Electronic Supplementary Information for

PEG-templated Mesoporous Silica Nanoparticles

exclusively target cancer cells

Catia Morelli, Pamela Maris, Diego Sisci, Enrico Perrotta, Elvira Brunelli, Ida Perrotta, Maria Luisa Panno, Antonio Tagarelli, Carlo Versace, Maria Francesca Casula, Flaviano Testa,

Sebastiano Andò, Janos B.Nagy and Luigi Pasqua
Electronic Supplementary Information, Methods

Synthesis

The reagents used in the MSNs synthesis were the neutral polyoxyethylene (10)octylphenylether, named Triton X-100 (Alfa Aesar, Karlsruhe, Germany) and tetraethylorthosilicate (TEOS, Aldrich). The molar composition of the synthesis mixtures investigated was: 1 SiO₂-0.52 decane - 0.324 Triton X-100-126.2 H₂O. For MSN preparation, 13.80 g of the surfactant Triton X-100 were completely dissolved in 150 g of distilled H₂O at room temperature. A solution of TEOS (13.8 g) in n-decane (99+% 10 g; Acros Organics, Geel Belgium) was slowly run along the vessel wall of the aqueous solution so that the upper organic phase was easily established. The mixture was aged at room temperature for 8 days under slow magnetic stirring. The suspension was filtered and the obtained material was dried at 80°C (MSN₄ną).

Aminopropyl functionalization

The hybrid silica source was (3-aminopropyl)-triethoxysilane (APTES, Aldrich). APTES (1.42 g, 0.0064 moles, Aldrich) were dissolved in ethanol (3 ml) at room temperature. Then a suspension of 700 mg of MSN₄ną in ethanol (700 mg in 2.5 ml) was added and the mixture stirred for 2 days. The resulting aminopropyl functionalized mesoporous silica nanospheres were named MSN-AP.

Folic acid coupling

For MSN-FOL preparation, folic acid (Sigma, 0.120 g, 0.27 mmoles) was dissolved in dimethylsulfoxide (DMSO) (3.0 ml) and triethylamine (0.06 ml, 0.43 mmoles). After dissolution, nitromethane (0.60 ml) and then MSN-AP (0.4 g) were added. Finally, for amide formation, diisopropylcarbodiimide (DIPC, Aldrich) (0.12 ml, 0.76 mmoles) was added. The so-obtained suspension was stirred at room temperature for 30 hours. The recovered material was washed with DMSO, dimethylformamide (DMF), dioxane, diethyl ether and water. The resulting fluorescent material was named MSN-FOL.
**Fluorescence labelling of MSN**

APTES (1.42g, 0.0064 moles, Aldrich) and Fluorescein isothiocyanate (FITC, Aldrich) (0.004 g; 0.0103 mmol) were stirred in ethanol (3 ml) at room temperature for 24 hours; then an ethanol suspension of 700 mg of MSN$_{surf}$ (700 mg in 2.5 ml) was added to the first solution and the whole mixture stirred for 2 days. The resulting fluorescent material was named MSN-FITC.

**Folic acid coupling on MSN-FITC**

MSN-FITC-FOL preparation was carried out according the procedure presented above in the section “Folic acid coupling”, using MSN-FITC instead of MSN-AP as starting material.

**Surfactant removal**

In order to preserve the integrity of the organic molecules, the surfactant was removed from the different materials (MSN-AP, MSN-FOL, MSN-FITC, MSN-FITC-FOL) by two extractions of 12 h each, using 1 g of material in 0.33 l of distilled water at room temperature.

The number of extractions to perform to reach a complete surfactant removal was established by monitoring (TG analysis) the total mass loss of little amounts of samples subjected to additional extraction steps, until a constant value was reached.

MSN$_{surf}$ was either extracted (MSN-E), as above reported, for the characterization of the porous system, or calcined in air at 550°C for 6 hours (MSN) and used in all experimental procedures as control vector.

**Characterization of MSNs**

X-Ray powder diffraction patterns were measured on a Philips PW1710 diffractometer using Cu-K$_\alpha$ radiation (40 Kv, 20 mA) over the range $1^\circ<2\theta<15^\circ$. The N$_2$ adsorption-desorption volumetric isotherms at 77 K were measured on a Micromeritics Asap 2010 apparatus. FT-IR spectra were acquired on a Bruker IFS 55 Equinox with a resolution of 2 cm$^{-1}$. The NMR spectra were recorded on a Varian 400 spectrometer. For $^{13}$C (100.6 MHz), a 5.0 µs ($\theta=\pi/6$) pulse was used with a repetition time of 15.0 s, and 5.0 ms contact time for the cross-
polarized spectra. The spinning speed 3330-3340 Hz. SEM micrograph was collected using a FEI Quanta 200 instrument.

Transmission electron microscopy (TEM) micrographs were recorded on a JEOL 200CX microscope operating at 200 kV. Prior to observations, the finely ground sample was deposited on a carbon-coated copper grid.

**Drug Loading**

For MSN-FOL-Cp preparation, 120 mg of Cisplatin (Cp, Sigma) (CAUTION: Cp is a mutagenic and carcinogenic agent) were dissolved in 6 ml of DMF for 2 hours at 37°C, then 250 mg of MSN-FOL were suspended in the Cp solution and stirred for 24 hours. Drug-loaded material was filtered and dried under vacuum, then washed with tetrahydrofuran (THF), dried and finally tested by TG analysis for checking the complete removal of DMF. The loading procedure was repeated two more times using a fresh Cp solution to increase the amount of Cp adsorbed on the surface.

**ICP Platin determination**

The ultrapure HNO₃ and HF (Normatont ultrapure, VWR prolabo) used in this work was acquired by analytical-reagent grade certified for the impurities. Platinum standard (Certipur, Merk, Darmstadt, Germany) was also analytical-reagent grade. Aqueous solutions were prepared using ultrapure water, with a resistivity of 18.2MΩcm⁻¹, obtained from a Milli-Q plus system (Millipore, Saint Quentin Yvelines, France). All glassware was decontaminated with nitric acid (2%, v/v) over night, rinsed with ultrapure water and dried.

For the quantitative analysis of platinum in MSN-FOL-Cp 20 mg of sample were dissolved in 1 ml of HF 40% and the resulting solution was made up to volume (50 mL) with ultrapure water. 2.5 ml of this solution were transferred a second time to a test tube, 1 ml of HNO₃ 65% was added and it was diluted to volume (50 ml) using ultrapure water.

Platinum determination was carried out utilizing an Elan DRC-e ICP-MS instrument (Perkin-Elmer SCIEX, Canada). Sample solution was introduced by means of a quartz nebulizer. The
ICP torch was a standard torch (Fassel type torch) with platinum injector. The performance of the ICP-MS instrument strongly depends on the operating conditions. A solution containing Rh, Mg, Pb, Ba and Ce (10 µgL\(^{-1}\)) was used to optimize the instrument in terms of sensitivity, resolution and mass calibration. The \(^{140}\text{Ce} / ^{16}\text{O}^{+}/^{140}\text{Ce}^{+}\) ratio was used to check the level of oxide ions in the plasma and, also, instrumental parameters such as RF power and carrier gas flow were optimized. Calibration curve was built on four different concentrations at a range of 100-2000 µgl\(^{-1}\). Standard solutions were prepared by diluting a solution of Pt at 1000 mgL\(^{-1}\). The signal of the isotope \(^{195}\text{Pt}\) has been monitored.

Electronic Supplementary Information, Discussion
Supplementary Figure 1 shows the XRD powder diffraction patterns of MSN$_{\text{surf}}$, MSN-FOL, MSN-FITC and MSN-FITC-FOL mesoporous materials. All the samples evidenced a broad single reflection arising from the lack of long range crystallographic order. This is due to disorder in the assembly of the surfactant-templated channels in adherence to the patterns observed for MSU family of materials. Furthermore the shoulders in XRD patterns (black and purple lines) are due to the formation of differently-sized micelles during the assembly of the material. The occurrence of heterogeneous formation of surfactant templating-micelles can be explained on the basis of solvent inclusion in the hydrophobic core of the micelles.

Supplementary Figure 2 shows FT-IR spectra of MSN$_{\text{surf}}$, MSN-FITC and MSN-FITC-FOL materials. The first one presents a very important surfactant contribution in the region around 2900 cm$^{-1}$ while the second one shows vibrations in the region 1020-1560 cm$^{-1}$, as expected for thiourea derivatives. However, MSN-FITC shows decreased intensity for surfactant vibrations probably because soaking in ethanol for a long time during FITC coupling partially removes surfactant. This is confirmed by thermogravimetric analysis which shows a decrease in total mass loss from 48.62 % for MSN$_{\text{surf}}$ to 25.8 % for the corresponding MSN-FITC material (data not shown). FT-IR spectrum of MSN-FITC-FOL shows typical vibrations of
folic acid: 793 cm$^{-1}$ N-H out of plane wagging; 1417 cm$^{-1}$ C-N amides stretching; 1531 and 1608 cm$^{-1}$ N-H bending; 1670-1700 cm$^{-1}$ C=O amides stretching; 3106 cm$^{-1}$ C-H aromatic stretching. This gives strong evidence of folic acid association on the functional silica matrix. Nevertheless, it is not possible to obtain a direct evidence of the chemical nature of the interaction. In fact, the expected spectroscopic evidence of an amide bond could be significant of a successful coupling reaction but the folic acid molecule contains itself an amide group. In order to solve this problem parallel experiments have been carried out in a previous work$^4$ to reveal the chemical structure of the folate-derivatized material (MSN-FOL).

**Supplementary Figure 2.** FT-IR spectra of MSNs. a, MSN$_{surf}$; b, MSN-FITC; c, MSN-FITC-FOL.
The cross-polarized $^{13}$C-NMR spectra of the MSN-FITC and MSN-FITC-FOL samples are shown in Supplementary Figures 3. The aminopropyl group is characterized by broad NMR line at $9.6$ ppm (Si-CH$_2$), $25.6$ ppm (-CH$_2$-) and $43.3$ ppm (-CH$_2$-NH$_2$). The NMR line at $70.6$ ppm is due to dioxane impurity in the MSN-FITC spectrum, while the narrow lines at $30.7$ and $31.7$ ppm stem from remaining n-decane molecules. The amide group (CO-NH-) is clearly identified showing that the fluoresceine moiety is covalently linked to the MSN derivatized surface. The aromatic carbon atoms are not well resolved at around $130$ ppm. The spectrum of the MSN-FITC-FOL sample shows the amide bands at ca. $160$ ppm. The possible aromatic moieties at ca. $115$-$130$ ppm of both fluoresceine and folic acid cannot be detected due to the overlap with the spinning side bands at $\pm 33.35$ ppm of the amide bands. Note that the impurities due to dioxane and n-decane have disappeared during the subsequent treatments. The covalent link between the folic acid and the aminopropyl group was demonstrated in a previous paper $^4$. 

**Supplementary Figure 3.** Cross polarized $^{13}$C-NMR spectra of MSNs. a, MSN-FITC and b, MSN-FITC-FOL.
The amount of aminopropyl function in MSN-AP material is $2.1 \times 10^{-3}$ mol/g, the amount of fluoresceine in MSN-FITC material is $9.6 \times 10^{-5}$ mol/g, the amount of folic acid in MSN-FOL material is $5.7 \times 10^{-4}$ mol/g, as determined through a set of TG-based experiments.

Following complete surfactant removal (as specified in the Methods section), MSN-FOL material exhibits a specific surface $S_{\text{BET}}$ of 460 $\text{m}^2\text{g}^{-1}$, a pore volume at $P/P_0=0.9$ of 0.48 $\text{cm}^3\text{g}^{-1}$ while MSN material exhibits a specific surface $S_{\text{BET}}$ of 778 $\text{m}^2\text{g}^{-1}$ and a pore volume at $P/P_0=0.9$ of 0.50 $\text{cm}^3\text{g}^{-1}$.

Supplementary Figure 4 shows nitrogen adsorption-desorption isotherm of MSN-E and MSN-FOL after solvent extraction. In both cases the samples show non-reversible type IV isotherms. The nitrogen adsorption-desorption isotherms obtained from material MSN-E confirms the formation of micelles with different diameters observed in the XRD pattern. It can be noted, in fact, (Supplementary Figure 4a) that the most important nitrogen uptake occurs in a reversible treat of the isotherm thus corresponding to the filling of mesopores with pore size lower than 32 Angstrom. Nevertheless, also a broad hysteresis loop (comprised between $P/P_0=0.43$ (the lowest hysteresis closure point for nitrogen) and $P/P_0=0.9$) can be observed and, although associated to a relatively low pore volume, it corresponds to the filling of mesopores produced by larger micelles. The occurrence of heterogeneous formation of surfactant templating-micelles can be explained on the basis of solvent inclusion in the hydrophobic core of the micelles.

The nitrogen adsorption-desorption isotherm obtained from MSN-FOL (Supplementary Figure 4b), where exhibits, a broad hysteresis loop comprised between $P/P_0=0.43$ and $P/P_0=0.9$, is present associated with the range the main nitrogen uptake that corresponds to not-reversible mesopore filling. In this case, the amount of nitrogen adsorbed in the reversible treat of the isotherm is very low. The BJH adsorption average pore size distributions, are shown in the insets. A main mesopore family possessing lower average size than around 32 Angstrom can be observed both in the case of sample MSN-E and in the case of MSN-FOL. In the latter case a broader peak is also present with a tail extended to the region of very large mesopores. The
variation in the pore systems of the materials is assigned to chemical treatments during folic acid coupling and chemical modification of the silica framework that probably induces a partial structure collapse producing larger mesopores. The interfacial synthesis procedure, in fact, adopted in the preparation of the materials, is carried out at room temperature and in the absence of catalysts. These two factors do not allow complete condensation of the silanols. The resulting material, for the presence of these defects, is slightly degradable upon water or solvents treatments during the preparation of the final system.
Supplementary Figure 4. N$_2$ adsorption-desorption isotherms and BJH adsorption pore size distribution (insets) of MSNs. a MSN-E and b, MSN-FOL.
Nitrogen adsorption-desorption isotherms obtained from material MSN-FITC-FOL (data not shown) is very similar to the already-discussed isotherm obtained from MSN-FOL.

The amount of Cp detected in MSN-FOL-Cp material, as determined by ICP measurement, resulted, to be 48.49% in weight. In Supplementary Figure 5 the TG (a) and DSC (b) curves of MSN-FOL and MSN-FOL-Cp are shown. The TG profiles concern the combustion of folic acid amide and free amino-propyl groups in the case of MSN-FOL material and the combustion of folic acid amide, free amino-propyl groups and the thermal decomposition of adsorbed Cp in the case of MSN-FOL-Cp material. The diffusion profile of Cp from MSN-Fol-Cp, in a simulated body fluid shows that cisplatin is gradually released during 6 h according to a kinetics very similar to the kinetics reported in a previous paper.
**Supplementary Figure 5.** TG (a) and DSC (b) curves of MSN-FOL and MSN-FOL-Cp materials.
References:

Supplementary Figure 6. MSNs are not toxic to human cells. Biocompatibility of MSNs was assessed by trypan blue exclusion assay (see Materials and Methods). In brief, HeLa cells were seeded in 12-well plates (10^5 cells/well) and grown overnight. The following day, cells were shifted to SFM for 24 h, treated for 1 h with differently functionalized MSNs and washed 2x with PBS before replacement with fresh medium supplemented with 1% FBS. After 1, 2 or 3 days cells were harvested, incubated in a 0.5% trypan blue solution and counted by using inverted microscope.
Supplementary Figure 7. MSNs endocytosis does not involve clathrin pathway neither Golgi network. MSN-FITC-FOL (green) intracellular localization in HeLa cells, relatively to: a, Rab5, Rab7 and Lamp1 (red), markers of the clathrin endocytic pathway for early endosomes, late endosomes and lysosomes, respectively, and b, Golgi complex (red). Confocal images were captured at different time points following treatments, at 6000x magnification. BF: Bright Field.
**Supplementary Figure 8.** MSN-FOL are mostly exocytosed from the cells within 48h. HeLa cells were opportunely seeded in 60mm culture dishes, and treated the following day with 30μg/10⁶ cells MSN-FOL for 1h or left untreated; cells were then washed 2x with PBS, the medium was replaced with fresh growing medium and collected after 48h. Size distribution of particles dispersed in a, control sample (medium only) and b, MSN-FOL treated sample was determined by DLS.