Supporting Information for Publication

Drug-loaded Photosensitizer-Chitosan Nanoparticles for Combinatorial Chemo- and Photodynamic-Therapy of Cancer

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1. Synthesis of Nucleophilic Amino-chlorin Intermediate (TPC_{N1P}). To efficiently synthesize the amphiphilic photosensitizer (TPC_{N1P}), we followed the previously optimized procedure (Supplementary Figure 1). Tetraphenylporphyrin (TPP) and p-amino-tetraphenylporphyrin (TPP were prepared as described in our previous publication[1]. For the current synthesis of TPCN1P the procedure was scaled up 8 times to synthesize 3.6 g of 5-(4-(2-(1-piperazinyl)acetyl)aminophenyl)-10,15,20-triphenylchlorin (TPC_{N1P}). The yields in each step were not affected. TPC_{N1P} is present in two isomeric forms, depending on the position of the reduced double bond in the porphyrin system. These will be present in approximately equal proportions [1].^{1}H NMR ($CDCl_3$):δ = 9.34, 9.39 (s, 1H), 7.86–8.65 (m, 16H), 7.66–7.73 (m, 9H,), 4.18–4.19 (br s, 4H), 3.29 (s, 2H), 3.17 (m, 4H), 2.81 (m, 4H), −1.37 (br s) ppm. ^{13}C NMR ($CDCl_3$):δ = 168.37, 167.48, 152.61, 143.14, 142.22, 140.86, 139.20, 138.32, 137.19, 136.99, 135.33, 134.64, 133.98, 133.01, 132.37, 132.12, 131.96, 128.17, 127.69, 126.81, 123.56, 123.38, 122.79, 122.08, 119.22, 117.94, 112.41, 111.65, 62.63, 53.50, 45.59, 35.90 ppm. HRMS (ESI): *m/z* calculated for $C_{50}H_{44}N_7O$ ($[M+H]^+$), 758.3602; found 758.3613.

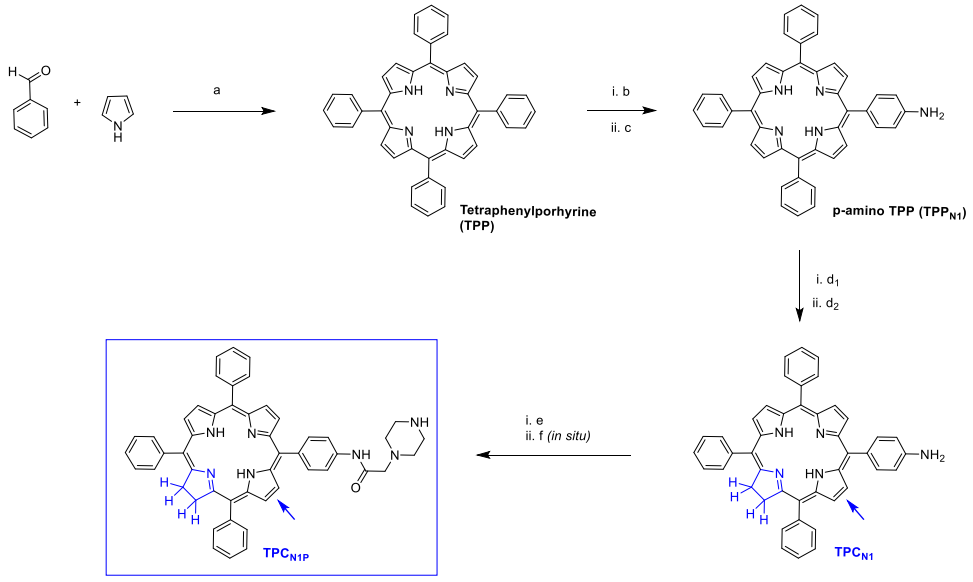

Figure S1. Synthesis of Nucleophilic Intermediate **4** (TPC_{N1P}). Reagents and conditions: (a) Propionic acid, reflux, 30 min (20%); (b) $NaNO_2$ (1.8 equiv), TFA, 25°C, 3 min; (c) $SnCl_2.2H_2O$, concentrated HCl, 60 °C, 1 h (54%); (d_1) *p*-Toluenesulfonyl hydrazide, K_2CO_3, pyridine, reflux, 24 h; (d_2) *o*-Chloranil, CH_2Cl_2, 25 °C (80%); (e) Chloroacetyl chloride, Et_3N, CH_2Cl_2, 25°C, 2 h; *in situ*-(f) Piperazine, CH_2Cl_2, 25°C, 12 h (65%). Only one of the two isomers of TP_{CN1} and TPC_{N1P} is shown. The arrow indicates the position of the reduced double bond in the other isomer.

2. Synthesis of TPC-CS conjugate polymers with varying amounts of TPC.

Details of the different syntheses are given in 2.1-2.6 below; all structures described are shown in Figure S2.

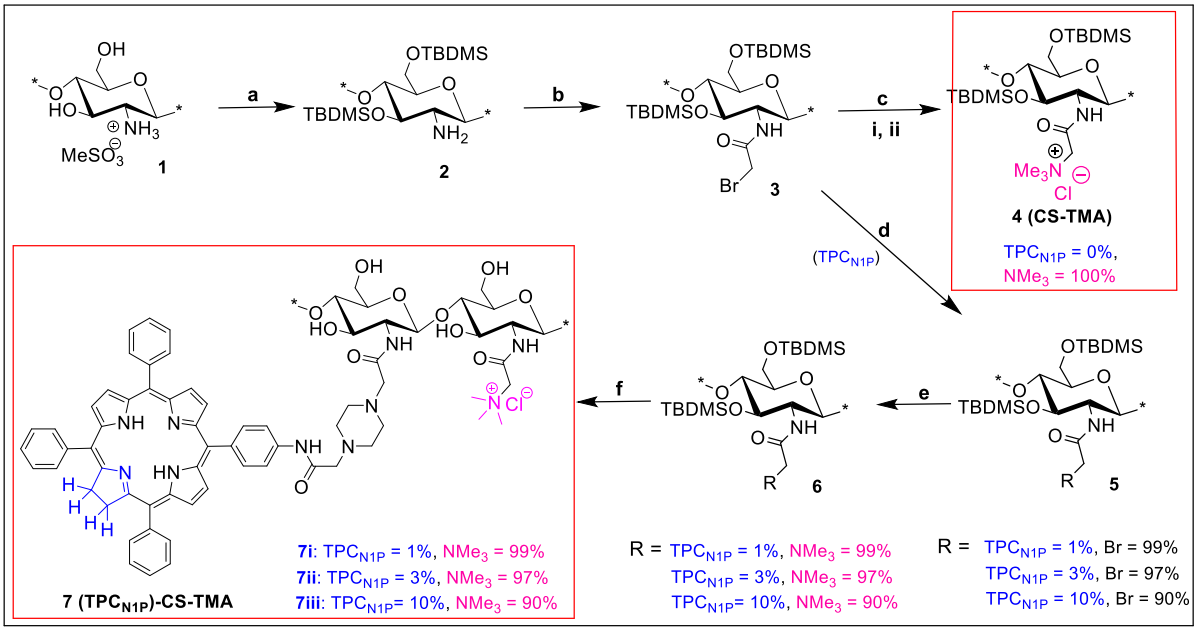

Figure S2. Synthetic scheme for (TPC$_{N1P}$)-chitosan-TMA conjugates: **(a)** TBDMSCl, imidazole, DMSO, 25 °C, 24 h; **(b)** Bromoacetyl bromide, Et_3N, CH_2Cl_2, –20 °C, 1 h; **(c)(i)** NMe_3 (31–35 wt % in EtOH, 4.2 M), CH_2Cl_2, 25 °C, 24 h; **(ii)** 1.25 M HCl-MeOH, 25 °C, 12 h. **(d)** TPC$_{N1P}$, Et_3N, CH_2Cl_2, 25 °C, 2 h; **(e)** NMe_3 (31–35 wt % in EtOH, 4.2 M), CH_2Cl_2, 25 °C, 24 h; **(f)** 1.25 M HCl-MeOH, 25 °C, 12 h.

2.1. Synthesis of 3,6-*O*-di-tert-butyldimethylsilyl (TBDMS) chitosan (2). TBDMS chitosan (compound **2**) was synthesized from chitosan mesylate salt **1** using a previously reported method [2].

2.2. Synthesis of *N*-bromoacetyl-3,6-di-*O*-tert-butyldimethylsilyl-chitosan (3). Compound **3** was synthesized using a previously published method [3]. Briefly, compound **2** (1 g, 2.53 mmol) was dissolved in dry CH_2Cl_2 (15 ml) under N_2 atmosphere. This solution was cooled to −20 °C and Et_3N (1.76 ml, 12.66 mmol) was added, followed by a slow dropwise addition of

bromoacetyl bromide (0.89 ml, 10.13 mmol). The stirring was continued for 1 hat −20 °C before the reaction mixture was diluted with CH_2Cl_2 (20 ml) and concentrated in vacuo. The obtained crude residue was triturated and stirred with CH_3CN (25 ml), filtered and washed with fresh CH_3CN (3 × 20 ml), and dried. The dry material was dissolved in CH_2Cl_2 (50 ml), and the organic phase was washed with H_2O (3 × 20 ml) and brine (25 ml), dried over Na_2SO_4, and concentrated in vacuo to afford the bromoacetyl compound **3** (1.1 g, 92%) as a faint yellow powder. This material was carried over to the next step without further purification.

2.3 Synthesis of *N*-[(2-(*N*,*N*,*N*-trimethylammoniumyl)acetyl)] chitosan (CS-TMA) (4).Compound **4** was synthesized using a previously published method [3].Freshly prepared bromoacyl compound **3** (2.0 g, 3.87 mmol) was dissolved in CH_2Cl_2 (20 mL) under N_2 atmosphere. Excess Me_3N (4.2 molar in EtOH) (15 ml) was added, and the resulting mixture was stirred for 24 h at 25 °C. The reaction mixture was concentrated *in vacuo* to isolate the corresponding compound *N*-(2-(*N*,*N*,*N*-trimethylammoniumyl)acetyl)-3,6-di-*O*-TBDMS-chitosan. Deprotection of this material using 1.25 M HCl-MeOH (20 mL) at 25°C for 12 h, followed by ion-exchange, dialysis and freeze-drying afforded *N*-(2-(*N*,*N*,*N*-trimethyl ammoniumyl)acetyl) chitosan **4** as the light brown solid. Yield: 0.90 g, 87%. ^{1}H NMR (400 MHz, D2O): δ 2.08 ($NCOCH_3$), 3.36 [$N(CH_3)_3$], 3.54-3.88 (H-2–H-6), 4.21 (s, CH_2CO), 4.66 (H-1) ppm.

2.4. General procedure for synthesis of [*N*-(2-(4-(*N*-(4-(10,15,20-Triphenylchlorin-5-yl)-phenylamino) carbonyl methyl) piperazin-1-yl)acetyl)][*N*-(2-bromoacetyl)] chitosan (TPC_{N1P})(Br) chitosan (5i-iii).

This synthesis was performed using a previously published method [1]. Compound **3** (1 equiv) and the nucleophilic amino-chlorin intermediate (TPC_{N1P}) (0.01, 0.03 and 0.10 equiv) were dissolved in CH_2Cl_2 (25 ml) under N_2 and with protection from light. An exact equimolar quantity of Et_3N with respect to TPC_{N1P} was added, and the reaction mixture was stirred at 25 °C for 24 h. The full consumption of the starting material was confirmed by TLC as previously reported. The reaction mixture was diluted with CH_2Cl_2 (55 ml) and washed with H_2O (2 × 25 ml) and brine (25 ml). The organic phase was dried over Na_2SO_4 and concentrated *in vacuo* to afford

compound **5i-iii** as a brown solid. This material was carried over to the next step without further purification.

2.5. General procedure for synthesis of [*N*-(2-(4-(*N*-(4-(10,15,20-Triphenylchlorin-5-yl)phenylamino) carbonyl methyl)piperazin-1-yl)acetyl)][*N*-(2-(*N*,*N*,*N*-trimethyl ammoniumyl)acetyl)]-3,6-di-*O*-tert-butyldimethylsilyl-chitosan bromide (TPC_{N1P})-Di-TBDMS-chitosan-TMA (6i-iii).

This synthesis was performed using a previously published method [1].Compound **3** (1 equiv) was dissolved in CH_2Cl_2 (15 ml) under N_2 with protection from light. Excess Me_3N (31−35 wt % in EtOH, 4.2 M) (15 ml) solution was added to the reaction mixture, and it was stirred at 25 °C for 24 h. The reaction mixture was concentrated *in vacuo*, and the crude material was completely dried under high vacuum, yielding the crude compound **6i-iii** as a brown solid. The material **6i-iii** was used directly for the next step without further purification.

2.6. Synthesis of [*N*-(2-(4-(*N*-(4-(10,15,20-Triphenylchlorin-5-yl)phenylamino) carbonyl methyl)piperazin-1-yl)acetyl)][*N*-(2-(*N*,*N*,*N*-trimethylammoniumyl)acetyl chitosan chloride (TPC_{N1P})-chitosan-TMA (7i-iii).

Compounds **6i-iii** were stirred in 1.25 M HCl-MeOH (25 ml) at 25 °C for 12 h. The reaction mixture was then diluted and ion-exchanged by the addition of 5% NaCl (aqueous) (40 ml) to the solution. It was then stirred for 1 h before it was dialyzed against 8% NaCl (aqueous) for 24 h, and then again against deionized water for 2 days. The clean brown solution was subsequently freeze-dried to afford the corresponding final nanoconjugates **7i-iii** as brown fluffy material. In some cases, the reaction was repeated in order to get rid of traces of TBDMS groups from the chitosan backbone. ^{1}H NMR (DMSO-d_6/D_2O, 96:4): δ = 7.82–8.59 (m, β-pyrrole-CH, triphenyl-Ho & R-NHTPC-phenyl-Ho,m), 7.69–7.74 (m, triphenyl-Hm,p), 4.46 (br s, H-1), 4.09–4.11 (m, CH_2CONGlc and chlorin β-pyrrole-CH_2), 3.31–3.70 (br m, partially overlapped with HDO peak, H-2 GlcNAc, H-3, H-4, H-5, H-6, H-6´, H-2 GlcNHCO, TPCNHCOCH_2-pip, piperazine ring-CH_2), 3.20–3.24 (s, $^+N(CH_3)_3$)) ppm.

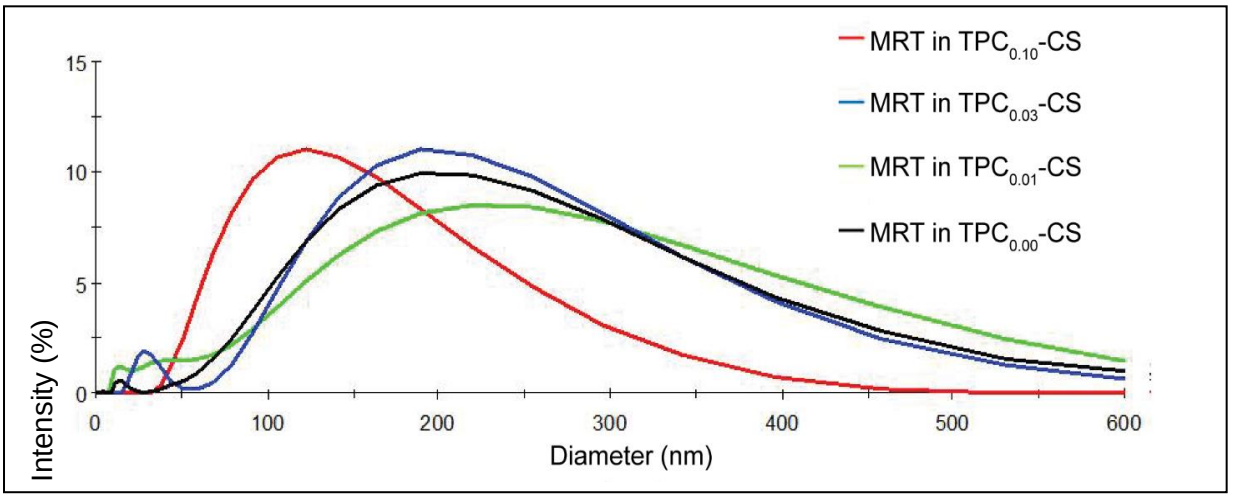

Figure S3. Size distributions of MRT-loaded TPC-CS NPs containing different amounts of bound PS bound, i.e. 10%, 3% or 1% of side chains of CS were conjugated to TPC. Polydispersity index, PDI= 0.25 (0.10), 0.45 (0.03 and 0.01).

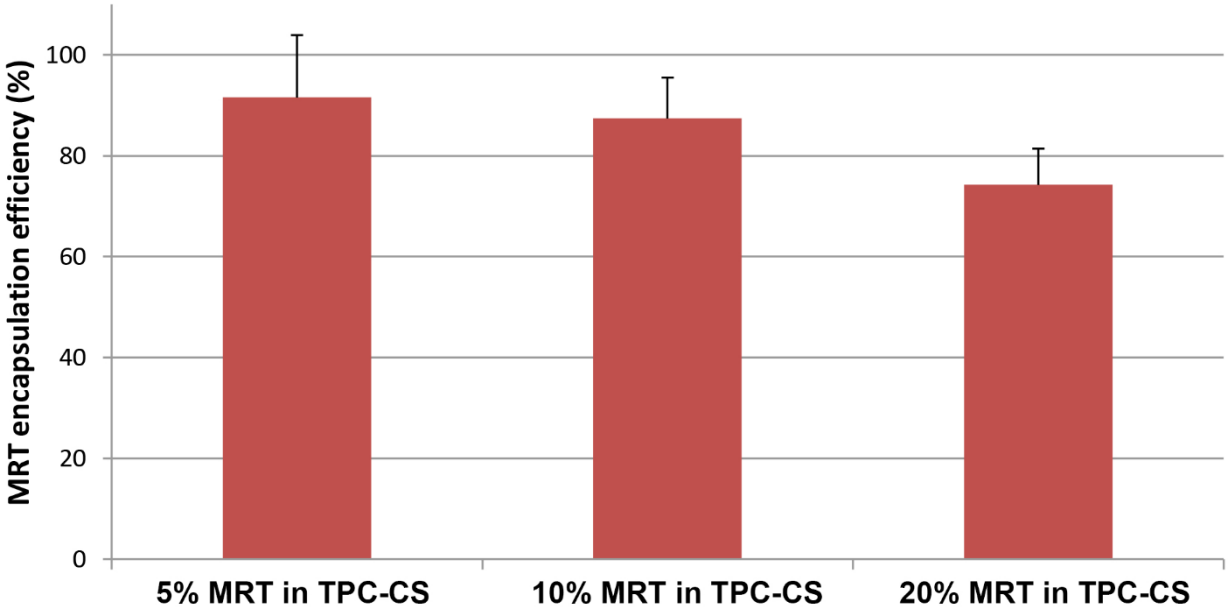

Figure S4. Increased loading concentration of MRT (5-20% (w/w) relative to TPC-CS) during preparation of TPC-CS NPs resulted in slightly decreased MRT encapsulation efficiency. Mean MRT ± SD (n=3) measured in solubilized micellar formulations using HPLC.

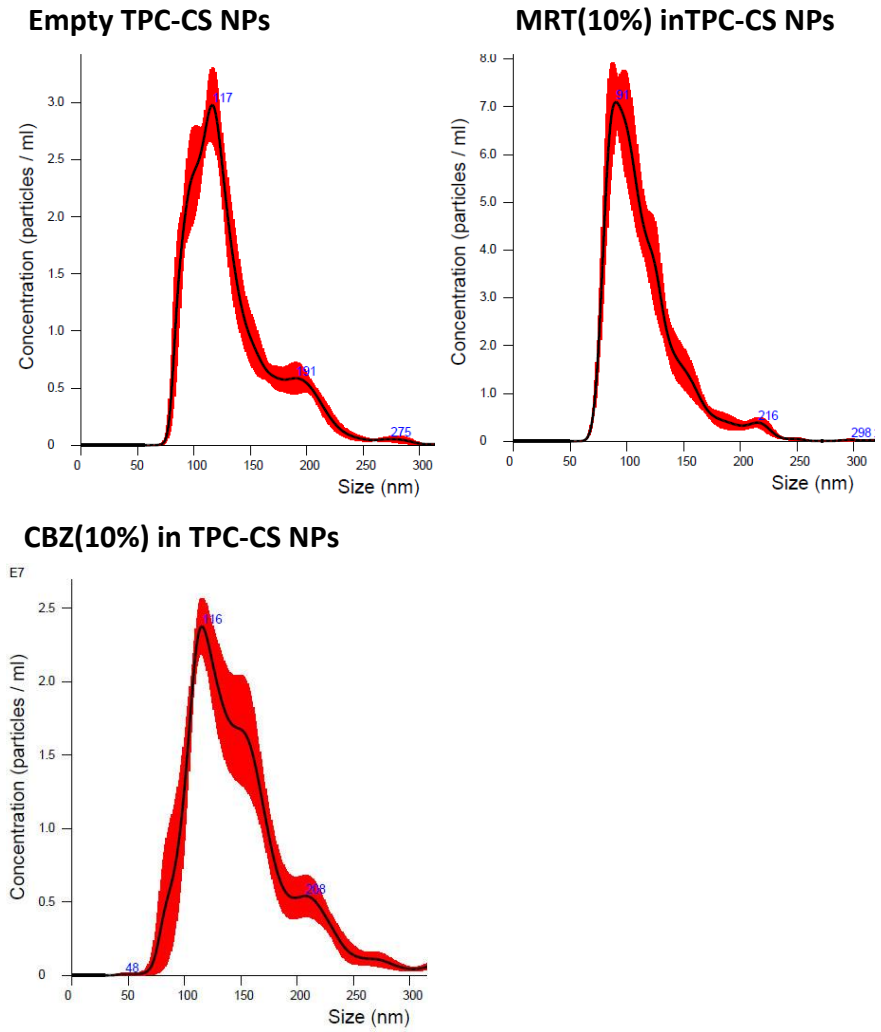

Figure S5. Size distribution of the empty, MRT- and CBZ-loaded TPC-CS NPs measured by NanoSight instrument. The drug loaded NPs contained 10% (w/w) of the drugs. The NP formulations were stored for 5 days and sonicated immediately prior to NTA measurement. In red, standard deviations calculated by the NTA software.

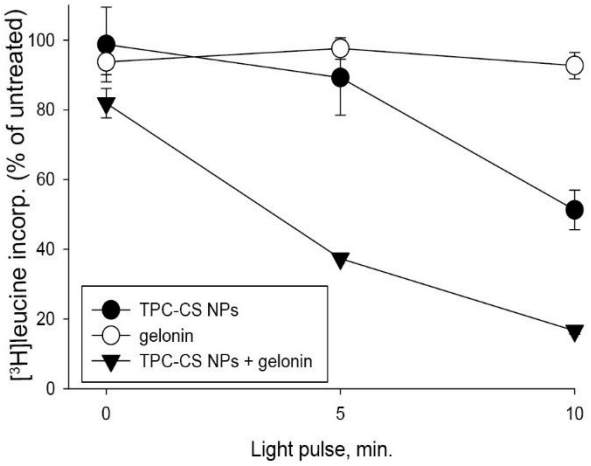

Figure S6. TPC-CS NPs mediate PCI of the toxin gelonin. MDA-MB-231 cells were incubated with TPC-CS NPs (0.20 µg/ml), gelonin (3 µg/ml) and TPC-CS NPs + gelonin at 37 ºC for 18 h. The cells were washed and chase at 37 ºC for 2 h prior to light exposure. Cytotoxic effect was evaluated 30 min post light exposure by measuring protein synthesis (incorporation of [^{3}H]leucine) as previously described [4].Mean values ± SD (n=3).

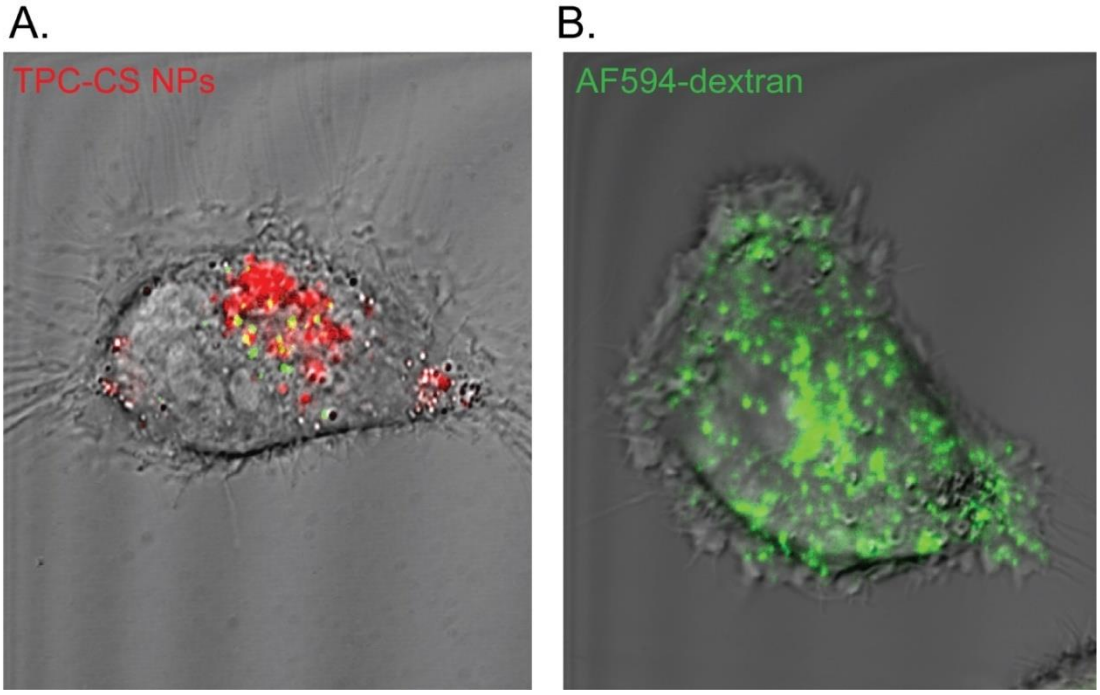

Figure S7. Live-cell imaging of TPC-CS NPs (in red) and AF594-dextran (green) in HeLa cells. The cells were incubated with (A) TPC-CS NPs (25 µg/ml) and AF594-dextran (50 µg/ml), or (B) only AF594-dextran for 1 h at 37 °C. Then, the cells were washed and chased for 2 h at 37 °C before time-lapse confocal microscopy imaging: laser excitation at 405 nm and 561 nm with 20 sec. imaging interval for 10 min. Plasma membrane blebs appeared after 60 sec and expanded up to 160 sec. Fluorescence of both TPC and AF594-dextran began to fade at the same time and disappeared after 300 sec (A), whereas the AF594-dextran fluorescence were stably retained over the laser illumination period (10 min) in the cells without uptake of TPC-CS NPs (B).

References

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