Supporting Information

MOF-Based Hybrid Electrodes for Multi-Enzyme Cascade Reactions with Stabilized Mediator Immobilization

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Experimental section

ZIF67-NQS/CNT synthesis

To prepare the ZIF67-NQS/CNT, 0.24 g of polyvinylpyrrolidone (PVP, K-90, Fujifilm Wako Pure Chemical Corp., Japan) was dissolved in 5 mL of methanol. Subsequently, 600 mg of carbon nanotubes (CNTs; Thin MWCNT, >95% C purity, Ref: NC3100, Nanocyl, Belgium) was added to the solution, followed by sonication for 30 minutes using an ultrasonic homogenizer. The sonicated mixture was then added to a ligand solution containing 0.56 g of 2-methylimidazole (Fujifilm Wako Pure Chemical Corp., Japan) dissolved in methanol and stirred for 15 minutes. A metal ion solution containing 0.3 g of cobalt nitrate hexahydrate (Fujifilm Wako Pure Chemical Corp., Japan) was then introduced into the ligand-CNT mixture. The combined solution was stirred for 1 hour and subsequently incubated at room temperature for 24 hours. After the reaction, the resulting precipitate was collected via centrifugation, followed by repeated washing with methanol to remove unreacted species. The washed precipitate was dried at 60 °C and ground using a mortar and pestle to obtain a fine ZIF67-CNT powder.

To synthesize ZIF67-NQS/CNT, the vacuum-dried ZIF67-CNT powder was dispersed in a 640 μM solution of 1,2-naphthoquinone-4-sulfonate (NQS, ≥97.0%, Fujifilm Wako Pure Chemical Corp., Japan) and treated with an ultrasonic homogenizer. The mixture was incubated for 24 hours to allow NQS incorporation. The resulting ZIF67-NQS/CNT was collected by centrifugation and repeatedly washed with distilled water to remove excess NQS. Finally, the washed material was redispersed in fresh distilled water and homogenized via ultrasonic treatment to yield the ZIF67-NQS/CNT ink.

Enzymes activity evaluation

Prior to preparing the enzyme mixture for electrode modification, the activity of each enzyme was assessed following previously reported methods.[5,6] For instance, LOx activity was evaluated using a peroxidase-coupled assay, where hydrogen peroxide generated from lactate oxidation (in the presence of oxygen) was utilized by horseradish peroxidase (HRP) to catalyze the oxidative condensation of N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline (TOOS) and 4-aminoantipyrine (4-AA) to form a quinoneimine dye, which was detected by measuring absorbance at 555 nm. The calculated kinetic parameters (k_{cat} and K_M) for LOx were 42.7 ± 0.6 s^{-1} and 0.97 ± 0.03 mM, respectively.[5]

The activity of PDC was determined using an alcohol dehydrogenase (ADH)-coupled assay. In this reaction, pyruvate is decarboxylated by PDC to produce acetaldehyde, which is subsequently reduced to ethanol by ADH with the consumption of NADH. The rate of pyruvate decarboxylation was monitored by the decrease in NADH absorbance at 340 nm, using a molar extinction coefficient of 6,220 M^{-1} cm^{-1} for NADH. The obtained kinetic parameters for PDC were k_{cat} = 39.0 ± 3.9 s^{-1} and K_M = 0.79 ± 0.22 mM.[6]

The activity of ALDH was assayed using dichlorophenolindophenol (DCPIP) as an electron acceptor. The reaction was carried out in 20 mM Tris buffer (pH 7.5) containing 1 mM $CaCl_2$, 0.1 mM DCPIP, 0.025 μM ALDH, and varying concentrations of acetaldehyde at 37 °C. The rate of ALDH activity was calculated from the decrease in absorbance at 600 nm, using a molar extinction coefficient of

21,000 M^{-1} cm^{-1} for DCPIP. The kinetic parameters obtained were k_{cat} = 25.3 ± 0.5 s^{-1} and K_M = 1.29 ± 0.07 mM.[6]

Cascade enzyme mixture solution preparation

The desalinated solution of each enzyme (20 mg/mL, RIKEN Centre for Integrative Medical Sciences was diluted by half (to 10 mg/mL) by phosphate buffer solution (PBS). Subsequently, LOx, PDC, and ALDH were mixed in a tube at a mass ratio of 1:2:1, corresponding to volumes of 0.5 µL, 1.0 µL, and 0.5 µL, respectively. This ratio was determined based on the enzymatic activities of the respective enzymes, where the PDC exhibited the lower activity. Specifically, the enzyme activities were approximately 63.4 ± 0.9 U/mg for LOx[5] and 38.0 ± 3.8 U/mg for PDC.[6] For ALDH, the enzymatic activity was 37.0 ± 0.7 U/mg when evaluated using DCPIP[6] and 930 ± 100 U/mg when evaluated with ferricyanide (FeCN).[7] 0.0021 g of $(NH_4)_2SO_4$ (≥ 99.5 %, Fujifilm Wako Pure Chemical Corp., Japan) was dissolved into the mixture of enzymes as the salting-out agent (0.77 M final concentration). After that, 0.2 µL of 0.2 mM thiamine pyrophosphate chloride (TPP, > 98.0 %, Tokyo Chemical Industry Co., Ltd., Japan) solution, 0.2 µL of 5 mM magnesium chloride hexahydrate ($MgCl{\cdot}6H_2O$, ≥ 98.0 %, Fujifilm Wako Pure Chemical Corp., Japan), and 0.2 µL of 20 mg/mL SPGE (Denacol EX-614B, Nagase Co., Ltd., Japan, distilled with PBS), a crosslinker, were mixed with the enzyme solution. Resulting the total final volume mixture of 2.6 µL. In this system TPP serves as a coenzyme for PDC, and magnesium ions promote the reaction of PDC as cofactors and materials to help TPP bound to the active sites. The final solution was vortexed for 20 seconds and left to stand at room temperature for one hour.

Electrode Fabrication

ZIF67-NQS/CNT Modified Enzyme Electrode

A glassy carbon electrode (GCE, diameter = 3 mm) was cleaned by polishing the surface with an alumina slurry prior to use. First, 3.0 µL of 0.5% chitosan solution was drop-cast onto the GCE surface and allowed to dry. Then, 6.5 µL of ZIF67-NQS/CNT ink was applied, followed by drying. Next, 3.0 µL of 0.25% chitosan solution was dropped onto the dried ink layer. The second layer was formed by drop-casting 0.5 µL of ZIF67-NQS/CNT ink, followed by an additional 3.0 µL of 0.25% chitosan solution. Chitosan, applied in this procedure, functions as a binder that helps retain the material on the electrode surface through hydrogen bonding and electrostatic interactions facilitated by its –OH and $–NH_2$ functional groups. Furthermore, it improves the homogeneity of the material coverage on the electrode surface. Finally, 2 µL of the enzyme mixture solution was drop-cast, and the electrode was incubated for 24 h to complete the crosslinking reaction.

The enzyme loading was calculated to be 286 µg cm^{-2} based on the electrode surface area. The relatively low enzyme loading was intentionally selected to enhance the efficiency of the cascade reaction by minimizing the presence of enzymes not involved in the cascade, reducing the dominance of any single enzyme, and mitigating substrate intra-diffusion limitations

GCE and CNT-COOH Modified Enzyme Electrodes

To prepare the GCE-modified enzyme electrode, a cleaned commercial glassy carbon electrode (3 mm diameter, GCE, EC Frontier, Japan.) was modified by drop-casting 1.0 µL of 1,2-naphthoquinone (25 mM in acetonitrile, Tokyo Chemical Industry Co., Ltd. (TCI), Japan). The resulting electrode (GCE/NQ) was then coated with 2.0 µL of the enzyme mixture solution and incubated for 24 h to complete the crosslinking process.

For the CNT-COOH-modified enzyme electrode, 4 mg of carboxyl-functionalized carbon nanotubes (CNT-COOH; Thin MWCNT, >95% C purity, NC3103, Nanocyl, Belgium) was dispersed in 1 mL of N-methyl-2-pyrrolidone (NMP) using an ultrasonic homogenizer. A 3.0 µL aliquot of the homogeneous dispersion was drop-cast onto a cleaned GCE and dried at 60 °C for 3 h to evaporate the solvent. Subsequently, 2.0 µL of the enzyme mixture solution was applied and incubated for 24 h to allow crosslinking

Material characterization

Morphological observations were conducted using a Field-Emission Scanning Electron Microscope (FE-SEM, Hitachi SU-8020). Samples were either in powder form or mechanically scratched from the electrode surface, then mounted onto conductive carbon tape for imaging. Fourier Transform Infrared (FTIR) spectroscopy was carried out using a Jasco FT/IR-4X spectrometer. Sample powders were mixed with finely ground potassium bromide (KBr) and pressed into pellets. Transmittance spectra were recorded in the range of 400–4000 cm^{-1}.

Electrochemical measurement

All electrochemical measurements were conducted using a three-electrode system. A GCE or CNT-COOH modified or ZIF67-NQS/CNT modified enzyme electrode was used as the working electrode, while an Ag/AgCl electrode and a platinum wire served as the reference and counter electrode, respectively. Measurements were performed in 0.1 M PBS (pH 7.0). The catalytic current obtained from amperometric data was recorded at 300 seconds.

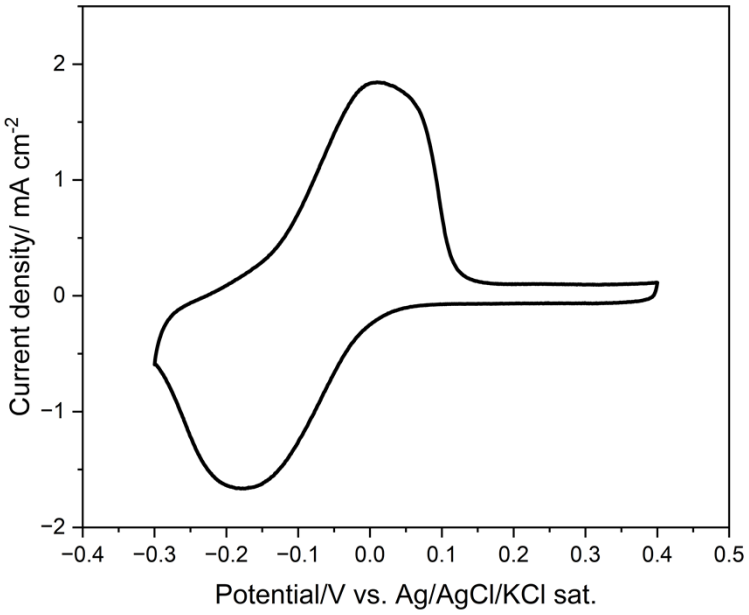

Fig. S1. Voltammogram of ZIF67-NQS/CNT in 0.1 M PBS pH 7

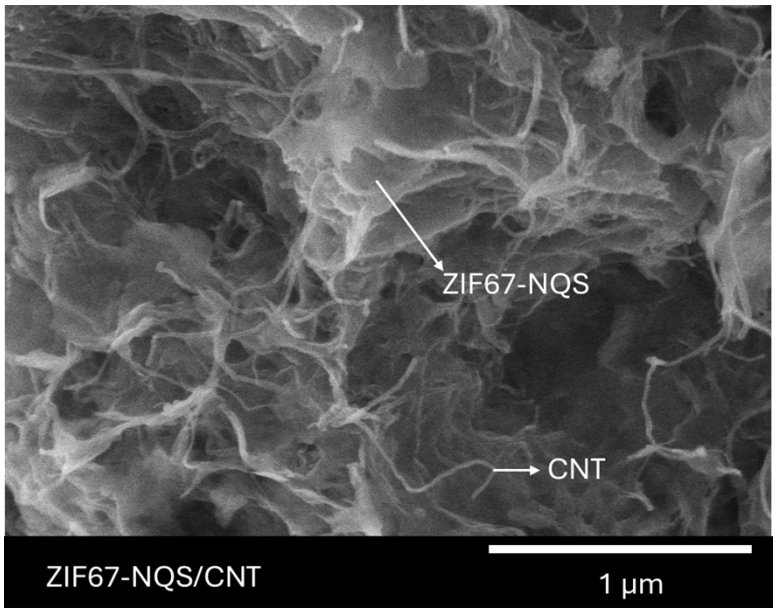

Fig. S2. SEM image of ZIF67-NQS/CNT

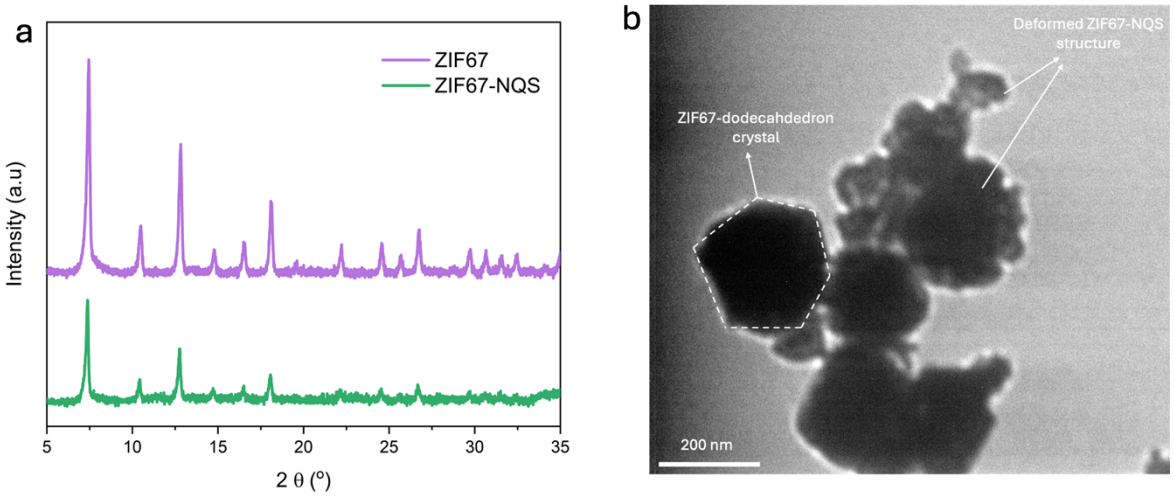

Fig. S3. a) Powder X-ray diffraction (PXRD) patterns of ZIF-67 and ZIF67–NQS, showing the reduction in crystallinity upon NQS integration; b) TEM image illustrating the well-defined rhombic dodecahedral crystals of ZIF67 and the deformed, irregular structure of ZIF67–NQS.

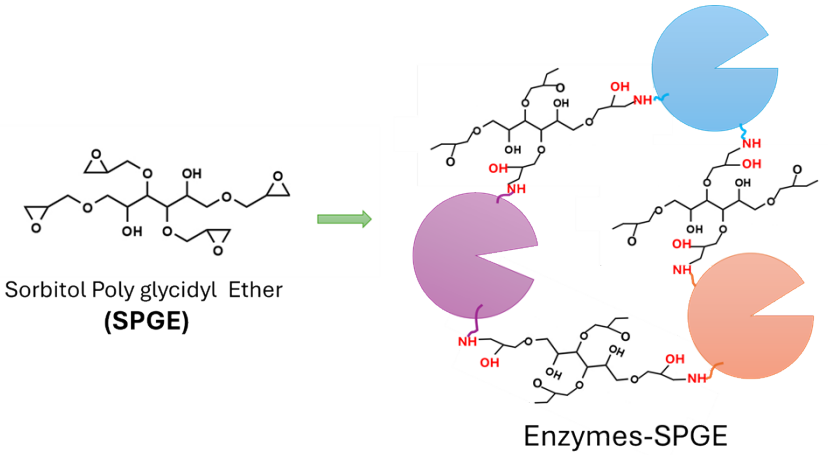

Fig. S4. Epoxy ring opening of sorbitol polyglycidyl ether (SPGE) upon reaction with the amine groups of the enzymes; crosslinks the enzymes, bringing them closer together and enhancing the efficiency of the cascade reaction. The sizes were adjusted for clearer visualization of the interactions.

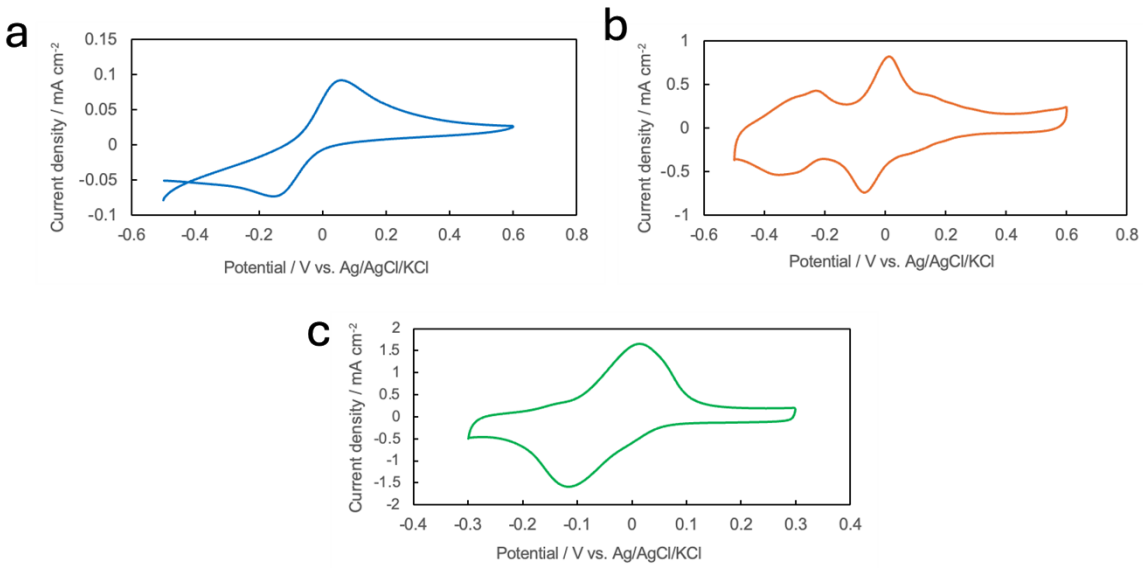

Fig. S5. Cyclic voltammetry (CV) in 0.1 M PBS (10 mV/s of scan rates) of a) GCE/NQ, b) CNT-COOH/NQ, and c) ZIF67-NQS/CNT; represents the higher redox active surface coverage of ZIF67-NQS/CNT compared to GCE/NQ and CNT-COOH/NQ.

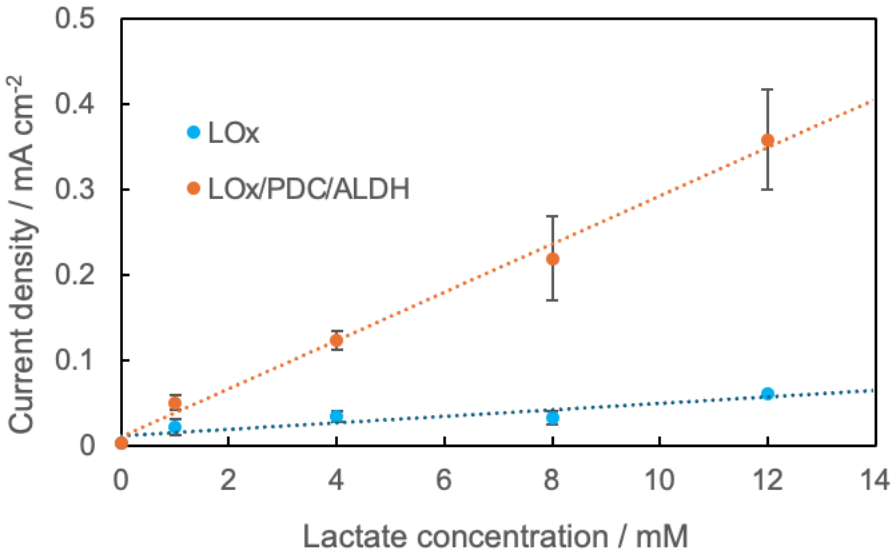

Fig. S6. Dependency of lactate concentration towards the achieved current density of the single-enzyme and the three-enzyme system.

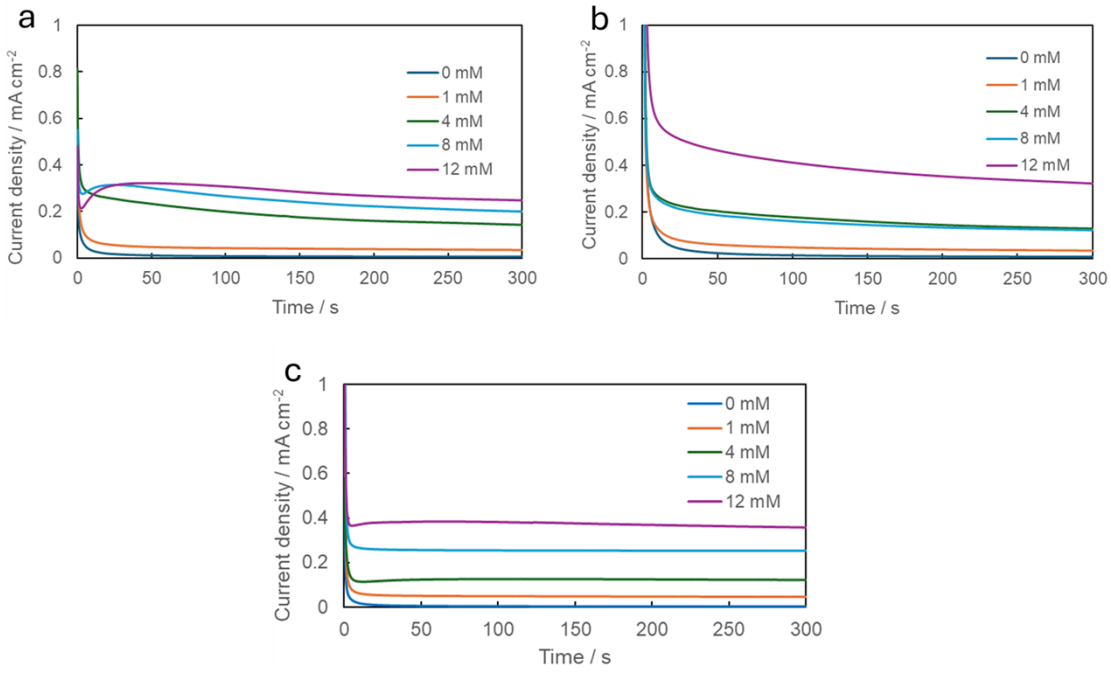

Fig. S7. Cascade enzyme electrode (LOx/PDC/ALDH) current response and stability towards the addition of lactate modified on; a) GC/NQ, b) CNT-COOH/NQ, and c) ZIF67-NQS/CNT. Measurements were conducted at 0.3V in 0.1M PBS.

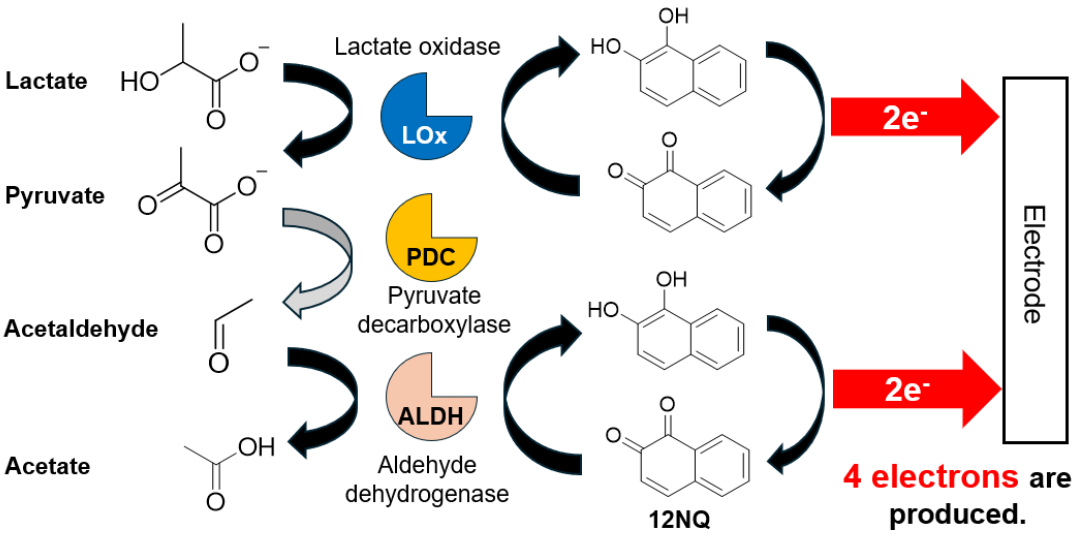

Fig. S8. Schematic illustration of electron production in mediated electron transfer reaction of cascade enzymes.

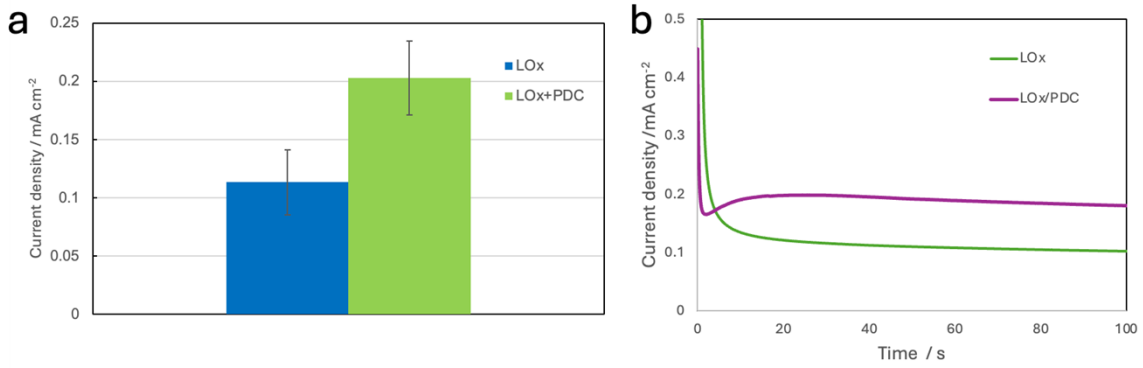

Fig. S9. a) Comparison of the current responses of LOx and LOx/PDC electrodes in 12 mM lactate in 0.1 M PBS (n=3), highlighting the current enhancement achieved by incorporating PDC. This enhancement is attributed to the enzymatic removal of pyruvate, a byproduct of lactate oxidation by LOx (measured without external pyruvate addition). b) Amperometric response at 0.3 V reveals an immediate current enhancement achieved by the LOx/PDC electrode compared to the LOx-electrode, indicating rapid initiation of the cascade reaction.

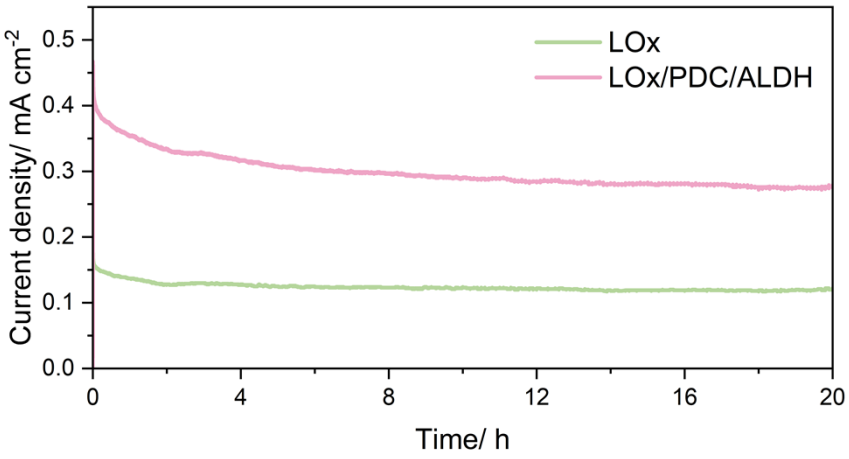

Fig. S10. Continuous operational stability of the multi-enzyme cascade electrode on the ZIF67-NQS/CNT platform over 20 h in 12 mM lactate in 0.1 M PBS at constant applied potential of 0.3V.