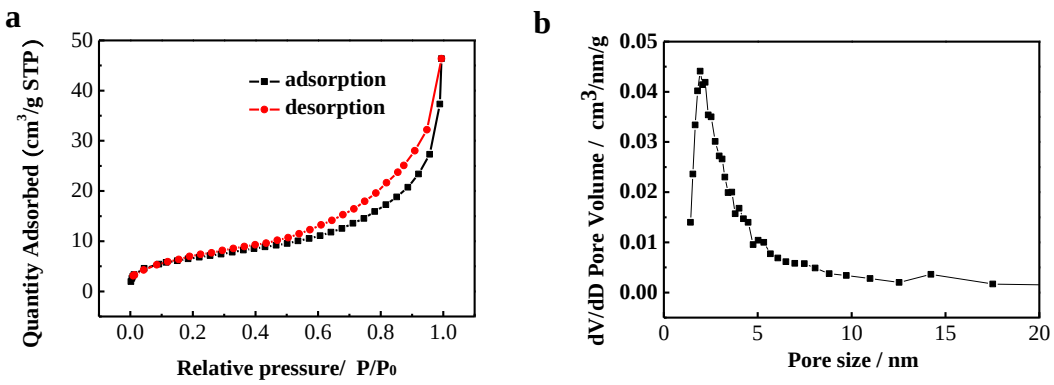

Fig. S4 (a) N_2 adsorption-desorption isotherms of CL. (b) Pore size distribution of CL.

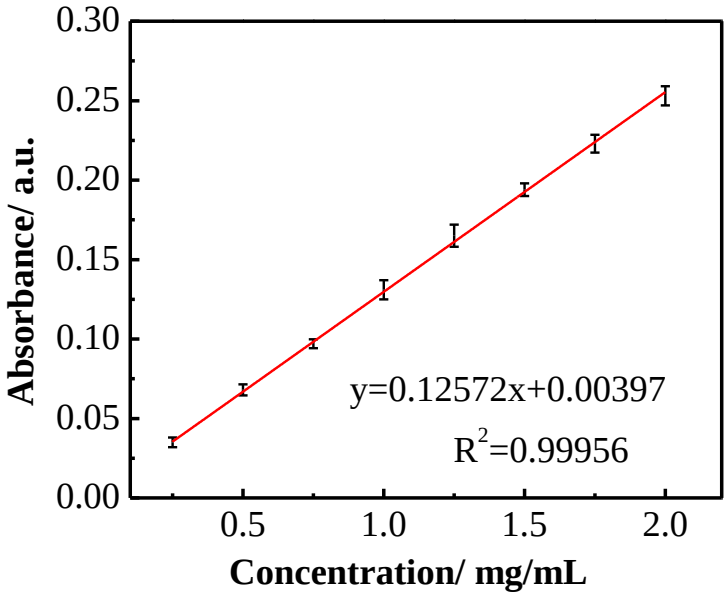

Fig. S5 Standard absorbance curve of GOx with different concentrations.

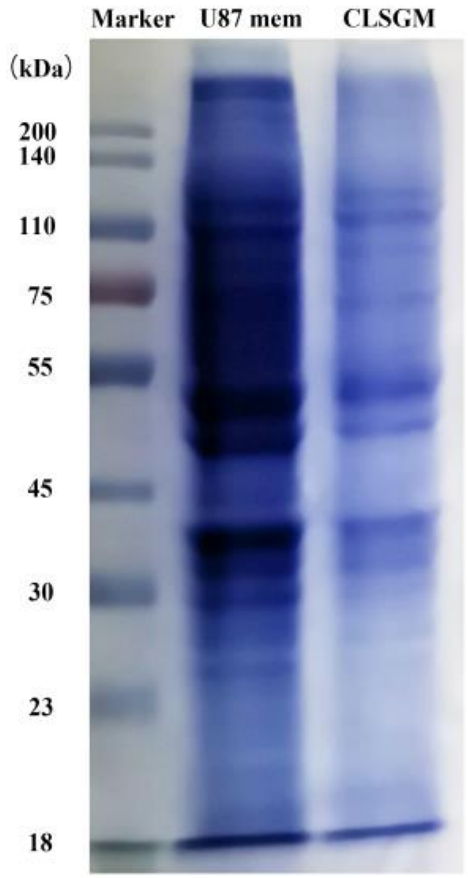

Fig. S6 SDS-PAGE protein analysis of U87 MG cell membrane and CLSGM.

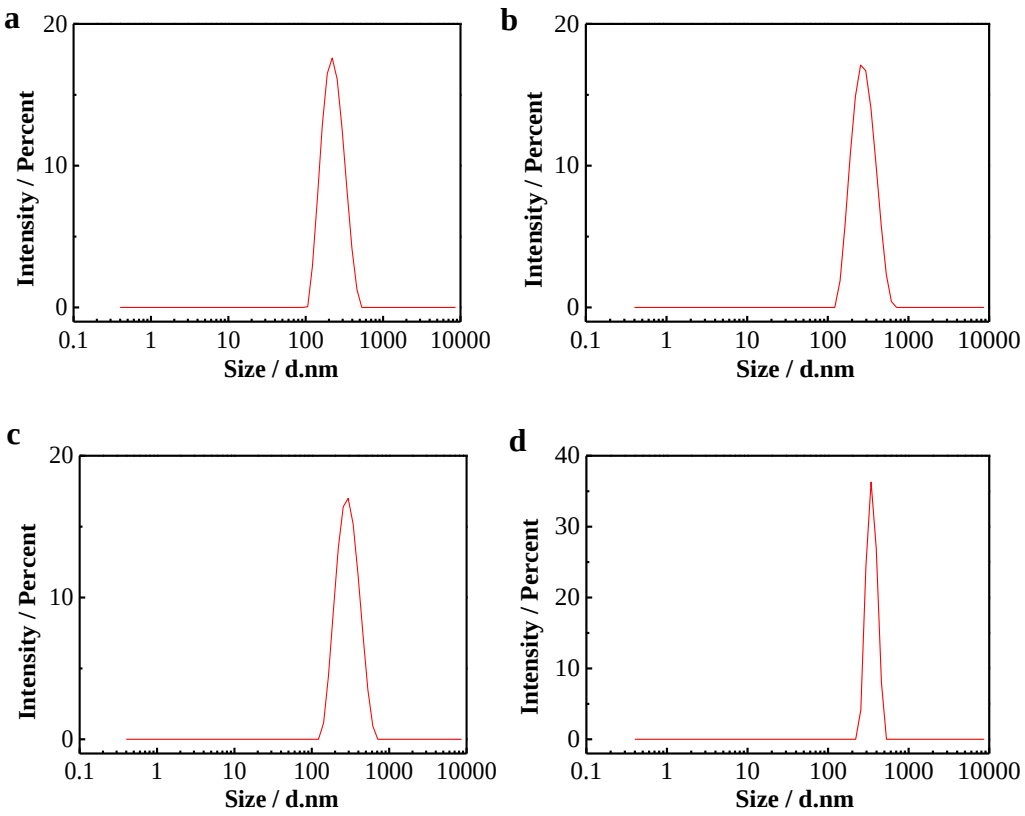

Fig. S7 Size distribution of CL (a), CLS (b), CLS-NH_2 (c) and CLSGM (d) by DLS measurements.

Table S1. Z-average of corresponding nanoparticles.

	Z-Average / nm	**PDI**
CL	214.3±10.9	0.092
CLS	266.5±8.6	0.121
CLS-NH_2	282.5±11.2	0.186
CLSGM	301.4±15.2	0.091

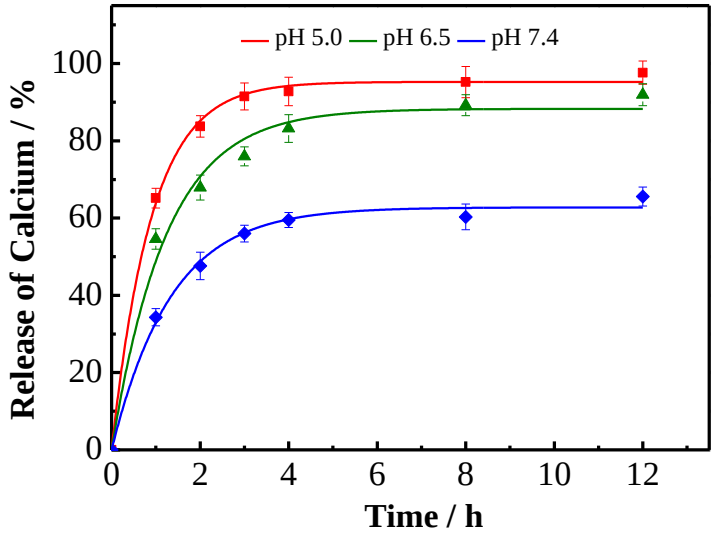

Fig. S8 Time-dependent release profile of Ca^{2+} from CL in Tris-HCl buffer at different pH (5.0, 6.5 and 7.4).

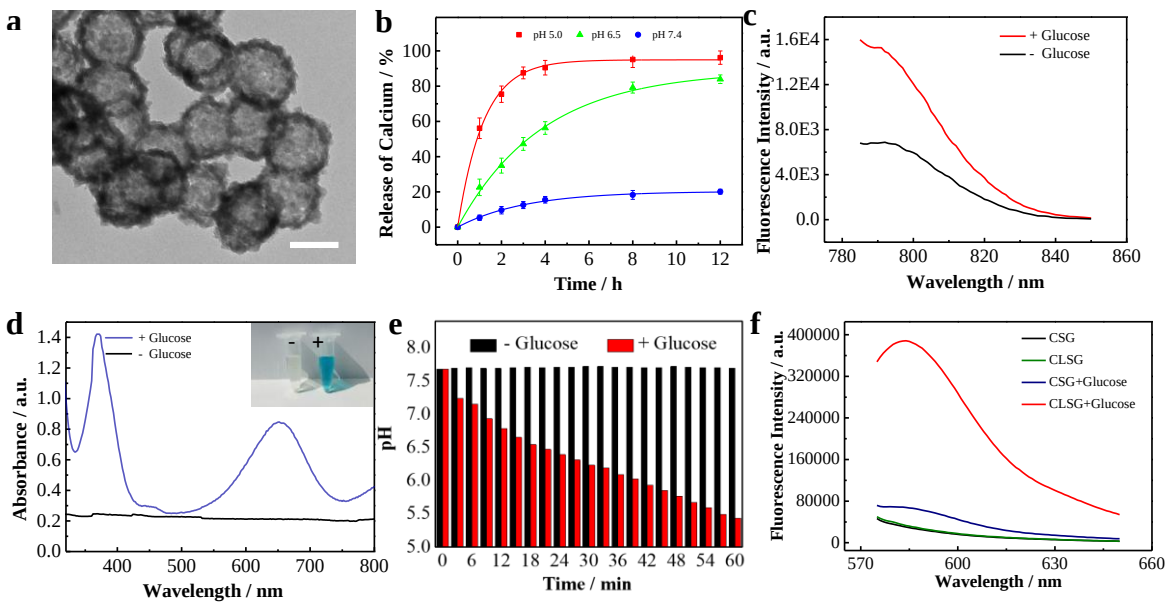

Fig. S9 Functions verification of CLSGM *in vitro*. a) TEM image of CLS after incubation in Tris-HCl buffer at pH 5.0 for 12 h. Scale bar is 100 nm. b) Time-dependent release profile of Ca^{2+} from CLSM in Tris-HCl buffer at different pH (5.0, 6.5 and 7.4). c) Fluorescence spectra of probe Cy-O-Eb incubated with CLSG in the presence of glucose or not (1 mg/mL). d) UV-Vis absorption spectra of TMB incubated with CLSG and HRP for the detection of H_2O_2 in the presence or absence of glucose (1 mg/mL). The inset shows the corresponding digital pictures. e) The pH value changes of CLSG solution in the presence or absence of glucose (0.2 mg/mL). f) Fluorescence spectra of RhBS incubated with different groups: CSG, CLSG, CSG with glucose and CLSG with glucose.

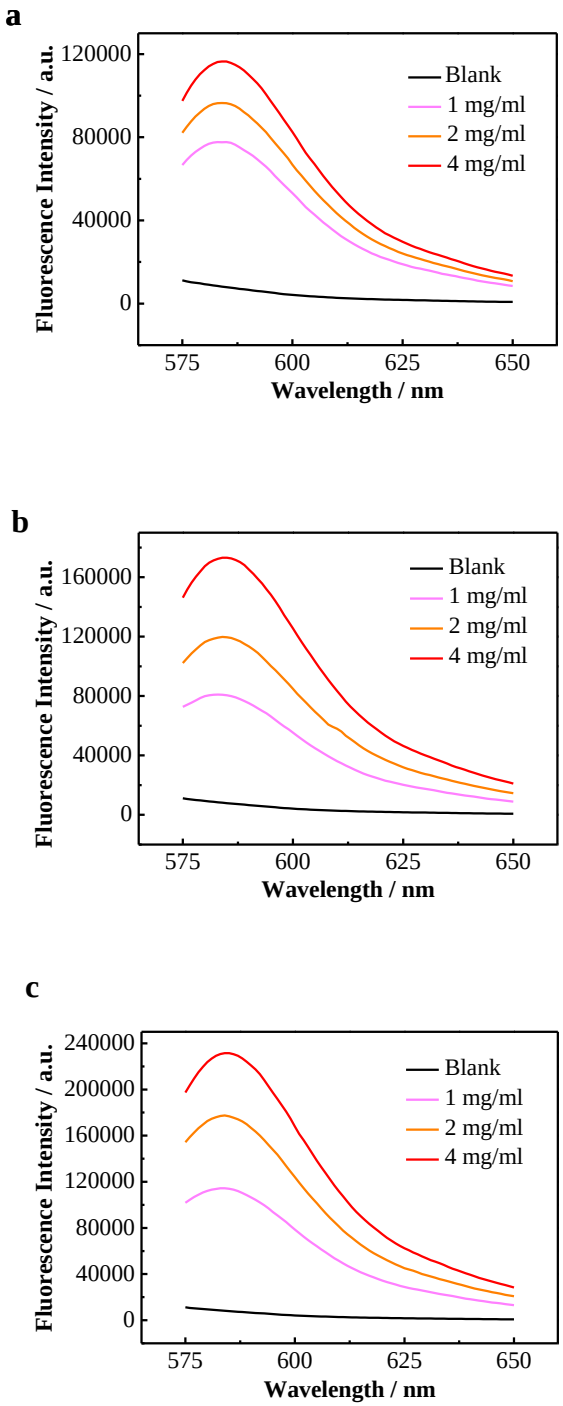

Fig. S10 Fluorescence spectra of RhBS (10 μM) incubated with different concentration of CLSG (0.1 mg/mL for a, 0.5 mg/mL for b and 1.0 mg/mL for c) in the presence of glucose (1, 2, 4 mg/mL).

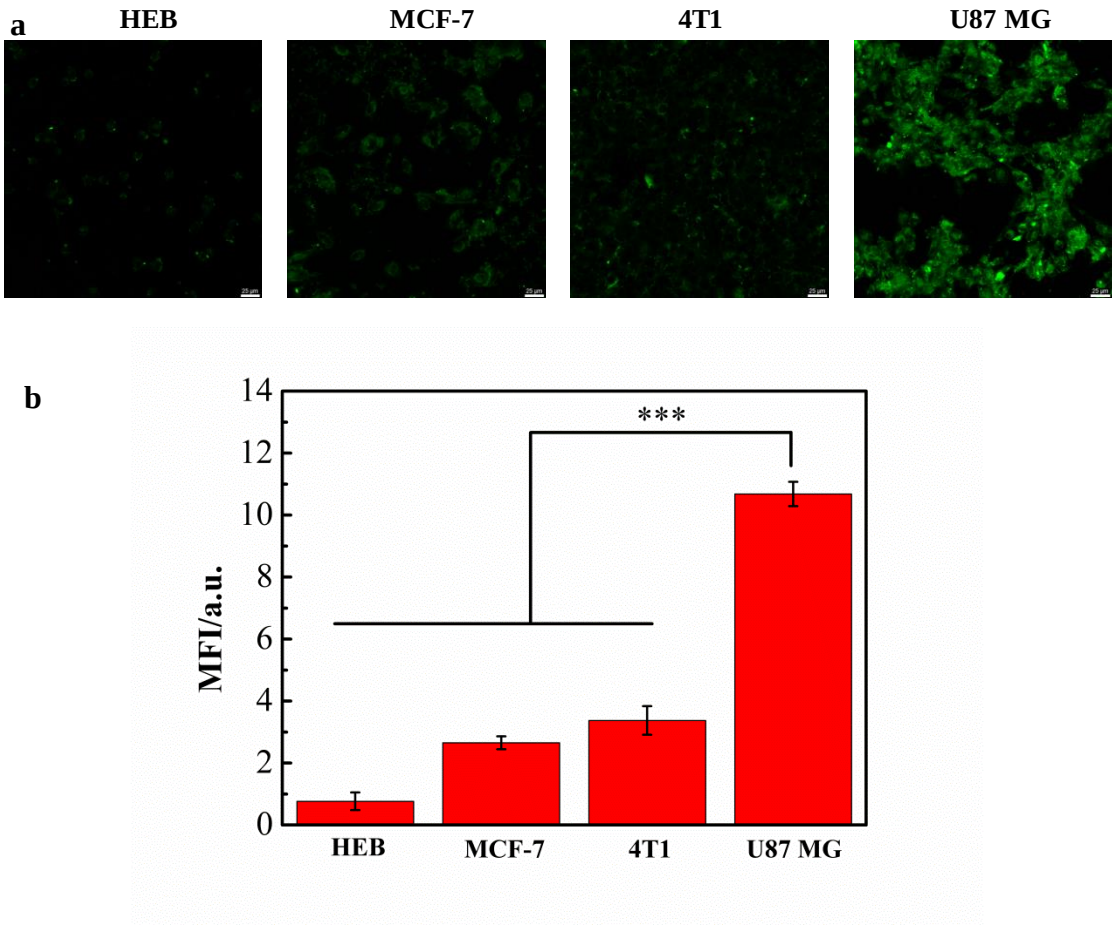

Fig. S11 (a) Confocal images of HEB cells, MCF-7 cells, 4T1 cells and U87 MG cells after incubated with CLSRM for 12 h. (b) Statistical fluorescent quantitative analysis of cells in a.

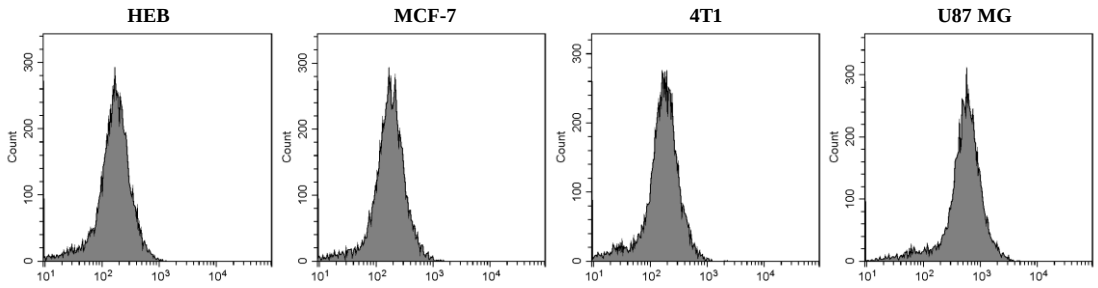

Fig. S12 Flow cytometry analyse of the flurescence intensity of HEB cells, MCF-7 cells, 4T1 cells and U87 MG cells after incubated with CLSRM for 12 h.

Table S2. Pearson correlation coefficients in Fig. 3a.

Incubation Time	Pearson correlation coefficients
1h	0.37
2h	0.52
4h	0.73
6h	0.69

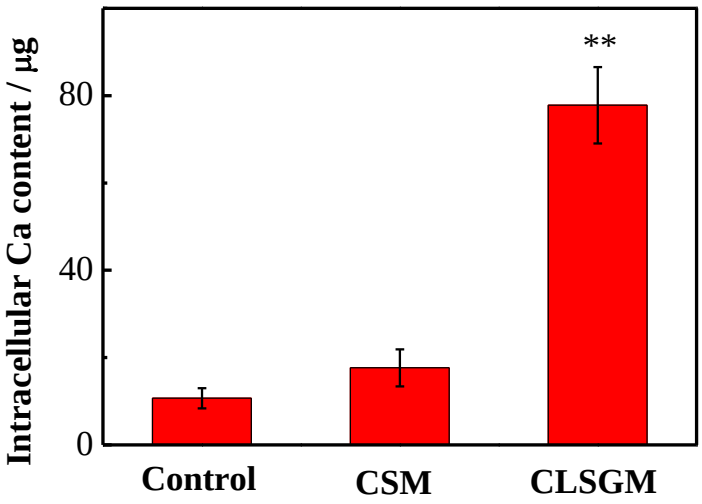

Fig. S13 Intracellular Ca content after U87 MG cells (5.0×10^7 cells) with different treatments *via* ICP-MS analyse. **p<0.01

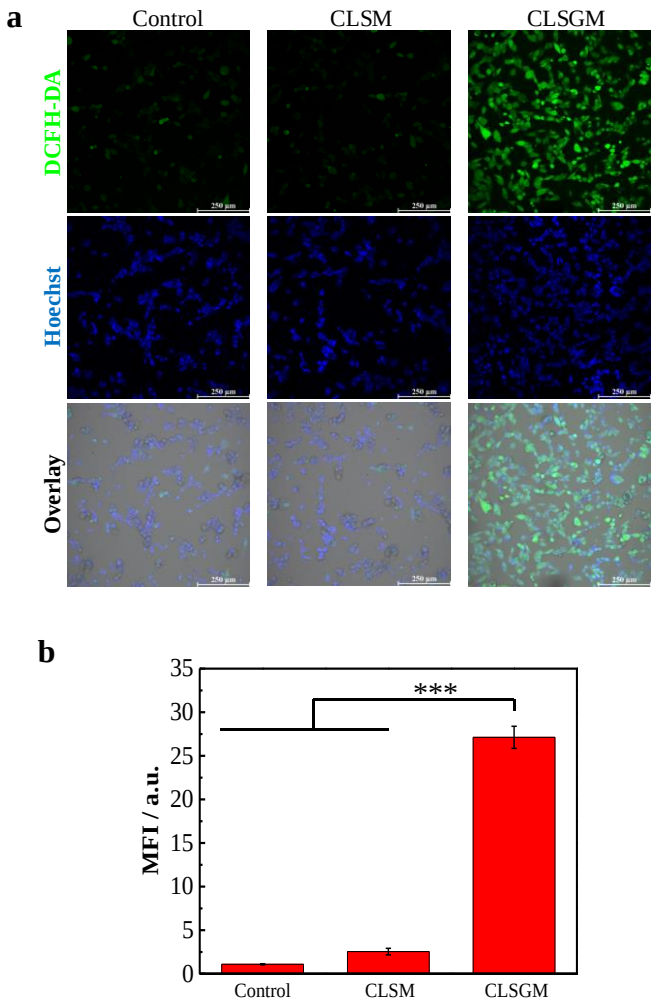

Fig. S14 (a) Confocal images of DCFH-DA stained U87 MG cells received treatment of CLSM or CLSGM for the detection of intracellular ROS. (b) Quantitative fluorescence intensity of U87 MG cells in a. ***p<0.001.

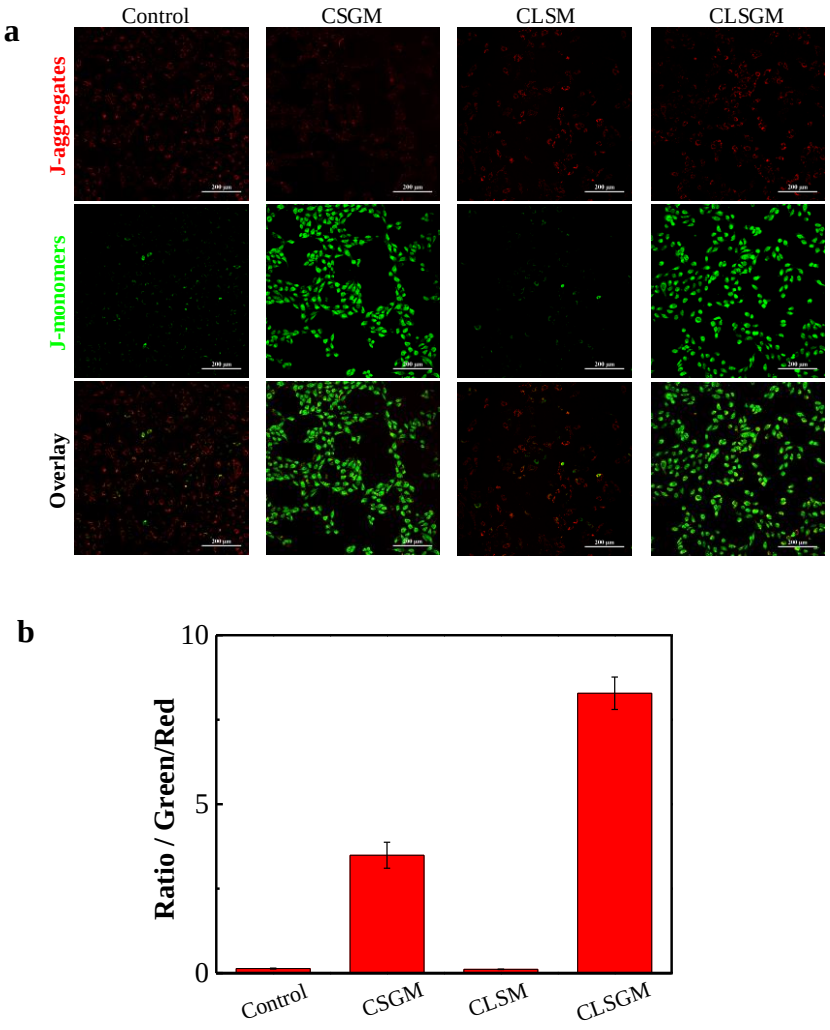

Fig. S15 (a) Confocal images of U87 MG cells under different treatments for the display of mitochondria membrane changes by JC-1 analysis. (b)Ratio of quantitative green/red fluorescence intensity of U87 MG cells with different treatments in a.

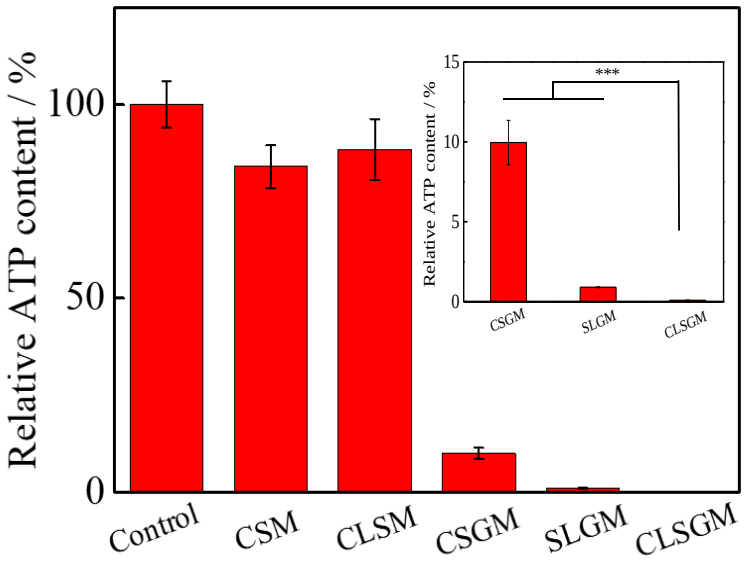

Fig. S16 Intracellular ATP content of U87 MG cells with different treatments (control, CSM, CLSM, CSGM, SLGM and CLSGM). ***p<0.001.

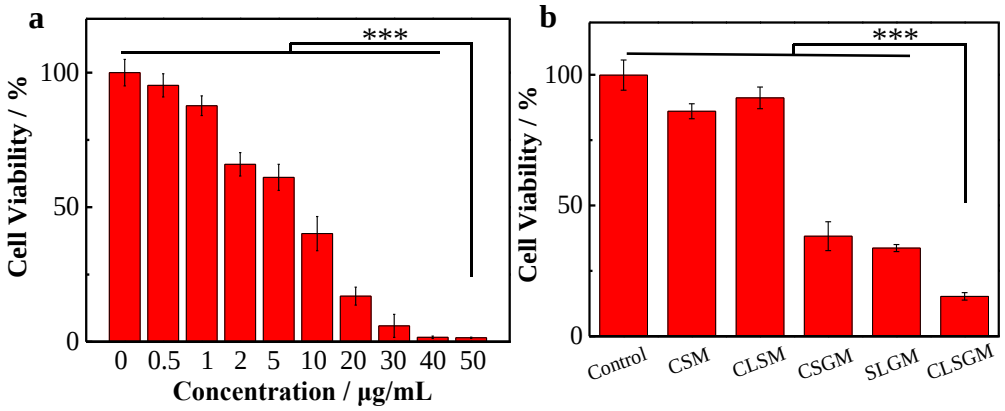

Fig. S17 (a) The viability of U87 MG cells after incubated with CLSGM with various concentrations for 12 h. (0, 0.5, 1, 2, 5, 10, 20, 30, 40, 50 μg/mL). ****p*<0.001. (b) The viability of U87 MG cells received different incubation of control, CSM, CLSM, CSGM, SLGM and CLSGM with a concentration of 20 ug/mL. ****p*<0.001.

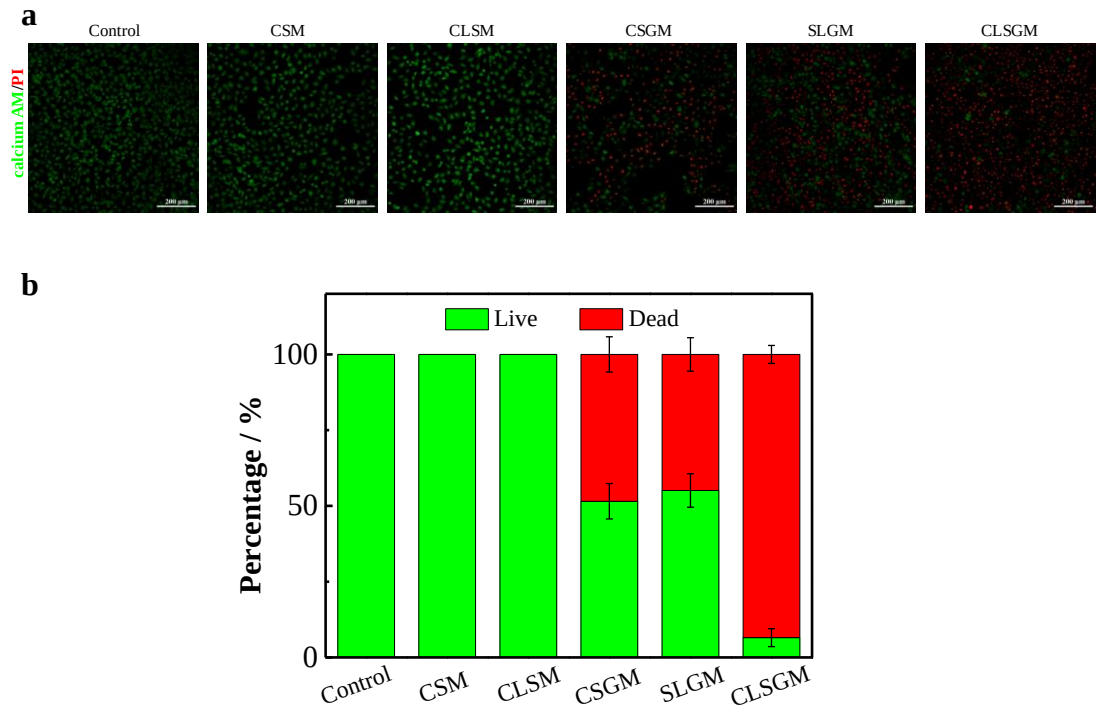

Fig. S18 (a) Confocal images of U87 MG cells stained with calcein AM/PI for distinguishing live/dead cells with different treatments. (b) Percentage of Calcein AM (green fluorescence for live cells)/PI (red fluorescence for dead cells) double stained U87 MG cells with different treatments in a.

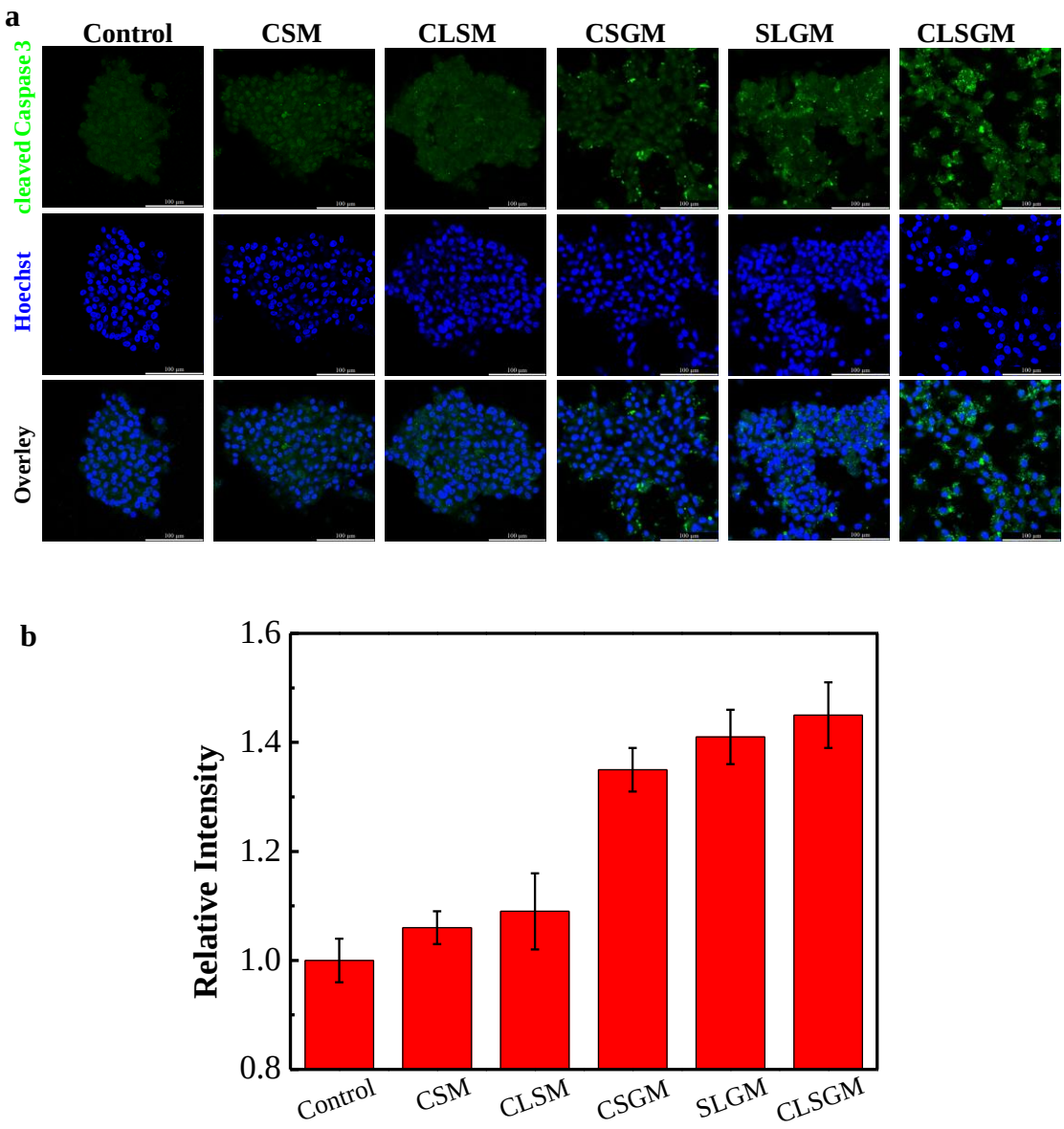

Fig. S19 (a) Confocal images of U87 MG cells after different treatments (Control, CSM, CLSM, CSGM, SLGM, CLSGM) to detect the expression of cleaved caspase 3. (b) Relative fluorescence intensity of the corresponding images in (a) through quantitative fluorescence analysis.

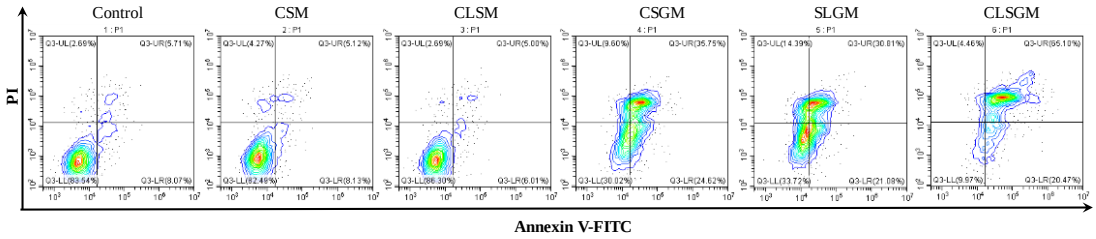

Fig. S20 Flow cytometry analysis of U87 MG cells with Annexin V-FITC/PI staining after different treatments.

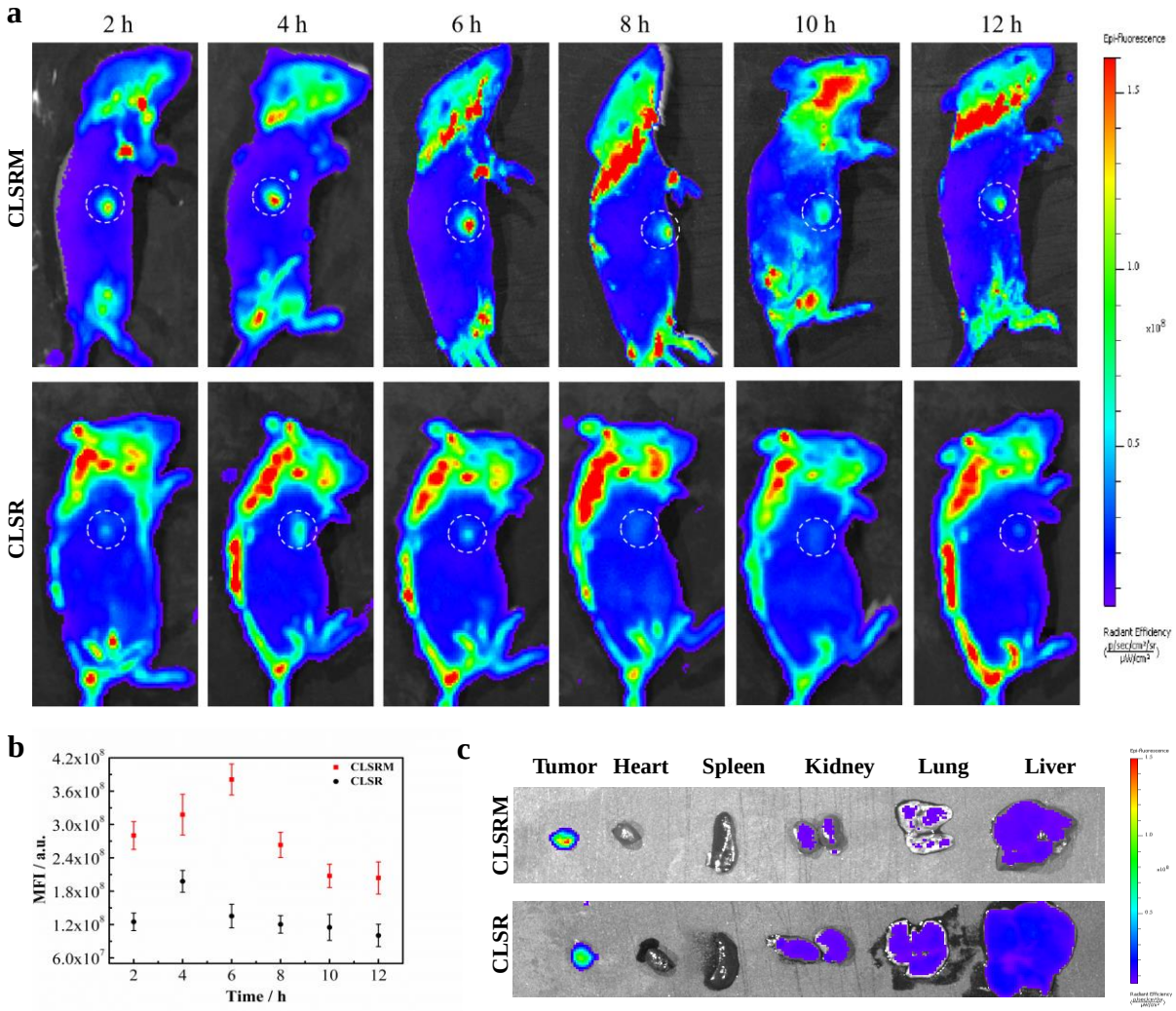

Fig. S21 *In vivo* targeting experiments. a) Time dependent fluorescence imaging of the mice bearing U87 MG xenografted tumor with intravenous injection of CLSRM (top) and CLSR(bottom) for the targeting analysis. The dotted circles are tumors. b) Quantitative fluorescence intensity of tumors in a. c) Fluorescence images of tumors and major organs extracted from the mice treated with CLSR or CLSRM at 6 h post intravenous injection.

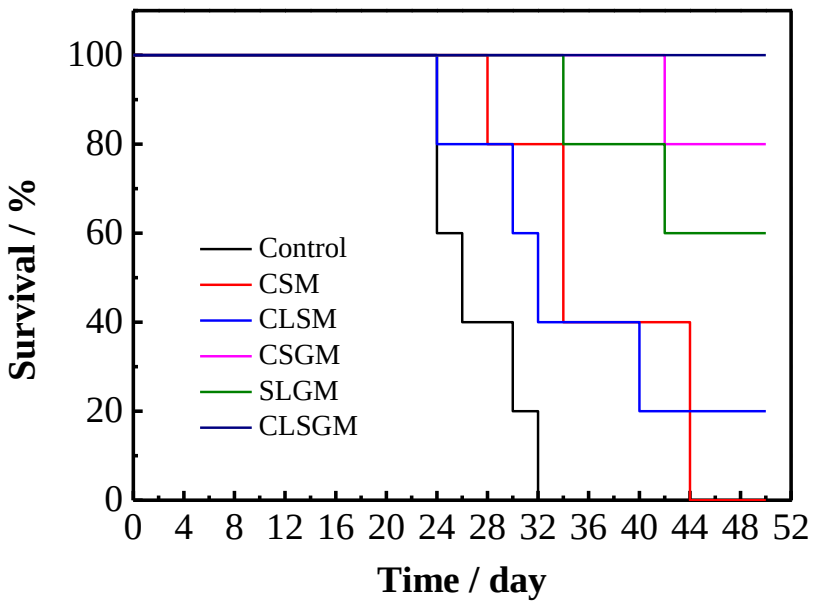

Fig. S22 Survival rates of mice in each group after receiving different treatments.

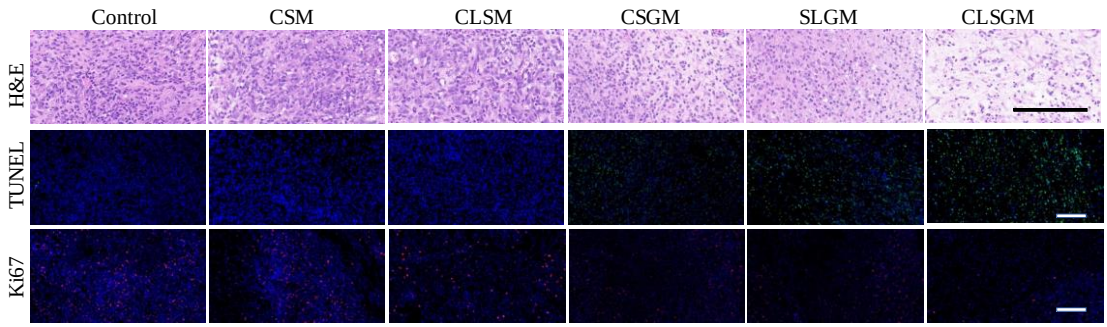

Fig. S23 H&E, TUNEL and Ki67 staining images of tumor sections with different treatments at 1 day post injection. Scale bars are 100 μm.

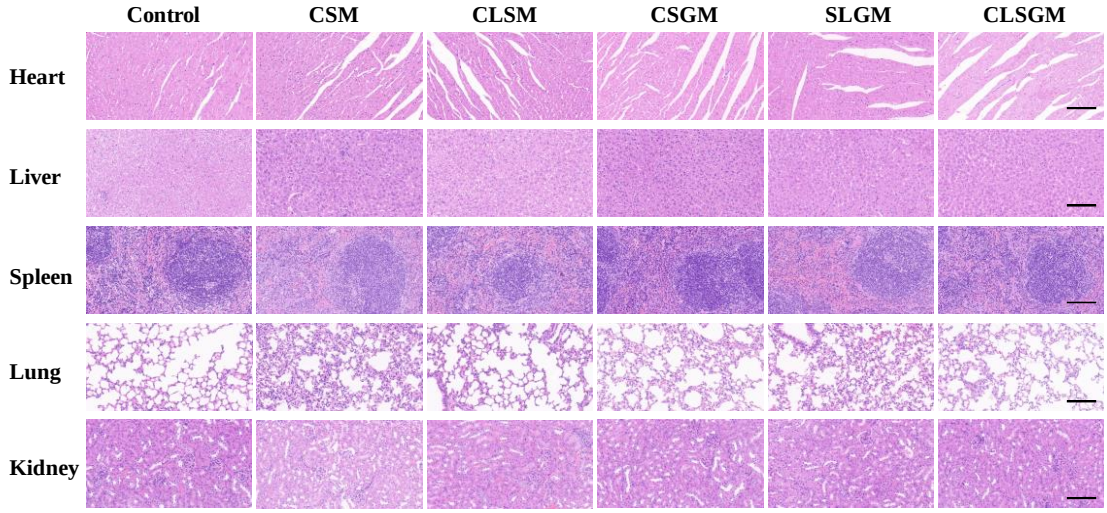

Fig. S24 H&E staining images of five major organs after the mice were treated with CSM, CLSM, CSGM, SLGM and CLSGM. (Scale bars are 100 μm)

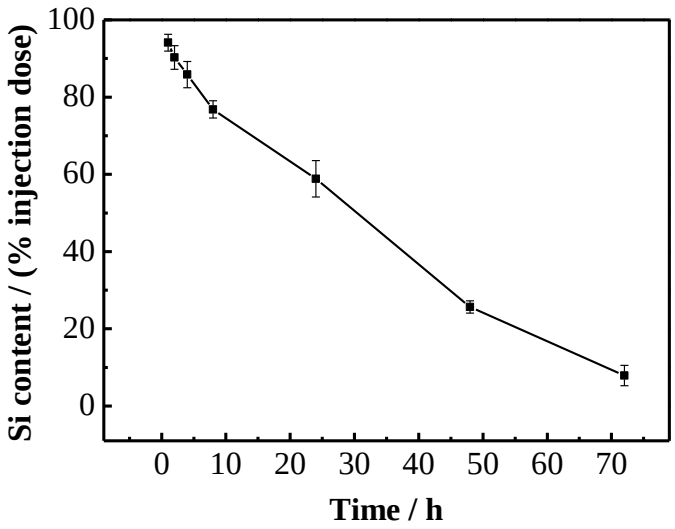

Fig. S25 Metabolism analysis by measuring the retention of CLSGM in the body at different time *via* ICP-OES.