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Supporting Information

pH-operated Hybrid Silica Nanoparticles with Multiple H-bond Stoppers for Colon Cancer Therapy

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Experimental Section

General methods: Reagents were used as received. Solvents were dried using MB SPS-800 apparatus. FTIR data were obtained on a Perkin Elmer FT-IR system Spectrum BX spectrophotometer equipped with GladiaATR. Liquid ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AC-400 spectrometer at room temperature with $CDCl_3$ and DMSO as solvents and tetramethylsilane (TMS) as an internal reference. ^{29}Si NMR spectra were obtained using a Bruker DSX 300 MHz spectrometer using cross-polarization and magic-angle spinning techniques (CP-MAS). High resolution mass spectra (Q-TOF ES+) were measured on a JEOL MS-DX 300 mass spectrometer. Discontinuous nitrogen sorption isotherms were measured at 77.15 K using a Micromeritics 2010 apparatus. Specific surface areas were derived using the BET transform of the sorption isotherms, taking 0.162 nm^2 as the cross-section area for nitrogen. No microporosity could be detected using the t-plot method, taking Aerosil200 as reference.

Synthesis: Stalk was prepared according to literature [1, 2]. Cap is commercial Cyanuric Acid.

Preparation of Porous MCM-41 Silica Particles: Cetyltrimethylammonium bromide (CTAB) 0.315 g was added to 150 mL of deionized H_2O. 2 M NaOH (1.1 mL) was added to the solution (pH > 12.0) inducing the complete dissolution of CTAB. The solution was heated to 80°C while stirring to create a homogenous solution. Tetraethylorthosilicate (TEOS) 1.4 mL was added to the solution dropwise. The reaction was allowed to complete over 2 h. The CTAB surfactant was removed by a solution of 9 ml of HClc in 160 mL of EtOH for 6 h at 60°C. The nanoparticles were finally washed 2 times with EtOH.

Nanoparticle (NP) preparation: Step 1: Grafting Silylated Precursor on MCM-41: 80 mg of Stalk (0.1 mmol) were added to a suspension of 80 mg of MCM-41 in 10 mL of dry toluene.

The solution was heated at 80°C during one day. Then the nanoparticles were collected by centrifugation and washed twice with EtOH and 5 times with water. ^{29}Si CP MAS Solid State NMR: -57.0; -102.4; -108.9. Step 2: Loading. Nanoparticles were added to a 2 mM solution of PI in water for fluorescent dye or to a15 mM solution of CPT in DMSO. To maximize dispersion, the suspension was sonicated during 20 min and then stirred overnight to allow the cargo to enter inside the pores. After centrifugation, the particles were washed one time with DMSO. Solutions of cargo molecules: with Propidium Iodide (PI) : 11 mg of PI in 8 mL of water and 35 mg of nanoparticles; or with Camptothecin: 35 mg of CPT in 8 mL of DMSO and 35 mg of nanoparticles. Step 3: Capping. Nanoparticles were dispersed in a solution of CA (13 mg in 8 mL of DMSO) and stirred 3 days at room temperature. After centrifugation, nanoparticles were washed with DMSO until the solution of washing was colorless and then 3 times with water. The last washing with water did not show the presence of the cargo molecule by UV-Vis. Nanoparticles were finally dried overnight under vacuum at 90°C giving NP_{PI} and NP_{CPT}.

In the case of NPs, prepared without cargo molecule, nanoparticles obtained in step 1 were directly closed with the solution of CA before the washings with DMSO and water. Finally, NPs were dried overnight under vacuum at 90°C.

Camptothecin assay: To evaluate the CPT loaded in nanoparticles, NPs containing or not the drug were dissolved at 5 $mg.mL^{-1}$ in an acidic solution of DMSO (pH 2). After 15 min sonication at room temperature, nanoparticles were centrifuged (10 min, 10,000 rpm). The absorbances of supernatants were read at 340 nm in a microplate reader. Titration gave 40 $\mu g.mg^{-1}$ of CPT loaded in the NP_{CPT}.

Cell culture conditions: Human colorectal cancer cells (HCT-116) purchased from ATCC (American Type Culture Collection, Manassas, VA) were cultured in McCoy's 5A culture medium supplemented with 10% fetal bovine serum and 50 $\mu g.mL^{-1}$ gentamycin. Normal fibroblasts were cultured in DMEM culture medium added with 10% of FBS and 1% of Penicillin/Streptomycin. These cells grew in humidified atmosphere at 37°C under 5% CO_2.

Visualization of fluorescent nanoparticles in living cells: Primary culture cells and HCT-116 cell line were seeded onto glass dishes (Word Precision Instrument, Stevenage, UK) at a density of 10^6 $cells.cm^{-2}$. One day after seeding, cells were incubated 20 h with 40 $\mu g.mL^{-1}$ fluorescent loaded nanoparticles NP_{PI}. Then, cells were counterstained using Hoechst 33342

(Invitrogen, Cergy-Pontoise, France) at 5 $\mu g.mL^{-1}$ during 15 min. For membrane labeling, a wheat germ agglutinin Alexa Fluor 488 conjugate (Invitrogen) was used as described by the manufacturer. For the lysosomal labeling, 2 h before the end of the experiment, 50 nM of lysotracker green DND-26 (Invitrogen) was added. Before visualization, cells were washed gently with phenol red-free medium. Cells were then scanned with a LSM 5 LIVE confocal laser scanning microscope (Carl Zeiss, Le Pecq, France), with a slice depth (Z stack) of 0.62 µm.

In vitro cytotoxicity experiments: HCT-116 cells were seeded into 96-well plates at 2.10^4 cells per well in 200 µL culture medium. NPs were freshly suspended in ethanol at a concentration of 5 $mg.mL^{-1}$ and submitted to an ultrasonic bath until a homogeneous solution was obtained. One day after seeding, cells were incubated 72 h with different concentrations of nanoparticles (0 to 10 $\mu g.mL^{-1}$). Then, a MTT assay was performed to evaluate the toxicity. Briefly, cells were incubated for 4 h with 0.5 mg.mL-1 MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Promega) in media. The MTT/media solution was then removed and the precipitated crystals were dissolved in EtOH/DMSO (1:1). The solution absorbance was read at 540 nm.

For normal cells study, the same protocol was used except for the concentrations of NPs and CPT. Experiments were conducted with normal fibroblasts incubated 72 h with NPs (5 to 50 $\mu g.mL^{-1}$). Non tumorigenic (BMEL) and tumorigenic (BMEL-Ras) hepatic progenitors were cultured for 20 h with NP_{PI} (40 $\mu g.mL^{-1}$), collected and fluorescence was quantified on a FACSCalibur flow cytometer using CellQuestPro software (BD Biosciences).

Ex vivo primary cell cultures: Primary cell cultures were obtained from human colon carcinoma pieces. For these experiments, informed and signed consent was obtained from patients. The cell culture protocol was established especially for growth *in vitro*. The biopsies were collected in Dulbecco's Modified Eagle Medium (DMEM) 2 mM L-glutamine, 4.5 $g.L^{-1}$ glucose, supplemented with 300 IU penicillin and 300 $\mu g.mL^{-1}$ streptomycin. Tissues were gently washed in Hank's buffer (NaCl 8.0 $g.L^{-1}$, KCl 0.4 $g.L^{-1}$, KH_2PO_4 60 $mg.L^{-1}$, glucose 1 $g.L^{-1}$, Na_2HPO_4 48 $mg.L^{-1}$, $MgSO_4$ 98 $m.L^{-1}$, $CaCl_2.2H_2O$, 185.4 $mg.L^{-1}$, $NaHCO_3$ 350 $mg.L^{-1}$) and sliced with scalpel on sterile Petri dishes. Then, the mixture was suspended in 20 mL DMEM culture medium containing 2 mM L-glutamine, 4.5 $g.L^{-1}$ glucose, 15% fetal bovine serum, 25 $\mu g.mL^{-1}$ gentamycin, 50 IU penicillin and 50 $\mu g.mL^{-1}$ streptomycin, 0.25 µg of

amphotericin B and 0.205 µg of sodium deoxycholate. For enzymatic digestion, 1 mL of collagenase type II 670 $U.mL^{-1}$ (Sigma-Aldrich, Buchs, Switzerland) was added and the mix was stirred at 37°C for 2 h. The mixture was filtered on a 300 Mesh filter, centrifuged (10 min, 10 000 rpm) and cell pellet was washed with culture medium. Finally, cells were seeded into Petri dishes 100 mm diameter (Sarstedt, Marnay, France) and imaging µ-dishes ibitreat 35 mm diameter (Ibidi, Martinsried, Germany) and allowed to grow in humidified atmosphere at 37°C under 5% CO_2.

Statistical analyses: They were performed using the Student's t test to compare paired groups of data. A p-value < 0.05 was considered as statistically significant.

Results

The pores closing of NP_{PI} at neutral pH 7 was verified by time-resolved fluorescence spectroscopy for 4h. During this time no PI was observed in the solution demonstrating the H-bond stability and confirming the absence of premature release of the cargo molecule (Figure S1) at neutral pH.

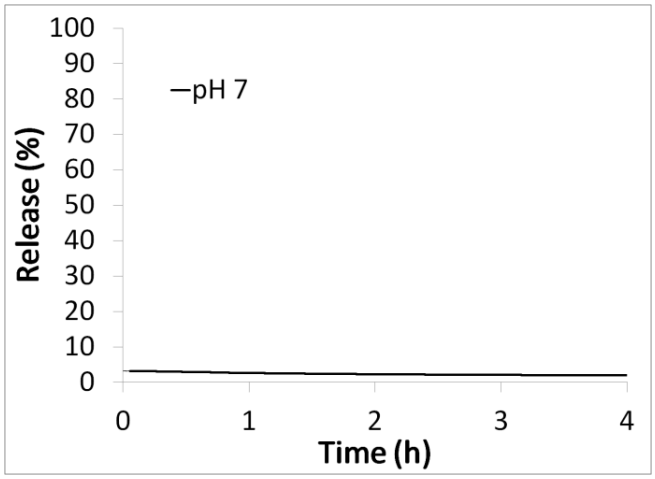


Figure S1. Release experiments of CA at pH 7.

The level of CPT in NP_{CPT} was determined. In this experiment, NP_{CPT} were dissolved in DMSO instead of ethanol in order to ensure an optimal dissolution of CPT in the solvent during the opening of NP_{CPT}. Data in Table S1 show that NP_{CPT} contains about 40 µg CPT per mg NP_{CPT}.

Table S1. Measurement of CPT contained in NP_{CPT}. Three independent experiments were realized with 5 $mg.mL^{-1}$ NPs to verify the reproducibility of the dosage. CPT release from NP_{CTP} was induced by acidic conditions (DMSO pH 2). CPT concentration was determined by UV-Vis and expressed in $\mu g.mg^{-1}$ of NPs. Absorbance of empty NP was measured (Background of NP).

Experiment	1	2	3	Average
NP ($mg.mL^{-1}$)	5	5	5	
Background of NP	0.13	0.24	0.12	0.16 ± 0.06
Released CPT µg CPT/ mg NP_{CPT}	42.0	38.0	39.5	40 ± 2

Human colon cancer cells and normal fibroblasts were treated with high concentrations of NP_{CPT} (from 0 to 50 $\mu g.mL^{-1}$) in contrast of experiments presented in Figure 4 (from 0 to 10 $\mu g.mL^{-1}$). Results presented in Figure S2 demonstrate that 50 $\mu g.mL^{-1}$ $NP_{CPT,}$ fibroblasts show a decrease of 23%. This is a very small effect in comparison with the effect observed on HCT-116 cancer cells which present an identical cell death level for 200 time less NP_{CPT} (0.25 $\mu g.mL^{-1}$) as shown in Figure 4.

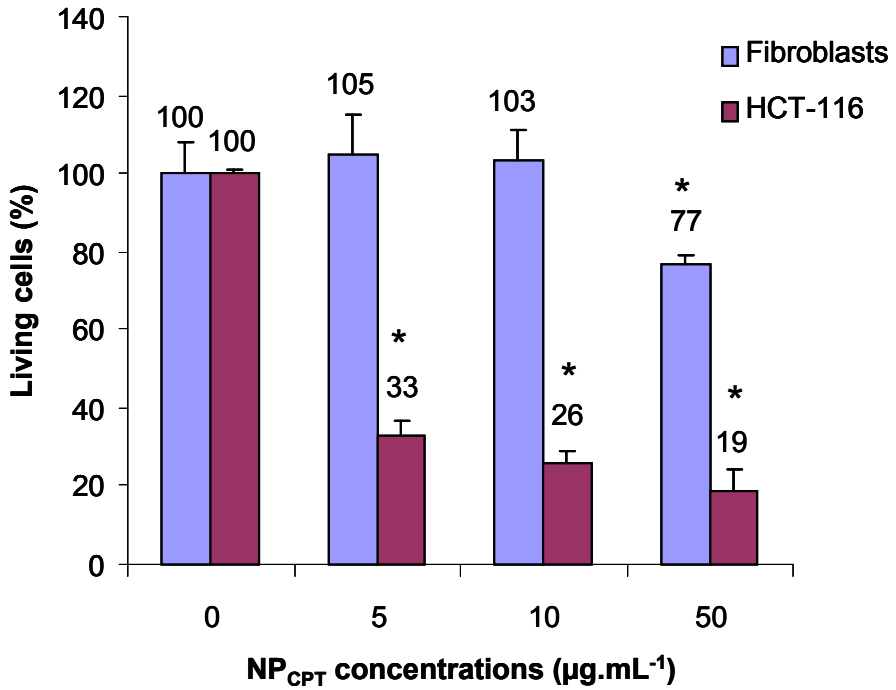


Figure S2. Viability of normal fibroblasts and colon cancer cells treated 72 h with increasing doses of NP_{CPT} (from 0 to 50 µg.mL^{-1}) and submitted to MTT assay. Values are expressed as % of control cells treated with vehicle alone. Values represent mean ± SD of 3 experiments. * $p < 0.05$ for treatment with NP_{CPT} compared to control (0 µg.mL^{-1}) using Student's t test.

In addition, a kinetic experiment on BMEL cells tumorigenic or not was realized and demonstrated the higher potential of tumorigenic cells to internalize NP_{PI} (Figure S3).

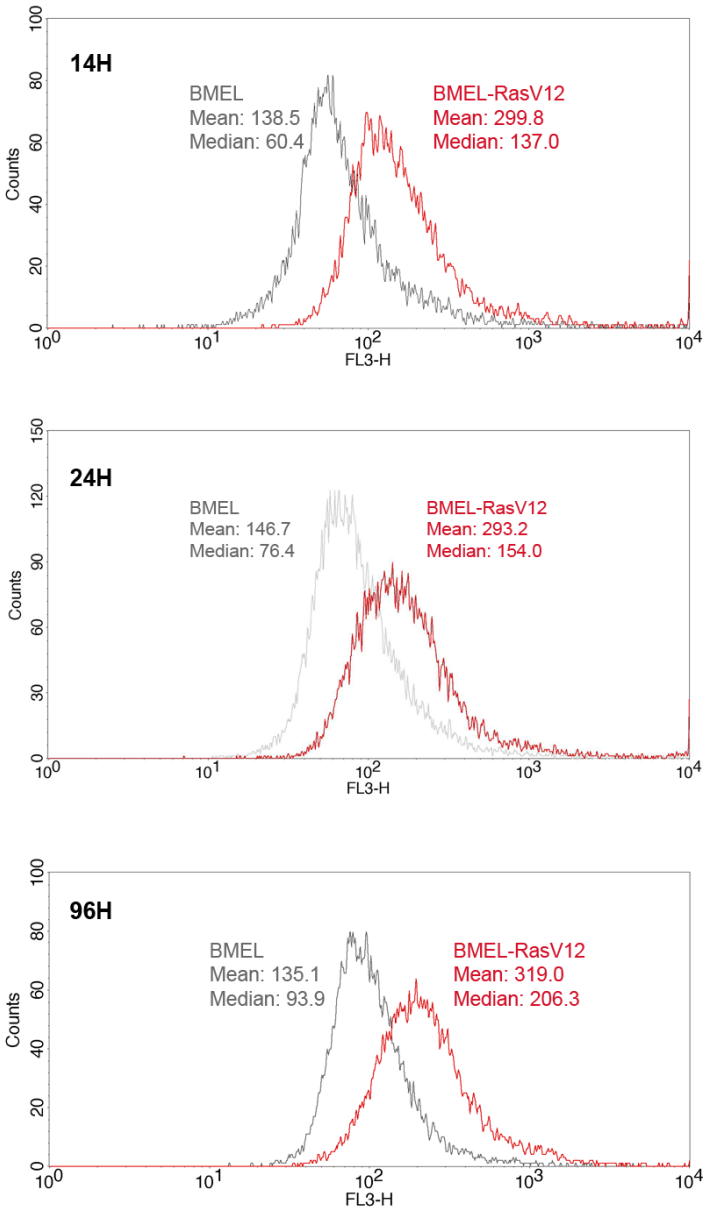


Figure S3. Fluorescence quantification by FACSCalibur of non tumorigenic (BMEL) and tumorigenic (BMEL-Ras) hepatic progenitors incubated 14, 24, and 96 h with NP_{PI} (40 µg.mL^{-1}). Results reproduced in 4 independent experiments with three independent sets of cell lines.

It is important to note that this cytotoxic effect on HCT-116 cells was neither due to the empty NPs nor cyanuric acid used for the capping (Figure S4).

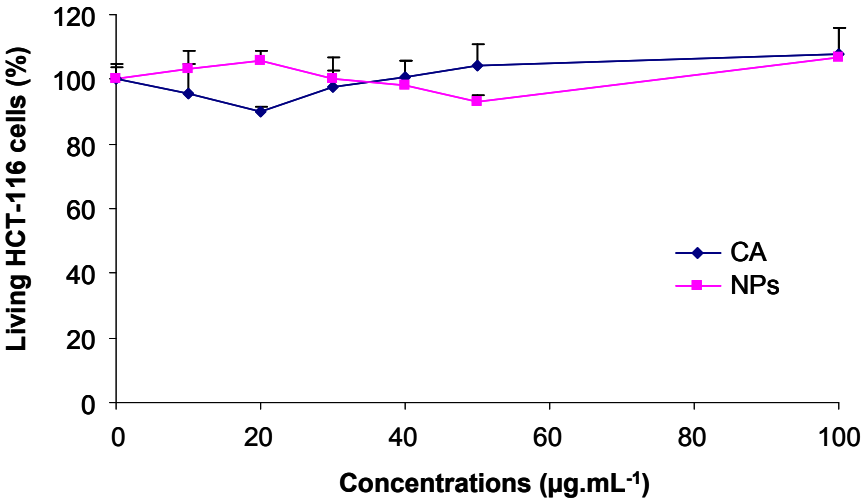
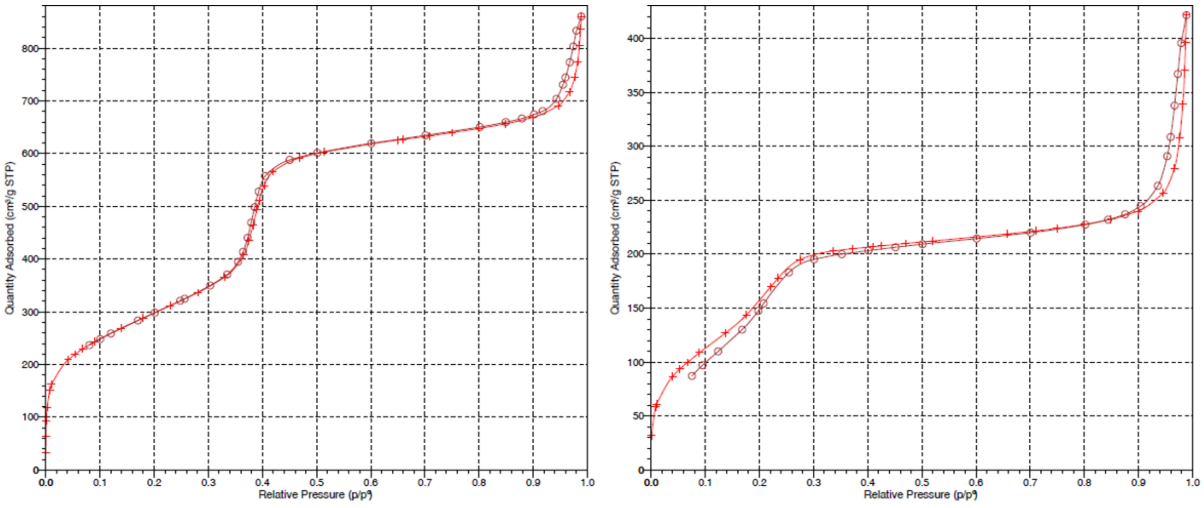


Figure S4. Cytotoxicity study of cyanuric acid (CA) and empty NPs. HCT-116 colorectal cancer cells treated 72 h with increasing concentrations of CA or NPs (10 to 100 µg.mL^{-1}) and submitted to MTT assay. Values are expressed as % of control cells treated with vehicle alone. Values represent mean ± SD of 3 experiments.

(A) (B)



	BET Surface Area (m^2/g)	Total pore volume (cm^3/g)	Adsorption average pore width (Å)
MCM-41	1085,772	1,330754	49,0252
MCM-41+stalk	686,9339	0,652077	37,9703

Figure S5: BET specific surface areas, pore volumes, and pore sizes calculated from N_2 adsorption-desorption isotherms of MCM- 41 (A) and MCM-41 post-functionalized with stalk (B)

[1] L. Fertier, C. Théron, C. Carcel, P. Trens, M. Wong Chi Man, *Chem Mater* **2011**, *23*, 2100.

[2] C. Théron, A. Gallud, C. Carcel, M. Gary-Bobo, M. Maynadier, M. Garcia, J. Lu, F. Tamanoi, J. I. Zink, M. Wong Chi Man, *Chemistry- A European Journal* **2014**, *20*, 9372.