Supporting Information

A Versatile Chemoenzymatic Nanoreactor that Mimics NAD(P)H Oxidase for the In Situ Regeneration of Cofactors

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1. Materials
Vinyl imidazole (VIm, MW 94.11 g mol\(^{-1}\), d 1.039 g ml\(^{-1}\), >99 %, Sigma), N-hydroxyethyl acrylamide (HEAAm, MW 115.13 g mol\(^{-1}\), 97 %, Sigma), N,N'-Methylenebisacrylamide (BIS, MW 157.17 g mol\(^{-1}\), 99 %, Sigma), ammonium persulfate (APS, 98%, Sigma), sucrose (>99.5 %, Sigma), N,N,N',N'-Tetramethylethlenediamine (TEMED, d 0.775 g ml\(^{-1}\), 99 %, Sigma), hemin chloride (>98 %, ACROS organics), D-glucose (≥99.5 %, Sigma), N,N,N',N'-Tetramethylethlenediamine (TEMED, d 0.775 g ml\(^{-1}\), 99 %, Sigma), hemin chloride (≥98 %, ACROS organics), D-glucose (≥99.5 %, Sigma), hydrogen peroxide (30 %, v/v, in water, Carl Roth), benzyl alcohol (99 %, ABCR), sodium L-lactate (>98 %, Alfa Aesar), sodium pyruvate (>99 %, Sigma), \(\beta\)-Nicotinamide adenine dinucleotide (NAD\(^+\), >97 %, PanReac AppliChem), reduced \(\beta\)-Nicotinamide adenine dinucleotide (NADH, >9 %, PanReac AppliChem), reduced \(\beta\)-Nicotinamide adenine dinucleotide phosphate (NADPH, >95 %, PanReac AppliChem), 1-benzyl-1,4-dihydrionicotinamide (BNAH, 95 %, ABCR), glucose oxidase from \textit{Aspergillus niger} (GOx, MW of 160 kDa, E.C. 1.1.3.4, 108 U mg\(^{-1}\), Amresco), catalase from bovine liver (MW of 250 kDa, E.C. 1.11.1.6., >10,000 U mg\(^{-1}\), Sigma), horseradish peroxidase (HRP, MW of 44 kDa, E.C. 1.11.1.7, >1100 U mg\(^{-1}\), Alfa Aesar), L-lactate dehydrogenase recombinant from \textit{Escherichia coli} (LDH, MW of 45.3 kDa, E.C. 1.1.1.27, >90 U mg\(^{-1}\), Sigma). Alcohol dehydrogenase from \textit{Bacillus (Geobacillus) stearothermophilus} (BsADH, MW of 147 kDa, E.C. 1.1.1.1) was produced in the laboratory. Acetonitrile (MeCN, VWR), 2-isopropanol (IprOH, PanReac AppliChem), dimethyl sulfoxide (DMSO, Sigma), and methanol (MeOH, Fischer) of analytical grade were used. All buffers were freshly prepared and filtered through 0.22 \(\mu\)m filters prior to use. Stock solutions of sodium phosphate 300 mM and pH 7.3 (7.988 g l\(^{-1}\) of Na\(_2\)HPO\(_4\)·H\(_2\)O and 64.89 g l\(^{-1}\) of Na\(_2\)HPO·7H\(_2\)O) and pH 8.0 (1.868 g l\(^{-1}\) of NaH\(_2\)PO\(_4\)·H\(_2\)O and 76.77 g l\(^{-1}\) of Na\(_2\)HPO·7H\(_2\)O) were prepared. Working solutions were prepared by the dilution of the stocks with water and the pH was adjusted with H\(_3\)PO\(_4\) or NaOH if needed. Filter membranes (30 kDa MWCO, Amicon®Ultra 0.5 ml and 15 ml) and dialysis membranes (10 KDa MWCO, SnakeSkin, ThermoFisher) were used for concentration and purification of the nanogels and the BsADH enzyme. HisPur NTA-Ni resin (Thermostifer) was used for the purification of BsADH.[1]

2. Instrumentation
2.1. Dynamic Light Scattering (DLS)
Dynamic Light Scattering measurements were performed on a Malvern Zetasizer Nano ZS. The dispersant refractive index was set at 1.33 and the viscosity at 1.0041 cP. The experiments were performed at 22 °C and 15 readouts were taken in three independent measurements for each sample prepared in sodium
phosphate buffer (30 mM, pH 8.0). Free GOx and GOx nanogels were measured at 1.6 µM. Heterogeneous NanoNOx200 sample was measured as obtained after the synthesis and purification protocol, at 0.69 µM.

2.2. Attenuated Total Reflection Fourier Transform Infrared Spectroscopy (ATR-FTIR)

The FTIR spectra were measured with a PerkinElmer Frontier spectrometer equipped with an ATR sampling stage. 2 µl of each sample (1.6 µM stocks of the nanogels and the free enzyme in water) were drop-casted on silicon wafers and subsequently dried at room atmosphere. The dropping process was repeated 3 times to obtain a readable sample. All spectra were measured with 20 scans from 600 to 4000 cm⁻¹ with a resolution of 4 cm⁻¹. Each sample was measured three times and the results were averaged.

2.3. X-ray Photoelectron Spectroscopy (XPS)

XPS experiments were performed in a SPECS Sage HR 100 spectrometer with a non-monochromatic X-ray source (Aluminum Kα line of 1486.6 eV energy and 300 W), placed perpendicular to the analyzer axis and calibrated using the 3d₅/₂ line of Ag with a full width at half maximum (FWHM) of 1.1 eV. The selected resolution for the spectra was 15 eV of Pass Energy and 0.15 eV/step. All measurements were made in an ultra-high vacuum (UHV) chamber at a pressure around 8·10⁻⁸ mbar. CasaXPS was used for deconvoluting data. C 1s peak from C-C sp³ of adventitious carbon was employed for charge correction fixing its binding energy (BE) to 284.8 eV. A Shirley background was applied. Peaks were assigned according to NIST DATABASE and xpsfitting.com. Survey spectrum was employed to calculate relative atomic composition in the samples. Samples were prepared in the same way as for ATR-FTIR.

2.4. Scanning Electron Microscopy (SEM) and Energy-Dispersive X-ray spectroscopy (EDX)

The Helios NanoLab 450S (FEI) scanning electron microscope was used to analyze the size and morphology of the samples. The microscope was operated in immersion mode with an acceleration voltage of 5.0 kV and a current of 0.2 nA. The working distance was set to 3 mm. Size estimation was performed using the microscope measurement module. The EDX module (Octane Elect EDS, AMETEK) was used to conduct the elemental analysis. To prepare the samples, a drop (10 µl of a stock solution of the nanogels of 1.6 µM in water) was spin-coated onto a 1 x 1 mm silicon wafer at 30 rps for 30 s. The silicon wafers were previously cleaned with acetone and isopropanol in an ultrasonic cleaner.

2.5. UV-Vis spectroscopy

UV-Vis spectroscopy studies were performed using an Epoch 2 Microplate Spectrophotometer (BioTek). UV-Vis spectra of the samples (6.2 nM in 5 mM Tris-HCl buffer, pH 7.0) were monitored over the spectral window from 250 nm to 700 nm with a step size of 2 nm. The NanoDrop™ 2000 (Thermo Scientific™) spectrophotometer was used for the NanoNOx recycling experiments.
2.6. *Circular Dichroism (CD)*

CD spectra were performed with the Jasco J-815CD spectropolarimeter. CD spectra were acquired in a 1 mm pathlength quartz cuvette. All CD spectra were recorded with a bandwidth of 1 nm increments, a mean time of 10 s, and 10 accumulations over a wavelength range of 190-260 nm. Samples were prepared in Tris-HCl buffer (5 mM, pH 7) at a final concentration of 1.25 µM.

2.7. *Proton Nuclear Magnetic Resonance (¹H NMR)*

NMR spectra were obtained from a Bruker fourier 300 MHz. A total volume of 300 µL solution was prepared containing 10% DMSO in D₂O in a Norell® Select Series™ 5 mm NMR spectroscopy tube. Samples/reaction mixtures containing the cofactors, i.e., NAD(P) and BNA (0.5 - 1 mM) were filtered with 30 kDa MWCO, Amicon®Ultra (0.5 ml) to remove the catalysts from solution and mixed with 10 % (v/v) of D₂O to reach a final volume of 300 µL. The spectra were acquired with a 300 MHz radiofrequency pulse, room temperature, 128 scans, 12 min acquisition time, and 1 s relaxation delay. Peaks were referenced against D₂O (δ = 4.79 ppm).

2.8. *High performance liquid chromatography (HPLC)*

HPLC measurements were carried out on a HPLC Nexera Lite (SHIMADZU), equipped by a SIL-40C autosampler (SHIMADZU), ACE 3 C18-PFP (length: 150 mm, inner diameter: 4.6 mm) column, and SPD-M40 photo diode array detector (SHIMADZU). Reaction samples containing benzyl alcohol (BnOH) were filtered (30 KDa MWCO, 10 min, 10000 rcf) and diluted (1:10) in methanol (final concentration of 10 %, v/v) prior to injection. The injection volume was 5 µl and the flow rate of the mobile phase (35:65; MeOH:H₂O) was set at 1 ml min⁻¹. The column oven temperature was set to 40 °C and the UV detector wavelength was fixed at 215 nm. Consistent results were obtained by measuring the remaining BnOH in the reaction. Conversely, the monitor of benzaldehyde (product of the reaction) gave several inaccuracies in the calculation of the conversions due to its high volatility in the reaction conditions.

L-lactate/pyruvate samples were filtered (30 KDa MWCO, 10 min, 10000 rcf) and diluted (1:10) in the mobile phase (4:96 acetonitrile:phosphate (0.12 M, pH 1.5), phosphate solution composed of 49.64 mg ml⁻¹ of Na₂HPO₄·12H₂O and 2.4 % H₃PO₄) before injection. The injection volume was 20 µl and the flow rate of the mobile phase was set at 0.3 ml min⁻¹. The column oven temperature was set to 35 °C and the UV detector wavelength was fixed at 210 nm.
3. Cell culture and BsADH purification

A clone containing the *adh* gene was kindly provided by Prof. Fernando López-Gallego (CICBiomagune, Spain). BsADH was overexpressed in competent *Escherichia coli* BL21 following a protocol optimized in the laboratory.[1] Briefly, 10 ml of an overnight culture of transformed *E. Coli* was used to inoculate 750 ml of Luria-Bertani (LB) medium containing kanamycin (30 µg ml⁻¹). The resulting culture was aerobically incubated at 37 °C with orbital shaking at 200 rpm until the optical density (OD₆₀₀) reached 0.6. Then, the protein expression was induced overnight with 1 mM of Isopropyl β-D-1-thiogalactopyranoside. After the induction time, cells were harvested by centrifugation at 4000 rpm for 30 min at 4 °C. Supernatants were discarded and the pellet was resuspended in sodium phosphate buffer (37.5 ml of 25 mM at pH 7.0). DNAse (2 µl of a stock solution of 1 mg ml⁻¹) and lysozyme (100 µl of a stock solution of 100 mg ml⁻¹) were added and incubated at 4°C for 1h. Then, cells were lysed by sonication using an Ultrasonic sonicator (Bandelin Sonoplus), at 25 % amplitude and 5 s pulse (on/off) for 6 minutes in an ice-water bath. The suspension was then centrifuged at 10000 rpm for 30 min at 4 °C.

For the purification of the enzyme, 10 volumes of the collected supernatant (10 ml) were mixed with 1 volume of Ni²⁺ affinity resin (TALON), which was previously cleaned with the extraction buffer (25 mM of imidazole and 25 mM of sodium phosphate, pH 8.0), for 2 h at 4 °C. The flow-through was discarded and the Ni²⁺ resin was cleaned 3 times with 10 volumes of phosphate buffer (25 mM, pH 8.0). 3 volumes of elution solution (300 mM of imidazole in sodium phosphate buffer 25 mM, pH 8.0) were added and the resin was incubated for 1h at 4 °C. Finally, BsADH was eluted and thereafter dialyzed against sodium phosphate buffer (50 mM, pH 8.0) at 4°C (4 x 1 l buffer exchanges over 40 h). BsADH concentration was calculated with the NanoDrop™ 2000 spectrophotometer (ε₂₈₀mm = 133000 M⁻¹ cm⁻¹; M_w = 147 KDa) using the Lambert-Beer equation.[2] 10 ml stocks (concentration of 6.8 µM of BsADH in 50 mM sodium phosphate buffer, pH 8.0) were stored at -80°C. Prior their use, stock aliquots were thawed overnight at 4°C.
4. Synthesis of NanoNOx

The synthesis of NanoNOx entails two steps: (i) the synthesis of imidazole decorated GOx nanogels and (ii) the addition of hemin for the polymer activation and the nanogel assembly.

4.1. Synthesis procedure of imidazole decorated GOx nanogels

A solution of GOx (10 mg) was prepared in a mixture of sodium phosphate buffer (520.8 µl from a 300 mM stock, pH 8.0), water (878.6 µl) and DMSO (411 µl). Then, HEAAm (5.75 mg, 57.56 µl taken from a 10%, m/v, stock prepared in sodium phosphate 50 mM, pH 8.0), VIm (23.5 mg, 22.6 µl from >99% solution), and BIS crosslinker (5.78 mg, 57.8 µl from a 10%, m/v, stock solution prepared in DMSO), were added to the enzyme solution. The final concentration of GOx in the polymerization reaction was 20 µM. Sucrose (156 mg, final concentration of 5%, m/v, in the reaction mixture) was added to enhance the non-covalent interactions between the enzyme surface and the monomers. The reaction mixture (3.12 ml) was bubbled with N₂ for 1 h and the radical polymerization started upon the addition of previously degassed TEMED (1.44 mg, 1.87 µl from a 99% solution) and ammonium persulfate (5.705 mg, 57.05 µl from a 10%, m/v, stock prepared in sodium phosphate 50 mM, pH 8.0). The polymerization was performed by stirring the mixture at 750 rpm at room temperature for 2 h. The non-reacted monomers were removed by 2 consecutive dialysis steps of 2 l each against 50 mM phosphate buffer pH 8.0 using a 10 kDa MWCO SnakeSkin dialysis membrane. The samples were washed 3 times and concentrated using filter membranes (30 kDa MWCO). As the spectroscopic measurement of the concentration of nanogels is not possible, we have considered that each nanogel bears one GOx molecule,[3] and measured the concentration of GOx at 450 nm (ε450 nm = 2.61x10⁴ M⁻¹ cm⁻¹), by tracking the concentration of FAD in each nanogel.[4] Finally, the nanogels were stored at 4°C in sodium phosphate buffer (50 mM, pH 8.0) at a concentration of 62.5 µM (corresponding to 10 mg ml⁻¹ of GOx enzyme) for further modifications.

4.2. Assembly of imidazole decorated GOx nanogels

Imidazole decorated nanogels (1.5 ml of a stock solution prepared at 1.6 µM in 40 mM of Tris-HCl buffer, pH 7.0) were mixed with an excess of hemin chloride (molar excess from 0 to 350, details in section 6.1) at 32°C for 16 h. Hemin stock was freshly prepared at a concentration of 30 mM in a NaOH solution (0.23 M). After incubation, the samples were centrifuged at 10000 rcf for 3 min. The supernatant was completely removed, and the solid was washed with water (1.5 ml). The cleaning process was repeated a total of 3 times and the NanoNOx were suspended in 1.5 ml of water and stored at room temperature.
5. Characterization of imidazole decorated GOx nanogel

5.1. Nanogel size determination by DLS (volume)

The average hydrodynamic diameters of free GOx and synthetized nanogels were measured by DLS, revealing hydrodynamic diameters of $9.2 \pm 1.2$ nm and $12.3 \pm 1.5$ nm, respectively. Nanogels show a polymeric shell average thickness of $1.5$ nm, calculated by the subtraction of the contribution of the enzyme core itself from the nanogel diameter, divided by 2. The activity of the encapsulated enzyme is not compromised with nanogels thinner than $2$ nm.$^{[3]}$

![Figure S1. DLS measurements of the hydrodynamic diameter of free GOx and GOx-nanogels.](image-url)
5.2. Assessment of the nanogel formation by ATR-FTIR spectroscopy

The embedment of the free enzyme within the nanogel was clearly evidenced by ATR-FTIR. The typical protein Amide I infrared band in the nanogel was more intense than in free GOx and shifted from 1637 cm$^{-1}$ (ii) to 1643 cm$^{-1}$ (i) (see inset from Figure S2). This fact was attributed to the incorporation of pHEAA, that shows a peak at 1654 cm$^{-1}$, and the interaction with the polymer.$^{[5]}$ Furthermore, the formation of the imidazole-bearing polymerization network was confirmed by the newly raised peaks at 920 cm$^{-1}$ (iii) and 664 cm$^{-1}$ (iv) related to imidazole bending.$^{[6]}

Figure S2. ATR-FTIR spectra of free GOx and GOx nanogels.
6. NanoNOx morphological and chemical characterization

6.1. NanoNOx composition and fabrication optimization

The concentration of hemin in the synthesis mixture was key for the assembly and the performance of the material. We observed that the composition of the final material and, thus, the catalytic performance thereof, was different for each of the assembly conditions applied. In order to study this divergence, we evaluated the relative GOx-nanogel and hemin loads for each of the NanoNOx synthesis.

- Composition of NanoNOx (I): GOx-nanogels content

The loads of GOx-nanogel in the solid material were calculated by measuring the glucose oxidase activity of the synthesized NanoNOx solids. We referred their activity to that measured for the GOx-nanogel solution before the assembly, which was set as the 100% of the recovered activity. Glucose oxidase activity was measured in 96 well-plates at room temperature. NanoNOx dispersion (2 µl of dispersed solid, calculated NanoNOx concentrations from 0.11 to 1.04 µM, as per immobilization yield) or control sample (2 µl soluble GOx-nanogel at 1.6 µM) were added to the reaction mixture that contained glucose (100 mM), ABTS (1 mM) and horseradish peroxidase enzyme (1.5 µl of a 45 µM stock solution, final concentration of 0.34 µM) in sodium acetate buffer (100 mM, pH 6.0). Final volume in each well was set to 200 µl. The oxidation of ABTS was tracked at 416 nm (ε_{416} = 36 mM⁻¹ cm⁻¹). Initial velocities (defined as µM of oxidized glucose per minute) measured for each reaction are plotted in Figure S3A (left axis). Protein immobilization yields (Figure S3A, right axis) were estimated as a % of the activity showed by the control sample.

- Composition of NanoNOx (II): hemin content

In order to calculate the amount of hemin molecules complexed within heterogeneous NanoNOx, we compared the UV-Vis spectra of the washed precipitates (and dispersed in 1.5 ml of water) vs. each of the supernatants (SN, non-bound material) after the first centrifugation step (rest of the SN were not measurable). We tracked the Soret band of the coordinated hemin at 410 nm to calculate the relative amount of hemin in each of the samples. After blank subtractions, we determined that the maximum hemin content was achieved for NanoNOx200 sample, with almost 400 nmol of hemin measured in the 1.5 ml solution (Figure S3B).
Figure S3. Effect of hemin concentration on the nanogel assembly. (A) Calculation of the glucose oxidase activity of NanoNOx used to estimate the protein immobilization yield (GOx-nanogel loads). Initial velocities (Vi) of the glucose oxidase activity each NanoNOx composition were represented in the left axis as μM of oxidized glucose per minute. Calculated protein immobilization yields are plotted on the right axis. B) Calculation of the hemin that is incorporated into the nanogels in each of the assembly conditions (nmol measured in 1.5 ml). Low hemin content samples could not be measured using this protocol. C) Image of dispersed NanoNOx samples (TOP) and after centrifugation (BOTTOM). The highest amount of material was produced between ratios 100 and 200. Out from this range the amount of precipitate decreased gradually.

- Composition of NanoNOx (III): Effect on the heterogenicity of the material

Different materials were obtained, either solid or soluble materials, as per the excess of hemin that is added to the assembly procedure. By adding a hemin:nanogel molar ratio ($n_{\text{hemin}}/n_{\text{nanogel}}$) from 10 to 350, iron porphyrins behaved as molecular bridges, triggering the aggregation of the nanogels, which eventually precipitated as heterogeneous catalysts. At $n_{\text{hemin}}/n_{\text{nanogel}}$ lower than 100, the incorporation of the GOx-nanogel into the material increased along with the hemin:nanogel ratio, reaching a maximum immobilization yield of 45% with an excess of hemin of 100, which was maintained up to a $n_{\text{hemin}}/n_{\text{nanogel}}$
of 200 (Figure S3C). At the same time, the highest amounts of precipitate were produced under these conditions. However, at \( n_{\text{hemin}}:n_{\text{nanogel}} \) higher than 200, both the immobilization yield and the amount of precipitate decreased with growing hemin concentrations. The loss of heterogeneous NanoNOx at higher hemin ratios could be attributed to a complete capping of imidazole residues of the nanogels by iron cations, which hampers the aggregation of the nanogels. Above a \( n_{\text{hemin}}:n_{\text{nanogel}} \) of 400, all the resulting materials are soluble.

- Composition of NanoNOx (IV): Heterogeneous vs Homogeneous NanoNOx in terms of activity

We obtained \( \text{app} k_{\text{cat}} \) values for homogeneous (NanoNOx200) and heterogenous NanoNOx (NanoNOx500) of \( 614 \pm 31 \) and \( 498 \pm 14 \text{ min}^{-1} \), respectively. From these data, one could assume that the homogeneous NanoNOx are more active than the heterogeneous. However, when the activity is calculated per hemin loads (Figure S3B, 0.26 and 0.77 \( \mu \text{mol ml}^{-1} \) for heterogeneous and homogeneous NanoNOx, respectively), the heterogeneous NanoNOx is more active (ca. 2.4 times) than the homogeneous version of NanoNOx. Therefore, it seems that, despite bearing less metalorganic catalyst, the configuration of the hemin in heterogeneous NanoNOx200 sample enhances the performance of the metalorganic catalyst.

6.2. NanoNOx size determination by DLS (volume)

The hydrodynamic diameter of NanoNOx was measured by DLS. The heterogeneous NanoNOx \( (n_{\text{hemin}}:n_{\text{nanogel}} = 200) \) showed a hydrodynamic diameter of \( 3079 \pm 199 \text{ nm} \) and a polydispersity index (PdI) of 0.272. This means that heterogeneous NanoNOx are made up of thousands of nanogels (individual nanogel size of \( 12.3 \pm 1.5 \text{ nm} \)) bridged through the hemin. On the contrary, homogeneous NanoNOx \( (n_{\text{hemin}}:n_{\text{nanogel}} = 400) \) exhibited a lower hydrodynamic diameter of \( 66.2 \pm 21.9 \text{ nm} \) and a PdI of 0.275. In this case, NanoNOx are composed of few nanogels and are therefore soluble in the medium.
Figure S4. DLS measurements of the hydrodynamic diameter (volume) of nanogels, heterogeneous NanoNOx (n_{hemin}:n_{protein} = 200), and homogeneous NanoNOx (n_{hemin}:n_{nanogel} ≥ 400).
6.3. SEM and EDX of NanoNOx

The morphology of solid NanoNOx ($n_{\text{hemin}}:n_{\text{nanogel}} = 200$) was unveiled by SEM (Figure S5A-C). NanoNOx are irregular aggregates build-up from spherical nanogels. The size determined by SEM was slightly smaller than that determined by DLS (see Figure S4). This size difference might be attributed to the sample preparation process. The spin-coating technique works well to separate particles individually, but at the same time it might disrupt their real morphology. On the other hand, the vacuum generated inside the microscope chamber facilitates the drying and shrinking of the nanogels.

EDX spectroscopy confirmed the presence of the nanogel (carbon (C), nitrogen (N), and oxygen (O)) and the iron porphyrin (Fe, C, N, and O) (Figure S5D). The silicon (Si) peak corresponds to the wafer used for sample preparation.

Figure S5. SEM images and EDX spectrum of NanoNOx ($n_{\text{hemin}}:n_{\text{nanogel}} = 200$). (A) 3000x magnification; (B) 15000x magnification, in which the irregular and non-homogeneous shape of NanoNOx is observed; (C) 150000x magnification, high magnification micrograph of NanoNOx, showing the spherical-like nanogels that form the structure. Scale bar: 1 µm. (D) EDX spectrum that evidences the presence of nanogels (C, N, O) and iron porphyrins (Fe, C, N, and O) within NanoNOx.
6.4. XPS of NanoNOx

The relative atomic composition of NanoNOx was calculated by XPS. As seen in Table S1, Fe from iron porphyrin was present in NanoNOx (0.4 %), confirming the EDX results (Figure S5D). C, O, and N corresponds to the enzyme, nanogel, and the porphyrin itself. The presence of phosphorous (P) is attributed to the phosphate buffer used in the polymerization reaction, as it can be retained by the nanogel due to its hydrogel nature.

Table S1. The relative atomic composition of NanoNOx determined by XPS.

|     | C (at%) | O (at%) | N (at%) | Fe(at%) | P(at%) |
|-----|---------|---------|---------|---------|--------|
|     | 52.2    | 35.5    | 9.2     | 0.4     | 2.7    |

The hemin used to form NanoNOx is composed of Fe$^{3+}$ and was partially reduced (50%) once dissolved in NaOH (Figure S6A). However, the Fe 2p region of NanoNOx ($n_{\text{hemin}}:n_{\text{nanogel}} = 200$) showed 90:10 (Fe$^{2+}$:Fe$^{3+}$) molar composition (Figure S6B). Therefore, the iron has been reduced upon coordination of imidazole molecules in the nanogel. Most likely, hemin was converted to heme B and incorporated into the nanogel in the same way as occurs in several heme proteins.\cite{7} Fe-imidazole coordination also enabled the stabilization of the organometallic catalyst inside the nanogel, as myoglobin does.

![Figure S6. XPS Fe 2p region of hemin (A) and NanoNOx (B) with relative peak fitting. The iron oxidation state changed from Fe$^{3+}$ to Fe$^{2+}$ upon NanoNOx formation (90% Fe$^{2+}$).](image)

Fortunately, the Fe-imidazole interaction was observed in the N 1s region of NanoNOx (Figure S7). The N 1s region of the spectrum of hemin showed two types of nitrogen atoms present in the pyrroles. The C-
NH-C bond is observed at a binding energy of 400.6 eV (-N-) and the C=N-C type bond at a binding energy of 398.4 eV (-N=). The higher intensity of the C-NH-C bond was attributed to the resonance between the pyrrole type nitrogen. On the other hand, the NanoNOx N 1s spectrum showed an additional peak at 401.4 eV (NH+), assigned to the positively charged nitrogen. This peak originated from the pyridine-type nitrogen of imidazole, which transferred the lone pair of electrons to Fe$^{3+}$ of hemin, and eventually reducing Fe$^{3+}$ to Fe$^{2+}$. [8]

Figure S7. XPS N 1s region of hemin (A) and NanoNOx (B) with relative peak fitting.
The iron-imidazole coordination in NanoNOx was also demonstrated by UV-Vis spectroscopy (Figure S8). The hemin (dashed line) dissolved in NaOH showed 3 characteristic peaks at 353 (i), 386 (ii) and 603 nm (vi). Upon the formation of NanoNOx, the Soret band of hemin at 386 nm shifted to 410 and 406 nm (iii), as per the excess of hemin with respect to protein that is incorporated. In addition, the Soret band satellite peak at 603 nm from hemin, which is related to Fe$^{3+}$, disappeared in the spectrum of the heterogeneous NanoNOx and two new peaks arose at 525 nm (iv) and 558 nm (v). These peaks, which are also present in homogeneous NanoNOx, resemble those from heme Soret band satellite peaks present in many hemoproteins. Interestingly, homogeneous NanoNOx also displayed a shoulder ca. 600 nm, which might arise from hemin molecules unspecifically attached to the nanogel. This fact is consistent with the shoulder shown by homogeneous NanoNOx at 386 nm, related to the presence of iron porphyrins not coordinated to imidazole.

![Figure S8](image.png)

**Figure S8.** UV-Vis spectra of heterogeneous NanoNOx ($n_{\text{hemin}}$:$n_{\text{nanogel}}$= 200), homogeneous NanoNOx ($n_{\text{hemin}}$:$n_{\text{nanogel}}$= 400) and free hemin.
6.6. CD spectrum of NanoNOx

The CD spectrum of NanoNOx was dominated by the characteristic signal of a α-helix secondary structure, confirming the structural integrity of GOx enzyme after all the chemical modifications.

Figure S9. CD of free GOx and NanoNOx.
7. Assessment of the catalytic performance of NanoNOx

General conditions for the measurement of NOx-like activity: Unless otherwise specified in the text, the catalytic performance of NanoNOx was determined spectroscopically by monitoring the NADH oxidation at 340 nm \((e_{340nm} = 6.22 \text{ mM}^{-1}\text{cm}^{-1})\) under aerobic conditions. Typically, reactions were performed in 96 well-plates at 37 °C. NanoNOx (2 µl from stock solution, ranging from 0.11 to 1.04 µM) were added to a 200 µl of HEPES buffer solution (100 mM, pH 7.3) containing glucose (25 mM) and NADH (1 mM). The reaction was stirred and monitored at 340 nm every 20 s up to a maximum of 80 min.

7.1. NOx-like activity measurement of heterogeneous NanoNOx and assessment of long-term stability of the material

Different NanoNOx compositions, synthesized from different hemin:nanogel molar rations \((n_{\text{hemin}}:n_{\text{nanogel}}\) from 0 to 400, synthesis description in Section 6.1), were tested for their NADH oxidation ability. For that, 2 µl of NanoNOx (washed heterogeneous NanoNOx dispersed in 1.5 ml of water) were added in each well, using the conditions mentioned above.

Figure S10 A and B ("fresh" data series), clearly shows that the heterogeneous NanoNOx fabricated with a hemin molar excess of 200 (NanoNOx200, \(n_{\text{hemin}}:n_{\text{nanogel}}\) of 200) showed the best performance among all the samples (3.2 µM min\(^{-1}\)). The reason for the high efficiency of the NanoNOx200 is related to the larger amount of precipitate formed under these synthesis conditions, which means a higher loading of both the hemin and GOx within the material (Figure S3A-C). For this reason, NanoNOx200 were selected for further experiments.

- Long-term stability of NanoNOx

The long-term stability of NanoNOx was assessed by measuring the NOx-like activity of samples of NanoNOx stored for 12.5 months at room temperature. Same reaction conditions as those applied for fresh samples were utilized. As observed in Figure S10B, no significant effect in the activity were found after long storage of NanoNOx. Moreover, regarding the integrity of the material, we observed that the “old” nanogels resuspended very easily, raising stable dispersions (Figure S10C).

- Assessment of the reproducibility of NanoNOx in terms of the NOx-like activity

To assess the reproducibility of NanoNOx synthesis procedure, three batches of NanoNOx200 were synthesized and their activity measured as described above. As shown in Figure S10D, the achieved
materials showed no-significant variations in terms of activity. We measured standard deviations of 1.7-2.8 % for each of the measurements and an insignificant deviation of 1.03 % among the samples.

Figure S10. NOx-like activity of heterogeneous NanoNOx formed from different hemin molar ratios \( (n_{\text{hemin}} : n_{\text{nanogel}} \) from 0 to 400). (A) NADH depletion kinetics of different NanoNOx \( (n_{\text{hemin}} : n_{\text{nanogel}} = 0 \ldots 400) \) monitored at 340 nm. (B) Calculated initial reaction velocities \( (V_i) \) of each NanoNOx composition for NOx-like activity of freshly prepared and “old” (12.5 months) samples represented in gray and black dots, respectively. The initial velocity is represented as \( \mu \text{M of oxidized NADH per minute.} \) Sample with \( n_{\text{hemin}} : n_{\text{nanogel}} = 400 \) yielded no activity because here we are evaluating the performance of the solid materials, and the use of an excess of hemin of 400 raised a soluble material. (C) Picture shows the easy dispersibility of the 12.5-months old samples. (D) Reproducibility measurements in terms of activity in which three batches of NanoNOx200 were synthesized.
7.2. Calculation of the apparent Michaelis-Menten constant ($^{app}K_M$) of NanoNOx

The measurements of the apparent Michaelis-Menten constant of NanoNOx were performed in 96-well plates (final volume of 200 µl), at 37 °C, in HEPES buffer (100 mM, pH 7.3). To measure the $^{app}K_M$ of NanoNOx for glucose, we performed the catalytic reactions at growing concentrations of glucose, from 0 to 100 mM, while keeping the NanoNOx and the NADH concentration constant (50 nM and 1 mM, respectively). In this experimental series, an $^{app}K_M$ of 0.39 ± 0.08 mM of glucose was obtained (Figure S11A). An equivalent experiment was carried out to determine the $^{app}K_M$ for NADH. For that, we varied the NADH concentration from 0 to 800 µM while fixed the glucose and NanoNOx concentrations to 5 mM and 50 nM, respectively. In this case an $^{app}K_M$ of 57.85 ± 17.8 µM of NADH was obtained, which is in the range of natural NOx enzymes (Figure S11B).\[11\] In both cases enzyme-like first order kinetic behavior was observed.

![Figure S11](image_url)
Figure S11. Measures performed for the calculation of the Apparent Michaelis-Menten constant ($^{app}K_M$) of NanoNOx in 100 mM HEPES pH 7.3 at 37 °C. (A) $^{app}K_M$ of NanoNOx for glucose using 1 mM of NADH and 50 nM of NanoNOx. (B) $^{app}K_M$ of NanoNOx for NADH using 5 mM of glucose and 50 nM of NanoNOx.

7.3. Calculation of the apparent turnover number ($^{app}k_{cat,NADH}$) of NanoNOx

The apparent turnover number ($^{app}k_{cat,NADH}$) of NanoNOx was measured at a fixed concentration of glucose and NADH (25 mM and 1 mM, respectively) in a HEPES buffer solution (100 mM, pH 7.3). Reactions were performed at 37°C in a 96-well plate (final volume per well of 200 µl) and the oxidation of NADH monitored at 340 nm. Each reaction was tested in a range of concentrations of NanoNOx, from 0 to 38
nM. We calculated an $appk_{cat,NADH}$ of almost 500 min$^{-1}$, which demonstrated that NanoNOx200 show higher activity than other materials used for the same purpose.

**Figure S12.** Calculation of the apparent turnover number ($appk_{cat,NADH}$) of NanoNOx200 using NADH and glucose as substrate (1 mM and 5 mM, respectively).
7.4. NanoNOx activity in the presence of organic solvents

The activity of NanoNOx was tested in the presence of organic solvents (50%, v/v). Reactions were performed in a 96-well plate (final volume of 208 µl) and monitored at 340 nm. NanoNOx (8 µl of a stock solution of 1.6 µM), glucose (5 mM), NADH (1 mM), and organic solvents (methanol (MeOH), acetone (ACE), tetrahydrofuran (THF), or acetonitrile (MeCN), 50%, v/v) were mixed in sodium phosphate buffer (100 mM, pH 8.0). The NADH depletion was also monitored in the absence of organic solvents for comparison. As observed in Figure S13, NanoNOx were remarkably active despite high concentrations of organic solvents.

**Figure S13.** NADH oxidase activity of NanoNOx in the presence of 50% (v/v) of organic solvents (MeOH, ACE, THF, and MeCN).
7.5. Reusability of NanoNOx

The reusability of NanoNOx was assessed by an ON-OFF experiment. The ON phase started with a reaction mixture that contained NADH (2.5 mM), glucose (5 mM) and NanoNOx (62.5 nM) in a HEPES buffered solution (100 mM, pH 7.3, final volume of 2 ml). Every minute the absorbance at 340 nm was measured using the Nanodrop™ 2000 (Thermo Scientific) spectrophotometer. The absorbance values at 340 nm were transformed into NADH concentration units using the Lambert-Beer equation (ε = 6.22 mM⁻¹cm⁻¹; l = 0.1 cm). After 5 minutes, the reaction mixture was centrifuged at 10000 rcf for 3 minutes, the supernatant was removed, and its absorbance measured every minute (OFF phase). After 4-5 minutes, the supernatant and NanoNOx were mixed and resuspended, repeating the cycle 9 times more. Importantly, before starting cycle 9, extra 5 mM glucose were added in the solution. The reaction velocity (defined as oxidized NADH (µM) per minute) of each ON phase is indicated in Table S2.

Table S2. Reaction rate of every ON phase. ‘*’ means extra addition of 5 mM glucose

| ON phase | Slope (µM min⁻¹) |
|----------|------------------|
| 1        | 3.25             |
| 2        | 4.66             |
| 3        | 4.59             |
| 4        | 4.98             |
| 5        | 3.05             |
| 6        | 2.93             |
| 7        | 2.24             |
| 8        | 0.46             |
| 9*       | 2.94             |
7.6. Biocatalytic test to confirm NAD⁺ bioavailability

We corroborated that the biologically active NAD⁺ cofactor was achieved in the oxidation reaction of NADH by NanoNOx (Figure S14). NanoNOx (8 µl of a stock solution of 1.6 µM) were added to 200 µl of a reaction mixture that contained glucose (25 mM) and NADH (1 mM) in sodium phosphate buffer (100 mM, pH 8.0) and incubated at 37 °C for 90 minutes. As depicted in Figure S14, the system reached a complete depletion of NADH. Thereafter, NanoNOx were removed from the reaction mixture by centrifugation (10000 rcf, 3 min) and BsADH enzyme (5 µl of an 54 µM stock solution) was added together with isopropanol (20 µl of a 100 mM stock solution) as substrate for the BsADH. The recovery of NADH indicated that it could be used by the NAD-dependent enzyme, confirming thereby the yield of the biologically active NAD⁺ by the NanoNOx catalyst.

![Figure S14](Image)

**Figure S14.** Complete NADH oxidation catalyzed by NanoNOx followed by the NAD⁺ reduction accomplished by BsADH using isopropanol as substrate. In the last enzymatic reaction (enzyme addition point marked with an arrow), around 85% NADH was obtained, which demonstrates that NanoNOx oxidized NADH into the biologically active NAD⁺.
7.7. Calculation of NanoNOx turnover number ($k_{\text{cat,NADPH}}$) with NADPH as substrate

The apparent turnover number ($^{\text{app}}k_{\text{cat,NADPH}}$) of NanoNOx for NADPH substrate was measured at a fixed concentration of glucose and NADPH (25 mM and 1 mM, respectively) in a HEPES buffer solution (100 mM, pH 7.3). Reactions were performed under aerobic conditions at 37°C in a 96-well plate (final volume per well of 200 µl) and the oxidation of NADPH was monitored at 340 nm ($e_{340\text{nm}} = 6.22 \text{ mM}^{-1}\text{cm}^{-1}$). Each reaction was tested in a range of concentrations of NanoNOx, from 0 to 44 nM. We calculated an $^{\text{app}}k_{\text{cat,NADPH}}$ of almost 300 min$^{-1}$, which demonstrated that the NanoNOx show an NADPH oxidation ability similar to other enzymes used for the same purpose, i.e., mutant phenylacetone monooxygenase.$^{[12]}$

![Graph showing the apparent turnover number of NanoNOx with NADPH as substrate.](image)

**Figure S15.** The NanoNOx apparent turnover number ($^{\text{app}}k_{\text{cat,NADPH}}$) calculation using NADPH and glucose as substrates.
7.8. BNAH oxidation by NanoNOx

The ability of NanoNOx to oxidize synthetic BNAH was tested in the presence of glucose and under aerobic conditions in 96-well plates fixing the final reaction volume to 204 µl. For that, NanoNOx (4 µl of a stock solution of 1.6 µM) was added to 200 µl of the reaction solution composed by BNAH (0.5 mM) and glucose (25 mM) in sodium phosphate buffer (100 mM, pH 8.0). The reaction was tracked at 361 nm over 60 minutes and a total conversion of BNAH was observed (Figure S16A). Additionally, UV-Vis spectra were measured before and after the reaction. The typical band of BNAH at 361 nm was completely disappeared after the reaction (Figure S16B). A new peak arose at 265 nm after the reaction, which is attributed to the biologically active oxidized species (BNA+).[13]

![Figure S16](image-url)

**Figure S16.** Oxidation of the BNAH synthetic cofactor by NanoNOx. (A) The reaction was monitored at 361 nm and full conversion was observed after 60 min. (B) UV-Vis spectra before (solid line) and after (dashed line) NanoNOx-catalyzed oxidation of 0.5 mM of BNAH.
7.9. Calculation of NanoNOx turnover number (k<sub>cat,BNAH</sub>) with BNAH as substrate

The apparent turnover number (app\(k_{\text{cat,BNAH}}\)) of NanoNOx for BNAH substrate was measured at a fixed concentration of glucose and BNAH (25 mM and 1 mM, respectively) in a HEPES buffer solution (100 mM, pH 7.3). Each reaction was tested in a range of concentrations of NanoNOx, from 0 to 44 nM. Reactions were performed under aerobic conditions at 37°C in a 96-well plate (final volume per well of 200 µl) and the oxidation of BNAH was monitored at 361 nm (ε<sub>361nm</sub> = 7.24 mM<sup>-1</sup>cm<sup>1</sup>).<sup>[14]</sup> We calculated an app\(k_{\text{cat,BNAH}}\) of almost 400 min<sup>-1</sup>, which is up to 100 times higher than reported artificial BNAH oxidation systems.

![Graph](image)

**Figure S17.** NanoNOx turnover number (app\(k_{\text{cat,BNAH}}\)) calculation using BNAH as substrate.
8. NanoNOx performance coupled with NAD-dependent dehydrogenases

8.1. Reaction conditions and chromatograms of the coupled NanoNOx-BsADH reaction (tests with 10 mM of BnOH) (from Figure 3B)

Figure S18 shows the overlay chromatograms for the calculation of the oxidation yields of 10 mM BnOH upon addition of a total concentration of 20 mM glucose. Red chromatograms show the BnOH in the absence of catalysts. Blue chromatograms show the depletion of BnOH in the presence of catalysts (BsADH and NanoNOx). Chromatograms corresponding to the obtained maximum yields for each reaction conditions are herein showcased.

Reaction conditions of the in situ NanoNOx-BsADH reaction using 10 mM of BnOH

- **Simultaneous glucose addition (chromatograms A and B)**

  Reaction solution contained NanoNOx (9.4 µM), BsADH (1.4 µM), BnOH (10 mM), NAD+ (1 mM), glucose (20 mM), and DMSO (5%, v/v) in sodium phosphate buffer (100 mM, pH 8.0, final volume of 500 µl). The reactions were performed in 2 ml safe lock Eppendorf tubes at 37 °C and constant agitation (40 rpm in carousel). The experiments were repeated in triplicates. Chromatograms displayed in Figure S18A correspond to reactions in which no recycling system was added (reaction time: 2 h). In Figure S18B, conversely, both NanoNOx and BsADH were added (reaction time: 2 h).

- **Sequential glucose addition (chromatograms C and D)**

  Reaction solution contained NanoNOx (9.4 µM), BsADH (1.4 µM), BnOH (10 mM), NAD+ (1 mM), and DMSO (5%, v/v) in sodium phosphate buffer (100 mM, pH 8.0, final volume of 500 µl). The reactions were performed in 48-well plates at 37 °C and constant orbital agitation. Glucose aliquots (5 µl containing 0.042 µmol of glucose) were added periodically with a sample injector until a final concentration of glucose of 20 mM was reached in each case (evaporation of the reaction mixture is also considered). Glucose was supplied either every 5 min for 8 h or 15 min for 24 h. Chromatograms shown below were taken when the sequential additions reached a final concentration of glucose of 20 mM (8 and 24 h for Figure S18C and Figure S18D, respectively). The experiments were repeated in triplicates.
Figure S18. Overlay chromatograms for the calculation of the oxidation of 10 mM of BnOH upon addition of a total concentration of 20 mM of glucose. In red, the reactions in the absence of catalysts. In blue, the reactions in the presence of catalysts. (A) BnOH oxidation in the absence of NanoNOx (reaction time: 2 h). (B) Simultaneous addition of 20 mM of glucose (reaction time: 2 h). (C) Sequential addition of 0.2 mM glucose every 5 min (reaction time: 8 h). (D) Sequential addition of 0.2 mM glucose every 15 min (reaction time: 24 h).
8.2. NanoNOx concentration optimization for coupled BsADH-NanoNOx BnOH oxidation

The influence of the concentration of NanoNOx on the oxidation of BnOH using coupled BsADH-NanoNOx system was evaluated. Different NanoNOx concentrations were utilized (0-1200 mU ml\(^{-1}\)) while BsADH concentration was fixed to 0.012 U ml\(^{-1}\). The conditions utilized were those described before in “Simultaneous glucose addition” section (Figure S18B) running the reaction for 2h. Under these conditions, a plateau was reached in the conversion of BnOH at 250 mU ml\(^{-1}\) of NanoNOx. For this reason, 0.25 U ml\(^{-1}\) were utilized for subsequent experiments.

![Graph](image)

**Figure S19.** Effect of NanoNOx concentration on the coupled BsADH-NanoNOx BnOH oxidation. BsADH concentration was fixed to 0.012 U ml\(^{-1}\) while NanoNOx concentration varied from 0 to 1200 mU ml\(^{-1}\).
8.3. Evaluation of the cofactor after BnOH oxidation (simultaneous glucose addition)

The reaction products of the BnOH oxidation with the simultaneous addition of glucose at 2 h were elucidated by $^1$H NMR. As control, the coupled reaction was also executed without glucose (red). Under non-fueled conditions, NanoNOx were not able to oxidize NADH and it accumulated in the reaction, mixed with NAD$^+$ (red). The depletion of NADH was only observed when the coupled reaction was carried out in the presence of glucose, which means that the cofactor remains in its oxidized form (blue).

![Figure S20. $^1$H NMR spectra of the BnOH oxidation products (experiments on the simultaneous addition of glucose) in the presence of glucose (blue) and in the absence of glucose (red).](image-url)
8.4. Evaluation of the biocatalyst after oxidation of BnOH upon simultaneous addition of glucose

After 2h of reaction in presence of 20 mM of glucose, NanoNOx were separated from BsADH by centrifugation (10000 rcf for 5 min). The activity of the biocatalyst was determined and compared with the activity of the control sample (no glucose added). To measure the recovered activity of BsADH, a sodium phosphate solution (50 mM, pH 8.0) with 2-isopropanol (10 mM) and NAD$^+$ (1 mM) was used. We observed a drop of activity 37.9% of the recycled enzyme compared to the control. Therefore, it was demonstrated that the H$_2$O$_2$ formed by the oxidation of glucose caused the inactivation of the enzyme.

![Figure S21](image)

**Figure S21.** Evaluation of the activity of the biocatalyst after the oxidation of BnOH using the simultaneous addition of glucose (20 mM) approach. We monitored the NAD$^+$ reduction carried out by recycled BsADH in the presence of glucose (blue) and in the absence of glucose (red).

8.5. H$_2$O$_2$ effect in BsADH activity

The sensitivity of the BsADH enzyme towards H$_2$O$_2$ was checked evaluating its enzymatic activity in the presence of different concentrations of H$_2$O$_2$ (0-100 mM). For that, BsADH enzyme (2 µl of a 6.8 µM stock solution) was added to a reaction solution prepared in HEPES buffer (50 mM HEPES pH 7.3) that contained 2-isopropanol (10 mM), NAD$^+$ (1 mM), and a range of concentrations of H$_2$O$_2$, from 0 to 100 mM. Reactions were performed in a 96-well plate at 37°C, fixing the final volumes to 200 µl.

The results from Figure S22 show that BsADH is overly sensitive towards H$_2$O$_2$. In fact, 5 mM of H$_2$O$_2$ were enough for diminishing BsADH catalytic activity around 35%.
Figure S22. Effect of H₂O₂ concentration in the BsADH enzymatic activity. (A) BsADH reaction evolution in the presence of different concentrations of H₂O₂ (0-100 mM). (B) Relative activity of BsADH in the presence of different H₂O₂ concentrations (100% of activity corresponds to the activity in absence of hydrogen peroxide).

8.6. Evaluation of NanoNOx after oxidation of BnOH upon simultaneous addition of glucose

After 2h of reaction in presence of 20 mM of glucose, NanoNOx were separated from BsADH by centrifugation (10000 rcf for 5 min). The activity of NanoNOx was determined and compared with the activity of the control sample (no glucose added). We determined a loss of 29.4% of the NOx-like activity compared to the control NanoNOx. Moreover, we observed that the hemin is leached to the medium after the reaction due to the deterioration of the material (inserted picture in Figure S23).

Figure S23. Evaluation of the activity of NanoNOx after the oxidation of BnOH using the simultaneous addition of glucose (20 mM) approach. We monitored the NADH oxidation carried out by recycled NanoNOx in the presence of glucose (blue) and in the absence of glucose (red). Inset picture shows the leached hemin to the medium after the reaction completion.
8.7. Assessment of the $H_2O_2$ directional channeling of NanoNOx

The directional channeling ability of NanoNOx was evaluated measuring its NADH oxidase activity (using glucose or $H_2O_2$ as fuel) in presence of catalase. For that, reaction solutions that contained either glucose or $H_2O_2$ (0.5 mM), NADH (0.5 mM), NanoNOx (0.19 µM) and catalase enzyme (0.4 nM) in sodium phosphate buffer (100 mM, pH 8.0) were prepared. Reactions were carried out at 37 °C in 96 well-plates (final volume of 200 µl) and tracked at 340 nm. When $H_2O_2$ was used as fuel, NanoNOx showed very low activity (Figure S24). The reason is that the catalase present in the reaction medium can disproportionate $H_2O_2$ before it reaches the organometallic catalyst, thus blocking the oxidation of NADH. On the contrary, when glucose was used as fuel, the NADH oxidation rate increased 4-fold. This increase arose from the directional channeling of $H_2O_2$ from GOx to the organometallic catalyst. Therefore, we conclude that, at low fuel concentration, the $H_2O_2$ required to accelerate the oxidation of NADH is mainly transported from the nanogel core to the organometallic catalyst, preventing its diffusion into the medium and the eventual denaturation of other biomolecules.

Figure S24. NADH oxidase activity of NanoNOx in presence of catalase. Either glucose or $H_2O_2$ were utilized as fuel for NanoNOx.
8.8. Reaction conditions and chromatograms of the coupled NanoNOx-BsADH reaction (50 mM BnOH)

The reaction solution was prepared in a sodium phosphate buffer (100 mM, pH 8.0) that contained BnOH (50 mM), NAD$^+$ (1 mM), DMSO (5%, v/v), NanoNOx (9.4 µM, 0.27 U ml$^{-1}$) and BsADH (1.36 µM, 0.015 U ml$^{-1}$). Reactions (final volume of 0.75 ml) were performed in 24-well plates at 37 °C and constant orbital agitation. Fuel injections of glucose (5 µl aliquots containing 0.042 µmol of glucose) were added every 5 min. The experiments were repeated in triplicates.

In Figure S25 the overlay chromatograms of the depletion of BnOH can be observed. The red chromatogram shows the presence of 50 mM of BnOH in the absence of both catalysts. The blue chromatogram shows the depletion of BnOH caused by the action of the BsADH-NanoNOx system. BnOH oxidation reached around 50% of conversion in 24 h.

**Figure S25.** Overlay chromatograms for the calculation of 50 mM BnOH conversion upon addition of 0.2 mM glucose every 5 min for 24 h. The red chromatogram was obtained in the absence of catalysts. The blue chromatogram refers to the reaction in the presence of the catalysts.
8.9. Reaction conditions and chromatograms of the coupled NanoNOx-LDH reaction

The reaction solution was prepared in a Tris-HCl buffer (500 µl, 100 mM, pH 8.0) that contained L-lactate (50 mM), NAD\(^+\) (1 mM), NanoNOx (9.4 µM, 0.27 U ml\(^{-1}\)) and LDH (4.4 µM, 1.52 U ml\(^{-1}\)). Reactions were performed in 24-well plates at 37 °C and constant orbital agitation. Fuel injections of glucose (5 µl aliquots containing 0.042 µmol of glucose) were added every 5 min. After 8 h, the reaction was stopped and analyzed by HPLC (Figure S26). Experiments were repeated in triplicates.

In Figure S26A, the reaction scheme that involves the production of pyruvate from L-lactate is depicted. In Figure S26B the overlay chromatograms of the reaction products can be observed. The red chromatogram shows the reaction products in the absence of both catalysts. The blue chromatogram shows the reaction product, pyruvate, and the non-reacted substrate, L-lactate, in the presence of both catalysts i.e., LDH and NanoNOx. L-lactate oxidation reached about 20 % of conversion in 8 h. Pyruvate formation was observed at 5.1 min (TTN of 10).

![Figure S26. Coupled NanoNOx-LDH reaction. (A) Reaction scheme of the oxidation of L-lactate to pyruvate using LDH and the NanoNOx cofactor-regeneration system. NanoNOx generates NAD\(^+\) from NADH using glucose and O\(_2\). (B) Overlay chromatograms for the calculation of 50 mM L-lactate oxidation to pyruvate upon sequential addition of glucose. Red chromatogram represents the enzymatic reaction without recycling system and blue chromatogram shows the production of pyruvate (min 5.1) in presence of the LDH-NanoNOx system (reaction time 8 h).](image-url)
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