Supplementary Information

Acetylcholine-responsive cargo delivery using acetylcholinesterase-capped nanomaterials

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1. Chemicals

Tetraethyl orthosilicate (TEOS), n-cetyltrimethylammonium bromide (CTABr), sodium hydroxide (NaOH), tris(2,2'-bipyridyl)dichlororuthenium(II) hexahydrate ([Ru(bpy)₃]Cl₂), (3-glycidoxypropyl)trimethoxysilane, 3-aminophenyl boronic acid, acetylcholine esterase from Electrophorus electricus, acetylcholine chloride, dopamine hydrochloride, serotonin hydrochloride, glycine, aspartic acid, glutamic acid, γ-aminobutyric acid, L-norepinephrine hydrochloride, epinephrine hydrochloride, acetylthiocholine iodide, 5,5′-dithiobis(2-nitrobenzoic acid and methyl red were purchased from Sigma-Aldrich and used without purification. Sodium dihydrogen phosphate monohydrate, disodium hydrogen phosphate heptahydrate, sodium sulfate anhydrous and solvents were provided by Scharlau. Bradford dye reagent was provided by Bio-Rad. Deuterium oxide (99.8%) was acquired from Thermo Fisher Scientific. Doxorubicin hydrochloride was purchased from CarboSynth. For cell experiments, HeLa human cervix adenocarcinoma cells were purchased from the German Resource Centre for Biological Materials (DSMZ). Dulbecco’s Phosphate Buffered Saline (PBS), Dulbecco’s Modified Eagle’s Medium (DMEM), Fetal Bovine Serum (FBS), and Hoechst 3342 were purchased from Sigma-Aldrich. WST-1 cell proliferation reagent WST-1 was purchased from Roche Applied Science.

2. General methods

Powder X-ray diffraction (PXRD), N₂ adsorption-desorption isotherms, transmission electron microscopy (TEM), solid state nuclear magnetic resonance (NMR), Fourier-transform infrared spectroscopy (FTIR), dynamic light scattering (DLS), elemental analysis, inductively coupled plasma mass spectroscopy (ICP-MS), UV-visible and fluorescence spectrophotometry, and scanning transmission electron microscopy coupled with energy dispersive X-ray spectroscopy (STEM-EDX) were employed for materials characterization. PXRD measurements were performed on a Seifert 3000TT diffractometer using CuKα radiation. N₂ adsorption-desorption isotherms were recorded...
3. Synthesis of Mesoporous silica nanoparticles (S0)

Firstly, the surfactant n-cetyltrimethylammonium bromide (CTABr) 1.00 g (2.74 mmol) was dissolved in 480 mL of distilled water and heated. Once the temperature reached 40 °C, 2.00 M of NaOH in distilled water (3.5 mL) was added to the CTABr solution, and then the temperature was further increased up to 80 °C. Then, TEOS 5.00 mL (22.4 mmol) were added dropwise. The mixture was kept under stirring for 2 h at 80 °C. The resulting white precipitate was isolated by centrifugation, washed with distilled water several times until pH 7.5 and dried at 70 °C overnight. Finally, the surfactant template was removed by calcination for 5 h at 550 °C, yielding the solid S0.

4. Synthesis of S1

In order to functionalize the outer surface of S0, an excess of (3-glycidyloxypropyl)trimethoxysilane (500 μL, 2.26 mmol) was added to 100 mg of S0, and the suspension was stirred for 5.5 h at room temperature. The resulting solid was isolated by centrifugation, washed with acetonitrile and toluene, and dried at room temperature overnight. Then, the previously prepared solid and 154 mg (1.13 mmol) of 3-aminophenyl boronic acid were dispersed in 15 mL of toluene and was stirred for 24 h. Afterward, the nanoparticles were isolated by centrifugation, washed with toluene and acetonitrile and dried at room temperature, which yielded the solid S0-I. Next, 100 mg of solid S0-I and 150 mg of [Ru(bpy)3]Cl2 were suspended in 15 mL of acetonitrile and stirred for 24 h at room temperature in order to load the pores. Finally, the
solid was isolated by centrifugation, washed with toluene and dried, yielding the functionalized and loaded nanoparticles S1.

5. Synthesis of S2

In order to cap the nanoparticles with the enzyme acetylcholinesterase, 5 mg of S1 were mixed with 0.5 mg of enzyme in 500 μL of phosphate buffer pH 7.5 (50 mM). The mixture was stirred overnight at 4 °C. Then, the resulting solid (S2) was isolated by centrifugation, exhaustively washed with phosphate buffer, and kept in the refrigerator (4 °C) in phosphate buffer until use.

6. Synthesis of S2_{dox}

For cells experiments, we prepared S2_{dox} (a solid like S2 but loaded with the cytotoxic drug doxorubicin). In this case, the loading process was performed in water. Briefly, 5 mg of the boronic functionalized solid (S0-I) was dispersed in 500 μL of phosphate buffer pH 7.5 (50 mM) containing 2.5 mg of doxorubicin, and the mixture was stirred overnight. Then, 0.5 mg of enzyme were added and the solution was further stirred for 24 h at 4 °C. Then, the S2_{dox} was isolated by centrifugation, exhaustively washed with phosphate buffer, aliquoted, and kept in the freezer until use.
7. Materials characterization

7.1 Solid-state NMR

Figure SI-1. Solid-state $^{13}$C-NMR of the 3-aminophenylboronic-functionalized nanoparticles (S0-I), showing the corresponding carbon signals from the organic functionalization.

Figure SI-2. Solid-state $^{11}$B-NMR of the 3-aminophenylboronic-functionalized nanoparticles (S0-I), confirming the presence of B.
Figure SI-3. FTIR spectra of the MS nanoparticles (S0, black line), 3-aminophenylboronic-functionalized nanoparticles (S0-I, red line), and cargo-loaded nanoparticles (S1, blue line).

Figure SI-3 showed the FTIR spectrum of S0, S0-I and S1 nanoparticles. As could be seen, the FTIR spectrum of S0 showed the typical appearance for MS nanoparticles with a major band at ca. 1050 cm$^{-1}$ corresponding to the Si-O stretching vibrations. After functionalization with 3-aminophenylboronic moieties, new peaks appear at around 3300 cm$^{-1}$, 1650-1350 cm$^{-1}$, and 955 cm$^{-3}$ which can be ascribed to the vibration of hydroxyl groups, C-C aromatic bonds and aliphatic C-C bonds, respectively. After cargo ([Ru(bpy)$_3$]Cl$_2$) loading, new peaks corresponding to bipyridine rings between 1400-1470 cm$^{-1}$ are observed.
7.3 UV-Vis

Figure SI-4. UV-visible spectrum of the final nanodevice S2 (0.2 mg mL$^{-1}$ in aqueous solution, pH 7.5), which shows a peak at 453 nm corresponding to the encapsulated dye.

7.4 Dynamic light scattering (DLS) studies

Figure SI-5. Z-potential curves for S0 (green, -30.5 mV), S1 (red, -12.7 mV) and S2 (blue, -24.1 mV).
Figure SI-6. Hydrodynamic diameter distribution of the final nanodevice S2 determined by DLS.

### 7.5 Elemental analysis

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Boronic ligand content = \( \frac{0.545 \text{ g of } N}{100 \text{ g of solid}} \times \frac{1 \text{ g of ligand}}{0.102 \text{ g of } N} = \frac{0.053 \text{ g of ligand}}{g \text{ of solid}} \)

Cargo content = \( \frac{3.54 \text{ g of } N}{100 \text{ g of solid}} \times \frac{1 \text{ g of } [Ru(bpy)_3]Cl_2}{0.131 \text{ g of } N} = \frac{0.27 \text{ g of } [Ru(bpy)_3]Cl_2}{g \text{ of solid}} \)

### 7.6 Enzymatic activity assay

Acetylcholinesterase activity on S2 was measured based on the Ellman’s assay. An enzymatic unit (U) transforms 1 µmol of substrate per minute. Acetylcholinesterase is capable of hydrolysing acetylthiocholine into thiocholine and acetic acid. The generated thiocholine reacts with the Ellman’s reagent (5,5′-dithiobis(2-nitrobenzoic acid), DTNB), cleaving its disulfide bond to give 2-nitro-5-thiobenzoate (TNB\(^2\)) which has a characteristic yellow colour. In this assay, the formation of TNB\(^2\) as a function of time is followed by monitoring absorbance at 412 nm during 2 minutes using a UV-visible spectrophotometer. The rate of transformation is directly proportional to the enzymatic activity of the sample.
In a typical assay, 900 µL of 100 mM sodium phosphate buffer (pH 7.5), 30 µL of 10 mM DTNB solution and 12 µL of acetylthiocholine (75 mM) where placed in a quartz cuvette. Then, 5 µL of either buffer (for blank) or S2 nanoparticles (1 mg·mL$^{-1}$) were added. The mixture was shaken and absorbance at 412 nm was monitored (Figure SI-7). When S2 was added, the solution turned yellow quickly as a consequence of TNB$^{2-}$ formation due to S2’s enzymatic activity.

**Figure SI-7.** Monitoring of TNB$^{2-}$ formation due to acetylcholinesterase activity upon addition of buffer (black points) and upon addition of S2 (red points).

The acetylcholinesterase activity on S2 was estimated to be 4784 U·g$^{-1}$, by applying the following formula:

$$\frac{\text{Enzymatic Units}}{g} = \frac{(\Delta - \Delta_{\text{blank}}) \times V_T}{\varepsilon_{\text{TNB}} \times l \times V_{NPs} \times C_{NPs}}$$

Where,

$\Delta$ is the slope of the graph (min$^{-1}$)

$\Delta_{\text{blank}}$ is the slope of the graph for the blank (min$^{-1}$)

$V_T$ is the total volume in the cuvette

$\varepsilon_{\text{TNB}}$ is the molar extinction of TNB$^{2-}$ at 412 nm (13.7 mM$^{-1}$·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$^{-1}$).
7.7 Bradford protein assay

For quantification of the protein content on the nanoparticles, using the Bradford method, we followed the technical procedure suggested by the provider. For obtaining the calibration line, different concentrations of commercial acetylcholinesterase were prepared by dilution of a 0.1 mg mL\(^{-1}\) stock solution in phosphate buffer (50 mM) and mixed with 200 µL of Bradford dye reagent concentrate (final volume 1 mL). For sample analysis, 10 µL of a suspension of nanoparticles (10 mg mL\(^{-1}\)) were diluted in 790 µL of phosphate buffer and mixed with 200 µL of Bradford dye reagent concentrate. The solutions were vortexed, and stirred at 25 °C for 45 min. Then, the absorbance at 595 nm was measured with the spectrophotometer.

![Figure SI-8. Bradford assay calibration for different concentrations of acetylcholinesterase.](image)

From the equation of the calibration line \((y = 0.0158x + 0.4839, r^2=0.998)\) and the signal of the sample assay, the amount of protein on the nanodevice S2 was quantified as 58.5 µg mg\(^{-1}\).


### 7.8 STEM-EDX

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<tr>
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*Figure SI-9.* Additional images of STEM-EDX mapping of S2.
8. Release experiments

For the release experiments indicated in the manuscript, S2 nanoparticles were taken from a refrigerated stock solution of 10 mg mL\(^{-1}\), washed three times by centrifugation with an aqueous solution at pH 7.5 (20 mM Na\(_2\)SO\(_4\)), re-suspended and brought to a concentration of 2 mg mL\(^{-1}\) in aqueous solution containing the corresponding concentration of ACh or other neurotransmitters (for selectivity studies). The samples were stirred at 25 °C. At the programmed times, aliquots were taken in 1.5 mL tubes and centrifuged for 2 min at 12000 rpm (to precipitate the solid) and the fluorescence of the supernatant corresponding to the released [Ru(bpy)$_3$]Cl$_2$ dye was measured ($\lambda_{\text{em}} = 595$ nm, $\lambda_{\text{exc}} = 453$ nm). Amount of cargo released was calculated from a calibration line and maximum release efficiency (total amount of loaded cargo) was calculated by via several extractions of the dye content in water at acidic pH (3).

![Graph](https://via.placeholder.com/150)

**Figure SI-10.** Cargo release from S2 (2 mg mL\(^{-1}\)) in aqueous media (pH 7.5) as a function of ACh concentration after 10 min.

Furthermore, we also conducted an additional experiment using a higher concentration of S2 (6.6 mg mL\(^{-1}\)) in order to test if the difference could be seen to the naked eye (see Figure SI-11).
Figure SI-11. Visualization of [Ru(bpy)$_3$]Cl$_2$ released from S2 nanoparticles in the absence (left) and in the presence (right) of ACh after 10 min. It can be noted that nanoparticles form a pellet at the bottom after centrifugation, and that there is a remarkable release of dye in the supernatant for the sample in medium containing ACh.

For evaluating the acetylcholinesterase activity on S2 after the release, nanoparticles were collected after 10 min in the absence or presence of ACh, washed 3 times with phosphate buffer and redispersed. Then, the enzymatic assay was conducted as explained above. As can be observed in Figure SI-12, the remaining acetylcholinesterase activity on S2 (or increase in absorbance at 412 nm) was much lower (0.4 U mg$^{-1}$) when S2 had been in the presence of ACh (red line) compared to when it had been stirred in the absence of ACh (4.5 U mg$^{-1}$).

Figure SI-12. Enzymatic assay (monitoring of TNB$^{2-}$ formation) for evaluating the remaining acetylcholinesterase activity on S2 after a release experiment. Plotted lines for the sample that had been exposed to ACh (red) and for the control (black, stirred in the absence of ACh).
9. Gating mechanism experiments

In a first step, in order to confirm that the enzyme is attached through the phenylboronic linker and not through simple physical adsorption to the silica surface, we carried out additional control experiments. To this end, we compared the amount of enzyme that attaches to phenylboronic-functionalized-MS-nanoparticles versus the amount of enzyme that attaches to non-functionalized-MS-nanoparticles. First, non-functionalized-MS-nanoparticles were loaded with the ruthenium dye (following the same procedure as described above). Then, 1 mg mL$^{-1}$ of enzyme solution were added to the different solids: (i) phenylboronic-functionalized-MS-nanoparticles ($S_1$) and (ii) non-functionalized-MS nanoparticles. Following the procedure as described above for preparing $S_2$, after 24 h of incubation, the solids were isolated by centrifugation and exhaustively washed with phosphate buffer.

In the second case (without the linker), a much lower amount of enzyme was found on the nanoparticles as measured by acetylcholinesterase-specific assay and by protein (Bradford) assay. In particular, ca. 10-times less AChE-activity was found when nanoparticles are non-functionalized with the ligand (Figure SI-13). Additionally, whereas the amount of protein attached to phenylboronic-functionalized-MSN was found to be 58.5 µg·mg$^{-1}$ (according to the Bradford protein assay quantification), the amount for the non-functionalized-MSN was below the LOD of the method. Furthermore, after the enzyme-attachment procedure, a significant larger cargo leakage is observed for the solution of non-functionalized nanoparticles (Figure SI-14), which is ascribed to the absence of enzyme molecules blocking the pores.

These results support the formation of ester linkages between diol/saccharide-groups (of the enzyme) and phenyl boronic ligands, as previously reported by other authors (see reference 19 in the manuscript).
Figure SI-13. Acetylcholinesterase activity assay on the different solids (after enzyme attachment procedure) for: (i) non-functionalized-MS nanoparticles (black), and (ii) phenyl-boronic-functionalized-MS-nanoparticles (red).

Figure SI-14. Absorbance of the cargo leakage from nanoparticles after enzyme attachment procedure for: (i) non-functionalized-MS nanoparticles (black), and (ii) phenyl-boronic-functionalized-MS-nanoparticles (red).

In a further step, we checked the pH variation after release experiments using an indicator solution of methyl red. Methyl red is a pH indicator that turns red in pH under 4.4, yellow in pH over 6.2 and orange in between. 10 µL of a saturated solution of methyl red (1 mg in 0.3 mL of ethanol and 0.2 mL of DI water) were added to the supernatant of aliquots (200 µL) after release.
experiments (10 min) in the presence and in the absence of ACh. A clear difference was observed (Figure SI-15), showing the acidification of the solution to pH below 4.4 after the release in the presence of ACh. This observation correlates with the formation of acetic acid by the enzyme.

**Figure SI-15.** Measurement of the pH of the supernatant solution after the release experiments (10 min) using methyl red indicator. It can be noted that for the control (absence of ACh) the pH of the solution remains neutral, whereas it is acidified to below 4.4 in the presence of ACh due to the enzyme-mediated formation of acetic acid.

**Figure SI-16.** Cargo release from S2 in aqueous media at pH 7.5 (black) and at pH 4.5 (red).
Figure SI-17. Cargo release from S2 in aqueous media under different conditions: pH 7.5 in the absence of ACh (black), pH 6.5 in the absence of ACh (blue), pH 7.5 in the presence of ACh (1 mM, red), and pH 6.5 in the presence of ACh (1 mM, green).

Figure SI-18. Cargo release from S1 in the absence (black) and in the presence (1 mM, red) of ACh.

In order to confirm the cleavage of the linker under acidic conditions, we also carried out $^1$H-NMR experiments using a model system (Figure SI-19). First, we recorded the spectra in D$_2$O of pure 3-aminophenylboronic acid (APB) and APB with D-glucose (model saccharide) after 24 h incubation. As showed in Figure SI-19-A-B, new signals appear due to the linkage of APB with D-glucose. However, upon addition of acetic acid (10 µL) to these samples, only the signals corresponding to the free (protonated) APB are observed which indicates the cleavage of the boronic ester linker.
Figure SI-19. $^1$H-NMR study of a model system in order to confirm the cleavage of the linker under acidic conditions. Samples containing (A) pure APB and (B) APB incubated with glucose, and corresponding spectra upon addition of acetic acid (C-D).

10. Cell experiments

In vitro release of the cytotoxic drug doxorubicin

Release experiments with $S_2$ dox were carried out as explained above for $S_2$. In this case, the amount of released cargo was determined by measuring the absorption of doxorubicin at 485 nm with the spectrophotometer.

Figure SI-20. Kinetics of doxorubicin release from $S_2$ dox in aqueous media (pH 7.5) in the absence (black curve) and in the presence of ACh (1 mM, red curve).
**Cell culture conditions**

HeLa human cervix adenocarcinoma cells were purchased from the German Resource Centre for Biological Materials (DSMZ) and were grown in DMEM supplemented with 10% 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 assays**

HeLa cells were seeded in a 24-well plate at 50,000 cells per well and incubated at 37 °C for 24 h. Then, cells were incubated with $\text{S2}_{\text{dox}}$ (50 µg·mL$^{-1}$) for 30 min, washed with PBS, and further incubated for 24 h in DMEM medium supplemented with 10% FBS in the absence or in the presence of ACh (50 mM). Finally, cell viability was assessed by incubation with the cell proliferation WST-1 reagent for 1 h and measuring the absorbance at 595 nm in the Wallac Workstation. Three independent experiments containing triplicates were carried out. Similar experiments with the equivalent amount of doxorubicin (16 µM) were also carried out.

**Confocal microscopy imaging**

HeLa cells were seeded on glass coverslips at 300,000 cells·mL$^{-1}$ in 6-well culture plates and incubated at 37 °C for 24 h. Then, cells were incubated with $\text{S2}_{\text{dox}}$ (75 µg·mL$^{-1}$) for 30 min, washed with PBS, and further incubated for 2 h in the absence or in the presence of ACh (50 mM). Then, cells were washed several times with PBS, stained with DNA marker Hoechst 33342 by addition at 2 µg·mL$^{-1}$, and placed in fresh media. Finally, samples were visualized using a confocal microscope Leica TCS SP8 AOBS.

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