Supporting Information

Stimulus-Responsive Nanomotors Based on Gated Enzyme-Powered Janus Au-Mesoporous Silica Nanoparticles for Enhanced Cargo Delivery

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1. Chemicals

Tetraethyl orthosilicate (TEOS), *n*-cetyltrimethylammonium bromide (CTABr), sodium hydroxide (NaOH), paraffin wax, (3-mercaptopropyl)trimethoxysilane, hydrogen tetrachloroaurate(III) ($HAuCl_4 \cdot 3H_2O$), sodium citrate tribasic dihydrate, 3-mercaptopropionic acid, tris(2,2'-bipyridyl)dichlororuthenium(II) hexahydrate ($[Ru(bpy)_3]Cl_2 \cdot 6H_2O$), 2,2'-dipyridyl disulfide, *O*-(2-Mercaptoethyl)-*O*'-methyl-hexa(ethylene glycol) (OEGSH), *N*-(3-dimethylaminopropyl)-*N*$^{\prime}$-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), catalase from bovine liver, hydrogen peroxide (30%), *L*-glutathione reduced (GSH), and sodium hydrogen phosphate monohydrate were provided by Sigma-Aldrich. Disodium hydrogen phosphate heptahydrate, methanol, absolute ethanol, chloroform and acetonitrile were provided by Scharlau. For cell culture, Dulbecco's Phosphate Buffered Saline (PBS), Dulbecco's Modified Eagle's Medium (DMEM) - high glucose, and Fetal Bovine Serum (FBS) and Hoechst 33342 were purchased from Sigma-Aldrich. HeLa human cervix adenocarcinoma cells were purchased from the German Resource Centre for Biological Materials (DSMZ).

2. General methods

UV-visible measurements were recorded with a JASCO V-650 Spectrophotometer. Powder X-ray diffraction (PXRD) measurements were carried using a Seifert 3000TT diffractometer using CuK_α radiation. N_2 adsorption-desorption isotherms were recorded using a Micromeritics TriStar II Plus automated analyzer. Transmission electron microscopy (TEM) images were acquired using a JEOL TEM-1010 Electron microscope. Scanning transmission electron microscopy coupled with

energy dispersive x-ray spectroscopy (STEM-EDX) was carried out using a JEM 2100F instrument. Elemental analysis was performed using a LECO CHNS-932 Elemental Analyzer. Dynamic light scattering (DLS) experiments were performed using a ZetaSizer Nano ZS (Malvern). Nanoparticle tracking experiments were performed using a Nanosight NS300 (Malvern). Confocal microscopy imaging was performed with a Leica TCS SP8 AOBS inverted laser scanning confocal microscope.

3. Synthesis of mesoporous silica nanoparticles (MSNPs)

First, the surfactant CTABr (1.00 g, 2.74 mmol) was dissolved in 480 mL of deionized water at 30 °C. Then, the media was basified by addition of 3.5 mL of a NaOH solution (2 M). Next, the temperature was increased to 80 °C and 5 mL of TEOS (22.4 mmol) were added to the stirring solution. The mixture was further stirred for 2 hours at 80 °C. Afterward, the mixture was cooled down at room temperature and the white precipitate was isolated by centrifugation. The resulting solid was washed several times with deionized water and dried in an oven at 70 °C overnight. Finally, the surfactant was removed by calcination at 550 °C in an oxidant atmosphere for 5 hours, which yielded the starting **MSNPs**.

4. Synthesis of gold nanoparticles (AuNPs)

100 mL of $HAuCl_4 \cdot 3H_2O$ aqueous solution (0.0116%) were brought to reflux under magnetic stirring. Then, 1.57 mL of freshly prepared 1% sodium citrate aqueous solution were added to induce the reduction of Au(III) and the formation of 20 nm gold nanoparticles. The initial faint yellow colour turned to blue-black and finally to red wine in a few minutes. The colloidal suspension was let to cool at room temperature. This process was repeated four times, yielding the as-synthesized **AuNPs**.

5. Synthesis of Janus Au-MSNPs (S0)

180 mg of **MSNPs** were dispersed in 9 mL of aqueous solution (6.7% ethanol). Then, 1 µM (final concentration) of CTABr were added. The mixture was stirred and heated in a water bath at 75 °C, followed by addition of 1 g of paraffin wax. Once the paraffin was melted, the mixture was emulsified by vigorously stirring using an Ultra-Turrax T-25 homogenizer (IKA) for 15 minutes. Afterward, the mixture was further stirred for 1 h at 75 °C using a magnetic stirrer. The resulting Pickering emulsion was then cooled to room temperature, mixed with 9 mL of methanol and

treated with 180 µL of (3-mercaptopropyl)trimethoxysilane for 3 hours. After this time, the solid was collected by centrifugation, washed twice with methanol, and dispersed in 68 mL of methanol. This solution containing the **MSNPs** partially functionalized with thiol moieties was added over 400 mL of the as-synthesized **AuNPs**. The mixture was stirred overnight. Afterward, the solid was isolated by filtration and exhaustively washed with ethanol and chloroform in order to remove the paraffin. The solid powder was dried at room temperature and ground. This process finally yielded the Janus Au-MSNPs (**S0**).

6. Synthesis of gated nanoparticles S1

First, 50 mg of **S0** were dispersed in 5 mL of EtOH containing 70 µL of 3-mercaptopropionic acid and magnetically stirred for 1 h at room temperature, in order to functionalize the gold face with carboxylic groups. The solid was collected by centrifugation, washed with ethanol, and dried at room temperature. Next, in order to load the cargo in the pores, the solid was dispersed in an acetonitrile solution (5 mL) containing 25 mg of $[Ru(bpy)_3]Cl_2 \cdot 6H_2O$. The mixture was stirred overnight and the solid was collected by centrifugation and washed with toluene. The resulting solid was redispersed in toluene (1.7 mL) and treated with 97 µL of (3-mercaptopropyl)trimethoxysilane. After 5.5 h, 110 mg of 2,2'-dipyridyl disulfide (0.5 mmol) were added and the mixture was stirred overnight. Afterward, the solid was isolated by centrifugation, washed with toluene and dried under vacuum overnight. Finally, this solid was dispersed in 3.3 mL of toluene and reacted with 50 µL of *O*-(2-Mercaptoethyl)-*O*'-methyl-hexa(ethylene glycol) (OEGSH), which capped the pores by the formation of disulfide bonds via substitution of dipyridyl moieties. The solid was exhaustively washed with toluene, acetonitrile and phosphate buffer and dried at room temperature. This process finally yielded the capped nanoparticles **S1**.

7. Preparation of enzyme-functionalized gated nanoparticles ($S1_{cat}$)

8 mg of **S1** were dispersed in 4 mL of sodium phosphate buffer (50 mM, pH 7.5). Then, 4 mg of EDC, 4 mg of NHS and 2 mg of commercial catalase were added and the suspension was stirred overnight in an ice bath. The solid was isolated by centrifugation and exhaustively washed with buffer. The solid was divided in several aliquots of 4 $mg \cdot mL^{-1}$ that were kept refrigerated until use. This process finally yielded the final nanobots $\mathbf{S1_{cat}}$.

8. Preparation of $S1_{dox\text{-}cat}$

For the preparation of **S1**$_{\text{dox-cat}}$, the procedure was similar to the one used for **S1**$_{\text{cat}}$ but using the drug doxorubicin (dox) as the cargo. Briefly, 50 mg of **S0** were suspended in 5 mL of ethanol and treated with 70 µL of 3-mercaptopropionic acid for 1 hour, in order to functionalize the gold face. The sample was then collected by centrifugation, washed with ethanol and suspended in 5 mL of phosphate buffer containing 25 mg of dox. The mixture was stirred overnight, redispersed in toluene and then 97 µL of (3-mercaptopropyl)trimethoxysilane were added. After 5.5 h, 110 mg of 2,2'-dipyridyl disulfide (0.5 mmol) were added and the mixture was further stirred for 24 h. Finally, the particles were collected by centrifugation, resuspended in 3.3 mL of toluene and capped by addition of 50 µL of *O*-(2-Mercaptoethyl)-*O*'-methyl-hexa(ethylene glycol) (OEGSH). The stirring was kept overnight, followed by centrifugation and washing of the solid with acetonitrile and sodium phosphate buffer (50 mM, pH 7.5). The capped solid (**S1**$_{\text{dox}}$) was dried, and further functionalized with catalase following the same procedure as described above. This process finally yielded the dox-loaded nanobots **S1**$_{\text{dox-cat}}$.

9. Materials characterization

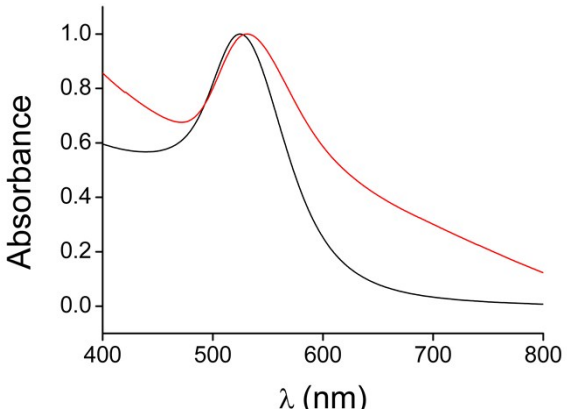

Figure SI-1. Normalized absorbance spectra of the as-synthesized **AuNPs** (black) and Janus particles **S0** (red).

The UV-visible spectra of the as-synthesised Au nanoparticles showed the characteristic absorption band with a maximum at 525 nm, which was red-shifted to 531 for the Janus particles **S0** due to the increase in refractive index around the Au surface induced by silica (Figure SI-1). On the other hand, powder X-ray diffraction (PXRD) at low angles of MSNPs showed a main peak around 2.4° which is characteristic of mesoporous nanoparticles with an MCM-41 type structure (Figure SI-2).[1] The preservation of this characteristic peak in the PXRD patterns of **S0** and **S1**

indicated that the cargo loading and functionalization processes had not damaged the mesoporous scaffolding. Furthermore, PXRD patterns at high angles (35-80°) of **S0** and **S1** showed four main peaks that can be indexed as the (100), (200), (220) and (311) Bragg peaks corresponding to the presence of cubic Au crystals.[2]

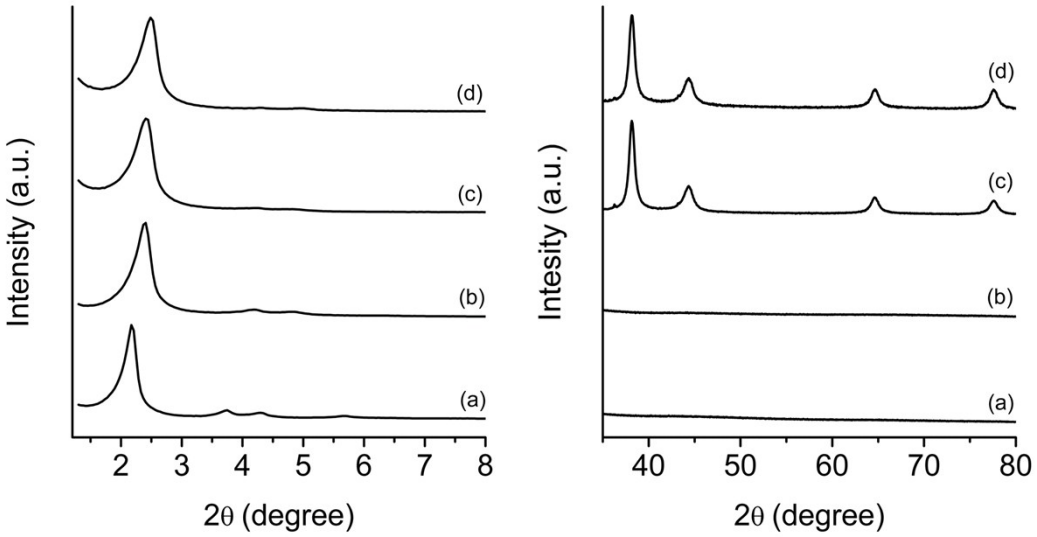

Figure SI-2. PXRD patterns at low angles (left) and at high angles (right) of: (a) as-synthesized **MSNPs**, (b) calcined **MSNPs**, (c) starting Janus Au-MSNPs (**S0**) and (d) gated nanoparticles (**S1**).

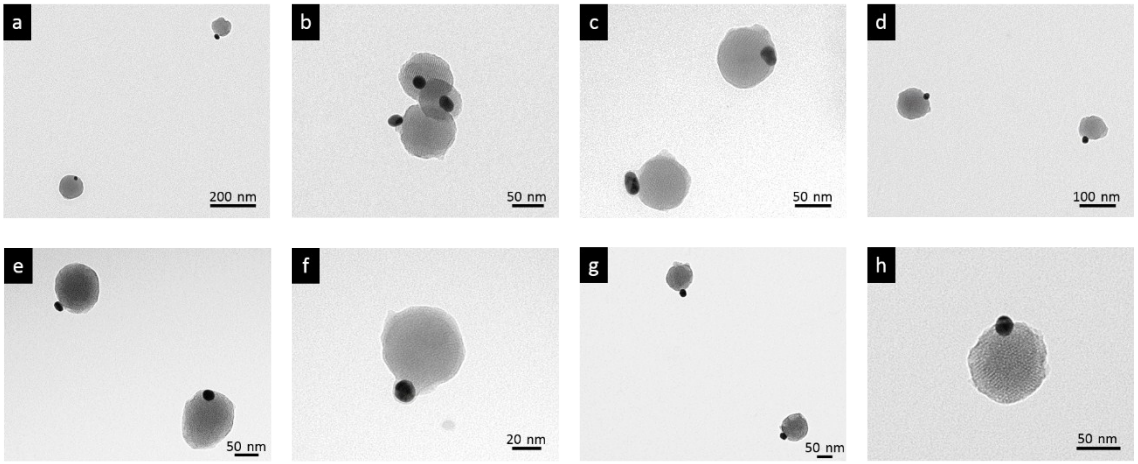

Figure SI-3. Representative TEM images of the Janus nanoparticles.

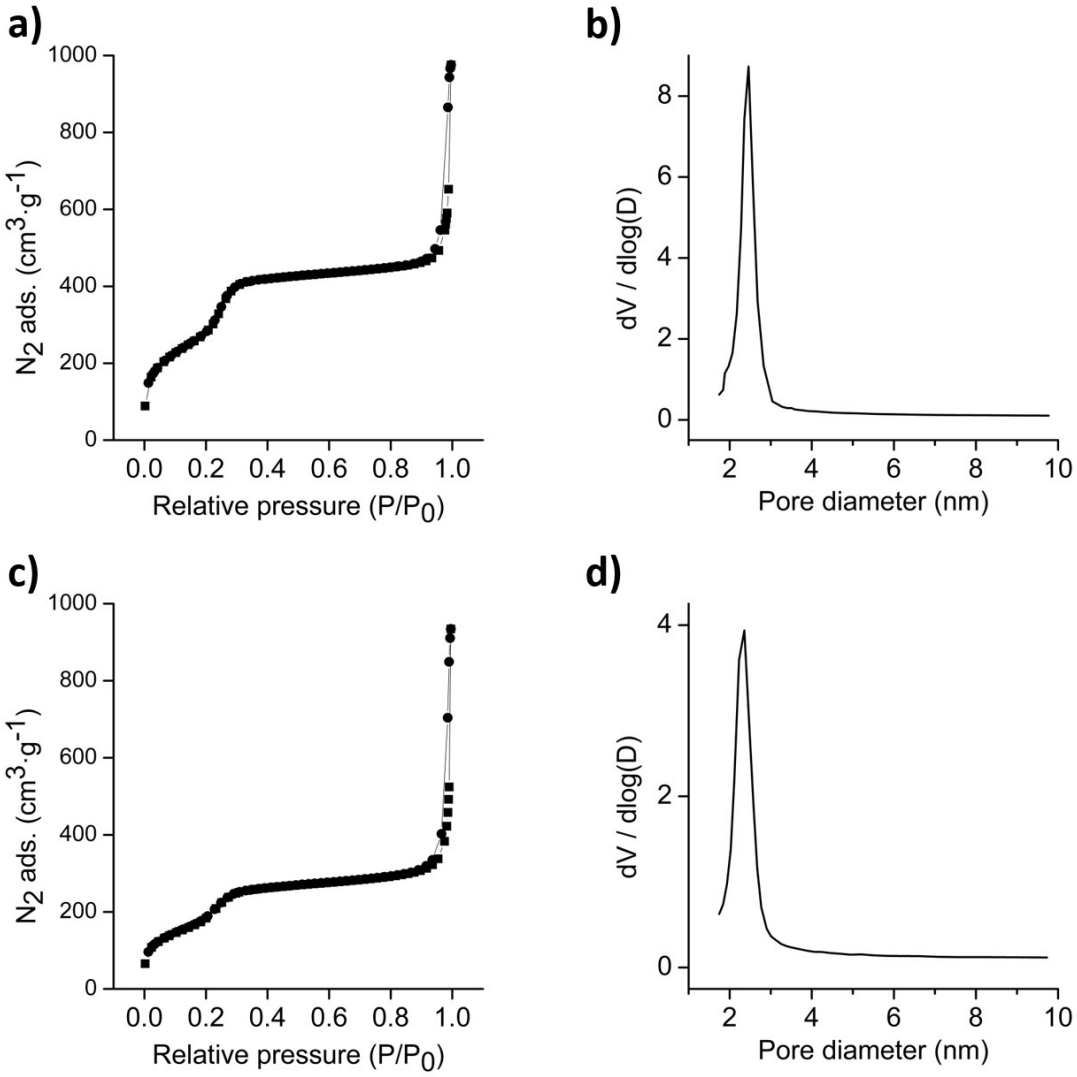

Figure SI-4. a) N_2 adsorption-desorption isotherms for the calcined **MSNPs** and (b) corresponding pore diameter distribution. c) N_2 adsorption-desorption isotherms for the Janus Au-MSNPs (**S0**) and (d) corresponding pore diameter distribution. Pore diameter distributions were determined by applying the BJH model on the adsorption branch of the isotherm for $P/P_0 < 0.8$, which is associated with the surfactant generated mesopores.

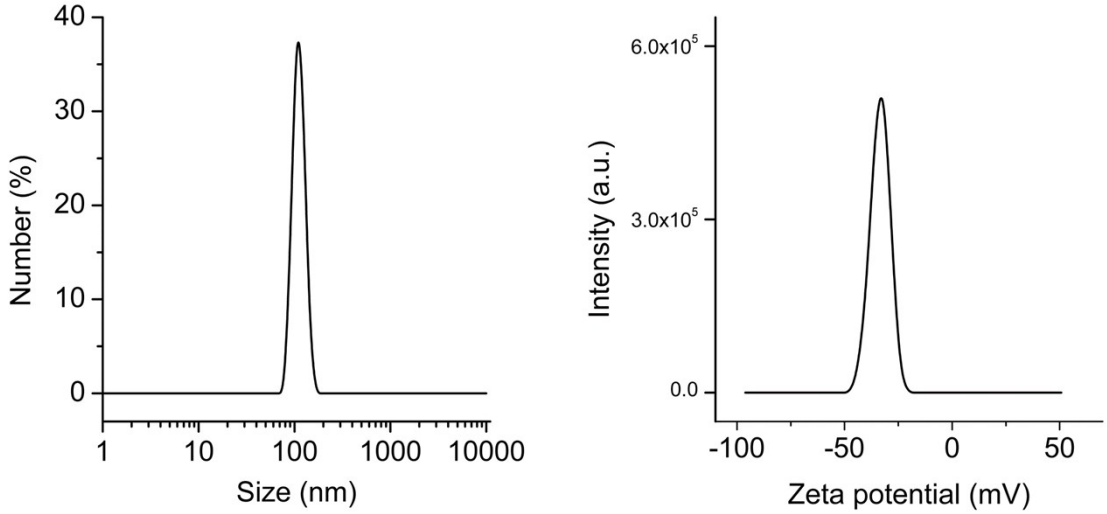

Figure SI-5. Hydrodynamic diameter and zeta potential distributions of the nanodevice (**S1$_{cat}$**) determined by DLS.

10. Catalase activity assay

Catalase activity was measured by adapting a procedure previously reported.[3] Hydrogen peroxide absorbs light at 240 nm. The assay is based on monitoring the transformation of H_2O_2 by catalase which can be followed by the decrease in absorbance at 240 nm due to the following reaction:

$$2\,H_2O_2 \overset{catalase}{\rightarrow} H_2O + 2\,O_2$$

In a typical assay, 850 µL of 50 mM sodium phosphate buffer (pH 7.5) and 100 µL of H_2O_2 0.35% where placed in a quartz cuvette. Then, the mixture was completed with either buffer (10 µL, for blank) or stock of nanoparticles (10 µL, 0.5 mg·mL^{-1}). The mixture was shaken and the absorbance at 240 nm was monitored during one minute in intervals of 5 seconds. Whereas a flat line was observed for the blank and **S1** nanoparticles, **S1$_{cat}$** nanobots shown a linear variation (decrease) in H_2O_2 absorbance which confirmed their catalase-activity (see Figure 2A). From the slope of the graph (absorbance *vs.* time, Figure 2A), the catalase activity was determined by applying Supplementary Equation 1.

Supplementary Equation 1:

$$\frac{Enzyme\ Units}{g} = \frac{(\Delta - \Delta_{blank}) * V_T * F_D}{\varepsilon_{H_2O_2} * l * V_{NPs} * C_{NPs}}$$

Where,

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

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

V_T is the total volume in the cuvette

ε_{H2O2} is the molar extinction of H_2O_2 at 240 nm (0.0435 $\mu M^{-1} \cdot cm^{-1}$)

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 the nanoparticle stock solution added ($g \cdot mL^{-1}$).

From this formula, the activity of immobilized catalase on **$S1_{cat}$** was calculated as 152 U per g of nanoparticles. Additionally, the corresponding activity for the commercial enzyme was calculated to be 10138 $U \cdot mg^{-1}$, which corresponds to 15 mg of native enzyme per g of nanoparticles.

11. Motion analysis

As previously described,[4] we used Nanoparticle Tracking Analysis (NTA) for evaluating the motion of the nanoparticles. These experiments were performed on a Nanosight NS300 instrument equipped with an sCMOS camera. This camera is mounted on an optical microscope in order to track the light scattered by the particles that are present in the focus of the beam generated by a single mode laser diode with a 55 mW blue light illumination. Samples were diluted in PBS buffer (1X) at 0.02 $mg \cdot mL^{-1}$, passed through PTFE 0.22 µm syringe filters, and introduced into the Nanosight chamber at 25 °C using a 1 mL syringe. The Brownian motion of the nanoparticles was tracked with 25 frames $\cdot s^{-1}$. NTA 3.0 software was used to track the particles which gives the x, y coordinates of each particle as a function of time and allows for the calculation of their mean square displacement (MSD) by applying Supplementary Equation 2.

Supplementary Equation 2:

$$MSD = \langle (r)^2 \rangle = \frac{1}{N} \sum_{i=0}^{N} (r_i(t) - r_i(0))^2$$

The apparent diffusion coefficient can be extracted by the linear fitting of MSD *vs.* Δt by applying the well-known equation MSD = 2 d · *D* · t (where d = dimensionality (2 in the case of 2-dimensional videos).

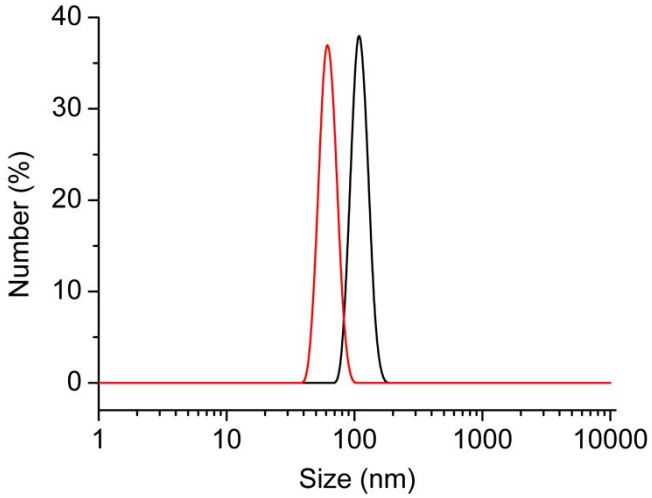

Figure SI-6. Apparent hydrodynamic size distribution of nanobots (**S1$_{cat}$**) before (black) and after the addition (red) of fuel (0.3% v/v H_2O_2) as measured by DLS.

13. Release experiments

For release experiments, refrigerated stocks of **S1$_{cat}$** were diluted with sodium phosphate buffer (10 mM, pH 7.5), washed a couple of times, and divided into fractions of 0.5 mg. Then 1 mL of phosphate buffer (10 mM, pH 7.5) containing the corresponding amount of glutathione and H_2O_2 was added. Release experiments were conducted at room temperature. At scheduled times, aliquots of 150 µL were taken with a pipette, centrifuged to remove the nanoparticles (12000 rpm, 2 min) and the supernatant absorbance was measured at 453 nm. Three independent experiments were carried out. The amount of released $[Ru(bpy)_3]Cl_2$ dye was determined by applying Beer-Lambert's law (Supplementary Equation 3).

Supplementary Equation 3:

$$Absorbance\ (453\ nm) = \varepsilon_{453} \cdot L \cdot C$$

Where ε_{453} is the molar extinction of $[Ru(bpy)_3]Cl_2$ at 453 nm (14600 M^{-1} cm^{-1}), L is the optical path of the cuvette (1 cm), and C is the concentration of $[Ru(bpy)_3]Cl_2$.

Release experiments using nanoparticles **S1** (without the catalase) were also carried out. These results are depicted in Figure SI-7 and SI-8.

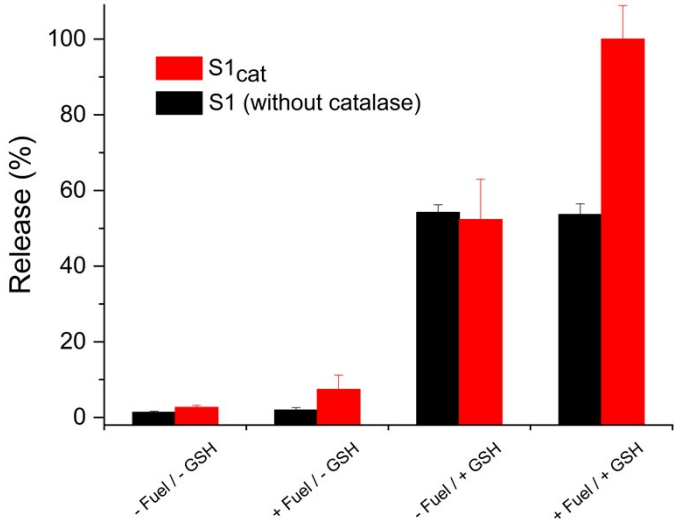

Figure SI-7. Cargo release from nanoparticles **S1** (without the catalase) and nanobots **S1$_{cat}$** in phosphate buffer (10 mM, pH 7.5) after 60 min under different conditions: (i) control (no fuel and no GSH), (ii) presence of H_2O_2 (0.3%), (iii) presence of GSH (10 mM), and (iv) presence of GSH (10 mM) and H_2O_2 (0.3%). Error bars represent s. d. from three independent experiments.

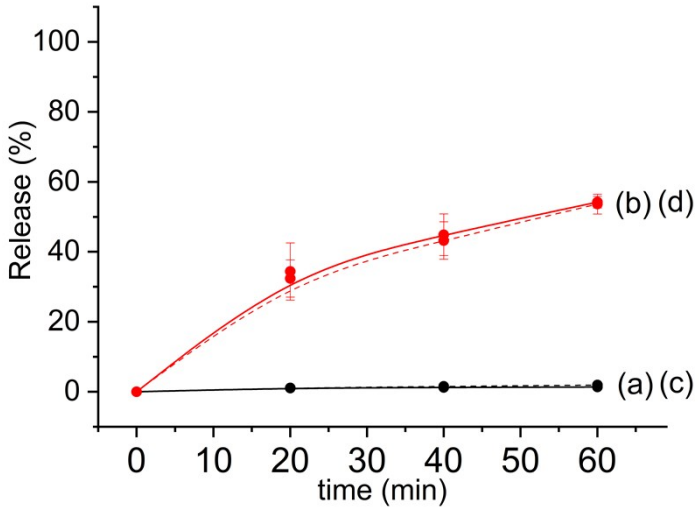

Figure SI-8. Cargo release from nanoparticles **S1** (without the catalase) as a function of time in phosphate buffer (pH 7.5) under different conditions: (a, continuous line) control (no fuel and no GSH), (b, continuous line) presence of GSH (10 mM) and no fuel, (c, dashed line) presence of fuel (H_2O_2 0.3%) and no SGH, and (d, dashed line) presence of GSH (10 mM) and fuel (H_2O_2 0.3%). Error bars represent s. d. from three independent experiments.

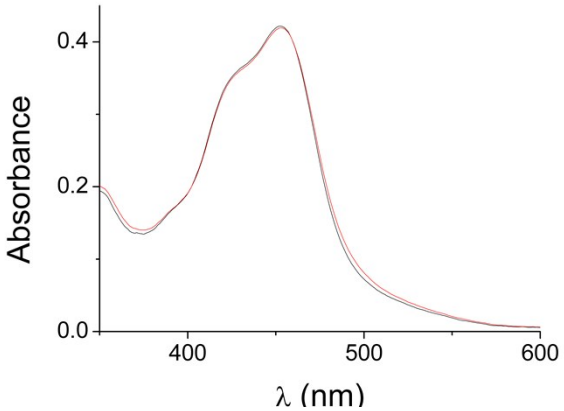

Figure SI-9. Absorbance spectra of the $[Ru(bpy)_3]Cl_2$ dye (30 µM) in phosphate buffer (10 mM, pH 7.5) in the absence (black curve) and in the presence of H_2O_2 (0.3%) (red curve) after 60 min of incubation.

14. Experiments with HeLa cells

Cell culture conditions

HeLa human cervix adenocarcinoma cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal 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.

Confocal microscopy studies of doxorubicin controlled release in HeLa cells

Internalization and cargo delivery of $\mathbf{S1_{dox\text{-}cat}}$ in HeLa cells was studied by confocal microscopy. For this purpose, Hela cells were seeded over glass coverslips at 300.000 cells·mL^{-1} in 6-well culture plates and incubated at 37 °C for 24 h. Then, $\mathbf{S1_{dox\text{-}cat}}$ was added to HeLa cells at 50 µg·mL^{-1} and incubated at 37 °C for 1 and 6 h in the absence or presence of H_2O_2 (0.2 mg·mL^{-1}). Afterwards, cells were washed several times with PBS to remove non-internalized nanoparticles and stained with DNA marker Hoechst 33342 (2 µg·ml^{-1}). Finally, slides were visualized using a Leica TCS SP2 AOBS confocal microscope. ImageJ software was used for fluorescence quantification. Two independent experiments were carried out, which gave similar results. A control experiment using $\mathbf{S1_{dox}}$ (without the catalase) was also carried out.

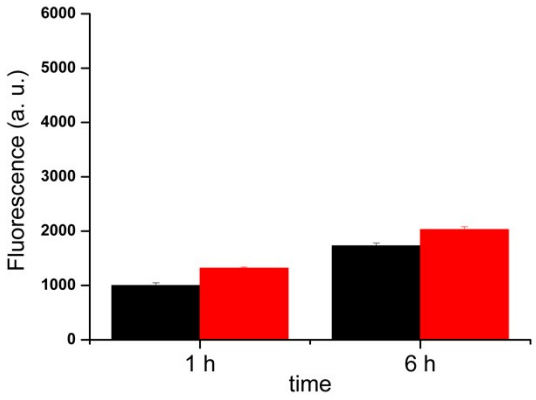

Figure SI-10. Quantification of dox-fluorescence in cells incubated with **S1$_{dox}$** (without catalase) in the absence (black) and in the presence of H_2O_2 (red), using the *ImageJ* software.

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[1] C. T. Kresge, W. J. Roth, *Chem. Soc. Rev.*, 2013, **42**, 3663-3670.

[2] D.V. Leff, L. Brandt, J.R. Heath, *Langmuir*, 1996, **12**, 4723-4730.

[3] (a) R. F. Jr., Beers, I. W. Sizer, *J. Biol. Chem.*, 1952, **195**, 133-140; (b) K. G. Stern, *J. Biol. Chem.*, 1937, **121**, 561-572; (c) Sigma-Aldrich (2018). Enzymatic Assay of Catalase. http://www.sigmaaldrich.com/technical-documents/protocols/biology/enzymatic-assay-of-catalase.html (accessed 30 October 2018).

[4] (a) D. A. Wilson, R. J. M. Nolte, J. C. M. van Hest, *Nat. Chem.*, 2012, **4**, 268-274; (b) Y. Tu, F. Peng, X. Sui, Y. Men, P. B. White, J. C. M. van Hest, D. A. Wilson, *Nat. Chem.*, 2017, **9**, 480-486.