

## Supporting Information

### **Zwitterionic Chitooligosaccharide Modified Ink-blue Titanium Dioxide Nanoparticles with Inherent Immune Activation for Enhanced Photothermal Therapy**

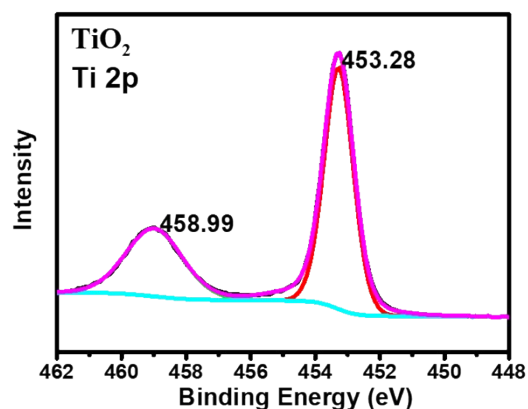
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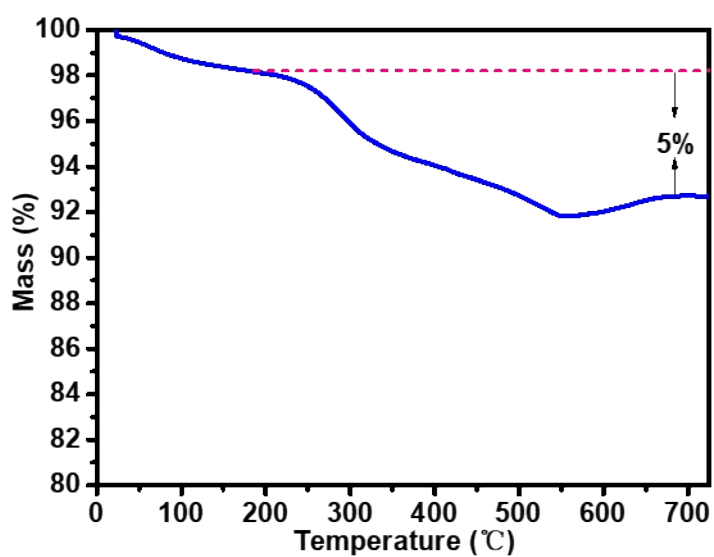
\* E-mail: zhiy@nankai.edu.cn.



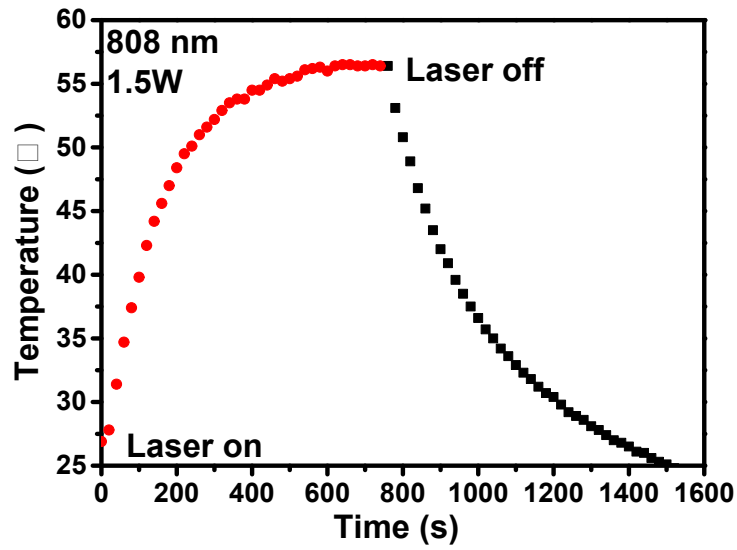
**Figure S1.** Fitted Ti 2p XPS spectra of white titanium dioxide (P25).

**Table S1.** The zeta potential of the BTiO<sub>2</sub>-COS NPs at pH 7.4 and pH 6.8

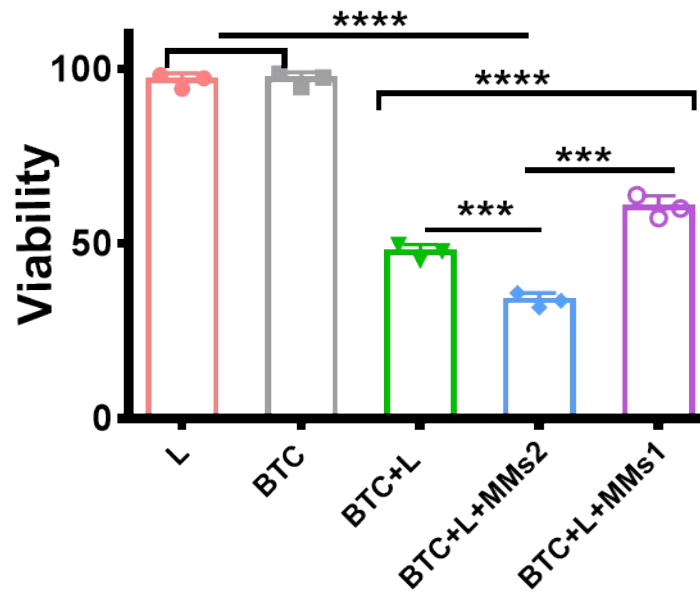
sample	-COOH(%)	-NH <sub>2</sub> (%)	COOH:NH <sub>2</sub>	ξ (pH 7.4)	ξ (pH 6.8)
BTiO <sub>2</sub> -COSA	24.7	91	0.27	-10 mV	+9.5 mV
BTiO <sub>2</sub> -COSB	37.6	83.9	0.45	-15 mV	+3.5mV
BTiO <sub>2</sub> -COSC	50.98	70	0.72	-20 mV	-2.5mV



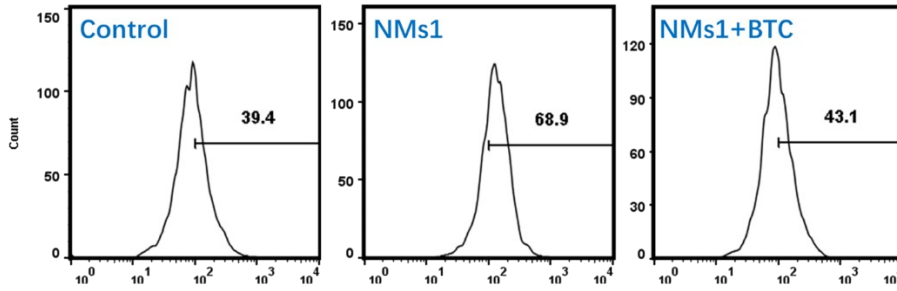
**Figure S2.** The thermogravimetric analysis of BTC NPs.



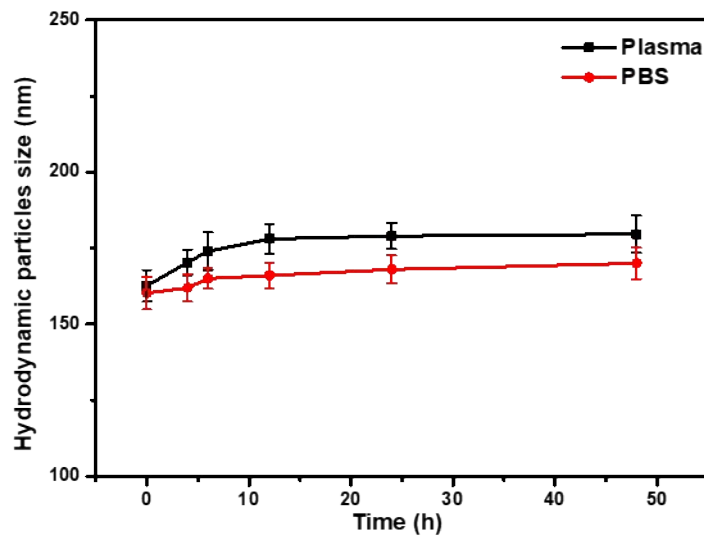
**Figure S3.** Time constant for heat transfer calculated from the cooling period of BTiO<sub>2</sub>



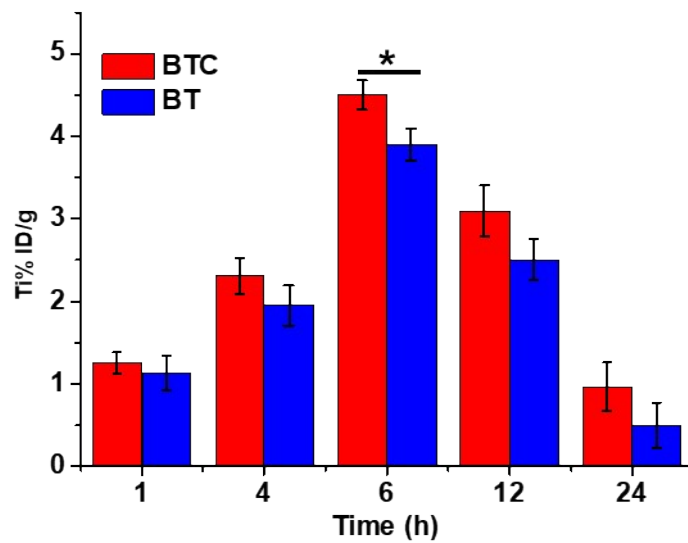
**Figure S4.** Relative cell viability of H22 cells after different treatment. The experiment was performed three times in parallel and is presented as the mean  $\pm$  SD. \*\*\*  $P < 0.001$   
\*\*\*\*  $P < 0.0001$



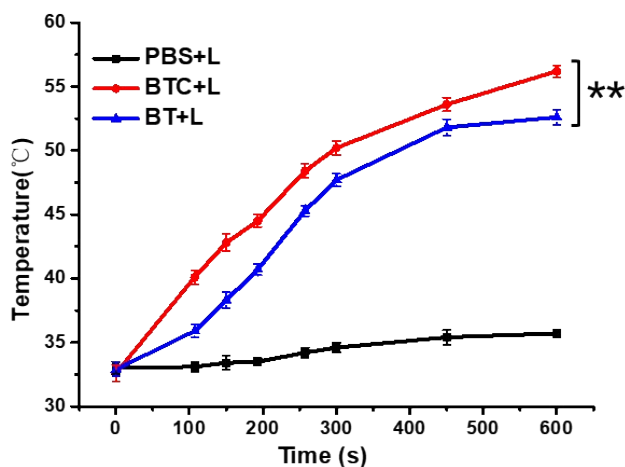
**Figure S5.** Flow cytometric analysis of expression of CD206 (M2 related marker) after treatment with necrotic cell secretion (NMs1) and BTC.



**Figure S6.** Particle stability of BTC NPs in PBS, rat ex-vivo plasma at 37 °C. Data were presented as mean  $\pm$  SD (n = 3).



**Figure S7.** Ti element bio-distribution in tumor at different time points post-intravenous injection of BTC and BT. Data represent three separate experiments and are presented as the mean  $\pm$  SD. \*  $P < 0.05$



**Figure S8.** The curve of tumor area temperature as a function of irradiation time. Data represent three separate experiments and are presented as the mean  $\pm$  SD. \*\*  $P < 0.01$

## Experimental Section

*Preparation of B-TiO<sub>2</sub> NPs:* Oxygen-deficient blue titanium dioxide nanoparticles were prepared by a high temperature reduction method with reference to previously published literature. The specific method is as follows: 1 g of P25 titanium white powder and 400 mg of NaBH<sub>4</sub> are taken and thoroughly mixed in an agate mortar. The mixed powder was then transferred to a small porcelain boat and placed in an argon-filled tube furnace, heated to 300 °C at a rate of 10 °C per minute and maintained for 1 h, finally cooled to room temperature. The burned product was dissolved in 200 mL of water and stirred for 12 h to completely remove the remaining NaBH<sub>4</sub>. The aqueous solution was centrifuged, and the precipitate was further dissolved in 50 mL of water. After ultrasonication for 2 min, it was resuspended by centrifugation. After repeating this step for 3 times, the precipitate obtained by the last centrifugation was vacuum dried for 12 h to obtain blue titanium dioxide nanoparticles.

*Synthesis of zwitterionic oligochitosan derivatives with different degrees of carboxymethyl substitution:* 2g COS was ultrasound dissolved in 10 mL deionized water, then add a certain amount of NaOH aqueous solution (20% w), the mixture was magnetically stir for 1h, then certain amount of chloroacetic acid aqueous solution (0.1g / mL) was dropwisely added into the above solution, finally the mixed solution was heated to 60 ° C and reacted for 6 h. In order to obtain different carboxymethyl substituted products, the amount of chloroacetic acid added was 10 mL, 20 mL and 40 mL, respectively, and the added mass of NaOH was 1.5 times that of chloroacetic acid. The obtained reaction product was added to 100 mL ethanol for precipitation, and the crude product was obtained by suction filtration, the crude product was dissolved in 50 mL of water, and precipitated again in ethanol. After three times of precipitation purification, the reaction cake was vacuum dried for 24 h to obtain zwitterionic oligochitosan derivatives with different degrees of carboxymethyl substitution.

*Fabrication of zwitterionic oligochitosan modified ink-blue titanium dioxide nanoparticles:*20 mg of COS was dissolved in 20 mL of deionized water, and then an aqueous blue titanium dioxide nanoparticle solution (1 mg/mL) was dropwise added to the chitooligosaccharide derivative solution under magnetic stirring. After the addition was completed, the mixture was placed in a probe-type ultrasonic for 5 min, and finally the reaction was continued for 10 h. After the reaction was completed, the resulting solution was centrifuged (10000 rpm, 10 min), and the precipitate was re-ultrasonic dispersed to deionized water and freeze-drying to obtain BTC NPs.

*Cell culture:*Mouse macrophage RAW 264.7, mouse liver cancer H22 cancer cells and

human hepatoma bel-7402 cell were cultured in RPMI 1640 (10% FBS, 1% penicillin–streptomycin) in Thermo Scientific CO<sub>2</sub> Incubators at 37°C and 5% CO<sub>2</sub> atmosphere. Bel-7402 cells were plated in 6-well plates, wait for cells to adhere for 24 h, add BTiO<sub>2</sub> (0.4 mg/mL), continue to culture for 24 h, then aspirate the supernatant, wash twice with PBS, add fresh medium, and irradiate with 808 nm laser (1.5 W, 10 min). Next, after incubating for 12 h in an incubator, the supernatant medium was collected and centrifuge to remove excess nanoparticles to obtain NTC-Ms1. The untreated bel-7402 cell supernatant was collected as a control group NTC-Ms0.

*In vitro macrophage re-polarization ability of BTC:* First, the primary undifferentiated macrophages (M<sub>0</sub>) was incubated with the inducible factor il-4 (100 ng/mL) for 48 h to obtain M2 type macrophages. M2 type macrophages were centrifuged and plated to 6-well cell culture plates. After waiting for 24h cell attachment, BTiO<sub>2</sub> (0.19 mg/mL), COS (0.01 mg/mL), BTC (0.2 mg/mL) were added to the wells, and after 24 h of continuous culture, the cells were subjected to flow immunofluorescence staining. The specific steps were as follows: the cells were re-suspended in PBS and centrifuged again, and then with a paraformaldehyde (4%) fix for 30 min, then re-suspended and washed twice with PBS. The cells were equally divided into two tubes, and CD206 primary antibody and CD86 primary antibody diluted by antibody dilution were added, then incubated at 4 ° C for 1 h. Next, wash twice with cold PBS, then the diluted secondary antibody was added, and continue to incubate at 4 ° C for 30 min, then centrifuge twice with cold PBS, and finally re-suspend at 200 µL PBS, then the fluorescence intensity was measured by flow cytometry (LSR Fortessa, BD).

Primary undifferentiated macrophages were plated into 6-well plates, cytokine il-4 (100 ng/mL) was added, and cultured for 48 h. The obtained cells were centrifuged and plated into a 6-well cell culture plate. After 24 h of cell attachment, BTiO<sub>2</sub> (0.19 mg/mL), COS (0.01 mg/mL), BTC (0.2 mg/mL) were added to the wells. After 24 h, the supernatant was collected and centrifuged to remove excess nanoparticles. The concentration of il-12 and il-10 in the obtained cell supernatant was measured using ELISA kit (Invitrogen). Primary undifferentiated macrophages were plated into 6-well plates, NTC-MS1 was added, cultured for 24 h, cell supernatants were collected, and the supernatants of the obtained cells were assayed for il-12 and il-10 concentrations by ELISA (Invitrogen).

*The M2-polarization effect of molecules released from necrotic tumor cell (NTC-MS):* The undifferentiated primary macrophage M0 was plated in a 6-well plate, and the supernatant was aspirated, and then incubated with NMs1 and NMs0 (control) for 24 h, and the cells were subjected to inverted fluorescence microscopy and flow cytometry. The cell supernatant of the NMs1 treatment group was collected and recorded as MMs1. Then, in the NMs1 culture group, BTC nanoparticles were added for further culture for 24 h, and the cell supernatant was collected and recorded as MMs2.

The H22 cells were plated into 24-well plates. After 24 h, the supernatant was removed by a plate-bending machine, BTC (0.2 mg/mL) diluted in the medium was added, and the cells were further cultured for 24 h, washed twice with PBS, and then added with NTC-MS1 and MMs1, followed by 808 nm laser irradiation (1.5W, 10 min). After incubation for 24 h in the incubator, cell viability assays were tested using cell counting



kit-8 (Beijing Solarbio Science & Technology Co., Ltd.).

The H22 cells were plated into 6-well plates. After 24 h, the supernatant was aspirated, BTC (0.2 mg/mL) diluted in the medium was added, and after 24 h of incubation, the supernatant was aspirated, washed twice with PBS, and MMs1 and MMs2 were added, respectively. Then, flowed by laser irradiation (808 nm, 1.5 W, 5 min). After 3 h of incubation, Annexin V-FITC/PI double staining was performed. As in the above procedure, the cells were changed to bel-7402 for AO/EB staining.

*Study on the ability of BTC NPs to remodel tumor-associated macrophage TAM:* H22 liver tumors were inoculated under the armpit of ICR mice. After 10 days of growth, the tumor volume was approximately 200-250 mm<sup>3</sup>. The mice were randomly divided into three groups, PBS, BTC and BT were injected into the tail vein respectively, maintain BTiO<sub>2</sub> concentration as 4 mg/mL. After 6 h of injection, the tumor site was irradiated with 808 nm laser (1.5 W, 10 min). Two days later, the mice were sacrificed by cervical dislocation, soaked in medical alcohol for 3 min, then dried, and placed in a clean bench to carefully remove the tumor. The tumor was cut into pieces and ground on a cell sieve to obtain a mixed cell suspension, and thee TAMs were isolated by using a mouse tumor macrophage extraction kit (Hao Yang biological manufacture, Tian Jin). TAMs were subjected to flow immunofluorescence staining of CD11b-CD45-F4/80-CD206 and CD11b-CD45-F4/80-CD86. Parallel mice were sacrificed, tumors were removed, dehydrated with sucrose, and then embedded. Finally, the tumors were sliced and subjected to tissue immunofluorescence staining.

*Tumor inhibition ability of BTC NPs:*H22 ascites cells were inoculated into the right

forelimb of ICR mice, and when the tumor volume was  $\sim 100 \text{ cm}^3$ , the mice were randomly divided into 5 groups. Next, PBS, COS, BTC, BT were injected into the tail vein respectively, the BT and BTC groups were followed with NIR laser irradiation (1.5W, 10 min). The tumor volume changes and weighed mice were observed every two days, after 23 days, mice were sacrificed and tumor were removed for histological H&E staining.

### **Materials and Characterization**

The chemicals applied in this study were P25 titanium white powder (AEROXIDE),  $\text{NaBH}_4$  (Acros organics), chloroacetic acid (Energy chemical), oligochitosan (Mw=800-1000, Dalian Meilun Biotechnology Co., Ltd) and cytokine IL-4 (genscript, USA). The antibodies used in the immunofluorescence staining were rat anti F4/80 antibody (17-4801-82, ebioscience), Ms CD11B PerCP-Cy5.5 (BD), Ms CD45 PE 30-F11(BD). The anti CD206 antibody (bs-4727R), and the anti CD86 antibody (bs-1035R) were all provided by Biosynthesis Biotechnology Inc. (Beijing, China). All fluorescently labeled secondary antibodies are from Biosynthesis Biotechnology Inc. (Beijing, China). AO-EB double staining kit was supported by Beijing Solarbio Science & Technology Co., Ltd. Annexin V-FITC/PI staining kit was purchased from Dalian Meilun Biotechnology Co., Ltd.

The optical absorption of materials was measured by UV-Vis spectrophotometer (SHIMADZU UV-2550 with an integrating Sphere), and the white barium sulfate solid powder was selected as a blank control. The morphology and dry size of the

nanoparticles were observed by Transmission electron microscopy (JEM-2800). The DLS and zeta potential measurements were performed on a Nano-ZS90 instrument (Malvern). The crystal ratio of the materials was obtained by X-ray diffraction (XRD) (D/max 2550, Rigaku) with Cu-K $\alpha$  (1.5406 Å) radiation. The surface electron spectroscopy was studied on an Axis Ultra DLD spectrometer (Kratos Analytical Ltd.). The Infrared spectroscopy was detected by Fourier transform infrared spectrometer (TENSOR II, Bruker). The Near-infrared photothermal experiments were completed by a semiconductor laser (Emission wavelength of 808 nm, WG1533D3) and a thermal imager (FOTRIC, US).