

**Multifunctional organic nanoparticles with aggregation-induced emission
(AIE) characteristics for targeted photodynamic therapy and RNA
interference therapy**

Guorui Jin,^{a,b} Guangxue Feng,^c Wei Qin,^d Ben Zhong Tang,^{d,e} Bin Liu^{*a,c} and Kai Li^{*a,f}

^aInstitute of Materials Research and Engineering, A*STAR, 3 Research Link, Singapore, 117602. E-mail: kai_li_cn@hotmail.com

^bCurrent Address: The Key Laboratory of Biomedical Information Engineering of Ministry of Education, School of Life Science and Technology, Xi'an Jiaotong University, Xi'an, China, 710049

^cDepartment of Chemical and Biomolecular Engineering, National University of Singapore, 4 Engineering Drive 4, Singapore 117585. E-mail: cheliub@nus.edu.sg

^dDepartment of Chemistry, Institute for Advanced Study, Institute of Molecular Functional Materials, Division of Biomedical Engineering, Division of Life Science, and State Key Laboratory of Molecular Neuroscience, The Hong Kong University of Science & Technology, Clear Water Bay, Kowloon, Hong Kong, China

^eGuangdong Innovative Research Team, SCUT-HKUST Joint Research Laboratory, State Key Laboratory of Luminescent Materials and Devices, South China University of Technology, Guangzhou, China, 510640

^fCurrent Address: Department of Radiology and Molecular Imaging Program at Stanford (MIPS), Stanford School of Medicine, Stanford, United States, 94305

Experimental Section

Materials. 2-(2,6-Bis((E)-4-(phenyl(4'-(1,2,2-triphenylvinyl)-[1,1'-biphenyl]-4-yl)amino)styryl)-4H-pyran-4-ylidene) malononitrile (TTD) was synthesized according to our previous report.¹ Thiolated cyclic (Arg-Gly-Asp-D-Phe-Lys(mpa)) peptide (c(RGDfK)) was customized from GL Biochem Ltd (Shanghai). 1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[amino(polyethylene glycol)] (DSPE-PEG₂₀₀₀-NH₂) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[maleimide(polyethylene glycol)] (DSPE-PEG₅₀₀₀-Mal) were purchased from Avanti Polar Lipids, Inc.. Dulbecco's modified Eagle's medium (DMEM, low glucose), fetal bovine serum (FBS), penicillin-streptomycin solution, CellROX[®] deep red reagent and 3-(2-pyridyldithio)propionate (SPDP) linker were commercial products from Life Technologies, Invitrogen, Singapore. Tetrahydrofuran (THF), dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 9,10-anthracenediyl-bis(methylene)dimalonic acid (ABDA) were purchased from Sigma-Aldrich (Singapore). The RNeasy Mini Kit and the GAPDH, and *VEGF* primers were purchased from Qiagen, Singapore. siVEGF (sense strand, 5'-GGAGUACCCUGAUGAGAUCTT-3' and antisense strand, 5'-GAUCUCAUCAGGGUACUCCTT-3') modified with thiol at 5' end and siVEGF modified with Cy3 and thiol at 3' and 5' end, respectively, were customized products from Shanghai GenePharma Co., Ltd.

Conjugation of siVEGF to DSPE-PEG-NH₂. DSPE-PEG-siVEGF was synthesized by conjugating DSPE-PEG-NH₂ and siVEGF using SPDP linker (100:1:10 mol/mol/mol) in DEPC-treated sodium borate buffer (50 mM, pH 8.2) for 24 h in nitrogen atmosphere at room temperature. The reaction mixture was then purified using centrifugal filters with MWCO 10 kDa (Amicon ultra-4.5 mL, Ultracel-10 membrane, 10 kDa). The success of conjugation was verified by using reverse-phase high performance chromatography (HPLC, Agilent HPLC 1200, Agilent, Tokyo, Japan) with C18 column (Agilent Zorbax SB-C18) as described previously.² Mobile phase A composed of 5% acetonitrile in 0.1 M triethylammonium acetate (TEAA) buffer, pH 7, and mobile phase B of 15% of acetonitrile in 0.1 M TEAA were applied to carry out the analyses with a flow rate of 1.0 mL min⁻¹. SiVEGF, DSPE-PEG-NH₂ and DSPE-PEG-siVEGF were separated by C18 column (Agilent Zorbax SB-C18) and detected at 260 nm, with a gradient elution from 20% B to 57.7% B in 15 min, followed by

stabilizing at 20% B for 5 min. The column temperature is kept at 60 °C throughout the analysis.

Synthesis of cRGD-siVEGF-TTD NPs. To prepare TTD (AIE photosensitizer) loaded siVEGF-TTD NPs, weighed amount of TTD, DSPE-PEG-siVEGF and DSPE-PEG-Mal (1 mg each) were dissolved in THF and quickly injected into water, followed by continuous sonification using a probe sonicator at 12 W output (XL2000, Misonix Incorporated, NY). The suspension was then stirred vigorously at room temperature overnight to evaporate THF and yield siVEGF-TTD NPs in water (5 mL). The formed suspension was then filtered through a 0.2 μm syringe filter. The NPs (2 mL) were further mixed with thiol-functionalized cRGD (5×10^{-2} M) in the presence of DMSO (1 mM). After overnight reaction at room temperature, the solution was dialysed against MilliQ water for 2 days to eliminate the excess cRGD. The obtained cRGD-siVEGF-TTD NPs were collected for further use. cRGD-TTD NPs without surface siVEGF were also synthesized through the same procedures using DSPE-PEG-Mal as the encapsulation matrix.

Characterization. The UV-vis spectra were recorded on a Shimadzu UV-1700 spectrometer. The fluorescence spectra were measured using a fluorometer (LS-55, Perkin Elmer, USA). The average particle size and size distribution were determined by dynamic light scattering with a DynaPro[®] Plate Reader II (WYATT technology, USA) at room temperature. The morphology of cRGD-siVEGF-TTD NPs was studied by a high-resolution transmission electron microscope (TEM, JEM-2010, JEOL, Japan).

Calculation of singlet oxygen quantum yield. The singlet oxygen quantum yield of cRGD-siVEGF-TTD NPs in water (Φ_{TTD}) was determined using Rose Bengal (RB) as the standard reference. To conduct the experiment, ABDA was added into cRGD-siVEGF-TTD NPs or RB aqueous solution to make a working concentration of 5 μL . The mixture was exposed to white light (400-800 nm, 0.25 W cm^{-2}) for designated time. The degradation of ABDA was access by the means of its absorbance changes at 378 nm. And the singlet oxygen quantum yield is calculated using the following equation:

$$\Phi_{\text{TTD}} = \Phi_{\text{RB}}(K_{\text{TTD}} A_{\text{RB}})/K_{\text{RB}}A_{\text{TTD}}$$

where K_{TTD} and K_{RB} represent 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_{RB} and A_{TTD} refer

to the light absorbed by cRGD-siVEGF-TTD NPs and RB, respectively, calculated by the integration of their absorption spectra from 400 to 800 nm, Φ_{RB} is the singlet oxygen quantum yield of RB, which is 75%.

Detection of ROS in solution: Photo-induced singlet oxygen generation from cRGD-siVEGF-TTD NPs under light irradiation was determined by photobleaching of the fluorescent indicator, 9,10-anthracenediyl-bis(methylene)dimalonic acid (ABDA). ABDA solid was dissolved in DI water (50 μM) with cRGD-siVEGF-TTD NPs (2.5 and 5 $\mu\text{g mL}^{-1}$) and was exposed to light irradiation for different time intervals at the power density of 0.2 W cm^{-2} . ABDA solution without cRGD-siVEGF-TTD NPs was used as control. The decomposition of ABDA was monitored by the maximum absorbance at 400 nm.

siVEGF release: Cy3 conjugated siVEGF was used to synthesize the siVEGF-TTD NPs. The concentration of siVEGF conjugated on the surface of siVEGF-TTD NPs (5 $\mu\text{g mL}^{-1}$) was quantified by the absorbance of Cy3 at 547 nm comparing with a standard curve and determined to be 80 nM. Dialysis tubes (MWCO 30 kDa) was applied to contain 2 mL of siVEGF-TTD NPs (5 $\mu\text{g mL}^{-1}$) and immersed in 1 \times PBS buffer (PH 7.4) with or without glutathione (GSH). The assemblies were kept at 37 $^{\circ}\text{C}$ under constant shaking. At designated time points, incubation medium outside dialysis bags was collected and replaced by fresh 1 \times PBS buffer. The siVEGF concentrations in the collected release mediums were quantified by measuring the absorbance of Cy3 at 547 nm.

Cell culture. The breast cancer cell line MDA-MB-231, MCF-7 and SKBR-3 were provided by American Type Culture Collection (ATCC). The cells were cultured in DMEM (Invitrogen, Carlsbad, CA) containing 10% heat-inactivated FBS (Invitrogen), 100 U mL^{-1} penicillin, and 100 $\mu\text{g mL}^{-1}$ streptomycin (Thermo Scientific) and maintained in a humidified incubator at 37 $^{\circ}\text{C}$ with 5% CO_2 . Before experiment, the cells were precultured until confluence was reached.

Intracellular ROS generation. ROS generation inside cells under light irradiation was monitored using CellROX[®] Deep Red Regent (Life technologies, Singapore). MDA-MB-231 cells were cultured in a 4-well confocal chamber at 37 $^{\circ}\text{C}$. After 80% confluence, the culture medium was removed and cells were washed twice with PBS buffer. Following incubation with cRGD-siVEGF-TTD NPs (5 $\mu\text{g mL}^{-1}$) for 4 h in the dark, the cells were washed with 1 \times PBS and further cultured with CellROX[®] Deep Red Regent (5 μM) in dark for 30 min. The

medium was then discarded and fresh medium was added. After exposure to white light (OSL2 Fiber Illuminator, Photonitech Pte Ltd, Singapore) irradiation for 10 min at the power density of 0.2 W cm^{-2} , the cells were washed with $1\times$ PBS twice and fresh medium was added. The living cells were then imaged under a laser confocal microscope.

Intracellular delivery of siVEGF. Cy3 conjugated siVEGF and green fluorophore 4,7-Bis[4-(1,2,2-triphenylvinyl)phenyl]benzo-2,1,3-thiadiazole (BTPETD)³ with aggregation-induced emission (AIE) was used to form cRGD-Cy3-siVEGF-BTPETD NPs by the same method as preparing the cRGD-siVEGF-TTD NPs and applied for visualizing cellular uptake of the VEGF siRNA. MDA-MB-231 cells were cultured on 15 mm coverslip located in 24-well plate at 37°C . After 80% confluence, the culture medium was removed and washed twice with $1\times$ PBS. Following incubation with BTPETD NPs ($5 \mu\text{g mL}^{-1}$) for 4 h in the dark, the medium was removed and the cells were washed twice with $1\times$ PBS. The cells on the coverslips were fixed by 4% paraformaldehyde and then mounted onto a rectangle glass slide, followed by fluorescence imaging under a laser confocal microscope.

siVEGF transfection efficiency. MDA-MB-231 cancer cells were seeded in 6-well tissue culture plates at 2×10^5 cells/well and incubated at 37°C in 5% CO_2 for 24 h. The cells were then incubated with cRGD-siVEGF-TTD NPs ($5 \mu\text{g mL}^{-1}$) for 4 h in the dark, followed by washing with $1\times$ PBS and further incubation in fresh medium. After 48 h, the supernatants were collected and analyzed by Human VEGF Quantikine ELISA Kit (R&D system, Singapore) following the manufacturer's instructions. The expression of VEGF protein in the supernatant was revealed by the absorbance value recorded using a microplate reader (infiniteM200, Tecan) at 450 nm wavelength with 540 nm as reference. VEGF mRNA expression level was then analyzed by qRT-PCR. Total RNA was isolated using RNeasy mini-kits (Qiagen, Singapore) according to supplier's protocol. VEGF mRNA level was quantified using iScript[®] one-step RT-PCR kit with SYBR green (Bio-Rad) and Bio-Rad real-time PCR detection system according to the manufacturer's protocol. Relative gene expression level was calculated using $\Delta\Delta\text{CT}$ method with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene expression level as a reference. Data are normalized to VEGF expression level of control cells without any treatment.

Photocytotoxicity studies. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were used to assess the metabolic activity of SKBR-3, MCF-7 and MDA-MB-231 cells. The cells were seeded in 96-well plates (Costar, IL, USA) at a density of 5×10^3

cells mL^{-1} . After 24 h incubation, the medium was replaced with medium containing cRGD-siVEGF-TTD NPs ($5 \mu\text{g mL}^{-1}$) and incubated at $37 \text{ }^\circ\text{C}$. Following incubation with the NPs for 4 h, cells were washed twice with $1\times$ PBS and then exposed to white light (OSL2 Fiber Illuminator, Photonitech Pte Ltd, Singapore) irradiation for 10 min at the power density of 0.2 W cm^{-2} . The cells were further incubated for 24 and 48 h and washed twice with $1\times$ PBS buffer. Freshly prepared MTT ($100 \mu\text{L}$, 0.5 mg mL^{-1}) solution in culture medium was then added into each well. The MTT medium solution was carefully removed after 3 h incubation at $37 \text{ }^\circ\text{C}$. DMSO ($100 \mu\text{L}$) was then added into each well and the plate was gently shaken to dissolve all the precipitates formed. The absorbance of MTT at 570 nm was monitored by the microplate reader (infiniteM200, Tecan). Cell viability was expressed by the ratio of absorbance of the cells incubated with NPs to that of the cells incubated with culture medium only.

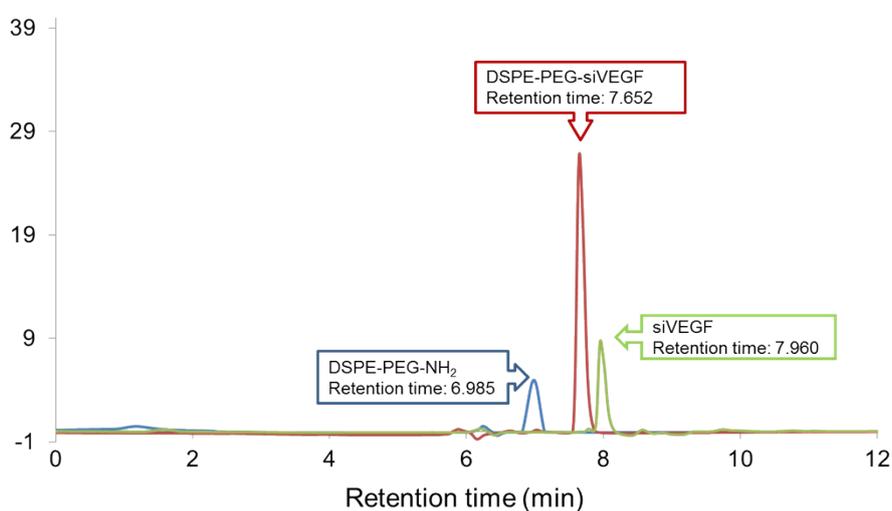


Figure S1. High performance liquid chromatography (HPLC) spectrum of DSPE-PEG-NH₂, DSPE-PEG-siVEGF and siVEGF.

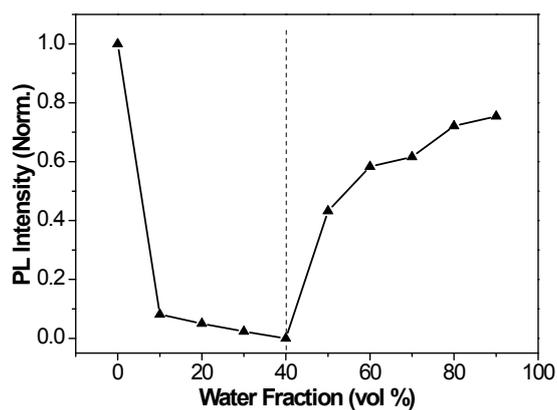


Figure S2. Change of PL intensity of 5 μM of TTD in THF-water mixtures with different water fractions (f_w) at the same total volume.

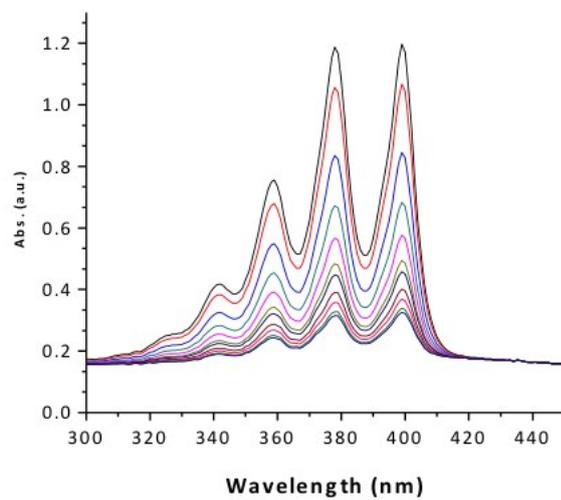


Figure S3. Photodegradation of ABDA with TTD NPs.

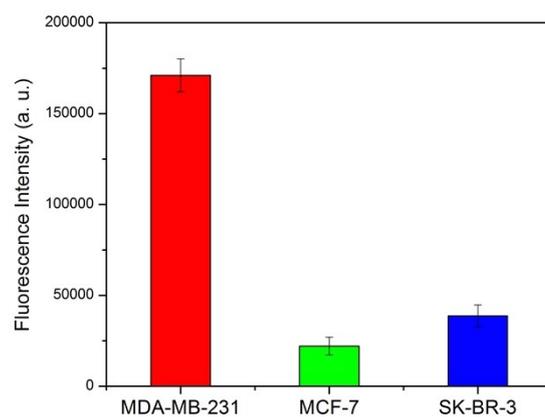


Figure S4. The mean fluorescence intensity quantitatively analyzed by ImageJ program from confocal images of MDA-MB-231, MCF-7, and SK-BR-3 cells after incubation with cRGD-siVEGF-TTD NPs ($5 \mu\text{g mL}^{-1}$ of TTD) for 4 h at 37°C .

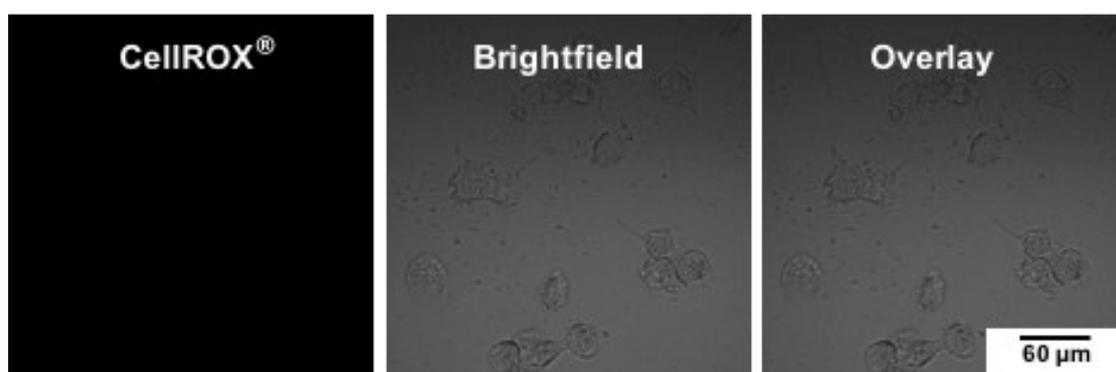


Figure S5. Confocal image of MDA-MB-231 cells treated with CellROX deep[®] red reagent after incubation with cRGD-siVEGF-TTD NPs ($5 \mu\text{g mL}^{-1}$) without light irradiation. No fluorescence was detected from the CellROX[®] deep red reagent ($\lambda_{\text{ex}} = 633 \text{ nm}$ with a 650 nm above longpass filter).

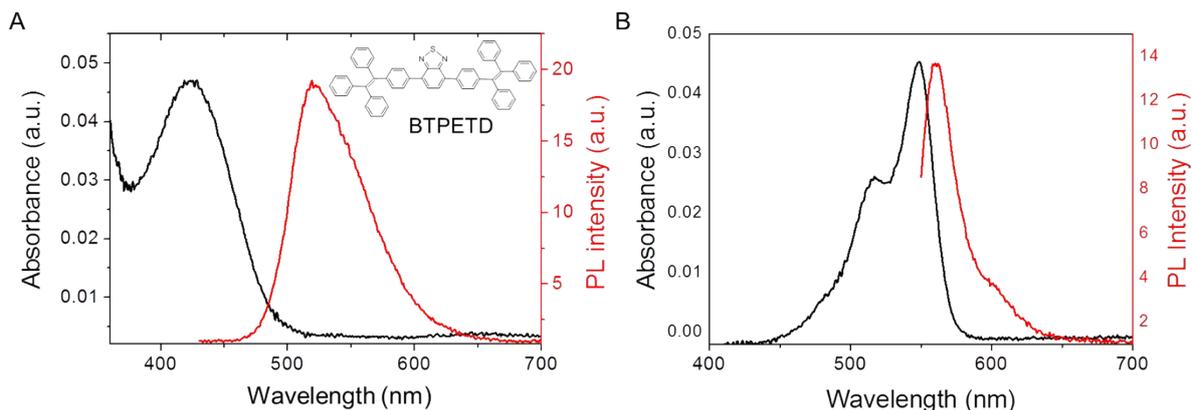


Figure S6. UV-vis absorption and emission spectra of (A) cRGD-siVEGF-BTPETD NPs in water ($\lambda_{\text{ex}} = 428$ nm) and (B) Cy3 conjugated siVEGF in water ($\lambda_{\text{ex}} = 549$ nm). The inset in (A) shows the chemical structure of BTPETD.

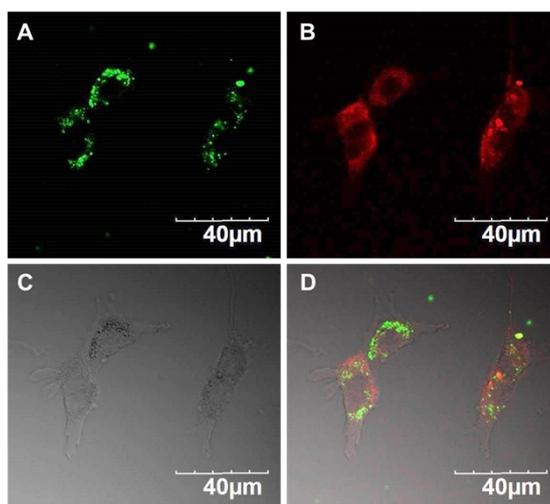


Figure S7. Confocal images of the MDA-MB-231 cells after incubation with cRGD-Cy3-siVEGF-BTPETD NPs for 4 h at 37 °C. The confocal images represent for (A) green BTPETD ($\lambda_{\text{ex}} = 405$ nm with a 520-550 nm band-pass filter), (B) Cy3 conjugated siVEGF ($\lambda_{\text{ex}} = 543$ nm with a 560 nm above longpass filter), (C) bright field image and (D) overlay image, respectively.

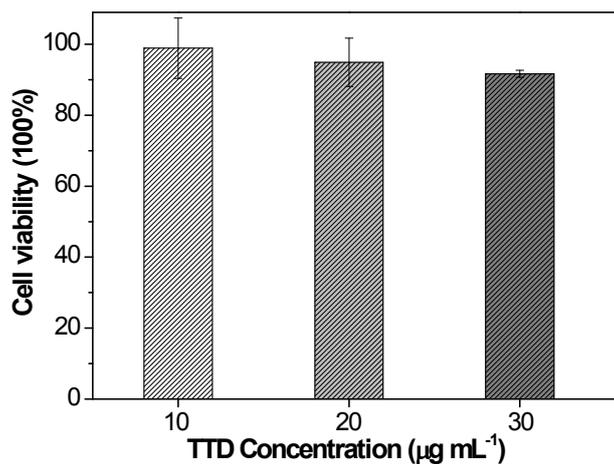


Figure S8. Relative viabilities of MDA-MB-231 cancer cells after incubation with cRGD-TTD NPs at various concentrations of TTD for 24 hours. Data represent mean values \pm standard deviation, $n = 3$.

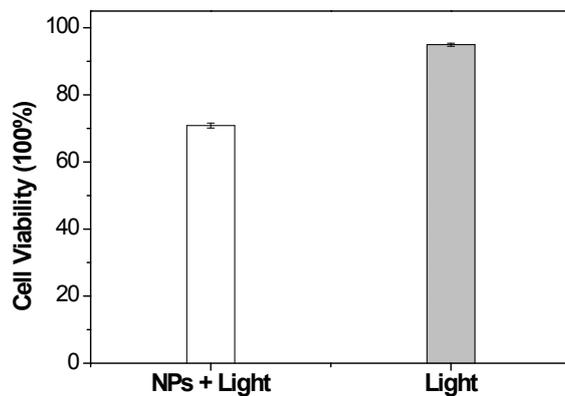


Figure S9. Viability data of MDA-MB-231 cells with cRGD-TTD NPs treatment (NPs + Light) and cells without NP treatment (Light) after light irradiation (0.20 W cm^{-2} , 10 min) and further incubation for 48 h. Data present mean values \pm standard deviation, $n = 3$.

Reference

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