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Supporting Information

Lactate-Sensitive Nanomachines for Enzyme-Controlled Drug Delivery in Cancer Therapy

Marta González-Jiménez,[a] Teresa Moreno,[a] Beatriz Mayol,[a] Esther Garcia-Díez,[a] Soraya Salido,[a] Alfredo Sánchez-Sánchez,[a] Paloma Martinez-Ruiz,[a] Narcisa Martínez-Quiles,[b] Diana Vilela[a*] and Reynaldo Villalonga [a*]

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[a] *Nanosensors and Nanomachines Group, Department of Analytical Chemistry, Faculty of Chemistry, Complutense University of Madrid, 28040 Madrid, Spain. E-mail: divilela@ucm.es, rvillalonga@quim.ucm.es*

[b] *Department of Immunology, Ophthalmology and ENT, School of Medicine,*

[c] *Complutense University, 28040 Madrid, Spain.*

Materials and Methods

1. Materials

Lactate oxidase (LOx), tetraethoxysilane (TEOS), (3-isocyanatopropyl)triethoxysilane (ICPTES), β-cyclodextrin (β-CD), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, tosyl chloride, 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), iodine, horseradish peroxidase (HRP), Bradford reagent, Bovine Serum Albumin (BSA), urea, hydrogen peroxide (30%), and deuterated chloroform were purchased from Sigma-Aldrich (USA). Hexadecyltrimethylammonium bromide (CTAB), N-hydroxysuccinimide (NHS), Sodium L-lactate and D-glucose were purchased from Alfa Aesar (USA). Copper (II) chloride dihydrate, silica, copper (II) sulfate pentahydrate were purchased from Merck chemicals (Germany). Magnesium sulfate was purchased from Quality Chemicals (Spain). Sodium hydroxide was purchased from Gerbu Biotechnik (Germany). Aniline was purchased from Panreac (Spain). Tris (2,2’-bipyridine)ruthenium (II) chloride hexahydrate was purchased from Acros organic (belgium). Isocove´s modified Dubecco’s medium (IMDM), phosphate buffer saline (PBS), Dulbecco´s modified Eagle medium (DMEM) fetal bovine serum (FBS), Wheat germ agglutinin (WGA) conjugated to Alexa Flour-488 and Hoechst 33342 were purchased from Thermo Fisher. Doxorubicin (Doxo) was acquired from Sequoia Research Products Ltd. (USA). Sodium phosphate buffer (PB) was prepared. Solvents were provided by Scharlau (Spain).

2. General techniques

Nitrogen adsorption/desorption isotherms and pore size distribution were determined using a physisorption analyzer (ASAP 2020, Micromeritics, USA). Infrared spectra were collected via Fourier-transform infrared spectroscopy (FT-IR) using a Spectrum 400 Series instrument (Perkin Elmer, USA). The crystalline phases of the samples were examined by powder X-ray diffraction (XRD) using a X'Pert MRD diffractometer (PANalytical B.V., The Netherlands). Nuclear magnetic resonance (NMR) spectroscopy for both ^{1}H and ^{13}C nuclei was carried out on a Bruker DPX 300 MHz spectrometer (USA). Thermal properties were evaluated using a SDT-Q600 thermal analysis system (TA Instruments, USA). Transmission electron microscopy (TEM) images were obtained with a JEM-2100 microscope (JEOL Ltd., Japan). UV-visible absorption spectra were measured using an Ultrospec™ 8000 dual-beam spectrophotometer (Biochrom, UK).

Confocal Fluorescence images were recorded using Olympus FluoView-1200 confocal laser scanning Microscope (Olympus Life Science). Cell viability % measurements were measured using a Biotek ELX800 plate reader.

3. Synthesis of mono-6-O-tosyl-β-cyclodextrin (CD-OTS)[1]

According to the previous reported method,[2] 11.3 g (10 mmol) of β-CD was dissolved in 500 mL of water in a 3 L three-necked round-bottom flask connected to a reflux condenser. Then, 7.5 g (30 mmol) of copper (II) sulfate pentahydrate was dissolved in 750 mL of water and then added to the flask along with 10.1 g (250 mmol) of NaOH dissolved in 250 mL of water. Subsequently, a solution of 15 g (79 mmol) of tosyl chloride in 100 mL of acetonitrile was added dropwise using an addition funnel, and the mixture was stirred for 3.5 hours.

The mixture was neutralized to pH 7 by dropwise addition of 1 M HCl solution. As the pH decreased, the solution color changed from dark to light blue and stirring was stopped to allow phase separation: the upper, colorless aqueous phase contained the cyclodextrin, whereas the lower phase contained the precipitated blue copper salts. The mixture was left for 12 h to ensure complete separation.

The supernatant was transferred under pressure via a cannula to a 1L Erlenmeyer flask, and the blue residue was filtered and washed with 50 mL of hot water.

After two thirds of water were evaporated, the solution was stored at 4°C for 12h to allow crystallization. Then, the resulting crystals were collected by vacuum filtration using a Buchner funnel and washed twice with 40 mL of acetone and 30 mL of diethyl ether, respectively.

A total of 2.76 g of product was obtained, corresponding to a yield of 24.7%.

^{1} H-RMN (DMSO- d6 , 300 MHz): δ = 7.78 (d, 2H), 7.48 (d, 2H), 5.95-5.55 (m,14H), 4.91-4.70 (m, 7H), 4.63-4.38 (m, 6H), 4.33 (m, 1H), 4.19 (m, 1H), 3.67-3.20 (m, 42H), 2.42 (s, 3H) ppm

4. Synthesis of mono-6-ethylendiamino-6-deoxy-β-cyclodextrin (CD-NH$_2$)[2]

In a two-necked round bottom flask equipped with a reflux condenser, 1.5 g of mono-6-O-tosyl-β-cyclodextrin and 7 mL of distilled ethylenediamine were added under an Ar atmosphere. The mixture was heated at 70 °C for 24 h, obtaining an amber-colored solution containing ethylendiamine-functionalized cyclodextrin.

For product recovery, acetone was gradually added to the solution, inducing the formation of a white precipitate. The solid was purified by recrystallization, involving dissolution in 30 mL of water followed by the addition of acetone until a stable white suspension was obtained. Finally, the purified solid was collected by vacuum filtration yielding 0.9713 g of product (70.9%).

^{1} H-RMN (D$_2$O, 300 MHz): δ = 6.20-5.23 (br, 14H), 4.80 (m, 7H), 4.10-3.50 (m, 51H), 2.60-2.90 (m, 6H) ppm.

5. Preparation of mesoporous silica nanoparticles (S$_0$)

MSNs (S$_0$) nanoparticles were synthesized following a previously reported procedure.[3] Briefly, 1.0 g of CTAB was first dissolved in 0.48 L of distilled water in a 1 L three-necked round-bottom flask. Then, 3.5 mL of 2.0 M NaOH solution were added. The mixture was connected to a cooling system and immersed in a silicone bath maintained the temperature at 80 °C, after which 5.0 mL of TEOS was gradually added dropwise under vigorous stirring. The mixture was allowed to stir for 2 h under the same conditions. After cooling to room temperature, the resulting white solid was filtered and washed with water and methanol, being finally dried at 60 °C for 18 h. The obtained solid was finely ground and calcined at 550 °C for 5 h.

6. Quantification of immobilized Lactate Oxidase.

The concentration of lactate oxidase immobilized on silica nanoparticles was determined using the Bradford colorimetric assay.[4] For calibration, bovine serum albumin (BSA) was used as the protein standard, generating a calibration curve based on measured absorbance values. The amount of enzyme anchored to the nanoparticles was calculated by subtracting the concertation of lactate oxidase found in the first wash supernatant from the total amount initially added.

Following the addition of the Bradford reagent, samples were gently agitated and incubated in the dark for 10 min. Absorbance was measured at 595 nm using a spectrophotometer, with all measurements performed in triplicate. The concentration of immobilized enzyme was then determined by interpolating the absorbance values into the established calibration curve.

7. Enzimatic activity measurement

Lactate oxidase (LOx) activity was measured using a colorimetric assay using horseradish peroxidase (HRP) as coenzyme.[5] ABTS oxidation, catalyzed by HRP in the presence of H_2O_2 generated during the LOx-mediated oxidation of L-lactate to pyruvate, was monitored by absorbance at 405 nm. Enzymatic activity was calculated using the molar extinction coefficient of oxidized ABTS (ε_{ABTSox} = 36.8 $mM^{-1}\,cm^{-1}$).

8. Release assays of S_2 with H_2O_2

Controlled release studies of S_2 were carried out to evaluate the operational mechanism of the molecular gate. A nanoparticle concentration of 8 mg mL^{-1} was employed in a final volume assay of 200 µL per tube. Three blank controls and three replicates were prepared for each of hydrogen peroxide concentrations: 100, 500 and 1000 mM. The release profiles of the encapsulated cargo were monitored by UV-Vis spectroscopy, with absorbance recorded at 454 nm every 30 min over a total duration of 270 min.

9. Cell Culture

HeLa human cervical adenocarcinoma cells (ATCC) were cultured in Iscove's modified Dulbecco's medium (IMDM) containing 25 mM D-glucose, supplemented with 10% fetal bovine serum (FBS, Fisher) and antibiotics (penicillin 100 U mL^{-1} and streptomycin 100 µg mL^{-1}, Invitrogen, Thermo Fisher). Cells were incubated at 37 °C and a humid atmosphere with 5% CO_2 atmosphere and subcultured twice weekly by trypsinization.

List of Figures:

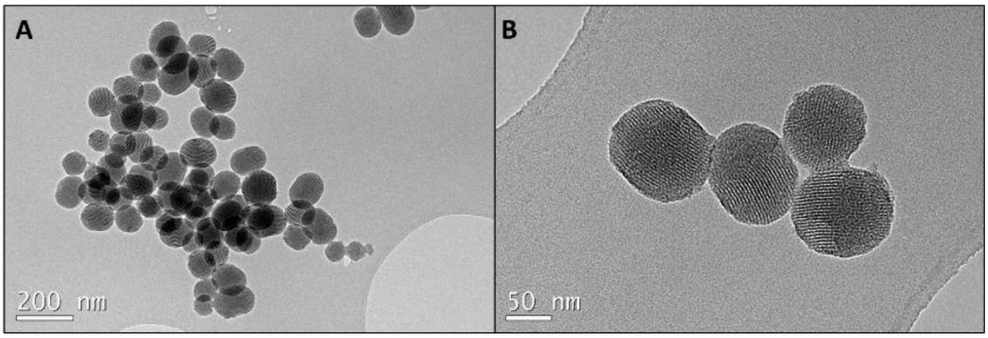

Fig S1. TEM images of solid S_0

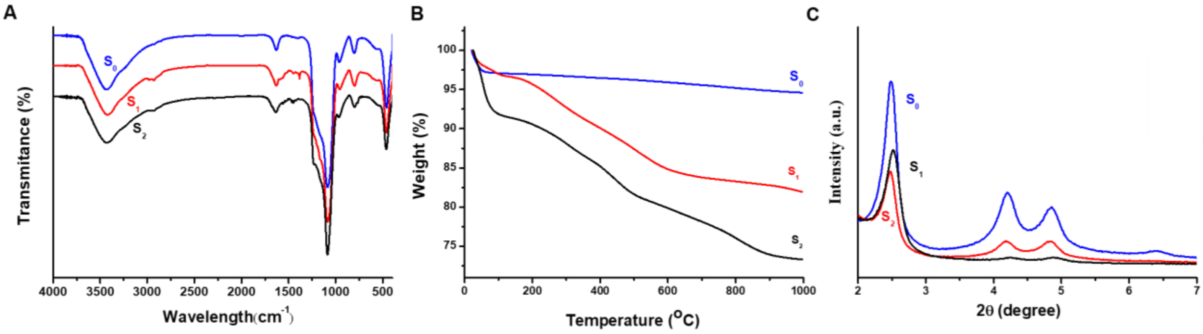

Fig S2. Characterization of S_0-S_2 using FT-IR analysis (**A**) thermogravimetric analysis (**B**) and XRD analysis (**C**).

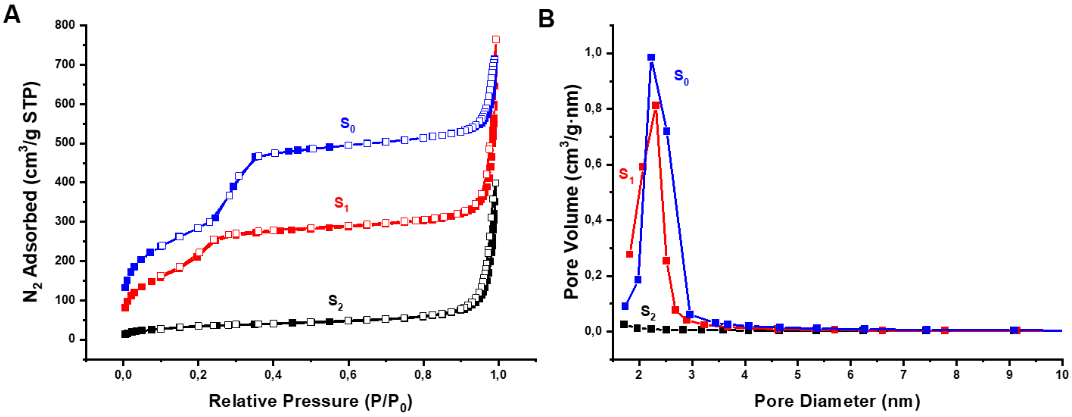

Figure S3: Nitrogen adsorption-desorption isotherms (**A**) and pore size distribution (**B**) using BET and BJH methods.

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