# Electronic Supplementary Information

## Self-Sustaining Enzyme Nanocapsules Perform Chemical Reactions On-Site

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### Materials

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), *Micrococcus lysodeikticus* and the enzymes horseradish peroxidase Type I, essentially salt-free, lyophilized powder, 50-150 U/mg solid, glucose oxidase from Aspergillus niger Type VII, lyophilized powder, ≥100.000 U/g solid and lysozyme from chicken egg white, lyophilized powder, protein ≥90 %, ≥40.000 U/mg protein were received from Sigma. Lutensol AT50 was purchased from BASF, polyglycerol polyricinoleate (PGPR) from Danisco, 2,4-toluene diisocyanate (TDI) from TCI Chemicals, bicinchoninic acid (BCA) from Alfa Aesar, dextran from Carl Roth and luminol from Acros Organics. Human blood was taken at the Department of Transfusion Medicine Mainz from ten healthy donors after physical examination and after obtaining informed consent in accordance with the Declaration of Helsinki. Human citrate plasma or serum were pooled into one batch and stored at - 80 °C. Sartorius™ Vivaspin™ 20 centrifugal filter devices were purchased from Thermo Fisher Scientific (300 000 molecular weight cut-off). The commercial glucose HK assay was purchased from Sigma-Aldrich and the glucometer from Adia.

### Methods

#### Synthesis of the Enzyme Nanoreactors by Ultrasonication

The enzyme nanoreactors were prepared by a polyaddition reaction in an inverse miniemulsion. In a typical experiment, the aqueous phase was prepared by dissolving 30 mg of enzyme and 1 mg of NaCl in 0.3 mL PBS-buffer (pH 7.0). The organic continuous phase was prepared by dissolving 75 mg of polyglycerol polyricinoleate (PGPR) in 3 mL toluene. The aqueous and organic phases were combined and subjected to ultrasound (Branson sonifier 450 equipped with ½" tip) for 3 min (70% amplitude, 20 s pulse, 10 s pause). The emulsion was divided into three parts of 1 mL. A solution of 25 mg PGPR and 5 or 10 mg of crosslinking

agent (TDI) in 0.5 mL of toluene was added dropwise to 1 mL of the miniemulsion over a period of 2 min. The reaction was stirred over 20 h at room temperature.

**Enzymatic activity assay**

The activity of HRP and the HRP-nanoreactors was determined using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) as substrate. Briefly, the ABTS was first dissolved in 100 mM potassium phosphate buffer (pH 5.0) at a final concentration of 5 mg/mL. HRP or the HRP-nanoreactors were diluted to a protein concentration of 0.002 mg/mL in a solution of 40 mM PBS buffer (pH 6.8) containing 0.5% of Triton X-100. 0,190 mL of the ABTS solution were mixed with 3.3 µL of the enzyme solution in a 96-well plate. The reaction was started by the addition of 6.6 µL of 0.3% (w/w) hydrogen peroxide solution ($H_2O_2$) and the increase of absorbance was detected at 405 nm by UV/Vis spectrophotometry.

The activity of GOx was measured using o-anisidine as substrate. In a typical measurement, 24 mL of o-dianisidine (0.21 mM in 50 mM sodium acetate buffer, pH 5.1) were mixed with 5 mL of a 10% (w/v) β-D-(+) glucose solution in water. The pH of this reaction mixture was adjusted to 5.1 with 1 M HCl. Native horseradish peroxidase (5-150 U/mL) was dissolved in sodium acetate buffer (50 mM, pH 5.1) with a final concentration of 1.2 mg/mL. Native GOx or the GOx-nanoreactors were diluted to a concentration of 0.05 mg/mL in sodium acetate buffer (50 mM, pH 5.1). The enzymatic reaction was initiated by mixing 190 µL of the glucose/dianisidine mixture, 3.3 µL of the HRP-solution and 6.6 µL of GOx/ GOx-nanoreactors suspension in a 96-well plate. The increase of absorbance at 500 nm was detected for five minutes using a UV/Vis spectrophotometer.

The activity of lysozyme was measured by using a suspension of *Micrococcus lysodeikticus* as substrate. Briefly, *Micrococcus lysodeikticus* were suspended in 50 mM potassium phosphate buffer, pH 6.2 at a concentration of 0.5 mg/mL. A solution of native lysozyme or lysozyme-nanoreactors was prepared in 50 mM potassium phosphate buffer, pH 6.2 with a final concentration of 0.1 mg/mL. In order to initiate the enzymatic reaction, 150 µL of the *Micrococcus lysodeikticus* suspension were mixed with 6.0 µL of enzyme-solution and the increase in absorbance at 450 nm was detected for five minutes.

The HRP/GOx enzyme cascade reaction activity was quantified by using o-anisidine as substrate. 24 mL of o-dianisidine (0.21 mM in 50 mM sodium acetate buffer, pH 5.1) were mixed with 5 mL of a 10% (w/v) $\beta$-$_D$- glucose solution in water. The pH of this reaction mixture was adjusted to 5.1 with 1 M HCl. The HRP/GOx-nanoreactors were diluted to a total protein concentration of 0.05 mg/mL in 50 mM sodium acetate buffer. The enzymatic reaction was initiated by mixing 190 µL of the reaction mixture and 6.6 µL of HRP/GOx-nanoreactors

suspension in a 96-well plate. The time-dependent change of absorbance at 500 nm was detected for five minutes by using a UV/Vis spectrophotometer.

Enymatic reaction was also analyzed using Michaelis Menten kinetic model. For each nanocapsule the appropriate enzymatic assay was performed using different amount of substrate [S] and a constant concentration of enzyme [E]. The initial rate of product formation ($v$) obtained from the enzymatic assay was used to calculate the catalytic rate constant ($k_{cat}$) using the Michaelis-Menten equation:

$$v = \frac{V_{max}[S]}{K_M + [S]} \qquad \text{(S1)}$$

where $K_M$ is the Michaelis constrant and $V_{max}$ is the maximal conversion rate of the enzymatic system given by

$$V_{max} = k_{cat}[E] \qquad \text{(S2)}$$

The ratio $k_{cat}/K_M$ was used as an indicator of the catalytic efficiency.

**CLSM imaging**

The images for the intracellular localization of the particles were taken using a commercial setup (LSM SP5 STED Leica laser scanning confocal microscope, Leica, Olympus, Germany), consisting of an inverse fluorescence microscope (DMI 6000 CS) equipped with a multi-laser combination, in addition to five detectors operating in the range of 400–800 nm. An HCX PL APO CS 63×/1.4–0.6 oil-immersion objective was used in this study. HRP capsules were detected at 625-670 nm, which corresponds to red in color.

**Conjugation of luminol to dextran**

The synthesis was carried out according to literature.[1] Briefly, dextran (MW 40 000, 30 g) was dissolved in water (400 mL) and potassium periodate (30 g) was added. The reaction mixture was stirred at room temperature overnight. Then, the reaction mixture was dialyzed against water for 3 days and freeze-dried.

The oxidized dextran (100 mg) was dissolved in dimethyl sulfoxide (DMSO) (10 mL) at 100 °C. The solution was cooled to 60 °C and glacial acetic acid (3.2 mL) and luminol (48 mg) were added to the solution subsequently. The reaction proceeded at 60 °C overnight. In order to precipitate the product, the reaction mixture was poured into methanol (100 mL) and filtered. Then, another 50 mL of methanol were added to the precipitate to remove unreacted luminol. After stirring for 30 minutes, the product was collected by centrifugation. The modified dextran

was dissolved in ethylene glycol (30 mL) and excess of sodium borohydride (400 mg) was added to the ice-cooled mixture. After reacting at room temperature for 4 hours, the reaction was continued at 4 °C overnight without stirring. The obtained polymer was precipitated in acetone, washed three times with methanol and dissolved in water. The product was further purified by dialysis against water (pH 8). After freeze-drying a pale yellow powder was obtained (50 mg). The covalent immobilization of luminol on the dextran chains was confirmed by HPLC, and the purity was assessed by NMR.

### Circular Dichroism

The CD measurement was carried out on a J-1500 JASCO circular dichroism spectrophotometer using a cuvette with a path length of 0.1 cm. The spectra were measured between 260 nm to 190 nm at an enzyme concentration of 0.03 mg/mL.

### Scanning electron microscopy (SEM)

For the measurement, 8.0 µL of the purified sample in toluene was dropped on a silica wafer and allowed to dry under ambient temperature. The SEM measurements were performed with a 1530 Gemini LEO (Zeiss) microscope.

### Additional Figures

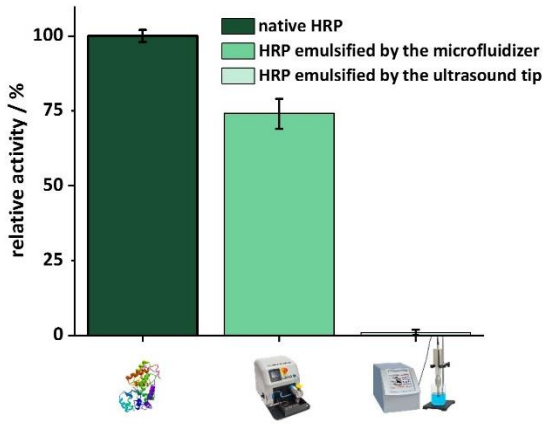


Figure S1. Influence of the emulsification process on the relative enzymatic activity of the HRP.

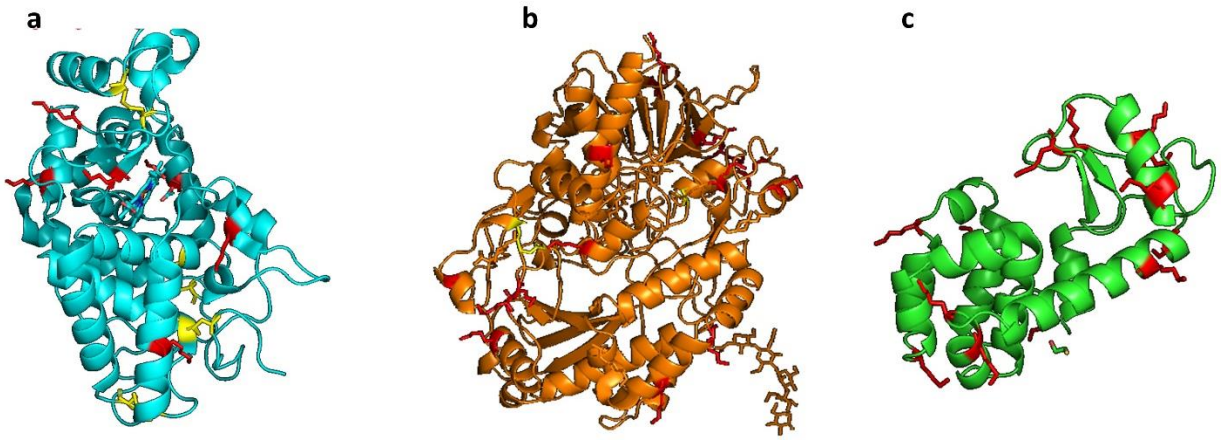


Figure S2. Enzyme folding with marked nucleophilic goups: lysine (red) and cysteine residues (yellow) for a) HRP, b) GOx, c) LYZ.

Table S1. Zeta-potential of the enzyme-nanoreactors.

| Sample | Zeta-pot / mV |
|---|---|
| HRP NCs, low crosslinking | - 10.1±0.8 |
| HRP NCs, high crosslinking | - 8.1±0.5 |
| GOx NCs, low crosslinking | - 9.7±1.2 |
| GOx NCs, high crosslinking | - 9.1±0.7 |
| LYZ NCs, low crosslinking | - 4.5±0.4 |
| LYZ NCs, high crosslinking | - 7.8±2.0 |
| HRP-GOx NCs, low crosslinking | - 9.2±1.2 |
| HRP-GOx NCs, high crosslinking | - 9.8±0.2 |

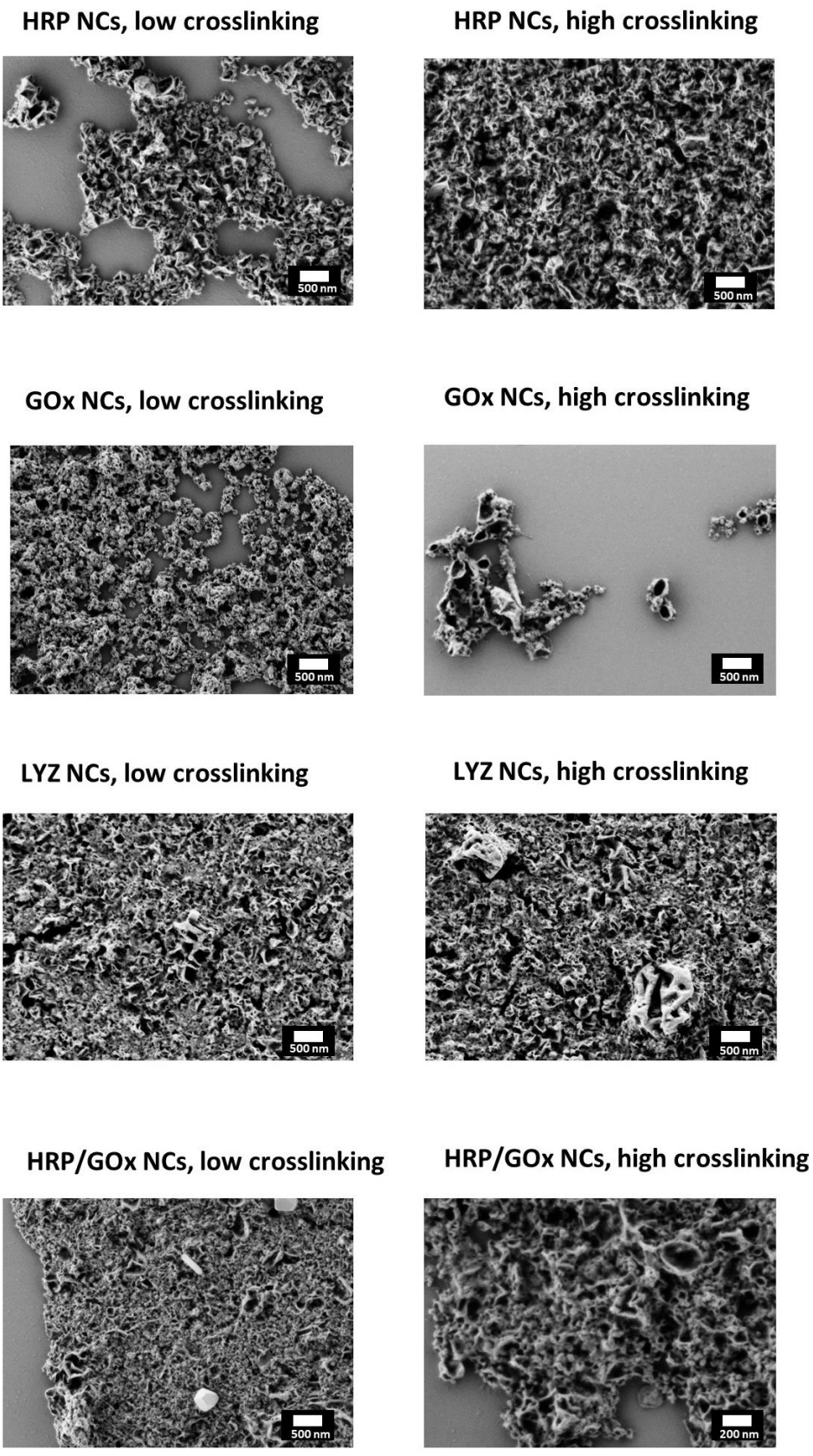


Figure S3. SEM-images of the enzyme-nanoreactors.

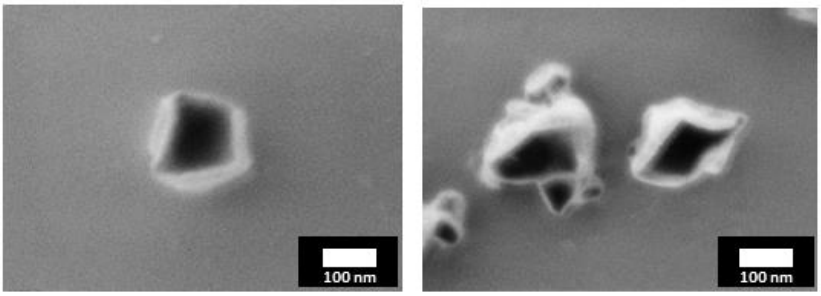


Figure S4. SEM images of HRP-nanoreactors obtained after drying a very diluted suspension of nanoreactors

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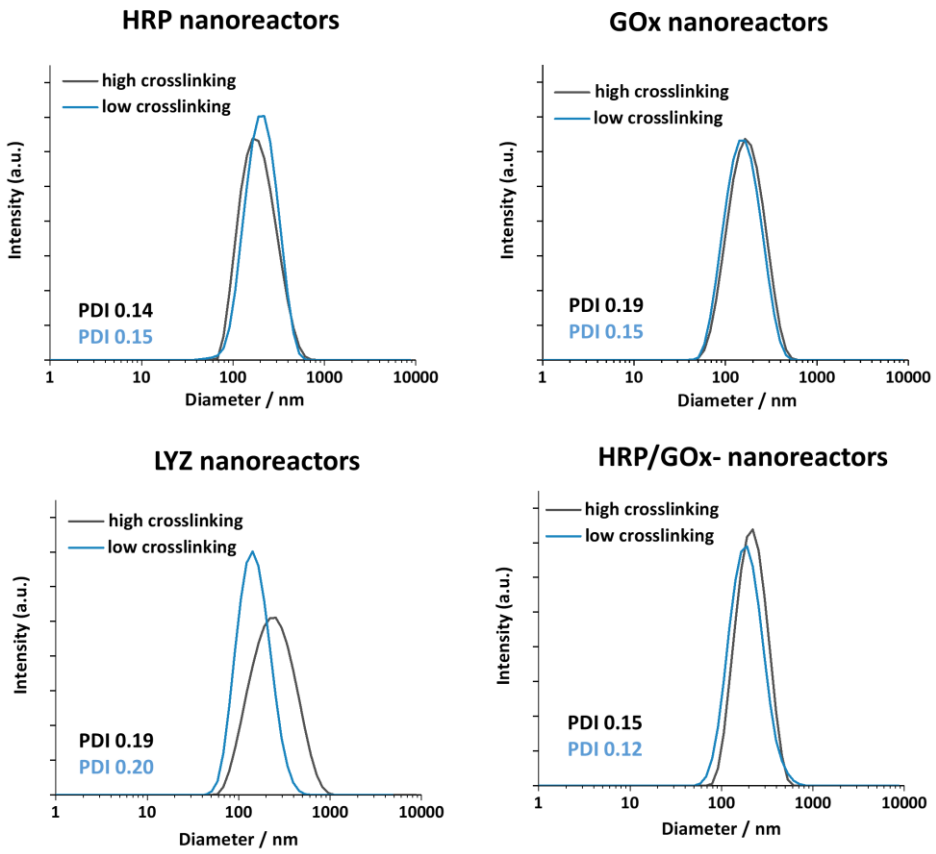



Figure S5. DLS-measurements of the enzyme-nanoreactors in aqueous suspensions.

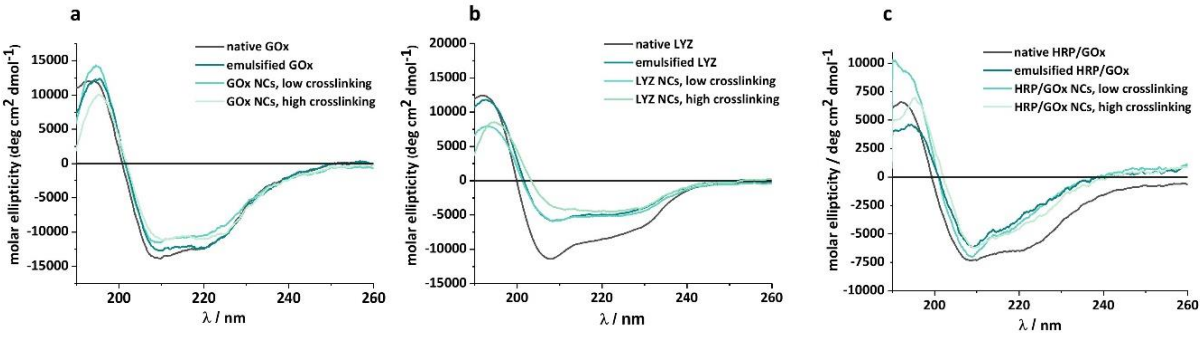



Figure S6. CD-spectra of a) GOx, b) LYZ, c) GOx/HRP after emulsification and crosslinking.

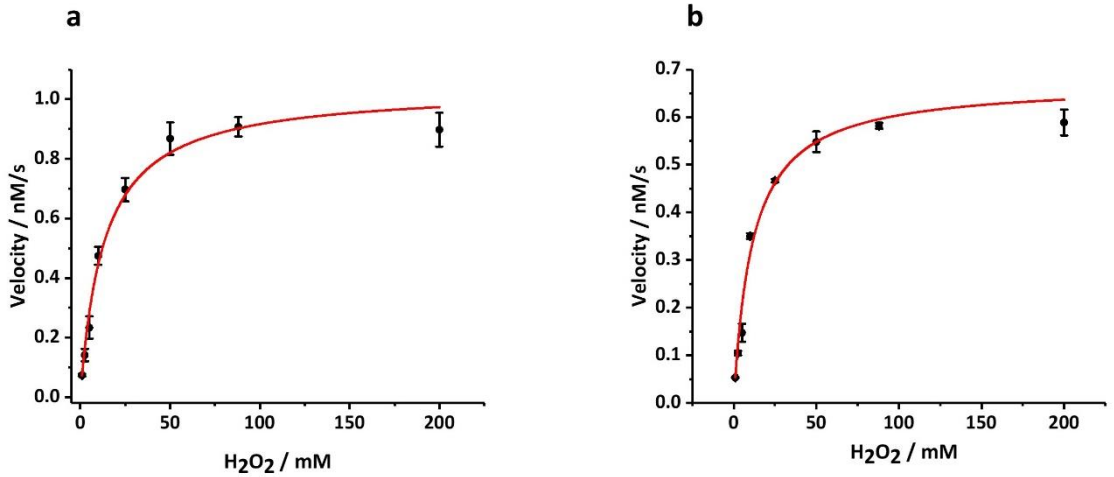
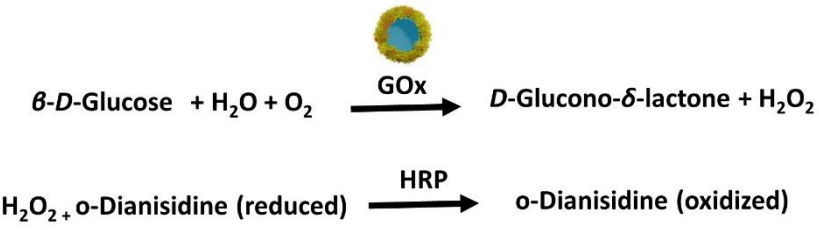
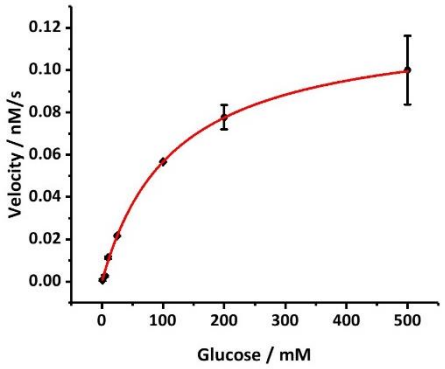



Figure S7. Michaelis Menten plots for a) native HRP, b) emulsified HRP.

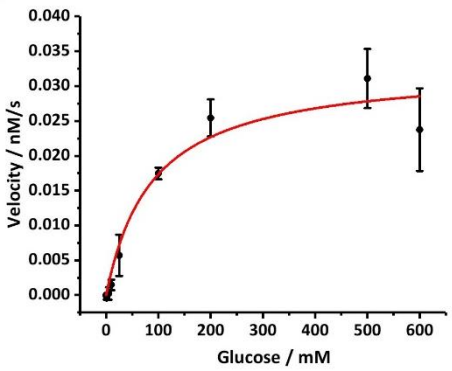
a




b




c




d

| | Native GOx | Emulsified GOx | GOx Nanoreactors |
|---|---|---|---|
| $V_{max}$ (nM/s) | 0.122 ± 0.002 | 0.068 ± 0.003 | 0.032 ± 0.003 |
| $K_m$ (mM) | 116 ± 4 | 78 ± 11 | 89 ± 30 |
| $k_{cat}$ ($S^{-1}$) | 0.00305 ± 0.00005 | 0.00170 ± 0.00008 | 0.00080 ± 0.00008 |
| $k_{cat}/K_m$ ($mM^{-1} s^{-1}$) | 0.00003 ± 0.00001 | 0.00002 ± 0.00001 | 0.0000089 ± 0.0000009 |

Figure S8. a) Oxidation of glucose by glucose oxidase followed by the oxidation of o-Dianisidine by horseradish peroxidase, b) Michaelis Menten plot of native GOx, c) Michaelis Menten plot of GOx- nanoreactors, d) Michaelis Menten parameters for native, emulsified and crosslinked GOx.

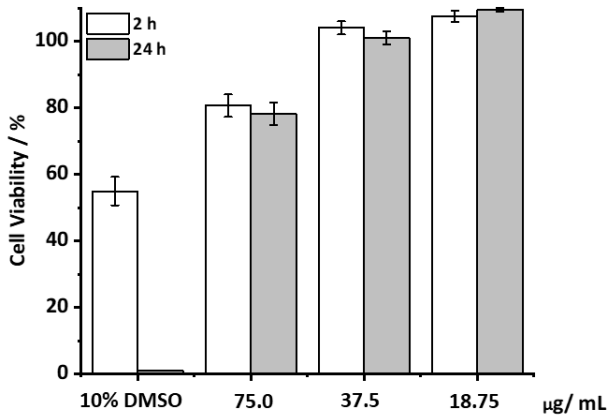



Figure S9. Cytotoxicity of the HRP nanoreactors. A solution containing the nanoreactors was prepared in cell culture media by successive two-fold dilution and cells were seeded in a 96 wells-plate in cell culture media (200 µL). After 2 or 24h, 100 µL of CellTiter Glo® solution was added to the wells and the cell viability was determine by luminescence according to the manufacturer protocol.

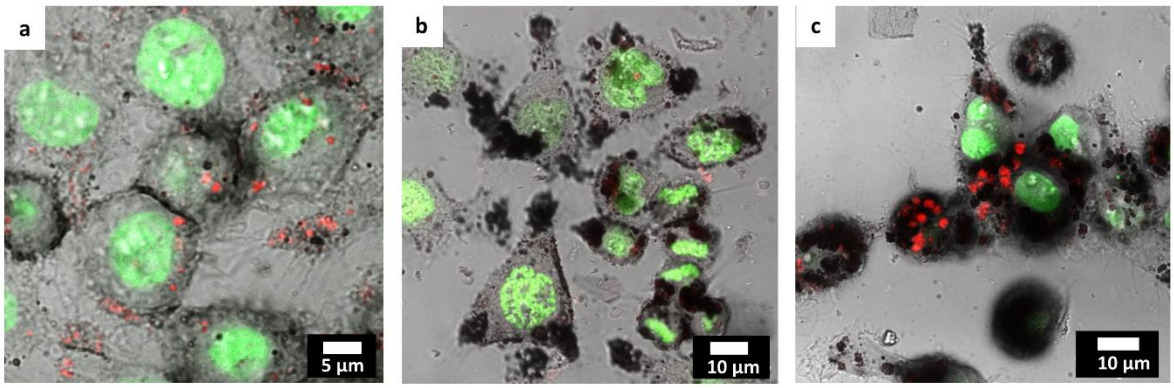



Figure S10. Confocal laser scanning fluorescence microscopy images of RAW264.7 cells (nucleus stained green) incubated with HRP nanoreactors (red) performing intracellular DAB polymerization (black islands) after a) 1 h , b) 4 h, c) 24 h of incubation.

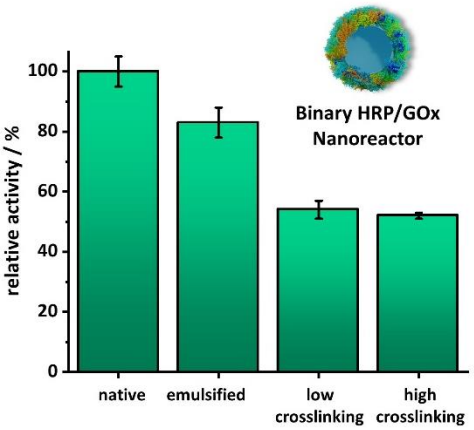



Figure S11. Relative activity of the HRP/GOx-nanoreactors.

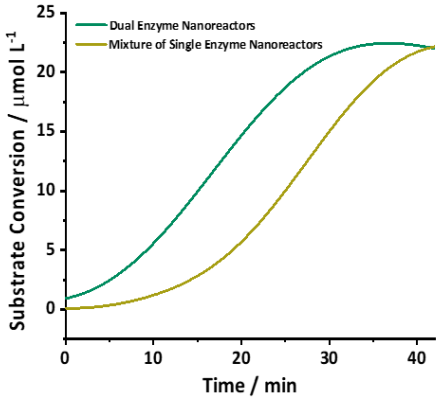



Figure S12. Kinetics of the substrate conversion of the dual HRP/GOx nanoreactors and a mixture of single nanoreactors.

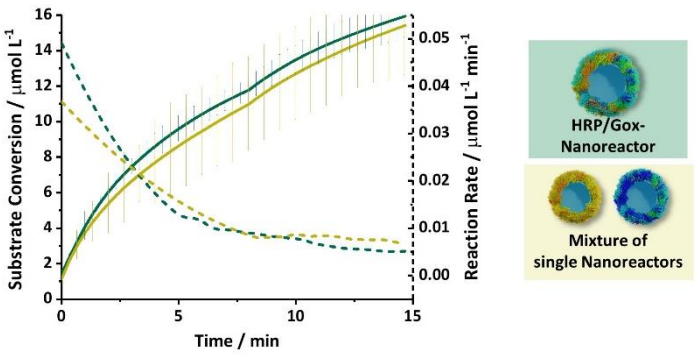



Figure S13. Kinetic of the substrate oxidation in the presence of either binary HRP/GOx-nanoreactors or a mixture of single-enzyme HRP and GOx nanoreactors during the direct oxidation of the substrate with the addition of peroxide.

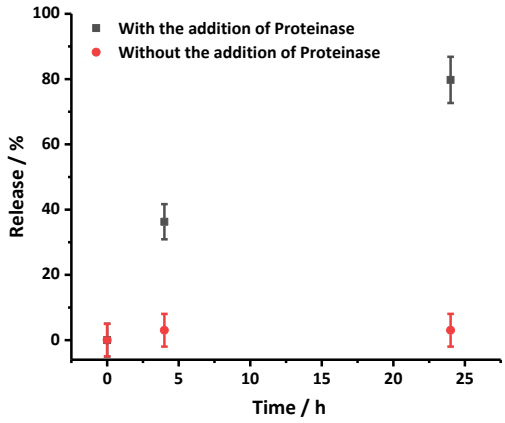



Figure S14. Release of labelled PEG (MW 5000) from crosslinked polypeptide nanocapsules in the presence of Proteinase K (0.1 u/ mg of nanocapsules) and without Proteinase K.

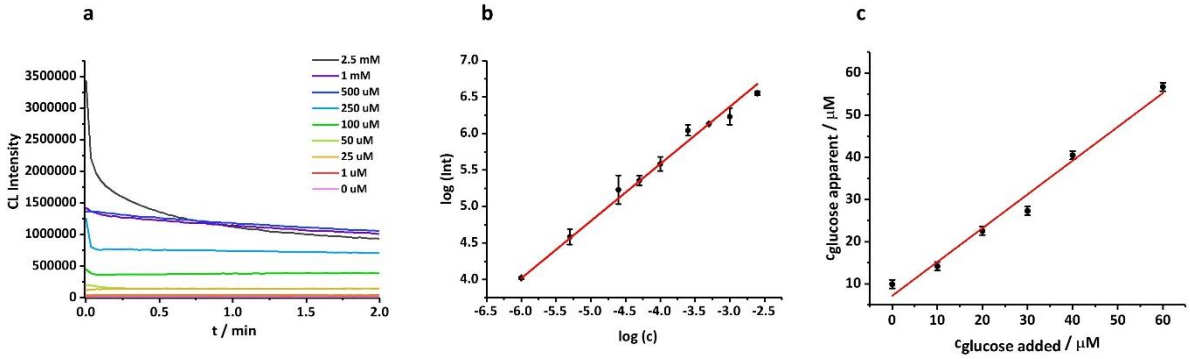



Figure S15. a) Time-dependent chemoluminescence curves of luminol loaded HRP/GOx-nanoreactors for various glucose concentrations, b) calibration curve for glucose sensing with HRP/GOx-luminol-nanoreactors and c) exemplary standard addition experiment for animal serum.

## References

1. H. Zhang, T. Shibata, T. Krawczyk, T. Kabashima, J. Lu, M. K. Lee and M. Kai, *Talanta*, 2009, **79**, 700-705.