Hierarchically encapsulating enzymes with multi-shelled metal-organic frameworks for tandem biocatalytic reactions

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**Supplementary Fig. 16** TEM images of GOx@ZIF-8@HRP@ZIF-8 a) immediately after preparation, b) after 10 day of storage at 4 °C in pure water, and c) after catalytic reaction post-storage.

**Supplementary Fig. 17** XRD pattern simulated from CIF file of ZIF-8 (black), XRD patterns of the GOx@ZIF-8@HRP@ZIF-8 after storage at room temperature for a time-interval of 10 day (blue) and after catalytic reaction post-storage (red).
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**Supplementary Fig. 19** TEM image of as-synthesized Pro@ZIF-8.

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Supplementary Fig. 27 TGA curves of Pro (black), ADH (red), NAD⁺ (blue), ZIF-8@ysZIF-8 (green) and Pro@ZIF-8@ADH/NAD⁺@ysZIF-8 (light blue).
Supplementary Fig. 28  a) FTIR spectra and b) magnified FTIR spectra of as-synthesized ZIF-8, Pro@ZIF-8@ADH/NAD^+@ysZIF-8, Pro, and ADH.

Supplementary Fig. 29 Simultaneously recorded a, d) AFM topography images and b, e) IR broadband images of Pro@ZIF-8 and ADH/NAD^+@ysZIF-8 particles, respectively. Representative point nano-FTIR spectra of c) Pro@ZIF-8 and f) ADH/NAD^+@ysZIF-8 particles.

Supplementary Fig. 30 Tandem biocatalytic reaction driven by incompatible enzymes and cofactor (Pro and ADH/NAD^+).
Supplementary Fig. 31  Standard curve for determination of acetaldehyde content using gas chromatography.

Supplementary Fig. 32  Catalytic efficiencies in Pro@ZIF-8@ADH/NAD⁺@ysZIF-8, ZIF-8@ysZIF-8 and Pro@ZIF-8@ysZIF-8. The concentrations of Pro, ADH and NAD⁺ used were 5.35, 19.3 and 17.1 μg mL⁻¹.

Supplementary Fig. 33  Catalytic efficiencies of Pro@ZIF-8@ADH/NAD⁺@ZIF-67@ZIF-8 with different etching time of 0 day, 1 day, 3 day, 7 day. The concentrations of Pro, ADH and NAD⁺ used were 5.35, 19.3 and 17.1 μg mL⁻¹.
NAD⁺-dependent ADH uses the interconversion of NAD⁺/NADH redox couple to catalyze the oxidation of alcohol to aldehyde. ADH exists as a dimer (that is, composed of two polypeptides), with each monomer containing a catalytic domain (that is catalytic zinc, which holds hydroxyl group on alcohol) and a coenzyme binding domain with a large cleft between the two. The active site is at the bottom of the cleft. According to previous studies⁴⁻⁶, the mechanism of NAD⁺-dependent ADH enzyme for the oxidation of alcohol to aldehyde is described as follows (summarized in Supplementary Fig. 34):

First, the ADH structure is initially open to facilitate access to the active site for NAD⁺ binding. When NAD⁺ and alcohol bind, ADH undergoes a global conformational change, which involves a rotation of the catalytic zinc domain relative to the NAD⁺ binding domain. This process closes up and isolates the active site from solvent, creating a hydrophobic environment for the productive holoenzyme complex (that is, \( E \rightarrow E\cdot NAD^+ \rightarrow E\cdot^*NAD^+ \rightarrow E\cdot^*NAD^+\cdot RCH_2OH \)).

Second, the resulting \( E\cdot NAD^+\cdot RCH_2OH \) complex is poised for hydrogen transfer, involving alcohol to deprotonate, and transfer the proton via Ser-48 and His-51 (His-51 contacts solvent water on the protein surface) to solvent. During the proton relay, the reduced nicotinamide ring may become puckered. This process leads to the formation of \( E\cdot NADH\cdot RCHO \).

Third, NADH and aldehyde dissociate from the abortive \( E\cdot NADH\cdot RCHO \) complex. Previous kinetics studies have shown that the dissociation of NADH is the rate-limiting step when ethanol is used as the substrate.

Overall, ADH relies on coenzyme dissociation and association that involves conformational changes and interconversion of NAD⁺/NADH redox couple to catalyze the oxidation of alcohol to aldehyde.

\[
\begin{align*}
E\cdot NAD^+ & \xrightleftharpoons[k_s][k_1] E\cdot^*NAD^+ & \xrightleftharpoons[k_5][k_2] E\cdot^*NAD^+\cdot RCH_2OH \\
E & \xrightleftharpoons[k_6][k_s] E\cdot NADH & \xrightleftharpoons[k_5][k_6] E\cdot NADH\cdot RCHO
\end{align*}
\]

**Supplementary Fig. 34** The mechanism of NAD⁺-dependent ADH enzyme for the oxidation of alcohol to aldehyde⁵.

**Supplementary Fig. 35** Time-dependent fluorescence at 330 nm as a result of the etching of ZIF-67 in Pro@ZIF-8@ADH/NAD⁺@ZIF-67@ZIF-8. Excitation wavelength: 280 nm.
Supplementary Fig. 36 Catalytic efficiencies of ADH/NAD⁺ in the absence (red) and in the presence (blue) of cobalt hydroxide. Note that the concentration of cobalt hydroxide was 1.1 mg mL⁻¹, which was calculated by assuming that ZIF-67 in Pro@ZIF-8@ADH/NAD⁺@ZIF-67@ZIF-8 was completely dissociated; the concentrations of ADH and NAD⁺ were 19.3 μg mL⁻¹ and 17.1 μg mL⁻¹, which was the same with respective enzyme concentrations in all control experiments of Pro-ADH/NAD⁺ cascade.

Supplementary Fig. 37 Comparison of long-term stability. Cascade activities of the Pro@ZIF-8@ADH/NAD⁺@ysZIF-8 and supernatant solution of Pro@ZIF-8@ADH/NAD⁺@ysZIF-8 immediately after fresh preparation (red) and after storage at room temperature for a time-interval of 10 day (blue), respectively.
Supplementary Fig. 38 TEM images of Pro@ZIF-8@ADH/NAD⁺@ysZIF-8 a) immediately after preparation, b) after storing at 4 °C for 10 day, and c) after catalytic reaction post-storage, respectively.

Supplementary Fig. 39 XRD pattern simulated from CIF file of ZIF-8 (black), XRD patterns of the Pro@ZIF-8@ADH/NAD⁺@ysZIF-8 after storing at 4 °C for 10 day (blue), and after catalytic reaction post-storage (red), respectively.

Supplementary Fig. 40 Leaching test of Pro@ZIF-8@ADH/NAD⁺@ysZIF-8 at 4 h, 10h and 20 h. The amount of leaching enzymes was determined by Bradford assay. The 100% standard solution at 0 minute was tested against 5.35 µg mL⁻¹ Pro, 19.3 µg mL⁻¹ ADH and 17.1 µg mL⁻¹ NAD⁺ in water.
Supplementary Fig. 41  The amine-reactive fluorophores are acylating reagents that form thioureas or carboxamides upon reaction with amino groups of enzymes. a) Reaction of a primary amine with an isothiocyanate in FITC. b) Reaction of a primary amine with an isothiocyanate in Rhodamine B isothiocyanate. c) Reaction of a primary amine with a succinimidy l ester in 7-hydroxycoumarin-3-carboxylic acid N-succinimidy l ester.
### Supplementary Table 1. Summary of encapsulation methods and enzyme activities of GOx-HRP@MOF cascade catalytic systems.

| GOx/HRP/MOF cascade catalytic systems | Encapsulation method | Application | Activity assay | Performance | References |
|--------------------------------------|----------------------|-------------|----------------|-------------|------------|
| GOx@ZIF-8@HRP@ZIF-8                   | Stepwise encapsulation of GOx and HRP by epitaxial shell-by-shell overgrowth | Biocatalytic cascades | Glucose + OPD | 1) Compared to mixture of unassembled single-enzyme-loaded ZIF-8, 9.1-fold activity improvement; 2) Compared to mixture of free enzymes, 5.8-fold activity improvement | This work |
| GOx&HRP@ZIF-8                        | one-pot encapsulation of both GOx and HRP into ZIF-8 | Biocatalytic cascades | Glucose + OPD | 1) Compared to mixture of unassembled single-enzyme-loaded ZIF-8, 10.0-fold activity improvement; 2) Compared to mixture of free enzymes, 6.9-fold activity improvement | Control in this work |
| GOx&HRP@ZIF-8                        | one-pot encapsulation of both GOx and HRP into ZIF-8 | Glucose biosensor | Glucose + ABTS² | 1) Compared to mixture of unassembled single-enzyme-loaded ZIF-8, ~3-fold activity improvement; 2) Compared to mixture of free enzymes, comparable activity (~0.8-fold activity) | 7 |
| GOx&HRP@PCN-888                      | stepwise encapsulation of enzymes into the largest (accommodate GOx) and medium cages (accommodate HRP) of hierarchical PCN-888 | Glucose detection | Glucose + ABTS² | 1) NA; 2) Compared to mixture of free enzymes, 1.24-fold activity improvement | 8 |
| GOx&HRP@ZIF-8                        | one-pot encapsulation of both GOx and HRP into ZIF-8 | Biocatalytic cascades | Glucose + Amplex Red | 1) NA; 2) Compared to mixture of free enzymes, 7.5-fold activity improvement | 9, 10 |
| Diffusional mixture of GOx@UiO-66-capsules and HRP@UiO-66-capsules | Pickering emulsions complementary coiled-coil forming peptide induced assembly of enzyme encapsulated ZIF-8A (ZIF-8A: 3-amino-1,2,4-triazole (Atz) functionalized ZIF-8 to enable further peptide functionalization) | Segregate incompatible species | Glucose + ABTS² | NA; improvement in recyclability | 11 |
| Chains of GOx/ZIF-8A and HRP/ZIF-8A | Stimuli-responsive biocatalytic cascades | | Glucose + ABTS² | 1) Compared to mixture of unassembled single-enzyme-loaded ZIF-8, 7.3-fold activity improvement; 2) NA | 12 |
| Diffusional mixture of GOx@ZIF-L-capsules and HRP@ZIF-L-capsules | separate encapsulation of GOx and HRP into ZIF-L-capsules via water-in-oil emulsions followed by metal-phenolic networks (MPNs) coating amino-acid-boosted one-pot encapsulation of both GOx and HRP into ZIF-8 | Artificial cells | Glucose + ABTS² | Improvement in the stability upon ultraviolet irradiation, thermal treatment, and proteolysis | 13, 14 |
| GOx&HRP@ZIF-8                        | one-pot encapsulation of both GOx and HRP into ZIF-8 | Glucose biosensor | Glucose + TMB | 1) NA; 2) Compared to mixture of free enzymes, ~3.2-fold activity improvement | 15 |
| GOx&HRP@ZIF-8                        | Gox biocatalysis therapy | | | |
| GOx&HRP@DNA/ZIF-8                    | DNA scaffold cross-linking GOx and HRP, followed by one-pot encapsulation | Glucose biosensor | Glucose + ABTS² | 1) NA; 2) Compared to mixture of free enzymes, 1.25-fold activity improvement | 17 |
**Supplementary Table 2.** The encapsulation efficiencies of enzymes in different systems. (E₀ refers that there is no enzyme.)

| Enzyme             | E@ZIF-8@E@ZIF-8 | Encapsulation efficiencies |
|--------------------|-----------------|----------------------------|
| GOx-FITC           | GOx-FITC@ZIF-8@E₀@ZIF-8 | 81.34%                      |
|                    | HRP@ZIF-8@GOx-FITC@ZIF-8 | 85.97%                      |
| HRP-RhB            | HRP-RhB@ZIF-8@E₀@ZIF-8 | 69.06%                      |
|                    | GOx@ZIF-8@HRP-RhB@ZIF-8 | 49.65%                      |
|                    | GOx@ZIF-8²@HRP-RhB@ZIF-8 | 53.01%                      |
|                    | GOx@ZIF-8³@HRP-RhB@ZIF-8 | 58.62%                      |
| Pro-RhB            | Pro-RhB@ZIF-8@E₀@ysZIF-8 | 77.38%                      |
|                    | ADH/NAD⁺@ZIF-8@Pro-RhB@ysZIF-8 | 87.05%                      |
| ADH-FITC           | ADH-FITC@ZIF-8@E₀@ysZIF-8 | 87.05%                      |
|                    | Pro@ZIF-8@ADH-FITC@ysZIF-8 | 55.20%                      |
|                    | Pro@ZIF-8²@ADH-FITC@ysZIF-8 | 59.33%                      |
|                    | Pro@ZIF-8³@ADH-FITC@ysZIF-8 | 63.13%                      |
| NAD⁺-coumarin      | NAD⁺-coumarin@ZIF-8@E₀@ysZIF-8 | 41.87%                      |
|                    | Pro@ZIF-8@NAD⁺-coumarin@ysZIF-8 | 48.90%                      |
|                    | Pro@ZIF-8²@NAD⁺-coumarin@ysZIF-8 | 53.71%                      |
|                    | Pro@ZIF-8³@NAD⁺-coumarin@ysZIF-8 | 60.29%                      |

Note that the differences in encapsulation efficiencies of multi-enzymes might be influenced by various factors, including enzyme-MOF interactions and loading spaces.¹⁰
Supplementary Table 3. Weight loss in ZIF-8, pure enzymes, and GOx@ZIF-8@HRP@ZIF-8.

|                     | Weight at 25 °C | Weight at 800 °C | Weight loss from 25 °C to 800 °C |
|---------------------|-----------------|------------------|----------------------------------|
| GOx                 | 100%            | 15.5%            | 84.5%                            |
| HRP                 | 100%            | 19.9%            | 80.1%                            |
| ZIF-8               | 100%            | 37.5%            | 62.5%                            |
| GOx@ZIF-8@HRP@ZIF-8 | 100%            | 27.6%            | 72.4%                            |

On the basis of enzyme loading characterization results and the final product weight, we determined the loadings of GOx and HRP to be 70.72 and 215.9 μg mg⁻¹; and weight percentage of GOx, HRP, and ZIF-8 in the final product to be 7.1 wt%, 21.6 wt%, and 71.3 wt%. Through the following equation, the weight loss in GOx@ZIF-8@HRP@ZIF-8 can be estimated as 67.9 wt%, which is similar to the corresponding measured weight loss in TGA curve (72.4 wt%). Based on the above results, we can conclude that the weight loss in TGA matches with the enzymes loading.

\[
\text{weight loss } X_{\text{GOx@ZIF-8@HRP@ZIF-8}}(\%) = m_{\text{GOx}} \times X_{\text{GOx}} + m_{\text{HRP}} \times X_{\text{HRP}} + m_{\text{ZIF-8}} \times X_{\text{ZIF-8}}
\]
\[
= 7.1\% \times 84.5\% + 21.6\% \times 80.1\% + 71.3\% \times 62.5\% = 67.9\%
\]

\(m\): weight percentage of each component; \(X\): weight loss of each component.
**Supplementary Table 4.** Weight loss in ZIF-8@ysZIF-8, pure enzymes, and Pro@ZIF-8@ADH/NAD⁺@ysZIF-8.

|                      | Weight at 25 °C | Weight at 800 °C | Weight loss from 25 °C to 800 °C |
|----------------------|-----------------|------------------|----------------------------------|
| Pro                  | 100%            | 12.5%            | 87.5%                            |
| ADH                  | 100%            | 18.5%            | 81.5%                            |
| NAD⁺                 | 100%            | 35.6%            | 64.4%                            |
| ZIF-8@ysZIF-8        | 100%            | 21.6%            | 78.4%                            |
| Pro@ZIF-8@ADH/NAD⁺@ysZIF-8 | 100%            | 27.6%            | 72.4%                            |

On the basis of enzyme loading characterization results and the final product weight, we determined the loadings of Pro, ADH, and NAD⁺ to be 53.49, 193.0, and 170.9 μg mg⁻¹; weight percentage of Pro, ADH, NAD⁺, and ZIF-8@ysZIF-8 in the final product to be 5.6 wt%, 19.3 wt%, 17.1 wt%, and 58.2 wt%. Through the following equation, the weight loss in Pro@ZIF-8@ADH/NAD⁺@ysZIF-8 can be estimated as 77.3 wt%, which is similar to the corresponding measured weight loss in TGA curve (72.4 wt%). Based on the above results, we can conclude that the weight loss in TGA matches with the enzymes loading.

\[
\text{weight loss } X_{\text{Pro@ZIF-8@ADH/NAD⁺@ysZIF-8}}(\%) = m_{\text{Pro}} \times X_{\text{Pro}} + m_{\text{ADH}} \times X_{\text{ADH}} + m_{\text{NAD⁺}} \times X_{\text{NAD⁺}} + m_{\text{ZIF-8@ysZIF-8}} \times X_{\text{ZIF-8@ysZIF-8}} = 5.6\% \times 87.5\% + 19.3\% \times 81.5\% + 17.1\% \times 64.4\% + 58.2\% \times 78.4\% = 77.3\%
\]

**Supplementary Table 5.** The size of GOx@ZIF-8, GOx@ZIF-8², and GOx@ZIF-8³, respectively.

|                 | GOx@ZIF-8 | GOx@ZIF-8² | GOx@ZIF-8³ |
|-----------------|-----------|------------|------------|
| size of the particle | ~120 nm   | ~160 nm    | ~240 nm    |

**Supplementary Table 6.** The size of Pro@ZIF-8, Pro@ZIF-8², and Pro@ZIF-8³, respectively.

|                 | Pro@ZIF-8 | Pro@ZIF-8² | Pro@ZIF-8³ |
|-----------------|-----------|------------|------------|
| size of the particle | ~140 nm   | ~180 nm    | ~260 nm    |
Supplementary Table 7. The amounts of enzymes added in the synthetic protocol for different systems.

| System                        | Enzyme loading             | The initially added enzyme amount |
|-------------------------------|----------------------------|-----------------------------------|
| GOx@ZIF-8@HRP@ZIF-8          | GOx: 70.72 μg mg⁻¹         | GOx: 3 mg, HRP: 3 mg               |
|                               | HRP: 215.9 μg mg⁻¹         |                                   |
| GOx@ZIF-8²@HRP@ZIF-8         | GOx: 65.95 μg mg⁻¹         | GOx: 3 mg, HRP: 2.8 mg             |
|                               | HRP: 200.6 μg mg⁻¹         |                                   |
| GOx@ZIF-8³@HRP@ZIF-8         | GOx: 60.25 μg mg⁻¹         | GOx: 3 mg, HRP: 2.5 mg             |
|                               | HRP: 180.9 μg mg⁻¹         |                                   |
| HRP@ZIF-8@GOx@ZIF-8          | HRP: 203.0 μg mg⁻¹         | HRP: 11 mg, GOx: 0.6 mg            |
|                               | GOx: 68.93 μg mg⁻¹         |                                   |
| GOx@ZIF-8                    | GOx: 65.08 μg mg⁻¹         | GOx: 0.6 mg                        |
| HRP@ZIF-8                    | HRP: 219.5 μg mg⁻¹         | HRP: 2.2 mg                        |
| Gox/HRP@ZIF-8                | Gox: 68.74 μg mg⁻¹         | Gox: 0.6 mg, HRP: 2.2 mg           |
|                               | HRP: 213.9 μg mg⁻¹         |                                   |
| Pro@ZIF-8@ADH/NAD⁺@ysZIF-8   | Pro: 53.49 μg mg⁻¹         | Pro: 3 mg, ADH: 3 mg, NAD⁺: 3 mg   |
|                               | ADH: 193.0 μg mg⁻¹         |                                   |
|                               | NAD⁺: 170.9 μg mg⁻¹        |                                   |
| Pro@ZIF-8²@ADH/NAD⁺@ysZIF-8  | Pro: 51.58 μg mg⁻¹         | Pro: 3 mg, ADH: 2.8 mg, NAD⁺: 2.7 mg |
|                               | ADH: 184.6 μg mg⁻¹         |                                   |
|                               | NAD⁺: 161.1 μg mg⁻¹        |                                   |
| Pro@ZIF-8³@ADH/NAD⁺@ysZIF-8  | Pro: 48.87 μg mg⁻¹         | Pro: 3 mg, ADH: 2.6 mg, NAD⁺: 2.4 mg |
|                               | ADH: 182.4 μg mg⁻¹         |                                   |
|                               | NAD⁺: 152.3 μg mg⁻¹        |                                   |
| ADH/NAD⁺@ysZIF-8@Pro@ZIF-8   | ADH: 174.1 μg mg⁻¹         | ADH: 9.5 mg, NAD⁺: 17.5 mg, Pro: 0.6 mg |
|                               | NAD⁺: 154.3 μg mg⁻¹        |                                   |
|                               | Pro: 54.98 μg mg⁻¹         |                                   |
| Pro@ZIF-8                    | Pro: 55.27 μg mg⁻¹         | Pro: 0.6 mg                        |
| ADH/NAD⁺@ysZIF-8             | ADH: 177.0 μg mg⁻¹         | ADH: 1.9 mg, NAD⁺: 3.5 mg          |
|                               | NAD⁺: 168.4 μg mg⁻¹        |                                   |
| Pro/ADH/NAD⁺@ysZIF-8         | Pro: 53.99 μg mg⁻¹         | Pro: 0.6 mg, ADH: 1.9 mg, NAD⁺: 3.5 mg |
|                               | ADH: 192.3 μg mg⁻¹         |                                   |
| Pro@ZIF-8@ADH/NAD+/ZIF-8 | ADH: 194.8 μg mg⁻¹ | Pro: 3 mg, ADH: 3 mg, NAD⁺: 3 mg |
|--------------------------|-------------------|---------------------------------|
| NAD⁺: 170.4 μg mg⁻¹     |                   |                                 |
| Pro: 54.62 μg mg⁻¹       |                   |                                 |
| ADH: 194.8 μg mg⁻¹       |                   |                                 |
| NAD⁺: 172.6 μg mg⁻¹     |                   |                                 |
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