# Supporting Information

## Effect of Surface Functionalization of MCM-41 Type Mesoporous Silica Nanoparticles on the Endocytosis by Human Cancer Cells

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### 1. Experimental:

### 1.1. Materials

Tetraethylorthosilicate was purchased from Gelest. 3-aminopropyltrimethoxysilane, 3-[*N*-(2-aminoethyl)amino]propyltrimethoxysilane, fluorescein isothiocyanate isomer I, folic acid, 1-*H*-pyrazole carboxamidine hydrochloride, diisopropyl ethylamine, and *N*-ethyl-*N*-(3-dimethylaminopropyl)carbodiimide hydrochloride were purchased from Aldrich. All chemicals were used as received.

### 1.2. Synthesis of fluorescein isothiocyanate-labeled mesoporous silica nanoparticles (FITC-MSNs)

FITC-MSN was prepared by reacting 500 mg (1.11 mmol) of fluorescein isothiocyanate with 0.2 mL (1.15 mmol) of (3-aminopropyl)trimethoxysilane (APTMS) for 2 h. The resulting product was introduced to a co-condensation reaction of 10.0 mL (43.9 mmol) tetraethylorthosilicate (TEOS), 2.04 g (5.32 mmol) cetyltrimethylammonium bromide (CTAB), 960 mL water, and 7.0 mL sodium hydroxide (2 M). The reaction mixture was heated at 80 ºC, under vigorous stirring for 2 h. The resulting orange colored solid was filtered, washed thoroughly with methanol and dried under vacuum for 20 h.

### 1.3. Synthesis of trimethoxysilylated FAP precursor (FAPTMS)

Following a literature procedure,[1] folic acid (66 mg, 0.15 mmol) was mixed with 150 mg (0.94 mmol) of *N*-ethyl-*N'*(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) in 10 mL of dimethylsulfoxide. After 30 minutes of stirring, APTMS (30 $\mu$L, 0.17 mmol) was added to the solution. The reaction was stirred at room temperature for 4 h. To avoid undesired hydrolysis of the FAPTMS, the DMSO solution was directly used for the grafting reaction described below without purification.

### 1.4. Synthesis of surface functionalized FITC-MSNs

3-Aminopropyl (AP), 3-[*N*-(2-aminoethyl)amino]propyl (AEAP), and 3-(*N*-folateamino)propyl (FAP) functionalized FITC-MSNs were prepared by grafting 6 mmol of the trimethoxysilylated precursors,

1. Leamon, C.P.; Low, P.S. *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 5572-5576.

APTMS, AEAPTMS (both were purchased from Aldrich), and the aforementioned FAPTMS, respectively, to the surface of FITC-MSN (1 g) in refluxing toluene (100 mL) for 20 h. The resulting materials were filtered, washed with methanol and dried under vacuum for 20 h. The success of the surface functionalization and the quantification of organic groups were examined by solid-state $^{13}C$ and $^{29}Si$ NMR spectroscopy (Section 2.4).

### 1.5. Removal of the CTAB surfactant from the organically functionalized FITC-MSNs

The CTAB surfactant was removed from each of the aforementioned materials by heating the mixture of 1.0 g of the MSN material in 100 mL of methanol and 1.0 mL of concentrated hydrochloric acid for 6 h at 60 ºC. The surfactant-removed materials were centrifuged, washed several times with methanol and dried under vacuum for 20 h.

### 1.6. Synthesis of GP- and GEGP-functionalized FITC-MSNs

**GP-functionalized FITC-MSN:** 3-Guanidiniopropyl (GP) derivatized FITC-MSN was prepared by stirring 0.5 g of the surfactant-removed AP-MSN with 0.3 g (2.0 mmol) of 1-*H*-pyrazole carboxamidine hydrochloride (HPCA), 0.36 mL (2.1 mmol) diisopropyl ethylamine in 5 mL of dimethylformamide (DMF) for 24 h. The resulting material was centrifuged, washed with methanol and dried under vacuum for 20 h.

**GEGP-functionalized FITC-MSN:** 3-[*N*-(2-guanidinioethyl)guanidinio]propyl (GEGP) derivatized FITC-MSN was prepared by stirring 0.5 g of the surfactant-removed AEAP-MSN with 0.6g (4.0 mmol) of 1-*H*-pyrazole carboxamidine hydrochloride (HPCA), 0.72 mL (4.1 mmol) diisopropyl ethylamine in 5 mL of dimethylformamide (DMF) for 24 h. The resulting material was centrifuged, washed with methanol and dried under vacuum for 20 h.

## 2. Characterizations

### 2.1. Nitrogen adsorption/desorption isotherms

Surface analysis of the materials was performed by nitrogen sorption isotherms in a Micromeritics ASAP2000 sorptometer. The surface areas were calculated by the Brunauer-Emmett-Teller (BET) and the pore size distribution were calculated by the Barrett-Joyner-Halenda method.

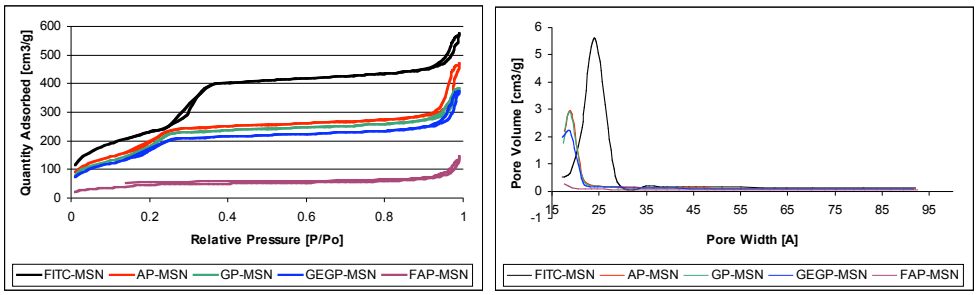


**Figure S1.** BET nitrogen adsorption/desorption isotherms (left), and BJH pore size distribution (right) of the MSN materials.

**Table S1.** Surface properties of the MSN materials

| Material | BET Surface Area [$m^2/g$] | Average Pore Width [Å] | Pore Volume [$cm^3/g$] |
|---|---|---|---|
| FITC-MSN | 850 | 24 | 0.799 |
| AP-MSN | 706 | 19 | 0.636 |
| GP-MSN | 645 | 19 | 0.570 |
| GEGP-MSN | 623 | 19 | 0.522 |
| FAP-MSN | 177 | <17 | 0.195 |

### 2.2. Powder X-ray diffraction

X-ray diffraction patterns were obtained in a Scintag XDS-2000 powder diffractometer using Cu Kα irradiation. All of the materials exhibit the hexagonal mesoporous structure, which is typical of MCM-41 with the characteristic (100) peak between 2.10 and 2.20 degrees (2θ). The high ordered (110) and (200) peaks are also observed in these organically functionalized FITC-MSNs.

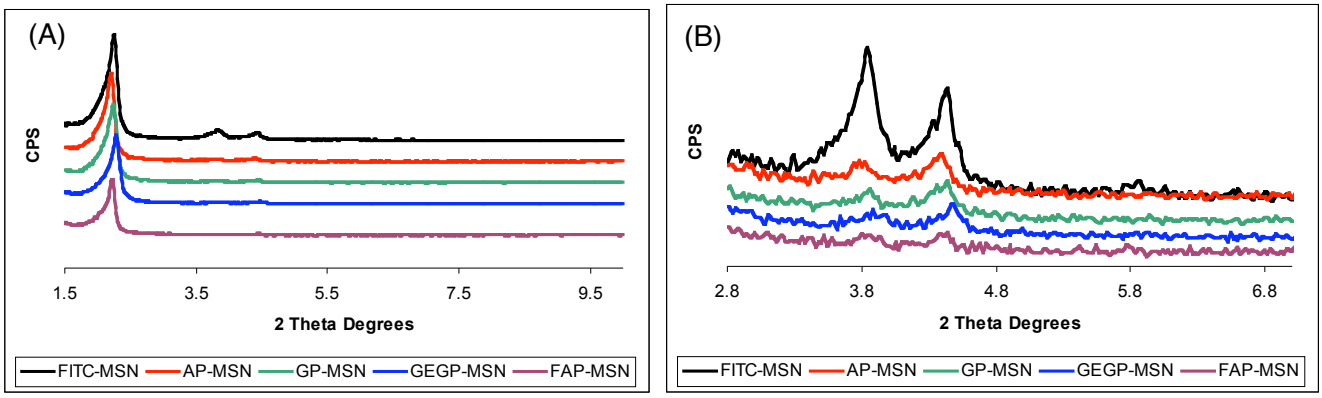

**Figure S2.** **(A)** The X-Ray diffraction patterns of the prepared materials from 1.5 to 10 degrees (2θ). **(B)** Enlarged view of the (110) and (200) peaks between 2.7 and 7.0 degrees (2θ).

### 2.3. ζ-Potential measurement

The ζ-potentials of these MSN materials were measured in a Malvern Nano HT Zetasizer. Each material was tested in triplicate. Suspensions (200 $\mu$g/mL) of each material in PBS buffer were prepared. The pH of the PBS buffer was 7.69 (40 mg KCl, 40 mg $KH_2PO_4$, 1.600g NaCl and 0.432g $Na_2HPO_4{\cdot}7H_2O$ per liter of nanopure water.) The ζ-potential was measured immediately after ultrasonication for 15 min. For the ζ-potential measurements of FITC-, AP- and FAP-MSN, 30 scans were taken per sample, while for the ζ-potential measurement of GP- and GEGP-MSN, the number of scans was reduced to 10 to avoid any deposition of the GP- and GEGP-MSNs on the surface of electrodes. Figure S3 shows zeta-potential graphs.

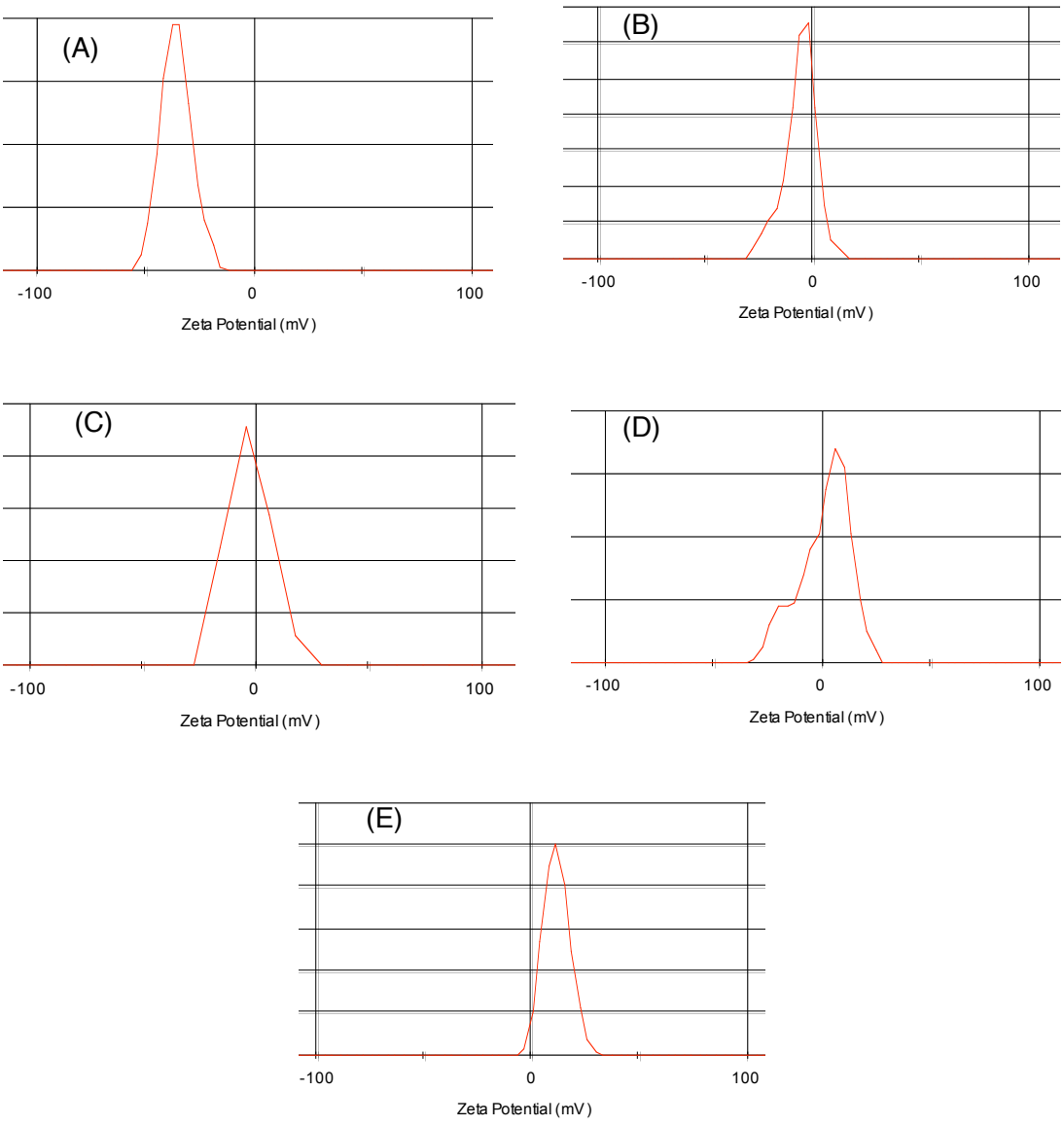

**Figure S3.** ζ-potential graphs of **(A)** FITC-MSN, **(B)** AP-MSN, **(C)** GP-MSN, **(D)** GEGP-MSN, and **(E)** FAP-MSN in 10 mM PBS buffer pH 7.69.

## 2.4. Solid state NMR

Solid-state $^{13}C$ and $^{29}Si$ crossed polarization and direct polarization magic angle spinning NMR (CP- and DP-MAS NMR) measurements of MSNs were conducted by using a Bruker MSL-300 spectrometer with a Bruker 4 mm rotor MAS probe.

### 2.4.1. MAS $^{13}C$-NMR

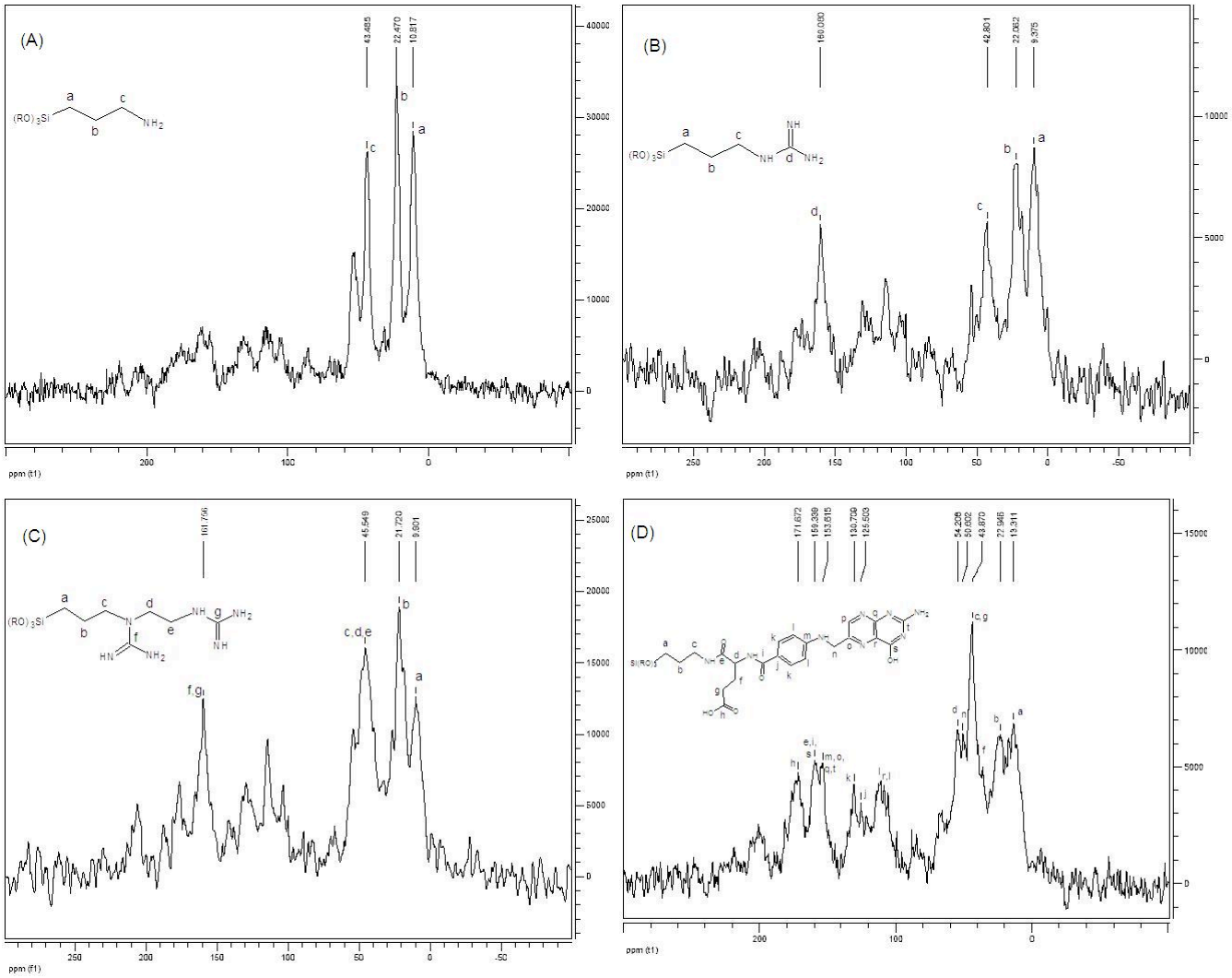


**Figure S4.** $^{1}H \rightarrow ^{13}C$ cross polarization (CP) solid-state NMR spectra of the functionalized materials: (A) AP-MSN, (B) GP-MSN, (C) GEGP-MSN and (D) FAP-MSN.

All spectra showed a small signal around 50 ppm, which is due to surface-adsorbed methanol from the washings. The smaller signals between 80 and 160 ppm correspond to the co-condensed FITC-APTMS. The signals at 160 and 161 ppm in (B) and (C), respectively, correspond to the guanidine carbons. No peaks of residual CTAB surfactant were observed in these measurements.

### 2.4.2. MAS DP $^{29}$Si-NMR

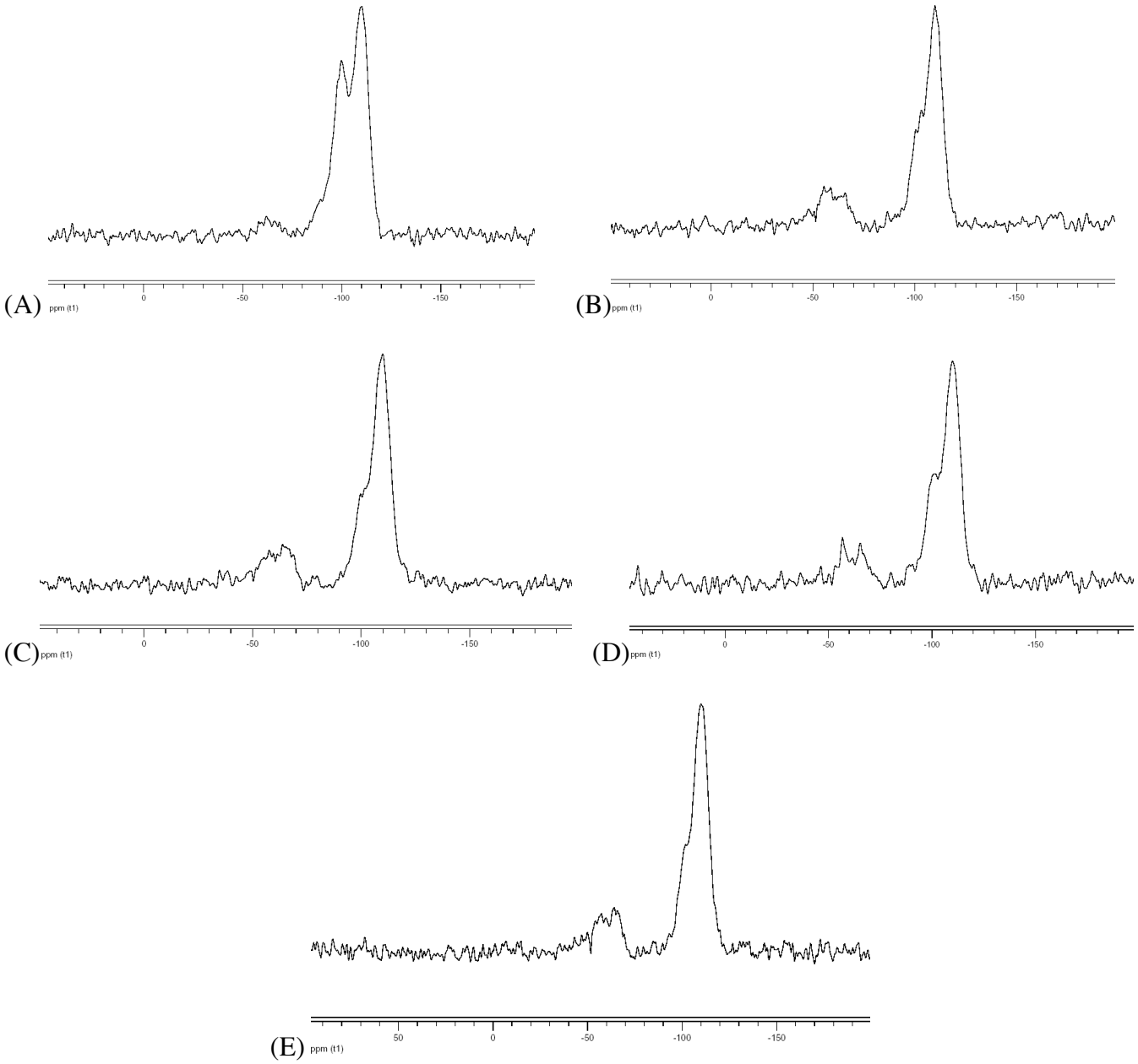


**Figure S5.** Direct polarization (DP) solid-state $^{29}$Si-NMR spectra of all the materials: (A) FITC-MSN, (B) AP-MSN, (C) GP-MSN, (D) GEGP-MSN and (E) FAP-MSN.

**Table S2.** Composition of the materials as calculated from the DP $^{29}$Si-NMR analysis.

| Material | Formula | Formula Mass | Loading [mmol/g] |
|---|---|---|---|
| FITC-MSN | $(SiO_2)_{100}(H_2O)_{25}(FITC^*)_5$ | 8,691.2290 | 0.575 |
| AP-MSN | $(SiO_2)_{100}(H_2O)_{13}(FITC)_5(C_3H_6NH_2)_{14}$ | 9,288.4912 | 1.507 |
| GP-MSN | $(SiO_2)_{100}(H_2O)_7(FITC)_5(C_3H_6NHC(NH)NH_2)_{13}$ | 9,668.8210 | 1.344 |
| GEGP-MSN | $(SiO_2)_{100}(H_2O)_{12}(FITC)_5(C_3H_6N(CNHNH_2)C_2H_4NHC(NH)NH_2)_{10}$ | 10,309.5545 | 0.970 |
| FAP-MSN | $(SiO_2)_{100}(H_2O)_5(FITC)_5(C_3H_6NHC_{19}H_{18}N_7O_5)_{13}$ | 14,590.3118 | 0.891 |

* FITC = $C_3H_6NHSC_{21}H_{11}NO_5$

## 2.5. Scanning electron microscopy

Particle morphology was investigated by using a JEOL 840A scanning electron microscope with a 10 kV acceleration voltage.

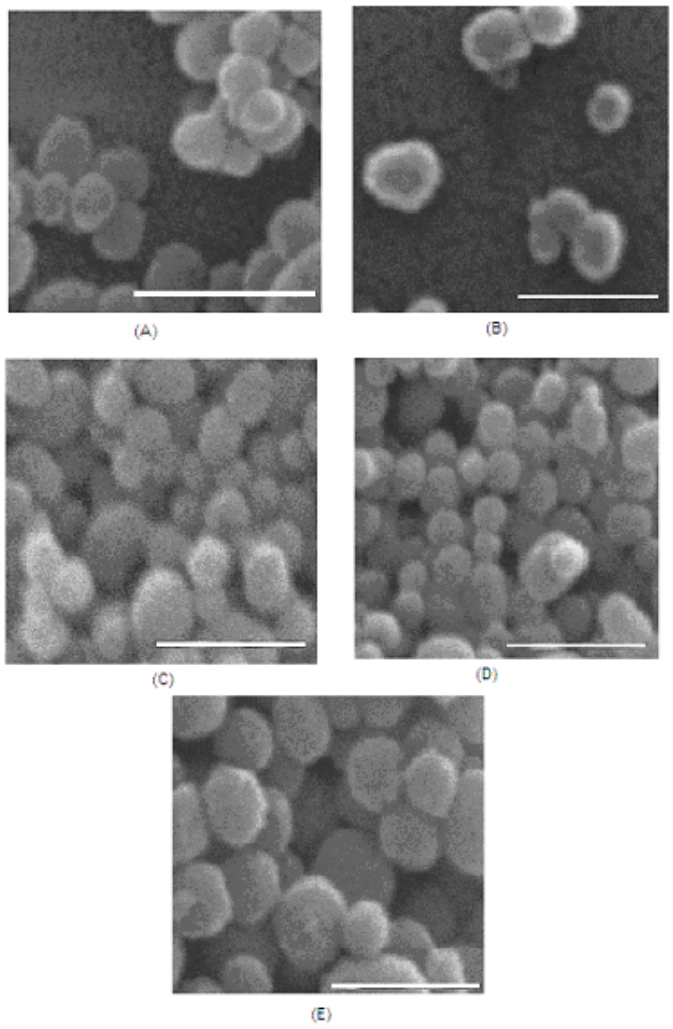


**Figure S6.** Scanning electron micrographs (SEMs) of (A) FITC-MSN, (B) AP-MSN, (C) GP-MSN, (D) GEGP-MSN, and (E) FAP-MSN. The scale bars are 500 nm. These materials are spherical particles ranging from 100 to 250 nm in diameter.

## 3. Measurement of uptake of the materials by HeLa Cells

The cellular uptake profiles of all MSNs were examined by FACS flow cytometry and fluorescence confocal microscopy.

### 3.1. Flow cytometry

For the flow cytometry assays, HeLa cells were seeded in six well plates with a density of $1 \times 10^5$ cells/mL in 3 mL of D-10 medium. The D-10 medium is a Dubelcco modified Eagle's medium (DMEM) containing 10% equine serum, *L*-alanylglutamine, gentamicin, and penicillin/streptomycin. After 34 h incubation, the D-10 medium was replaced by 3 mL of MSN suspensions at different concentrations ranging from 0.1 to 40 $\mu$g/mL in the serum-free DMEM medium for 10 h. Different concentrations of MSN suspensions were tested. All the tests were run in triplicate. The cells were washed then with medium and harvested by trypsinization. After centrifugation the cell pellets were resuspended in 0.4 % trypan blue PBS solution, and analyzed by flow cytometry with a Becton-Dickinson FACSCanto cytometer and BD-FACS Diva software.

To distinguish the true fluorescence generated by the FITC-MSN from the natural autofluorescence of cells, a threshold of fluorescence intensity was established by performing the flow cytometry analysis on HeLa cells incubated without any MSN. The threshold was set at a fluorescence intensity slightly above the highest value observed for control samples (HeLa Cells only). The number of cells with encytosed MSNs was determined by counting the cells showing fluorescence intensity higher than the threshold.

### 3.2. Inhibition and competition assays

HeLa cells were seeded at the density of $1 \times 10^5$ cells/mL in 6 well plates and allowed to attach for 48 h in 3 mL of D-10 medium. The D-10 medium was then replaced by 3 mL of: (a) DMEM medium for the wells used as control, (b) 1 mM folic acid in DMEM medium for the wells used in the competition assay, and (c) 450 mM sucrose in DMEM medium for the wells used in the clathrin inhibition assay. The cells were left in contact with the media for 30 min. The media were then replaced with 3 mL of MSN (40 $\mu$g/mL) in: (a) DMEM medium for the control, (b) 1 mM folic acid in DMEM medium for the competition assay, and (c) 450 mM sucrose in DMEM medium for the clathrin inhibition assay. After 1 h, the cells were washed with DMEM medium and harvested by trypsinization. After centrifugation, the cell pellets were resuspended in 0.4% trypan blue PBS solution, and analyzed by flow cytometry with a Becton-Dickinson FACSCanto cytometer and BD-FACS Diva software.

### 3.3 Cell growth and viability assays

Cell growth and viability of the HeLa cells in the presence and absence of MSN were studied by Guava ViaCount assay (Guava Technologies, Inc.; Hayward, CA). For that purpose HeLa cells were seeded in four six well plates with a density of $1 \times 10^5$ cells/mL in 3 mL of D-10 medium containing (a) no MSNs, (b) FITC-MSN, (c) AP-MSN, (d) GP-MSN, (e) GEGP-MSN and (f) FAP-MSN. The concentration of the MSNs in the culture media was 40 $\mu$g/mL. After seeding them the plates were set in an incubator at 37°C and 5% $CO_2$ and every other day, for a lapse of four days, one plate was removed from the incubator, the media of each well was discarded, each well was washed with fresh D-10 medium and the cells trypsinized, centrifuged and resuspended in D-10 medium. The cells in the resuspended media were then counted and their viability determined by the Guava ViaCount cytometry assay. Figure S7 shows the results of the cell counts at every day, the viability was found to be between 90 and 100 %.

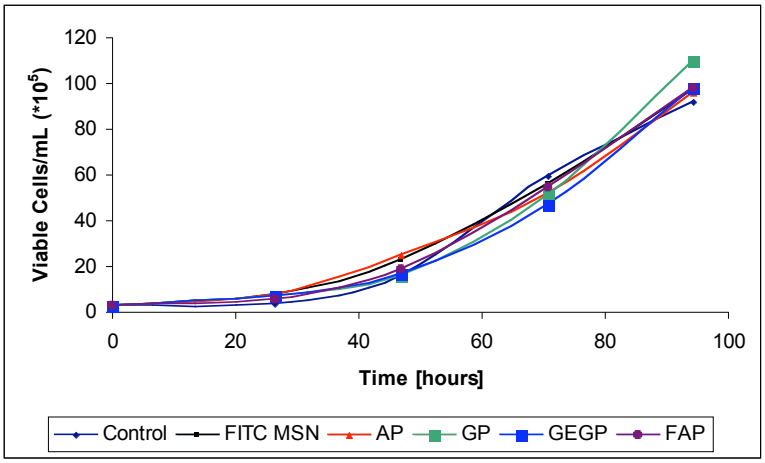


**Figure S7.** Cell counts performed with Guava ViaCount assay after incubation at different times in absence and in presence of 40 $\mu$g/mL of the MSNs in D-10 media.

### 3.4. Confocal fluorescence microscopy

#### 3.4.1. Localization of the MSNs inside the cells

For confocal fluorescence microscopy measurements, HeLa cells were seeded at the density of $1 \times 10^5$ cells per well in 6 well plates in 3 mL D-10 medium with coverslips at the bottom of the wells. After 34 h incubation, the D-10 medium was replaced by 3 mL of MSNs (40 $\mu$g/mL) in the serum-free DMEM medium for 10 h. The cell-plated coverslips were then washed with medium and soaked for 30 min in 3 mL of 0.4 % trypan blue in 100 mM PBS buffer (pH 7.4). The trypan blue solution was then replaced by a solution of 5.7 $\mu$M 4',6-Diamidino-2-phenyindole (DAPI) and 3.7 % formaldehyde in 100 mM PBS buffer pH 7.44. The DAPI-stained coverslips were placed in microscope slides and examined under a Leica TCS NT confocal fluorescence microscope system using a 100x oil immersion objective. As depicted in Figure S8, the blue fluorescent, DAPI-stained nuclei (right images) were clearly observed by exciting the cells with a UV laser at wavelengths from 340 to 458 nm. The green fluorescent FITC-MSN particles inside of the HeLa cells (middle images) were visualized by excitation at 488nm with an Argon Laser and the phase contrast images of the cells (left images) were obtained with a 568nm Krypton laser.

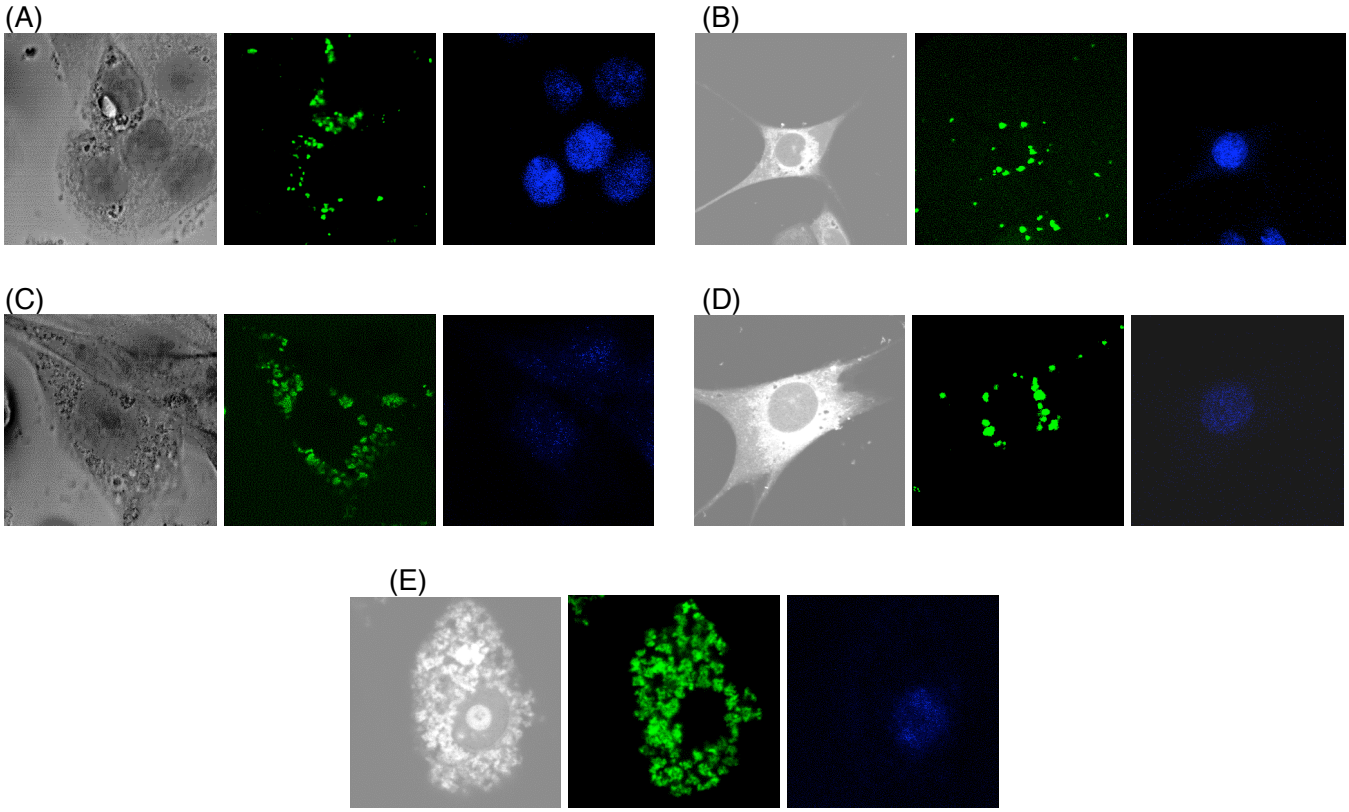


**Figure S8.** Confocal fluorescence images of HeLa cells loaded with the different organically functionalized MSNs: (A) FITC-MSN, (B) AP-MSN, (C) GP-MSN, (D) GEGP-MSN, and (E) FAP-MSN. The left images were taken with a Krypton laser source, the ones in the middle showed the endocytosed green fluorescent FITC-labeled MSN particles (Argon laser) and the right images exhibited the blue fluorescent, DAPI-stained nuclei (UV laser).

### 3.4.2. Localization of the MSNs inside the cells in presence of an endosome marker

HeLa cells were seeded at the density of $1 \times 10^5$ cells per well in 6 well plates in 3 mL D-10 medium with coverslips at the bottom of the wells. After 38 h incubation, the D-10 medium was replaced by 3 mL serum-free DMEM medium containing MSNs (40 $\mu$g/mL) and the endosome marker FM 4-64 (10$\mu$g/mL) and incubated for 6 h. The cell-plated coverslips were then washed with medium and soaked for 30 min in 3 mL of 0.4 % trypan blue in 100 mM PBS buffer (pH 7.4). The trypan blue solution was then replaced by a solution of 5.7 $\mu$M 4',6-Diamidino-2-phenyindole (DAPI) and 3.7 % formaldehyde in 100 mM PBS buffer pH 7.44. The DAPI-stained coverslips were placed in microscope slides and examined under a Leica TCS NT confocal fluorescence microscope system using a 100x oil immersion objective. Both, the MSNs and the endosome marker were excited at 488 nm with an Argon Laser, the MSNs were detected at wavelengths in the 515 to 590 nm range (observed green) while the endosome marker was detected at wavelengths in the 590 to 750 nm range (observed red). MSNs are considered to be inside of the endosomes in the spots where the green and the red spots are co-localized to give a yellow color. The phase contrast images of the cells (right images) were obtained with a 568nm Krypton laser.

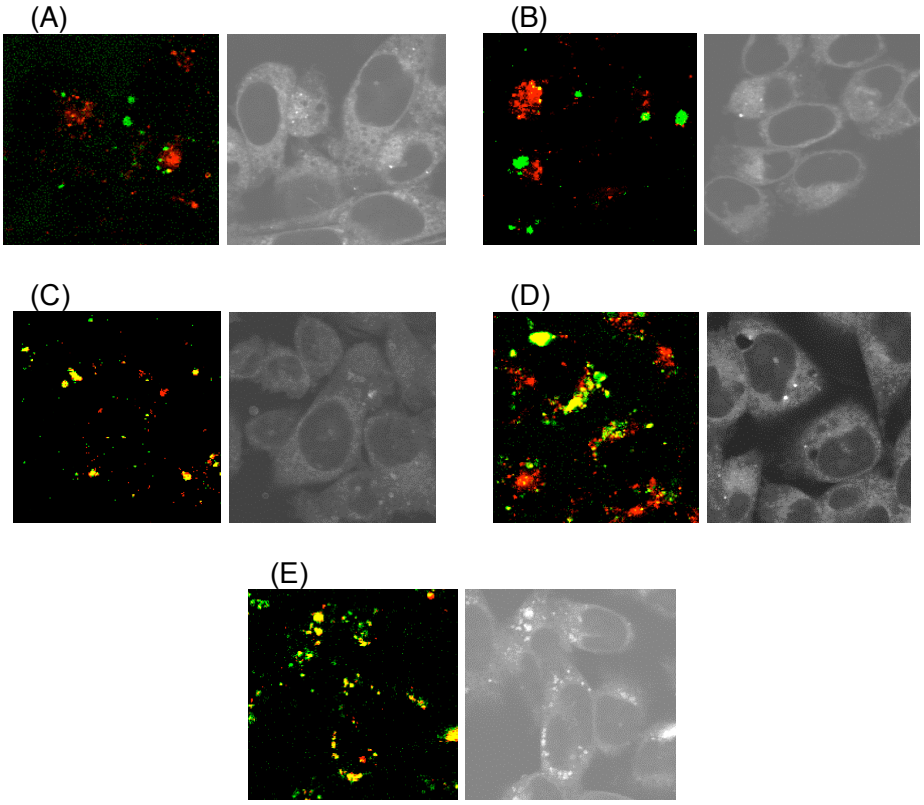


**Figure S9.** Confocal fluorescence images of HeLa cells loaded with the different organically functionalized MSNs: (A) FITC-MSN, (B) AP-MSN, (C) GP-MSN, (D) GEGP-MSN, and (E) FAP-MSN, and stained with the endosome marker FM 4-64. The images to the right were taken with a Krypton laser source, and the ones to the left show the Argon laser excited endocytosed green fluorescent FITC-labeled MSNs, the red fluorescent marked endosomes, and the yellow spots where the MSNs and endosomes are colocalized.