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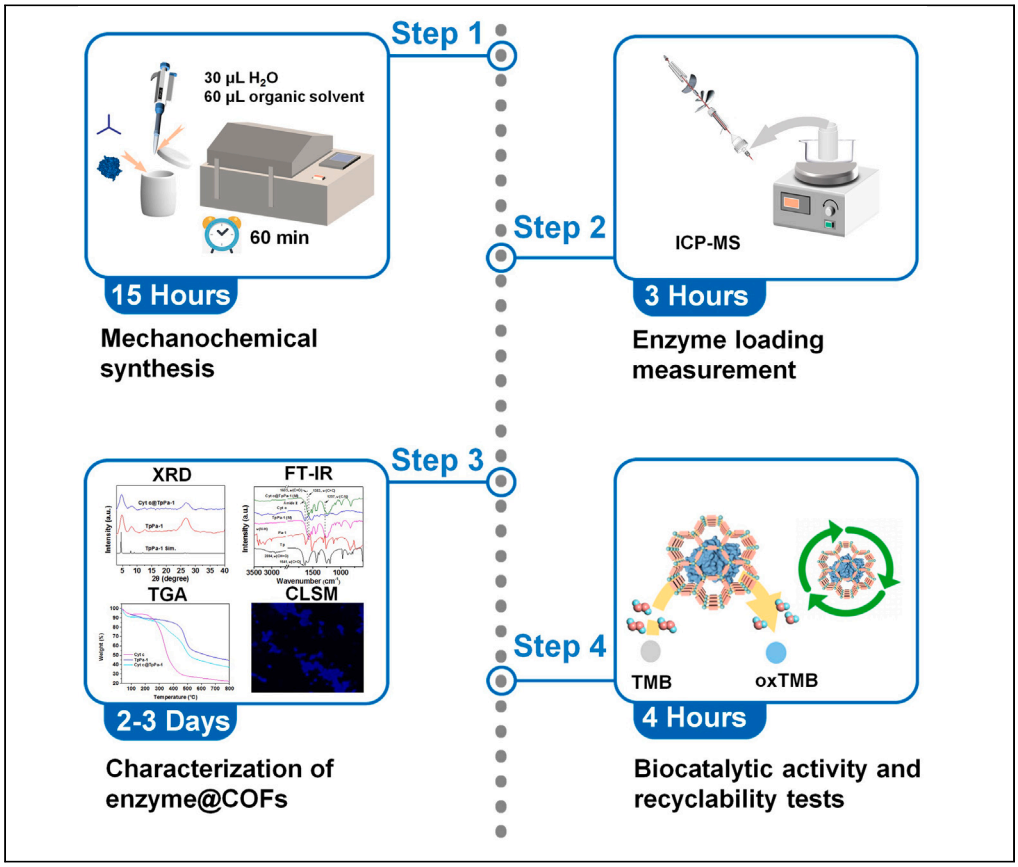
Protocol for mechanochemistry-guided assembly strategy for enzyme encapsulation using covalent organic frameworks



Enzyme immobilization into porous frameworks is an emerging strategy for enhancing the stability of dynamic conformation and prolonging the lifespan of enzymes. Here, we present a protocol for a *de novo* mechanochemistry-guided assembly strategy for enzyme encapsulation using covalent organic frameworks. We describe steps for mechanochemical synthesis, enzyme loading measurements, and material characterizations. We then detail evaluations of biocatalytic activity and recyclability.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Rui Gao, Xiaoxue Kou, Rongwei He, ..., Siming Huang, Guosheng Chen, Gangfeng Ouyang

huangsm@gzhmu.edu.cn (S.H.)
chengsh39@mail.sysu.edu.cn (G.C.)

Highlights

Detailed procedures of a mechanochemical COF strategy for enzyme encapsulation

Applicable for encapsulating multiple enzymes into COFs

Detailed steps for characterization of enzyme@COFs

Evaluation of the biocatalytic activity and recyclability of the biocomposites

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Protocol for mechanochemistry-guided assembly strategy for enzyme encapsulation using covalent organic frameworks

Rui Gao,[1] Xiaoxue Kou,[1] Rongwei He,[1] Yujiang Shen,[1] Lihong Guo,[1] Hao Wang,[1] Siming Huang,[2,*] Guosheng Chen,[1,3,4,*] and Gangfeng Ouyang[1]

[1]MOE Key Laboratory of Bioinorganic and Synthetic Chemistry, School of Chemistry, Sun Yat-sen University, Guangzhou 510275, China

[2]Guangzhou Municipal and Guangdong Provincial Key Laboratory of Molecular Target & Clinical Pharmacology, the NMPA and State Key Laboratory of Respiratory Disease, School of Pharmaceutical Sciences and the Fifth Affiliated Hospital, Guangzhou Medical University, Guangzhou 511436, China

[3]Technical contact

[4]Lead contact

*Correspondence: huangsm@gzhmu.edu.cn (S.H.), chengsh39@mail.sysu.edu.cn (G.C.)
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SUMMARY

Enzyme immobilization into porous frameworks is an emerging strategy for enhancing the stability of dynamic conformation and prolonging the lifespan of enzymes. Here, we present a protocol for a *de novo* mechanochemistry-guided assembly strategy for enzyme encapsulation using covalent organic frameworks. We describe steps for mechanochemical synthesis, enzyme loading measurements, and material characterizations. We then detail evaluations of biocatalytic activity and recyclability.

For complete details on the use and execution of this protocol, please refer to Gao et al. (2022).[1]

BEFORE YOU BEGIN

Encapsulation of enzymes within porous organic frameworks is a cutting-edge strategy for enhancing enzyme protection and reusability.[2–4] Covalent organic frameworks (COFs) with covalent-linked topology, highly tunable porosity and facilely tailored structures may serve as ideal hosts for accommodating bulky enzymes.[5,6] However, designing larger pores of COFs for wrapping bulky enzymes by pore encapsulation strategy is challenging, due to the limited library of COFs with suitable pore structure. Besides, the harsher synthetic conditions of COFs pose risks of enzyme denaturation, which can impede the assembling of proteins through de novo encapsulation. These continuous challenges significantly hinder the progress of enzyme-COFs system. Mechanochemistry, has gained popularity for large-scale porous organic frameworks synthesis.[7] Considering its solvent-free nature, mild synthesis conditions and rapid reaction time, de novo mechanochemical methods offer a hopeful technology to encapsulate fragile enzymes during the COFs crystallization. Therefore, this protocol presents a mild and straightforward mechanochemical strategy for de novo encapsulation of different enzyme guests into a series of host COFs. The structural robustness and large pore channels of COFs permit the bio-composites have higher stability and better catalytic efficiency.

Preparation of solution

Timing: 5 min (for step 1)

Timing: 5 min (for step 2)

Timing: 10 min (for step 3)

1. Preparation of 5% HNO_3 solution.
   a. Dropwise 56.7 mL 65% HNO_3 to 1000 mL of deionized water under vigorous stirring.
   b. Store in a glass container in a well-ventilated room, tightly closed and kept at 20°C–25°C.

⚠ CRITICAL: HNO_3 is a volatile strong acid, and has strong irritation to skin, which can cause severe irritation to the skin, mucous membranes, and eyes. Therefore, it is essential for the operator to wear protective masks and handle it in a fume hood to prevent any possible injuries.

2. Preparation of Fe or P stock solution.
   a. Weight 7.24 mg iron (III) nitrate nonahydrate or 4.38 mg potassium dihydrogen phosphate separately.
   b. Dissolve in 1 L of 5% HNO_3 to obtain 1 ppm Fe or P stock solution.
   c. Store the stock solution in a tightly closed glass container in a well-ventilated room at 20°C–25°C.
3. Preparation of 3,3′,5,5′-tetramethylbenzidine (TMB) solution.
   a. Dissolve 0.15 g TMB in 3 mL of DMSO, followed by 50 mL of glycerol and 0.95 g citric acid.
   b. Bring to volume with deionized water in a 500 mL volumetric flask.
   c. Store at 4°C in the dark.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
1, 3, 5-triformylphloroglucinol (Tp)	Aladdin	CAS:34374-88-4
P-phenylenediamine (Pa-1)	Aladdin	CAS:06-50-3
2, 5-dimethyl-p-phenylenediamine (Pa-2)	Aladdin	CAS: 6393-01-7
Benzidine (BD)	Aladdin	CAS: 92-87-5
Cytochrome C (Cyt c)	Aladdin	CAS: 9007-43-6
Horseradish peroxidase (HRP)	Aladdin	CAS: 9003-99-0
Lipase PS	Aladdin	CAS: 9001-62-1
Alexa Fluor 350 NHS ester (AF350)	Thermo Fisher	CAS:200554-19-4
Iron (III) nitrate nonahydrate	Aladdin	CAS: 7782-61-8
Potassium dihydrogen phosphate	Aladdin	CAS: 7778-77-0
Hydrogen peroxide	Aladdin	CAS: 7722-84-1
Potassium bromide	Aladdin	CAS: 7758-02-3
Tris-HCl buffer (pH 7.4)	Solarbio	N/A
Phosphate buffer saline buffer (pH 9.0)	Yuanye	N/A
Mesitylene	J&K	CAS: 108-67-8
Dioxane	J&K	CAS: 123-91-1
3,3′,5,5′-tetramethylbenzidine (TMB)	J&K	CAS: 54827-17-7
P-Nitrophenyl palmitate	Macklin	CAS: 1492-30-4
P-nitrophenol	Macklin	CAS: 100-02-7
Triton X-100	Macklin	CAS: 9002-93-1
Nitric acid (>65%)	Guangzhou Chemical Reagent	CAS: 7697-37-2
Isopropanol	Guangzhou Chemical Reagent	CAS: 67-63-0
Ethanol	Guangzhou Chemical Reagent	CAS: 64-17-5

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
Origin software	OriginLab, Co., Ltd	https://www.OriginLab.com
MDI Jade 6	Materials Data Inc.	https://materialsdata.com
Others		
Ultrafiltration centrifugal tube	Millipore Co., LTD	N/A
Ultrasonic cleaner	Kunshan Ultrasonic Instruments Co., LTD	KQ218
Centrifuge	Andreas Hettich GmbH & Co. KG	320
Hot plate	Shanghai Scigrace Biotech. Co., Ltd	N/A
Vacuum dryer	Shanghai Jinghong Co., Ltd	XMTD-8222
Powder X-ray diffraction (PXRD)	Bruker Co., Ltd	D8 Advance
Planetary ball mill	ChiShun Co., Ltd, (Nanjing, China)	PMQW2
Inductively coupled plasma mass spectrometer (ICP-MS)	Thermo Fisher Scientific Co., Ltd	iCAP RQ
Thermogravimetric analyzer (TGA)	TA Co., Ltd	Q50
Ultraviolet-visible (UV-vis) absorbance spectrophotometer	Sunny Hengping Co., Ltd	2800S
Quartz cuvetts	Purshee experiment Co., Ltd	N/A
Fourier transform infrared (FT-IR)	Bruker Co., Ltd	EQUINOX 55
Confocal laser scanning microscope (CLSM)	Carl Zeiss, Co., Ltd	880 NLO

MATERIALS AND EQUIPMENT

Reagents

5% HNO_3 solution

Reagent	Final concentration	Amount
65% HNO_3	5%	56.7 mL
DI water	N/A	943.3 mL
Total	**N/A**	**1000 mL**

Note: The 5% HNO_3 solution can be stored at 20°C–25°C for three days.

Fe stock solution

Reagent	Final concentration	Amount
$Fe(NO_3)_3 \cdot 9H_2O$	1 ppm	7.24 mg
5% HNO_3	N/A	1000 mL
Total	**N/A**	**1000 mL**

Note: The Fe stock solution can be stored at 20°C–25°C for three days.

P Stock solution

Reagent	Final concentration	Amount
KH_2PO_4	1 ppm	4.38 mg
5% HNO_3	N/A	1000 mL
Total	**N/A**	**1000 mL**

Note: The P stock solution can be stored at 20°C–25°C for three days.

TMB solution		
Reagent	Final concentration	Amount
TMB	1.25 mM	0.15 g
DMSO	85 mM	3 mL
Glycerol	1.37 M	50 mL
Citric acid	10 mM	0.95 g
DI water	N/A	447 mL
Total	N/A	500 mL

Note: The TMB solution can be stored at 4°C in the dark for one day.

Equipment

Setup of planetary ball mill

Perform the mechanochemical synthesis of COFs on a ChiShun PMQW2 planetary ball mill. Set the rotational speed of 550 rpm with foreward for 15 min, and then hold the speed of 550 rpm with reverse for 15 min, and repeat one more time.

STEP-BY-STEP METHOD DETAILS

Part 1. In situ growth of COFs onto enzyme

Timing: 15 h

The chemically stable COF is constructed through the Schiff-base condensation reaction of Tp with Pa-1, Pa-2, and BD by a mechanochemical approach. The enzymes are encapsulated into the COFs during the COF crystallization, resulting in the one-step formation of the enzyme@COFs bio-composites. The schematic diagram of the fabrication process for enzyme@COFs was shown in Figure 1. This section provides procedures for mechanochemical synthesis of enzyme@COFs.

⚠ CRITICAL: This step for the biohybrids synthesis is only applied to enzymes@TpPa-1, enzymes@TpPa-2, enzymes@TpBD.

1. Weigh out 10 mg of the enzyme and the appropriate amount of the organic linker, such as Tp (15.75 mg), Pa-1 (12.15 mg for TpPa-1), Pa-2 (15.3 mg for TpPa-2), or BD (20.7 mg for TpBD), into an agate mortar, and fully grind them with an agate pestle about 2 min.
2. Transfer the mixed powders using spoon into 25 mL zirconia jars with ten 3.5 mm diameter and forty 1 mm diameter zirconia balls (weighing 8.2 g) (Figure 2A).

Note: The large and small diameter zirconia balls need to be prepared into zirconia jars to ensure high crystallinity. It is important to note that the volume of the balls should not exceed one-third of the jar's volume for optimal grinding efficiency.

3. Inject 60 μL mesitylene and dioxane mixed solvent (1: 1) and 30 μL deionized water into the jar to assist the mechanical milling.

⚠ CRITICAL: In this step, a portion of water was included due to its ability to protect the enzyme's natural structure under harsh conditions.[8] Mesitylene and dioxane often are commonly used in the process of mechanochemistry-synthesized COFs. Considering this, the three solvent system of H_2O-mesitylene-dioxane is chosen as the solvent for the mechanical assembly of enzyme@COFs. The volume of organic solvent added needs to be precisely controlled to avoid negatively affecting COF growth or reducing enzyme encapsulation activity. In addition, the ratio of the volume of liquid additive to the weight

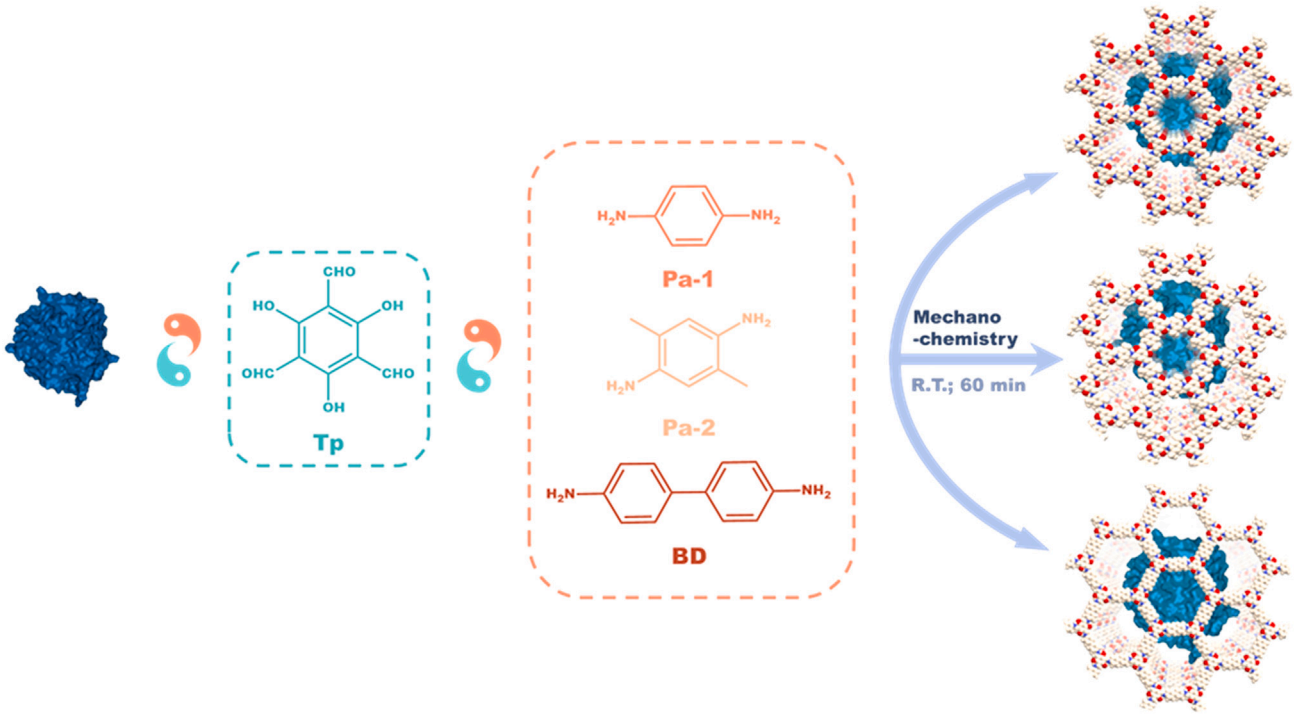

Figure 1. Schematic diagram of the fabrication process for enzyme@COFs

of reactants (η) is within a range of 1.94–2.37 during the process of mechanochemical synthesis, suggesting that the milling process falls under the category of slurry reaction.[9]

4. Place the zirconia balls in a planetary ball mill, and grind the mixture at a rotational speed of 550 rpm for 1 h at 20°C–25°C (Figure 2B).

⚠ CRITICAL: The zirconia jars should be placed symmetrically. When the instrument is running, the cover of equipment should be closed to avoid injuries.

5. Take out the zirconia jars from the equipment and add 5 mL deionized water into each jar.
6. Close the lid tightly and shake the jars to disperse any products that may be stuck to the zirconia balls or the walls of the jars.

⚠ CRITICAL: The lid of jars should be closed tightly before shaking the jars. Otherwise, the suspension will probably overflow. The ultrasound is not allowed for the jars, because it may crack the zirconia balls with cracks.

7. Transfer the suspension in a centrifuge tube using plastic pipettes and repeat step 5 and step 6 twice to ensure complete product collection (Figure 2C).
8. Place the centrifuge tubes into a centrifuge, and spin at 13200 *g* for 5 min at 20°C–25°C.
9. wash the sediment with deionized water three times (3 × 10 mL) and ethanol three times (3 × 10 mL) to remove any excess enzymes and unreacted regents (Figure 2D).

⚠ CRITICAL: It is recommended that ethanol is used to wash the precipitate before the final centrifugation, as it facilitates the drying process of the sample.

10. Dry the precipitate under 133 Pa using vacuum oven for 12 h at 20°C–25°C .

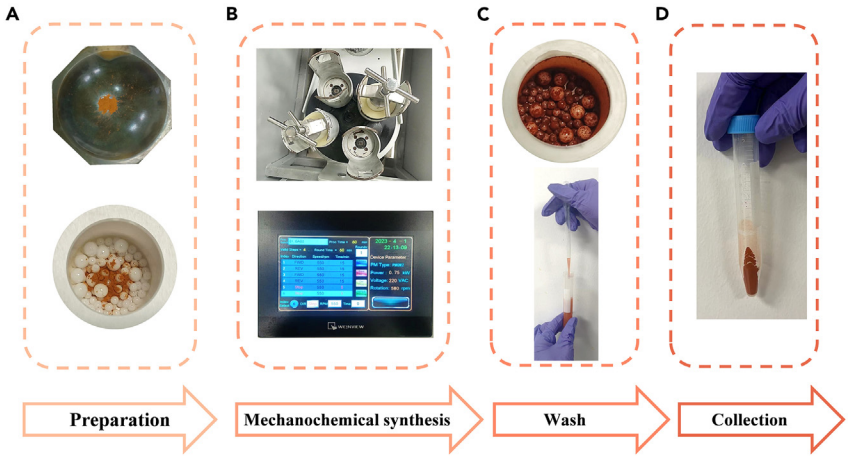

Figure 2. The procedure for *in situ* growth of COFs onto an enzyme by a mechanochemical approach
(A) Prepare the organic linker and enzyme mixed powders.
(B) Place the zirconia balls in a planetary ball mill, and start the program.
(C) Wash the product using deionized water and ethanol solution.
(D) Collect the product by centrifugation.

11. Store the product under dark at 4°C in the dark conditions.

Note: The obtained product can be stored at 4°C refrigerator in the dark conditions at least 7 days without noticeable loss of enzyme activity.

Part 2. Enzyme loading measurement

Timing: 3 h per sample

This section describes how to measure the enzyme loading content in the enzymes@COFs by ICP-MS.

⚠ CRITICAL: The content of encapsulated Cytochrome C (Cyt c) and Horseradish peroxidase (HRP) is measured based on ICP-MS method. Both Cyt c and HRP are hemoproteins and contain one Fe atom per enzyme. While lipase PS has one P atom per enzyme. According to the molecular weight of these enzyme from Protein Data Bank (https://www.rcsb.org, Cyt c: 12.37 KD, PDB: 1HRC; HRP: 34.52 KD, PDB: 1HCH; Lipase PS: 33.45 KD, PDB: 1HQD), the proportion of Fe element in Cyt c, HRP are is about 0.43% and 0.16% (w/w) respectively, while the proportion of P in Lipase PS is about 0.118% (w/w). Therefore, we can quantify these specific elements and figure out the content of encapsulated enzyme. This step for the enzyme loading measurement is only applied to Cyt c@TpPa-1, HRP@TpPa-1, Lipase PS @TpPa-1.

12. Weigh out 2 mg as-synthesized enzymes@COFs into a 10 mL glass vial, and then add 0.25 mL of 65% (w/w) nitric acid.

⚠ CRITICAL: The glass vial needs to be soaked in a solution of 5% (w/w) HNO_3 and wash it thoroughly with deionized water to avoid contamination of measured elements. In addition, concentrated HNO_3 (65% w/w, impurity $\leq$ 0.0005%) is recommended to reduce background signals

13. Put the grass vials into the oil 110°C until the HNO_3 is totally evaporated and then allow it to cool down to room temperature.

⚠ CRITICAL: Due to its volatile nature and strong acidity, HNO_3 can cause severe skin, mucous membrane, and eye irritation. Therefore, this step must be conducted in a laboratory fume hood. During the heating digestion process, the operator should wear protective masks and not get too close to the equipment to avoid burns and injuries.

14. Inject 4 mL of 5% HNO_3 into the glass vials, and sonicate the mixture for 1 time (5 min; 70 W) to form a homogeneous dispersion at room temperature.
15. Transfer the dispersion in a centrifuge, and spin at 13200 *g* for 5 min to separate the insoluble solid.

⚠ CRITICAL: Centrifugation is necessary because a small amount of solid will plug the injector of ICP-MS.

16. Before running the ICP-MS test, prepare 100 mL of 10 ppm Fe or P standard stock solution.
    a. Add 0.1 mL, 0.5 mL, 1 mL, 5 mL, 10 mL and 20 mL of 10 ppm stock solution to the 100 mL volumetric flasks using micropipettors,
    b. Fill the volume with 5% HNO_3 to obtain 100 mL of 1 ppb, 5 ppb, 10 ppb, 50 ppb, 100 ppb and 200 ppb stock solution for calibration.

⚠ CRITICAL: The 5% HNO_3 is used to dilute the bulk solutions. If the correlation coefficients (R^2) values of the calibration curve are less than 0.999, it is imperative to reconfigure the standard solutions.

17. Measure the concentration of Fe (for Cyt c and HRP) using Standard model or P (for Lipase PS) using Kinetic Energy Discrimination model by ICP-MS. Each concentration is measured with at least three replicates and then calculate the average value.

⚠ CRITICAL: Please insure the value is covered in the linear range of the standard curve. Otherwise, dilute the sample using 5% HNO_3 and repeat the Step 15.

18. Calculate the content of Fe or P in the digested solution by multiplying the volumes of the solution and the concentration measured.

⚠ CRITICAL: Based on the proportion of special element in the protein, determine the mass of encapsulated enzyme. Calculate the enzyme loading content by dividing the mass of encapsulated enzyme by the mass of the added enzymes@COFs. The loading of encapsulated Cyt c, HRP and Lipase PS in TpPa-1 measured by ICP-MS is about 22%, 28% and 13%, respectively.

Part 3. Characterization of enzyme@COFs

Timing: 10 min per sample (for step 19)

Timing: 20 min per sample (for step 20)

Timing: 30 min per sample (for step 21)

Timing: 48 h per sample (for step 22)

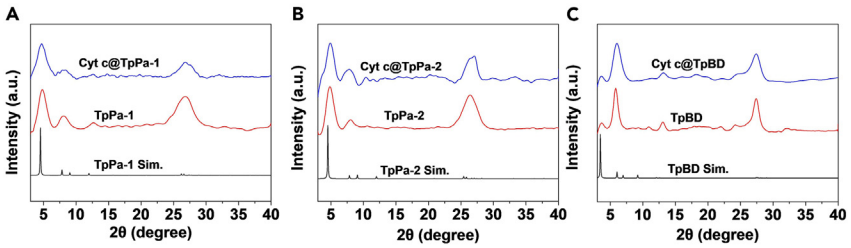

Figure 3. The PXRD characterizations of the enzyme@COFs

(A) PXRD characterization of Cyt c@TpPa-1.

(B) PXRD characterization of Cyt c@TpPa-2.

(C) PXRD characterization of Cyt c@TpBD. Reproduced with permission[1] Copyright 2022, Elsevier.

To assess the structure properties and gain insights into the spatial distribution of the encapsulated enzyme, powder X-ray diffraction (PXRD), N_2 adsorption, thermogravimetric analysis (TGA), Fourier-transform infrared spectroscopy (FTIR), and confocal laser microscopy (CLSM) experiment are employed. This section outlines procedures to characterize the enzymes@COFs using PXRD, N_2 adsorption, TGA, FTIR and CLSM.

19. PXRD characterizations of the enzyme@COFs (Figure 3).
    a. Transfer the obtained sample onto a clean silicon substrate, and press with a flat spoon.
    b. Place the silicon substrate with loading sample on a SmartLab powder X-ray Diffractometer (Cu K α Radiation, 3 kW).
    c. Set the working voltage at 40 kV and the working current at 30 mA, and measure the diffraction pattern from 3° to 40° with 0.02°/step.
20. FTIR characterizations of the enzyme@COFs (Figure 4A).
    a. Weigh out 1 mg sample and 500 mg potassium bromide into an agate mortar, and mix the powders for 2 min to ensure homogeneity.
    b. Dry the powders using an infrared-ray oven, and press the powders added into the pressing mold to obtain a transparent membrane using a tablet press.
    c. Place the raw mold onto the Bruker EQUINOX 55 spectrometer, and scan the spectrum from 4000 to 400 cm^{-1} with 32 scans at a resolution of 4 cm^{-1} to obtain the background spectrum.
    d. Place the sample-loaded mold onto the Bruker EQUINOX 55 spectrometer, and scan the spectrum from 4000 to 400 cm^{-1} with 32 scans at a resolution of 4 cm^{-1}.
    e. Subtract the background spectrum to obtain the FTIR spectrum of enzyme@COFs.
21. Thermogravimetric analysis of the enzyme@COFs (Figure 4B).
    a. Weigh out 5–10 mg dried enzyme@COFs into the sample pan of the TGA instrument, and flatten it.

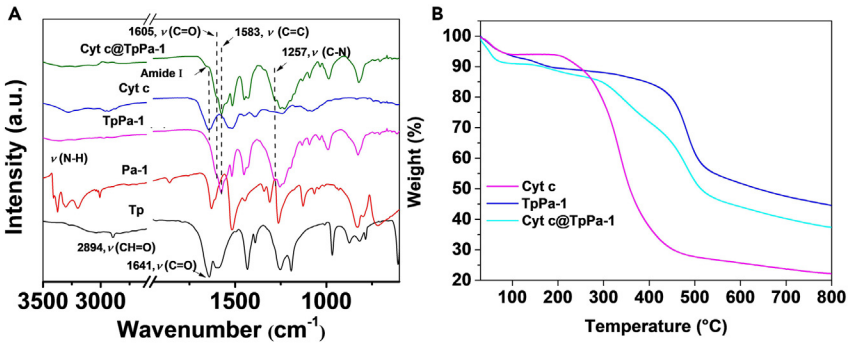

Figure 4. The FTIR and TGA characterizations of Cyt c@TpPa-1

(A) FTIR characterization of Cyt c@TpPa-1.

(B) TGA characterization of Cyt c@TpPa-2. Reproduced with permission1 Copyright 2022, Elsevier.

b. Load the pan into the TG, and set the temperature ranging from 30°C to 800°C under N_2 atmosphere (20 mL/min) with temperature increasing at 10 °C/min using a TA-Q50 system.

22. CLSM characterizations of the enzyme@COFs (Figure 5).

The TpPa-1, TpPa-2 and TpBD COFs inherently exhibit red fluorescence, but Cyt c has very limited fluorescence. In order to observe the spatial distribution of enzyme, we label the Cyt c with a blue fluorescent dye (AF350).

a. Weigh out 20 mg Cyt c into 10 mL of phosphate buffer (pH = 8.0, 0.5 M) in a 20 mL brown glass vial, and followed by introducing 1 mg AF350 into the vial.
b. Place the vial in a magnetic stirring apparatus and then stir for 4 h at 500 rpm in the dark.
c. Transfer the mixture to a 50 mL ultrafiltration centrifugal tube (molecular weight cut-off MWCO = 3 kDa).
d. Spin it at 10000 *g* for 10 min to separate the AF350-labelled Cyt c product, and add 10 mL deionized water into the centrifugal tube to disperse the product.
e. Repeat step 22 d 2 times to remove the excess reagents and salts.
f. Collect the AF350-labelled Cyt c, dry it by lyophilization (−50°C, 13 Pa) for one day and store in 4°C refrigerator.
g. Repeat the step 1–11 using AF350-labelled Cyt c instead of raw Cyt c to obtain the AF350-labelled Cyt c@COFs.
h. Add 1 mg of AF350-labelled Cyt c@COFs into 5 mL of ethanol solution, and disperse it by ultrasound for 1 time (5 min; 70 W).
i. Inject 2 μL of sample solution to a confocal dish, and dry it under vacuum at room temperature.
j. Place the confocal dish onto a CLSM. Locate the sample under bright field by × 100 magnification, and image the fluorescence of COF under the excitation wavelength of 561 nm and image the fluorescence AF350-labelled Cyt c of under the excitation wavelength of 405 nm.

⚠ CRITICAL: For a satisfactory outcome, we can see the blue fluorescence originated from the AF350-labeled Cyt c, completely overlaid with the red fluorescence of Cyt c@TpPa-1 (the right in Figure 5) scaffold due to the π conjugate system, confirming the Cyt c was encapsulated and uniformly distributed within the frameworks.

Part 4. Evaluation of the biocatalytic activity

Evaluation of the Cytc and HRP activity

Timing: 1 h per sample

TMB is selected as the peroxidase substrate to evaluate the activity test, in which Cyt c@COFs, HRP@COFs can catalyze the oxidation of TMB, resulting in the formation of a blue product, oxTMB (Figure 6A). The biocatalytic activity can be measured by monitoring the oxTMB at 650 nm UV absorbance. This section describes how to measure the Cytc and HRP activity of the enzymes@COFs.

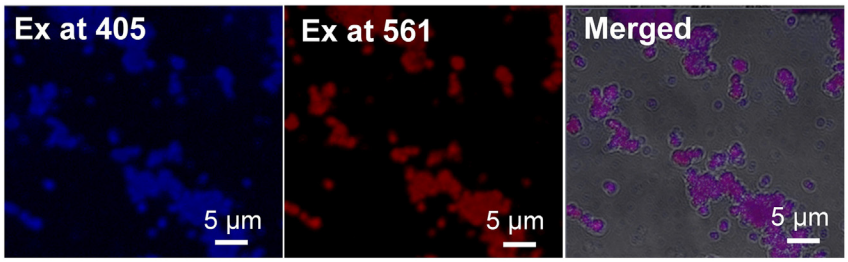

Figure 5. The CLSM characterizations of Cyt c@TpPa-1

CLSM images of Cyt c@TpPa-1 (M), in which Cyt c was labeled by the blue fluorescent AF350 dye and TpPa-1 (M) exhibited red fluorescence. Scale bar, 5 mm. Reproduced with permission[1] Copyright 2022, Elsevier.

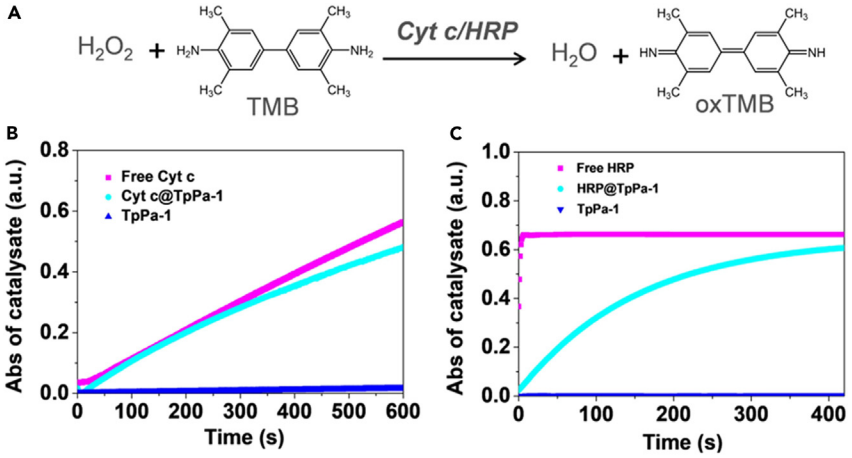

Figure 6. The biocatalytic activities of Cyt c@TpPa-1 and HRP@TpPa-1

(A) Reaction equation.

(B) The bioactivities of free Cyt c, TpPa-1, and Cyt c@TpPa-1 biohybrids through mechanochemistry-guided assembly, respectively.

(C) The bioactivities of free Cyt c, TpPa-1, and Cyt c@TpPa-1 biohybrids through mechanochemistry-guided assembly, respectively. Reproduced with permission[1] Copyright 2022, Elsevier.

⚠ CRITICAL: This activity test is only applied to Cytc@TpPa-1 and HRP@TpPa-1.

23. Weigh out the 1.15 mg Cytc@TpPa-1 or 0.95 mg HRP@TpPa-1 into 15 mL centrifugal tube, add 10 mL Tris buffer (pH = 7.4, 50 mM) and sonicate it.
24. Add 0.4 mL of dispersed catalyst into a Uv-vis quartz cuvette by pipette, followed by adding 0.2 mL prepared TMB solution as a substrate.
25. Place the cuvette in a UV-vis spectrophotometer, and immediately add 0.2 mL of 0.8 mM H_2O_2 (for Cytc@TpPa-1) or 0.08 mM H_2O_2 (for HRP@TpPa-1) to activate the catalytic reaction.
26. Collect the data using a time-scanning mode at 650 nm from 0 to 300 s, and set the sampling interval of 1 s.

⚠ CRITICAL: Because the enzymatic reaction is so fast that the measurement measuring parameters should be predefined. Start the test immediately after adding H_2O_2.

27. Calculate the slope of the kinetic curve in the initial phase using linear fitting processing on Origin software, and measure the reaction rate based on the average value of the slope of three replicates.

Evaluation of the lipase PS activity

Timing: 1 h per sample

p-nitrophenyl palmitate (p-NPP) is utilized as a substrate to assess the activity of Lipase PS@COFs. Lipase PS is capable of hydrolyzing the ester bond of p-NPP, generating a yellow-colored p-nitrophenol (Figure 7A). The concentration of p-nitrophenol can be monitored at 405 nm using a UV-vis spectrophotometer. This section describes how to measure the Lipase PS activity of the Lipase PS@TpPa-1.

⚠ CRITICAL: This activity test is only applied to Lipase PS@TpPa-1.

28. Weigh out the 2.5 mg Lipase PS@TpPa-1 into a 15 mL centrifugal tube, add 10 mL Tris-HCl buffer (pH = 7.4, 50 mM) and disperse it through ultrasound for 1 time (5 min; 70 W).

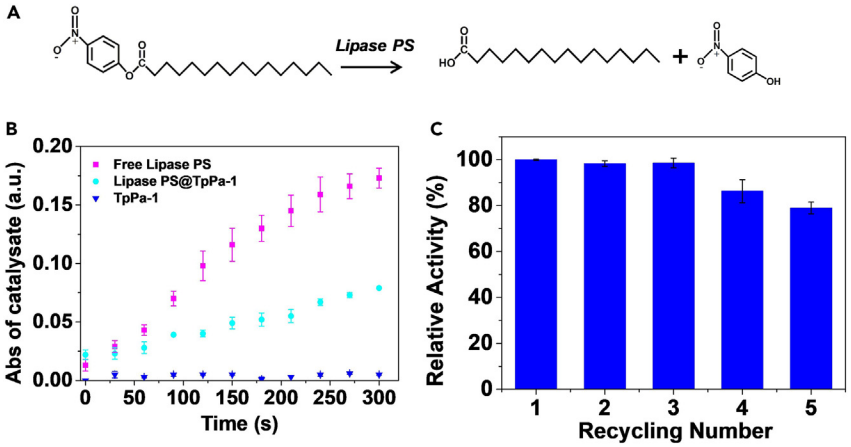

Figure 7. The biocatalytic activities of Lipase PS@TpPa-1 and recyclability tests

(A) Reaction equation.

(B) The bioactivities of free Cyt c, TpPa-1, and Lipase PS@TpPa-1 biohybrids through mechanochemistry-guided assembly, respectively. The error bars are representative of the standard deviation of triplicate experiments.

(C) The recyclability of our enzyme@TpPa-1 system. The error bars are representative of the standard deviation of triplicate experiments. Reproduced with permission[1] Copyright 2022, Elsevier.

29. Add 0.1 mL of dispersed catalyst into a 2 mL centrifugal tube, followed by adding 0.8 mL Tris buffer and 0.1 mL of a series concentration of p-NPP solution to activate the catalytic reaction.
30. Collect supernatant by centrifugation at 13200 *g* for 30 s every 1 min, and record the data at 405 nm.
31. Calculate the slope of the kinetic curve in the initial phase using linear fitting processing on Origin software.

Part 5. Recyclability tests

Timing: 3 h per sample

This section aims to investigate the recyclability of HRP@TpPa-1.

⚠ CRITICAL: This recyclability test is only applied to HRP@TpPa-1.

32. Weigh out 1 mg HRP@TpPa-1 into a 15 mL of centrifugal tube, add 4 mL of Tris-buffer (pH 7.4, 50 mM) and sonicate the mixture for 1 time (5 min; 70 W) in an ice bath to form a homogeneous dispersion.
33. Add 2 mL prepared TMB solution into the plastic tube, and activate the catalytic reaction by adding 2 mL 0.08 mM H_2O_2 at 25°C.
34. After 5 min reaction in the dark, collect the supernatant by centrifuge, and measure the absorbance at 650 nm by the UV-vis spectrophotometer.
35. Collect precipitate by washing the residual HRP@TpPa-1 with distilled water 3 times to remove the catalytic product and unreacted H_2O_2.

⚠ CRITICAL: When washing the precipitate, use the plastic pipettes transferring the supernatant carefully to avoid the loss of catalysts.

36. Disperse the collected HRP@TpPa-1 in 4 mL Tris-buffer (pH 7.4, 50 mM), and repeat the step 33 to step 35.

Note: The intensity of the catalytic product at 650 nm in each running relative to the first round is used for the evaluation of the recyclability.

EXPECTED OUTCOMES

This protocol presents a method for prepare the enzyme-encapsulated COFs biocomposites by a green and convenient mechanochemistry-guided assembly strategy, and describes the procedures for the evaluation of the biocatalytic activity and recyclability of enzyme@COFs. In this regard, the enzyme can be successfully wrapped into various COF hosts with diverse pore sizes *in situ*, including TpPa-1, TpPa-2, and TpBD COFs. The synthesized enzyme@COFs display high crystallinity and maintain high bioactivity (Figure 6B and 6C; Figure 7B).

This mechanochemistry-guided assembling approach can be extended to host different enzymes, including Cyt c, HRP and Lipase PS. The large pore structure of the enzyme@COFs allows the relatively large-sized substrates to enter into the cavity and activate the catalytic reaction of encapsulated enzymes. The covalent-linked topology of COFs affords protection against the denaturation of the guest enzymes[1]. In the HRP@TpPa-1 recyclability tests, the catalytic activity is retained at approximately 80% after five reuse cycles (Figure 7C). This work provides a mechanochemistry-guided assembly strategy toward novel COF biosystems and it will extend the biocatalytic reaction that can be explored in harsh conditions.

LIMITATIONS

The main limitation of this protocol lies in encapsulating certain highly dynamic and vulnerable enzymes that may be sensitive to mechanical milling. During the COF crystallization, the mechanical milling may cause the changes of enzyme's structure, leading to the denaturation of some enzymes, for example catalase.[1] In addition, the recyclability studies have only be carried out for the HRP@TpPa-1 biocomposite, while the recyclable performances for other enzyme counterparts remain unknown. Furthermore, the crystallinity of the obtained COFs product using mechanochemistry is inferior to that using traditional solvothermal method. The crystallinity of COFs biocomposite in this protocol can be ameliorated by adding 30 μL of 10 mM acetic acid to replace the deionized water during the mechanochemistry reaction.

TROUBLESHOOTING

Problem 1

The obtained enzyme@COFs have no crystallinity (steps 3).

Potential solution

Add 30 μL of 1 mM acetic acid solution into the organic linkers replacing the deionized water to slow down the Schiff-base reaction, and this can improve the crystallinity of obtained enzyme@COFs.

Problem 2

The yield of product obtained is not high (Steps 5–6).

Potential solution

Repeat the washing step to recycle the products absorbed on the zirconia balls and the wall of jars.

Problem 3

The enzyme loading contents obtained from ICP-MS are not accurate enough (Step 16).

Potential solution

Weight a range of different quality of raw enzymes and digest them as alternative standards for the ICP-MS quantification. For example, weigh out 0.2 mg, 0.5 mg, 1 mg, 1.5 mg, and 2 mg raw Cyt c into a 10 mL glass vial, respectively, then add 0.25 mL of concentrated nitric acid (65%, w/w), and repeat steps 11–13.

Problem 4

In FT-IR spectrum, the typical amide I band of enzyme are not observed (Step 18).

Potential solution

Dry the potassium bromide or the product under vacuum at 80°C 12 h before the FT-IR test.

Problem 5

The catalysts have low bioactivity (Step 25).

Potential solution

The product is hard to be re-dispersed after drying, which may decrease the bioactivity. Increase the sonication time to ensure the bio-composites are highly dispersive or directly store the collected product in deionized water at 4°C without undergoing the drying treatment.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for the resources are available from the lead contact, Guosheng Chen (chengsh39@mail.sysu.edu.cn).

Materials availability

This study did not generate unique materials.

Data and code availability

This study did not involve any code, and the data would be available upon request.

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AUTHOR CONTRIBUTIONS

R.G. designed and performed the experiments and drafted the manuscript. X.K. did the data analysis. R.H. and Y.S. drew the figures. L.G. and H.W. investigated the experiments. G.C. directed the projection and reviewed and edited the manuscript. G.C., S.H., and G.O. planned and supervised the entire study.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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