Bioresponsive Mesoporous Silica Nanoparticles for Triggered Drug Release

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Materials. All materials were obtained from Sigma Aldrich unless otherwise specified and were used as received. HeLa-GFP and HT-1080 cells were cultured in Dulbecco's modification of Eagl'es medium (DMEM, purchased from Invitrogen) with 10% bovine serum (Invitrogen), 5 I.U. penicillin, and 5 µg/mL streptomycin. 3T3-J2 Fibroblast cells were cultured in Dulbecco's modification of Eagl'es medium (DMEM, purchased from Invitrogen) with 10% bovine serum (Invitrogen), 5 I.U. penicillin, and 5 µg/mL streptomycin. All animal work was performed in accordance with the institutional animal protocol guidelines in place at the Massachusetts Institute of Technology, and it was reviewed and approved by the Institute's Animal Research Committee.

Synthesis of mesoporous silica nanoparticles: To a 1g CTAB in NaOH (14 mM) at 80 °C, was added 5 mL TEOS and 5 µL APTES. The solution was stirred at 80 °C for 2 h to produce the white MSNPs suspension. The product was washed with methanol and water several times and then refluxed for 6 h in HCl/methanol to extract the CTAB. The Final MSNPs were then again washed several times with methanol and water and dried in air.

Synthesis of on-surface polymer-coated silica nanoparticles: For synthesizing single coated MSNPs, a 2 mg/mL solution of MSNPs was prepared in distilled water (DI) and sonicated to disperse the particles until a uniform colloidal solution was observed. To this solution 1 M of N-(3-Aminopropyl)methacrylamide hydrochloride, or APMA (Polysciences, Inc.) was added and left on the shaker table overnight. The solution was then centrifuged at 13200 rpm for 10 minutes to remove excess APMA. Next a 6 mM total monomer solution with an appropriate amount of monomers in DI water was added to the reaction vial. . The total monomer concentration used in forming the shell was necessarily kept very low (<8 mM) to avoid encapsulation of multiple MSNPs in the polymer matrix. Monomers that were used included: poly(ethylene glycol)

diacrylate (PEGDA, molecular weight 700), N-Isopropylacrylamide (NIPAM), PEG-MMP-HD, PEG-MMP-LD. Amine functionality was incorporated by copolymerizing APMA, which was 10% by mole of the total monomer concentration. For polymer shell containing Coumarin, a hydrophobic, fluorescent comonomer 7-[4-(Trifluoromethyl) coumarin] methacrylamide dissolved in dimethyl sulfoxide was added to the reaction vial. For NIPAm based polymer shells, a molar composition of 80% monomer (NIPAM), 10% crosslinker (PEG-DA) and 10% comonomer was used. For PEG based polymer shells, 90% PEGDA and 10% of comonomer was used. The total monomer concentration used for all the polymer shell synthesis was 6 mM. After addition of all the monomers, 1.3% by volume of 0.1 M ammonium persulfate (APS) was added. After thorough mixing by vortex, the polymerization reaction was initiated by adding 1% by volume N,N,N′,N′-Tetramethylethylenediamine (TEMED). The reaction solution was stirred overnight. The solution was then centrifuged at 13200 rpm for 10 minutes and resuspended in DI water to remove excess unreacted monomers and initiators. The synthesized polymer coated MSNPs were cleaned by several such cycles of centrifugation/resuspension. The clean single-polymer coated MSNPs can then be used for synthesizing multiple polymer shells using the same procedure. The single-coated polymer-MSNPs can further be used to obtain core-loaded MSNPs, by adding doxorubicin hydrochloride to the cleaned nanoparticles. Doxorubicin was added at a concentration of 60 μg/mg MSNPs. A second polymer shell was then synthesized on the cleaned Dox loaded single-coated MSNPs by following the same procedure outlined above. If a Dox loaded polymer-MSNPs were used, then the second shell polymerization was carried out in a1mg/ml doxorubicin solution in DI water. For synthesizing the second polymer shell on unloaded polymer coated MSNPs, the reaction was carried out in just DI water. For shell-loaded MSNPs, the two polymer coats were synthesized first and the double-coated MSNPs were then

incubated with doxorubicin at a doxorubicin concentration of 60 μg/mg MSNPs. To create Vivotag 680®-tagged MSNPs, after the polymer shell synthesis with APMA as comonomer, 5X molar excess of amine reactive Vivotag 680® NHS were added to the cleaned polymer-MSNP pellet and reacted for at least 4 hours. After every synthesis step and doxorubicin loading steps, the MSNPs were cleaned via several cycles of centrifugation and resuspension to remove unreacted monomers, excess reagents, and soluble reaction byproducts. The particle size of the synthesized polymer-MSNPs was characterized by dynamic light scattering instrument (Zetasizer-Nano, Malvern, Inc.). The data presented is an average of 3 experiments with at least 50 measurements in each experiment. We found that the nanoparticle size and polymer thickness could be controlled by the monomer concentration used for polymerization (Figure S1a,b), thus providing synthetic flexibility.

Synthesis of MMP-sensitive acrylate–PEG–(peptide–PEG)m–acrylate Macromers: The bis-cysteine peptide sequences CGPQGIWGQGCR (highly degradable, HD, 1261.42 g/mol), CGPQGIAGQGCR (native collagen, NC, 1146.28 g/mol), and CGPQGPAGQGCR (least degradable, LD, 1130.23 g/mol) were custom synthesized by Aapptec (Louisville, KY). In a typical reaction, 183.8 mmol biscysteine peptide (HD, 231.6 mg) was reacted with a 1.6 molar excess of PEGDA (3400 Da, 1 g, 294.1 mmol) by dissolution in 10 mL 100 mM sodium phosphate, pH 8.0 (94.7 mM Na2HPO4, 5.3 mM NaH2PO4). The reaction was sterile filtered through a 0.22 mm PVDF membrane (Millipore, Billerica, MA), protected from light and proceeded on a circular shaker for 85 h at room temperature to yield acrylate–PEG–(peptide–PEG)m–acrylate macromers. The reaction mixture was dialyzed against DI water (Millipore) with regenerated cellulose dialysis tubing (MWCO 3500, Pierce, Rockford, IL) for 24 h with

water changes every 4 h. The dialyzed PEG–peptide conjugates were frozen overnight (-20 °C), lyophilized, and stored at -80 °C until use. The degradability of HD-MMP and LD-MMP in solution relative to native collagen was 800% and 0.5% respectively.

Transmission Electron Microscopy: The synthesized nanoparticles were imaged on a JEOL 200CX (200 kV) transmission electron microscope (TEM). All TEM samples were prepared by casting a drop of the nanoparticle solution (diluted 10 times) on a Formvar-coated Cu TEM grid (Ted Pella) placed on a Kimwipe. The grid was then dried overnight at ambient temperature.

UV-Vis and Fluorescence Spectroscopy: All absorption and fluorescence spectra were obtained in 96Well Clear Flat Bottom UV-Transparent and black 384 well microplates respectively using a Molecular Devices microplate Spectrophotometers.

Drug loading and release: Nanoparticles were incubated with doxorubicin hydrochloride while shaking overnight at a concentration of 60 µg/mg MSNPs. Following incubation, the nanoparticles were centrifuged at 13200 rpm for 10 minutes. The amount of doxorubicin loaded was calculated by obtaining the absorbance of the supernatant and the pellet at 490 nm. For release studies, the doxorubicin-loaded nanoparticle pellet was resuspended in the same volume of distilled water and incubated at room temperature or 37 ºC (for temperature triggered release). After 2 hours the solution was centrifuged at 13200 rpm for 10 minutes and the supernatant absorbance at 490 nm was measured to calculate the percentage of drug released. Release kinetics of the loaded doxorubicin from core loaded and shell loaded polymer-MSNPs (2 mg/mL) in PBS at 37^{o}C was measured by using the Slide-A-Lyzer MINI Dialysis Device (Invitrogen). The dialysis devices were kept in a stirring water bath. At each time point the Dox loaded MSNPs solution were removed and the Dox left in the MSNPs solution was monitored by

measuring the absorbance at 490 nm and the fluorescence at 590 nm (λex = 480 nm). For release of doxorubicin in the presence of collagenase, the nanoparticles were incubated with 0.2 mg/mL collagenase in PBS buffer (pH 7) solution at 37 ºC for specified time. The samples were removed and centrifuged at 13200 rpm for 10 minutes and the absorbance at 490 nm for the supernatant and the pellet were measured to calculate the percentage of drug released.

Cellular uptake study of Vivotag PEG-MSNPs: HeLa cells were cultured on cover slips in a 12-well plate to ~70-80% confluence. To the HeLa cell cultures, 100 µL of Vivotag PEG-MSNPs and bare MSNPs (2mg/ml) were added to each well and incubated for 4 h at 37 °C, after which the nanoparticles were removed and the cells were then rinsed three times with cell medium, fixed with 4% paraformaldehyde for 20 min. The cellular nuclei were stained with DAPI. The fixed and stained cells were then observed under the fluorescence microscope with UV filter cubes and Cy 5 filter cube was used.

Cytotoxicity of nanoparticle formulations: Cytotoxicity assessments were conducted on HeLa cells in 96-well plates grown to ~70-80% confluency. Cells were incubated in triplicate with specified concentrations of the nanoparticles in 10% FBS DMEM medium for 24 h. Cells were then washed three times with cell medium and assessed for viability using the Calcein assay (Invitrogen) and MTT assay according to manufacturer's instructions. Cell viability was expressed as the percentage of viable cells compared with controls (cells without nanoparticles).

In vivo circulation of nanoparticles: Vivo-Tag labeled polymer-MSNPs were injected in Swiss Webster mice through tail-vein injections. Blood (about 100 µl) was periodically drawn retro orbitally and the near-infrared fluorescence from the circulating nanoparticles was measured using the Odyssey imaging system (Li-COR Biosciences). While the PEG-coated MSNPs with 2

mol % PEG and 10 mol % PEG had a blood circulation half-lives of 15 and 45 min, respectively, >60% of the nanoparticles coated with 90% PEG were still in circulation 90 min post-injection.

Cellular cytotoxicity due to Doxorubicin release from MMP responsive MSNPs: 3T3-J2 fibroblast cells were cultured on a 96 well plate at a density of 5000 cells/well. After 36 hours, the MSNPs were added such that the doxorubicin concentration in each well was 8μg/mL. The cells were incubated with nanoparticles for 24h and 48h followed by washing of the cells with cellular medium three times. The cytotoxicity was measured by Alamar blue (invitrogen) assay according to the manufacturer's protocol. Cell viability was expressed as the percentage of viable cells compared with controls (cells without nanoparticles).

In vivo treatment of mouse tumors: All xenograft animal studies were conducted in accordance with guidelines from the MIT Committee on Animal Care with approved protocols. A human sarcoma cell line (HT-1080) were injected subcutaneously in flanks of 4- to 6-week-old NCr/nude mice (Charles River Laboratories) at 5 x 10^6 cells per mouse per tumor. Two weeks after injection, tumor establishment was confirmed by a well established tumor mass. The animals were randomly divided into five cohorts of at least three animals each. The nanoparticles were intratumorally injected at a dose of 2 mg DOX/kg body mass (200 μL in PBS solution). Animals were euthanized 60h after the injection, and tumors were harvested for immunoblotting and histological analyses.

Immunoblotting: Frozen tumor tissues were homogenized in a lysate buffer containing protease inhibitor cocktail (Roche) on ice. The tissue lysates were centrifuged at 12,000 rpm for 20 minutes at 4° C. The supernatants were collected, and their protein concentrations measured with BCA reagents (Pierce, Rockford, Illinois). The proteins in the lyasate were separated by

electrophoresis on a 4-20% acrylamide gel (Bio-Rad) and transferred to a poly(vinylidene diluoride) membrane, which were then blocked with 5% nonfat milk in 0.1% Tween 20-TBS for 2 h at room temperature. The membranes were immunoblotted with one of the primary antibodies for caspase-9 (cell signaling) and GAPDH (cell signaling). After further washing, the blots were incubated with the appropriate horseradish peroxidase–conjugated secondary antibody. Antibody binding was detected with the Western Blotting Reagent (Pierce).

Histological (TUNEL) analysis. For histological analysis, frozen sections of tumours were prepared. The sections were first fixed 4% paraformaldehyde and stained with TMR red *in-situ* cell death detection kit (Roche) according to the protocol provided by the manufactures. The slides were counterstained with DAPI and mounted on glass slides for microscopic analysis. At least three images from representative microscopic fields were analyzed for each tumour sample using the ImageJ software.

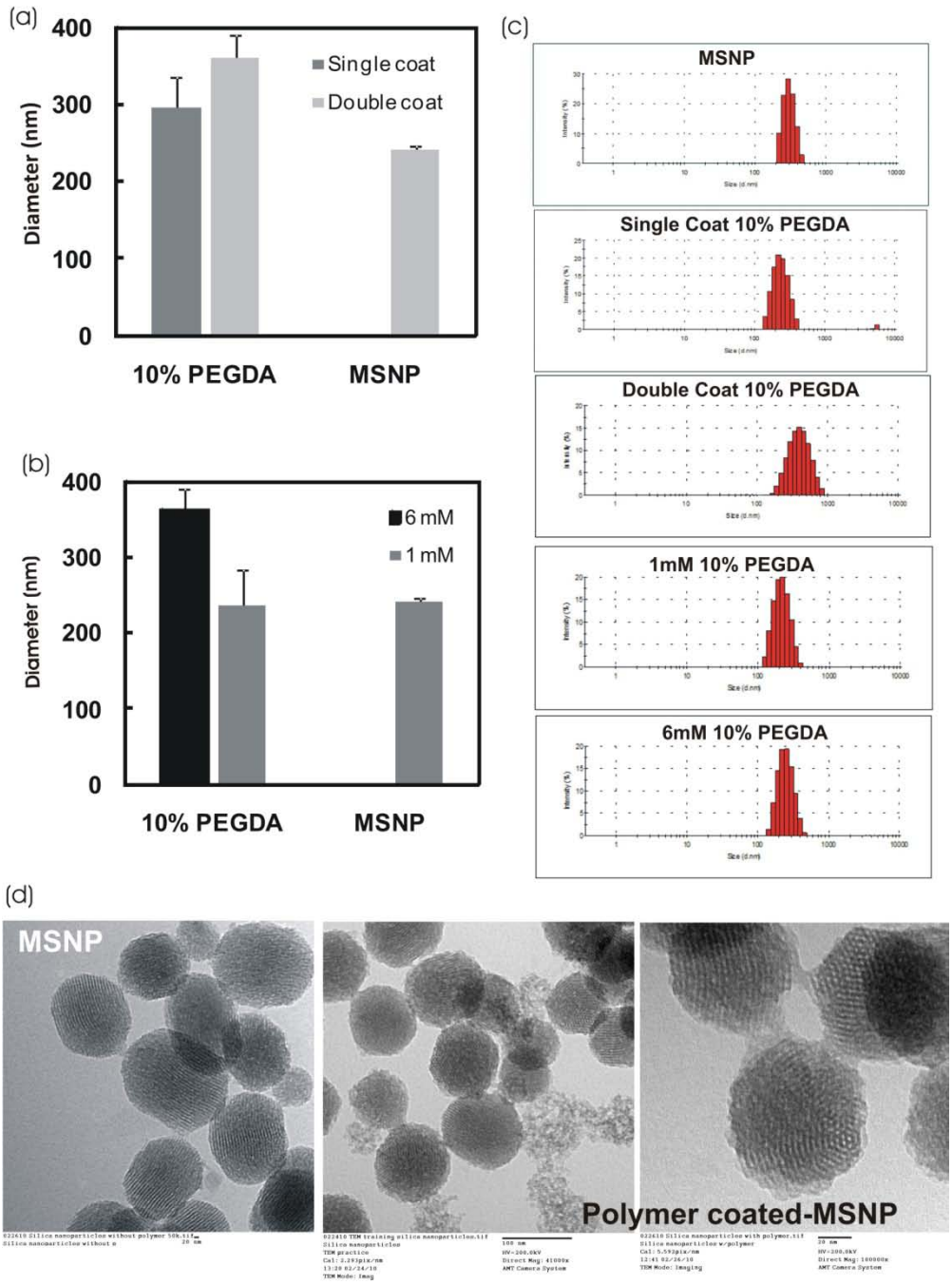

Fig S1: (a) Size of the MSNPs before and after single and double polymer shell as measured by DLS (b) Size of double shell MSNPs with varying polymer concentration. (c) Size histograms of the MSNPs before and after single and double polymer shell as measured by DLS (d) TEM images of polymer-coated MSNPs.

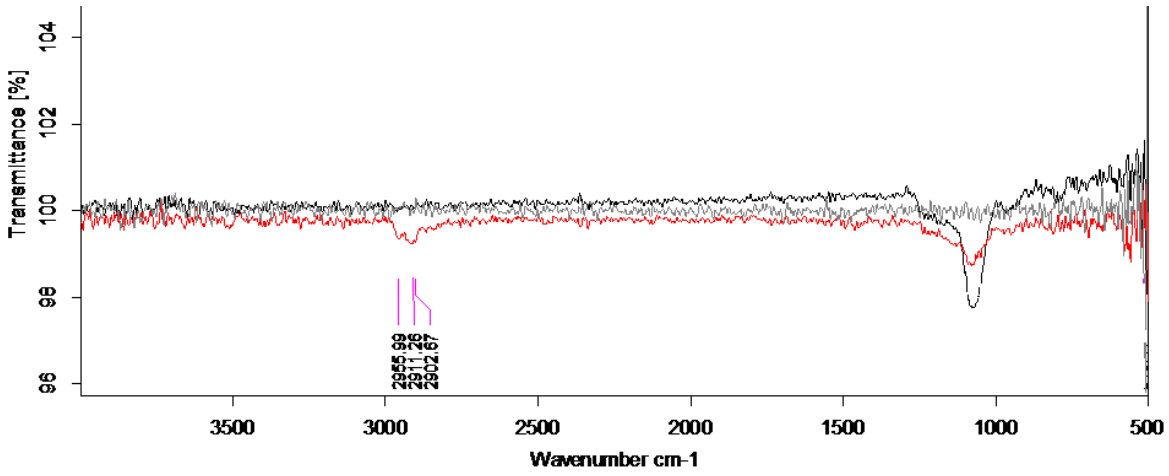

Fig S2: FT-IR spectra of MSNPs before (black) and after polymer shell coating (red).

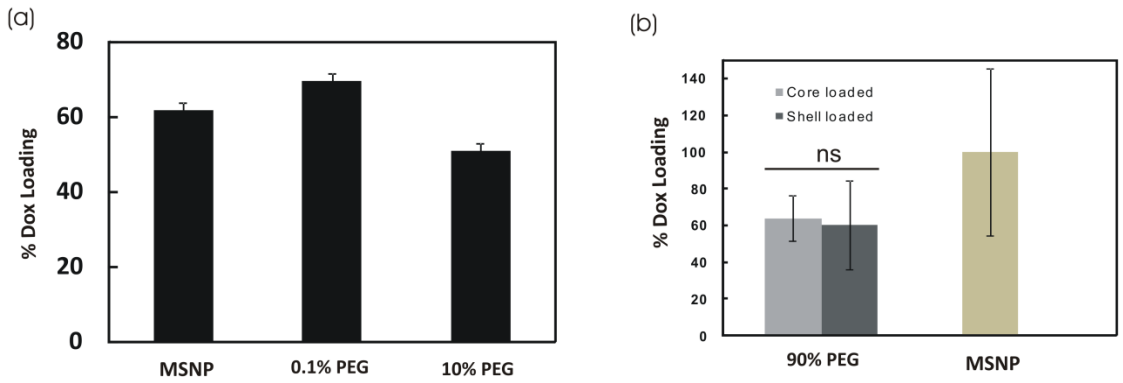

Fig S3: (a) Doxorubicin loading in (a) uncoated, 0.1% and 10% PEG coated MSNPs. (b) Relative Dox loading compared to bare MSNPs in PEG-MSNPs by core loading strategy (second shell synthesis after drug loading) and shell loading strategy (drug loading after both shell syntheses). The difference in % Dox loading was not significant (ns) compared to uncoated MSNP (p = 0.043, ANOVA; Tukey's test at 0.05 significance level)

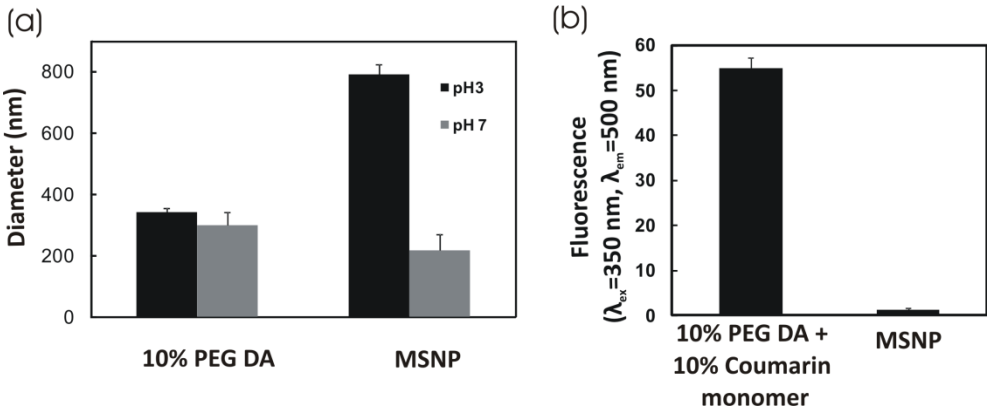

Fig S4: (a) Changes in the size of MSNPs at pH 3 and pH 7 before and after polymer coating. At pH 3 silanols (pKa 126 ≈ 3.5) on the surface of uncoated MSNPs become protonated and the negative repulsive interactions between the particles decrease significantly, resulting in aggregation. In contrast, the MSNPs coated with 10 mol % PEGDA display no significant increase in size at pH 3 compared with pH 7. (b) Fluorescence from coumarin–PEG coated MSNPs. The synthesis allows facile incorporation of comonomers that can add additional functionality to the shell. For example, a hydrophobic fluorescentmonomer (7-[4-(trifluoromethyl) coumarin] methacrylamide) was incorporated along with NIPAm and PEGDA onto the MSNPs

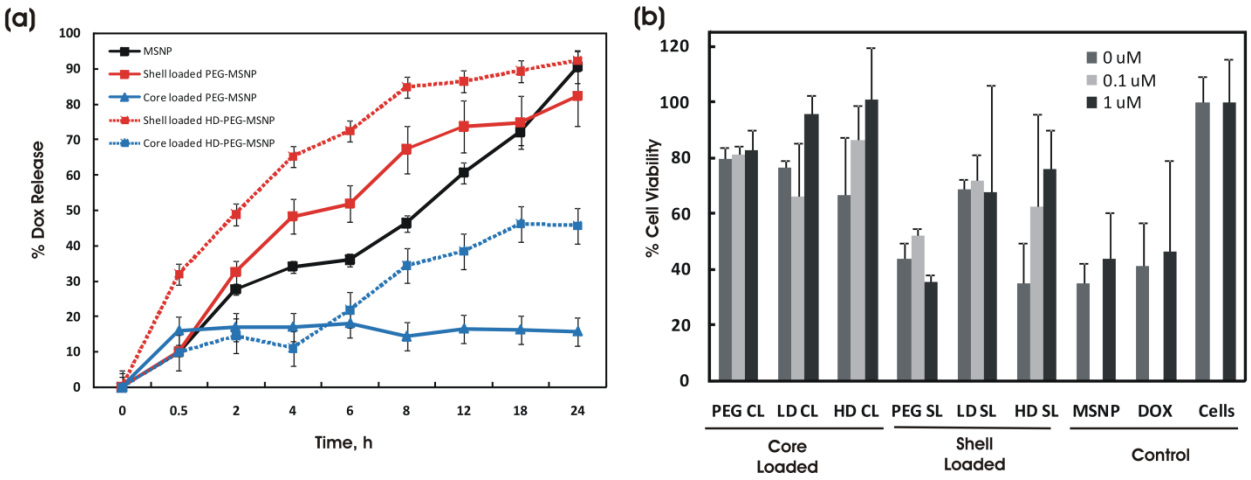

Fig S5: (a) Doxorubicin release profile in PBS at 37 °C for uncoated MSNPs, core-loaded and shell-loaded PEG-MSNPs, core-loaded and shell-loaded HD-PEG-MSNPs in presence of collagenases. (b) Cytotoxicity on J2-3T3 fibroblasts due to released doxorubicin from MMP-degradable PEG-MSNPs (HD: highly degradable; LD: low degradable; CL: core loaded; SL: shell loaded), PEG-MSNPs and uncoated doxorubicin loaded MSNPs in the presence of exogenous batimastat (MMP inhibitor).

Complete References:

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