

Supporting Information

Cell-induced Intracellular Controlled Release of Membrane Impermeable Cysteine from a Mesoporous Silica Nanoparticle-based Drug Delivery System

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Reagents and Materials: Tetraethylorthosilicate (TEOS) was purchased from Gelest. *N*-hexadecyl-trimethylammonium bromide (CTAB), 3-mercaptopropyltrimethoxy silane, 3-aminopropyl trimethoxy silane (AP-TMS), 2,2'-dipyridyl disulfide (Aldrithiol-2), dithiothreitol (DTT), cysteine (Cys), glutathione (GSH), fluorescein isothiocyanate (FITC), *N*-acetylcysteine (NAC), fluorescein isothiocyanate (FITC), tetramethylrhodamine isothiocyanate (TRITC), and trifluoroacetic acid (TFA) were purchased from Aldrich. Methanol, hydrochloric acid, tetrahydrofuran and acetonitrile were purchased from Fischer. All chemicals were used as received. Nanopure water (18.1 MHz), prepared from a Barnstead E-pure water purification system, was employed throughout all the experiments.

Synthesis of MCM-41-type mesoporous silica nanoparticles with mercaptopropyl functionality (MSN-SH): Following the procedure previously reported,¹ 1.0 g of *n*-hexadecyltrimethylammonium bromide (CTAB) was dissolved in 480 mL nanopure water and 3.5 mL NaOH_(aq) (2.0 M). The solution was stirred for 1 h at 353 K, followed by the dropwise addition of 5 mL of tetraethylorthosilicate (22.5 mmol, TEOS). Immediately after the injection of TEOS, 1 mL of 3-mercaptopropyl-trimethoxysilane (5.4 mmol, MPTMS) was introduced to the solution mixture. After stirring for 2 h at 353 K, the resulting solid product was filtered, dried under high vacuum. The CTAB surfactant was removed by extraction with a solution of 0.5 mL HCl (37.4%) and 50 mL of methanol for 6 h at 333 K.

Characterization of MSN systems: Powder X-ray diffraction patterns were collected with a Scintag XRD 2000 X-ray diffractometer using CuK_α radiation (2-theta: 1.5°-10°). Nitrogen sorption measurements at 77 K were performed using a Micromeritics TriStar sorptometer; BET specific surface areas have been calculated in the relative pressure range 0.05-0.15 and pore size has been evaluated following the BJH method.² A JEOL 840A Scanning Electron Microscopy, a Tecnai G² F20 transmission electron microscopy operating at 200 kV and a Malvern NANO ZS90 Zetasizer were used to determine the mesoporous particle morphology and ζ potential.

Physisorption of cysteine on the mesopore surface of MSN (MSN+Cys): MSN particles (100 mg) were dispersed in a PBS solution of Cys (1 mM). The suspension was stirred for 20 h at RT. The resulting material was washed several times with PBS solution and dried under high vacuum. The amount of Cys absorbed by MSN was quantified by HPLC as detailed below.

Preparation of cysteine-functionalized MSN via a disulfide linkage (MSN-S-Cys): MSN-SH particles (200 mg) were treated with a solution of 2,2'-dipyridyl disulfide (88 mg) in 200 mL PBS. After stirring for 8 h at RT, the resulting 2-pyridinyldisulfanylpropyl-functionalized MSN product was isolated and washed with copious methanol and PBS buffer. The purified material was then introduced to a 200 mL PBS solution of Cys (48 mg) to yield the desired MSN-S-Cys product.^{3,4} The amount of Cys bonded to the surface of MSN was quantified by UV/Vis spectroscopy and HPLC as detailed below.

Preparation of cysteine-functionalized MSN via thioether bond (MSN-Cys): Following a experimental procedure previously reported in the literature^{1,3} 1.0 g of CTAB was dissolved into 480 ml of nanopure water and 3.5 ml of NaOH_(aq) (2.0 M) and stirred for 1h at 353 K. Then, 5 ml of TEOS and 1 ml of allyltrimethoxysilane were then added drop wise. After stirring for 2 hours at 353 K, the products were filtered, washed, and dried under vacuum. To wash CTAB out from this material, 500 mg of the as-made MSN were dispersed in a solution of 0.5 ml HCl (37.4%) and 50 ml of methanol and stirred for 6h at 333 K. The material was washed several times with methanol and dried under high vacuum. Following the well known radical reaction between alkenes and thio compounds,⁵ 200 mg of this sample were subsequently added to Cys (0.4 mmol) and azobisisobutyronitrile (6 mg, AIBN) in 30 ml of dry tetrahydrofuran (THF) for 24 hours in nitrogen conditions with vigorous stirring and UV light irradiation(λ~350 nm).

Controlled release of cysteine: The release profiles were measured by soaking 30 mg of Cys-containing MSN sample in 30 mL PBS solution (10 mM, pH 7.3) at RT. Every 15 min, the sample was centrifuged and aliquots (0.5 mL) were taken to quantify the amount of Cys released from MSN by HPLC. Two different disulfide-reducing agents (dithiothreitol and glutathione) were used as triggers for the release of cysteine in the case of MSN-S-Cys.

Cys quantification: HPLC method. A Hitachi Organizer high-performance liquid chromatographic system controlled with a Hitachi L-7100 pump, a Hitachi L-7200 autosampler, a Hitachi L-7455 Diode Array Detector and a Hitachi L-7300 Column Oven was employed to evaluate the amount of Cys loaded and released by the samples. The mobile phase was 99.9% of

nanopure water and 0.1% TFA. It was pumped through a Supelco Discovery®C18 column with a flow-rate of 0.2 mL/min at RT. The detector was set at 320 nm and the peak ascribable to Cys in PBS solution appeared between 8.54 and 8.59 minutes. In the case of release with reducing agents both the peaks of GSH (19.1-19.5 minutes) and DTT (24.9-25.3 minutes) did not overlap with the Cys signal. To determine the Cys loaded by the materials the difference in the concentration of Cys in PBS solution before and after contact with the samples was calculated. The calibration curves have been determined using PBS solutions of Cys in the range of 0.01 mM – 5 mM.

UV-Vis method. The MSN-SH sample was treated in PBS firstly with 2,2'-dipyridyl disulfide and subsequently with Cys. This reaction afforded 2-thiopyridone as byproduct. The concentration of this molecule was used to quantify the surface coverage of chemically accessible thiol groups in MSN-SH and the amount of Cys linked to MSN in MSN-S-Cys by means of a HP 8453 UV-VIS system ($\lambda = 354$ nm). The calibration curve has been calculated using PBS solutions of 2-thiopyridone in the 0.01 mM – 0.1 mM concentration range.

Synthesis of MSN-S-Cys labeled with FITC: 200 mg of as-made MSN-SH sample were treated at first with a solution of 3-aminopropyltrimethoxysilane (0.23 mL) in 30 mL of dry toluene at 370 K for 24h. Then this material was added to a solution of FITC (7.75 mg) in 200 mL of methanol and stirred at RT for 24h. Afterwards the material was washed and Cys was linked to FITC labeled-MSN via disulfide bond, as reported above.

Synthesis of TRITC labeled MSN-S-Cys(FITC): FITC (39.8 mg, 100 μ mol) were added to 100 mg of MSN-S-Cys in dry DMSO and stirred for 12h. The product was collected by centrifugation and washed several times with DMSO, MeOH and water, and finally dried under high vacuum. Then, TRITC (1 mg, 2.25 μ mol) was reacted with AP-TMS (10 μ L, 56 μ mol) in DMSO (1.0 mL) for 6h at room temperature. To this solution 50 mg of previously prepared MSN-S-Cys(FITC) material dispersed in 4 mL of DMSO were added and the final mixture was allowed to react for 24 h at 353 K. The final product was recovered by centrifugation and washed several times with DMSO and water, and dried under high vacuum.

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Intracellular toxicity of MSN-S-Cys in HeLa cells: HeLa cells were seeded in six-well plates with a density of 1×10^5 cells mL^{-1} in 3 mL of D-10 medium (Dubelcco Modified Eagle's Medium with horse serum, L-alanyl-L-glutamine, gentamicin sulfate and penicillin-streptomycin solution), and incubated at 310 K with a 5% CO_2 atmosphere for 24 hours. Then, the cells were seeded with different concentration of NAC solution (ranging from 0.05 to 100 mM), MSN-SH, MSN + Cys, and MSN-S-Cys samples (ranging from 25 to 100 $\mu\text{g mL}^{-1}$) in D-10 serum for other 48 hours. After the incubation each well was washed with PBS and the cells were trypsinized, centrifuged, and re-suspended in D-10 medium. Viability was determined by the Guava ViaCount cytometry assay (Guava Technologies, Inc.).

Intracellular toxicity of MSN-S-Cys in HeLa cells in presence of GSH monoester⁶: HeLa cells were seeded in six-well plates with a density of 1×10^5 cells mL^{-1} in 3 mL of D-10 medium (Dubelcco Modified Eagle's Medium with horse serum, L-alanyl-L-glutamine, gentamicin sulfate and penicillin-streptomycin solution), and incubated at 310 K with a 5% CO_2 atmosphere for 24 hours. Then, the cells were seeded with MSN-S-Cys (100 $\mu\text{g mL}^{-1}$) in D-10 serum for other 24 hours. The cells medium was replaced by GSH monoester in D-10 serum (10 mM and 20 mM) and incubated for 2 h. After that the cells were washed with PBS and D-10 serum was added and incubated for other 22h. After the incubation each well was washed with PBS and the cells were trypsinized, centrifuged, and re-suspended in D-10 medium. Viability was determined by the Guava ViaCount cytometry assay (Guava Technologies, Inc.).

Measurement of the internalization of MSN-SH, MSN + Cys, and MSN-S-Cys in HeLa cells by flow cytometry: HeLa cells were seeded at the density of 1×10^5 cells per well in six-well plates in 3 mL D-10 medium. After incubation for 48 hours, the D-10 medium was replaced by 3 mL of FITC labeled material suspensions at different concentrations (1, 10, 25, 50, and 100 $\mu\text{g mL}^{-1}$) in the serum-free DMEM medium for 12 h. All the tests were run in triplicate. The cells were washed with medium, harvested by trypsinization and, after centrifugation, re-suspended in 0.4% trypan blue PBS solution in order to be analyzed by flow cytometry with a Becton-Dickinson FACSCanto cytometer with a DB-FACS Diva software. To distinguish the true fluorescence generated by the endocytosed FITC-labeled material from the natural autofluorescence of cells, a threshold of fluorescence intensity was established by performing the flow cytometry analysis on the cells incubated without FITC-labeled material. The threshold was set at an intensity of fluorescence slightly above the highest value observed for control samples (HeLa cells only). The number of cells with endocytosed FITC-labeled material was determined by counting those which show fluorescence intensity higher than the threshold.

Confocal fluorescence microscopy measurements: HeLa cells were seeded at the density of 5×10^4 cells per well in six-well plates in 3 mL of D-10 medium. After incubation for 24 h, the D-10 medium was replaced by 3 mL of either TRITC-labeled MSN-S-Cys(FITC) or TRITC-labeled MSN-Cys(FITC) material ($15 \mu\text{g mL}^{-1}$) in fresh D-10 medium for 12 h. Then the cell medium was replaced by a solution of GSH monoester in D-10 serum (10 mM) for 2 h. The cells were washed with PBS and the cells were incubated in D-10 medium for other 10h. In the case of the samples that were not treated with GSH monoester, the cells in presence of material were incubated for 24h. Finally, the cell-plates were then washed with PBS and 3 mL of D-10 medium were added to each well. An aqueous solution of Hoechst 33258 ($5 \mu\text{g/mL}$) was added to the wells and they were let to rest for 30 min at RT. After replacing the Hoechst-staining solution with D-10 serum, the TRITC-labeled MSN-S-Cys(FITC)/ TRITC-labeled MSN-Cys(FITC) materials inside of HeLa cells were visualized by means of a Leica TCS NT confocal fluorescence microscope system with 100x oil immersion objective.

References

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Table S1. Specific Surface Area (SSA), mesopore volume (V_p), pore size (D_{BJH}), d_{100} , cell parameter (a), ζ potential (ζ) and average particles diameter (D) values for MSN materials.

	SSA _{BET} (m ² /g)	V_p (cm ³ /g)	D_{BJH} (nm)	d_{100} (nm)	a (nm)	ζ (mV)	D (nm)
MSN	1093	1.014	3.02	3.98	4.59	-30.5	380
MSN-SH*	1012	0.578	2.20	3.83	4.43	-28.8	390

*EC₅₀ of MSN-SH = 4.3 $\mu\text{g/mL}$

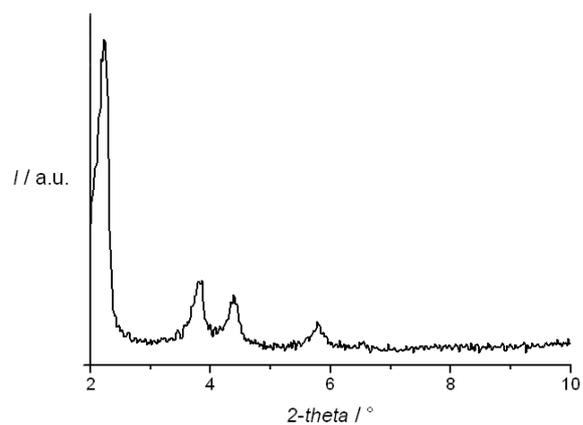
Table S2. Amount of pharmacological agent (Cys), ζ potential (ζ) and average particles diameter (D) of different MSN systems:

	Cys (mmol g ⁻¹)	ζ (mV)	D (nm)	EC ₅₀ ($\mu\text{g/mL}$)
MSN + Cys	0.41 ⁷	-19.2	390	4.0
MSN-S-Cys	0.45 ⁷	-20.1	395	3.6

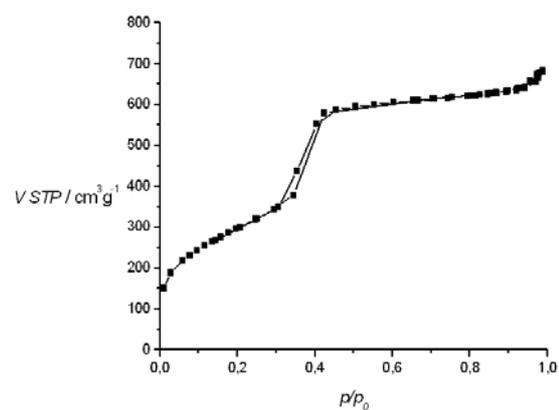
⁷ Evaluated by means of HPLC.

⁷ Evaluated by means of UV after aldrithiol-2 reaction.

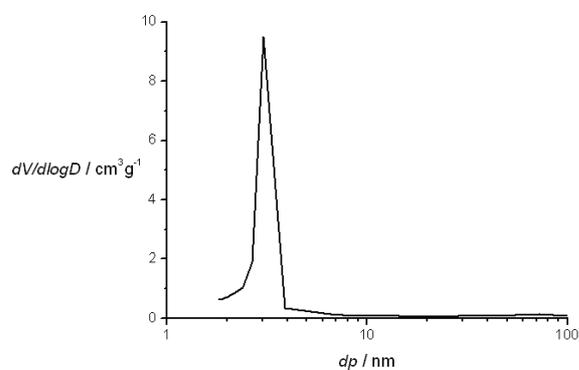
a)



b)



c)



d)

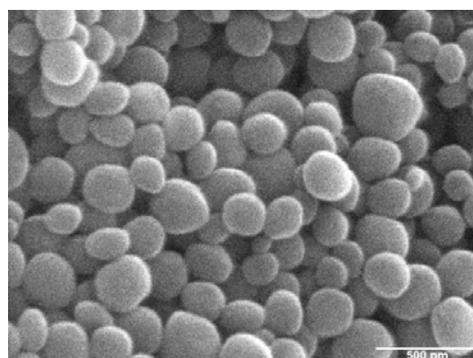
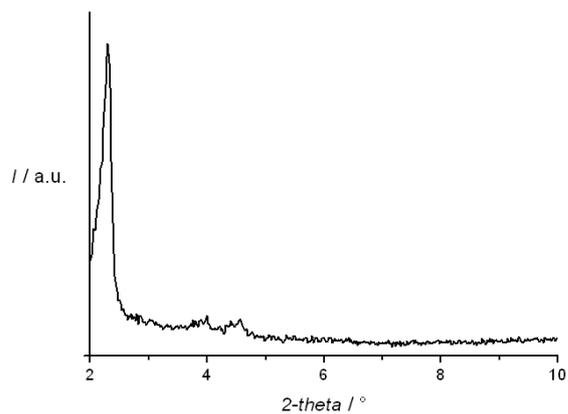
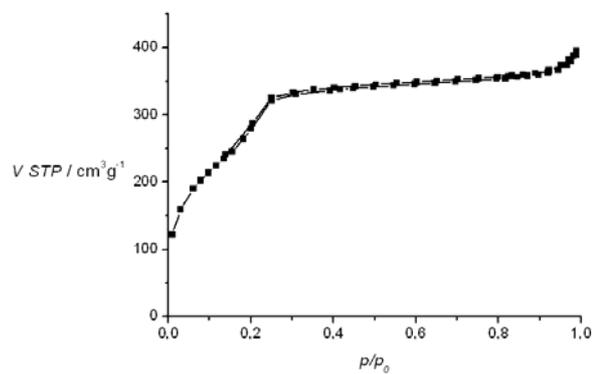


Figure S1. Characterization of MSN sample. a) XRD pattern, b) nitrogen sorption isotherms at 77 K, c) BJH pore size distribution and d) SEM picture. The material exhibited hexagonal type of mesoporous structure characteristic of MCM-41 with a pore size of 3.02 nm and particle size of 380 nm.

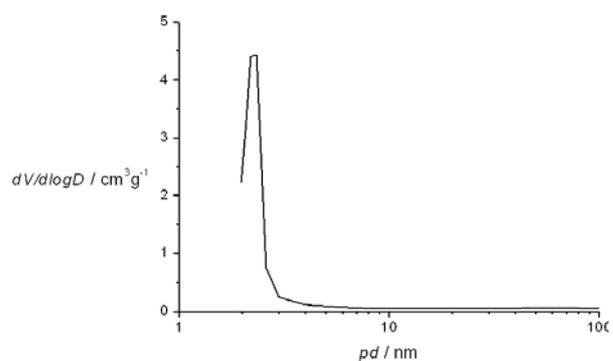
a)



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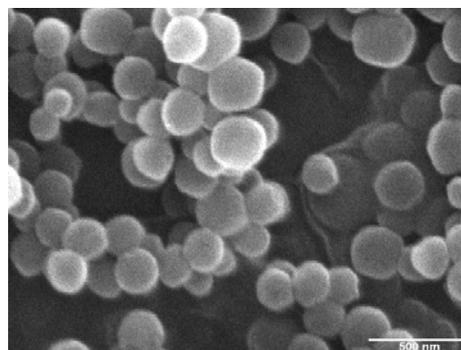


Figure S2. Characterization of MSN-SH sample. a) XRD pattern, b) nitrogen sorption isotherms at 77 K, c) BJH pore size distribution and d) SEM picture. The material exhibited hexagonal type of mesoporous structure characteristic of MCM-41 with a pore size of 2.2 nm and particle size of 390 nm.

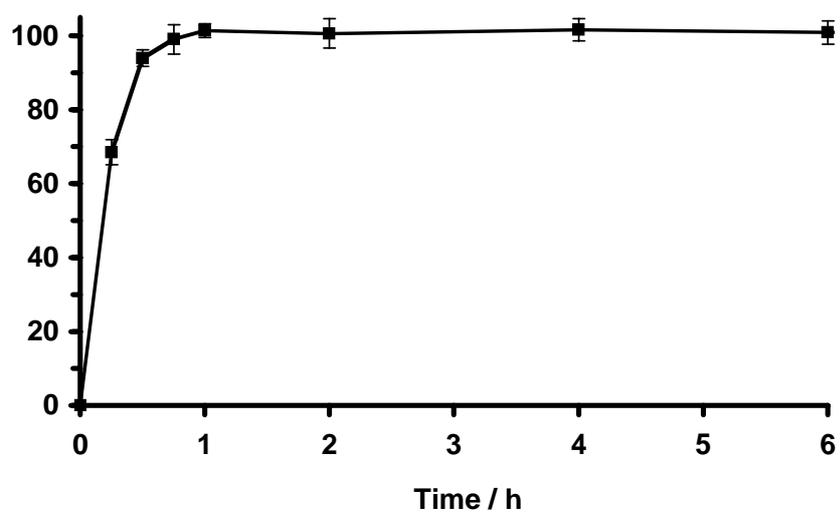


Figure S3. Percent release of mesopore-adsorbed cysteine from MSN+Cys material in PBS solution (10 mM, pH 7.3). The profile showed a fast release kinetics of Cys from MSN+Cys, in less than 60 min roughly 95% of Cys was released in PBS solution.

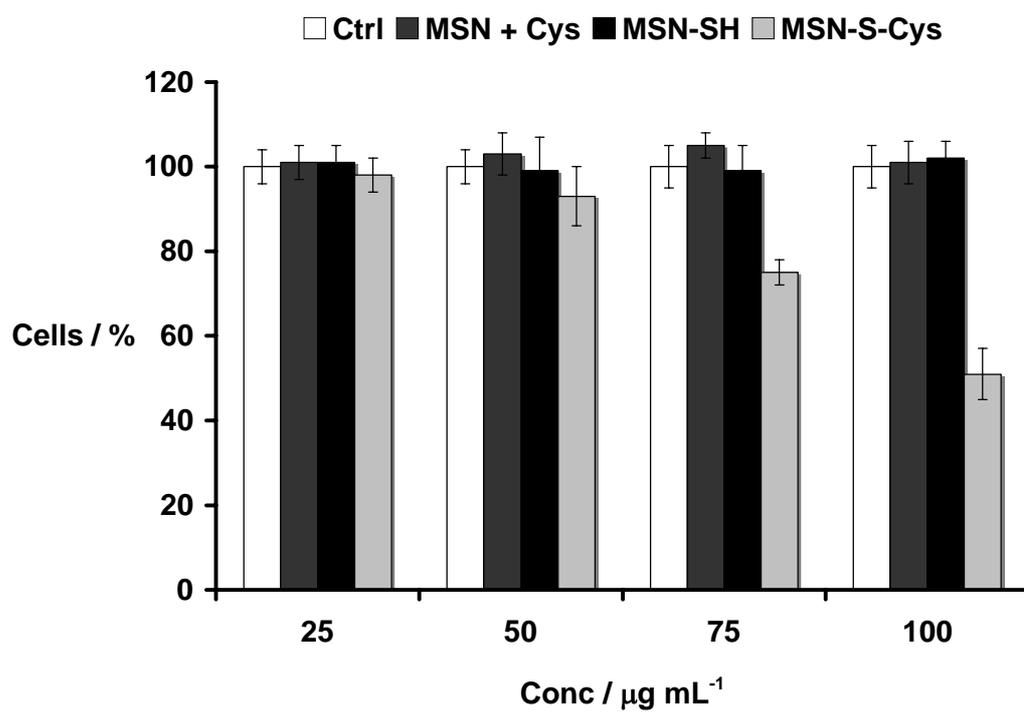


Figure S4. Proliferation of HeLa cells treated for 48 hours with different concentrations of MSN + Cys (dark gray boxes), MSN-SH (black boxes), and MSN-S-Cys (light gray boxes). Graph shows the percentage of cells after each treatment, pointing out the role played by the Cys released inside the cell membrane from MSN-S-Cys.

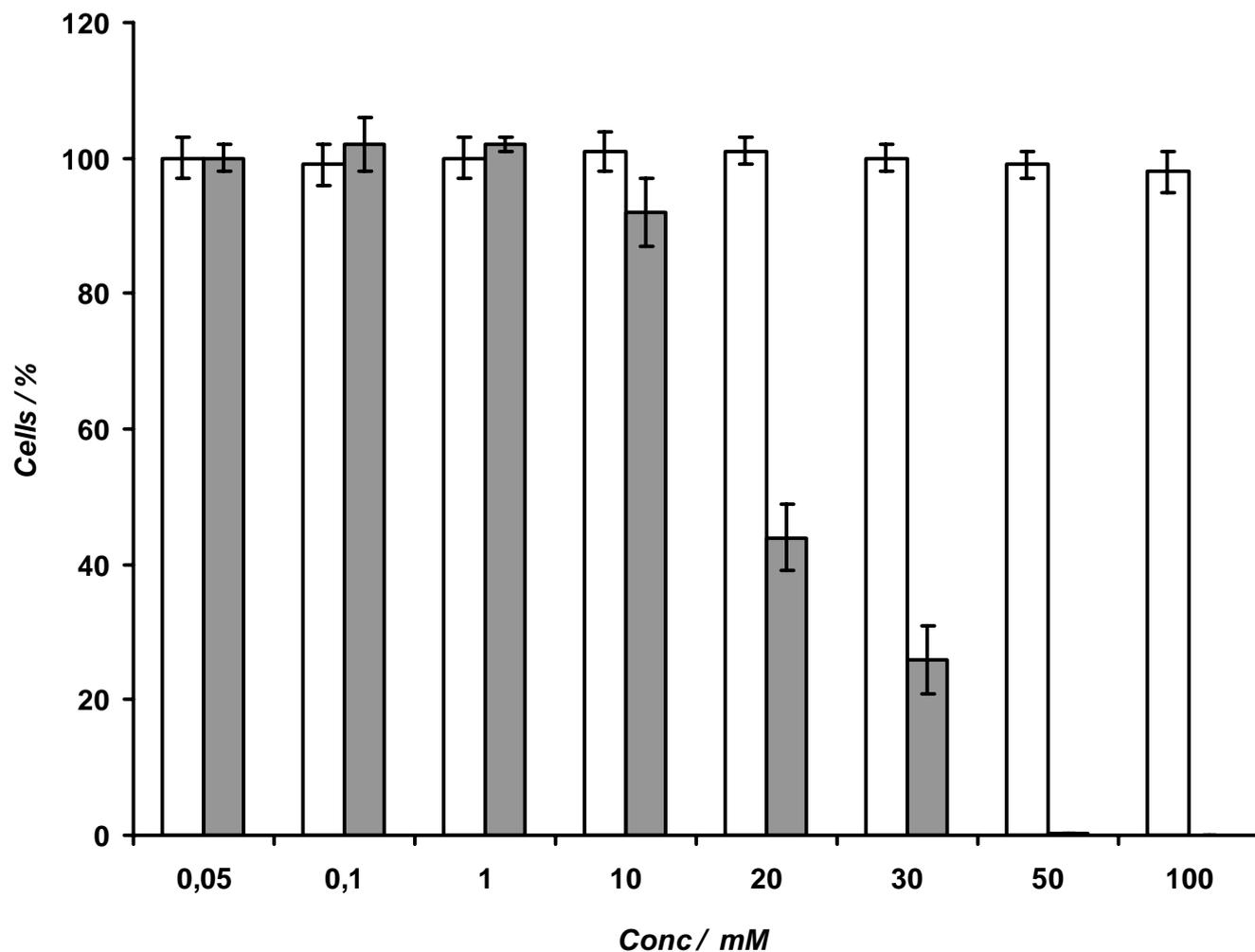


Figure S5. Proliferation of HeLa cells treated for 48 hours with solutions at different concentrations of NAC (dark gray boxes) compared to HeLa control (white boxes). Graph shows the percentage of cells after each treatment, stressing a noticeable reduction of viability above 10 mM solution.

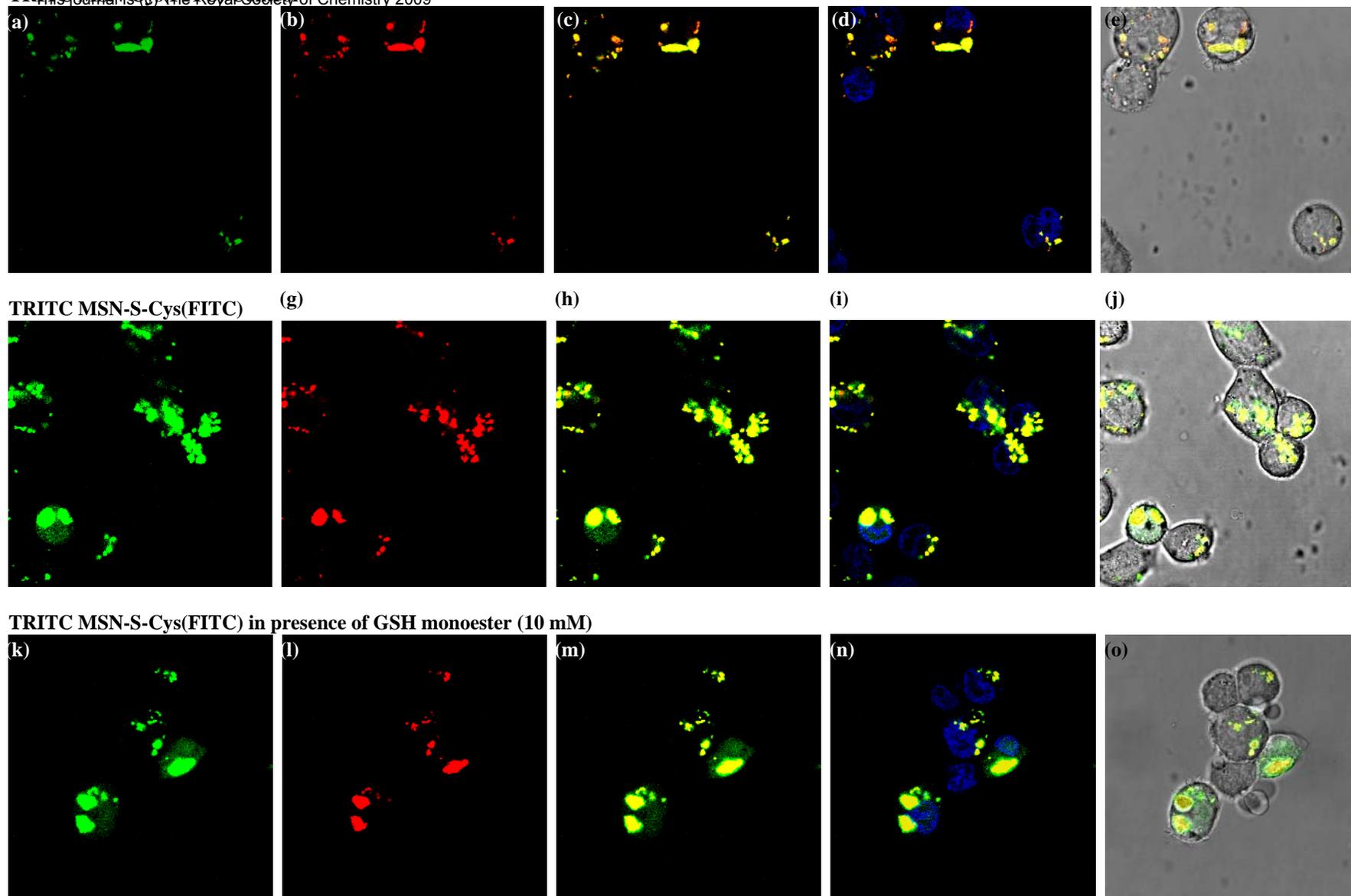


Figure S6. Confocal fluorescence images of HeLa cells loaded with TRITC-MSN-Cys(FITC) (**a-e**), TRITC-MSN-S-Cys(FITC) (**f-j**), and TRITC-MSN-S-Cys(FITC) in presence of GSH monoester (**k-o**). (**a, f, and k**) green fluorescence of FITC labeled Cys, (**b, g, and l**) red fluorescence of the endocytosed TRITC-labeled MSN, (**c, h, and m**) yellow spots corresponding to the overlapped image of green (Cys-FITC) and red (TRITC-MSN) micrographs, (**d, i, and n**) superimposed micrographs of green (Cys-FITC) and red (TRITC-MSN) images in presence of nuclei-staining dye Hoechst 33258, and (**e, j, and o**) overlapped image of green (Cys-FITC) and red (TRITC-MSN) with transmission image.

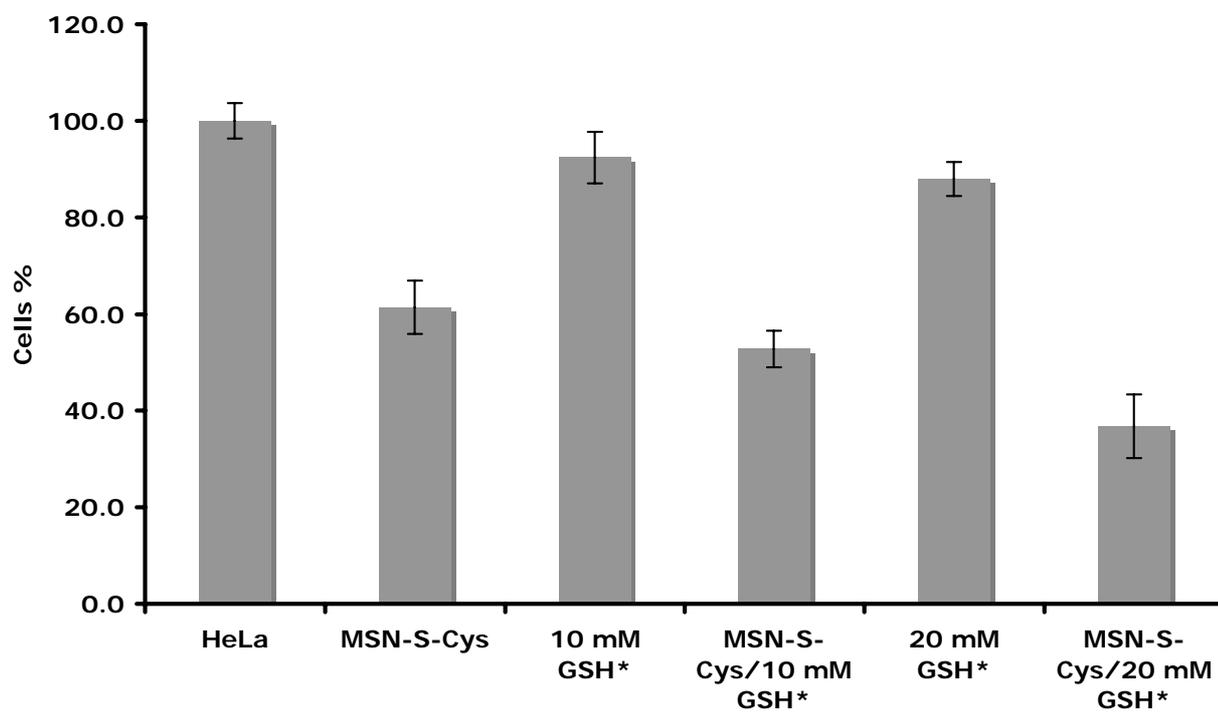


Figure S7. Cell proliferation inhibition of HeLa cells as a function of the concentration of glutathione monoester (GSH*). The glutathione monoester dose-dependent performance in the cell proliferation inhibition confirm its influence in the intracellular release of Cys from MSN-S-Cys.