Self-Immolative Polymers as novel pH-responsive gate keepers for drug delivery

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**Characterization Techniques**

**Power X-Ray Diffraction (XRD)** experiments were performed in a Philips X’Pert diffractometer equipped with Cu Kα radiation (wavelength 1.5406 Å). XRD patterns were collected in the 2θ range between 0.6° and 8° with a step size of 0.02° and counting time of 5 s per step.

**Nuclear Magnetic Resonance (NMR)** spectroscopy was carried out on a Bruker AV250 MHz.

**Fourier Transform Infrared (FTIR)** spectroscopy was carried out in a Nicolet Nexus (Thermo Fisher Scientific) equipped with a Golden gate attenuated total reflectance device, averaging 64 scans in the range of 4000-400 cm⁻¹ at a resolution of 1 cm⁻¹.

**Thermogravimetric (TG)** measurements were performed in a Perkin Elmer Pyris Diamond TG/DTA analyser by placing 10 mg of sample in an aluminium crucible and applying 5ºC/min heating ramps from room temperature to 600ºC.

**Scanning Electron Microscopy (SEM)** images were recorded on JEOL JSM 6335F (Electron Microscope Centre, UCM).

**Transmission Electron Microscopy (TEM)** images were recorded on a JEOL JEM 2100. Sample preparation was performed by dispersion in distilled water and subsequent deposition onto carbon-coated copper grids. (Electron Microscope Centre, UCM).

**Nitrogen adsorption and desorption** isotherms were obtained at 77K using a Micromeritics ASAP 2020. To perform the N₂ measurements, ca. 50 mg of each sample were previously degassed under vacuum for 24 h at 40ºC. The surface area \(S_{BET}\) was determined using the Brunauer-Emmett-Teller (BET) method and the pore volume \(V_p\) was estimated from the amount of N₂ adsorbed at a relative pressure of ca. 0.99. The pore size distribution was calculated from the desorption branch of the isotherm by means of the Barrett-Joyner-Halenda (BJH) method. The mesopore size was determined from the maximum of the pore size distribution curve.

**Zeta-Potential** analysis and **Dynamic Light Scattering (DLS)** were carried out in a Zetasizer Nano ZS (Malvern Instruments Ltd.) equipped with a 633 nm “red” laser. For this purpose, 10 mg of nanoparticles were added to 10 mL of water followed by 5 min of sonication to obtain a homogeneous suspension. In the case of Zeta-Potential measurements, the pH was adjusted by adding appropriated amounts of HCl 0.1 M or NaOH 0.1 M to the suspension under magnetic stirring. In both cases, measurements were recorded by placing ca. 1 mL of suspension (1 mg/mL) in DTS1070 disposable folded capillary cells (Malvern Instruments).

**Fluorescence** spectrometry was carried out in a BioTek Spectrofluorimeter.

**Elemental analysis** was performed in a CE Instrument EA-1110 CHN Elemental Analyzer.
Experimental Section

Chemicals:

The following compounds were purchased from Sigma-Aldrich Inc.: Tetraethyl orthosilicate (TEOS); Ammonium nitrate; Cetyltrimethylammonium bromide (CTAB); Fluorescein isothiocyanate isomer I (FITC); 4-Aminobenzyl alcohol; Phenyl chloroformate; N,N-Diisopropylethylamine (DIPEA); Dibutyltin dilaurate (DBTL); Tris(2,2’-bipyridyl)dichlororuthenium(II) hexahydrate; (3-Chloropropyl)trimethoxysilane (CS); Di-tert-butyl dicarbonate; Dimethyl sulfoxide (DMSO); N,N-Dimethylformamide (DMF); Tetrahydrofuran (THF). The rest of the chemicals (ethanol, heptane, etc) were of the best quality and were employed as received.

1. Synthesis of nanoparticles

Synthesis of Mesoporous Silica Nanoparticles (MSNs):

Mesoporous silica nanoparticles were synthesised following a modification of the Stöber method. First, CTAB (1 g, 2.74 mmol) was dissolved in H2O (480 mL) and NaOH (3.5 mL, 2M) in a 1 L round bottom flask with moderate magnetic stirring. Then, the solution was heated to 80°C and TEOS (5 mL, 22.39 mmol) was added dropwise and heated for further 2 hours at 80°C under magnetic stirring. After that, the solution was centrifuged and washed 2 times with water and one more with ethanol. The product was dried at room temperature (RT) under vacuum. The surfactant was removed by ionic exchange using a 500 mL of a solution of 10 mg/mL of NH4NO3 in EtOH (95%) at 70°C overnight. The product was centrifuged and washed 3 times with ethanol and dried under vacuum.

To synthesise fluorescein-labeled MSN (MSN-FITC), FITC (1 mg, 0.002 mmol) was reacted with APTES (2.2 μL, 0.009 mmol) in ethanol (115 μL) for 2h. Then the reaction mixture was added dropwise with TEOS (5 mL, 22.39 mmol) and the reaction was carried out as previously described.

The nanoparticles were characterized by thermogravimetric analysis (TGA), Scanning Electron Microscope (SEM), Transmission Electron Microscope (TEM), Fourier Transform Infrared spectroscopy (FTIR), N2 adsorption (BET), Zeta Potential, Dynamic Light Scattering (DLS) and X-ray diffraction (XRD) and elemental analysis techniques.

2. Synthesis of Self-Immolative Polymers

Self-immolative polymers were produced following a modification of a previously described method, as shown in Scheme S1:
a) Synthesis of phenyl (4-(hydroxymethyl)phenyl)carbamate (1)

Dry DMF (15 mL) and dry DIPEA (1.7 mL, 9.76 mmol) were added to 4-aminobenzyl alcohol (1 g, 8.12 mmol) in a round bottom flask immersed in an ice bath with magnetic stirring. Then, phenyl chloroformate (1.12 mL, 8.93 mmol) was added dropwise. After 15 minutes the ice bath was removed and the reaction was controlled to completion by thin layer chromatography. The organic phase was extracted in ethyl acetate and washed with saturated NH₄Cl solution and brine and finally dried over sodium sulfate. The resultant solution was precipitated in cold heptane and centrifuged twice to give a white solid. The product was dried under vacuum and characterized by proton nuclear magnetic resonance (¹H-NMR).

b) Synthesis of tert-butyl (4-(hydroxymethyl)phenyl)carbamate (2)

Dry THF (80 mL) and dry DIPEA (1.4 mL, 8.12 mmol) were added to 4-aminobenzyl alcohol (1 g, 8.12 mmol) and di-tert-butyl dicarbonate (1.8 g, 8.12 mmol). Then, the mixture was stirred and refluxed overnight and subsequently evaporated under vacuum to give a yellow oil. The oil was dissolved in ethyl acetate and precipitated in cold heptane to give a white solid. The product was dried under vacuum and characterized by ¹H-NMR.

c) Synthesis of poly(phenyl (4-(hydroxymethyl)phenyl)carbamate) (SIP) (3)

Dry DMSO (1.62 mL) was added to the as-synthesized monomer (1 g, 4.12 mmol). Then, the solution was heated to 85°C, DBTL (5% mol) was added and the reaction mixture was subsequently stirred for 2h30' at 85°C. After that, compound 2 (223 mg, 1 mmol) in dry DMSO (0.5 mL) was added and the solution was heated at 85°C for further 2 hours. Finally, the crude reaction mixture was precipitated in cold MeOH and centrifuged and washed 3 times to give a white solid. The product was dried under vacuum and characterized by ¹H-NMR.

3. Synthesis of self-immolative polymer-grafted mesoporous silica nanoparticles (MSN-CS-SIP)

Dry DMSO (7.5 mL) and dry DIPEA (77 µL, 0.44 mmol) separated in 3 different vials were added to compound 3 (1 g, 0.29 mmol) every 2 hours. Every mixture was stirred for 2 hours at RT and subsequently added dropwise to previously dried MSN-CS (175 mg) in dry DMSO (25 mL) in separated steps. After that, the solution was heated at 80°C overnight. Finally, the product was thoroughly washed with DMSO, water and ethanol. The product was dried under
vacuum and characterized by TGA, XRD, DLS, Zeta potential, elemental analysis, BET, SEM and TEM.

4. Release experiment of MSN-Ru and MSN-CS-Ru

a) Loading of MSN-CS with Tris(2,2′-bipyridine)dichlororuthenium(II) hexahydrate (MSN-CS-Ru)

A solution of Tris(2,2′-bipyridine)dichlororuthenium(II) hexahydrate (Ru) (260 mg, 0.33 mmol) in DMSO (25 mL) was added to MSN (175 mg) and the solution was stirred for 24 h at RT. The product was dried under vacuum.

b) Loading of MSN-CS-SIP with Tris(2,2′-bipyridine)dichlororuthenium(II) hexahydrate (MSN-CS-SIP-Ru)

A solution of Tris(2,2′-bipyridine)dichlororuthenium(II) hexahydrate (260 mg, 0.33 mmol) in DMSO (25 mL) was added to MSN-CS (175 mg) and the solution was stirred for 24 h at room temperature. Then, it was heated to 80°C and the protocol described in 3) was carried out again.

c) Release experiment

The evaluation of the pH responsiveness release behaviour of MSN-CS-Ru and MSN-CS-SIP-Ru was carried out via in-vial release experiments at two different pH values: 7.4 (0.01 M phosphate buffer) and 5 (0.01 M acetate buffer). For this purpose, 2 batches of 10 mg of each nanocarrier were prepared. First, all of them were suspended in 0.5 mL of phosphate buffer (pH 7.4) and then placed into the insert of a Transwell permeable support with 0.4 µm of polycarbonate membrane (3 replicas were performed). The external well was filled with 1.5 mL of the same medium and the suspension was kept under orbital shaking at 100 rpm and 37°C for all the experiment. After 2 hours, the phosphate buffer of one of them was replaced by the acetate buffer (pH 5). At every time point evaluated, the solution from the external well of the Transwell was taken out and replaced with fresh medium at pH 7.4 and 5 respectively. The amount of Ru released was determined by fluorescence spectrometry.

5. Cell experiments

a) Cell cultures

Cell culture tests were performed using the well-characterized mouse osteoblastic cell line MC3T3-E1 (subclone 4, CRL-2593; ATCC, Mannassas, VA) and an androgen-sensitive LNCaP cells, a human prostate cancer cell line (CRL-1740; ATCC, Mannassas, VA). The tested MSNs (at different concentrations) were placed into each well of 6- or 24-well plates (Corning, CULTEK, Madrid, Spain) after cell seeding. MC3T3-E1 and LNCaP cells were then plated at a density of 20,000 cells cm⁻² in 1 mL of alfa-minimum essential medium or Dulbecco’s modified Eagle’s medium (DMEM, Sigma Chemical Company), respectively, containing 10% of heat-inactivated fetal bovine serum (FBS, Thermo Fisher Scientific, Waltham,MA, USA) and 1% penicillin–streptomycin (BioWhittaker Europe, Verviers, Belgium) at 37°C in a humidified atmosphere of 5% CO₂, and incubated for different times. Some wells contained no MSNs as controls.

b) Cell growth

Cell proliferation was determined by addition of Alamar Blue solution (AbD Serotec, Oxford, UK) at 10% (v/v) to the cell culture at 24, 48 and 72h, following manufacturer’s instructions. Four hours after, 1 mL samples of the cell-conditioned medium were added to 24-well plates, and absorbance intensity was measured using excitation emission wavelengths of 570 and 600 nm, respectively, in a Unicam UV-500 UV–visible spectrophotometer.
c) Fluorescence microscopy
LNCaP cells were incubated with the nanoparticles (100 µg/mL) for 2h in serum-free culture medium. Then, the medium was withdrawn and cells were washed with PBS three times. Cells were fixed with ethanol for 2 min and stained with DAPI. Fluorescence microscopy images were taken to evaluate nanoparticles. Green channel was used to locate for nanoparticles and blue for cell nucleus, in a Evos FL Cell Imaging System equipped with tree Led Lights Cubes (IEX (nm); IEM (nm)): DAPY(357/44; 447/60), GFP (470/22; 525/50), RFP (531/40; 593/40) from AMG (Advance Microscopy Group).

d) Flow cytometry studies
LNCaP cells were cultured in each well of a 6-well plate. After 24h, the cells were incubated in the absence or presence of the tested MSNs (100 µg/mL). After 2 h, cells were washed twice with PBS and incubated at 37°C with trypsin–EDTA solution for cell detachment. The reaction was stopped with culture medium after 5 min and cells were centrifuged at 1.500 rpm for 10 min and resuspended in fresh medium. Then, the surface fluorescence of the cells was quenched with trypan blue (0.4%) to confirm the presence of an intracellular, and therefore internalized, fluorescent signal. Flow cytometric measurements were performed at an excitation wavelength of 488 nm, green fluorescence was measured at 530 nm (FL1). The trigger was set for the green fluorescence channel (FL1). The conditions for the data acquisition and analysis were established using negative and positive controls with the CellQuest Program of Becton–Dickinson and these conditions were maintained during all the experiments. Each experiment was carried out three times and single representative experiments are displayed. For statistical significance, at least 10,000 cells were analysed in each sample in a FACScan machine (Becton, Dickinson and Company, USA) and the mean of the fluorescence emitted by these single cells was used.
**Figures**

![Figure S1: Thermogravimetric analysis of MSN, MSN-CS and MSN-CS-SIP](image)

The successful elimination of the surfactant from within the mesopores was confirmed by TGA (continuous black line). The correct surface modification with CS was confirmed by the great weight loss in MSN-CS TGA (dashed line) as well as the successful incorporation of the polymer, shown as an even bigger weight loss in the dotted line.
Figure S2: FTIR spectra of MSN, MSN-CS and MSN-CS-SIP

The silica backbone in the nanocarriers was confirmed by the presence of the following vibration and stretching bands: Symmetric stretching Si-O, $\nu_{\text{SiO}}$, at 800 cm$^{-1}$, asymmetric stretching Si-O-Si, $\nu_{\text{SiOSi}}$, at 1085 cm$^{-1}$, stretching Si-OH, $\nu_{\text{SiOH}}$, at 960 cm$^{-1}$, stretching OH, $\nu_{\text{OH}}$, between 3000-3600 cm$^{-1}$, Si-O-Si bending, $\delta_{\text{SiOSi}}$, at 470 cm$^{-1}$ and HOH bending, $\delta_{\text{HOH}}$, at 1600 cm$^{-1}$.

The correct functionalization with CS was confirmed by the presence of the stretching band C-H at 2900 cm$^{-1}$, $\nu_{\text{CH}}$ and the successful incorporation of the polymer by the appearance of the C-O stretching vibration band at 1650 cm$^{-1}$, $\nu_{\text{CO}}$. 
Results from DLS measurements for sample MSN were in agreement with those obtained by SEM analysis (Figure 1a, 150 nm), although little differences arose from the fact that the DLS measurements were carried out in water (increase in hydrodynamic radius of the nanoparticles). The measurements were carried out at pH 7.4 and pH 5, and no significant changes were obtained among the different samples (MSN, MSN-CS, MSN-CS-SIP), as it was expected from the low molecular weight of the SIP.

**Figure S3:** DLS measurements of MSN, MSN-CS and MSN-CS-SIP at pH 7.4 and pH 5.
The correct synthesis and purification of compound 1 were confirmed by the signals observed in Figure S4: $^1$H NMR (250 MHz, DMSO) δ 10.20 (s, 1H), 7.25-7.45 (m, 9H), 5.12 (t, 1H), 4.45 (d, 2H).

Figure S5: $^1$H NMR spectrum of compound 2
The successful protection of 4-aminobenzyl alcohol with BOC$_2$O was confirmed by the signals observed in Figure S5: $^1$H NMR (250 MHz, DMSO) δ $9.29$ (s, 1H), $7.17-7.41$ (m, 4H), $5.05$ (t, 1H), $4.40$ (d, 2H), $1.48$ (s, 9H).

Figure S6: $^1$H NMR spectrum of compound 3

Instead of trying the transesterification of tert-butanol at the end of the polymer chain, we designed a trigger monomer (compound 2) that would polymerised in the same way that the rest of the monomers. The successful incorporation of compound 2 was verified by the presence of a signal at $1.48$ ppm (-O-[CH$_3$]$_3$): $^1$H NMR (250 MHz, DMSO) δ $9.82$ (s, 19H), $7.56-7.15$ (m, 96H), $5.15$(s, 41H), $4.43$ (d, 2H), $1.48$ (s, 9H).
**Figure S7**: $^1$H NMR spectrum of compound 3 treated with TFA

A small quantity of TFA in dichloromethane was added to the self-immolative polymer to confirm its self-immolative behaviour and the typical signals of compound 3 were readily lost, as it can be seen in figure S7.

**Figure S8**: Release experiment of naked mesoporous silica nanoparticles

A release experiment with nanoparticles without polymer was carried out to confirm the expected release behaviour of these mesostructured materials. Although at first the acid release was faster, finally both samples released the same amount of Ru, as it was expected.
Figure S9: LNCaP cell viability in contact with different concentrations of nanoparticles at 24, 48
and 72h of cell culture. *p<0.05 vs control without nanoparticles (Student’s t-test).

The in vitro cytotoxicity study was determined by the exposition of LNCaP human prostate
cancer cell line to different amounts of nanoparticles (50, 100, 200 and 300 µg/mL). Figure S9
shows that none of the studied concentrations, except 300 µg/mL, induced significant
cytotoxicity measured by an Alamar Blue assay.

Figure S10: TEM images of MSN-CPTMS-SIP.

The functionalisation of the nanoparticles with the polymer does not affect the dispersibility of
the nanocarrier, as can be seen from comparison with Figure 1b.