Electronic Supplementary Information

In-Situ Synthesis of Superorganism-Like Au NPs within Microgels with Ultra-Wide Absorption in Visible and Near-Infrared Region for Combined Cancer Therapy

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Materials and methods

1. Materials and Instruments

Gelatin, ethylene diamine tetraacetic acid disodium salt dihydrate (EDTA•2Na) and chloroauric acid ($HAuCl_4$) were purchased form Sinopharm Chemical Reagent Co. Ltd (China). Sodium borohydride was purchased from Shanghai Aladdin Bio-Chem Technology Co. Ltd (China). Genipin was obtained from Woka Company (Japan). Hexadecyltrimethylammonium bromide (CTAB), sodium carbonate (Na_2CO_3), calcium chloride dihydrate ($CaCl_2$•$2H_2O$), 9,10-anthracenediylbis(methylene) dimalonic acid (ABDA) and 2',7-dichlorodihydrofluorescein-diacetate (DCFH-DA) were purchased from Sigma-Aldrich without further purification. Penicillin, streptomycin, Dulbecco's modified Eagle medium (DMEM), and fetal bovine serum (FBS) were obtained from Invitrogen. All aqueous solutions were prepared using ultrapure water (18.2 MΩ•cm) from Milli-Q system (Millipore, USA).

Transmission electron microscopy (TEM) images were captured by JEOL JEM-2100 TEM at 200 kV. Scan electron microscopy (SEM) images were obtained from Hitachi-S4800 equipped with energy-dispersive X-ray spectroscopy (EDS). A Lecia SP-8 confocal microscope was used for cell imaging. Shimadzu UV-2600 spectrophotometer was used for recording UV-Vis absorption spectra with a quartz cell (1 cm optical length). HITACHI F-4600 spectrophotometer was used to record fluorescence emission spectra.

2. Methods

2.1. Preparation of $CaCO_3$ Doped with Gelatin.

$CaCO_3$ microparticles doped with gelatin were prepared by mixing $CaCl_2$•$2H_2O$ and Na_2CO_3 solution according to literature procedure. Gelatin (0.60 g) and $CaCl_2$•$2H_2O$ (1.47 g) were dissolved in DI water (100 mL) under stirring at 1200 rpm for 30 minutes at 60^{o}C. After cooling down to room temperature, the solution of Na_2CO_3 (1.06 g) in DI water (10 mL) was added under stirring at 500 rpm for 20 s. The prepared

microparticles were centrifuged at 8000 rpm for 2 minutes and then washed with DI water and ethanol for three times. Finally, the microparticles were vacuumed to dry and stored in 4°C refrigerator for further use.

2.2. Preparation of Au seeds@$CaCO_3$, HB@$CaCO_3$ and HB@Au seeds@$CaCO_3$.

Au seed solution was prepared by mixing of 1.5 mL 0.1M CTAB, 50 μL 0.01M $HAuCl_4$ and 120 μL 0.01M $NaBH_4$. For Au seeds@$CaCO_3$, 1.5 mL of Au seed solution mixed with 20 mg $CaCO_3$, and then shook for 30 minutes on a shaker. Au seeds@$CaCO_3$ were separated by centrifugation at 8000 rpm for 2 minutes. By repeating the loading process, Au seeds@$CaCO_3$ with different amounts of Au seeds were obtained. For HB@$CaCO_3$, 1.5 mL 100 μg/mL HB solution in acetone mixed with 20 mg $CaCO_3$, and then shook for 30 minutes. HB@$CaCO_3$ were separated by centrifugation at 8000 rpm for 2 minutes. The loading process was repeated until the absorption of HB solution had no obvious change before and after mixing with $CaCO_3$. For HB@Au seeds@$CaCO_3$, Au seeds were firstly loaded and then HB was loaded according to above procedure.

2.3. Preparation of Au@Gelatin, HB@Gelatin and HB@Au@Gelatin by "casting" strategy.

To obtain Au@Gelatin, HB@Gelatin and HB@Au@Gelatin, 20 mg $CaCO_3$ microparticles loaded with Au seeds, HB or both were mixed with 2 mL gelatin solution (20 wt%) at 60°C for 6 h. Then 8 mL DI water at 4°C was added quickly. The microparticles were separated by centrifugation at 8000 rpm for 2 minutes and 2 mL (0.625 wt%) genipin solution was added to cross-link gelatin for 16 h at room temperature. The cross-linked $CaCO_3$-gelatin was treated with 0.1 M ethylenediaminetetraacetic acid disodium salt (EDTA•2Na, PH=7.0), purified by centrifugation at 2500 rpm for 2 minutes and washed with DI water. These three hybrid colloidal spheres mentioned above were obtained and stored at 4°C for further use. For Au@Gelatin and HB@Au@Gelatin, a certain amount of Au growth solution was added to increase the size of Au NPs in hybrid colloidal spheres. With the addition of Au growth solution, the size of Au seeds in microgels gradually increased. After adding growth solution for 6 times, Au NPs size was about 50 nm within Au@Gelatin and HB@Au@Gelatin. The individual 50 nm Au NPs were prepared according to literature

procedure.[20] Au growth solution was prepared by mixing of 8 mL 0.1 M CTAB,400 μL 0.01M $HAuCl_4$, 100 μL 0.01M $AgNO_3$ and 80 μL 0.1M Vc.

2.4. Regulation of size and accumulation state of Au NPs in Au@Gelatin and HB@Au@Gelatin.

Firstly, Au seeds@$CaCO_3$ were prepared according to the experimental procedure described above. In order to improve Au content in $CaCO_3$, the procedure was repeated for 2, 4 and 6 times. If the repeating time was more than 6, the structure of $CaCO_3$ changed and some was broken. Au seeds@Gelatin with different contents of Au seeds were named Au seeds@$Gelatin_2$, Au seeds@$Gelatin_4$ and Au seeds@$Gelatin_6$. The size of Au seeds in gelatin microgels was 1-3 nm. Then 8 mL Au growth solution was mixed with Au seeds@Gelatin and then shook for 30 minutes on a shaker. Au@Gelatin were separated by centrifugation at 2500 rpm for 2 minutes. In order to increase the size and accumulation state of Au NPs in gelatin microgels, the addition of Au growth solution was also repeated for many times. When the repeated times was 6, the size of Au NPs increased to 50 nm and the size of Au NPs had no obvious change when more Au growth solution was added. Au NPs had occupied the whole gelatin microgels. The microgels were named Au@$Gelatin_2$, Au@$Gelatin_4$ and Au@$Gelatin_6$. For HB@Au@Gelatin, the same method was used. The size and accumulation state of Au NPs in microgels were adjusted by changing addition times of Au seed solution and Au growth solution.

2.5. Cellular uptake and ROS Detection.

For cellular internalization observation, Hela cells were seeded in plastic bottomed Ibidi μ-dishes (35 mm) and allowed to grow for 24 h. After incubation with the microparticles for 24 h, cells were washed three times with 1mL phosphate buffered saline (PBS, pH 7.4) and then stained with 1 mL Alexa Fluor 488 (1 μg/mL) in PBS for 10 min in dark. After washing with PBS for three times, cells were subjected to confocal microscopy observation (100×oil objective, 408/561 nm excitation).

To evaluate the ability of producing ROS in cells, Hela cells were incubated with HB@Gelatin and HB@Au@Gelatin at an equivalent gelatin concentration of 10 mg/mL for 24 h. After the medium was removed, cells were washed three times with

1mL PBS (pH 7.4). Then, the cells were incubated in medium solution containing DCFH-DA (10 μM) for 10 min at 37°C and then irradiated with a 680 nm laser (1 W/cm^2) for 5 min. After another 15 min of incubation at 37°C, the cells were washed three times with 1mL PBS (pH 7.4) and fluorescent images of DCFH-DA staining on cells were captured by using a Leica confocal microscope (100×oil objective, 488 nm excitation).

2.6. PTT or PDT effects of Au@Gelatin, HB@Gelatin and HB@Au@Gelatin on Hela cells.

For Calcein-AM/PI staining, Hela cells seeded in μ-dishes were incubated in medium solution containing 50 μL of gelatin, Au@Gelatin, HB@Gelatin and HB@Au@Gelatin at 10 mg/mL gelatin concentration. After incubating for 24 h in dark, Hela cells were washed three times with PBS. Then the cells were incubated in fresh culture medium and irradiated with a 680 nm laser (1 W/cm^2) for different times. After incubating for another 4 h at 37°C, the cells were treated with live/dead assay reagent. The cells were observed by using a Leica confocal microscope (10×objective, 488/561 nm excitation). Hela cells were seeded into 96-well plate at a density of 1×10^4 cells per well and maintained in DMEM medium (100 μL) for 24 h. The culture medium was then replaced with fresh culture medium (100 μL) containing gelatin, Au@Gelatin, HB@Gelatin or HB@Au@Gelatin. After incubation for 24 h, cells were washed three times with 100 μL PBS and refilled with 100 μL of fresh culture medium. The plates were irradiated with a 680 nm laser (1 W/cm^2) for 5 min. The cells were further cultured for 12 h and then culture medium in each well was replaced with 100 μL medium solution containing 10 μL CCK-8. After incubating for another 4 h, the absorbance intensity at 450 nm was recorded by using a microplate reader (Synergy H4, BioTek).

2.7. Measurement of photothermal conversion efficiency.

In order to compare photothermal conversion ability of Au seeds@Gelatin, Au@Gelatin, HB@Au@Gelatin and Au NPs (50 nm), 1 mL each sample solution (10 mg/mL in DI water) was placed in a quartz cell and irradiated with a 680 nm laser (1 W/cm^2) for 960 s. The solution temperature was real-time monitored by a thermocouple probe with a digital thermometer. After laser off, the solution temperature was still

monitored for another 960 s. For photothermal conversion stability test of Au@Gelatin, the above procedure was repeated for three times at different wavelengths. 0.1 M PBS (PH=8) was used as a control under the same condition. Photothermal conversion efficiency (η) of Au@Gelatin and HB@Au@Gelatin for different wavelengths was calculated based on reported methods.

2.8. The model of tumor-bearing mice.

All animal experiments were performed under the guidance of Chinese laws and regulations of experimental animal welfare and approved by Institutional Animal Care and Use Committee (IACUC). To obtain tumor-model mice, female nude mice (average weight 15-20 g) were injected 200 μL Hela cells suspension (1×10^7/mL in PBS) at the right hind hip or at the armpit. When tumor size grew to 50-100 mm^3, 100 μL PBS, HB@Gelatin, Au@Gelatin or HB@Au@Gelatin solution were injected into tumor sites. After 1 h, tumor sites were irradiated with a 680 nm laser ($0.5W/cm^2$) for 5 or 10 min. In infrared thermal imaging experiment, mice were captured pictures every minute for 5 minutes by using infrared thermal imager. Tumor size was calculated by using digital caliper with the formula volume = 1/2•ab^2（a = tumor length, b = tumor width）.

2.9. Calculation of the photothermal conversion efficiency

The photothermal conversion efficiency of Au@Gelatin and HB@Au@Gelatin was determined according to previous method[1-2]. Detailed calculation was given as following: based on the total energy balance for this system

$$\sum_i m_i C_{p,i} \frac{dT}{dt} = Q_{NPs} + Q_s - Q_{loss} \quad (1)$$

where m and C_p are the mass and heat capacity of solvent (water), respectively. T is the solution temperature.

Q_{NPs} is the photothermal energy input by Au@Gelatin or HB@Au@Gelatin:

$$Q_{NPs} = I(1 - 10^{-A_\lambda})\eta \quad (2)$$

where I is the laser power, A_λ is the absorption of Au@Gelatin at the wavelength of

680 nm, and η is the conversion efficiency from the absorbed light energy to thermal energy.

Q_s is the heat associated with the light absorbance of PBS solution.

Q_{loss} is thermal energy lost to the surroundings:

$$Q_{loss} = hA\Delta T \tag{3}$$

where h is the heat transfer coefficient, A is the surface area of the lighting point, and ΔT is the temperature change, which is defined as $T - T_{surr}$ (T and T_{surr} are the solution temperature and ambient temperature of the surroundings, respectively).

At the maximum steady-state temperature, the heat input is equal to the heat output, that is:

$$Q_{NPs} + Q_s = Q_{loss} = hA\Delta T_{max} \tag{4}$$

where ΔT_{max} is the temperature change at the maximum steady-state temperature. According to the Eq.2 and Eq.4, the photothermal conversion efficiency (η) can be determined:

$$\eta = \frac{hA\Delta T_{max} - Q_s}{I(1 - 10^{-A_\lambda})} \tag{5}$$

In this equation, only hA is unknown for calculation. In order to get the hA, we herein introduce θ, which is defined as the ratio of ΔT to ΔT_{max}:

$$\theta = \frac{\Delta T}{\Delta T_{max}} \tag{6}$$

Substituting Eq.6 into Eq.1 and rearranging Eq.1:

$$\frac{d\theta}{dt} = \frac{hA}{\sum_i m_i C_{p,i}}\left[\frac{Q_{NPs} + Q_s}{hA\Delta T_{max}} - \theta\right] \tag{7}$$

When the laser was shut off, the $Q_{NPs} + Qs = 0$, Eq.7 changed to:

$$dt = -\frac{\sum_i m_i C_{p,i}}{hA}\frac{d\theta}{\theta} \tag{8}$$

Integrating Eq.8 gives the expression:

$$t = -\frac{\sum_i m_i C_{p,i}}{hA} In\theta \quad (9)$$

Thus, hA can be determined by applying the linear time data from the cooling period vs -lnθ. Substituting hA value into Eq.5, the photothermal conversion efficiency (η) of Au@Gelatin or HB@Au@Gelatin can be calculated.

References

1. W. Ren, Y. Yan, L. Zeng, Z. Shi, A. Gong, P. Schaaf, D. Wang, J. Zhao, B. Zou, H. Yu, G. Chen, E. M. Brown and A. Wu, *Adv. Healthc. Mater.*, 2015, **4**, 1526-1536.
2. Y. Liu, K. Ai, J. Liu, M. Deng, Y. He and L. Lu, *Adv. Mater.* 2013, **25**, 1353-1359.

Figures

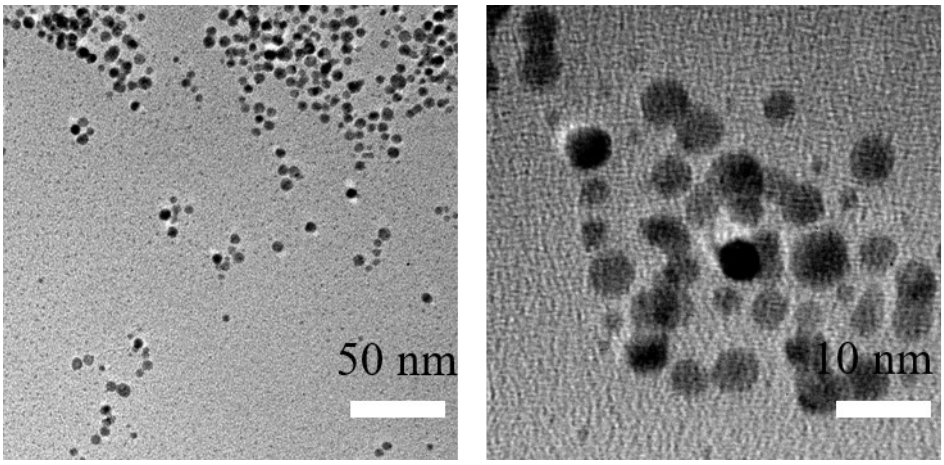

Figure S1. The TEM images of Au seeds.

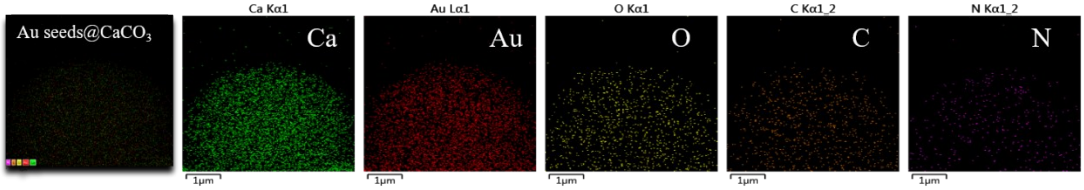

Figure S2. The element mapping of Au seeds@$CaCO_3$. The C, N and O element were from gelatin doped in the $CaCO_3$.

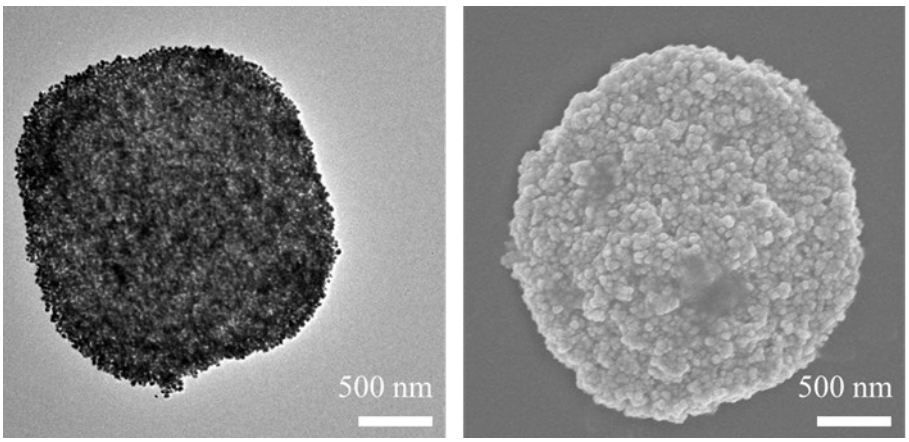

Figure S3. The TEM and SEM image of Au@Gelatin after irradiation for three cycles.

Figure S4. The digital images of the solution of Au NPs (50 nm) before and after irradiation with 680 nm laser for a cycle of heating.

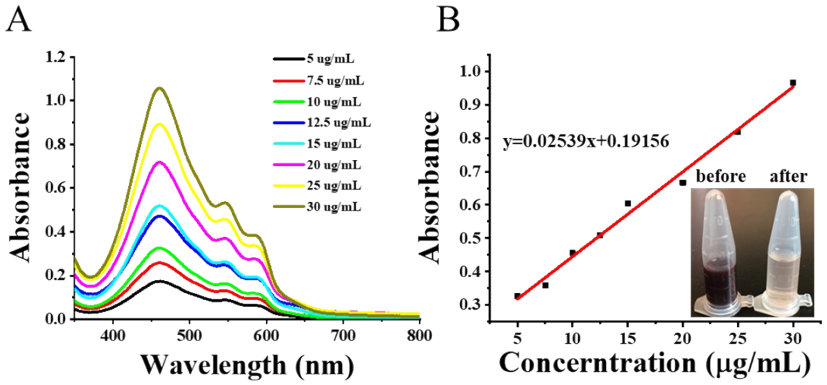

Figure S5. (A) UV-Vis spectrum of HB solution in acetone at different concentration. (B) is the standard cure of HB solution in acetone. The insert image showed the color change of solution after mixing with $CaCO_3$ indicating the HB was absorbed into the $CaCO_3$.

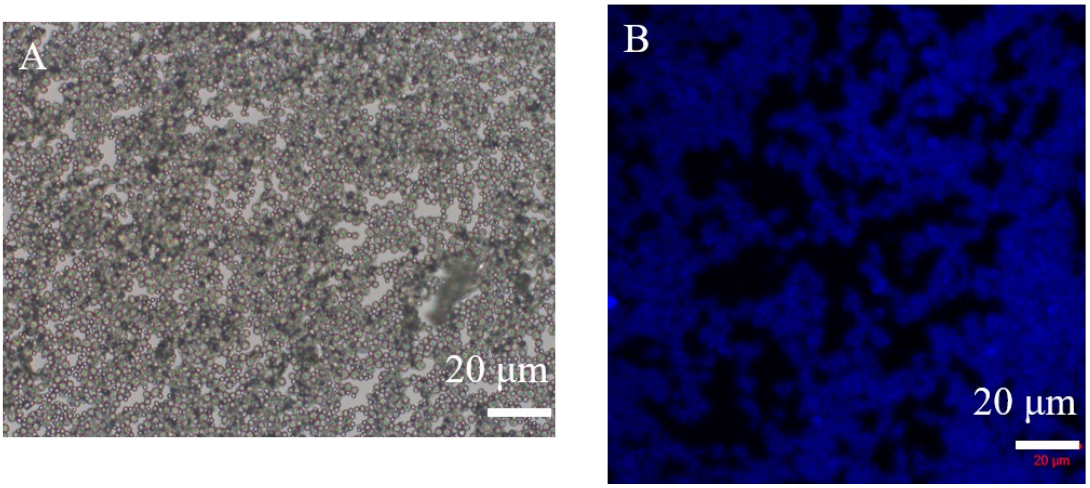

Figure S6. The CLSM images of HB@$CaCO_3$ excited by 405 nm. (A) is the image of light field. (B) is the image excited by 405 nm. The blue fluorescence indicated the HB had been loaded into the $CaCO_3$.

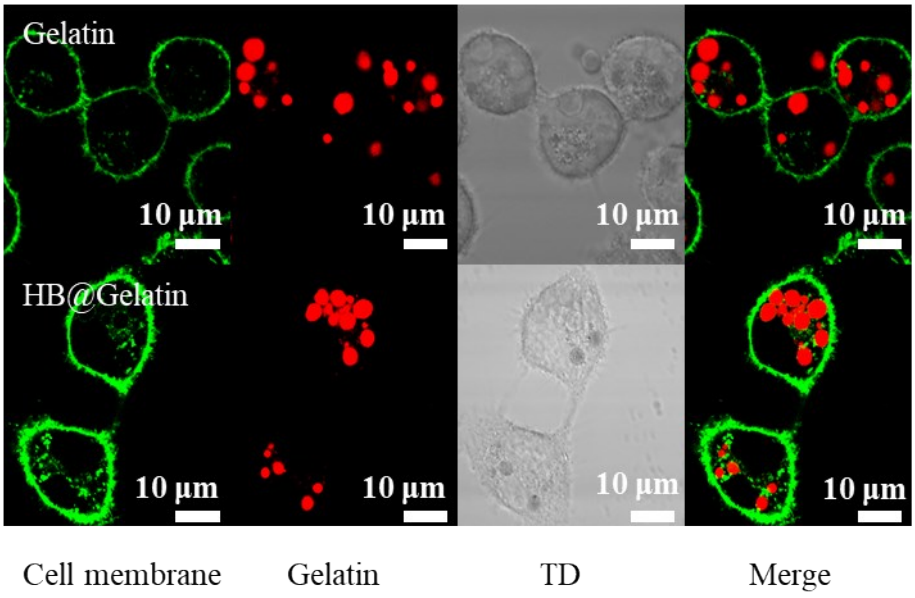

Figure S7. The CLSM images of Hela cells after Gelatin and HB@Gelatin being taken up into the cells. The membrane was stained with Alexa Fluor 488 (green fluorescence) and the gelatin was excited with 559 nm laser (red fluorescence).

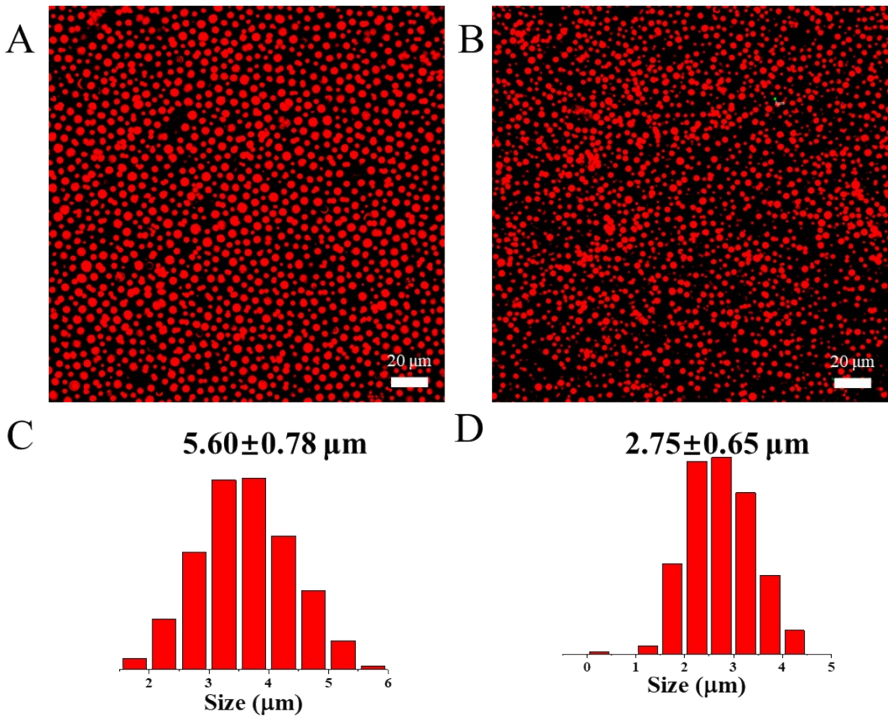

Figure S8. The CLSM images of Gelatin microgels A) in water and B) in PBS. The average size of Gelatin microgels C) in water and D) in PBS. The size of Gelatin microgels is calculated by using imageJ.

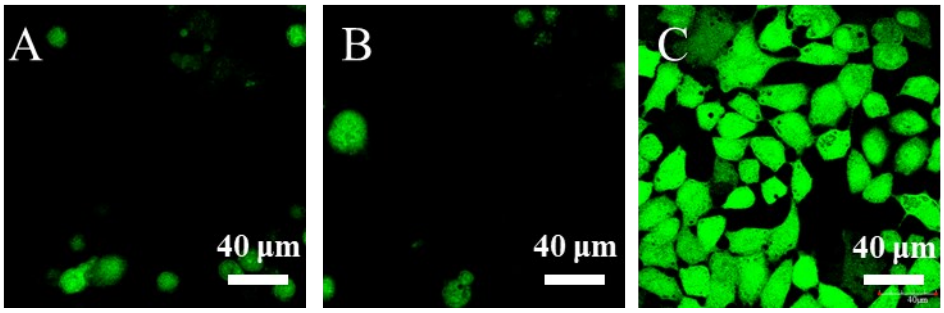

Figure S9. The CLSM images of Hela cells A) after incubating with HB@Gelatin but without irradiation B) after incubating with irradiation but without HB@Gelatin C) after incubating with HB@Gelatin and with irradiation. The green fluorescence was from DCF indicated that a large amount of ROS was produced.

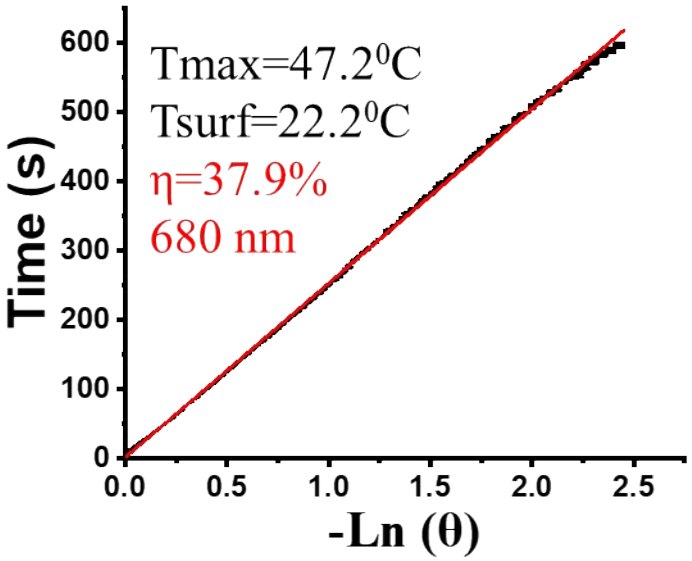

Figure S10. The time versus the negative natural logarithm of the temperature from the cooling test for HB@Au@Gelatin with 680 nm (1W/cm^2). The fitted values were used to calculate the photothermal conversion efficiency.

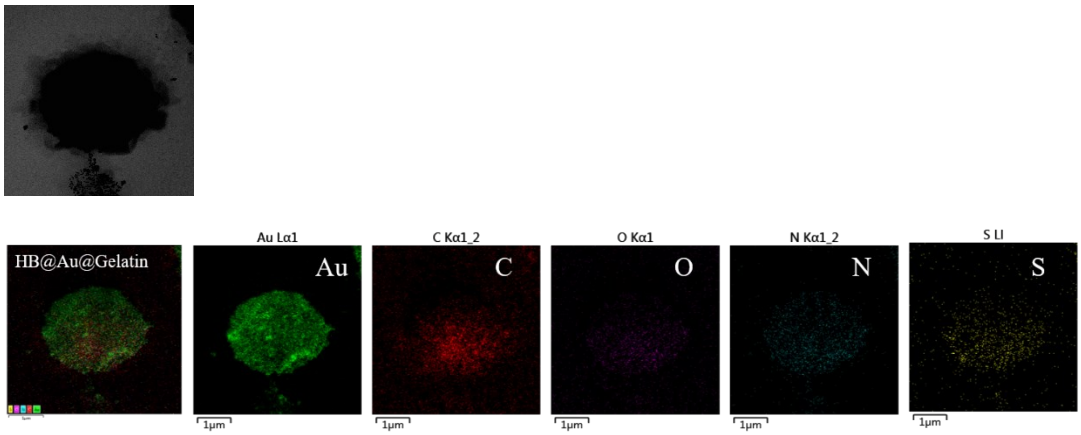

Figure S11. The TEM image (upper) and element mapping (lower) of HB@Au@Gelatin. The C, O, N and S element were from gelatin or HB.

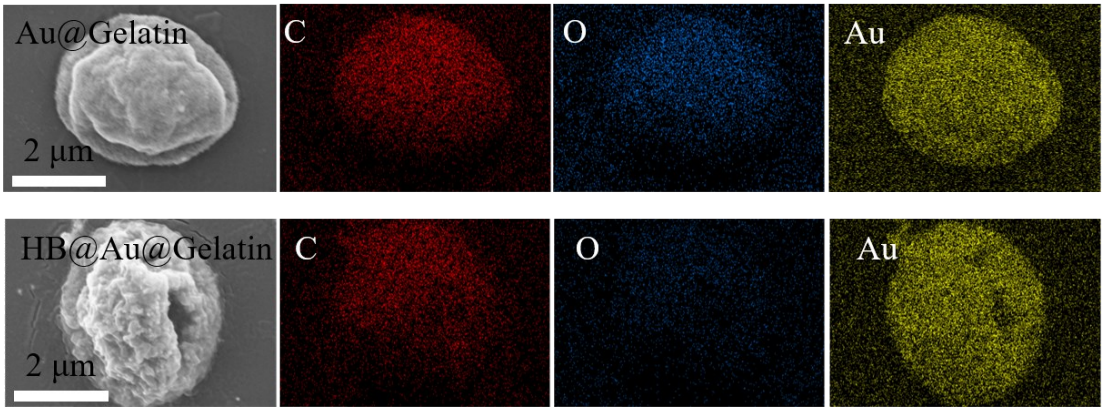

Figure S12. The SEM images and element mapping of Au@Gelatin, HB@Au@Gelatin for C, O and Au element.

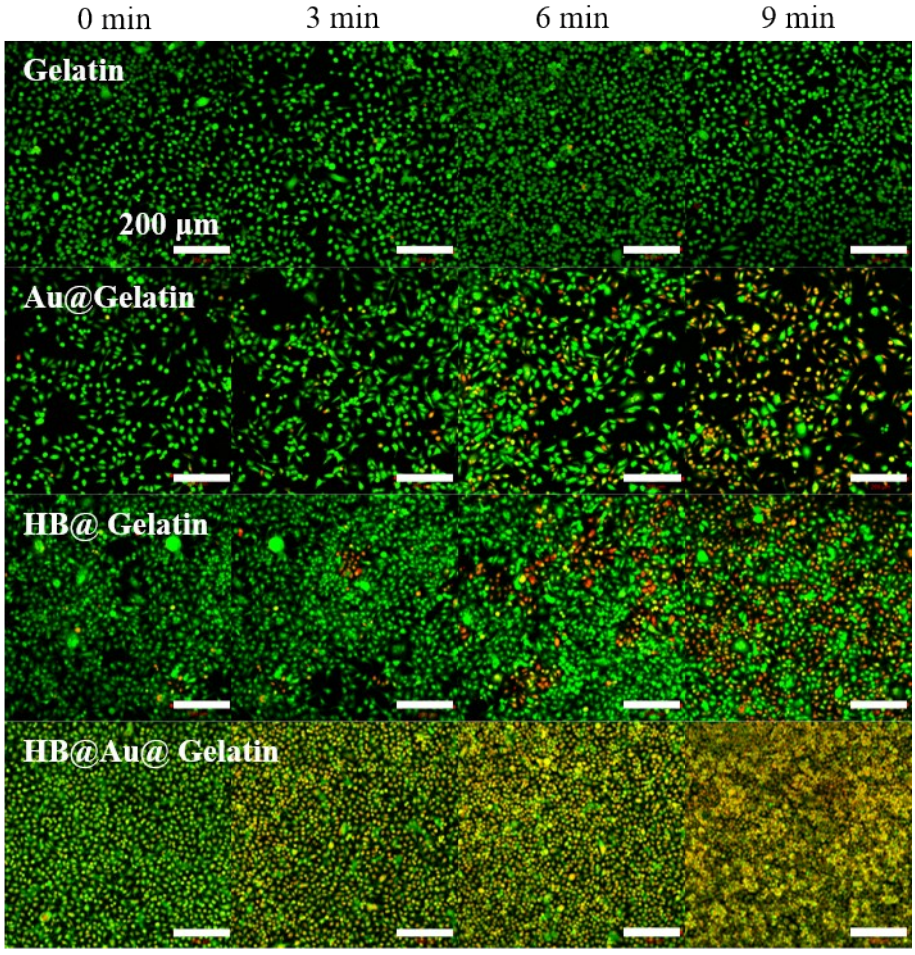

Figure S13. The Calcein-AM/PI staining of Hela cells after incubating with Gelatin, HB@Gelatin, Au@Gelatin and HB@Au@Gelatin (100 μg/mL) for 12 h and then irradiation with a 680 nm laser (1 W/cm^2) for different time. The scale bar is 200 μm.

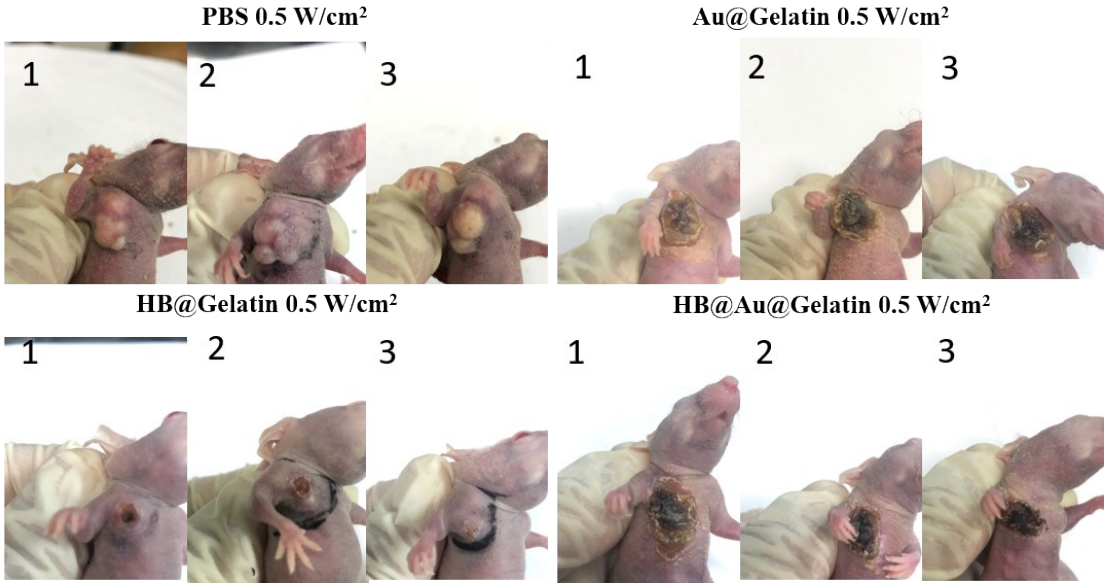

Figure S14. The digital images of different groups of mice the day before they were sacrificed.