Supplementary Information

Aqueous core and hollow silica nanocapsules for confined enzyme modules

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Materials and methods

Materials

Glucose oxidase from *Aspergillus niger* type VII (GOX; EC:1.1.3.4, 149 unit/mg), horseradish peroxidase (HRP; EC 1.11.1.7, 200 unit/mg), catalase from bovine liver (CAT; EC 1.11.1.6; 3000 unit/mg), NAD^+-dependent glucose dehydrogenase from Pseudomonas sp. (GDH; EC 1.1.1.47; 550 unit/mg), NADH-dependent lactate dehydrogenase recombinant from *E. coli* (LDH; EC 1.1.1.27, 96 unit/mg), D-glucose, tetraethyl orthosilicate (TEOS; 98%), trimethoxy(octyl)silane (octyl-TMS; 96%) bis(triethoxysilylpropyl)tetrasulfide (TESPT; 90%), potassium fluoride, cyclohexane, Amplex red (Ampliflu™), and hydrogen peroxide (35%) were purchased from Sigma-Aldrich (St. Louise, MO, United States). Nicotinamide adenine dinucleotide reduced form (NADH), nicotinamide adenine dinucleotide oxidized form (NAD^+), sodium pyruvate, and ammonia (25 wt%) were obtained from Carl Roth (Karlsruhe, Germany). (3-Aminopropyl)trimethoxysilane (APTMS; 97%) was purchased from Merck (Merck, Darmstadt, Germany). Polyglycerol polyricinoleate (PGPR) was kindly gifted from Danisco (Copenhagen, Denmark). Lutensol AT50 was purchased from BASF (Ludwigshafen, Germany). All other chemicals and solvents are in reagent grade.

Preparation of silica nanocapsules

The miniemulsion was prepared by a sonication method. Toluene (1.5 mL) as a continuous phase containing TEOS (30.2 mg) and PGPR (12 mg), and sodium phosphate buffer (50 µL, pH 7.4, 50 mM) as an aqueous phase containing APTMS (5.6 mg) and potassium fluoride (0.1 mM) were mixed together. Homogenization was performed by a tip type sonicator (Branson Ultrasonics, CT, USA) under 17% amplitude for 3 min (20 s pulse on/10 s pulse off) with iced bath. Then, silica precursors (described in **Table A**) were added to the emulsion and reacted for 24 h at ambient temperature under gentle orbital mixing. The resulting nanocapsule was purified by dispersing into cyclohexane (10 mL) and centrifugation (11000 rpm, 20 min).

The pellet was re-dispersed in pH 7.4 sodium phosphate buffer (250 μL; 10 mM) assisted with 0.25 wt% Lutensol AT50. The nanocapsule was stored at fridge (7 °C).

For encapsulation of enzymes, each aqueous phase contained GOX, HRP, CAT, GDH, or LDH, respectively (described in **Table B**). To obtain nanocapsules (ca. 600 nm) instead of microcapsules (1-2 μm), we further added sodium chloride (10 mM) in aqueous phase.

Table A. Amount of silica precursors to prepare silica nanocapsules.

	APTMS	TEOS	TESPT	Octyl-TMS
APTMS/TEOS (1 : 5)	5.4 mg	30 mg	-	-
APTMS/TEOS/TESPT (1 : 3.5 : 1.5)	5.4 mg	21 mg	25 mg	-
APTMS/TEOS/Octyl-TMS (1: 3: 2)	5.4 mg	18 mg	-	14 mg
APTMS/TEOS/Octyl-TMS (1: 2: 3)	5.4 mg	12 mg	-	21 mg
APTMS/TEOS/Octyl-TMS (1: 1: 4)	5.4 mg	6 mg		28 mg
APTMS/TEOS/Octyl-TMS (1: 4: 1)	5.4 mg	24 mg	-	7 mg
APTMS/TEOS/Octyl-TMS (1: 0: 5)	5.4 mg	-	-	35 mg
APTMS/TEOS/Octyl-TMS (0: 2: 3)	-	12 mg		21 mg
APTMS/TEOS/Octyl-TMS (0: 0: 5)	-	-	-	35 mg
APTMS/TEOS/Octyl-TMS (0: 5: 0)	-	30 mg	-	-

Table B. Amount of enzymes to encapsulate into silica nanocapsule.

	GOX	HRP	CAT	GDH	LDH
GOX@nanocapsules	0.2 mg	-	-	-	-
GOX/HRP@nanocapsules	0.2 mg	0.6 mg	-	-	-
GOX/CAT@nanocapsules	0.2 mg	-	0.6 mg	-	-
GDH/LDH@nanocapsules	-	-	-	0.4 mg	0.4 mg

Enzymatic assay for GOX

The reaction cocktail was composed of sodium phosphate buffer (1 mL, pH 7.4, 10 mM), Amplex red (3.3 μL, 1 mM) dissolved in DMSO, glucose solution (25 μL, 100 mM), and HRP solution (5 μL, 50 unit/mL). To assay the activity of GOX, the reaction mixture (100 μL) was placed in a well of a 96-well plate, then 2.5 μL of native GOX solution or GOX@nanocapsule dispersion was added, and the changes in fluorescence at excitation 555 nm and emission 595 nm were monitored by a TECAN plate reader (Infinite® M1000, Männedorf, Switzerland). Initial reaction velocity within 4 min was taken to compare the relative activity.

Enzymatic assay for HRP

The reaction cocktail was composed of sodium phosphate buffer (1 mL, pH 7.4, 10 mM), Amplex red (3.3 μL, 1 mM) dissolved in DMSO, hydrogen peroxide solution (10 μL, 0.35%). To assess the activity of HRP, the reaction mixture (100 μL) was placed inside a well of a 96-well plate, then 2.5 μL of a native HRP solution or GOX/HRP@nanocapsule dispersion was added, and the changes in fluorescence at excitation 555 nm and emission 595 nm were monitored by a TECAN plate reader (Infinite® M1000, Männedorf, Switzerland). Initial reaction velocity within 4 min was taken to compare the relative activity.

Enzymatic assay for CAT

The reaction cocktail was composed of sodium phosphate buffer (1 mL, pH 7.4, 10 mM), Amplex red (3.3 μL, 1 mM) dissolved in DMSO, hydrogen peroxide solution (10 μL, 0.35 %) and HRP solution (2 μL, 0.4 μU/mL). To assess the activity of CAT, the reaction mixture (100 μL) was placed in a well of a 96-well plate, then 2 μL of a native CAT solution or GOX/CAT@nanocapsule dispersion was added, and the changes in fluorescence at excitation 555 nm and emission 595 nm were monitored by a TECAN plate reader (Infinite® M1000, Männedorf, Switzerland). Initial reaction velocity within 4 min was taken to compare the relative activity. A standard curve in terms of different amounts of CAT was obtained. The CAT activity was evaluated in reliable ranges of the standard curve and estimate the activity.

Enzymatic assay for GDH

The assay was conducted by mixing NAD^+ (2 μL of 60 mM stock solution), glucose (5 μL of 100 mM stock solution) in sodium phosphate buffer (50 mM, pH 7.4). The total volume of the assay was 100 μL (in a 96-well plate). The addition of GDH or GDH/LDH@nanocapsule (5 μL) initiated the reaction. The changes in absorbance at 340 nm was monitored at intervals of 30 s.

Enzymatic assay for LDH

The assay was conducted by mixing NADH (2 μL of 60 mM stock solution) and pyruvate (5 μL of 100 mM stock solution) in sodium phosphate buffer (50 mM, pH 7.4). The total volume of the assay is 100 μL (in a 96-well plate). The addition of LDH or GDH/LDH@nanocapsule (5 μL) initiated the reaction. The changes in absorbance at 340 nm was monitored at intervals of 30 s.

CD measurements

Each enzyme solution was prepared in pH 7.4 sodium phosphate buffer (10mM) following concentration. GOX: 0.2 mg/mL, CAT: 0.2 mg/mL, HRP: 0.15 mg/mL, LDH: 0.15 mg/mL, GDH: 0.15 mg/mL. Each enzyme solution was also prepared with 0.1 mM potassium fluoride with the buffer or 3 M ammonia with the buffer and kept for 24 h at 25 °C. CD spectrum was measured in a range of 180 – 260 nm using a JASCO J-1500 CD spectrometer (MD, United States).

Thermogravimetric analysis

The dried silica nanocapsules (10 mg) was used for analysis. The measurement condition is described as follows: the heating rate: 10 °C/min, heating from 25 to 700 °C under N_2 atmosphere. Instrument: Mettler TG 50 (Mettler Toledo, Columbus, Ohio, United States).

Cascade reaction between GOX and HRP

The reaction cocktail was composed of sodium phosphate buffer (1 mL, pH 7.4, 10 mM), Amplex red (3.3 μL, 1 mM) dissolved in DMSO, glucose solution (25 μL, 100 mM). To assay the activity of GOX, the reaction mixture (100 μL) was placed in a well of a 96-well plate, then 2.5 μL of a GOX/HRP@nanocapsule suspension or GOX@nanocapsule + native HRP dispersion was added, and the changes in fluorescence at excitation 555 nm and emission 595 nm were monitored by a TECAN plate reader (Infinite® M1000, Männedorf, Switzerland). Initial reaction velocity within 4 min was taken to compare the relative activity. To adjust the same amount of GOX in each group, the activity of the co-loaded GOX and the single-loaded GOX was investigated by the decreasing pH-value as during the reaction gluconic acid is produced. To adjust the same amount of HRP in each group, the native HRP or GOX/HRP@nanocapsule was reacted with a reaction mixture composed of H_2O_2 (1 mM) and Amplex red (3.3 μM).

NAD^+/NADH regeneration by LDH/GDH@nanocapsules

Different aqueous stock solutions were prepared: NADH (60 mM), pyruvate (60 mM), glucose (60 mM) and LDH/GDH@nanocapsule suspension (7 mg/mL silica content). To 95 μL buffer solution in a 96-well plate (50 mM sodium phosphate, pH 7.4), NADH (1 μL) and pyruvate (1 μL) was added. LDH/GDH@nanocapsule (5 μL) was added to initiate the LDH reaction (NAD^+ production). The changes in absorbance at 340 nm was monitored at an interval of 60 s. After 1 h, glucose (1 μL) was added to initiate the GDH reaction (NADH production), then the absorbance was monitored for 1 h. This cycle was repeated three times (results are plotted in Figure 7).

Cell culture

HeLa cells were obtained from the ATCC (American Type Culture Collection, Manassas, VA, USA). The cells were cultured in Eagle's Minimum Essential Medium (EMEM; Lonza, Basel, Switzerland) media supplemented with 10% fetal bovine serum (Gibco, Waltham, Massachusetts, USA) and penicillin (100 U/mL) in a CO_2 incubator (37 °C, 5% CO_2). Dulbecco's buffered saline (DPBS) was used for all cell-washing steps. For cell detachment, trypsin/EDTA solution (Gibco) was used. Glucose-free DMEM media were purchased from Thermo Scientific (Waltham, Massachusetts, USA).

Cytotoxicity assay (cascade reaction between GOX and CAT)

The cells were cultured on a 96-well plate with 2000 cells/well of initial cell density. After a day culture the cells, the cytotoxic assay was performed. GOX@nanocapsule or GOX/CAT@nanocapsule (0.2 μL; stock: 7 mg/mL of silica concentration) was treated to the cells (each well) with 44 mM glucose in 100 μL total volume (serum-free media). For cell viability assay, CCK-8 (cell counting kit-8) assay was used (Sigma-Aldrich). Assay reagent (10 μL) was mixed with the serum media (90 μL), treated to the cells, then

the absorbance at 450 nm was measured by the TECAN plate reader. Cells kept with serum-free media was used as a standard for non-dead cells (control).

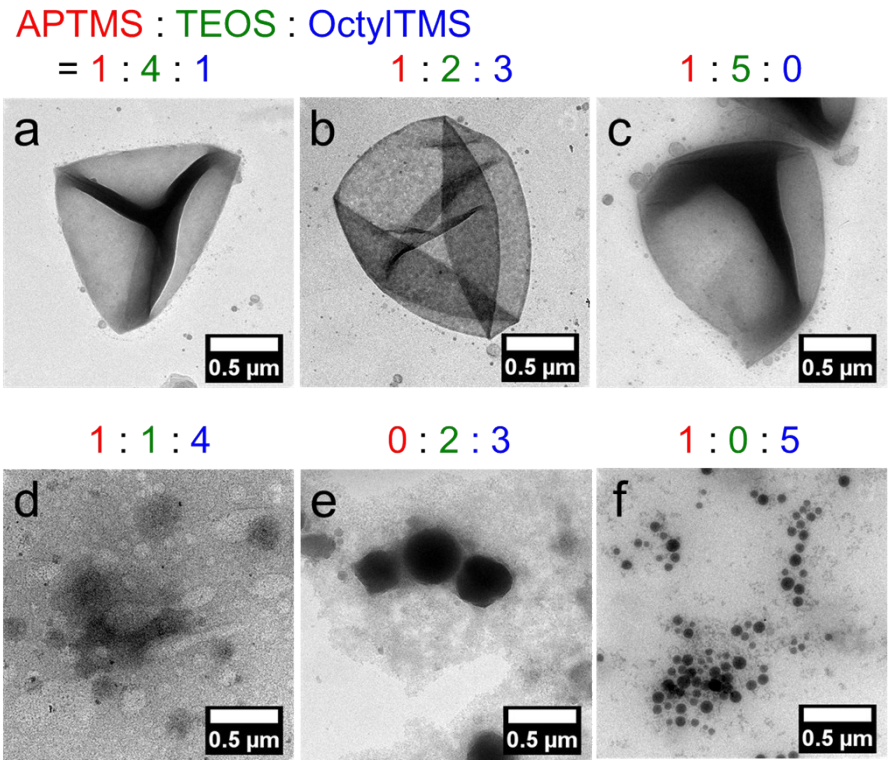

Figure 1. **Silica nanocapsules prepared by different molar ratios of silica precursors.** Molar ratio of APTMS, TEOS and octyl-TMS is 1:4:1 (**a**), 1:2:3 (**b**), 1:5:0 (**c**), 1:1:4 (**d**), 0:2:3 (**e**), 1:0:5 (**f**).

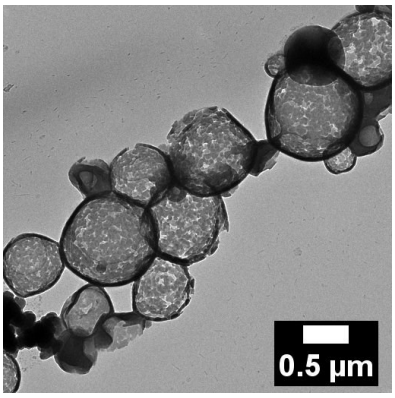

Figure S2. Silica nanocapsule composed of APTES and TEOS (1:5 molar ratio, NH_3 as catalyst).

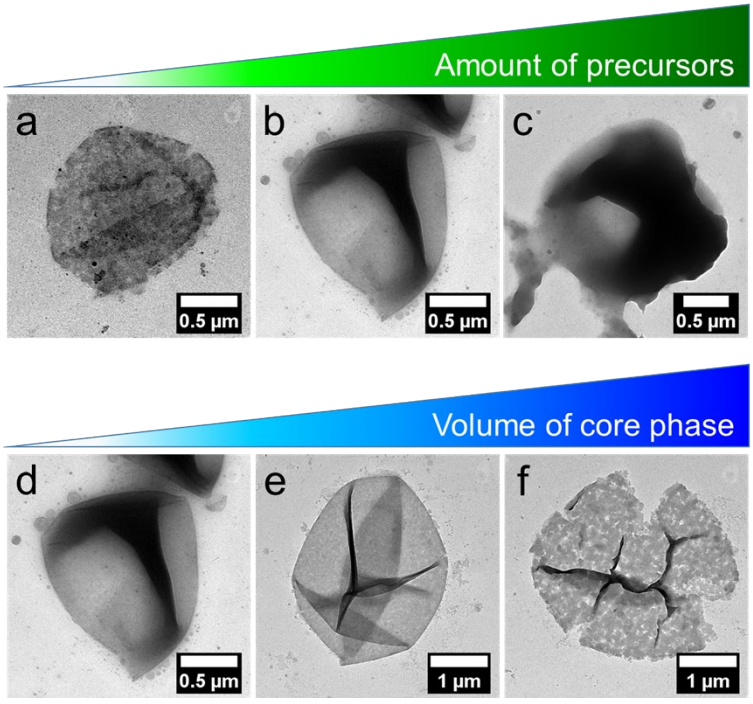

Figure S3. Preparation of silica nanocapsule composed of APTMS and TEOS (1:5 molar ratio) in different amount of total silica precursors: (a) 17.7 mg, (b) 35.4 mg, (c) 70.8 mg, and different volume of core (aqueous) phase, (d) 50 µL, (e) 100 uL, (f) 150 µL in 1.5 mL continuous phase.

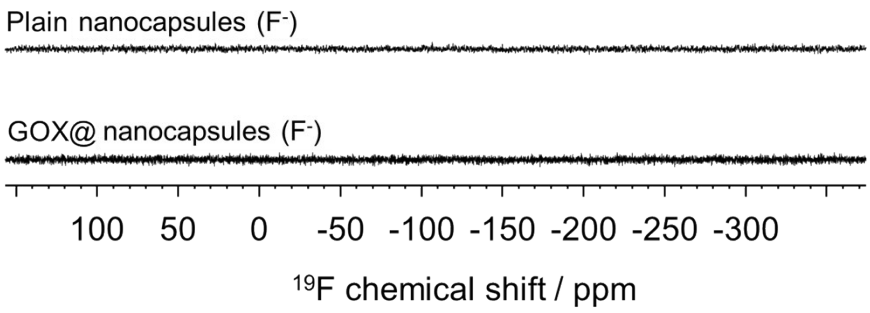

Figure S4. Solid-state ^{19}F NMR spectra of the silica capsules.

Table S1. Diameter and zeta potential of plain silica nanocapsules.

	Diameter (nm)	Zeta potential (mV)	Thickness of shells (nm)
APTMS/TEOS with ammonia	583 ± 64.6	0.78 ± 0.05	31 ± 6.3
APTMS/TEOS with fluoride	600 ± 86.5	0.26 ± 0.07	19 ± 4.3
APTMS/TEOS/TESPT with fluoride	527 ± 66.8	1.87 ± 0.08	25 ± 5.0

Table S2. Diameters of silica nanocapsules (fluoride catalyzed) measured by dynamic light scattering.

	Silica precursors	Diameter (nm)	Polydispersity index
GOX@nanocapsules	APTMS, TEOS	440	0.280
GOX@nanocapsules	APTMS, TEOS, TESPT	419	0.279
GOX/HRP@nanocapsules	APTMS, TEOS	267	0.156
GOX/CAT@nanocapsules	APTMS, TEOS	333	0.270
LDH/GDH@nanocapsules	APTMS, TEOS	364	0.211

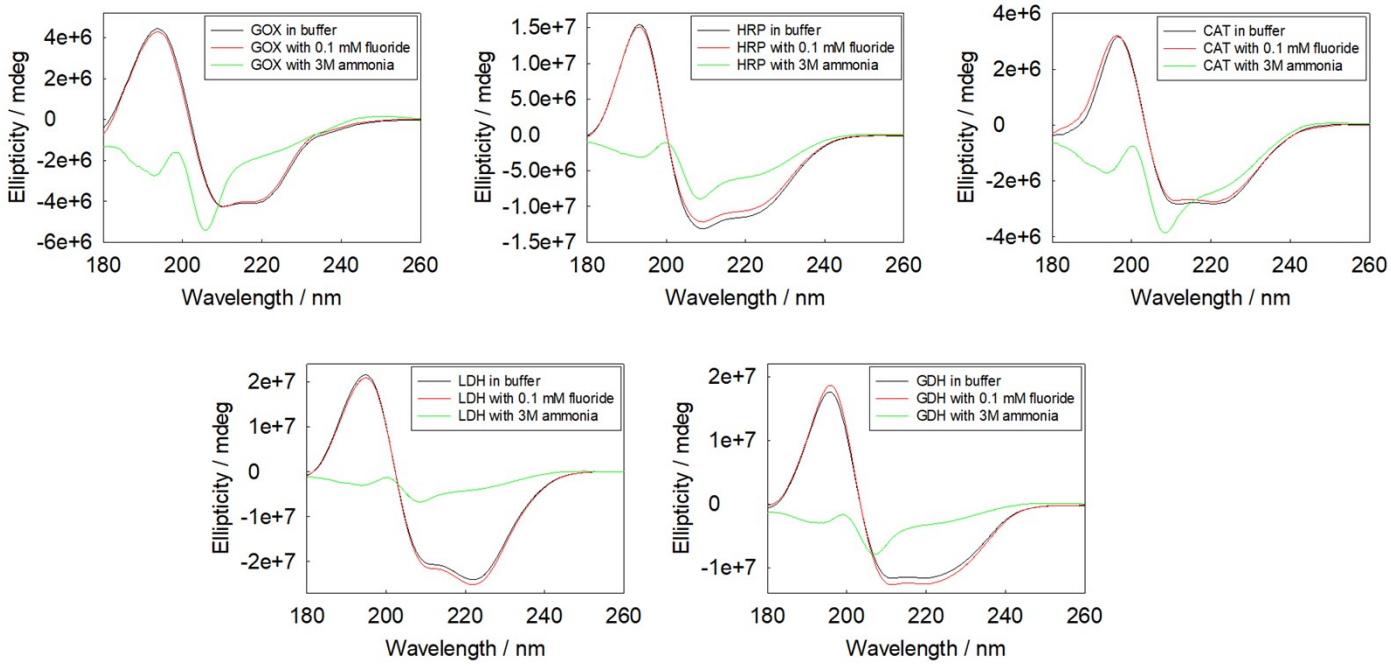

Figure S5. Circular dichroism (CD) spectra of enzymes after exposure to 0.1 mM fluoride and 3 M ammonia solution.

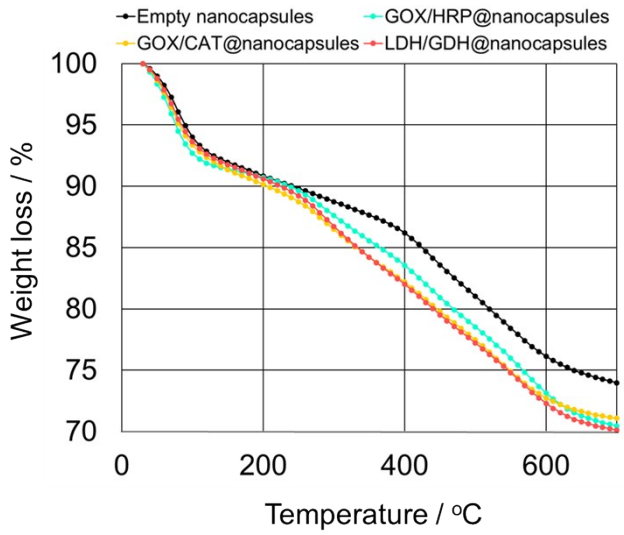

Figure S6. TGA of silica nanocapsules. Empty nanocapsules (black) lose 26% weight by 700 °C. Each enzyme@nanocapsule further losses their weight compared to empty nanocapsules: GOX/HRP@nanocapsules (3.5%; cyan), GOX/CAT@nanocapsules (2.9%; yellow) and LDH/GDH@nanocapsules (3.9%; red). (Note: loss of 4.5% weight indicates 100% loading of enzymes.) The loading efficiency of each enzyme was described in Table S1.

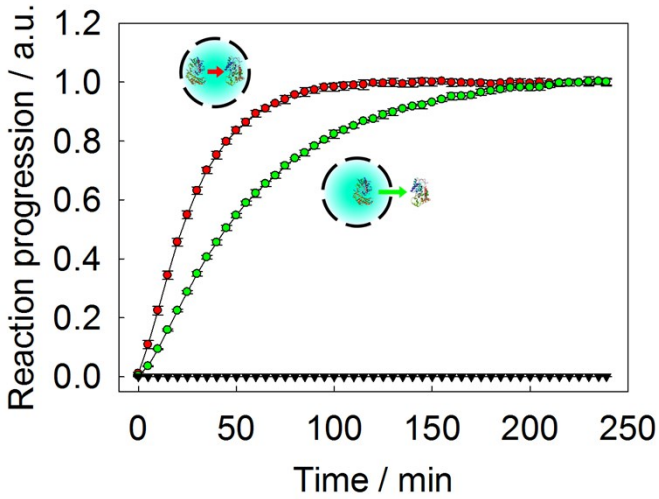

Figure S7. Comparison of cascade reaction inside the nanocapsule, and across the shell of the nanocapsule.