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Supporting Information

Au-Mesoporous Silica Nanoparticles Gated with Disulfide-Linked Oligo(Ethylene Glycol) Chains for Modulable Cargo Delivery Mediated by an Integrated Enzymatic Control Unit

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1. Chemicals

Tetraethyl orthosilicate (TEOS), *n*-cetyltrimethylammonium bromide (CTABr), sodium hydroxide (NaOH), (3-mercaptopropyl)trimethoxysilane, hydrogen tetrachloroaurate(III) ($HAuCl_4 \cdot 3H_2O$), sodium citrate tribasic dihydrate, paraffin wax, safranin O, 3-mercaptopropionic acid, 2,2'-dipyridyl disulfide, *O*-(2-Mercaptoethyl)-*O*'-methyl-hexa(ethylene glycol), acetylcholinesterase from *Electrophorus electricus*, acetylcholine chloride, acetylthiocholine iodide, diisopropylfluorophosphate (DFP), diethyl cyanophosphonate (DCNP), diethyl chlorophosphate (DCP), *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich. Sodium dihydrogen phosphate monohydrate, disodium hydrogen phosphate heptahydrate, ethanol, chloroform and acetonitrile were provided by Scharlau. Doxorubicin hydrochloride was purchased from Sequoia Research Products. For cell culture, Dulbecco's Phosphate Buffered Saline (PBS), Dulbecco's Modified Eagle's Medium (DMEM) - high glucose, Fetal Bovine Serum (FBS) and Hoechst 33342 were purchased from Sigma-Aldrich. Cell proliferation reagent WST-1 was obtained from Roche Applied Science. HeLa human cervix adenocarcinoma cells were purchased from the German Resource Centre for Biological Materials (DSMZ). The rest of chemicals were commercially available and used without further purification.

2. General methods

Powder X-ray diffraction (PXRD), transmission electron microscopy (TEM) , N_2 adsorption-desorption isotherms, elemental analysis and UV-visible and fluorescence spectrophotometry techniques were employed for materials characterization. PXRD measurements were performed on a Seifert 3000TT diffractometer using CuK_α radiation. TEM images were acquired using a JEOL TEM-1010 Electron microscope. N_2 adsorption-desorption isotherms were recorded on a Micromeritics TriStar II Plus automated analyzer. UV-visible spectra were recorded with a JASCO V-650 Spectrophotometer. Fluorescence measurements were carried out in a JASCO FP-8500 Spectrophotometer. Elemental analysis was performed in a CE Instrument EA-1110 CHN Elemental Analyzer. TEM coupled with energy dispersive X-ray spectroscopy (TEM-EDX) was used for element mapping using a JEOL TEM-2100F microscope. Cell viability measurements were taken with a Wallac 1420 workstation. Confocal microscopy imaging was performed with a Leica TCS SP2 AOBS (Leica Microsystems Heidelberg GmbH) inverted laser scanning confocal microscope.

3. Synthesis of mesoporous silica nanoparticles

1.00 g (2.74 mmol) of *n*-cetyltrimethylammonium bromide (CTABr) was dissolved in 480 mL of deionized water. Then, the pH was basified by adding 3.5 mL of a 2 $mol \cdot L^{-1}$ NaOH solution and the temperature was increased to 80 °C. Then, TEOS (5.00 mL, 22.4 mmol) was then added dropwise to this solution. Magnetic stirring was kept for 2 hours to give a white precipitate. Finally, the solid was isolated by centrifugation, washed several times with water and dried at 70 °C overnight (as-synthesized MCM-41). To obtain the final mesoporous nanoparticles, the as-synthesized solid was calcined at 550 °C using an oxidant atmosphere for 5 hours in order to remove the surfactant.

4. Synthesis of gold nanoparticles

Gold nanoparticles were synthesized based on the Turkevich-Frens method.[1] Briefly, 360 mL of a 3 μM $HAuCl_4 \cdot 3H_2O$ solution was brought to 100 °C under stirring and refluxing. Then, 5.4 mL of a 10^{-2} % sodium citrate solution was added to synthesize 20 nm gold nanoparticles. The initially faint yellow colour turned to blue-black and finally to red wine in 10 min. After this, the colloidal suspension was let to cool at room temperature.

5. Synthesis of Janus Au-MS nanoparticles (S1)

Janus nanoparticles were synthesized by adapting a method previously reported in the literature.[2] MCM-41 mesoporous silica nanoparticles (180 mg) were dispersed in 9 mL of aqueous solution (6.7 % ethanol) and *n*-cetyltrimethylammonium bromide (CTABr) was added for a 1 µM final concentration. The mixture was heated at 75 °C, and then 1 g of paraffin wax was added. Once the paraffin was melted, the mixture was vigorously stirred for 15 minutes using an Ultra-Turrax T-8 homogenizer (IKA). Afterward, the mixture was further stirred for 1 hour at 1500 rpm and 75 °C using a magnetic stirrer. The resulting Pickering emulsion was then cooled to room temperature, diluted with 9 mL of methanol and reacted with 180 µL of (3-mercaptopropyl)trimethoxysilane for 3 hours. The solid was collected by centrifugation and washed with methanol. For gold attachment, the partially mercapto-functionalized MCM-41 nanoparticles were dispersed in 75 mL of methanol and added over 360 mL of the as-synthesized gold nanoparticles. The mixture was stirred overnight. Then, the solid was isolated by filtration and exhaustively washed with ethanol and with chloroform. The solid was dried and ground. This process finally yield the Janus Au-MS nanoparticles (**S1**).

6. Synthesis of S2

For the preparation of **S2**, 80 mg of **S1** and safranin O dye (24 mg, 0.07 mmol) were suspended in distilled water (5 mL) in a round-bottomed flask. The mixture was stirred for 24 hours at room temperature, filtered off and dried under vacuum. Afterwards, in order to protect the gold face, this loaded solid was re-suspended in ethanol (8 mL) and treated with 100 µL of 3-mercaptopropionic acid for 1 hour. The solid was isolated by centrifugation, washed twice ethanol and allowed to air dry. Next, 50 mg of this solid was re-suspended in acetonitrile (1.7 mL) and (3-mercaptopropyl)trimethoxysilane (93 µL, 0.5 mmol) was added. The suspension was stirred for 5.5 hours at room temperature and then, 2,2'-dipyridyl disulfide (110 mg, 0.5 mmol) was added to the reaction mixture. After stirring overnight at room temperature, the resulting solid was centrifuged, washed once with acetonitrile and dried under vacuum. Finally, a mixture of this prepared solid and O-(2-Mercaptoethyl)-O'-methyl-hexa(ethylene glycol) (50 µL, 0,14 mmol) were suspended in acetonitrile (3.33 mL) in the presence of an excess of safranin O. The mixture was stirred for overnight and the final capped Janus nanoparticles **S2** were isolated by centrifugation and washed with abundant acetonitrile and dried under vacuum.

7. Preparation of S3

8 mg of **S2** were suspended in 4 mL of 50 mM sodium phosphate buffer at pH 7.5. Then, 2.5 mg of EDC, 2.5 mg of NHS and 1 mg of commercial acetylcholinesterase were added and the suspension was stirred overnight in an ice bath. The solid was isolated by centrifugation and washed several times with cold 50 mM sodium phosphate buffer (pH 7.5). The resulting **S3** was kept wet in refrigerator until use.

8. Preparation of $S3_{DOX}$

For the preparation of $\mathbf{S3_{DOX}}$, the procedure was similar to that used for **S3** but using doxorubicin as the cargo. Briefly, 50 mg of **S1** and 24 mg of doxorubicin were suspended in 3.2 mL of water and stirred overnight. The particles were then collected by filtration and dried at 36 °C overnight. Next, the solid was suspended in 5 mL of ethanol and treated with 70 µL of 3-mercaptopropionic acid for 1 hour, in order to functionalize the Au face. The sample was then collected by centrifugation, washed twice with ethanol and dried at room temperature. Afterwards, the particles were suspended in acetonitrile and further functionalize with (3-mercaptopropyl)trimethoxysilane (93 µL for 5.5 hours at room temperature). Then, 110 mg of 2,2'-dipyridyl disulfide (110 mg, 0.5 mmol) were added and the mixture was stirred overnight. The resulting solid was centrifuged, washed once with ethanol and dried under vacuum overnight. Finally, the prepared solid, 50 µL of O-(2-Mercaptoethyl)-O'-methyl-hexa(ethylene glycol) and 5 mg of doxorubicin were suspended in acetonitrile (3.33 mL). The doxorubicin loaded solid was washed with acetonitrile 4 times and dried overnight. 8 mg of this solid was functionalized with acetylcholinesterase as described above.

9. Characterization

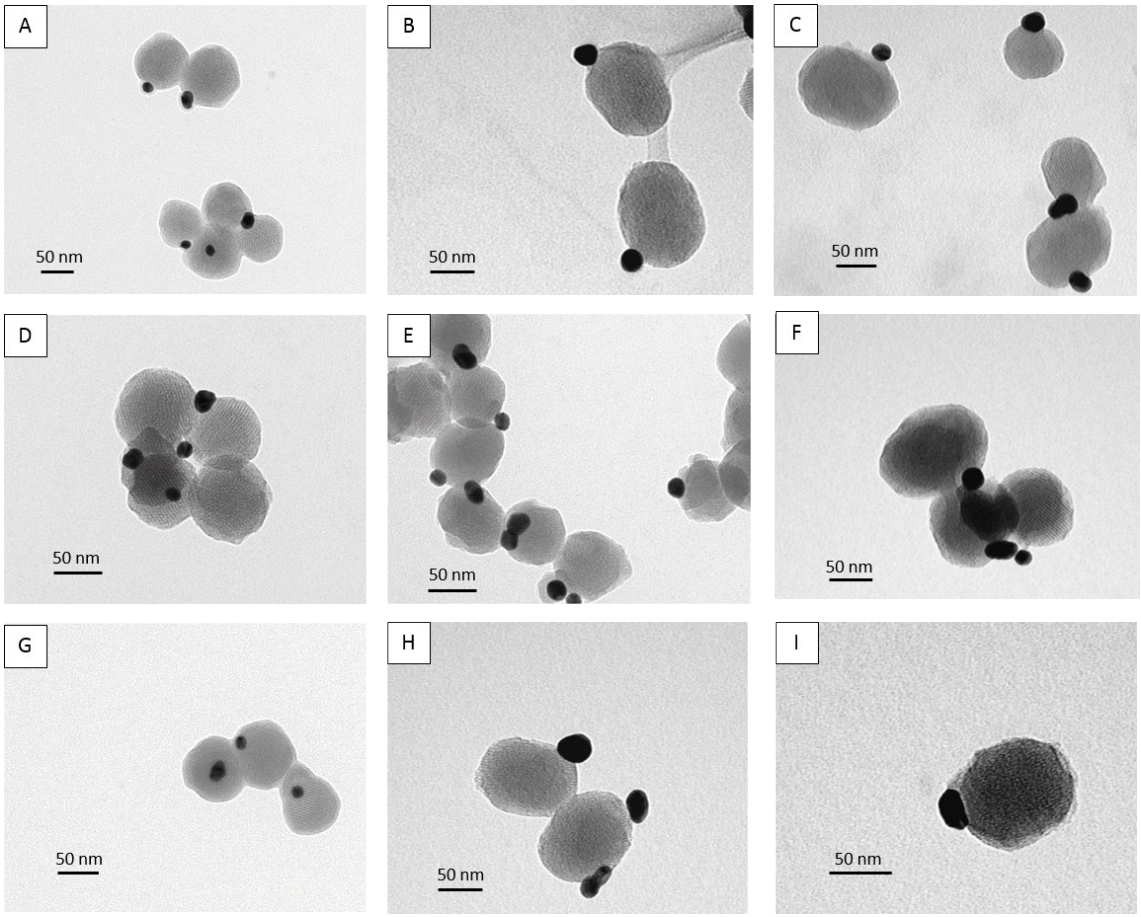

Figure SI-1. Additional TEM images of the Janus Au-mesoporous silica nanoparticles.

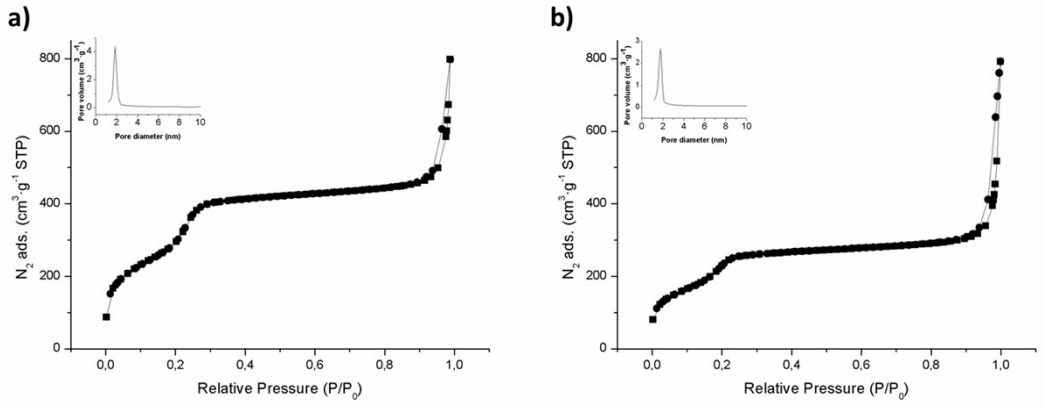

Figure SI-2. The N_2 adsorption-desorption isotherms for (a) the calcined mesoporous material and (b) Janus Au-MS nanoparticles **S1**. Inset: Pore size distribution.

Table SI-1. BET specific surface values, pore volumes and pore sizes calculated from N_2 adsorption-desorption isotherms for selected materials.

Solid	S_{BET} [$m^2\ g^{-1}$]	Pore Volume [$cm^3 g^{-1}$]	Pore size [nm]
Mesoporous nanoparticles	1075.90	0.76	2.30
S1	907.73	0.52	2.28

The N_2 adsorption-desorption isotherms of the calcined mesoporous nanoparticles and Janus nanoparticles **S1** show the typical curve for mesoporous solid; i.e. an adsorption step at intermediate P/P_0 value 0.25 (see Figure SI-2). This step is related to the nitrogen condensation inside the mesopores by capillarity. The absence of a hysteresis loop in this interval and the narrow BJH pore distribution suggest the existence of uniform cylindrical mesopores. The application of the BET model results in a value for the total specific surface of 1075.90 $m^2 \cdot g^{-1}$ for calcined mesoporous nanoparticles and 907.73 $m^2 \cdot g^{-1}$ for **S1**. The pore size was estimated by using the BJH model applied on the adsorption band of the isotherm for $P/P_0 < 0.5$. Pore size for calcined mesoporous nanoparticles and **S1** are 2.30 and 2.28 nm respectively, which are in agreement with the typical values for these materials and can associated to the surfactant generated mesopores. Total pore volume was also calculated according to BJH model. BET specific values, pore volumes and pore sizes calculated from N_2 adsorption-desorption isotherms for selected materials are listed in Table SI-1.

Table SI-2. Elemental analysis for MCM-41, **S1** and **S2**.

Solid	% C	% H	% N	% S
Mesoporous nanoparticles	0.13	0.60	0.14	0.01
S1	3.14	2.32	0.11	0.29
S2	5.22	1.63	0.53	1.52

From elemental analysis measurements, the amount of safranin O loaded in **S2** was estimated to be 52.6 mg·g^{-1}. Besides, the amount of oligo(ethylene glycol) grafted onto the MS face was estimated to be 137.0 mg·g $^{-1}$.

Acetylcholinesterase activity on **S3** was checked by the Ellman's assay.[3] The test is based on the fact that thiols react with Ellman's reagent (5,5'-dithiobis(2-nitrobenzoic acid), DTNB), cleaving the disulfide bond to give 2-nitro-5-thiobenzoate (TNB^{2-}). This TNB^{2-} has a characteristic yellow colour that can be monitored by UV-visible spectrophotometry. Acetylthiocholine is used as the enzyme substrate which when hydrolysed produces the resulting thiocholine as the thiol containing molecule.

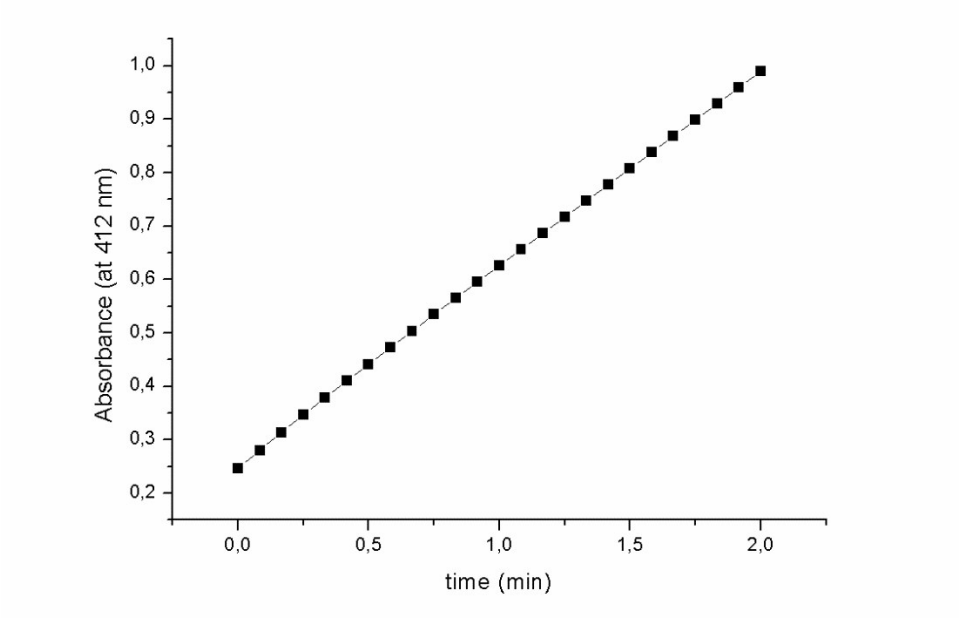

Figure SI-3. Monitoring of TNB^{2-} formation due to acetylcholinesterase activity on **S3**.

In a typical assay, 900 µL of 100 mM sodium phosphate buffer (pH 7.5), 30 µL 10 mM DTNB solution and 12 µL of acetylthiocholine (75 mM) where placed in a quartz cuvette. Then, 6 µL of either buffer (for blank), **S3** (5.3 mg·mL^{-1}) or commercial enzyme (0.1 mg/mL) were added. The mixture was shaken and absorbance at 412 nm was monitored. When **S3** was added, the solution turned yellow quickly as a consequence of TNB^{2-} formation due to the acetylcholinesterase activity. The acetylcholinesterase activity on **S3** was estimated to be 797 U·g^{-1}, by applying the following formula:

$$\frac{Enzyme\ Units}{g} = \frac{(\Delta - \Delta_{blank}) * V_T * F_D}{\varepsilon_{TNB} * l * V_{NPs} * C_{NPs}}$$

Where,

Δ is the slope of the graph (min^{-1})

Δ is the slope of the graph for the blank (min^{-1})

V_T is the total volume in the cuvette

ε_{TNB} is the molar extinction of TNB^{2-} at 412 nm (13,700 $M^{-1} \cdot cm^{-1}$)

L is the optical path in the cuvette (1 cm)

V_{NPs} is the volume of nanoparticles added (mL)

C_{NPs} is the concentration of nanoparticles suspension added (g/mL).

Taking into account that the activity for the commercial acetylcholinesterase was determined to be 217 U/mg, the amount of enzyme immobilized on **S3** can be estimated as 3.51 $mg \cdot g^{-1}$.

TEM-EDX mapping of the gold side of the nanodevice shows that this area was rich in sulphur atoms (see Supporting Information), thus strongly suggesting the preferential localization of the enzyme in the gold face (see Figure SI-4).

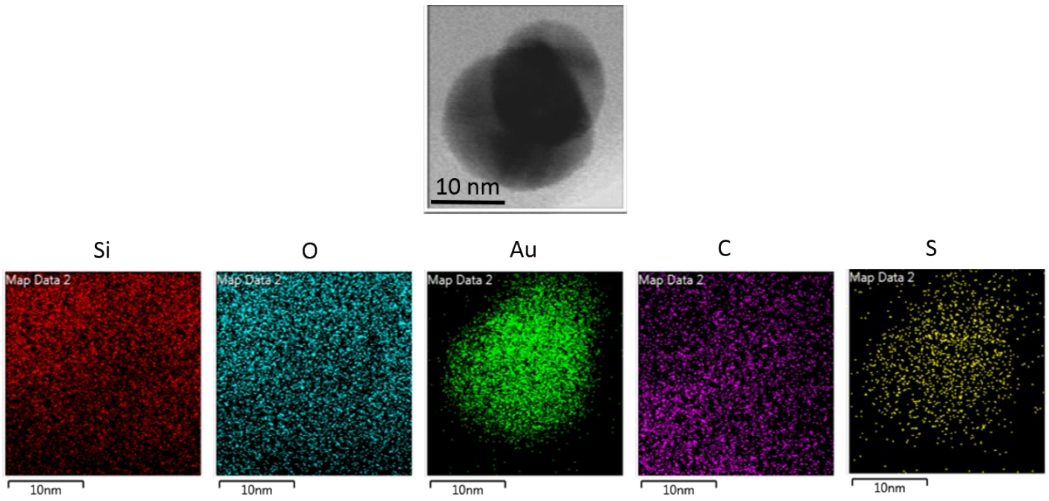

Figure SI-4. TEM-EDX element mapping of the gold region in the final nanodevice **S3**. Top: mapped area. Bottom: mapping of different atoms.

10. Release Experiments

In a typical release experiment, refrigerated solutions of **S3** where aliquoted and suspended in 1.5 mL of aqueous solution (50 mM sodium phosphate buffer, pH 7.5). Nanoparticles were washed and brought to a concentration of 0.9 $mg \cdot mL^{-1}$ for each release experiment. For inhibition experiments, the corresponding amount of inhibitor was added to the suspension. Samples were incubated for 75 min and then 15 µL of acetyltiocholine were added for a total

concentration of 1 mM. Aliquots were taken at scheduled times and centrifuged to remove the nanoparticles. Then, the fluorescence at 580 nm of the safranin O dye released was measured (λ_{exc} = 520 nm). For release experiments in different media, 50 mM sodium phosphate buffer at pH 6.5 and 8.5, phosphate buffered saline 1x (PBS 1x) and simulated body plasma were prepared. PBS 1x consisted of 137 mM NaCl, 1.47 mM KH_2PO_4, 7.85 mM Na_2HPO_4, 2.68 mM KCl (pH 7.5) and simulated body plasma (SBP) consisted of NaCl 137 mM, $NaHCO_3$ 27 mM, KCl 3 mM, $K_2HPO_4 \cdot 3H_2O$ 1 mM, $MgCl_2 \cdot 6H_2O$ 1.5 mM, $CaCl_2$ 2.5 mM, Na_2SO_4 0.5 mM, $NH_2C(CH_2OH)_3$ 50 mM and HCl 45 mM (final pH 7.25).

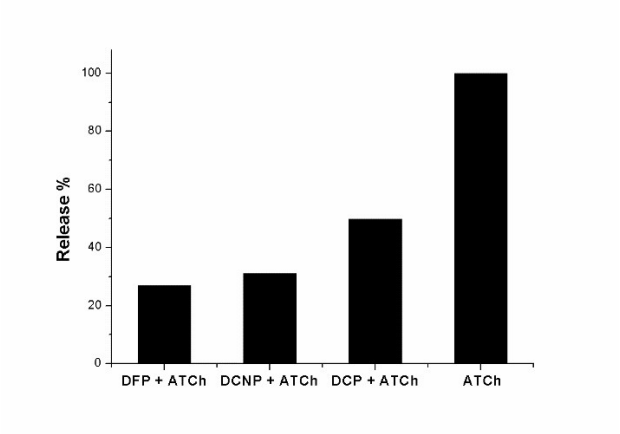

Figure SI-5. Normalized cargo release after 60 min from **S3** in the presence of 1 mM acetylthiocholine (ATCh) determined by measuring safranin O fluorescence at 585 nm (λ_{exc} = 520 nm) in aqueous solution (50 mM sodium phosphate buffer, pH 7.5) previously incubated (75 min, 5 mM) with DFP, DCNP, DCP and without inhibitor.

11. Cell Experiments

Cell culture conditions

HeLa human cervix adenocarcinoma cells were and were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum (FBS). Cells were incubated at 37 °C in an atmosphere of 5% carbon dioxide and 95% air and underwent passage twice a week.

Cell viability studies with S3

To discard any intrinsic toxicity of the nanodevice, the toxicological profile of the safranin O-loaded nanodevice **S3** solid was tested in vitro in Hela cells. For this purpose, Hela cells were seeded in a 96-well plate at 50,000 cells/well and treated at different solid concentrations (0, 25, 50, 100 and 200 μg·mL^{-1} in PBS). Cells were incubated for 24 hours and the viability was determined by the WST-1 cell proliferation assay. Finally, the cell viability was measured at 595 nm in the Wallac Workstation***.*** Three independent experiments containing triplicates were carried out. The results demonstrated that in the presence of **S3** nanoparticles were well-tolerated by HeLa cells at concentrations up to 200 μg·mL^{-1} after 24 hours of exposure.

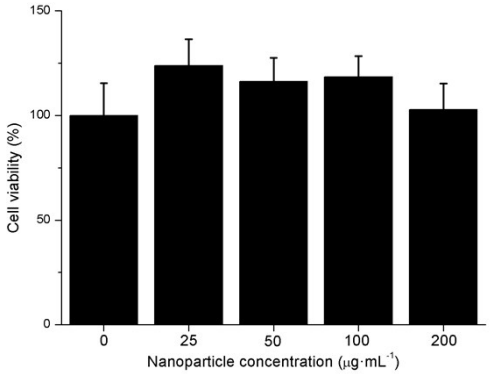

Figure SI-6. Cell viability studies in the presence of **S3** at different concentrations (0, 25, 50, 100 and 200 μg·mL^{-1}) after 24 hours of incubation. WST-1 reagent was added and cell viability was measured. Data are expressed as mean ± σ.

Delivery of doxorubicin in HeLa cells

The performance of the nanodevice **S3$_{DOX}$** to deliver the cytotoxic agent doxorubicin in HeLa cells was tested. For this purpose, Hela cells were seeded in a 24-well plate at 50,000 cells/well and incubated at 37 °C for 24 hours. **S3$_{DOX}$** was added at a concentration of 50 and 100 μg·mL^{-1} and after 30 min of incubation, cells were washed with PBS in order to remove non-internalized nanoparticles and new media was added. The nanoparticles were incubated for 24 hours in the absence or in the presence of 40 mM of acetylthiocholine. Finally, doxorubicin delivery was evaluated by incubation with the cell proliferation WST-1 reagent for 1 hour and measuring the absorbance at 595 nm.

Confocal microscopy studies of controlled release from S3 in HeLa cells

Internalization and cargo delivery of **S3** in HeLa cells was studied by confocal microscopy. For this purpose, Hela cells were seeded over glass coverslips at 300,000 cells/ml in 6-well culture plates and incubated at 37 °C for 24 hours. Then, **S3** was added to HeLa cells at 100 $\mu g \cdot mL^{-1}$ and cells were incubated at 37 °C for 30 min. Afterwards, cells were washed several times with PBS to remove non-internalized nanoparticles and incubated in fresh media for 2 hours in the absence or presence of 40 mM of acetylthiocholine. After 2 hours, cells were washed several times with PBS and DNA marker Hoechst 33342 was added at 2 $\mu g \cdot ml^{-1}$. Finally, slides were visualized using a confocal microscope Leica TCS SP2 AOBS.

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