

Supplementary materials

Targeted Modulation of Cell Differentiation in Distinct Regions of the Gastrointestinal Tract via Oral Administration of Differently PEG-PEI Functionalized Mesoporous Silica Nanoparticles

*Diti Desai, Neeraj Prabhakar, Veronika Mamaeva, Didem Şen Karaman, Iris A.K. Lähdeniemi, Cecilia Sahlgren *, Jessica M. Rosenholm *, Diana M. Toivola *⁺*

Materials

Aminopropyl trimethoxysilane (APTMS), Hexadecyltrimethylammonium chloride (CTAC), Cyclohexane, N,N-diisopropylethylamine (DIPEA), dimethyl formamide (DMF), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), Folic Acid (FA), O-(7-azabenzotriazol-1-yl)-N,N,N,N-tetramethyluronium hexa fluorophosphate (HATU), hexamethylene diisocyanate (HMDI), poly(ethylene glycol) methyl ether (mPEG; 5000 mW), N-hydroxysuccinimide (NHS), tetramethyl orthosilicate (TMOS), toluene were obtained at the highest purity available from Sigma-aldrich. Maleimide-PEG hydroxyl (5000 mW) obtained from JenKem technology, China. Absolute ethanol (99%) was obtained from the company ALTIA. The anhydrous chloroform and diethyl ether were from Riel-de Haen and J.T.Baker respectively. Milli-Q water (18.2 Ω) was used all through the study. Dulbecco's modified eagle medium (DMEM) and supplements such as 1% L-glutamine, penicillin (100 IU/mL) and streptomycin (100 μ g/mL) for cell culture were purchased from Sigma, USA. Fetal calf serum (FCS) was purchased from BioClear, Wiltshire, UK. O.C.T compound (Tissue-Tek® O.C.T. Compound, Sakura® Finetek), disposable plastic cryomolds (Tissue-Tek® Cryomolds® Sakura® Finetek), charged superfrost/plus microscope slides (Fisher Brand), premium cover glasses Thickness 1 (17 mm) (Fisher scientific), 4% para-formaldehyde (PFA), 0.2% nonident p-40 (NP-40), bovine serum albumin (BSA) and ribonuclease A (RNase A) were obtained from Sigma. All materials were used as received.

Synthesis of MSNs

In the synthesis of MSNs methanol was used as co-solvent and cetyltrimethylammoniumchloride (CTAC) as structure directing agent. FITC-conjugation was done by adding fluorescein isothiocyanate (FITC) in the synthesis step to create inherently fluorescent particles. A typical synthesis solution consisted of a molar ratio of 0.9 TMOS: 0.1 APTMS: 1.27 CTAC: 0.26 NaOH: 1439 MeOH: 2560 H₂O. After the synthesis, the surfactant template was removed by sonication extraction three times in acidic (HCl) ethanol (12.5 %) for 30 minutes, washed with ethanol and dried under vacuum at room temperature (RT).

Synthesis of PEI-functionalized MSNs

Surfactant-extracted MSNs were poly (ethylene amine; PEI) functionalized by surface hyperbranching polymerization of PEI, to yield PEI-MSNs. In brief, the extracted and dried MSNs (0.250 g) were further vacuum-dried at 40 °C and subsequently subjected to argon atmosphere. Then, particles were dispersed in toluene under inert atmosphere and catalytic amounts of acetic acid (13 μ L) and aziridine (130 μ L) were added,

and the reaction mixture was stirred overnight at 75 °C under reflux. After the reaction, the functionalized particles were separated by centrifugation and washed with copious amounts of toluene and ethanol, and vacuum-dried at RT for 24 hours.

Synthesis of folic acid conjugated MSNs

To obtain FA conjugated MSNs, a 100 µg portion of FA was dissolved in ethanol, to which 5 µL of EDC crosslinker was added to react with the carboxyl groups of FA. To increase the yield of EDC-mediated amine coupling, 250 µL of a 1 mg/mL NHS-solution was added and kept stirring for one hour at RT. This solution was then mixed with the particle suspension (50 mg in MES buffer; 10 mM, pH 5.5) and was agitated for 4 hours at RT. Afterwards, the particles were centrifuged, washed and vacuum dried to yield FA-MSNs and FA-PEI-MSNs.

Synthesis of FA-PEG- and FA-PEG-PEI- functionalized MSNs

Particles (50 mg) were dispersed in chloroform after which 12.5 mg mal-PEG-HMDI and 5 µL DIPEA were added, and the suspension was kept overnight at 60 °C under reflux. Afterwards, the reaction mixture was centrifuged, washed with ethanol and the particles were resuspended in HEPES buffer (pH 7.2). 1 mg/mL FA-SH (in-house prepared) dissolved in HEPES buffer and added to the particle suspension, the reaction mixture was agitated for 4 hours at RT. The resulting particle suspension was centrifuged, washed and vacuum dried.

Synthesis of HMDI-mPEG

To obtain HMDI activated PEG, 100 mg of mPEG (5 kDa) was dissolved in 10 mL of chloroform in a schlenk tube and 336.34 µL HMDI was added under stirring, whereafter the mixture was heated up to 60 °C under reflux and stirred overnight. Subsequently, the polymer was precipitated by addition of diethyl ether and the precipitates were collected by centrifugation and dried under vacuum to yield HMDI activated mPEG.

Particle characterization

Full redispersibility of dried, extracted and surface functionalized particles was confirmed by redispersion of dry particles in 25 mM HEPES buffer at pH 7.2 and Milli-Q water, and subsequent dynamic light scattering (DLS) measurements (Malvern ZetaSizer NanoZS). Successful modification of PEI and further modifications with PEG and FA were further confirmed by zeta potential measurements (Malvern ZetaSizer NanoZS, Malvern Instruments Ltd., Worcestershire, UK). Thermogravimetric analysis (Netzsch TG 209, Germany) was used to determine the accumulated of PEI and PEG amount at temperature intervals 170 °C – 770 °C. Successful incorporation of FITC was determined by fluorescence spectrometry (Perkin Elmer LS 50B, PerkinElmer, Waltham, MA, USA) of particles dispersed in HEPES at a concentration of 1 mg/mL by excitation at 492 nm and determining the fluorescence intensity at wavelength 520 nm. The mesoscopic ordering of the nanoparticles was further confirmed by transmission electron microscopy (TEM; JEM 1400-Plus, JEOL Ltd., Tokyo, Japan) operating at 120 kV with a LaB6 filament and a 2k × 2 k CCD camera), scanning electron microscopy (SEM; Jeol JSM-6335 F, JEOL Ltd., Tokyo, Japan) and powder-XRD (SAXS) using a Kratky compact small-angle system (MBraun, Nottinghamshire, UK). The structural parameters related to the mesoporosity (surface area, pore size, pore volume) were determined by nitrogen sorption measurements (Autosorb 1, Quantachrome, Boynton Beach, FL, USA).

Determination of folic acid content

The FA amount in weight% with respect to whole particle system was determined by the spectrophotometric method. The standard curve of FA was prepared in 1M sodium hydroxide (NaOH) by UV-Vis spectroscopy at $\lambda=285$ nm (NanoDrop 2000c, Thermo Scientific, USA). The particles were dissolved in 1 M NaOH by sonication and overnight stirring and the absorbance of solution was measured by UV-Vis spectroscopy using respective particle without FA as a blank. The amount of FA per mg of particle was then calculated from the standard curve of FA.

In vitro cytotoxicity assay

Caco-2 cells were transferred to 96-well plates (6×10^3 cells/well) and allowed to attach and grow. After 24 hours, the medium was removed and replaced with 100 μ L medium containing different concentrations of MSNs (10, 25, 50, 100 μ g/mL) and incubated for 48 hours. After the incubation period, 10 μ L of WST-1 reagent (Roche Applied Science, Upper Bavaria, Germany) was added to each well and the plate was incubated for 90 minutes at 37 °C. The absorbance of the colored formazan was measured at 430 nm using the Varioskan microplate reader (Thermo Scientific, Logan UT, USA).

Cellular uptake studies by FACS

MSNs were suspended in cell medium at 10 μ g/mL concentration. After 30 minutes sonication in water bath, the medium containing the particles or control medium was applied to confluent (12×10^4 cells/well) as well as non-confluent (8.8×10^4 cells/well) Caco-2 cells in 12- well plate and incubated for 12 hours at 37 °C. Uptake of MSNs was also studied in the HT-29 cells (9.0×10^4 cells /well in 12- well plates) for 4 and 12 hours at 37 °C. After the incubation cells were washed with PBS, trypsinized and the extracellular fluorescence was quenched by resuspension in 200 μ g/mL trypan blue for 7 minutes at RT. The cells were washed once and resuspended in PBS. The amount of endocytosed particles inside cells was analyzed by BD FACS Calibur flow cytometer (FL-1, BD Pharmingen). The mean fluorescence intensity (MFI) of the cells at FL-1 channel was measured. The data was analyzed with BD CellQuest Pro™ software for total amount of MSNs uptake by 10000 cells.

Cellular uptake studies by confocal microscopy

For microscopical studies HT-29 and Caco-2 cells were seeded on glass-bottom chamber slide (Lab-Tek™, Brendale, Australia) and incubated with MSNs (10 μ g/mL) for 12 hours. After 12 hours cell media was removed, and cells were washed with PBS and incubated with trypan blue (200 μ g/mL). Cells were washed and fixed with 4 % paraformaldehyde, permeabilized using 0.1 % Triton X-100, blocked with 2.5 % BSA and incubated with primary antibody (E-cadherin; Cell Signaling Technology®), then cells were washed and labeled with secondary antibody Cy 5 (Life technologies™). The cell nucleus was stained after treatment with RNase using propidium iodide (1 mg/mL, Invitrogen) and finally coverslips were mounted using ProLong® Gold antifade reagent (Molecular Probes®). The cells were viewed with Leica TCS SP5 confocal microscope (Wetzlar, Germany; 63X oil objective, 488 nm/ 543 nm/633 nm excitation).

DAPT retention and stability of DAPT-loaded MSNs in the gastrointestinal fluid

The HPLC measurements were made using Agilent-HPLC system and Purospher Star 55 X 4 mm, C18e (end-capped; Merck) column. The chromatographic conditions were 1 ml/min flowrate, solvents- milliQ water (solvent A), methanol (solvent B) and detection at 222 nm. Gradient conditions were - at time 0 min Solvent B 70%, at 5 min Solvent B 100%, at 7 min Solvent B 100% and at time 7.1 min Solvent B 70%. The chromatographic analysis of DAPT/PEG-PEI-MSNs at 0 time point was made after elution of DAPT in ethanol. The samples of SGF were collected after 2 hours and injected for the HPLC analysis. Further particles were collected after end of incubation period with SIF (8 hours) and centrifuged; DAPT remaining in the particles was eluted with ethanol and analyzed with HPLC.

Fluorescence spectroscopy

Tissue samples frozen in liquid nitrogen were thawed on dry ice and freeze-thaw cycle was repeated 2-3 times. Each tissue sample was weighed and transferred to polystyrene tube (Falcon) and PBS (51 μ L per mg of tissue weight) was added to each tube. Samples were homogenized using LabGEN[®] 7 Series Homogenizer for 30-60 seconds. Then they were transferred to the eppendorf tubes and centrifuged at 13000 rpm for 30 minutes at 4 °C. The supernatant was collected and fluorescence intensity was measured at 520 nm (Perkin Elmer LS 50B). The results were expressed as fluorescence unit per gram of tissue.

Texas Red-Phalloidin staining procedure

Tissue sections were washed with PBS (2-3 times) and cell membrane was permeabilized using 0.2 % NP-40 in PBS for 5 minutes at RT. Then sections were washed with PBS and incubated with blocking buffer (2.5 % w/v BSA in PBS) for 10 minutes. Afterwards the buffer solution was removed and samples were incubated with Texas Red[®]-X Phalloidin (Molecular Probes[®]) in blocking buffer (1:40) for 30 minutes at RT in the dark. Then they were washed with PBS (2-3 times) and treated with RNase (0.2 mg/mL) for 5 minutes. Subsequently samples were washed with PBS and nucleus was stained using DRAQ5[®] (Cell Signaling Technology) for 5 minutes and coverslip was mounted using ProLong[®] Gold antifade reagent. The sample was viewed with Zeiss LSM510 Meta confocal microscope (Jena, Germany; 63X oil objective, 488 nm/ 543 nm/633 nm excitation).

Supplementary Figures

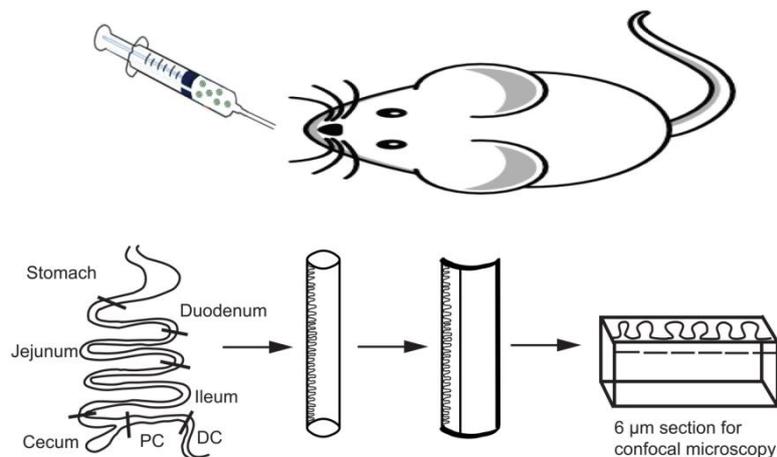


Figure S1. Schematic of tissue collection and marking system for the *in vivo* oral gavage uptake experiment. PC; proximal colon, DC; distal colon.

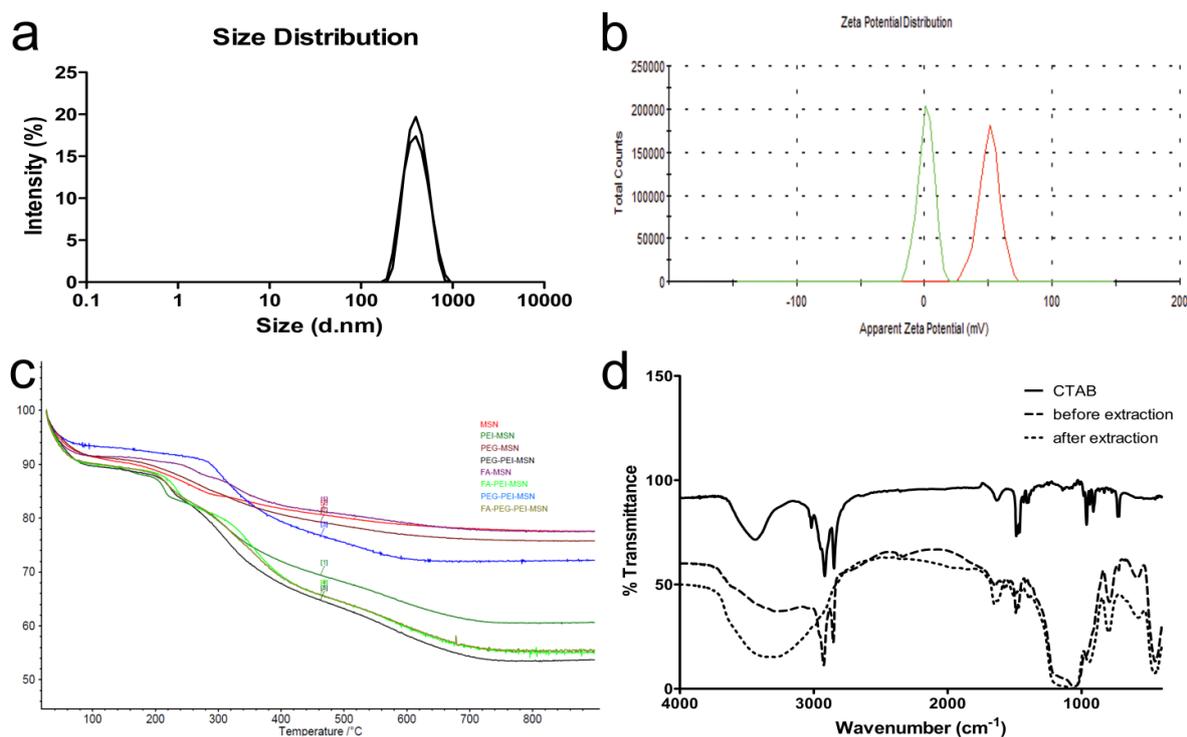


Figure S2. Characterization of functionalized MSNs. (a) DLS size distribution of MSNs measured in HEPES buffer. (b) Zeta potential measurement of FITC-MSN (green) and PEI-MSN (red) in HEPES buffer (pH 7.2). (c) TGA analysis of MSNs (green), PEI-MSNs (red), PEG-MSNs (blue). (d) FT-IR spectra of structure directing agent, CTAB (Cetyl trimethyl ammonium chloride); MSNs before extraction of surfactant; after extraction of surfactant.

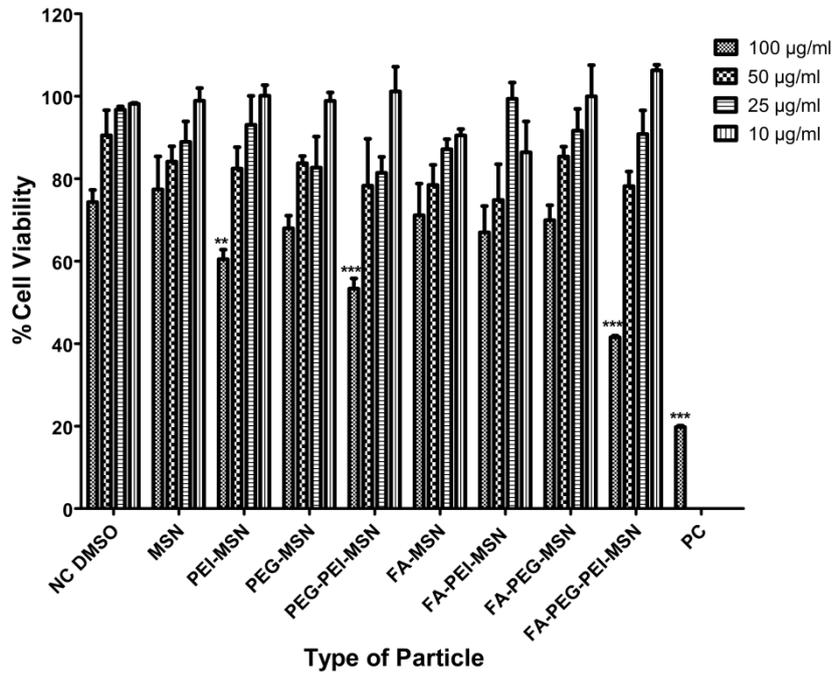


Figure S3. Functionalized MSNs are non-toxic to Caco-2 cells. The viability of Caco-2 cells after 48 hours incubation with functionalized MSNs at different concentrations was assessed using the WST-1 assay at indicated MSN concentrations. Error bars represent SD ($n \geq 3$). Statistical analysis was done by ANOVA followed by a Dunnett's post-test. All data sets were compared with a negative cytotoxicity control cell sample treated with particle vehicle DMSO alone (NC DMSO), and the toxin caliculin A was used as positive control (PC). The level of significance was set at probability of $**p < 0.01$, $***p < 0.001$ as compared to respective DMSO control.

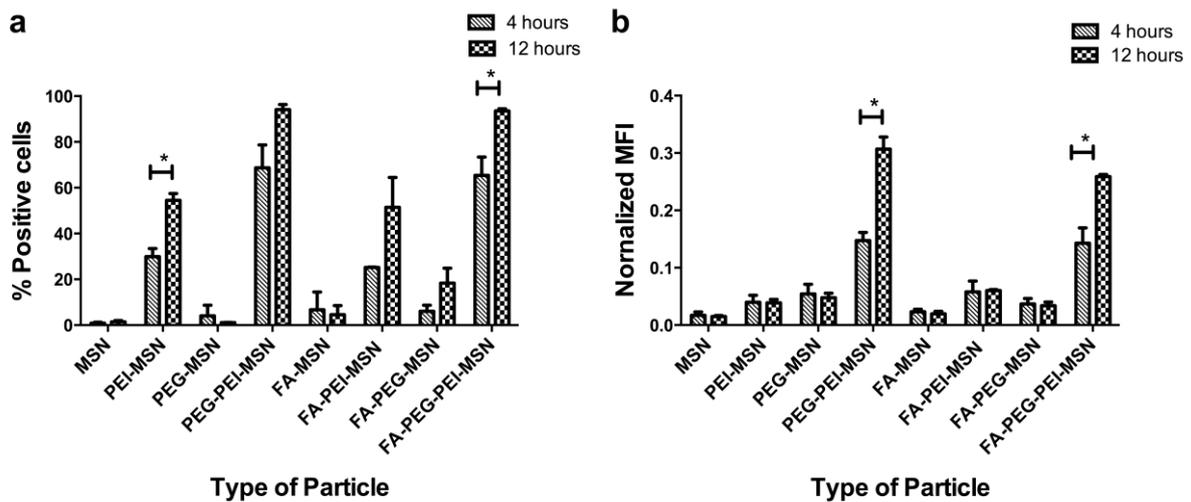


Figure S4. Internalization of functionalized MSNs in HT-29 colon cancer cells. (a) Flow cytometric analysis of the uptake of functionalized and FITC-labeled MSNs in HT-29 cells after 4-hour and 12-hour incubation. (b) Normalized fluorescence intensity of FITC-labeled functionalized MSNs in HT-29 cells is shown at indicated time points. Error bars represent SD ($n \geq 4$). $*p < 0.05$

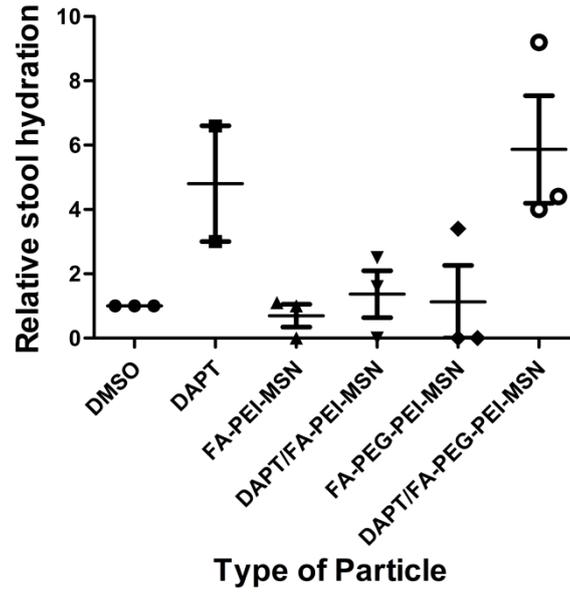
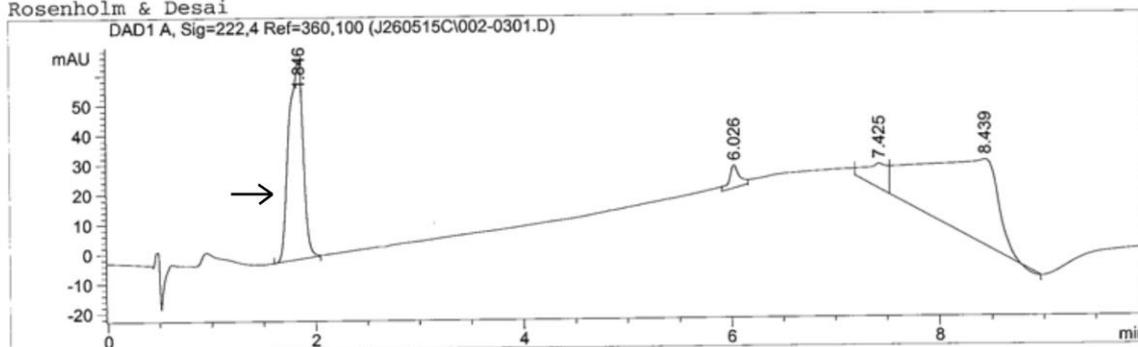


Figure S5. Level of water in stool after treatment of mice with functionalized MSNs. The level of water in stool (stool hydration) as measure of diarrhea and goblet cell proliferation was measured in stool from mice fed by oral gavage DMSO-vehicle solutions, free DAPT, control particles (FA-PEI-MSNs, FA-PEG-PEI-MSNs) and DAPT-loaded particles (DAPT/FA-PEI-MSNs, DAPT/FA-PEG-PEI-MSNs). The % water content (stool hydration) was determined from stool wet and dry weights (n=3).

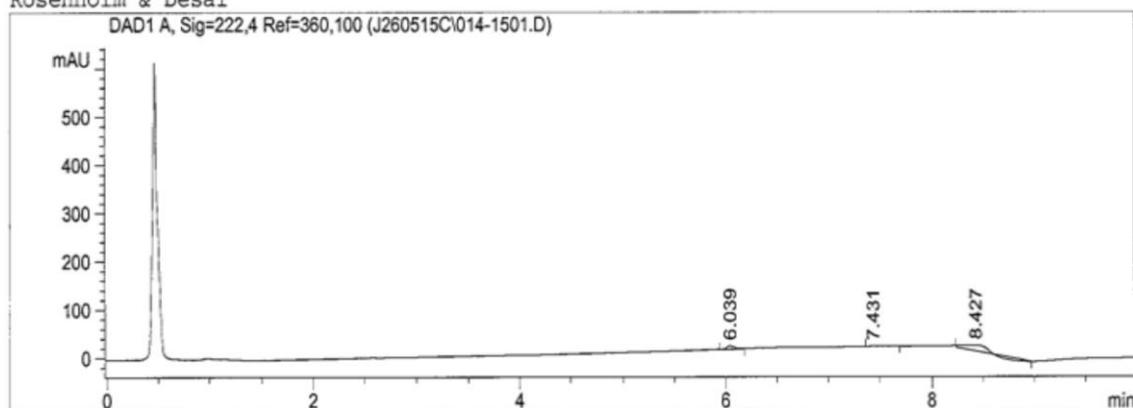
a) Chromatogram OF DAPT/ PEG-PEI-MSN in Ethanol

Separation of DAPT using Purospher Star 55 x 4 mm
Rosenholm & Desai



b) Chromatogram OF DAPT/ PEG-PEI-MSN after 2 hour incubation in SGF

Separation of DAPT using Purospher Star 55 x 4 mm
Rosenholm & Desai



c) Chromatogram OF DAPT/ PEG-PEI-MSN eluted in Ethanol after 2 hour incubation in SGF and 6 hour incubation in SIF

Separation of DAPT using Purospher Star 55 x 4 mm
Rosenholm & Desai

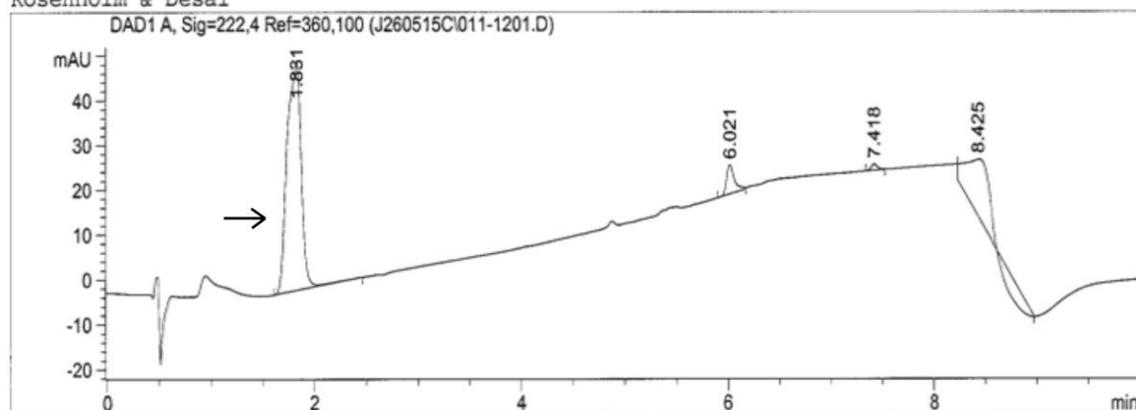


Figure S6. DAPT retention and stability when loaded in MSNs after exposure to SGF and SIF as measured by HPLC. A) Chromatogram of DAPT (tR- 1.846 min) eluted from starting MSNs in ethanol. B) Chromatogram of DAPT released in SGF after 2 hours, showing no release. C) Chromatogram of DAPT (tR- 1.831 min) remaining in MSNs after incubation in SGF and SIF, eluted in ethanol. Arrows showing DAPT peak in the chromatogram.

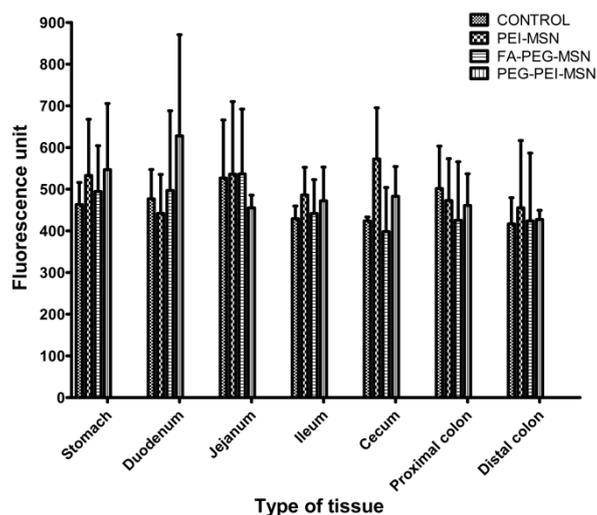


Figure S7. Quantitative estimation of MSN internalization by fluorescence spectroscopy. Fluorescence intensities per gram of different gastrointestinal tissue samples analyzed by fluorescence spectroscopy. Error bars represent SD (n=2).

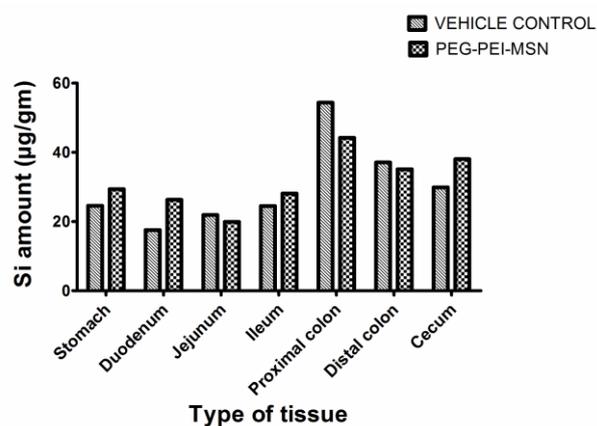


Figure S8. Quantitative estimation of Silicon content by ICP-MS. Silicon content was analyzed by inductively coupled plasma - mass spectrometry as µg/g in tissue sections of different gastrointestinal tissue after oral gavage of vehicle control (HEPES) and PEG-PEI-MSNs (n=1).