Electronic Supporting Information

Cellular Endocytosis and Trafficking of Cholera Toxin B-Modified Mesoporous Silica Nanoparticles

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Materials and Methods. All chemical reagents were purchased from Aldrich and used without further purification. All the organelle-specific staining dyes such as Lysotracker® Red DND-99, Hoechst 33342, ERtracker™ Blue-White DPX and BODIPY-TR™ Ceramide were obtained from Life Technologies. RPMI 1640 medium, fetal bovine serum (FBS), 0.25% Trypsin (2.21 mM EDTA, 1X) and penicillin-streptomycin solution were bought from Corning. Pierce™ BCA protein quantification assay was purchased from ThermoFisher. Nano-W™ was purchased from Nanoprobes Inc. Thermogravimetric analysis (TGA) was carried out with a Mettler Toledo TGA/SDTA851 instrument equipped with a platinum pan and using a heating rate of 5 °C/min under nitrogen. A JEOL JEM 2100 LaB6 Transmission Electron Microscope (TEM) was used to corroborate particle size and morphology. Each TEM sample was prepared by suspending the nanoparticles in methanol. A drop of the suspension was placed on a TEM carbon grid 200 mesh

and the solvent was allowed to evaporate overnight. For the negative-staining with Nano-W™ (Nanoprobes, Inc.), the CTxB-MSN solution was dropped on the Lacey carbon grid and allowed to dry for few seconds, before the sample dried completely on the grid, one drop of the Nano-W™ was added. Another drop of Nano-W™ was added before the first drop dried. Finally, the grid was allowed to air dry for few hours before imaging with TEM. Dynamic light scattering (DLS) and ζ-potential measurements were carried out using a Malvern Instrument Zetasizer Nano. The N_2 sorption isotherms were determined in a NOVA 2200e Quantachrome surface area and pore size analyzer. A BD LSRFortessa™ cell analyzer was used for the fluorescence-activated cell sorting (FACS) experiments. An Olympus Fluoview FV 1000 confocal fluorescence microscope system was used for the LCSM experiments.

Synthesis of heterobifunctional carboxylic acid poly(ethylene glycol) (MW = 2K) (PEGC) polymer silane

The heterobifunctional PEGC linker (**Scheme S1**) was synthesized using a three-step approach. In the first step; dehydrated poly(ethylene glycol) (MW = 2K) (3 mmol, 6 g, PEG 2K) was dissolved in dry tetrahydrofuran (THF; 300 mL) under N_2 atmosphere. To this solution sodium hydride (9 mmol; 363.3 mg, NaH) was added and stirred at RT for 30 min. After that *tert*-butyl bromoacetate (7.5 mmol; 1.1 mL, *t*BBA) was added, and the final solution was stirred for further 24 h under N_2 atmosphere at RT. The products were concentrated by rotary evaporation, precipitated with diethyl ether and isolated by gravity filtration. The monosubstituted product was purified by silica column chromatography using a solvent mixture of methanol and dicloromethane (MeOH:CH_2Cl_2; 1:9 vol.). The monosubstituted product was concentrated by rotary evaporation and dried using a lyophilizer. Yield: 1.91g (32 %wt.). ^{1}H NMR (300MHz, $CDCl_3$, ppm): δ 1.45 (s, 9H, $C(CH_3)_3$), 3.46 (s, 1H, OH), 3.58−3.75 (m, 180H, OCH_2), 3.99 (s, 2H, $CH_2C(O)OtBu$); ^{13}C

NMR (300MHz, $CDCl_3$, ppm): δ 28.21 ($C(CH_3)_3$), 69.12 ($CH_2C(O)OtBu$), 70.64 (OCH_2), 81.62 ($C(CH_3)_3$), 169.68 (C(O)OtBu); FT-IR (cm^{-1}): 1104 (C-O), 1241 ($C(CH_3)_3$), 1466 (CH_2,CH_3), 1745 (C=O), 2881 (C-H), 3512 (O-H). The monosubstituted heterobifunctional carboxylic acid PEG polymer was generated by deprotection of the monosubstituted product in the presence of trifluoroacetic acid (TFA) in CH_2Cl_2 (3:7 v/v) at RT for 24 h. The resulting solution was concentrated by rotary evaporation and redissolved in a dispersion of 150 mg Amberlyst A21 in 15 mL of CH_2Cl_2. The dispersion was stirred for further 24 h at RT, followed by filtration to get rid of Amberlyst A21. The solid polymer was obtained by rotary evaporation and was further dried using a lyophilizer for 18 h. ^{1}H NMR (300MHz, $CDCl_3$, ppm): δ 3.58−3.75 (m, 180H, OCH_2), 4.15 (s, 1H, C(O)OH); ^{13}C NMR (300MHz, $CDCl_3$, ppm): 68.81 ($CH_2C(O)OH$), 70.35 (OCH_2), 172.59 (C(O)OH); FT-IR (cm^{-1}): 1103 (C-O), 1466 (CH_2,CH_3), 1738 (C=O), 2883 (C-H), 3456 (O-H). Finally, the PEGC polymer silane was obtained through the reaction of heterobifunctional polymer with triethoxysilylpropyl isocyanate (TESPI, 1:1.1 mol) in CH_2Cl_2. The reaction was carried out in an ice bath for 48 h under stirring conditions. The product was concentrated by rotary evaporation and dried by lyophilization.^{1}H NMR (300MHz, $CDCl_3$, ppm): δ 0.85 (t, 2H, CH_2Si), 1.23 (t, 9H, OCH_2CH_3), 1.26 (m, 2H, CH_2CH_2Si), 2.69 (t, 2H, $CH_2NHC(O)O$), 3.4 (q, 6H, OCH_2CH_3), 3.5−3.75 (m, 180H, OCH_2), 3.85 (s, 2H, CH_2COOH), 6.41 (s, 1H, $CH_2NHC(O)O$); ^{13}C NMR (300MHz, $CDCl_3$, ppm): 13.10 (CH_2Si), 29.76 (CH_3CH_2OSi), 53.52 ($CH_2NHC(O)O$), 68.81 ($CH_2C(O)O$), 70.89 (OCH_2), 157.44 (NHC(O)O), 172.18 (C(O)OH); FT-IR (cm^{-1}): 705, 719, 797 (SiCH), 1100 (Si-O), 1103 (C-O), 1466 (CH_2,CH_3), 1533 (NHC=O), 1690 (C=O), 2886 (C-H), 3330 (N-H).

Synthesis of FITC-labeled CTxB chemically attached to Rhodamine B-labeled mesoporous silica nanoparticles (FCTxB-RMSNs)

The synthesis of FCTxB-RMSNs was carried out by following the same protocol as described in the Experimental section (2.1, 2.2 and 2.3), but using Rhodamine B isothiocyanate instead of FITC and FITC-labeled CTxB instead of the regular CTxB protein.

The amount of protein grafted to PEGC-RMSNs was determined using a calibration curve for the fluorescence of FITC-labeled CTxB (λex = 495 nm; λem = 515 nm). For this, the total protein content of the reaction supernatant was found to be 7.95 μg FCTxB/mg of RMSN material.

Determination of CTxB protein using BCA protein quantification assay.

The amount of protein grafted to PEGC-FMSNs was determined using a colorimetric Pierce BCA protein quantification assay from the protein remaining in reaction supernatant following collection of PEGC-CTxB-FMSN material. For this, the total protein content of the reaction supernatant was found to be 167.62 μg, giving the extent of protein attachment to be 8.23 μg CTxB/mg of FMSN material.

Double and triple endocytosis inhibition assays

For the double endocytosis inhibition assays, the cell medium was removed and the cultured cells were treated with 2 mL of Wortmannin (240 nM) and Chloropromazine (10.66 μg/mL) for 30 min. After that, the same experimental procedure was followed as the one described for the single endocytosis inhibition assays.

For the triple endocytosis inhibition assays, the cell medium was removed and the cultured cells were treated with 2mL of Wortmannin (240 nM), Chloropromazine (10.66 μg/mL) and Genstein (50 μg/mL) for 30 min. After that, the same experimental procedure was followed as the one described for the single endocytosis inhibition assays.

FACS data analysis. The FACS data was analyzed with BD FACSDiva software. To determine the extent of changes in the uptake of the different materials, the mean fluorescence of the population of cells negative for nanoparticle uptake was subtracted from the mean fluorescence of the positive population and normalized the control. Prior to harvesting, all cells were washed 2x with PBS between each treatment. The experiments were run in triplicate for each endocytosis inhibition assay.

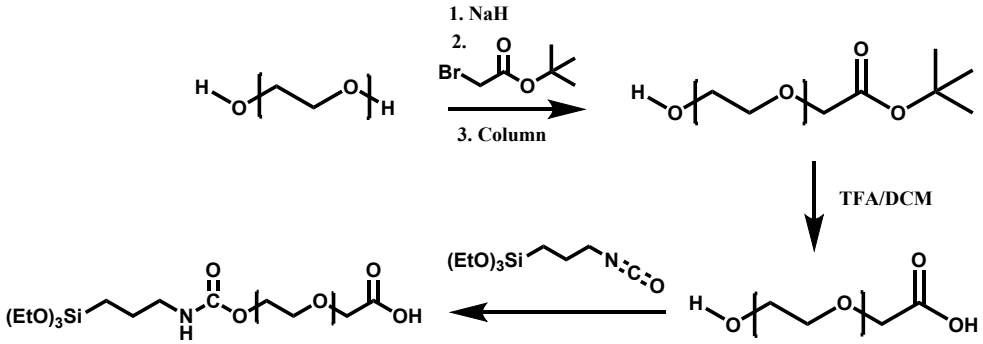
Cholera Toxin Subunit B Receptor Specificity Assay

For competitive binding inhibition using free CTxB protein, cells were seeded in 6-well tissue culture plates at $2x10^5$ cells/well and incubated for 36 h at 37 ^oC/5% CO_2. The medium was aspirated and 4.1 μg of CTxB in 2 mL of RPMI 1640 serum-free medium were added to each well. The HeLa cells were incubated for 30 min at 37 ^oC/5% CO_2, afterwards the medium was aspirated and replaced with 2 mL of MSN, PEGC-MSN, or CTxB-MSN dispersion (25 μg/mL) in RPMI 1640 serum-free medium. The cells were incubated in the presence of the FMSN materials for 1 h at 37^oC/5% CO_2. After which the medium was aspirated and replaced with RPMI 1640 medium supplemented with FBS and incubated for an additional 2 h in the same conditions. The cells were then prepared for FACS experiments using the aforementioned procedure.

Internalization of FCTxB-RMSNs by LSCM experiments

After growing HeLa cells as described in section 2.4, the cell medium was removed and replaced with 300 μL of serum-free medium containing RMSN, or FCTxB-RMSN materials at a concentration of 12.5 μg/mL. The, cells were incubated for 60 min at 37 °C/5% CO_2. Afterwards, the medium was removed, cells were washed with PBS and fixed with

paraformaldehyde solution (4% (v/v)) for 1 h. After that, the fixing solution was removed and wells were washed with PBS. A nuclei-staining dye, Hoescht 33342 (300 µL, 5µg/ml) was added and the cells were incubated for 30 min 37 °C/5% CO_2. Finally, the staining solution was aspirated and the cells were washed with PBS. After the addition of 300 µL PBS to each well, the intracellular localization of all materials was determined by LSCM.



Scheme S1. Synthesis of PEGC silane derivative.

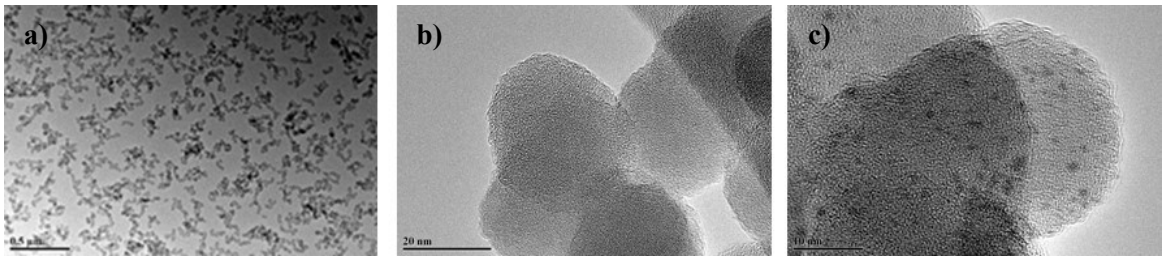


Fig. S1 TEM images of a) PEGC-FMSNs, b) CTxB-FMSNs and c) negatively-stained (Nano-W) CTxB-FMSNs.

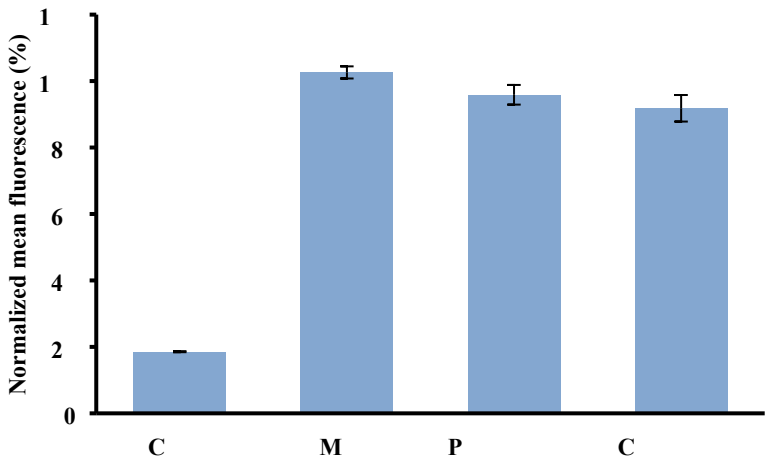


Fig. S2 Competitive binding effect of CTxB for the internalization of CTxB protein, FMSNs, PEGC-FMSNs and CTxB-FMSNs. Data represent means ± SEM of triplicates from 3 independent experiments. Data are represented in relation to untreated control (100%).

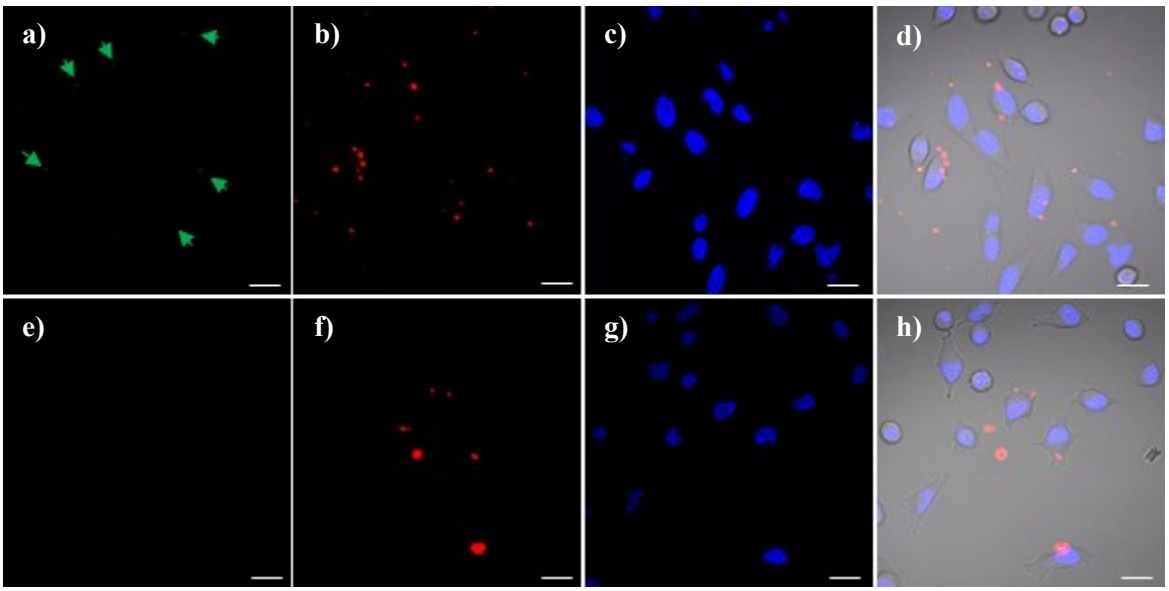


Fig. S3 LSCM images of HeLa cells inoculated with FCTxB-RMSNs. (**a & e**) FCTxB (green). (**b & f**) RMSNs (red). (**c & g**) Nuclei-stained image. (**d & h**) A super-imposed micrograph of the previous images with the DIC channel. All the scale bars are 10 μm in size.

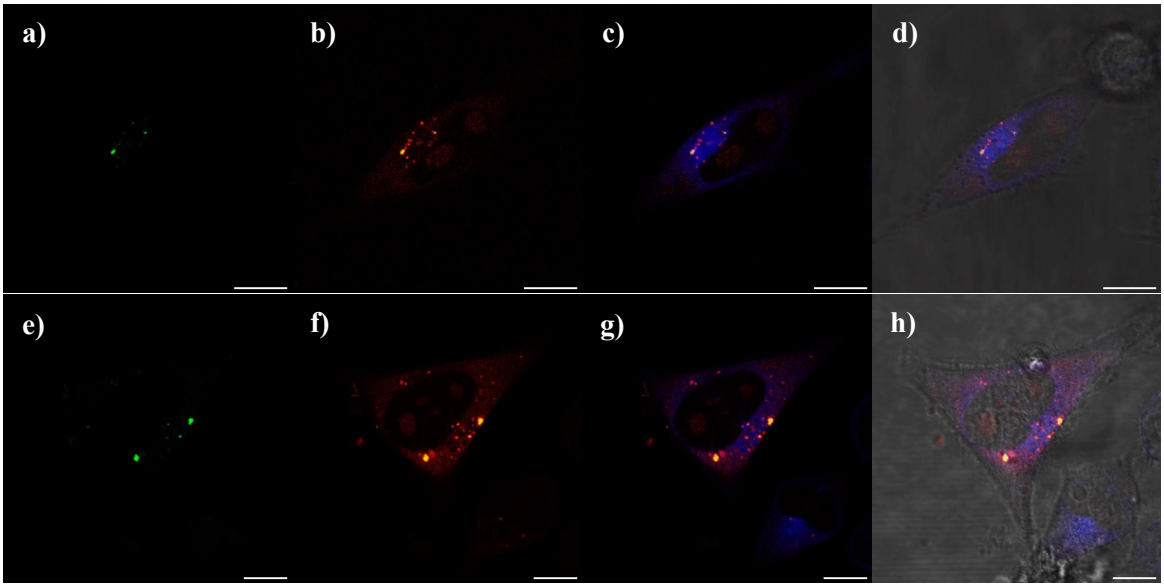


Fig. S4 LSCM images of HeLa cells inoculated with PI-loaded CTxB-FMSNs. (**a & e**) CTxB-FMSNs (green). (**b & f**) CTxB-FMSNs super-imposed with PI molecules (red). (**c & g**) ER-stained image super-imposed with CTxB-FMSNs. (**d & h**) A super-imposed micrograph of the previous images with the DIC channel. All the scale bars are 10 μm in size.