

Supplementary Information

Signal processing and generation of bioactive nitric oxide in a model prototissue

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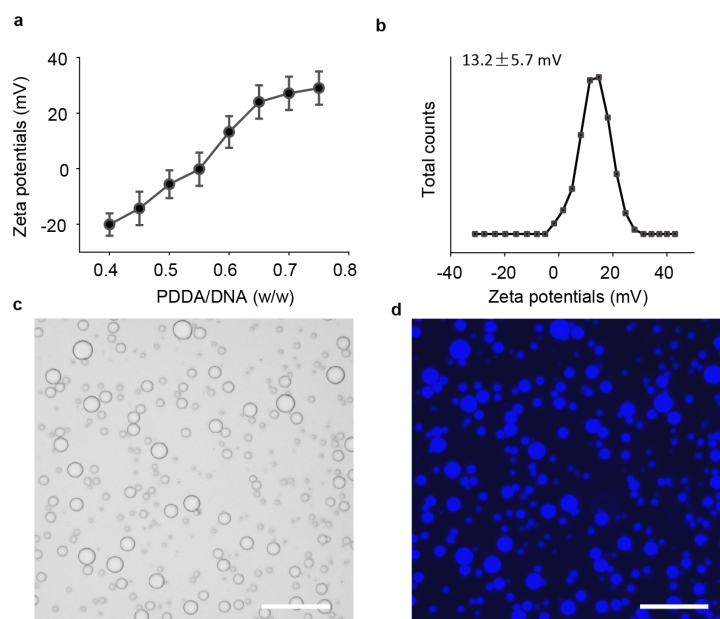
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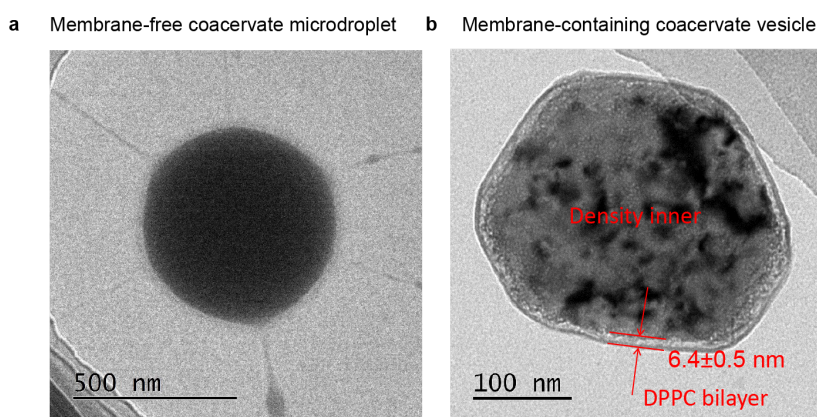
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Supplementary Figures

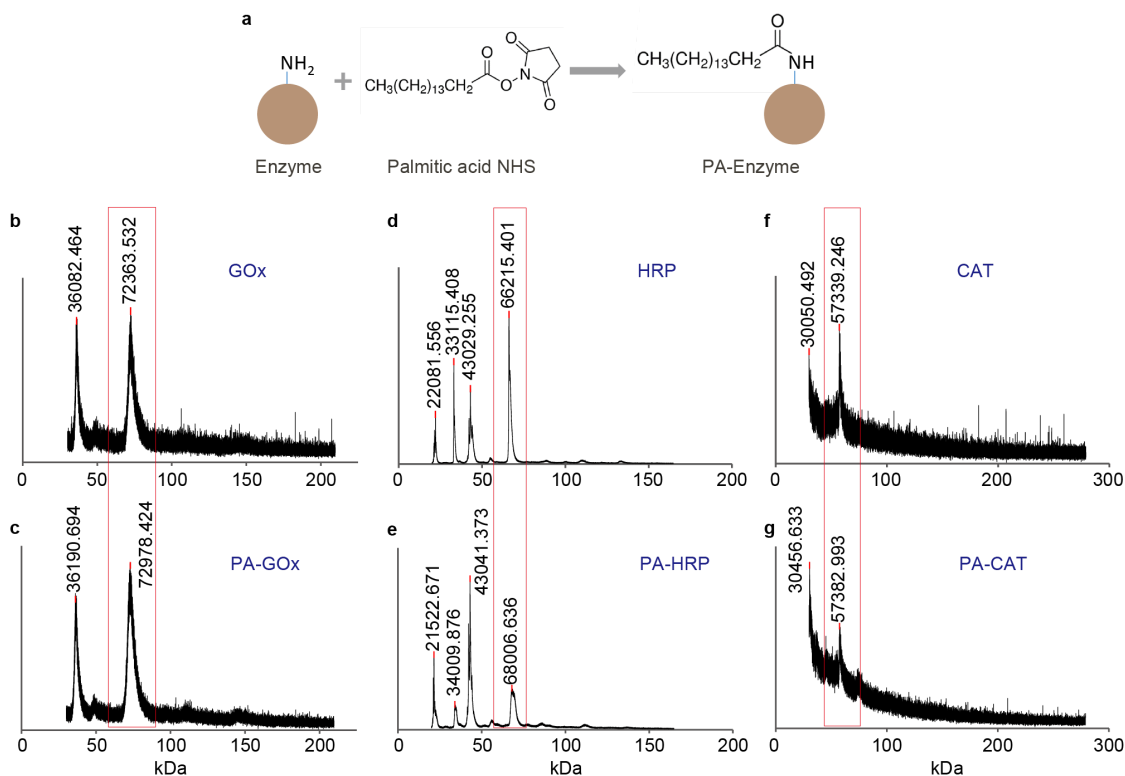


Supplementary Figure 1. Characterization of PDDA/DNA coacervate microdroplets. **(a)** Zeta potentials of PDDA/DNA coacervate microdroplets produced at different polyelectrolyte weight ratios (0.4–0.8, w/w). Data are presented as mean $\pm$ s.d. ($n=3$ independent experiments). **(b)** A typical zeta potential profile of an aqueous dispersion of PDDA/DNA coacervate microdroplets (PDDA=8.0 mg mL⁻¹, DNA=5.0 mg mL⁻¹, PDDA/DNA = 0.6:1, w/w; $\zeta = +13.2 \pm 5.7$ mV) at pH=8. **(c, d)** Optical bright field **(c)** and corresponding blue fluorescence **(d)** images of DNA-binding Hoechst-stained PDDA/DNA coacervate microdroplets (PDDA/DNA = 0.6:1, w/w); scale bar, 10 μ m. 3 times each experiment was repeated independently with similar results **(c, d)**.

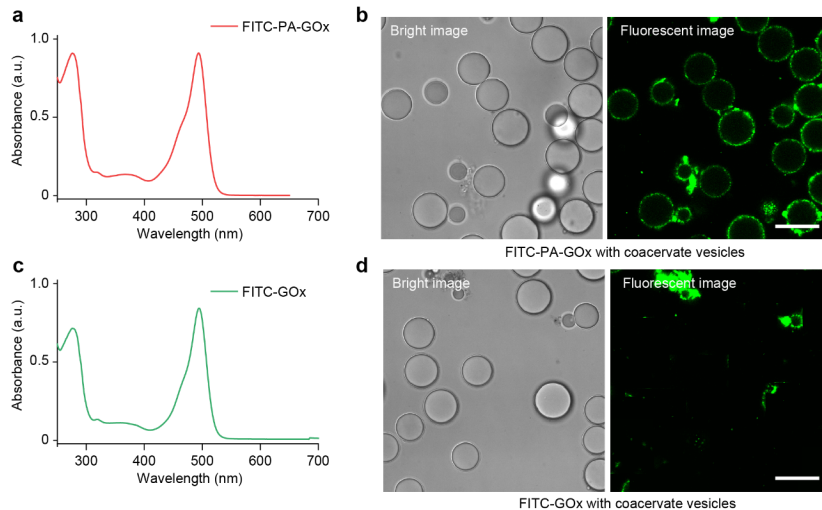


Supplementary Figure 2. TEM images of a single membrane-free coacervate microdroplet **(a)** and membrane-enveloped coacervate vesicle **(b)**. The membrane-free coacervate microdroplet shows homogeneous electron density, while the membrane-enclosed coacervate vesicle shows an electron dense interior and outer membrane structure. The non-spherical shape of the coacervate vesicle is likely to be a drying artefact. The small membrane-free coacervate microdroplets are from the supernatant solution after centrifugation of an aqueous dispersion of coacervate microdroplets (PDDA=8.0 mg mL⁻¹, DNA=5.0 mg mL⁻¹, PDDA/DNA = 0.6:1, w/w) for 5 min at 1,000 rpm. 3 times each experiment was

repeated independently with similar results.

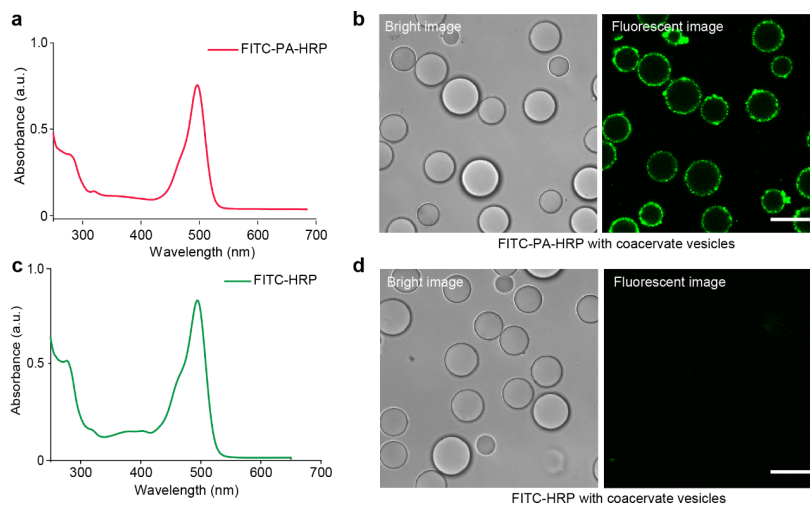


Supplementary Figure 3. Characterization of palmitic acid (PA)-modified enzymes (PA-Enzyme: PA-GOx, PA-HRP, and PA-CAT). **(a)** Reaction scheme. **(b-g)** MALDI-TOF mass spectra of protein enzymes and PA-Enzyme conjugates; GOx **(b)**, PA-GOx **(c)**, HRP **(d)**, PA-HRP **(e)**, CAT **(f)** and PA-CAT **(g)**. The average numbers of hexadecenoic chains attached per enzyme molecule were 4.3 (PA-GOx), 6.2 (PA-HRP) and 1.6 (PA-CAT).

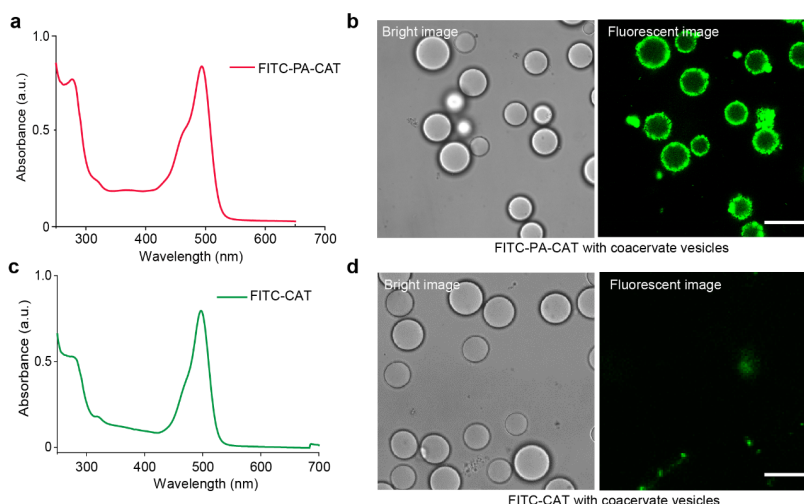


Supplementary Figure 4. **(a, c)** UV-Vis absorbance spectra of FITC-PA-GOx (red line, **a**) and FITC-GOx

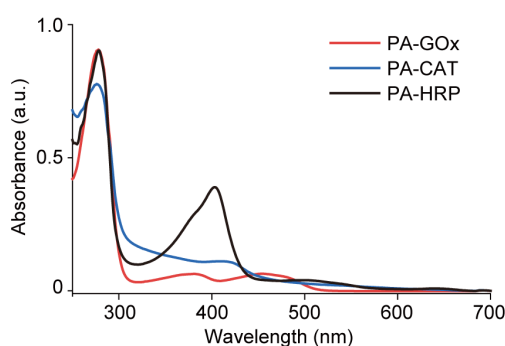
(green line,c). The fluorescein/protein molar ratio (F/P) was determined by measuring the absorbance of the conjugate at 280 nm and 495 nm, and using Supplementary Equation S1). F/P values were 5.3 and 7.0 for FITC-PA-GOx and FITC-GOx, respectively. **(b, d)** Fluorescent imaging of FITC-PA-GOx **(b)**, and FITC-GOx **(d)** in the presence of DOPC-CVs. A high level of FITC-PA-GOx attachment to the coacervate vesicles is observed **(b)**. In contrast, FITC-GOx exhibits minimal interaction with the DOPC-CVs **(d)**. FITC-PA-GOx and FITC-GOx (0.01 mg mL^{-1}) were separately incubated with an aqueous dispersion of DOPC-CVs (8.0 mg mL^{-1}) for 60 min. The coacervate vesicles were imaged directly by a Nikon fluorescence confocal microscope (Ex: blue excitation, Em: FITC channel). Scale bars **(b, d)**, $10 \mu\text{m}$. 3 times each experiment was repeated independently with similar results **(b, d)**.



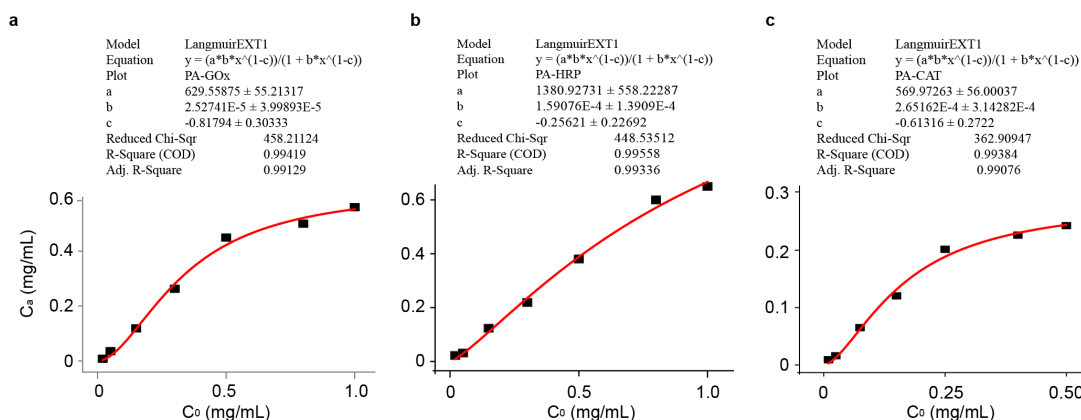
Supplementary Figure 5. **(a, c)** UV-Vis absorbance spectra of FITC-PA-HRP (red line in **a**) and FITC-HRP (green line in **c**). The fluorescein/protein molar ratio (F/P) was determined by measuring the absorbance of the conjugate at 280 nm and 495 nm. F/P values were 4.5 and 5.1 for FITC-PA-HRP and FITC-HRP, respectively. **(b, d)** Fluorescent imaging of FITC-PA-HRP **(b)**, and FITC-HRP **(d)** in the presence of DOPC-CVs. A high level of FITC-PA-HRP attachment to the coacervate vesicles is observed **(b)**. In contrast, FITC-HRP exhibits minimal interaction with the DOPC-CVs **(d)**. FITC-PA-HRP and FITC-HRP (0.01 mg mL^{-1}) were separately incubated with an aqueous dispersion of DOPC-CVs (8.0 mg mL^{-1}) for 60 min. The coacervate vesicles were imaged directly by a Nikon fluorescence confocal microscope (Ex: blue excitation, Em: FITC channel). Scale bars **(b, d)**, $10 \mu\text{m}$. 3 times each experiment was repeated independently with similar results **(b, d)**.



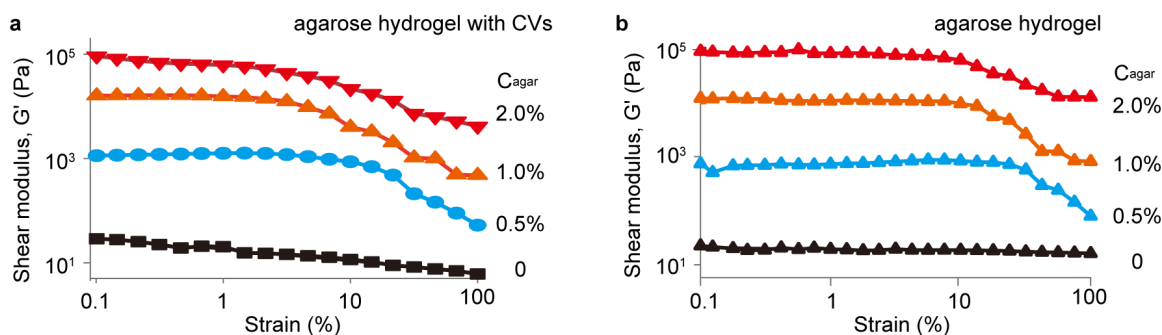
Supplementary Figure 6. (a, c) UV-Vis absorbance spectra of FITC-PA-CAT (red line in a) and FITC-CAT (green line in c). The fluorescein/protein molar ratio (F/P) was determined by measuring the absorbance of the conjugate at 280 nm and 495 nm. F/P values were 2.8 and 3.7 for FITC-PA-CAT and FITC-CAT, respectively. (b, d) Fluorescent imaging of FITC-PA-CAT (b), and FITC-CAT (d) in the presence of DOPC-CVs. A high level of FITC-PA-CAT attachment to the coacervate vesicles is observed (b). In contrast, FITC-CAT exhibits minimal interaction with the DOPC-CVs (d). FITC-PA-CAT and FITC-CAT (0.01 mg mL^{-1}) were separately incubated with an aqueous dispersion of DOPC-CVs (8.0 mg mL^{-1}) for 60 min. The coacervate vesicles were imaged directly by a Nikon fluorescence confocal microscope (Ex: blue excitation, Em: FITC channel). Scale bars (b, d), $10 \mu\text{m}$. 3 times each experiment was repeated independently with similar results (b, d).



Supplementary Figure 7. UV-Vis absorbance spectra of different PA-enzymes. Characteristic absorption peaks are observed at 280 and 450 nm (PA-GOx), 280 and 403 nm (PA-HRP) and at 280 nm and 405 nm (PA-CAT). The extinction coefficients for GOx (PA-GOx) at 280 and 450 nm are 2.67×10^5 and $2.61 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$, respectively.^[1] The extinction coefficient for HRP (PA-HRP) at 403 nm is $1.0 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$.^[1] The extinction coefficient for CAT (PA-CAT) at 405 nm is $3.2 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$.^[2]

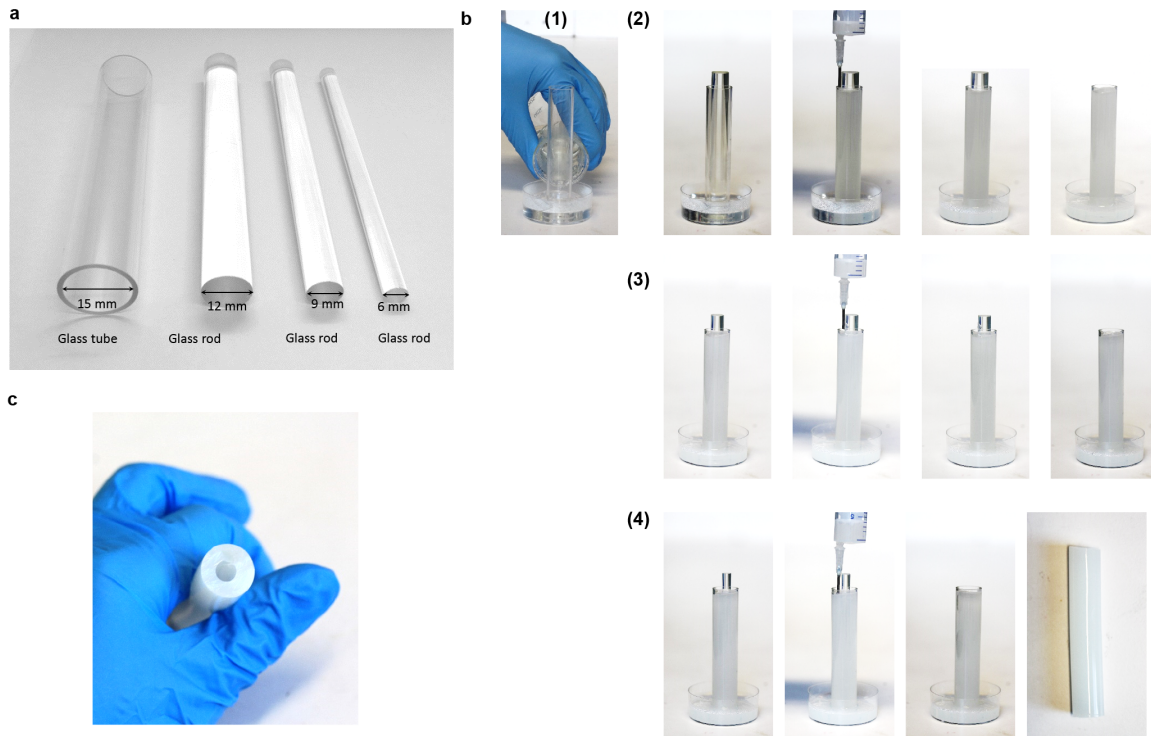


Supplementary Figure 8. Adsorption curves for PA-enzyme attachment to DOPC-CVs. Attachment of the hydrophobized enzymes onto the coacervate droplets is mediated by lipophilic adsorption of the PA-enzymes onto the DOPC lipid membrane of the protocells. Large volumes of the PA-enzymes (PA-GOx, PA-HRP, and PA-CAT) at different concentrations (C_0 , PA-GOx, 0-1.0 mg mL⁻¹; PA-HRP, 0-1.0 mg mL⁻¹; PA-CAT, 0-0.5 mg mL⁻¹) were incubated with dispersions of DOPC-CVs (8.0 mg·mL⁻¹, pH=8.0). After incubation for 60 min, the DOPC-CVs were obtained after centrifugation for 5 min at 500 rpm. The collected enzyme-decorated DOPC-CVs were dissociated after treatment with 2.0 mol L⁻¹ NaCl. The amount of enzyme adsorbed onto the coacervate vesicles (C_a) was determined from the specific absorbance peaks of the different enzymes. The extinction coefficients for GOx at 280 and 450 nm are 2.67×10^5 and 2.61×10^4 M⁻¹ cm⁻¹, respectively.^[1] The extinction coefficient for HRP at 403 nm is 1.0×10^5 M⁻¹ cm⁻¹.^[1] The extinction coefficient for CAT at 405 nm is 3.2×10^5 M⁻¹ cm⁻¹.^[2] The adsorption curves were fitted for each of the PA-enzymes. Non-standard Langmuir isotherms were obtained in each case. A deviation from the standard Langmuir curve is likely due to the uneven sizes of the DOPC-CVs and multi-layer adsorption of the hydrophobized enzymes. (a), 0.50 mg·mL⁻¹ PA-GOx in a dispersion of DOPC-CVs (8.0 mg·mL⁻¹, pH=8.0) resulted in the formation of PA-GOx CVs (loading efficiency = 88%) with ~0.4 mg mL⁻¹ PA-GOx immobilized on the surface of the protocell (0.4 mg mL⁻¹ GOx in 4.0 mg·mL⁻¹ GOx-CVs for most of the experiments). (b), 0.50 mg·mL⁻¹ PA-HRP in a dispersion of DOPC-CVs (8.0 mg·mL⁻¹, pH=8.0) resulted in the formation of PA-HRP CVs (loading efficiency = 82%) with ~0.4 mg mL⁻¹ PA-HRP immobilized on the surface of protocell (0.4 mg mL⁻¹ HRP in 4.0 mg·mL⁻¹ HRP-CVs for most of the experiments). (c), 0.25 mg·mL⁻¹ PA-CAT in a dispersion of DOPC-CVs (8.0 mg·mL⁻¹, pH=8.0) resulted in the formation of PA-CAT CVs (loading efficiency = 84%) with ~0.2 mg mL⁻¹ PA-CAT immobilized on the surface of protocell (0.2 mg mL⁻¹ CAT in 4.0 mg·mL⁻¹ CAT-CVs for most of the experiments).



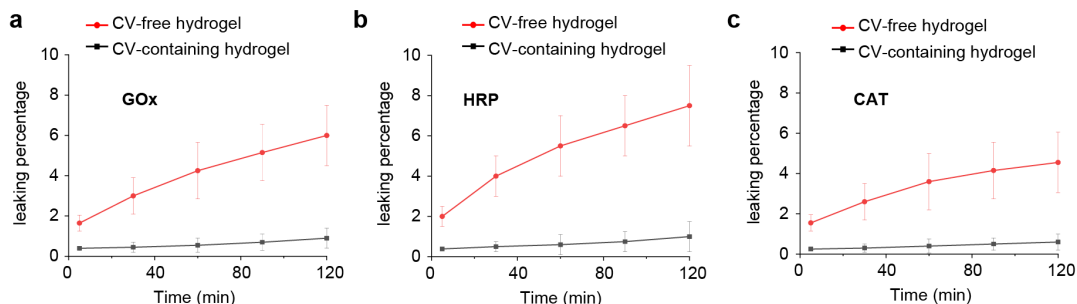
Supplementary Figure 9. Rheometry dynamic strain sweep of the storage modulus (G') for protocell-loaded hydrogels (a) and protocell-free hydrogels (b), prepared at different agarose

concentrations (red, 2.0 wt%; orange, 1.0 wt%; blue, 0.5 wt%; black 0 wt%); frequency, 1 rad·s⁻¹.

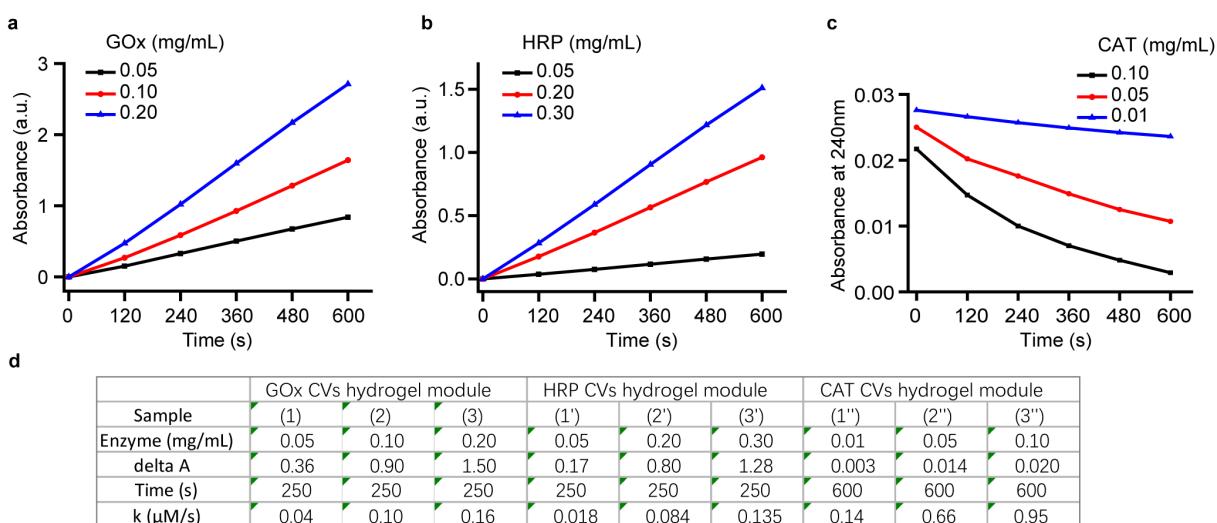


Supplementary Figure 10. Assembly of a tubular prototissue vessel from a three-layer concentric arrangement of protocell-loaded hydrogel modules. **(a)** Photograph of a 15 mm-diameter glass tube, and 12 mm-, 9 mm- and 6 mm-diameter glass rods used as physical templates for the preparation of the modular tubular prototissue vessel. Length of glass tube, 100 mm; glass rod length, 90 mm. **(b)** The prototissue vessel was assembled from three concentric hydrogel modules containing GOx-CVs, HRP-CVs, or CAT-CVs arranged respectively from the exterior to interior of the vessel using a gel perfusion method involving four steps. **Step (1):** The glass tube is sealed at one end by a plastic stopper and stood upright in an agarose hydrogel matrix. **Step (2):** The 12 mm-diameter glass rod is placed in the center of the glass tube and 10 mL of a hot (40 °C) aqueous agarose suspension containing PA-GOx-CVs added to fill the empty space between the glass tube and inserted glass rod, and then cooled to 4 °C for 30 min in a fridge to induce hydrogelation. The 12 mm-diameter glass rod is then carefully removed to produce a tubular outer layer comprising a hydrogel/PA-GOx-CV module. **Step (3):** The 9 mm-diameter rod is placed into the center of the glass tube, and 7 mL of a hot (40 °C) aqueous agarose suspension containing PA-HRP-CVs is added to fill the empty space between the outer PA-GOx-CV-containing hydrogel layer and inserted glass rod, and then cooled to 4 °C for 30 min in a fridge to induce hydrogelation. The 9 mm-diameter glass rod is then carefully removed to produce a tubular middle layer consisting of a hydrogel/PA-HRP-CV module. **Step (4):** The 6 mm-diameter glass rod is placed in the center of the glass tube and 2 mL of a hot (40 °C) aqueous agarose suspension containing PA-CAT-CVs is added to fill the empty space between the middle HRP-GOx-CV -containing hydrogel layer and inserted glass rod, and then cooled to 4 °C for 30 min in a fridge to induce hydrogelation. The 6 mm-diameter glass rod is then carefully removed to produce a tubular inner layer consisting of a hydrogel/CAT-HRP-CV module. Finally, the resulting tubular three-layer prototissue vessel is obtained from the glass tube as a self-supporting material by removing the plastic stopper and gently removing the 15 mm-diameter glass tube. **(c)** Photograph showing the prototissue vessel viewed end-on. The three-layer hydrogel module arrangement has an inner channel with a diameter of 6 mm, and an outer diameter of 15 mm. Each

hydrogel layer is 1.5 mm in thickness and contains single populations of GOx, HRP or CAT-decorated DOPC-CVs. The prepared prototissue vessel was stored in a fridge.

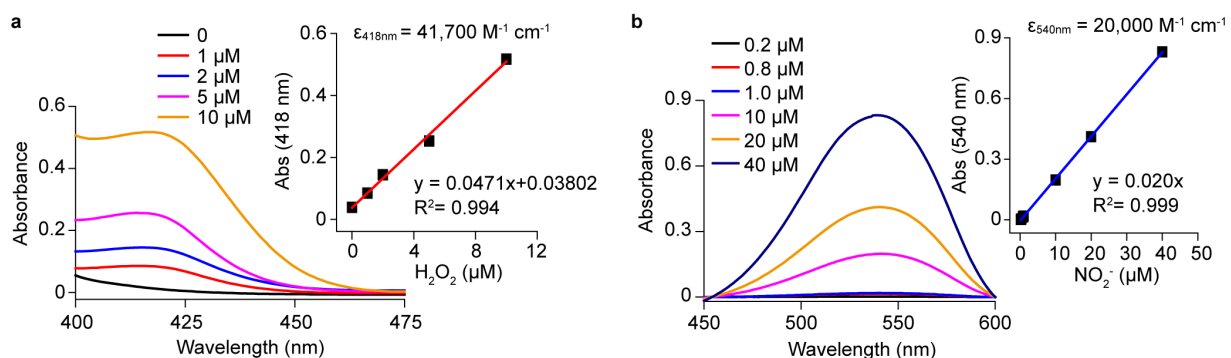


Supplementary Figure 11. Enzyme leakage experiments. Time-dependent plots of enzyme concentrations in the external solution (% leakage) for coacervate vesicle (CV)-free hydrogels containing immobilized free hydrophobized enzymes (red line) or hydrogels containing CVs with surface attached hydrophobized enzymes; (a) PA-GOx, (b) PA-HRP, (c) PA-CAT. The bulk hydrogels were immersed in water and the leakage determined from UV-Vis absorbance spectra of the different enzymes as shown in **Supplementary Figure 7**. Leakage of the PA-enzymes was inhibited for the CV-containing hydrogel matrix, which was attributed to attachment of the enzymes on the membrane surface of the CVs. Data are presented as mean $\pm$ s.d. (n= 3 independent experiments).

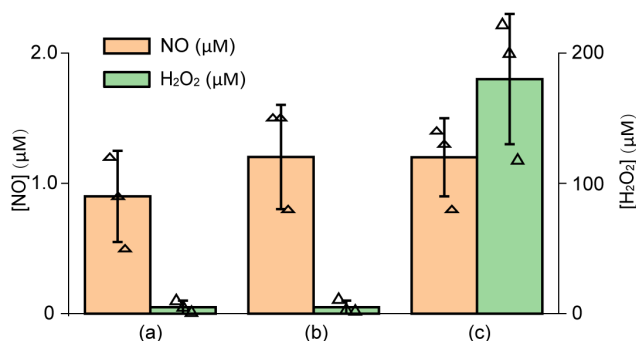


Supplementary Figure 12. Activity assays for different enzyme-CV hydrogel modules. (a) GOx activity in GOx-CV hydrogels; GOx-CV-containing hydrogel modules (50 μ L) loaded with 0.05 mg mL⁻¹ GOx (black), 0.10 mg mL⁻¹ GOx (red), or 0.20 mg mL⁻¹ GOx (blue) were placed in 1 mL PBS buffer (150 mM, pH=7.4) along with HRP (0.02 mg mL⁻¹) and ABTS (4.0 mM). Glucose (10 mM) was then added and time-dependent changes in absorption associated with the oxidation product (ABTS-diradical) at 418 nm ($\epsilon_{418} = 36,800 \text{ M}^{-1} \text{ cm}^{-1}$) in buffer were recorded. (b) HRP activity in HRP-CV hydrogels; HRP-CVs containing hydrogel modules (50 μ L) loaded with 0.05 mg mL⁻¹ HRP (black), 0.20 mg mL⁻¹ HRP (red) or 0.30 mg mL⁻¹ HRP (blue) were placed in 1 mL PBS buffer (150 mM, pH=7.4) along with ABTS (4.0 mM). H₂O₂ (10 mM) was then added and time-dependent changes in absorption associated with the oxidation product

(ABTS-diradical) at 418 nm ($\epsilon_{418} = 36,800 \text{ M}^{-1} \text{ cm}^{-1}$) in buffer were recorded. (c) CAT activity in CAT-CV hydrogels; CAT-CVs containing hydrogel modules (50 μL) loaded with 0.01 mg mL^{-1} CAT (blue), 0.05 mg mL^{-1} CAT (red) or 0.10 mg mL^{-1} CAT (black) were placed in 1 mL PBS buffer (150 mM, pH=7.4). H_2O_2 (10 mM) was then added and time-dependent changes in absorption associated with the change of H_2O_2 at 240 nm ($\epsilon_{240} = 35 \text{ M}^{-1} \text{ cm}^{-1}$)^[3] in buffer were recorded. (d) Table showing the average reaction rates (k) for different enzyme-CV-containing hydrogel modules under different experimental conditions, as shown in (a-c). The average reaction rates were estimated from H_2O_2 production or consumption in a given time interval.

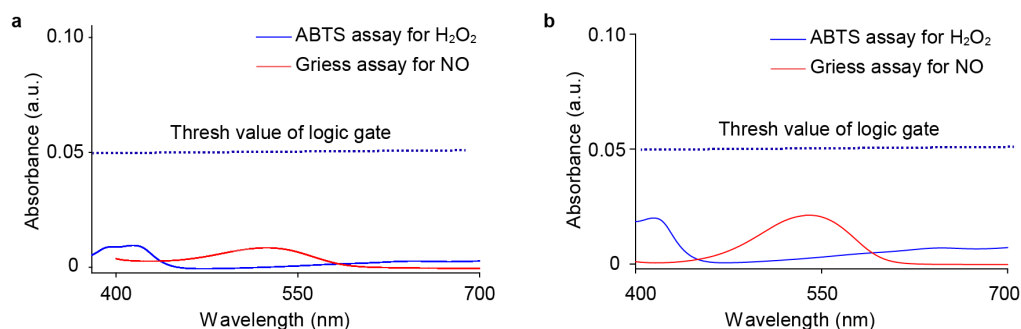


Supplementary Figure 13. Calibration curves for (a) ABTS colorimetric assay of H_2O_2 and (b) Griess colorimetric assay of NO . (a) Different concentrations of H_2O_2 (0-10 μM) were added into ABTS solution (2 mM) in the presence of HRP (100 nM) and incubated for 10 min. (b) Different concentrations of nitrite (NO_2^-) (0-40 μM) were mixed with equal volume of 1 $\times$ Griess agent (40 mg/ml) and incubated for 15 min.

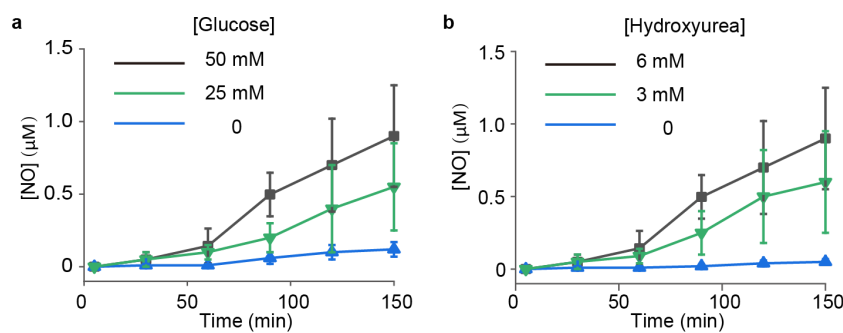


Supplementary Figure 14. Effect of changing the loading capacity in the middle (HRP-CV) and inner (CAT-CV) layers on prototissue model-mediated production of H_2O_2 and NO . Prototissue vessel composition: (a) 0.2 $\text{mg}\cdot\text{mL}^{-1}$ GOx (GOx-CV outer layer), 0.2 $\text{mg}\cdot\text{mL}^{-1}$ HRP (HRP-CV middle layer) and 0.1 $\text{mg}\cdot\text{mL}^{-1}$ CAT (CAT-CV inner layer); (b) 0.2 $\text{mg}\cdot\text{mL}^{-1}$ GOx (GOx-CV layer), 0.3 $\text{mg}\cdot\text{mL}^{-1}$ HRP (HRP-CV layer) and 0.1 $\text{mg}\cdot\text{mL}^{-1}$ CAT (CAT-CV layer); (c) 0.2 $\text{mg}\cdot\text{mL}^{-1}$ GOx (GOx-CV layer), 0.2 $\text{mg}\cdot\text{mL}^{-1}$ HRP (HRP-CV layer) and 0.01 $\text{mg}\cdot\text{mL}^{-1}$ CAT (CAT-CV layer). In each case 50 mM glucose and 6 mM hydroxyurea were added to the vessel exterior and the amounts of NO and H_2O_2 measured in the lumen were determined after 150

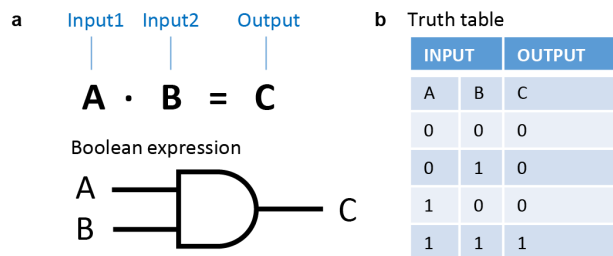
min. Reducing the catalase concentration in the inner layer has minimal effect on NO production but increases the amount of excess H_2O_2 produced by the micro-reactor. Data are presented as mean $\pm$ s.d. (n= 3 independent experiments).



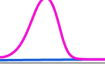
Supplementary Figure 15. Absorbance spectra recorded from HRP-mediated ABTS colorimetric assay for H_2O_2 (blue) and Griess reagent assay for NO (red). (a) A homogeneous mixture of GOx-CVs, HRP-CVs, and CAT-CVs in bulk solution. (b) Uniform distribution of the GOx-CVs, HRP-CVs, and CAT-CVs in a prototissue vessel. No apparent production of H_2O_2 and NO is observed in both cases. (GOx-CVs with $0.2 \text{ mg}\cdot\text{mL}^{-1}$ GOx, HRP-CVs with $0.2 \text{ mg}\cdot\text{mL}^{-1}$ HRP, and CAT-CVs with $0.1 \text{ mg}\cdot\text{mL}^{-1}$ CAT).



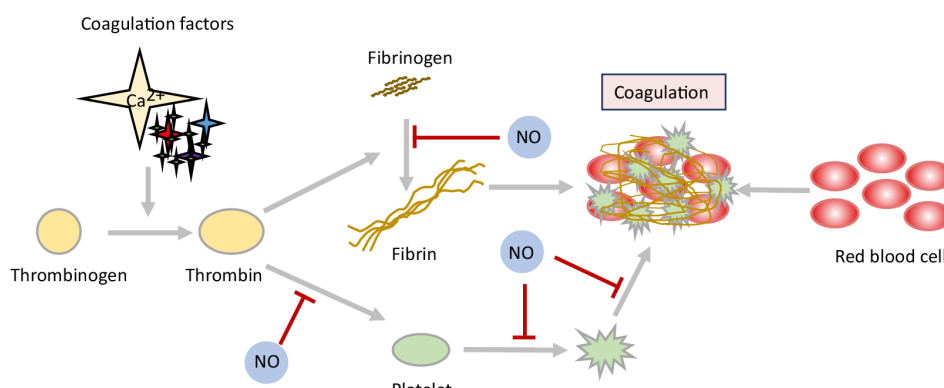
Supplementary Figure 16. Glucose-dependent (a) and hydroxyurea-dependent (b) NO generation in the interior lumen of the prototissue vessel. (a) addition of 0 -50 mM (black line, 50 mM; green line, 25 mM; blue line, 0 mM) glucose and 6 mM hydroxyurea to the exterior of the prototissue vessel. (b) addition of 50 mM glucose and 0-6 mM (black line, 6 mM; green line, 3 mM; blue line 0 mM) hydroxyurea to the exterior of the prototissue vessel. The NO concentrations were determined by NO-specific microelectrodes. Prototissue vessel composition: $0.2 \text{ mg}\cdot\text{mL}^{-1}$ GOx in GOx-CV layer, $0.2 \text{ mg}\cdot\text{mL}^{-1}$ HRP in HRP-CV layer, and $0.1 \text{ mg}\cdot\text{mL}^{-1}$ CAT in the CAT-CV layer. Data are presented as mean $\pm$ s.d. (n= 3 independent experiments).



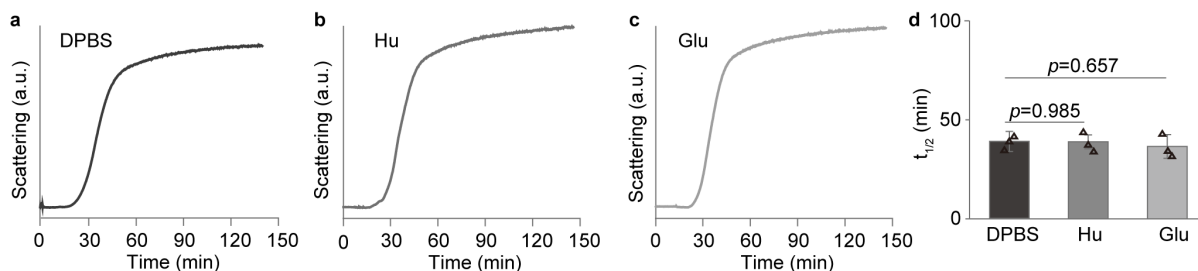
Supplementary Figure 17. (a) Boolean expression of an AND logic gate, and (b) truth table. A threshold absorption value above 0.04 at 540 nm (Greiss reagent, purple coloration with NO) was used as a verifiable (0,1) signal output.

Case	INPUT Exterior	SIGNAL PROCESSING Modular prototissue						OUTPUT Interior	INPUT	OUTPUT	Abs. spectra	Colorimetry	
									Glu, Hu	H ₂ O ₂ NO		ABTS	Greiss
I	Glu Hu							NO	1, 1	0, 1			
V	Glu Hu							H ₂ O ₂ NO	1, 1	1, 1			
VI	Glu Hu								1, 1	0, 0			
VII	Glu Hu								1, 1	0, 0			
VIII	Glu Hu							H ₂ O ₂	1, 1	1, 0			
IX	Glu Hu								1, 1	0, 0			
X	Glu Hu								1, 1	0, 0			

Supplementary Figure 18. Spatial organization and signal processing in dual input prototissue vessels. Seven different three-layer sequence arrangements of enzyme-CV hydrogel modules (case I (standard configuration; alternative configurations (cases V-X)) operating under dual substrate inputs are shown. Outputs in the interior lumen include NO (II), H₂O₂ (VIII), or mixtures of H₂O₂ and NO (V), depending on how the spatial sequence influences enzyme-mediated processing within the tubular micro-reactor. No outputs in the lumen are recorded for cases VI, VII, IX and X. Labels and column representations are as shown in Figure 3h of the main manuscript. Reaction conditions: Glu (50 mM); Hu (6 mM); GOx (GOx-CV layer, 0.2 mg·mL⁻¹); HRP (HRP-CV layer, 0.2 mg·mL⁻¹); CAT (CAT-CV layer, 0.1 mg·mL⁻¹).



Supplementary Figure 19. Biochemical pathway for the NO-mediated anticoagulation in blood plasma. The presence of NO inhibits fibrin formation as well as thrombin-mediated platelet activation, both of which normally induce blood coagulation. Solid arrows indicate activation; bar-headed arrows indicate inhibition.^[4,5]



Supplementary Figure 20. Control experiments. (a-c) Real-time light scattering of blood plasma anticoagulation in the central lumen of the prototissue vessel after incubation in the lumen for 50 min. (a) No substrates in the exterior (DPBS buffer as control), (b) Hu (6 mM) in the exterior, (c) Glu (50 mM) in the exterior. Coagulation is activated upon addition of CaCl_2 (0.02 mol L^{-1}) after incubation in the lumen for 50 min. Prototissue vessel composition: GOx (GOx-CV layer, $0.2 \text{ mg}\cdot\text{mL}^{-1}$); HRP (HRP-CV layer, $0.2 \text{ mg}\cdot\text{mL}^{-1}$); CAT (CAT-CV layer, $0.1 \text{ mg}\cdot\text{mL}^{-1}$). (d) Plots of half-life for plasma coagulation ($t_{1/2}$) in the lumen for samples (a-c). $t_{1/2}$ was obtained through fitting of a single exponential equation. Data are presented as mean $\pm$ s.d., two-sided Student's t-test ($n=3$ independent experiments).

Supplementary References

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