Electronic Supplementary Information

Enzyme-controlled Janus nanomachine for on-command dual and sequential release

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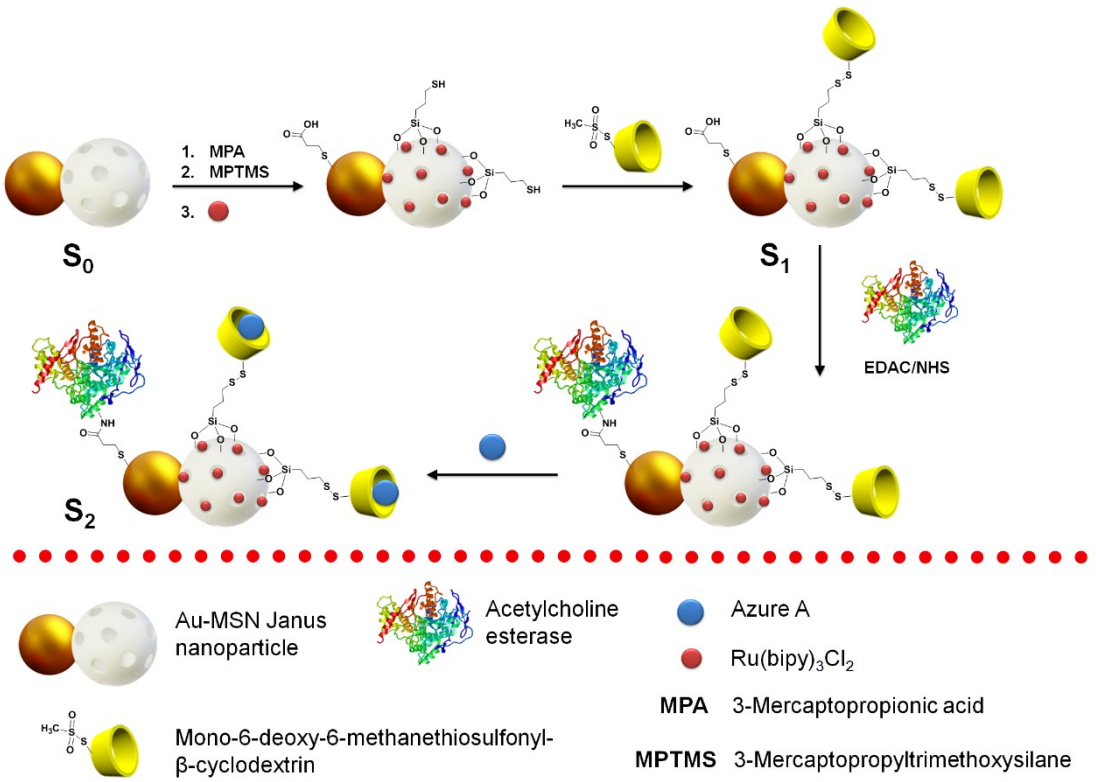
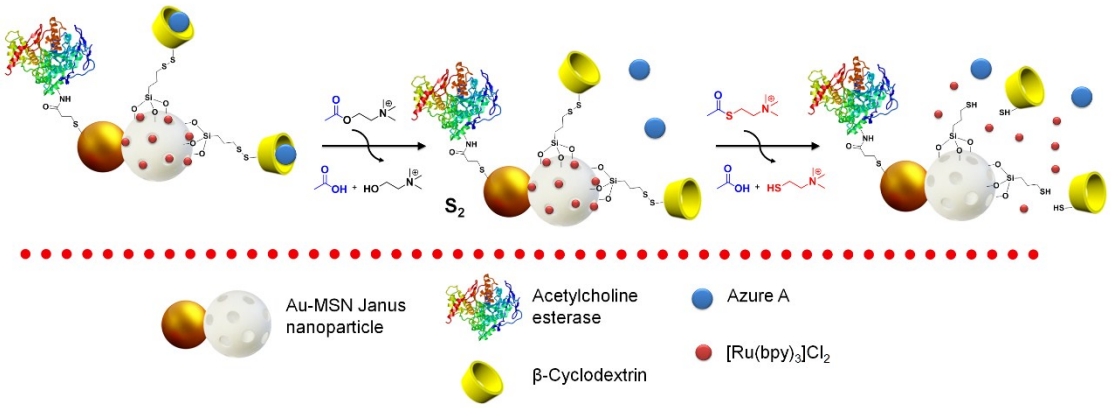


Scheme 1S. Schematic display of the processes involved in the preparation of the acetylcholine esterase-controlled nanomachine $\mathbf{S_2}$.



Scheme 2S. Performance of the enzyme-controlled Janus nanomachine for sequential delivery.

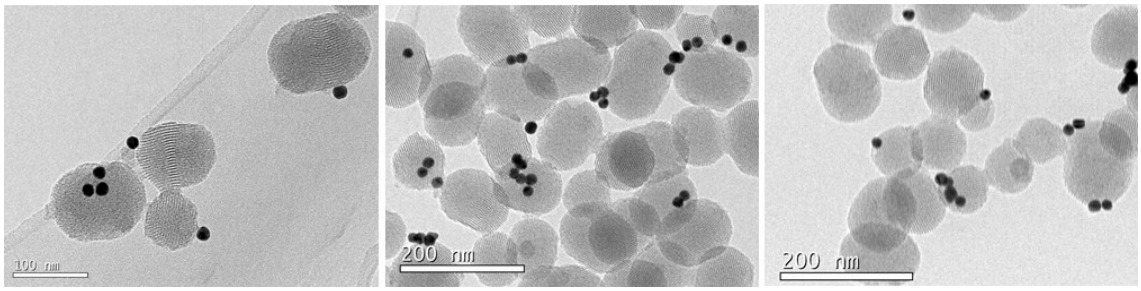


Figure 1S. Representative TEM images of solid $\mathbf{S_1}$.

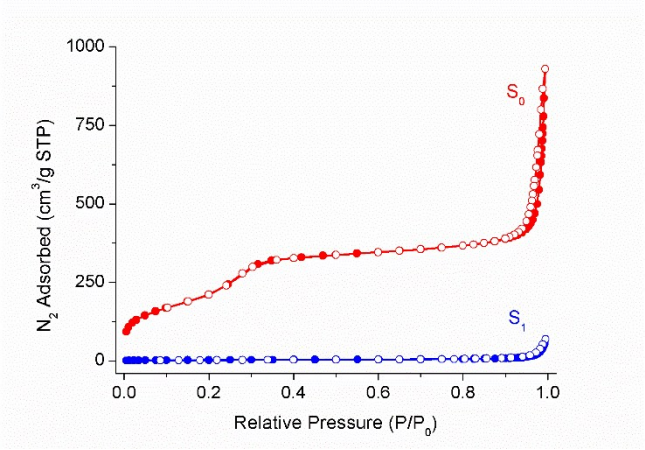


Figure 2S. Nitrogen adsorption (closed)/desorption (open) isotherms for solids $\mathbf{S_0}$ and $\mathbf{S_1}$.

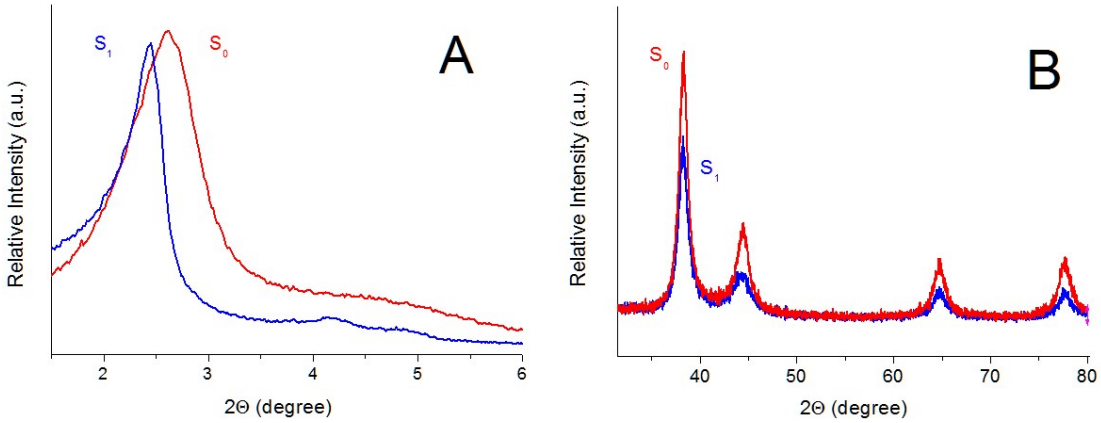


Figure 3S. Powder X-ray diffraction analysis for solids $\mathbf{S_0}$ and $\mathbf{S_1}$ at low (A) and high (B) diffraction angles.

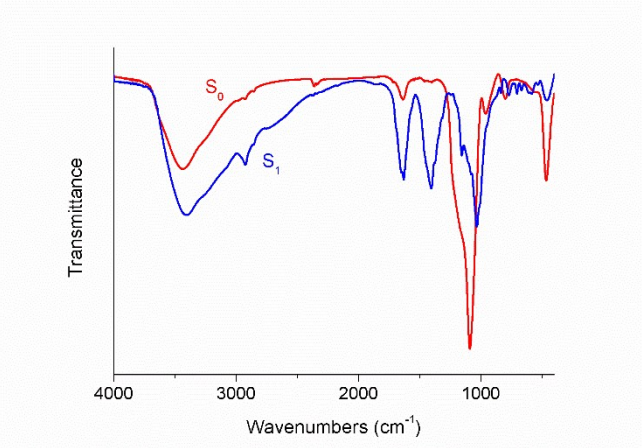


Figure 4S. FT-IR analysis for solids $\mathbf{S_0}$ and $\mathbf{S_1}$.

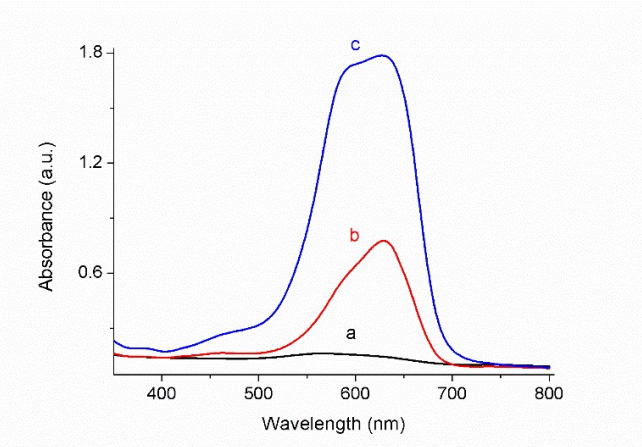


Figure 5S. UV/VIS spectra for $\mathbf{S_2}$ dispersion in 20 mM Na_2SO_4, pH 7.5 after 1 h incubation in the absence (a) and the presence of 150 mM acetylcholine (b) or acetylthiocholine (c).

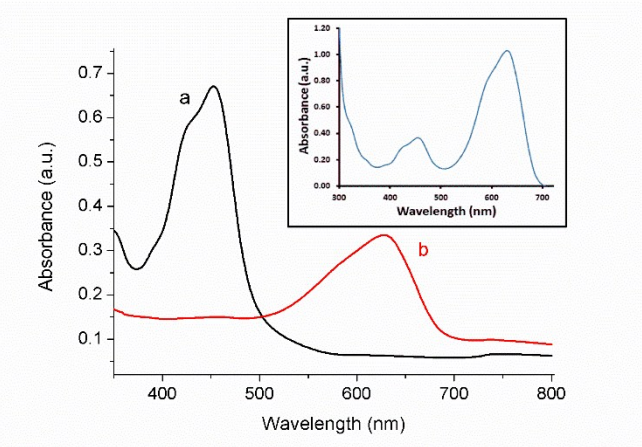


Figure 6S. UV/VIS spectra for 47 µM $Ru(bipy)_3Cl_2$ (a) and 5 µM Azure A (b) solutions in 20 mM Na_2SO_4, pH 7.5. Inset: UV/Vis spectrum for a mixture of 21 µM $Ru(bipy)_3Cl_2$ and 17 µM Azure A (b) in 20 mM Na_2SO_4, pH 7.5.

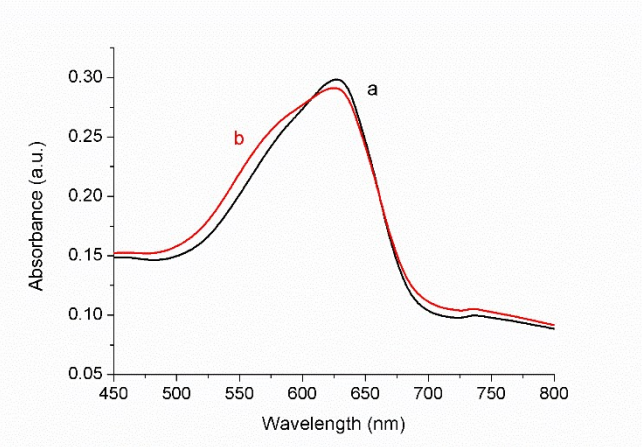


Figure 7S. VIS spectra for 5 µM Azure A solution in 20 mM Na_2SO_4, pH 7.5 before (a) and after addition of CD at 100 µM (b)

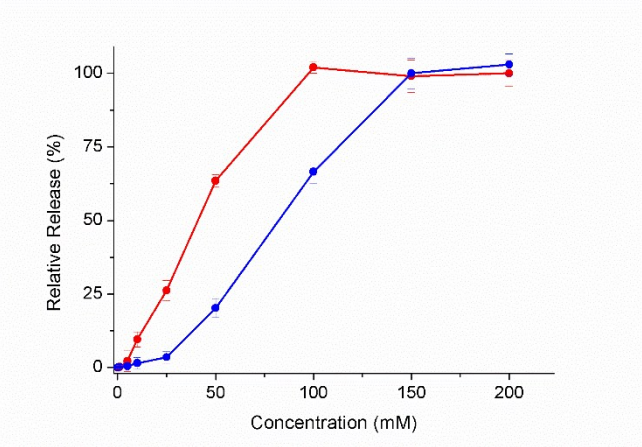


Figure 8S. Relative release of Azure A (blue) and $Ru(bipy)_3Cl_2$ (red) from **S_2** in 20 mM Na_2SO_4, pH 7.5 in the presence of 150 mM acetylcholine and 150 mM acetylthiocholine, respectively.

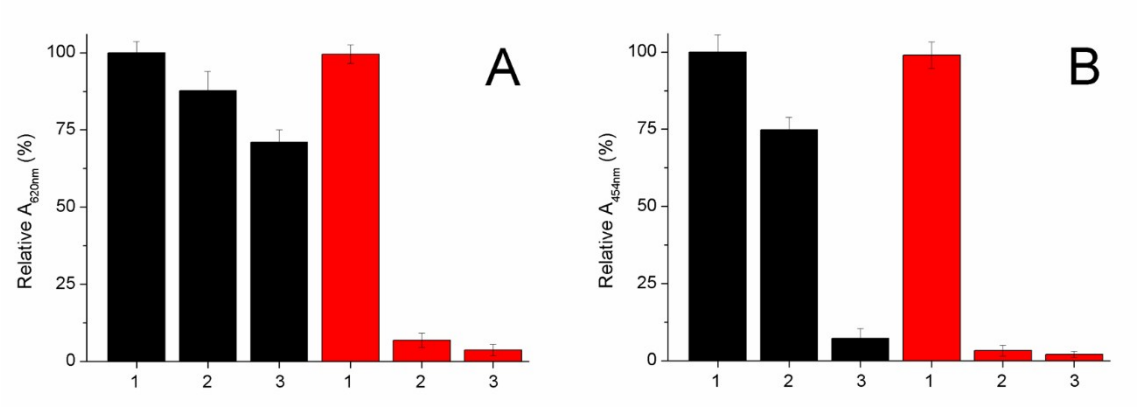


Figure 9S. Relative release of Azure A (A) and $Ru(bipy)_3Cl_2$ (B) from **S_2** (black) and thermal inactivated **S_2** (red) in 20 mM Na_2SO_4, pH 7.5 in the presence of 150 mM N-acetyl cysteine (1) acetylthiocholine (2) and acetylcholine (3).

1. Experimental Part

1.1. Chemicals

Tetraethoxysilane, cetyltrimethylammonium bromide, β-cyclodextrin, $NaSSO_2CH_3$, $HAuCl_4$, (3-mercaptopropyl) trimethoxysilane, 3-mercaptopropionic acid, tris(2,2′-bipyridyl)dichlororuthenium(II) hexahydrate, Azure A hydrochloride, acetylthiocholine chloride, acetylcholine chloride, acetylcholine esterase, 5,5′-dithiobis(2-nitrobenzoic acid) and p-toluenesulfonyl chloride were purchased from Sigma-Aldrich. Solvents were provided by Scharlau. All other reagents were of analytical grade.

1.2. Instruments and general techniques

Transmission electron microscopy (TEM) measurements were performed with a JEOL JEM-2100 microscope. Spectrophotometric measurements were performed using an Ultrospec 8000 UV/VIS spectrophotometer. Powder X-ray diffraction was performed with an X'Pert MRD diffractometer. Nitrogen adsorption/desorption isotherms and pore size distributions were determined with a Micromeritics ASAP 2020 automated analyzer. FT-IR spectra were obtained from KBr discs by using a Nicolet Nexus 670/870 spectrometer. ^{1}H-NMR analysis was performed with a Bruker AV 500MHz instrument. Acetylcholine esterase activity was determined by using acetylthiocholine as substrate in 10 mM sodium carbonate buffer, pH 10 at 25ºC.[1] The enzymatic product was detected at 412 nm after reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman reagent). One unit was defined as the amount of enzyme able to release 1.0 μmol thiocholine per minute under the cited conditions.

1.3. Synthesis of mono-6-deoxy-6-methanethiosulfonyl-β-cyclodextrin

Mono-6-iodo-6-deoxy-β-cyclodextrin was first synthesized as previously described.[2] To synthesize the mono-6-deoxy-6-methanethiosulfonyl-β-cyclodextrin derivative, a previous procedure was adapted.[3] To a solution of 0.5 g mono-6-iodo-6-deoxy-β-cyclodextrin in 5 mL DMF was added 72 mg $NaSSO_2CH_3$ and the mixture was stirred for 24 h at 50°C under reflux and N_2 atmosphere. The reaction mixture was concentrated in a vacuum to obtain a syrup-like mass, and 10 mL EtOH was added. The resulting solid was filtered, exhaustively washed with EtOH and dry under vacuum. Yield: 0.41 g (0.33 mmol). ^{1}H-RMN (DMSO-d6, 500 MHz): d = 2.42 (s, 3H), 2.95 (s, 3H), 3.20-3.67 (m, 40H), 4.16-4.20 (m, 1H), 4.32 (d, 1H), 4.37-4.39 (m, 1H), 4.45-4.48 (m, 2H), 4.52 (m, 3H), 4.77 (d, 2H), 4.84 (m, 5H), 5.64-5.85 (m, 14H). IR (KBr): 3527, 3360, 1651, 1020 cm^{-1}.

1.4. Preparation of solid S_0.[4]

Mesoporous silica nanoparticles (MSN) were prepared according to our previously adapted protocol.[4] Cetyltrimethylammonium bromide (1.0 g) was placed in a 1.0 L three-neck round-bottom flask, and 480 mL of double-distilled water were added. The surfactant was dissolved under sonication, and then 3.5 mL of 2.0 mol/L NaOH solution were added. The temperature of the mixture was adjusted to 80ºC and 5.0 mL tetraethoxysilane were added dropwise to the solution within 5 min under vigorous magnetic stirring. The mixture was allowed to react for 2 h. The resulting white solid was filtered, washed with water and methanol, and then dried in desiccator. To remove the surfactant template, the solid **S_0** was finally calcined at 550 ºC for 5 h.

In parallel, Au nanoparticles (20 nm) were prepared according to the Frens method[5] by heating 100 mL of 0.3 mM $HAuCl_4$ solution to boiling. Then, 5 mL of a 39 mM trisodium citrate solution were added and the mixture was heated for 10 min and further cooled to room temperature. This procedure was repeated four times to prepare the volume of Au nanoparticles required.

Au-MSN Janus nanoparticles were prepared according to our previously reported method.[4] Briefly, 200 mg MSN were dispersed in 10 mL of 1.0 μM of cetyltrimethylammonium bromide in 6.7% ethanol aqueous solution, and the mixture was heated at 75ºC. One gram of paraffin wax was added and the mixture was kept at 75ºC until the paraffin wax was melted. The mixture was vigorously stirred at 25000 rpm for 10 min using an Ultra Turrax T-18 homogenizer, and then stirred for 1 h at 4000 rpm and 75ºC using a magnetic stirrer. The resulting Pickering emulsion was then cooled to room temperature, mixed with 10 mL methanol and treated with 200 μL of (3-mercaptopropyl)trimethoxysilane. After 3 h under magnetic stirring, the silanized emulsion was filtered off, three-times washed with methanol and further dispersed in 400 mL of Au nanoparticles aqueous solutions. The mixture was stirred overnight, then filtered and washed two-times with ethanol, three-times with chloroform and dried overnight at 70ºC. The resulting solid **S_0** was exhaustively washed with ultrapure water, dried and kept in desiccators until use. Au-MSN Janus nanoparticles were prepared in good yield (56%), as revealed by TEM analysis. Au:MSN ratio in Janus nanoparticles

was estimated as 38%, 27%, 19% and 16% for 1:1, 2:1, 3:1 and +3:1 morphology, respectively.

1.5. Preparation of S_1.[4]

Solid $\mathbf{S_0}$ (100 mg) were dispersed in 5.0 mL MeOH and 100 µL 3-mercaptopropionic acid were added. The mixture was stirred for 1 h, then centrifuged and the solid was repeatedly washed with MeOH. The modified nanoparticle was further dispersed in 5.0 mL MeOH and mixed with 100 µL 3-mercaptopropyltrimethoxysilane. The mixture was stirred for 3 h at room temperature, then centrifuged and the solid was repeatedly washed with MeOH and finally dried in desiccator.

The modified nanoparticle (100 mg) and 60 mg of tris(2,2´-bipyridyl)ruthenium(II) chloride hexahydrate were suspended in 20 mL of anhydrous acetonitrile inside a round-bottom flask connected to a Dean-Stark trap under Ar atmosphere. The suspension was refluxed at 110 °C in azeotropic distillation, collecting about 4 mL in the trap in order to remove the adsorbed water. The mixture was stirred for 24 h at room temperature to load the dye into the MSN face pores. The resulting orange solid was filtered off, washed two times with 30 mL acetonitrile, and dried under Ar atmosphere.

Solid $\mathbf{S_1}$ was prepared by dispersing 50 mg of the dye-loaded solid in 5 mL of anhydrous toluene containing 100 mg t-BuOK. The suspension was stirred during 10 min, centrifuged and the solid was washed three times with anhydrous toluene. The solid was then dispersed in 3.0 mL DMF and mixed with 3.0 mL DMF containing 100 mg of mono-6-deoxy-6-methanethiosulfonyl-β-cyclodextrin. The mixture was stirred overnight, filtered off and sequentially washed with DMF, DMF:acetonitrile (1:1), acetonitrile and 100 mM sodium phosphate buffer, pH 7.0 until a clear solution is obtained. The solid $\mathbf{S_1}$ was then washed with ultrapure water, dried and kept in desiccators until use.

Tris(2,2´-bipyridyl)ruthenium(II) content was quantified by incubating 5 mg of solid $\mathbf{S_1}$ in 2 mL of 1 M NaOH during 1 h. The resulting solution was centrifuged and the absorbance at 454 nm was measured. In parallel, solutions of different concentrations of tris(2,2´-bipyridyl)ruthenium(II) in 1 M NaOH were treated under the same conditions to further construct a calibration plot for quantitative determination of the dye. All determinations were performed by triplicate.

1.6. Preparation of S_2.[4]

To prepare the solid $\mathbf{S_2}$, 20 mg of solid $\mathbf{S_1}$ were dispersed in 2.0 mL of cold 100 mM sodium phosphate buffer, pH 7.0 containing 10 mg 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) and 10 mg N-hydroxysuccinimide (NHS). The mixture was stirred for 1 h at 4ºC and then 150 µg acetylcholine esterase were added. The mixture was stirred overnight at 4ºC, centrifuged and the solid was repeatedly washed with cold sodium phosphate buffer. The solid was then suspended in 5.0 mL of cold 10 mM sodium phosphate buffer, pH 10 and 2.0 mg Azure A hydrochloride were added. The

mixture was stirred overnight at 4ºC in dark conditions, then centrifuged and exhaustively washed with cold sodium carbonate buffer until a clear solution is obtained. Solid $\mathbf{S_2}$ was then dried and kept in refrigerator until use. The Azure A content was determined by sequential incubation of 5 mg of solid $\mathbf{S_1}$ in three portions of 1 mL of 1 M HCl, and further measurement of absorbance at 620 nm in the resulting extracted solution. Dye was quantified by using a proper calibration plot. All determinations were performed by triplicate.

1.7. Release assays.

In a typical release assay, 5 mg $\mathbf{S_2}$ were suspended in 5 mL of 20 mM Na_2SO_4 solution at pH 7.5 and shaken over time at 25ºC. After 60 min incubation, the enzyme substrates acetylcholine or acetylthiocholine were added to a final concentration of 150 mM. Aliquots were taken at scheduled times, centrifuged and the absorbance at 454 nm and 620 nm was measured to detect the released tris(2,2′-bipyridyl)dichlororuthenium(II) and Azure A, respectively. As control experiments, $\mathbf{S_2}$ samples were suspended in similar buffer solution without addition of triggers. Released dyes were quantified by using 14335 M^{-1} cm^{-1} and 57500 M^{-1} cm^{-1} and as extinction coefficients for tris(2,2′-bipyridyl)dichlororuthenium(II) and Azure A, respectively.[6,7] For control experiments, thermal inactivated samples were prepared by boiling a 1 mg/mL dispersion of solid $\mathbf{S_2}$ during 15 min, and further washing until a clear solution is obtained.

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