Electronic Supplementary Material (ESI) for Biomaterials Science.

Supporting Information

A Near-Infrared Light-Excitable Immunomodulating Nano-photosensitizer for Effective Photoimmunotherapy

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Materials

1-Octadecene (ODE), oleic acid (OA), ammonium fluoride, rare earth oxides yttrium (III) oxide (Y_2O_3), ytterbium (III) oxide (Yb_2O_3), thulium (III) oxide (Tm_2O_3), 2,5-Dibromoterephthalaldehyde and N-pheny-4-(tetrahydro-2H-pyran-2-yi)oxy)aniline2-(Hexamethyleneimino) ethanol (C7A) were purchased from Energy Chemical Co., Ltd. (Shanghai, China). The rare earth chlorides were synthesized through the oxide dissolved in hydrochloric acid. Methacryloyl chloride, bis(dibenzylideneacetone)palladium(0) [$Pd_2(dba)_3$], 2-dicyclohexylphosphino-2',6'-diisopropoxybiphenyl (Ruphos), *N*-(3-Aminopropyl)-methacrylamine hydrochloride (Apm), *N, N'*-methylene bisacryamide (Bis), ammonium persulfate (APS), *N*,*N*,*N*',*N*'-tetramethylethylenediamine (TEMED), Rhodamine 123, 9,10-Anthracenediyl-bis(methylene)dimalonic acid (ABDA) and Rose Bengal were purchased from Aladdin (Tianjin, China). 3, 5-Bis(trifluoromethyl)phenylacetonitrile, 4-hydroxydiphenylamine, 1,4-dibromo-2,5-dimethylbenzene, tripheylphosphine, dichlorofluorescein diacetate (DCFH-DA), methacryloyl chloride, and 2-methacryloyloxyethyl phosphorylcholine (MPC) were purchased from Heowns (Tianjin, China), and 4, 6-diamidino-2-phenylindole dihydrochloride (DAPI) were purchased from Invitrogen (USA). Antibodies for confocal laser scanning microscope observation (CLSM) were purchased from Biolegend and Invitrogen as follow, calreticulin (CRT) antibody (anti-CRT from rabbit, Invitrogen), CD4 antibody (anti-CD4 from Rat, Biolegend), CD8 antibody (anti-CD8 from Rat, Biolegend), high-mobility group box 1 (HMGB1) antibody (anti-HMGB1 from mouse). Antibodies for flow cytometry were obtained from Biolegend. ATP assay kit, paraformaldehyde and triton X-100 were purchased from Solarbio. All ELISA kits were obtained from Elabscience (China). Trypsin, fetal bovine serum (FBS) and RPMI 1640 culture medium were purchased from Sigma-Aldrich (Shanghai, China). THF, HCl, methanol and other chemical reagents were purchased from local chemical companies.

Instruments

Up-conversion luminescence spectra in near-infrared (NIR) range were collected on an Edinburgh FLS920 spectrometer (collected at range of 400-880 nm) with 980 nm laser as the excitation source. NMR data were recorded on a Varian UNITY-plus 400 M NMR spectrometer. Dynamic light scattering (DLS) and zeta potential measurements were performed on a Brookheaven ZETAPALS/BI-200SM (Brookheaven Instrument, USA). Transmission electron microscopy (TEM) observations were performed on a FEI Talos F200X G2, AEMC electron microscope at an acceleration voltage of 200 kV. UV-Visible spectra were acquired with a NanoDrop Onec (Thermo Scientific, USA). Fluorescence spectra were measured on Hitachi F4600. Ultraviolet absorption was measured on a Tecan Spark plate reader. Flow cytometry analysis was performed on a BD LSRFortessa flow cytometry. Confocal laser scanning microscope (CLSM) images were captured on TCS SP8 confocal laser scanning microscopes (Leica, Germany). *Ex vivo* images were taken by IVIS Lumina imaged system (Caliper Life Sciences, USA).

Synthesis of (2Z,2'Z)-3,3'-(2,5-bis((4-methylacrylate)(phenyl)amino)-1,4-phenylene) bis(2-(3,5-bis(trifluoromethyl)phenyl)acrylonitrile) (AIEPS)

The AIEPS was synthesized according to previously reported method.[1] A flask was charged with 1 (5.0 mmol, 1.347 g), 2 (0.5 mmol, 0.381 g), $Pd_2(dba)_3$ (0.038 mmol, 0.035 g), Ruphos (0.15 mmol, 0.070 g), K_3PO_4 (5.0 mmol, 1.061 g) and toluene (2 mL). The mixture was refluxed under argon atmosphere for 18 h, after cooled to room temperature, then the water (30 mL) and chloroform (50 mL) were added to the above mixture. The organic layer was separated and washed with brine, and then purified by column chromatography. The intermediate product was purified by recrystallization from $CHCl_3$/ethanol. Subsequently, the intermediate product (3.0 mmol, 3.417g) was dissolved in THF (100 mL) and methanol (5 mL), and then 2N HCl (10 mL) was added drop-wisely into the solution mixtures and stirred at room temperature for 12 h. After the solvent was removed under reduced pressure, the residue was purified by silica gel chromatography (light petroleum:ethyl acetate = 5:1) to yield AIEPS, Yield: 89%, a red

solid. ^{1}H NMR (400 MHz, DMSO-d_6, TMS, ppm): δ 9.32 (s, 2H), 8.13 (s, 4H), 7.85 (s, 4H), 7.57 (s, 2H), 7.20 (t, J = 7.8 Hz, 4H), 6.95 (dd, J = 28.3, 8.2 Hz, 8H), 6.88–6.81 (m, 3H), 6.66 (d, J = 8.4 Hz, 4H).

Synthesis of Up-conversion Nanoparticles (UCNPs)

UCNPs ($NaYF_4$: Yb, Tm) was synthesized using oleic acid as the stabilizing agent according to the literature methods.[2] YCl_3 (0.747 mmol), $YbCl_3$ (0.25 mmol), and $TmCl_3$ (0.003 mmol) were dispersed in oleic acid (OA, 6 mL) and 1-octadecene (ODE, 15 mL), and then the mixture was heated to 150 °C for 30 min. After a homogeneous solution was formed, the mixture was cooled to room temperature, then, 5 ml of methanol containing NaOH (0.1 g, 2.5 mmol) and NH_4F (0.148 g, 4 mmol) was pipetted into the reaction mixture under stirring. The temperature was raised to 110 °C to evaporate methanol, then heated to 300 °C and maintained the temperature for 1 h under argon atmosphere. The resulting product was washed with acetone and cyclohexane three times and then finally dissolved in tetrahydrofuran (THF).

Preparation of NeINP

For the preparation of NeINP, the core was first prepared by modified nanoprecipitation methods.[3] 0.2 mg of AIEPS and 0.05 mg UCNPs were dissolved in THF (1 mL). Subsequently, the mixture was injected into 10 mL of MilliQ water under sonication at 12 W output for 60 s to form the core. Then the mixture was stirred overnight to evaporate THF. The solution was further concentrated by centrifugation.

NeINP was prepared by encapsulating core using a polymerization method according to our previous report.[4] Apm, C7A-MA, MPC, Apm and BIS were prepared as 10% (m/v) stock solution in DI-water or anhydrous DMSO firstly. The molar ratio between the monomers and core during the polymerization was listed as follow (Apm/core, 2500:1; MPC/core, 50000:1; C7A-MA/core, 30000:1; BIS/core, 8000:1). After the polymerization, the mixture was dialyzed against PBS, the purified NeINP was then obtained using a size exclusion column (Sepharose 6B).

Load and release of small molecular drug

10 mg of NeINPs were dispersed into celecoxib (CXB) or rhodamine 123 solution in phosphate buffer (pH 6.5, 5 mL), and kept stirring under dark for 24 h. The drug-loaded nanoparticles (denote as NeINP-CXB and NeINP-Rho123) were collected by centrifugation at 13,000 rpm. The release of CXB or rhodamine 123 from NeINP were then studied. Briefly, NeINP containing CXB or rhodamine 123 was transferred into a dialysis bag and immersed into 20 mL phosphate buffer at pH 6.5 and pH 7.4 at 37°C, respectively. To monitor the release of small molecular drug, certain amount of the release medium was repeatedly collected and replaced with fresh release medium of the same volume during the dialysis. Drug loading content (DLC) and drug loading efficiency (DLE) were calculated as following formulas:

$$\text{DLC (wt\%)} = (weight\ of\ loaded\ drug / total\ weight\ of\ NeINP) \times 100\%$$

$$\text{DLE (\%)} = (weight\ of\ loaded\ /\ weight\ of\ drug\ in\ feed) \times 100\%$$

ROS quantum yield measurements.

The ROS quantum yield of NeINPs in water (Φ) under 980 nm laser irradiation (2.5 mW/cm^2) was determined using 9,10-anthracenediyl-bis(methylene) dimalonic acid (ABDA) as an indicator and using Rose Bengal (RB) as the standard reference. ABDA solid (200 μM) was dissolved in DI water. The NeINPs (200 μg/mL) or RB (5 μg/mL) was then added in aqueous solution. The absorbance decrease of ABDA at 400 nm was recorded for different durations of light irradiation to obtain the decay rate of the photosensitizing process. And the ROS yield is calculated using the following equation:

$$\Phi_{NeINP} = \Phi RB\ (K\ NeINP \bullet ARB)/KRB \bullet ANeINP$$

Where K_{NeINP} and K_{RB} are the decomposition rate constants of the photosensitizing process determined by the plot ln (A_0/A) versus irradiation time. A_0 is the initial absorbance of ABDA while A is the ABDA absorbance after different irradiation times. A_{NeINP} and A_{RB} represent the light absorbed by NeINPs and RB, which are determined by integration of the absorption bands in the wavelength range of 400-800 nm. ΦRB is the ROS quantum yield of RB, which is 75% in water.

Cell culture

The mouse breast cancer cells (4T1) was purchased from the American Type Culture Collection. 4T1 cells were maintained in RPMI 1640 medium with 10% fetal bovine serum (v/v), 100 U/ml penicillin and streptomycin. The tumor cells were incubated in a humidified atmosphere of 5% CO_2 at 37 ℃. The cells were sub-cultivated approximately every 2 days at 80% confluence using 0.25% (w/v) trypsin at a split ratio of 1:5.

Intracellular ROS detection

ROS generation inside cells under irradiation was detected using DCFH-DA as an indicator. 4T1 cells were cultured in the plates at 37 ℃. After 80% confluence, the culture medium was removed and washed twice with PBS. Following incubation with NeINPs (50 μg/mL) for 3 h in the dark, the DCFH-DA was then added into the cells. After incubation for 15 min, cells were washed twice with PBS and then exposed to 980nm laser for different irradiation time (2.5 mW/cm^2). The fluorescence images of treated cells were acquired using CLSM.

The cytotoxicity and phototoxicity of NeINPs in vitro

The cytotoxicity of NeINP was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Briefly, 4T1 cells were seeded in the 96-well plate at a density of 5000 cells per well and grown to 70% confluence, followed by replacing the culture medium with the fresh ones containing NeINP. After 4 h incubation, the selected wells were exposed to laser irradiation (2.5 mW/cm^2, 60 s) and further cultured for 24 h. In the parallel experiment, cells were treated with NeINPs for 24 h in the dark. The wells were washed with PBS and replaced with freshly prepared MTT solution (0.5 mg/mL). After incubation, the solution in each well was carefully removed and 100 μL of DMSO was added. The plate was gently shaken for 10 min at room temperature and then the absorbance of MTT at 570 nm was monitored by the microplate reader in order to determine the cell viability.

Inducting immunogenic cell death by NeINPs in vitro

Immunofluorescence staining was used to investigate treatment-induced CRT exposure on the surface of the tumor cells. 4T1 cells were seeded in 35 mm confocal dishes (Φ =15 mm) at a density of 1 × 10^5 cells/well. The cells were incubated with NeINPs at a concentration of 50 μg mL^{-1} for 6 h. The selected cells were then washed twice and irradiated with 980 nm laser (2.5 mW/cm^2, 60 s). The cells were washed twice with cold PBS and fixed in 4% paraformaldehyde for 15 min. Nonspecific binding sites were blocked by the pre-incubation with 5% FBS in PBS for 30 min, followed by incubating with primary antibody for 1 h, and then incubated with the Alexa 594-conjugated secondary antibody for 30 min. Finally, the cells were stained with DAPI and examined by CLSM.

The level of ATP secretion was determined with a commercially available ATP assay kit. Briefly, 4T1 cells were seeded in 24-well plates at a density of 2 × 10^4 cells/well. The cells were incubated with NeINPs at a concentration of 50 μg/mL for 6 h. The selected cells were then washed twice and irradiated with 980 nm laser (2.5 mW/cm^2, 60 s). The supernatant of the cell culture was collected, and the ATP content was tested using an ATP assay kit according to manufacturer's instructions.

Intracellular HMGB1 distribution was tested using immunofluorescence staining. Briefly, 4T1 cells were seeded in 35 mm confocal dishes (Φ =15 mm) at a density of 1 × 10^5 cells/well. The cells were incubated with NeINPs at a concentration of 50 μg/mL for 6 h. The selected cells were then washed twice and irradiated with 980 nm laser (2.5 mW/cm^2, 60 s). The cells were washed twice with cold PBS and fixed in 4% paraformaldehyde for 15 min and permeabilized with 0.1% Triton X-100 for 10 min. Non-specific binding sites were blocked by pre-incubation with 5% fetal bovine serum in PBS for 30 min, followed by incubation with primary antibody for 1 h, and then with an Alexa Fluor 488-secondary antibody. The cells were further stained with DAPI for CLSM examination.

DC maturation in vitro

To investigate DC maturation in vitro, BMDCs were generated from the bone

marrow of 8-week old BALB/c mice. Immature DCs were co-cultured with NeINPs pretreated 4T1 cells for 24 h. After staining with anti-CD11c-APC, anti-CD80-FITC, anti-CD86-PE antibody, the DCs were analyzed using flow cytometry.

In vivo biodistribution of NeINP

The tumor-bearing mice were generated by subcutaneous injection of 1×10^6 4T1 cancer cells at the left mammary fat pad of female BALB/c mice. The mice were randomly divided into different groups. When the tumor volume was about 250 mm^3, the mice were intravenously injected with 100 μL NeINP. At 24 h post-injection, the mice were sacrificed, and the major organs and the tumors were collected for *ex vivo* imaging. *Ex vivo* images were taken by IVIS Lumina imaged system. The fluorescence images were analyzed using Living Image 3.1 (Caliper Life Sciences, USA).

The anti-tumor efficacy of NeINP

To investigate the anti-tumor efficacy of NeINP in vivo, female BALB/c mice were subcutaneous injected with 1×10^6 4T1 cancer cells at the left mammary fat pad. The mice with tumor volumes at around 50 mm^3 were randomized into four groups (n = 6) and intravenous injected with different nanoparticles every three days for 9 days. The mice which received the different interventions were then divided into two parts, and half of the mice received irradiation. The tumor volume was measured by a Vernier calipers and the volume (V) was calculated by using the formula:

$$V = \frac{length\ of\ the\ tumor * width\ of\ the\ tumor^2}{2}$$

Flow cytometry analysis

Freshly harvested tumor tissues were cut into small pieces and homogenized using the GentleMACs Dissociator followed by passing through a 70×10^{-6} m cell strainer for single-cell suspensions. Subsequentially, the cells were collected and diluted to 1×10^7 cells/mL. 100 μL cells were stained by adding a cocktail of fluorescent conjugated antibodies. For intracellular staining, cells were firstly permeabilized with 100 μL fixation/permeabilization buffer, and then stained with the antibody cocktail.

After the staining, the cells were fixed with 4 % paraformaldehyde and analyzed via flow cytometer.

Immunofluorescence staining

Immunofluorescence staining was performed on frozen tumor sections. Briefly, harvested tumors were first placed in 4% paraformaldehyde for 48 h at 4 ˚C, and then transferred to 15% then 30% sucrose solution (w/w) for dehydration. The tumors were embedded in optimal cutting medium, and frozen slices were made on a cryostat microtome. Immunofluorescence staining was performed by rinsing with PBS, permeabilization, followed by blocking in 5% FBS at room temperature for 1 h, and then stained with different primary antibodies including CD4 (anti-CD4 from Rabbit), CD8 (anti-CD8 from Rat) and COX-2 (anti- Cyclooxygenase 2 from Rabbit) overnight at 4 °C according to the manufacturer's instructions. Following by the addition of fluorescently labelled secondary antibodies, the slides were analyzed with a confocal microscope. All antibodies used in the experiments were diluted 200 times.

Analysis of memory T cells in spleen

To examine the memory T lymphocytes in spleens, the spleens of the mice were harvested and pressed gently to obtain a single cell suspension solution using a syringe piston. Subsequentially, the cells were collected and diluted to 1×10^7 cells/mL. 100 μL cells were stained by adding a cocktail of fluorescent conjugated antibodies. After the staining, the cells were fixed with 4% paraformaldehyde and analyzed via flow cytometer. For the analysis of T_{EM} ($CD3^+CD8^+CD44^+CD62L^-$) and T_{CM} ($CD3^+CD8^+CD44^+CD62L^+$), T lymphocytes in the spleen were stained with anti-CD3-APC, anti-CD8-PE, anti-CD62L-Percp-Cy5.5, anti-CD44-FITC according to the manufacturer's protocols.

Statistical Analysis

Statistical comparisons were achieved using one-way ANOVA with Dunnett post-test with GraphPad Prism 7.0. Data represent mean ± standard error of the mean (s.d.) deviation from at least three independent experiments ($n \geq 3$) and the significance levels

are *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001

Supplementary Fig.s

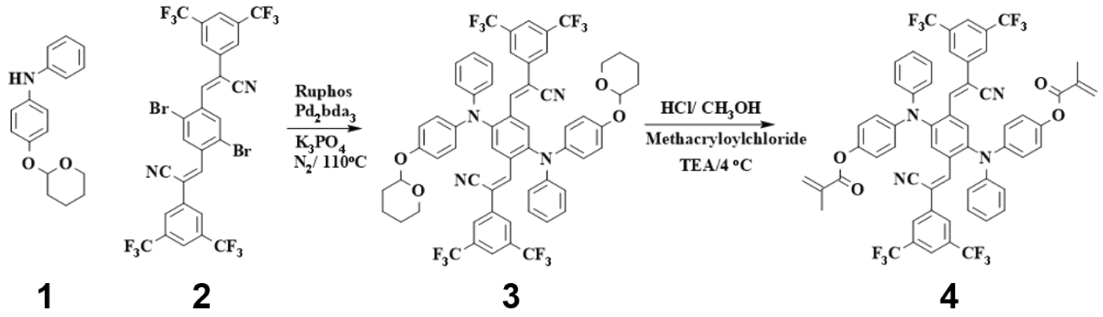

Fig. S1. The synthetic route of AIEPS

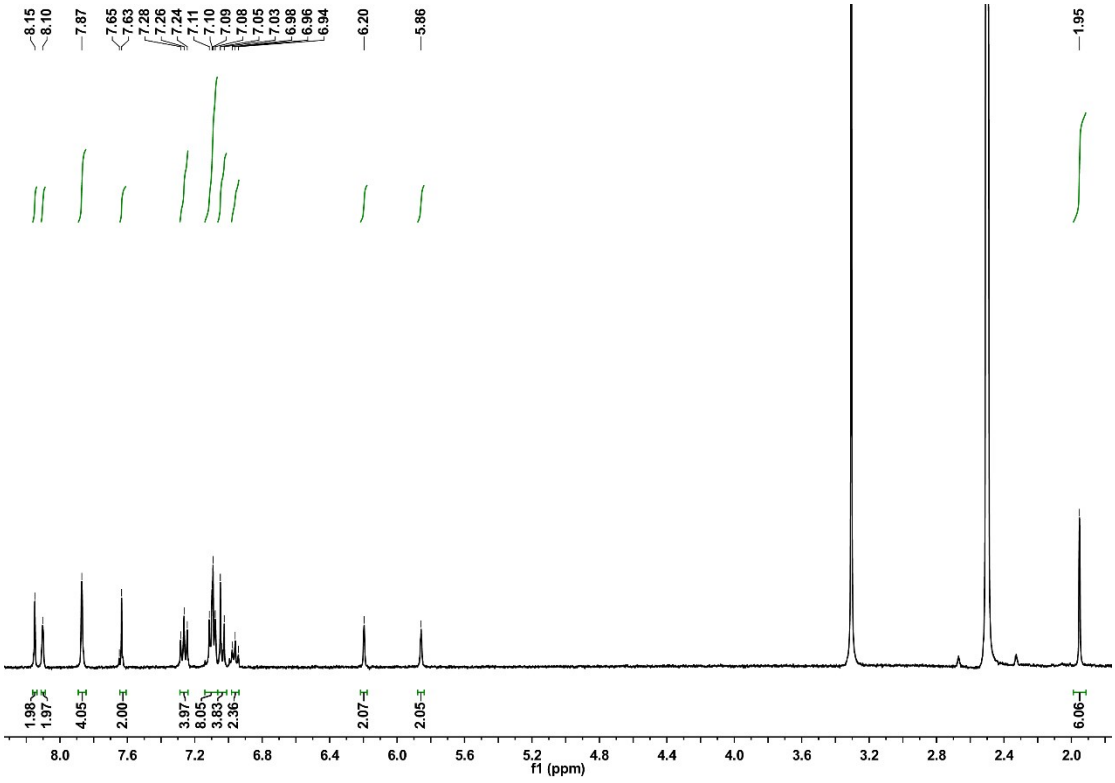

Fig. S2. ^{1}H NMR spectrum of AIEPS in DMSO-D_6, 400 MHz, 25 ˚C.

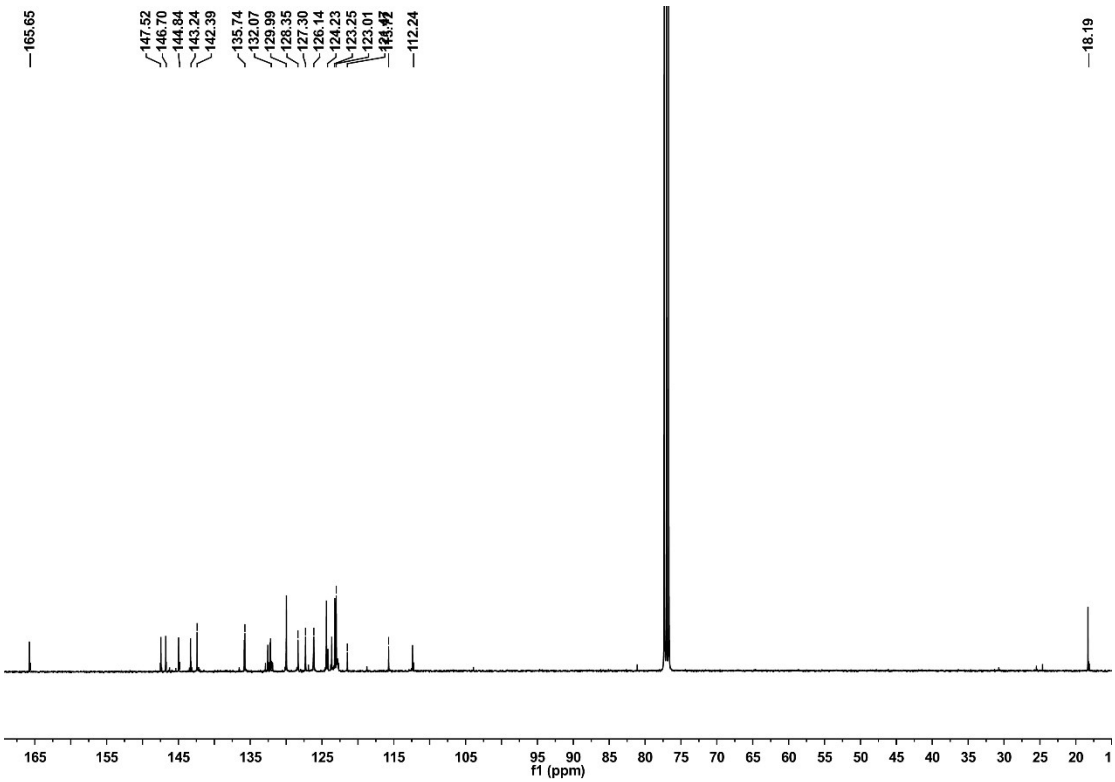

Fig. S3. ^{13}C NMR spectrum of AIEPS in $CDCl_3$, 100 MHz, 25 ˚C.

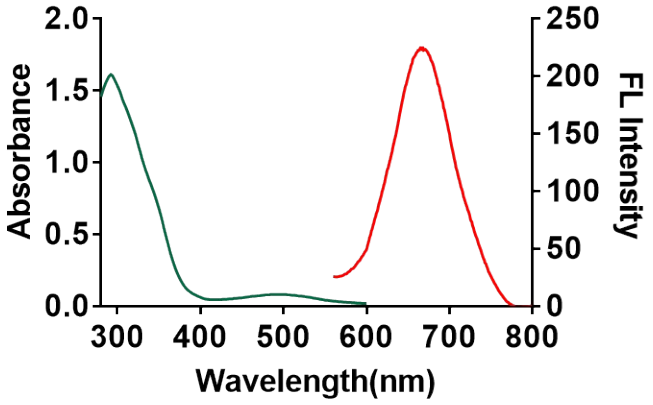

Fig. S4. UV-vis absorbance and FL spectra of AIEPS (25 μM) in THF/water mixture (*v*/*v* = 5/95).

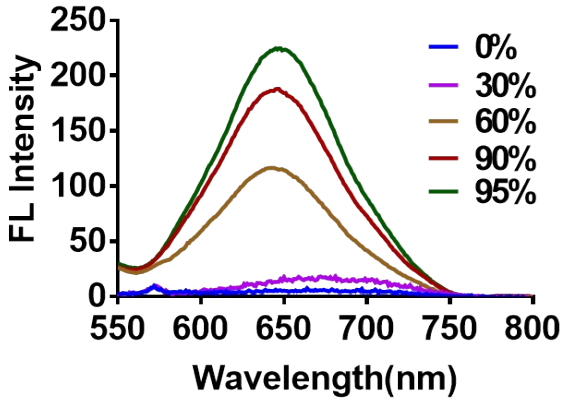

Fig. S5. FL spectra of AIEPS on the solvent composition of the THF/water mixture.

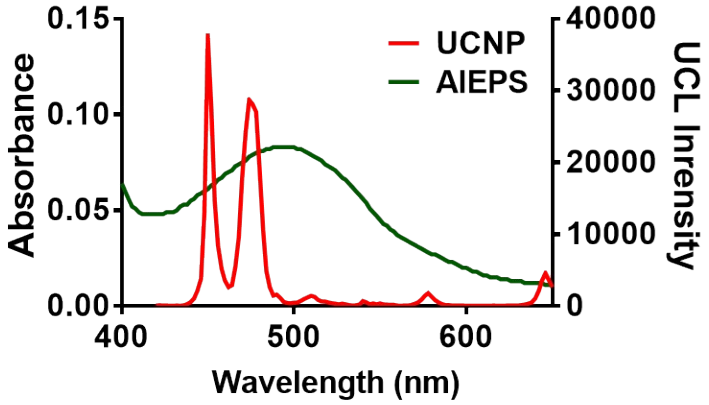

Fig. S6. Absorbance spectrum of AIEPS and UCL spectrum of the UCNP.

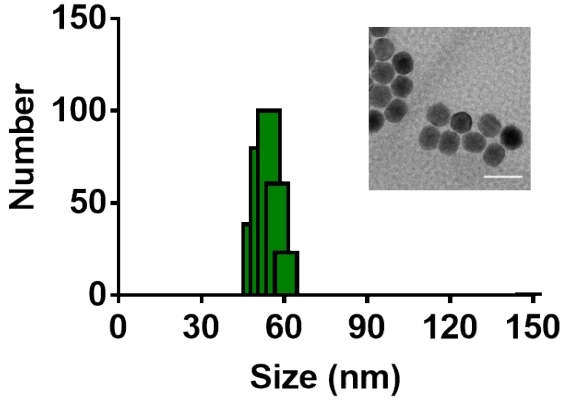

Fig. S7. DLS measurements and morphology assessment of the core using transmission electron microscopy (TEM) bright field (insert photo), the scare bar represents 100 nm.

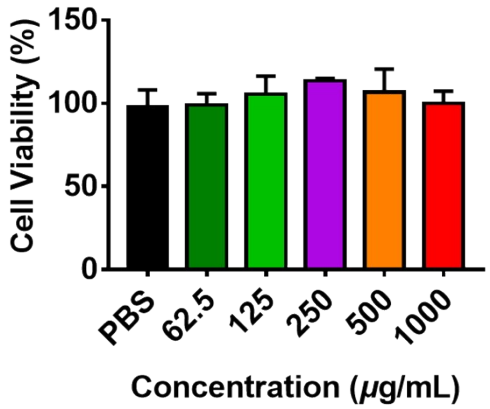

Fig. S8. Cell viability of 4T1 cancer cells incubation with NeINPs at various concentrations.

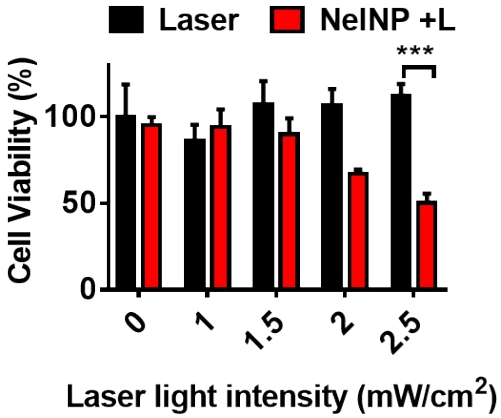

Fig. S9. Cell viability of 4T1 cancer cells incubation with NeINPs for 48 h under different laser intensity.

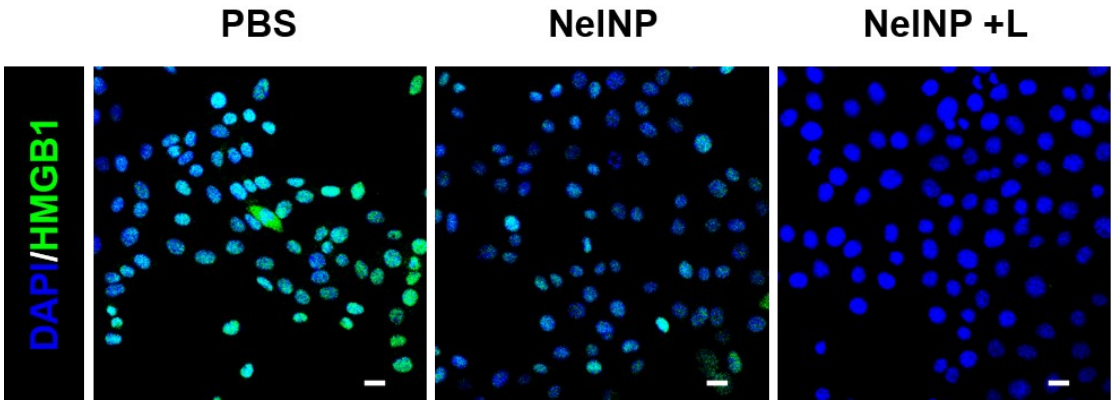

Fig. S10. Representative immunofluorescence images of HMGB1 release in 4T1 cells after different treatments. The treatment under irradiation denote as '+L'. PBS-treated 4T1 cells were used as the negative control. The scale bar represents 20 μm.

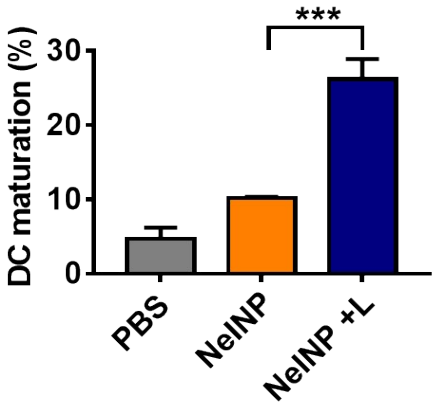

Fig. S11. Flow cytometric examination of DC maturation.

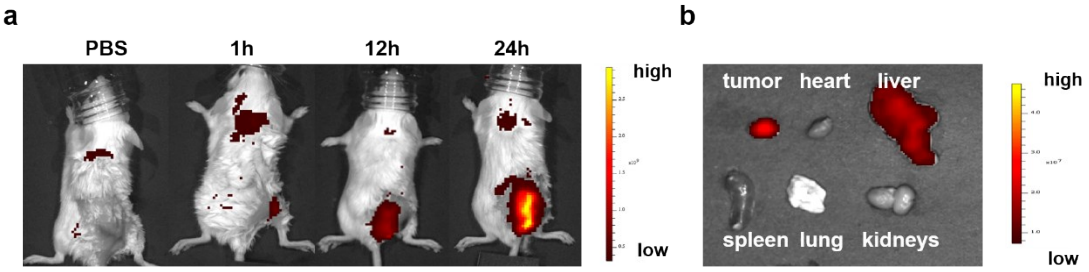

Fig. S12. a) *In vivo* fluorescence imaging of the BALB/C mice bearing 4T1 tumors at 1 h, 6 h and 24 h after intravenous injection of NeINP, b) *Ex vivo* fluorescence imaging of the tumor and normal tissues harvested from the euthanized 4T1 tumor-bearing mouse at 24 h post injection.

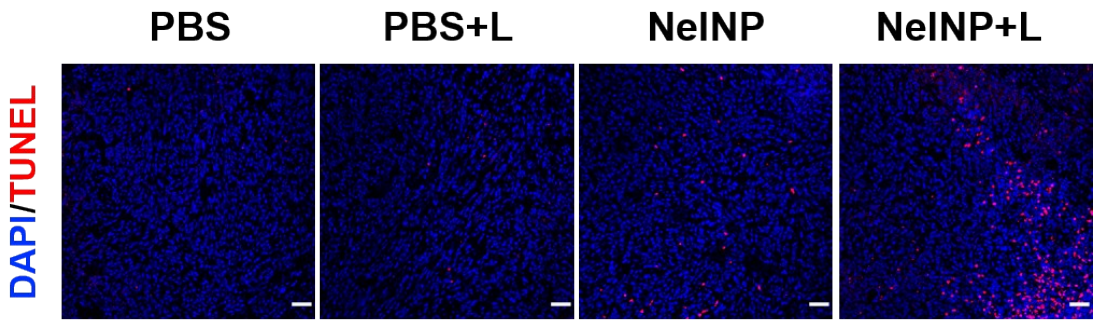

Fig. S13. TUNEL analysis from the mice in tumor tissues after different treatments. The scale bars represent 50 μm.

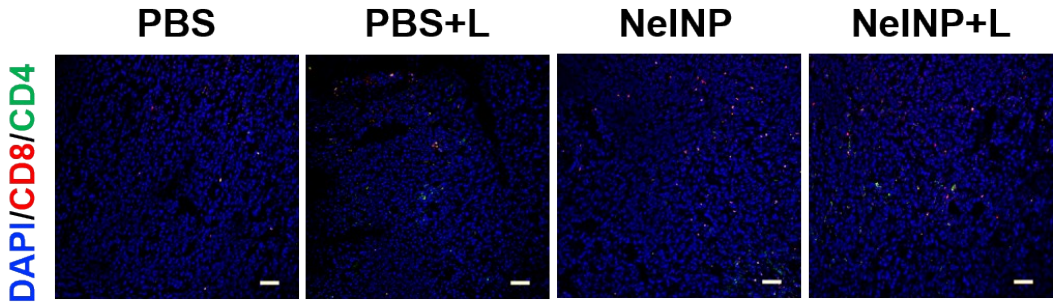

Fig. S14. Representative immunofluorescence images of tumors showing $CD4^+$ T cell and $CD8^+$ T cell infiltration from the mice after different treatments. The scale bars represent 50 μm.

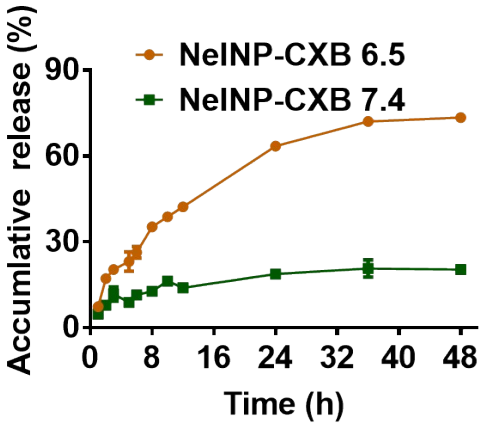

Fig. S15. The release profiles of celecoxib from NeINP-CXB at pH 6.5 and 7.4.

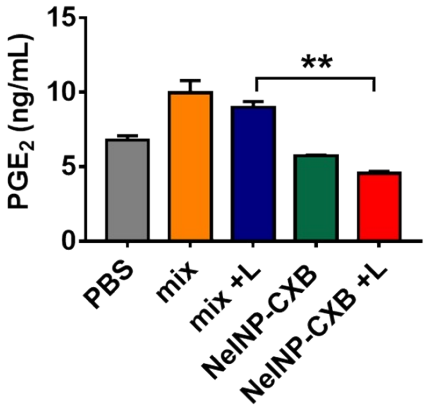

Fig. S16. The content of the level of PGE_2 in tumors.

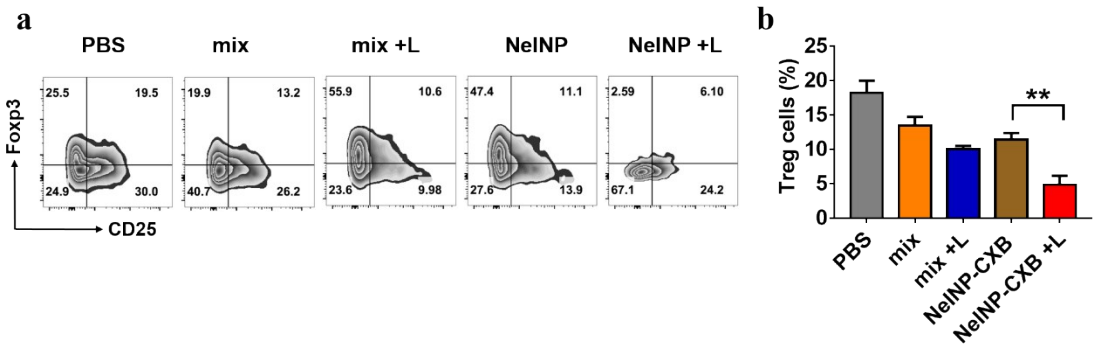

Fig. S17. Representative flow cytometric plots of the Treg (gating on $CD45^{+}CD4^{+}$ cells) population at day 10 post-treatment.

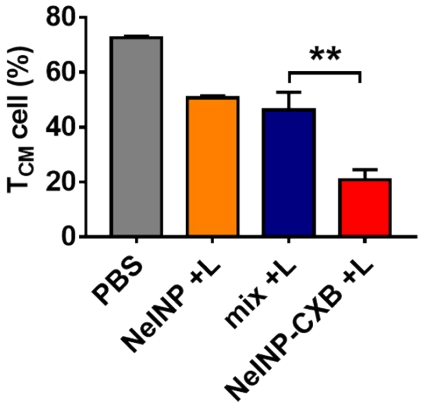

Fig. S18. The percent of central memory T cells (T_{CM}) in spleens collected from mice bearing 4T1 tumors following the indicated treatments.

Reference

1. Y. Zheng, H. Lu, Z. Jiang, Y. Guan, J. Zou, X. Wang, R. Cheng and H. Gao, *J. Mater. Chem. B*, 2017, **5**, 6277-6281.
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4. Y. Zheng, Z. Zhang, Q. Liu, Y. Zhao, C. Zheng, J. Hao, K. Yi, Y. Wang, C. Wang, X. Zhao, L. Shi, C. Kang and Y. Liu, *ACS Appl. Bio Mater.*, 2020, **3**, 4635-4642.