Supplementary Information

A charge-assisted hydrogen-bonded organic framework as host platform for enzymes immobilization and robust biocatalysis

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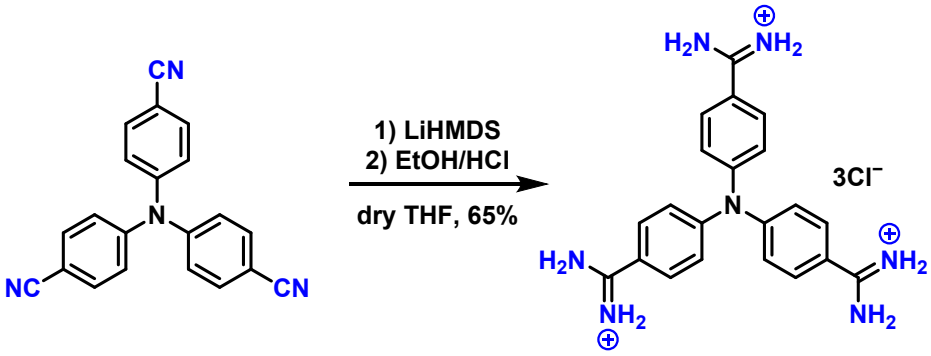
1. General methods

Reagent and materials: The enzymes including catalase (CAT) and α-amylase (α-Amy) were obtained from Meryer (Shanghai) Biochemical Technology Co., Ltd. The fluorescence dyes and other chemicals were purchased from Macklin Biochemical Technology Co., Ltd. (Shanghai, China). All reagents and solvents were purchased from commercial sources and used as received without further purification.

Characterization: ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AV 400 spectrometer, and the chemical shifts were reported in ppm with respect to reference standards. FT-IR spectra were taken on a Bruker AlPHA FT-IR spectrometer (with KBr pellets) in 4000–400 cm^{-1}. Powder X-ray diffraction (PXRD) patterns were recorded on a Bruker D8 Advance diffractometer (Cu-Kα, γ = 1.5406 Å), operating at 40 kV and 100 mA, and the diffraction intensity data were obtained by the continuous scans in the 2θ/θ mode with scan speed of 2 s/step and step size of 0.02°. The transmission electron microscope (TEM) and energy-dispersive X-ray spectroscopy (EDS) were performed on a Thermo Fisher Scientific Talos F200X scanning electron microscope, with the electron beam energy of 200 keV. The ultraviolet-visible (UV-Vis) absorbance measurements were performed with a TU-1900 spectrophotometer (Puxi, Beijing). Confocal laser scanning microscope (LSM 880 NLO, Carl Zeiss, Göttingen, Germany) was used to determine the distribution of enzyme within the nanosystem. Liquid chromatograph-mass spectrometer (LC-MS) of LTQ XL and multiscan spectrum of LSM 800 from Thermo Fisher Scientific were used. The metal concentrations were determined on a Perkin-Elmer Avio 200 inductively coupled plasma optical emission spectrometer (ICP-OES). Dynamic light scattering (DLS) measurements were conducted on the Nano ZS ZEN3600, Malvern.

2. Experiment section

2.1 Preparation of NBI·3HCl



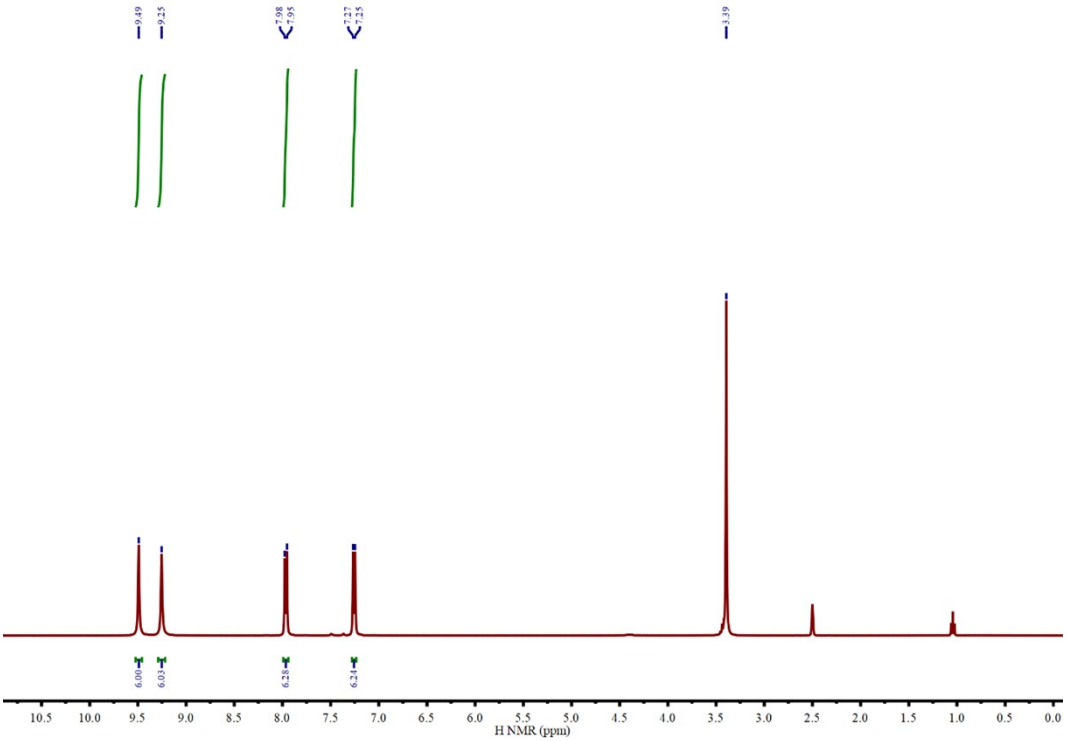
Tris(4-cyanophenyl)amine (0.32 g, 1 mmol) was dissolved in dry THF (10 mL). It was cooled to 0 ºC under an Argon atmosphere, and LiHMDS (1.0 M in THF, 8 mL) was added dropwise in 30 min resulting in the immediate formation of a precipitate. The mixture was allowed to warm to room temperature and stirred overnight, during which time the precipitate dissolved to give an orange solution. And then this was cooled to 0 ºC and ethanolic HCl (prepared by cautiously adding 3 mL of acetyl chloride to 8 mL ethanol) was added, which resulted in the formation of a yellow precipitate. And the mixture was stirred at 25 ºC overnight. The precipitate was then filtered, washed with ethyl acetate and ethanol. The powder of 4,4',4''-nitrilotribenzimidamide trihydrochloride (NBI·3HCl) was recrystallized from H_2O-EtOH mixture to get 0.3124 g light-yellow product (yield 65%).

^{1}H NMR (400 MHz, DMSO-d_6) δ 9.49 (s, 6H), 9.25 (s, 6H), 7.97 (d, J = 8.7 Hz, 6H), 7.26 (d, J = 8.7 Hz, 6H).

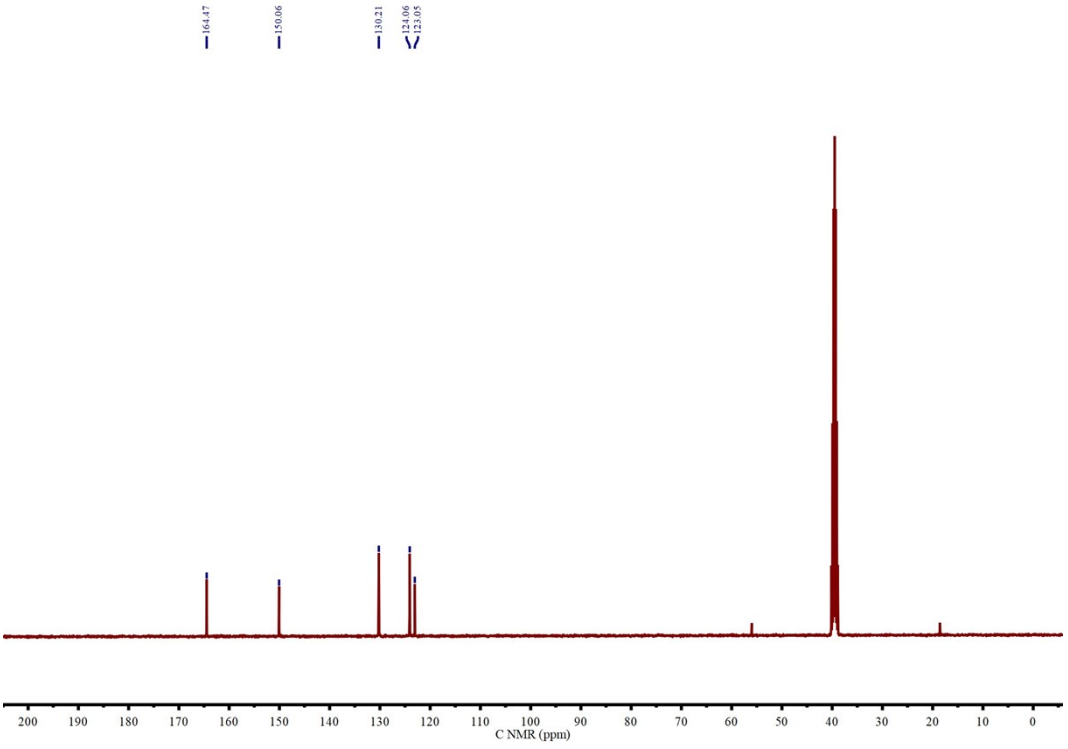
^{13}C NMR (101 MHz, DMSO-d_6) δ 164.47, 150.06, 130.21, 124.06, 123.05.

FT-IR: 1677 cm^{-1} (C=N stretch).

LC-MS (pos.) 372.30, calc. for $[C_{21}H_{22}N_7]^+$ (i.e. loss of $2H^+$ and $3Cl^-$): 372.4 Da.



(a)



(b)

Fig. S1. ^{1}H NMR (a) and ^{13}C NMR (b) spectra of NBI·3HCl.

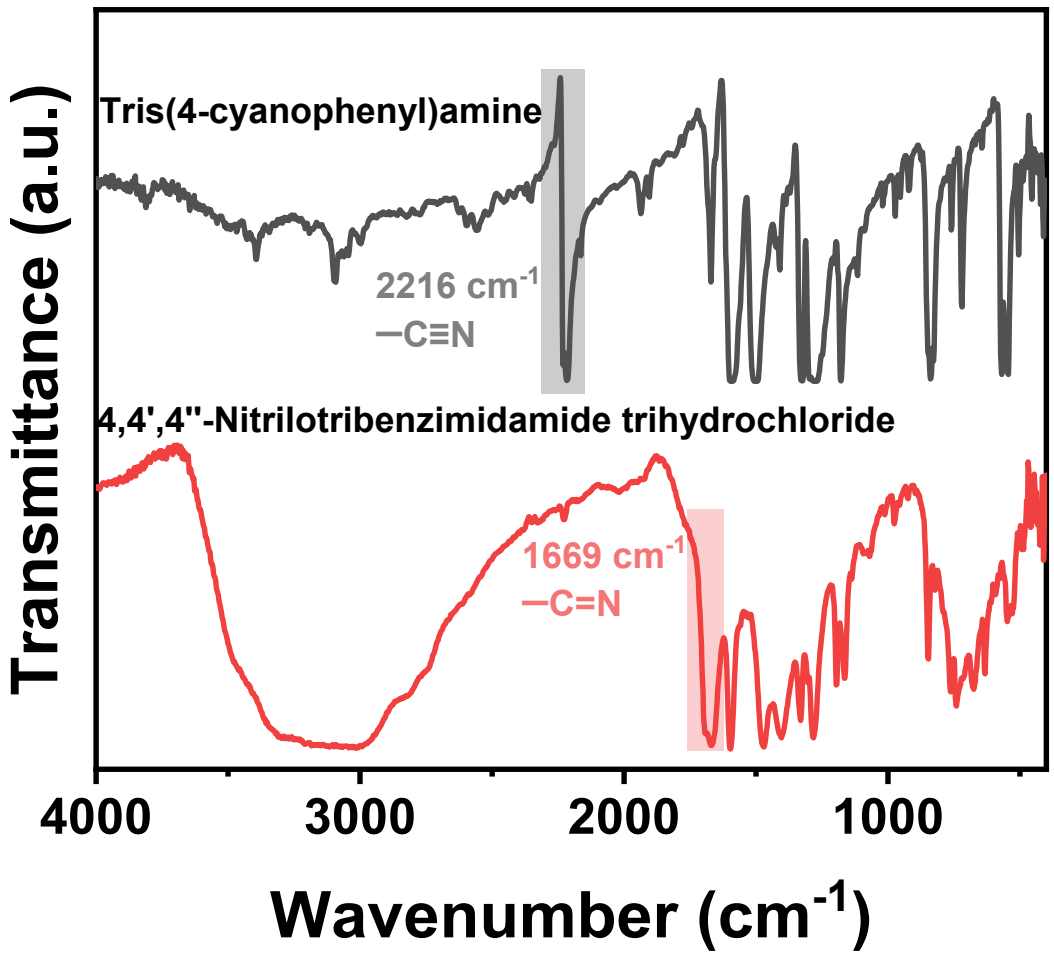
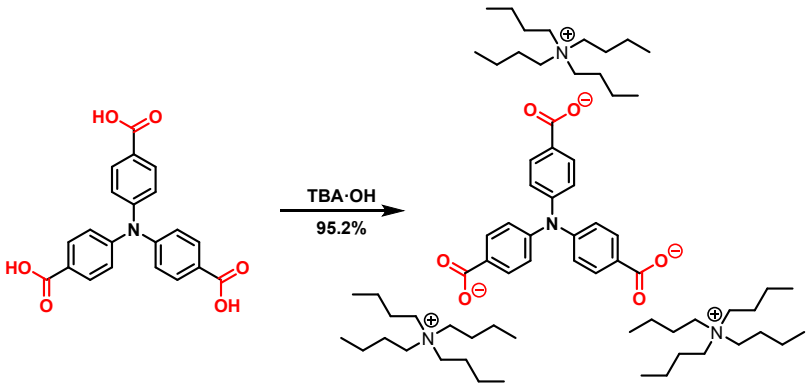


Fig. S2. FT-IR spectrum of Tris(4-cyanophenyl)amine and NBI·3HCl.

2.2 Preparation of TBA_3·TCPA



4,4',4''-Nitrilotribenzoic acid (TCPA) (0.1887 g, 0.5 mmol) was suspended in water (10 mL), and a solution of tetrabutyl ammonium hydroxide (TBA·OH) (1.0 M in methanol, 1.5 mL) was added and the mixture stirred for 1 hour at room temperature during which time everything dissolved to give a clear solution. It was taken to dryness under reduced pressure and dried thoroughly in vacuo to give product as a yellow powder of TBA_3·TCPA. Yield: 0.5242 g (0.48mmol, 95.2%).

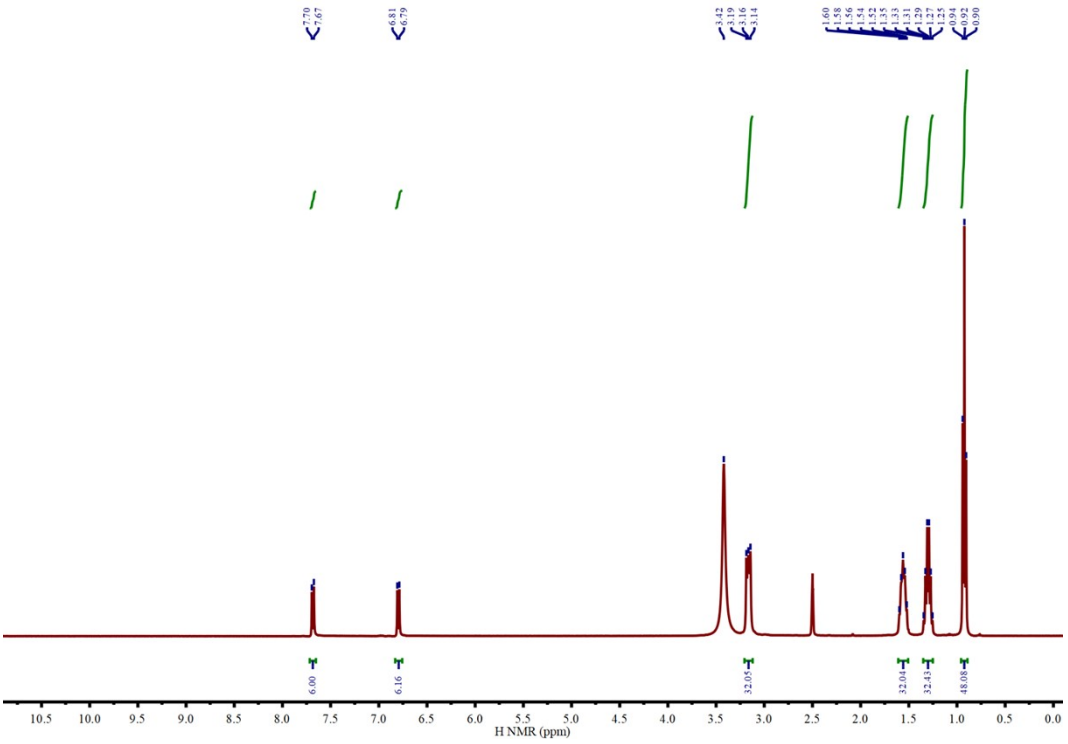
^{1}H NMR (400 MHz, DMSO-d_6) δ 7.68 (d, J = 8.5 Hz, 6H), 6.80 (d, J = 8.5 Hz, 6H), 3.21–3.12 (m, 32H), 1.56 (p, J = 8.0 Hz, 32H), 1.30 (h, J = 7.3 Hz, 32H), 0.92 (t, J = 7.3 Hz, 48H).

^{13}C NMR (101 MHz, DMSO-d_6) δ 147.76, 137.04, 130.48, 122.38, 58.03, 23.56, 19.67, 13.94.

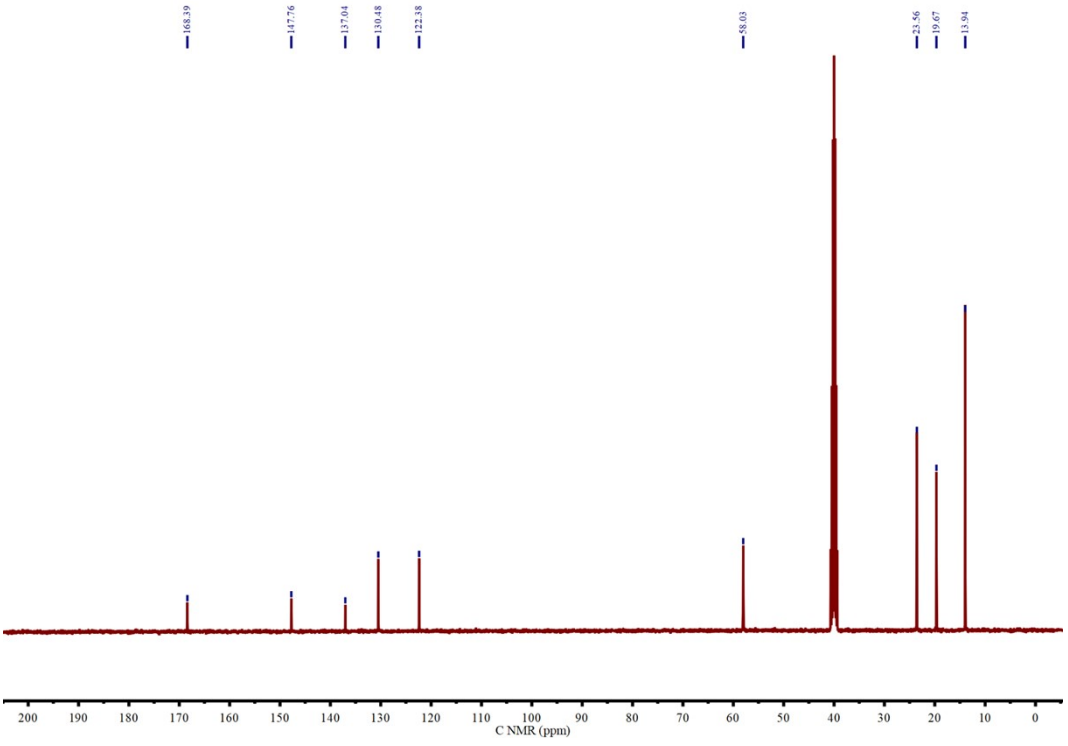
LC-MS (pos.): 242.36, calc. for $[C_{16}H_{36}N]^+$ (i.e. TBA^+): 242.36 Da.

LC-MS (neg.): 617.6, calc. for $[C_{37}H_{49}N_2O_6]^-$ (i.e. $N\text{-}COO^{3-}\cdot H^+\cdot TBA^+$): 617.6 Da;

376.43, calc. for $[C_{21}H_{14}NO_6]^-$ (i.e. $N\text{-}COO^{3-}\cdot 2H^+$): 376.43 Da.



(a)



(b)

Fig. S3. ^{1}H NMR (a) and ^{13}C NMR (b) spectra of TBA_3·TCPA.

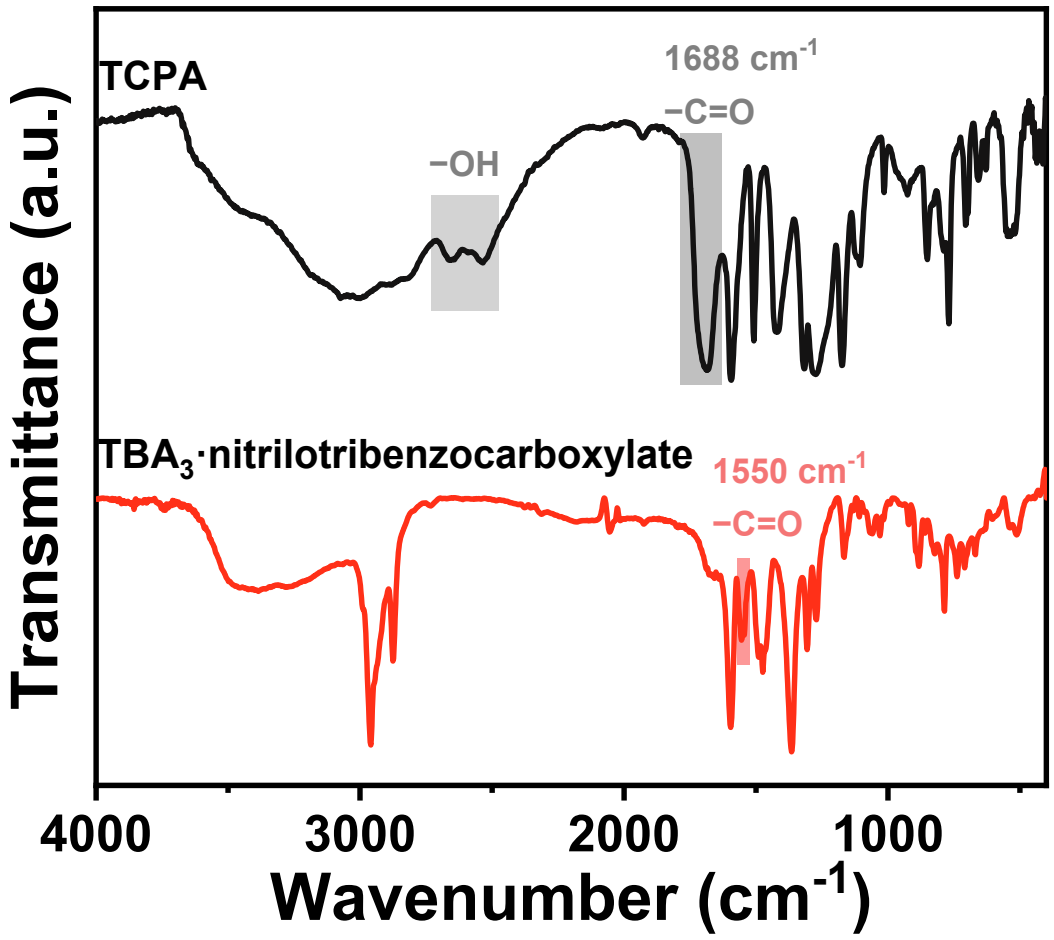
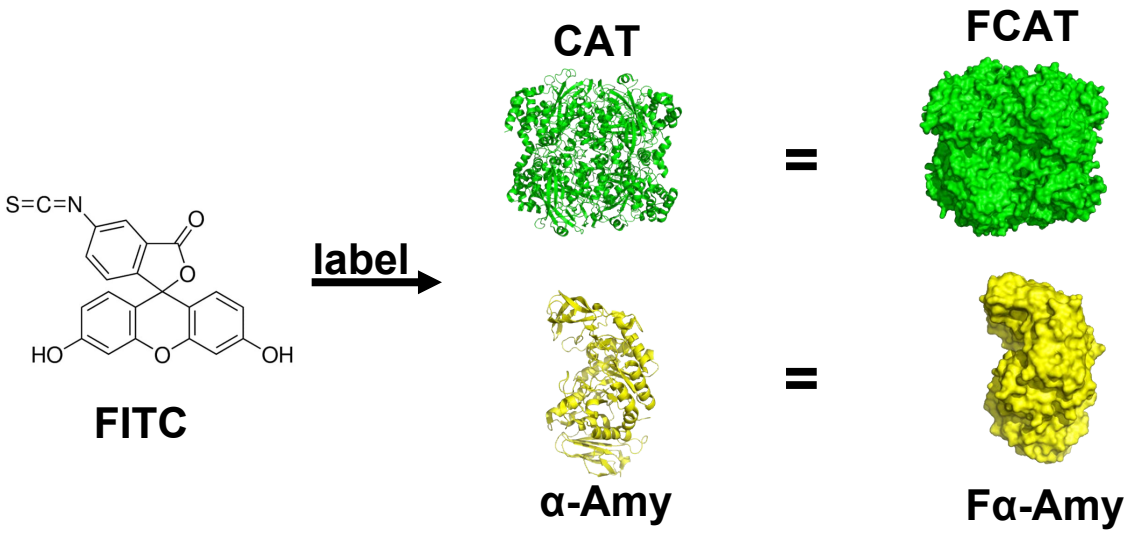


Fig. S4. FT-IR spectrum of TCPA and TBA_3·TCPA.

2.3 Preparation of TNU-14

NBI·3HCl (10 mg, 0.021 mmol) was dispersed in H_2O (3 mL) to form solution A. TBA_3·TCPA (23 mg, 0.021 mmol) was dissolved in H_2O (3 mL) to form solution B. Then, solution A was added dropwise to the stirred solution B at room temperature. After mixing, a yellow precipitate was formed immediately. Then, the mixture was aging for fifteen minutes in dark. The as-synthesized product (TNU-14) was collected, centrifuged (10000 rpm, 5 min), and washed three times with water to ensure the complete removal of unreacted precursors.

2.4 Fluorescence labeling of enzyme

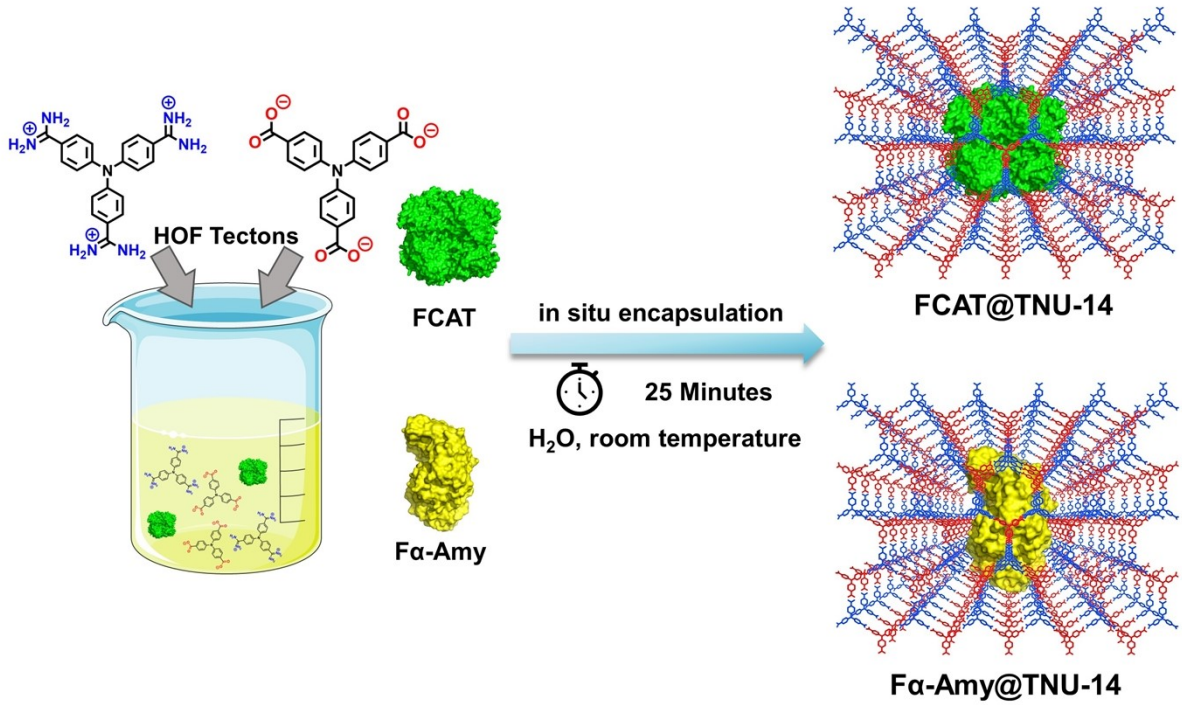


Fluorescence labeling of enzymes was based on the conjugation between the amino of lysine residue of enzymes and the Fluorescein isothiocyanate (FITC). In brief, 10 mg catalase (CAT; Sigma-Aldrich, catalase from bovine liver, 2000−5000 units mg^{-1} protein) or α-Amylase (α-Amy, Macklin, 4000 units g^{-1}) was dispersed into 10 mL carbonate buffer solution (pH = 9.0, 0.5 M), followed by adding 1 mg FITC. The mixed solution was then stirred for 12 h in the dark. Finally, the FITC-labelled enzyme was obtained through ultrafiltration by a centrifugal filter device (molecular weight cut-off MWCO = 10 kDa). The concentration-solvent-exchange process was repeated three times (4000 rpm for 20 min) to ensure the buffer salts were completely removed from

the solution. The obtained FITC-enzyme solution was stored in darkness at 4 °C.

2.5 Synthesis of Enzyme@TNU-14 Biocomposite

NBI·3HCl (10 mg) was dissolved in H_2O (1.5 mL) to form solution A. An aqueous solution of enzymes (FCAT and Fα-Amy 3 mg, 1.5 mL of 2 mg mL^{-1} stock solution) was added to solution A and stirred at room temperature for 10 min to form solution B. TBA_3·TCPA (23 mg) was dissolved in 3 mL of H_2O to form solution C. Solution C was then added dropwise to solution B under stirring. The mixture was then left to gently stir for 5 min and then aged for 10 min to ensure the completion of the reaction. Thereafter, Enzyme@TNU-14 composite was collected by centrifugation and then washed, dispersed, and centrifuged three times in H_2O to remove any unreacted precursors and loosely adsorbed enzyme.



2.6 Immobilization efficiency

The immobilization efficiency in Enzyme@TNU-14 was determined by fluorescence spectrophotometry using calibration curves of different enzymes labeled with

fluorescein isothiocyanate (FITC) dye. Immobilization efficiency was calculated by the following Eq. (1):

$$\text{Immobilization efficiency (\%)} = [(m - C{\cdot}V)/m] \times 100\% \quad (1)$$

where m is the mass of the enzyme initially added to the system (mg); C is the concentration of enzyme in the supernatant after centrifugation of materials synthesized (mg mL^{-1}); and V is the volume of the supernatant (mL).

2.7 Catalytic performance of FCAT and the FCAT@TNU-14

The calibration curve of the $Ti(SO_4)_2$ assay. H_2O_2 standard solutions at various concentrations (0, 10, 25, 50, 100, 200, 400, 600, 800, 1000 μM were mixed with the $Ti(SO_4)_2$ reagent (40 μL, obtained from 1.33 mL of 24% $Ti(SO_4)_2$ + 8.33 mL of H_2SO_4 in 50 mL of distilled water) and incubated for 30 min at room temperature before reading absorbance at 405 nm.

Catalytic activity of free FCAT and FCAT@TNU-14. In each test, the catalytic activity of FCAT or FCAT@TNU-14 was assessed by the amount of H_2O_2. Experiments were conducted using the property of $Ti(SO_4)_2$ colorimetric method. The FCAT concentration in each experiment was set at 5 μg mL^{-1}. Typically, the enzyme or enzyme mixture was dispersed into different pH conditions. The enzymatic assays were performed in hydrochloric acid (pH 2–3), acetate buffer (pH 4–5, 0.1 M), phosphate buffer (pH 6–8, 0.1 M), or glycine-HCl buffer (pH 9–10, 0.1 M), sodium hydroxide (pH 11–12) with H_2O_2 (1 mM). And 5 μL of the reaction solution was taken out at regular intervals during the reaction process of H_2O_2 concentration determination. After 40 μL of $Ti(SO_4)_2$ solution was added to stop the reaction. After stopping the reaction, the absorbance value can be detected at 405 nm with a UV-vis spectrophotometer and quantified by a standard calibration curve. The relative activity of FCAT or FCAT@TNU-14 was calculated as activity at pH 8/maximal FCAT activity.

Catalytic activity of TNU-14 and supernatant of FCAT@TNU-14. The assay was performed in phosphate buffer (pH 7.4, 100 mM) with TNU-14 material (1 mg/mL) or FCAT@TNU-14 supernatant (10 μL) (FCAT@TNU-14 was stored in H_2O, 2 mg/mL). The H_2O_2 concentration was 1 mM.

2.8 Catalytic performance of Fα-Amy and the Fα-Amy@TNU-14 biocomposite

Catalytic activity of Fα-Amy and the Fα-Amy@TNU-14 biocomposite. In each test, the catalytic activity of Fα-Amy or Fα-Amy@TNU-14 was assessed by the amount of starch hydrolyzed. Experiments were conducted using the characteristic of starch turning blue when encountering iodine. The Fα-Amy concentration in each experiment was set at 20 μg mL^{-1}. Typically, the enzyme or enzyme mixture was dispersed into different pH conditions. The enzymatic assays were performed in hydrochloric acid (pH 2–3), acetate buffer (pH 4–5, 0.1 M), phosphate buffer (pH 6–8, 0.1 M), or glycine-HCl buffer (pH 9–10, 0.1 M), sodium hydroxide (pH 11–12) with starch (0.5% w/v). And 925 μL of the reaction solution was taken out at regular intervals during the reaction process of starch hydrolyzed concentration determination. After 50 μL of KI-I_2 solution was added to stop the reaction. After stopping the reaction, the absorbance value can be detected at 598 nm with a UV-vis spectrophotometer. The relative activity of Fα-Amy or Fα-Amy@TNU-14 was calculated as activity at pH 7/maximal Fα-Amy activity.

Catalytic activity of TNU-14 and supernatant of Fα-Amy@TNU-14. The assay was performed in phosphate buffer (pH 7.4, 100 mM) with TNU-14 material (1 mg mL^{-1}) or Fα-Amy@TNU-14 supernatant (10 μL) (Fα-Amy@TNU-14 was stored in H_2O for one week, 2 mg mL^{-1}). The starch concentration was 0.5% w/v.

2.9 Stability and recyclability test

The stability test for FCAT@TNU-14 and Fα-Amy@TNU-14 was incubated in different

pH values (2–12) for 24 h, different temperatures (30–60 ºC) for 1 h, and a series of inhospitable conditions, such as trypsin (0.25%), urea (6 mol L^{-1}) for 2 h. Then, the catalytic reaction was performed to determine the residual activity. Recyclability tests were carried out in a fresh reaction solution (2 mL), and the activity for enzyme was measured in the supernatant after each reaction by using UV-vis.

3. Supplementary Tables

Table S1. Z average (d. nm) and zeta potential values (mV) for the FCAT and FCAT/TNU-14 ligand mixture.

Sample	Z average (d. nm)	Zeta potential (mV)
FCAT	193.5 ± 14.3	–13.3 ± 0.9
FCAT & NBI	**15456.7 ± 41.6**	**–4.0 ± 0.9**
FCAT & TBA_3·TCPA	248.7 ± 7.4	–21.6 ± 0.9

Table S2. Loading of FCAT in the FCAT@TNU-14.

Sample	Dosage[a] (mg/mL)	Loading[b] (%, w/w)
FCAT@TNU-14	0.5	17.90%
		17.04%
		17.48%

[a] The dosage (mg/mL) means the enzymes concentration in the Initial assembly system.

[b] The loading (w/w) of each enzyme was calculated based on the assembly experiment with fluorescence labelled enzymes.

Table S3. Calculated FCAT loadings in three assays of FCAT@TNU-14 using Bradford proteins assays.

FCAT@TNU-14	**No. 1**	**No. 2**	**No. 3**	**Average**
FCAT (wt%)	17.8	17.3	17.6	17.6

Table S4. *Z* average (d. nm) and zeta potential values (mV) for the F*α*-Amy and F*α*-Amy/TNU-14 ligand mixture.

Sample	Z average (d. nm)	Zeta potential (mV)
F*α*-Amy	1253.1 ± 27.6	−27.7 ± 4.0
F*α*-Amy & NBI	**11178.0 ± 2904.7**	**−5.7 ± 1.7**
F*α*-Amy & TBA_3·TCPA	5669.3 ± 864.7	−29.3 ± 2.3

Table S5. Loading of α-Amy in the α-Amy@TNU-14.

Sample	Dosage[a] (mg/mL)	Loading[b] (%, w/w)
α-Amy@TNU-14	0.5	18.21
		17.52
		17.90

[a] The dosage (mg/mL) means the enzymes concentration in the Initial assembly system.

[b] The loading (w/w) of each enzyme was calculated based on the assembly experiment with fluorescence labelled enzymes.

Table S6. Calculated Fα-Amy loadings in three assays of Fα-Amy@TNU-14 using Bradford proteins assays.

Fα-Amy@TNU-14	**No. 1**	**No. 2**	**No. 3**	**Average**
Fα-Amy(wt%)	17.7	18.4	17.8	17.9

4. Supplementary Figures

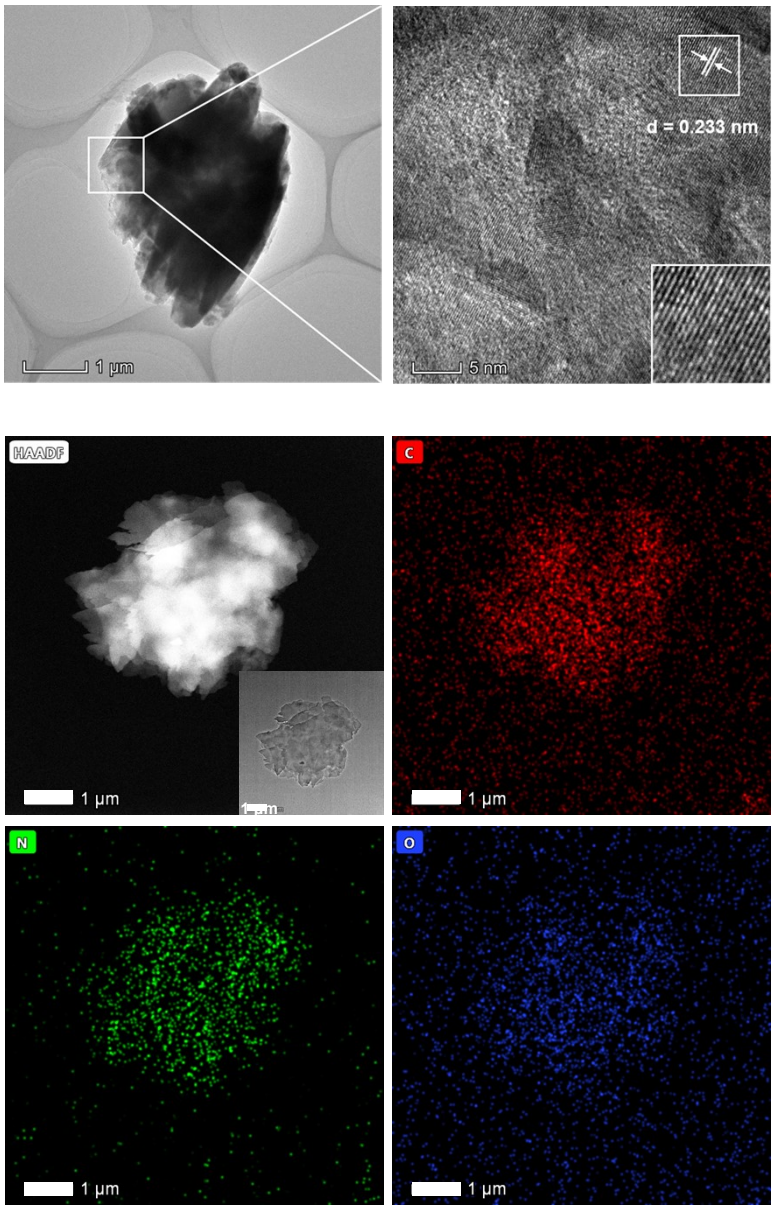


Fig. S5. HAADF-STEM image and EDS elemental mapping images of TNU-14.

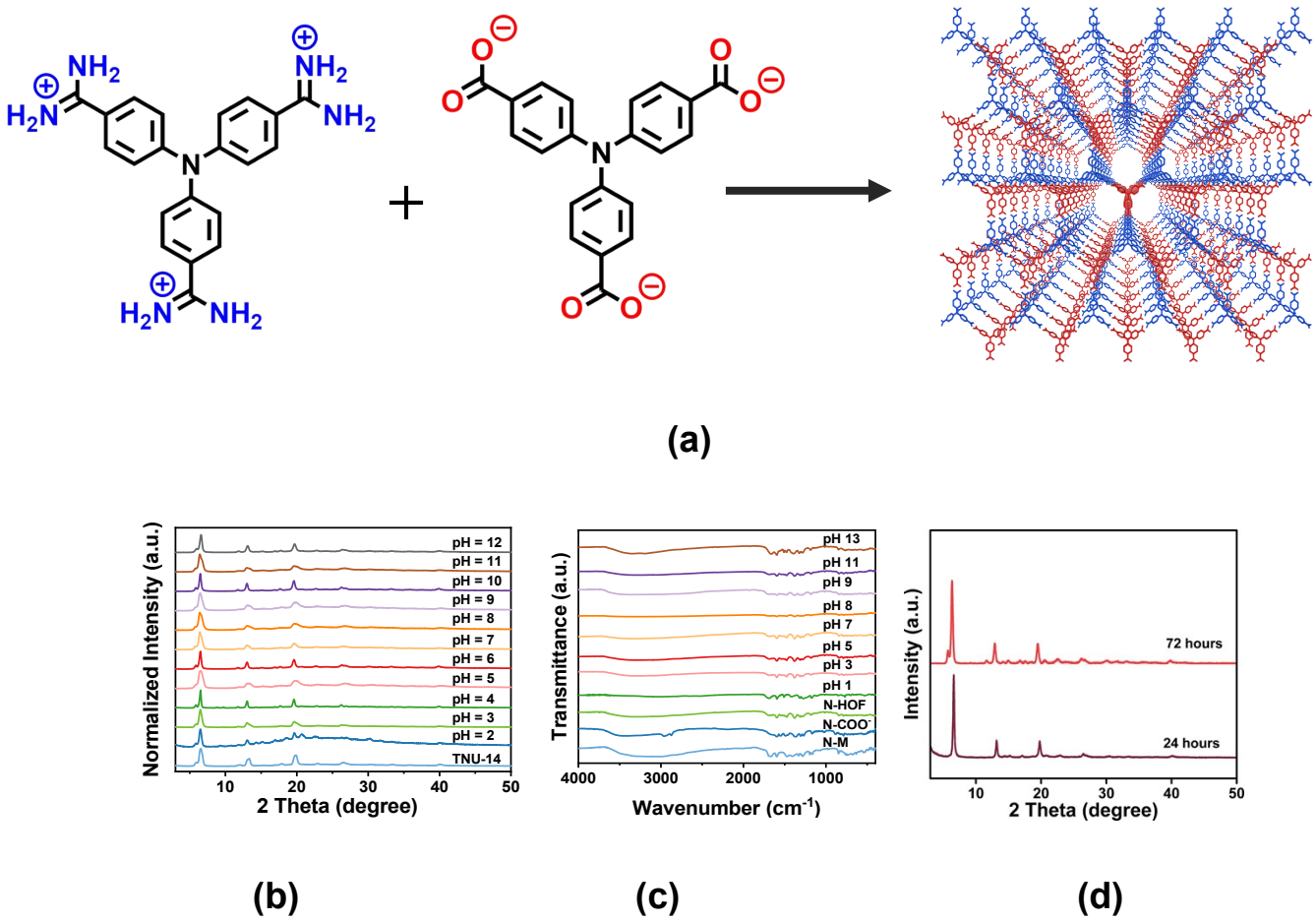


Fig. S6. (a) Schematic view of the preparation of TNU-14. (b) PXRD and (c) FTIR of TNU-14 after exposure to buffer solutions at different pH (from 2−12) for 24 h. (d) PXRD of TNU-14 after exposure at pH = 7 for 72 h.

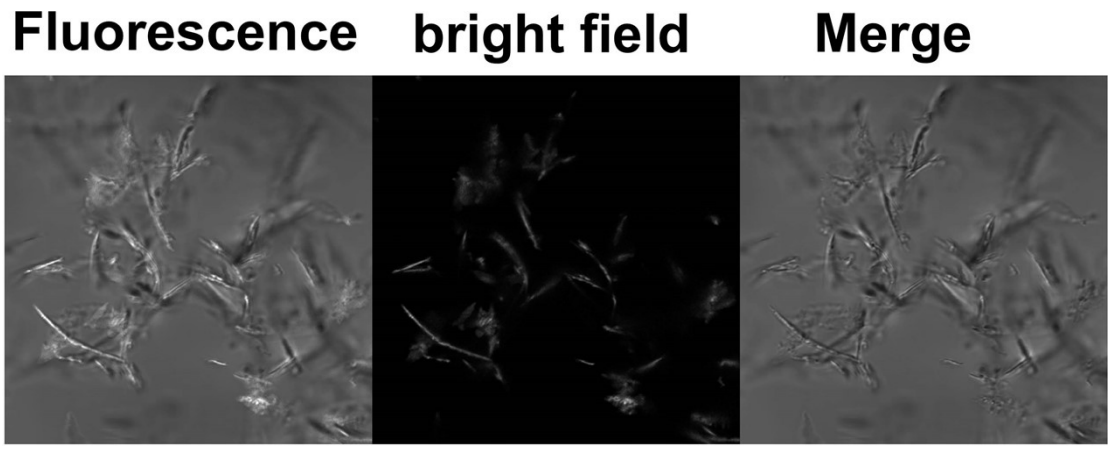


Fig. S7. The CLSM micrographs show the fluorescence (left), bright field (middle) and merge (right) images of TNU-14.

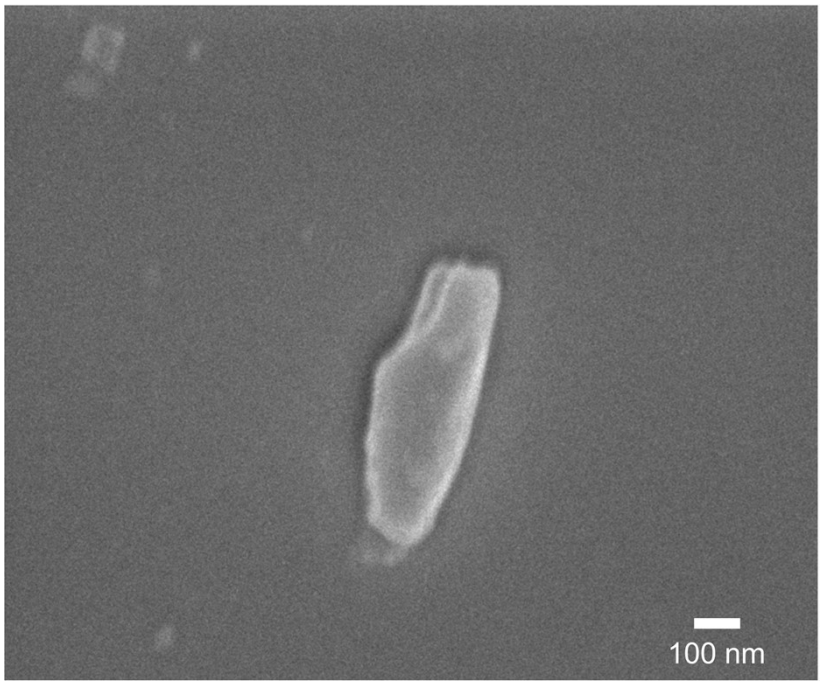
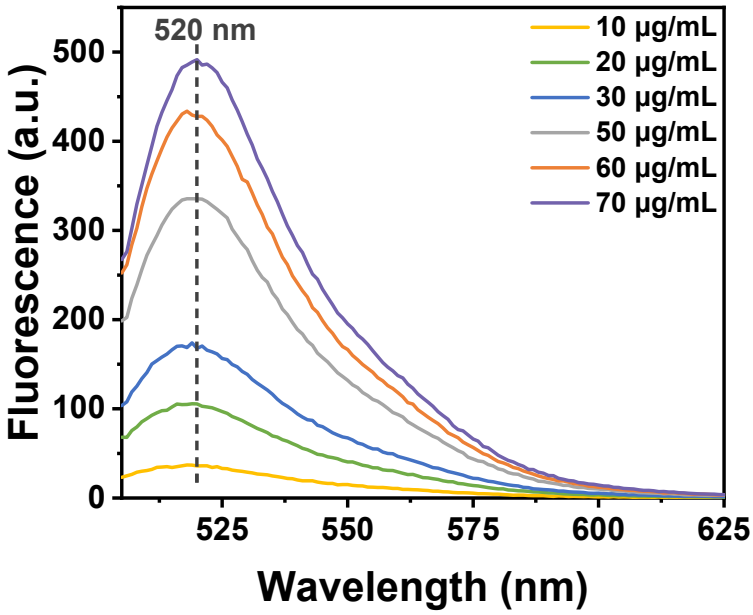
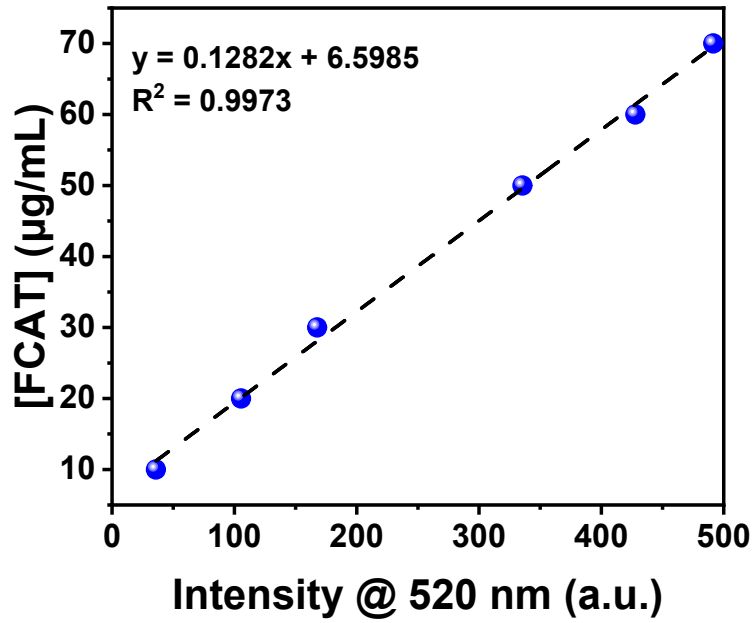


Fig. S8. The SEM image of the TNU-14.



(a)



(b)

Fig. S9. (a) Fluorescence spectra of FITC-CAT at different standard concentrations. (b) The fitted standard curves of the fluorescence spectra of FITC-CAT with different standard concentrations. The FITC-labeled enzyme was quantified at 520 nm (493 nm excitation).

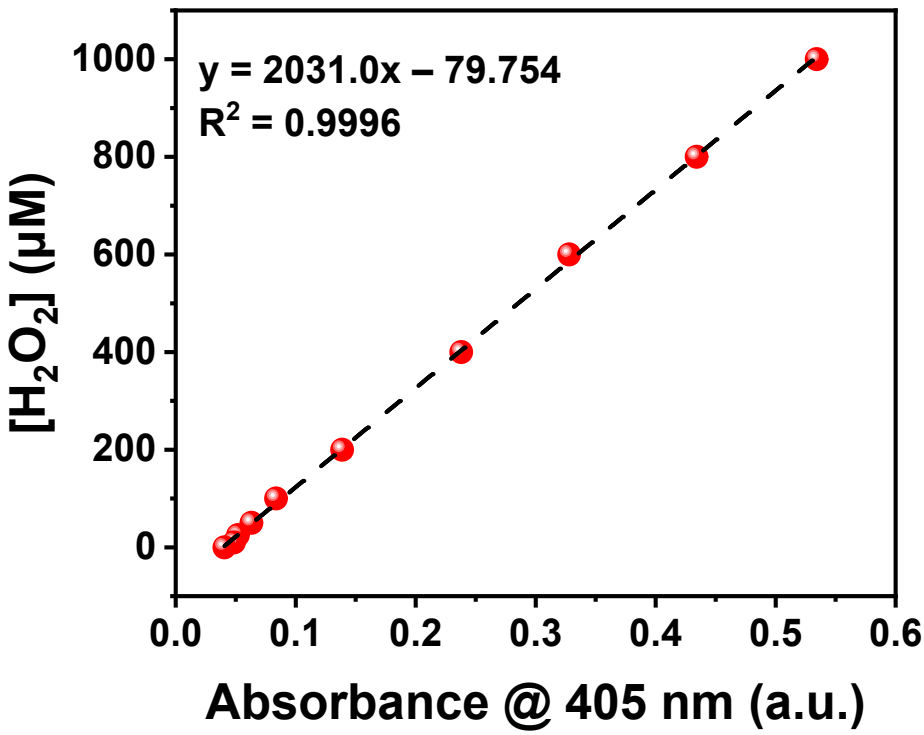


Fig. S10. The calibration curve of the $Ti(SO_4)_2$ assay.

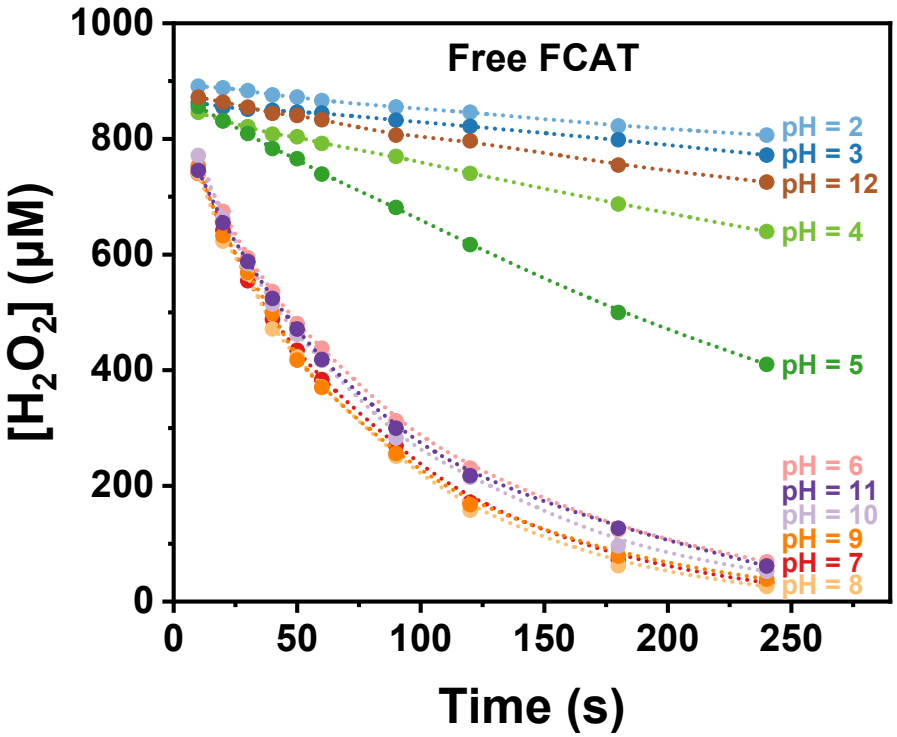


Fig. S11. Biological activity of free FCAT in different pH conditions.

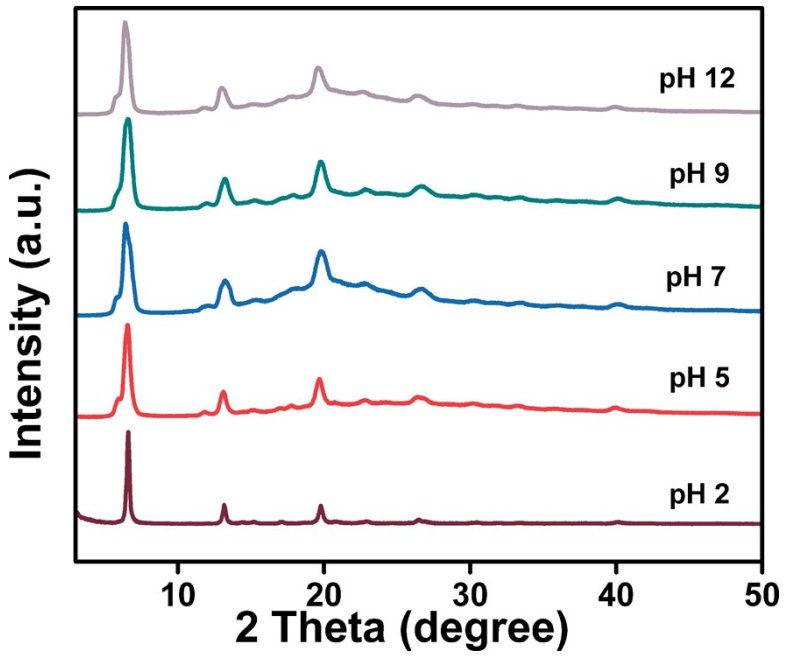
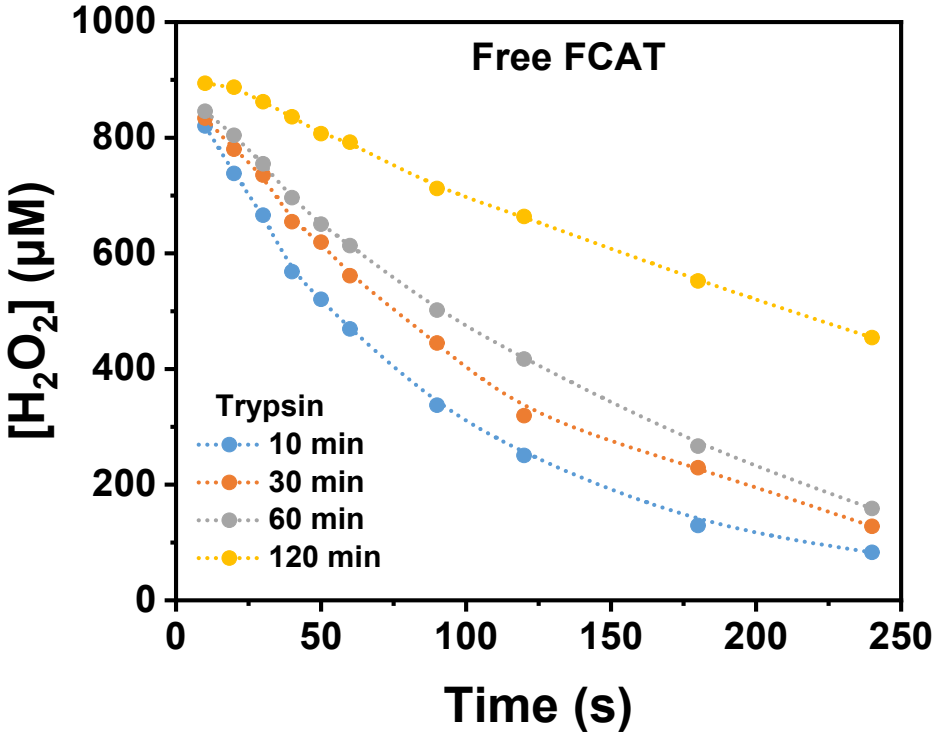


Fig. S12. PXRD of the FCAT@TNU-14 after exposure to buffer solutions at different pH (from 2−12) for 3 days.



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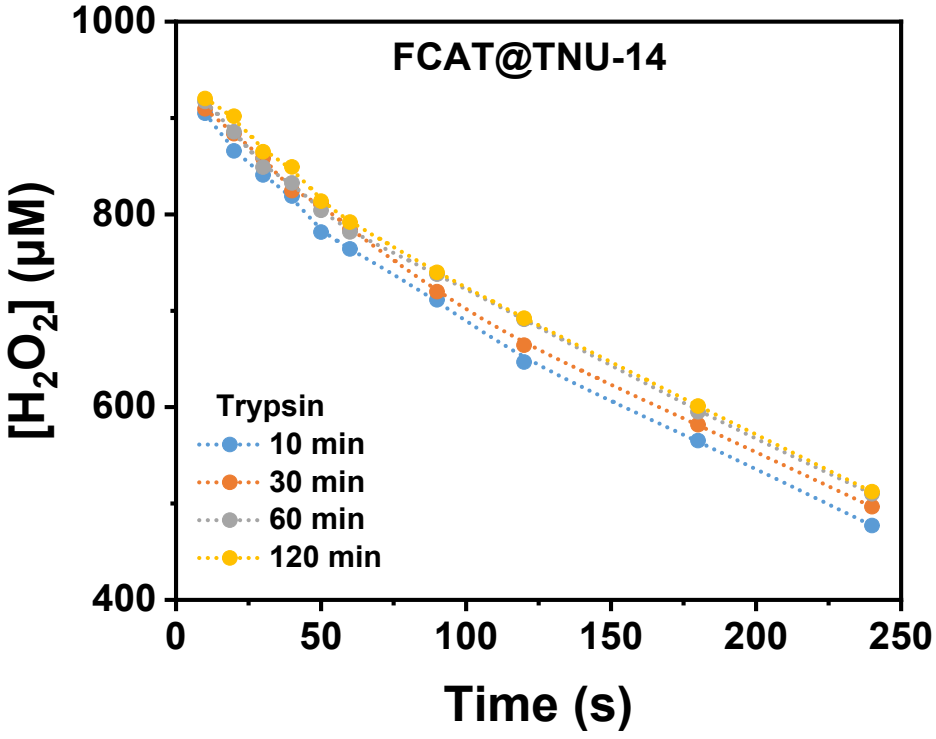
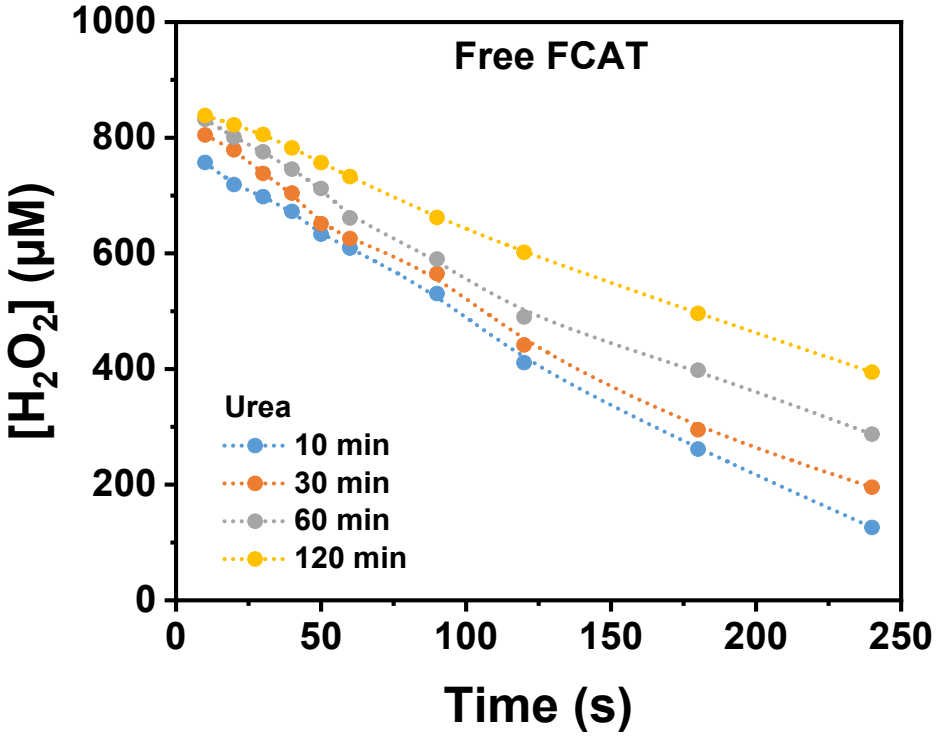
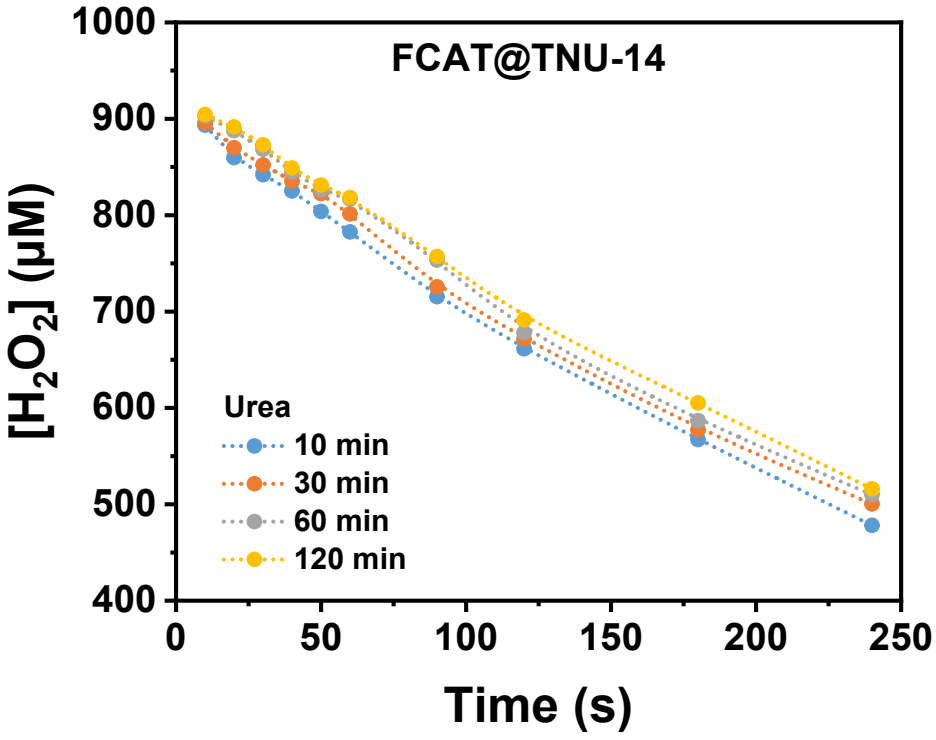


(b)

Fig. S13. S Biological activity of (a) free FCAT and (b) FCAT@TNU-14 after proteolytic agent treatment (0.25% trypsin in 0.1 M pH 8 phosphate buffer for 10–120 min).

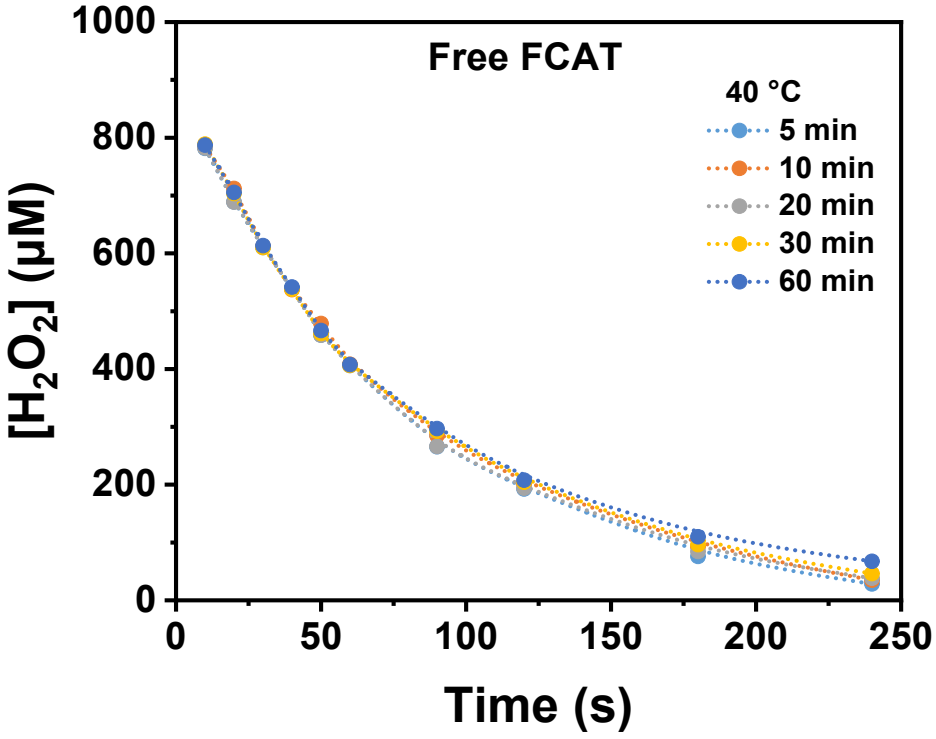


(a)

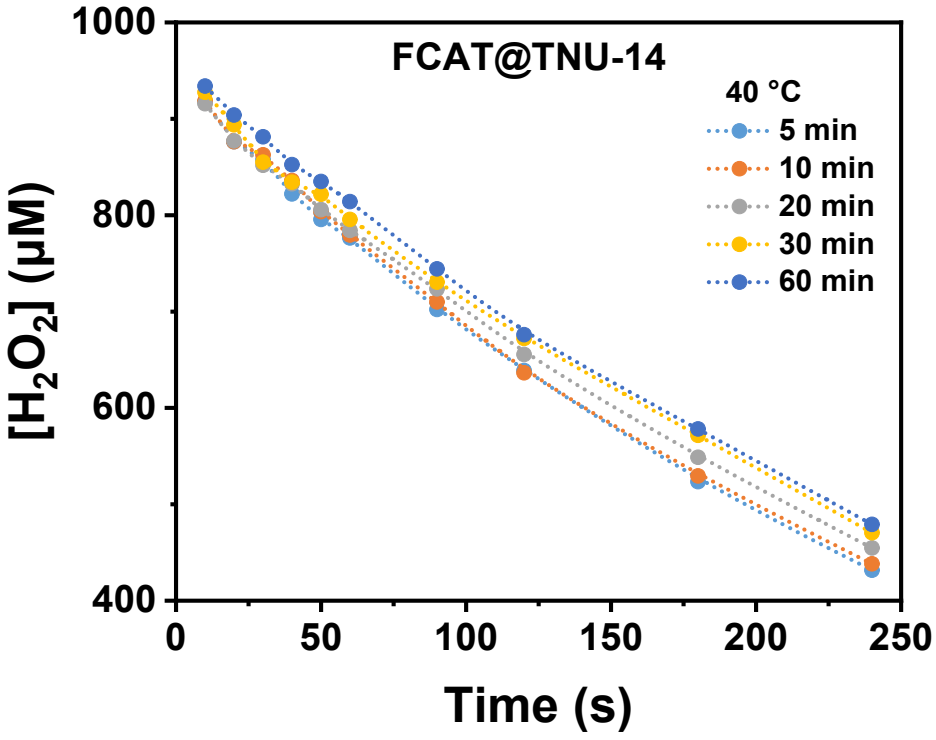


(b)

Fig. S14. Biological activity of (a) free FCAT and (b) FCAT@TNU-14 after treatment with an unfolding agent (urea (6 M) in phosphate buffer (pH 8, 0.1 M) for 10–120 min).



(a)



(b)

Fig. S15. Biological activity of (a) free FCAT and (b) FCAT@TNU-14 after treatment in water for 0–60 min at 40 °C.

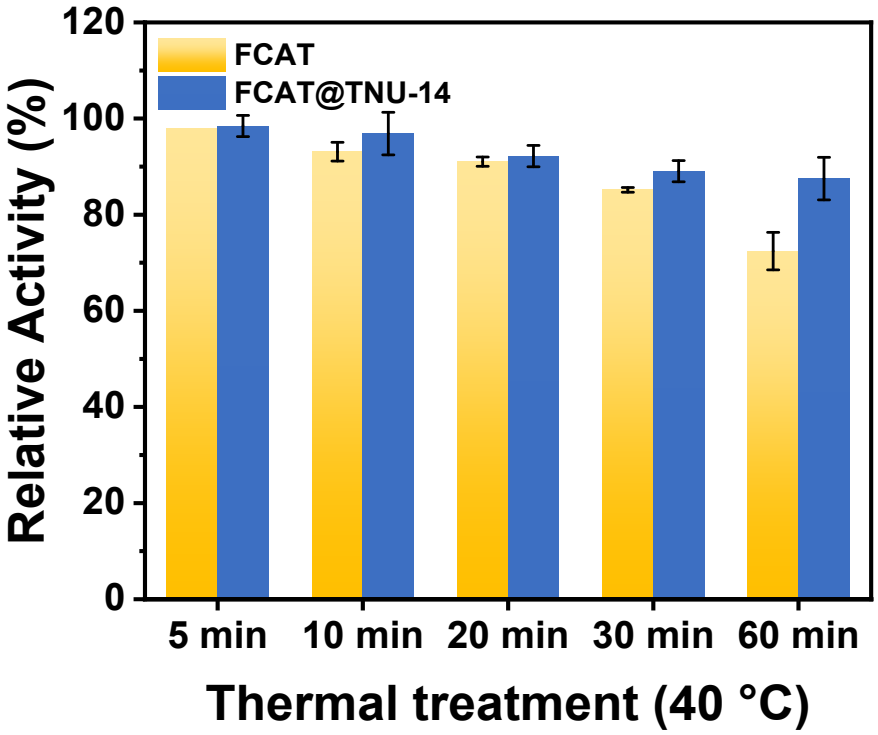
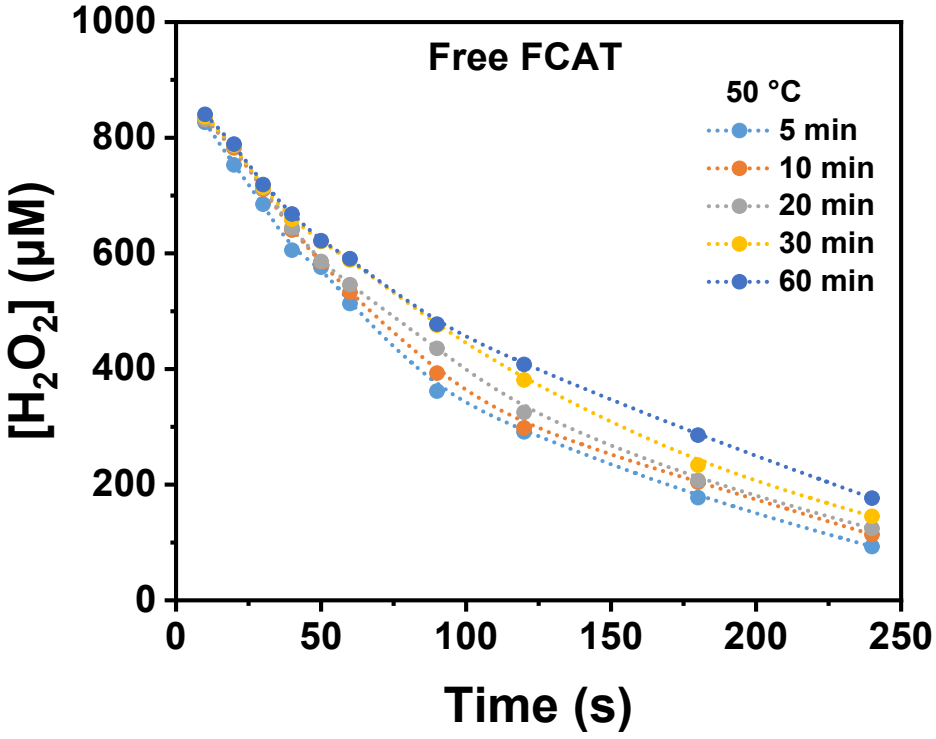
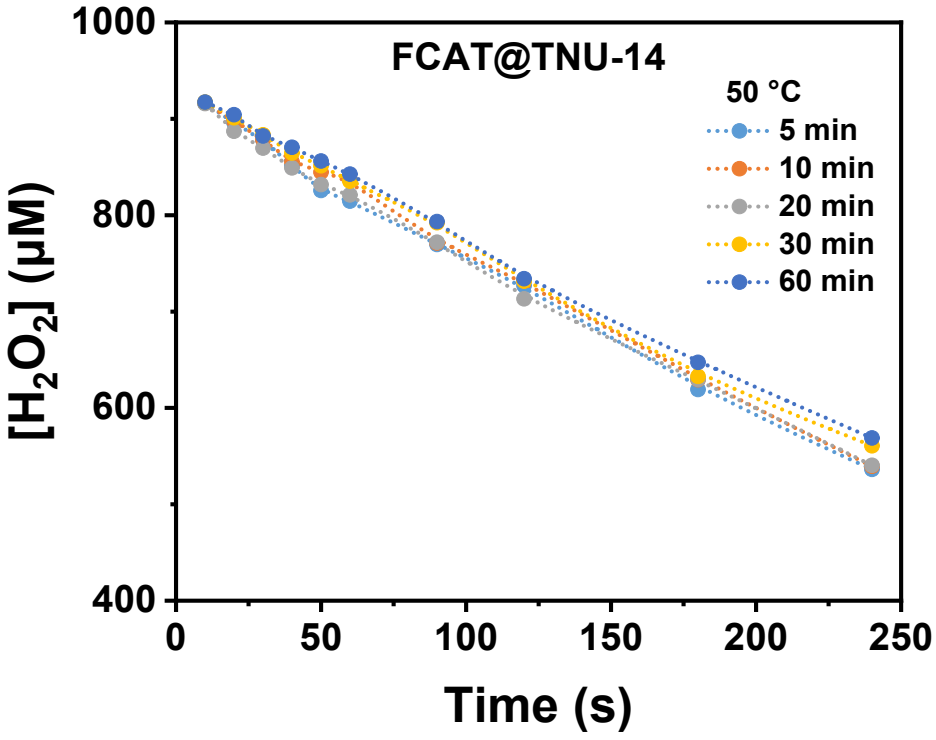


Fig. S16. Relative activity comparisons of free FCAT and FCAT@TNU-14 exposure to water for 0–60 min at 40 °C.



(a)



(b)

Fig. S17. Biological activity of (a) free FCAT and (b) FCAT@TNU-14 after treatment in water for 0-60 min at 50 °C.

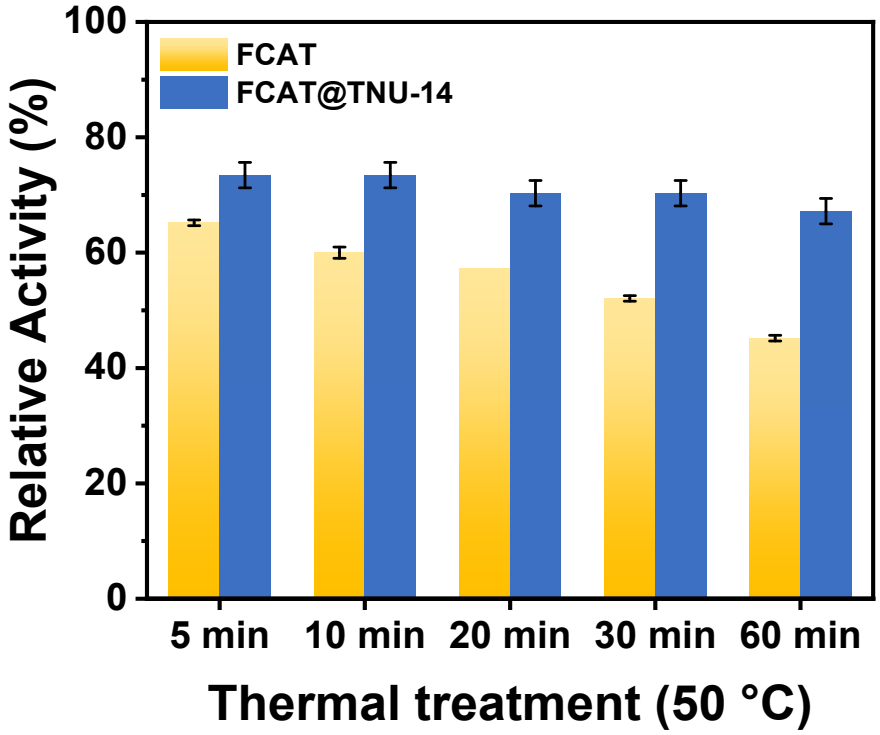
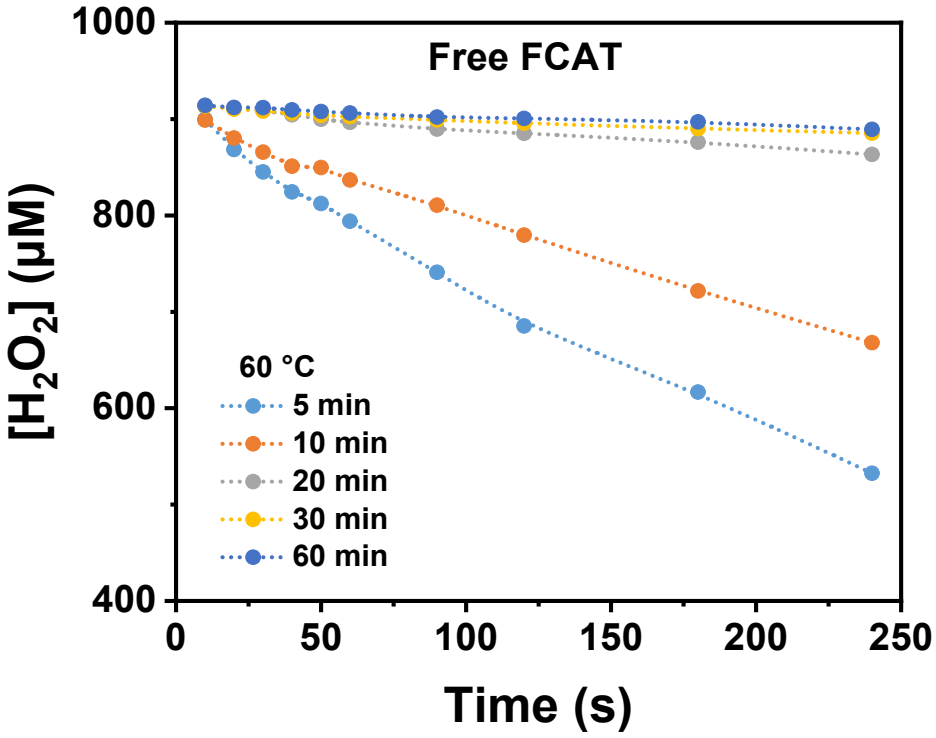
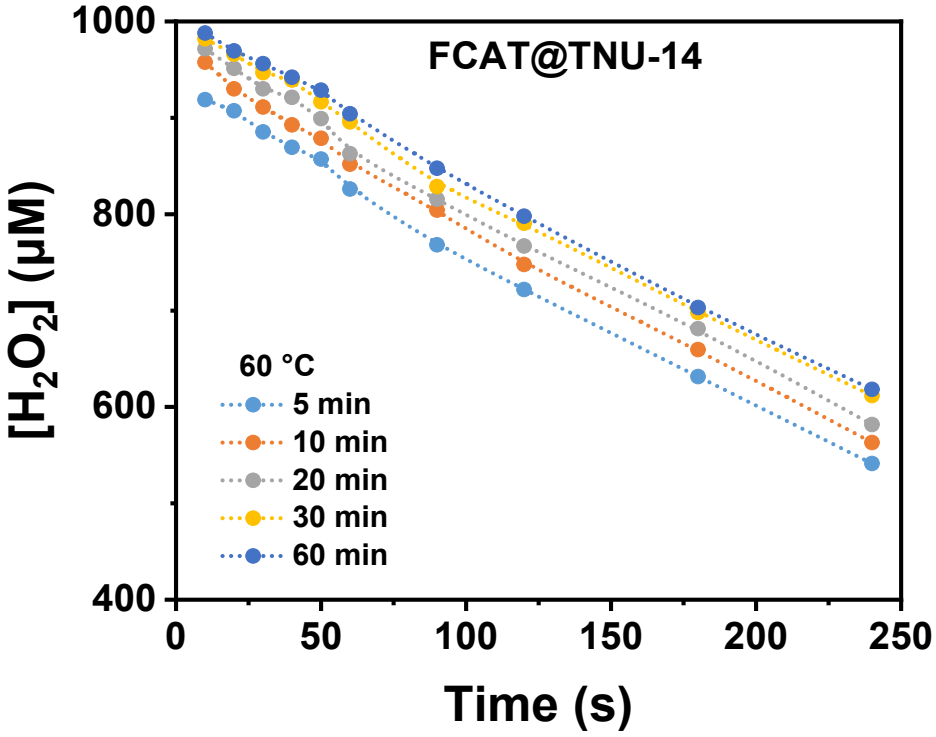


Fig. S18. Relative activity comparisons of free FCAT and FCAT@TNU-14 exposure to water for 0–60 min at 50 °C.



(a)



(b)

Fig. S19. Biological activity of (a) free FCAT and (b) FCAT@TNU-14 after treatment in water for 0-60 min at 60 °C.

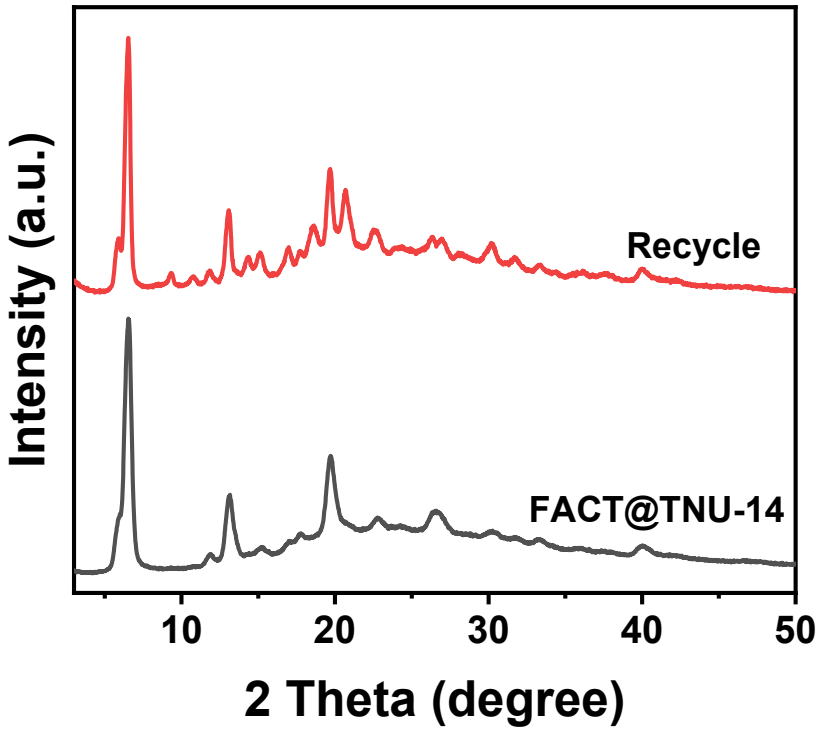
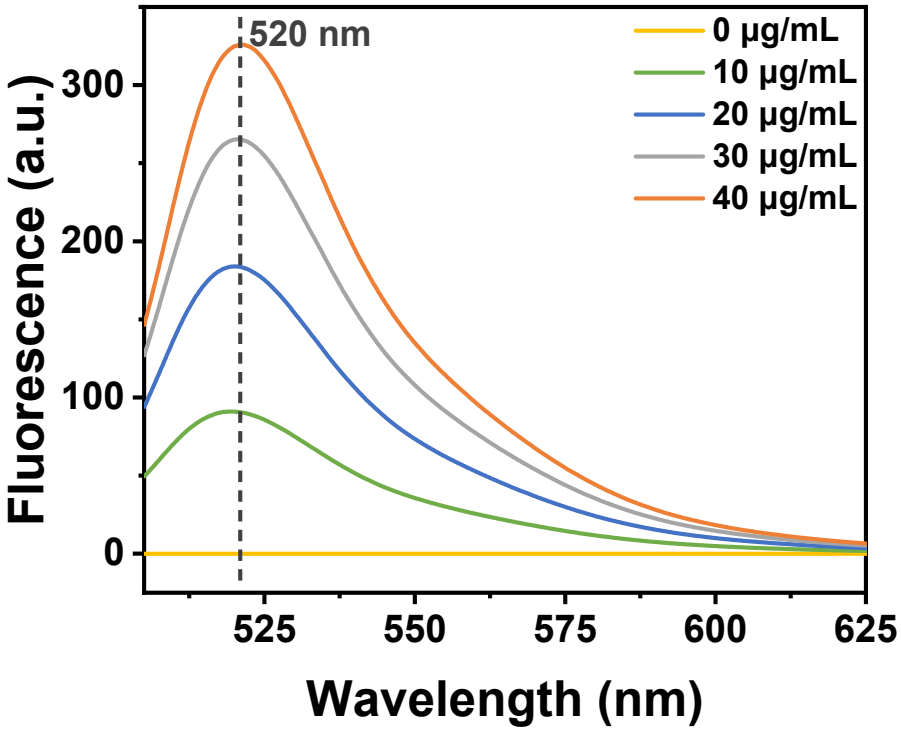
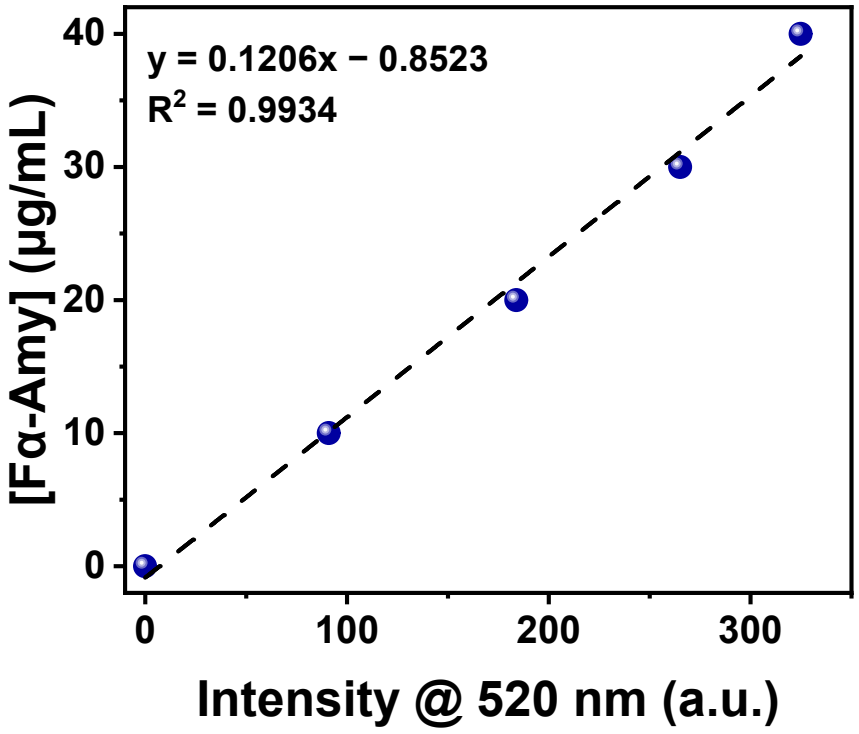


Fig. S20. PXRD of the FCAT@TNU-14 after 15 recycles.

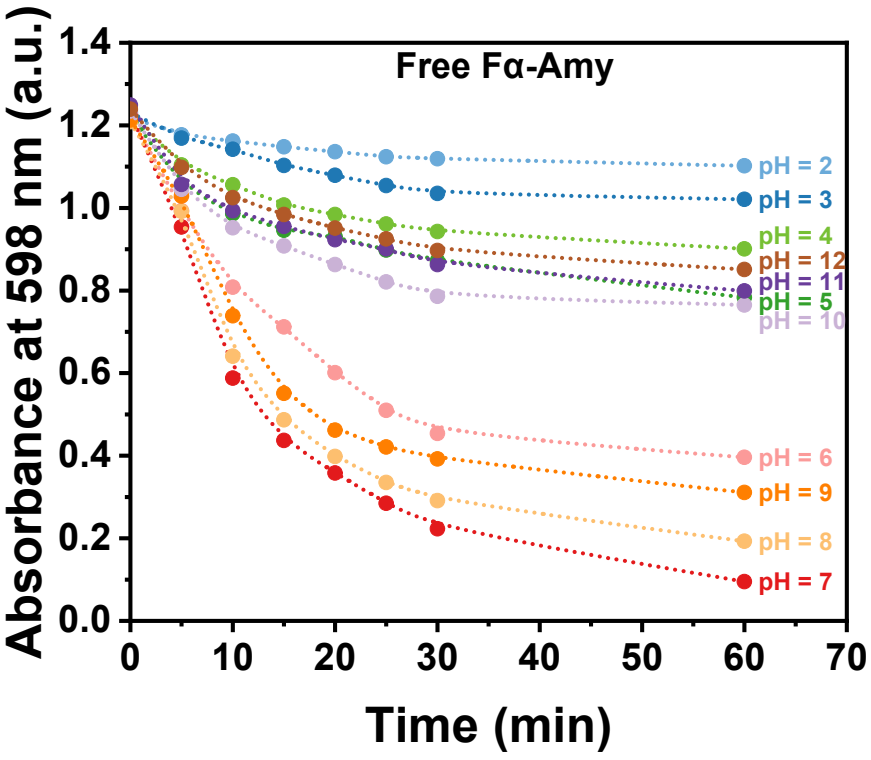


(a)

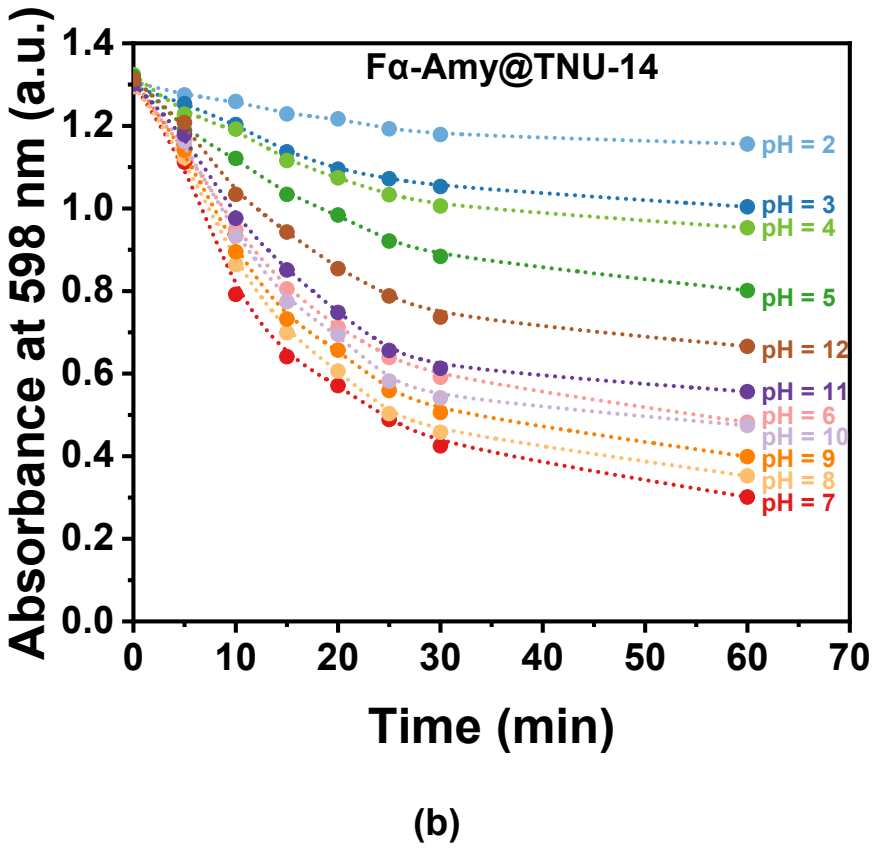


(b)

Fig. S21. (a) Fluorescence spectra of Fα-Amy at different standard concentrations. (b) The fitted standard curves of the fluorescence spectra of Fα-Amy with different standard concentrations. The FITC-labeled enzyme was quantified at 520 nm (493 nm excitation).



(a)



(b)

Fig. S22. Biological activity of (a) free Fα-Amy and (b) Fα-Amy@TNU-14 composites in different pH conditions.

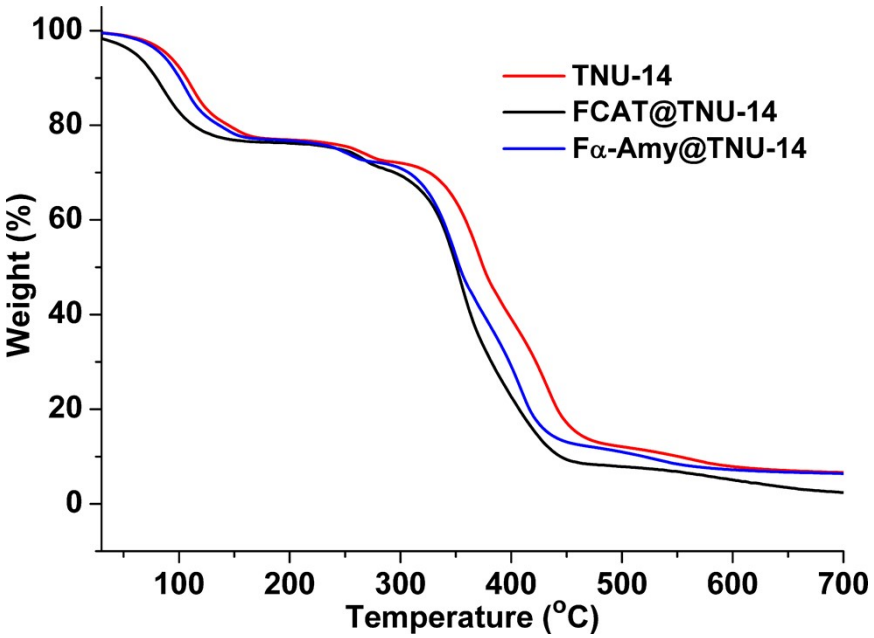
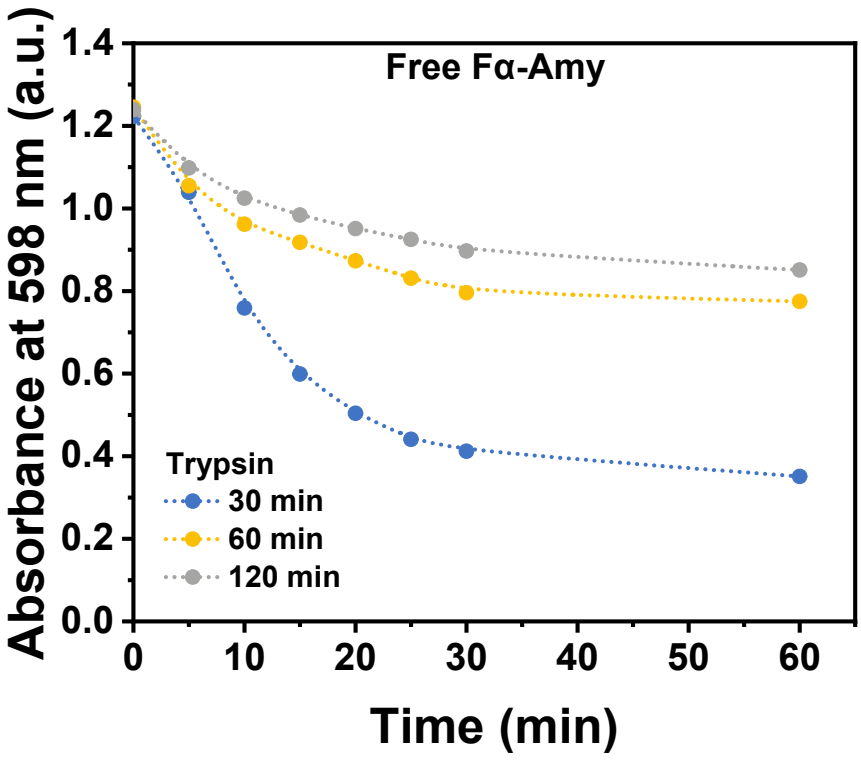
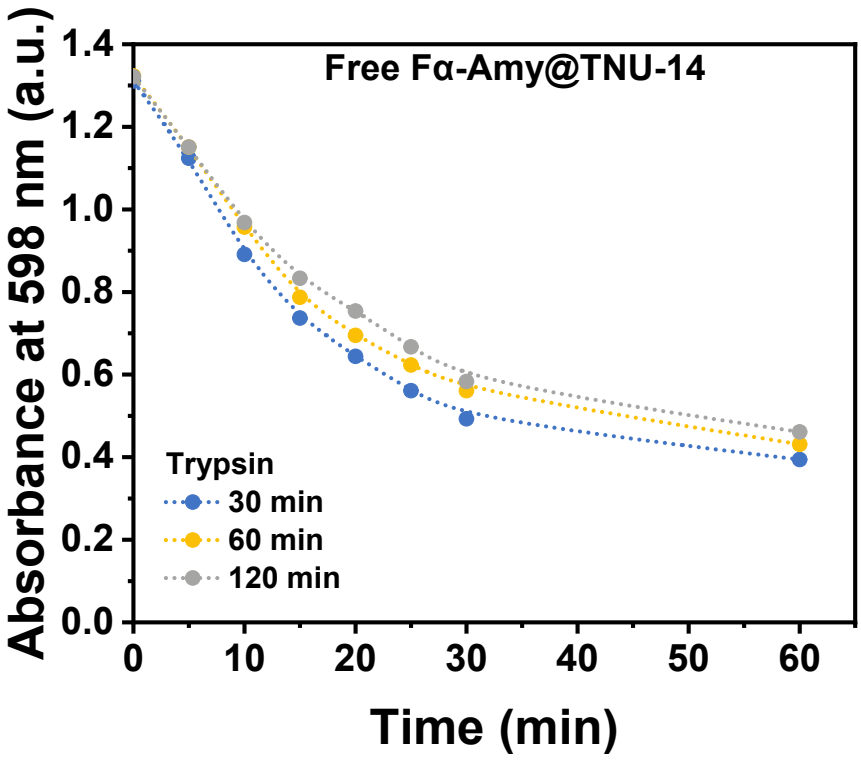


Fig. S23. TGA curves of TNU-14, FCAT@TNU-14 and Fα-Amy@TNU-14.

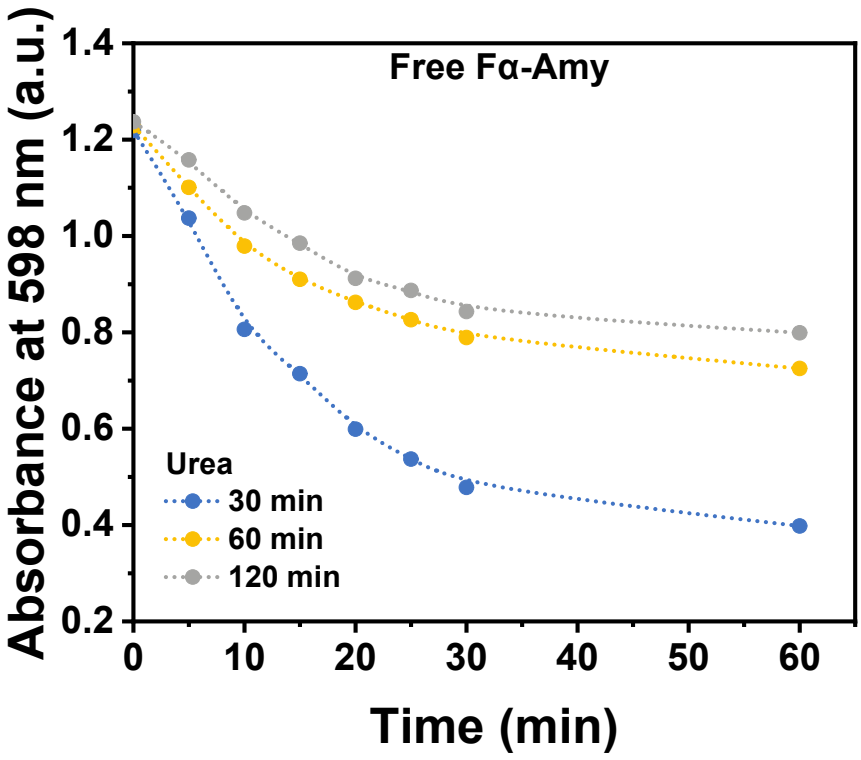


(a)

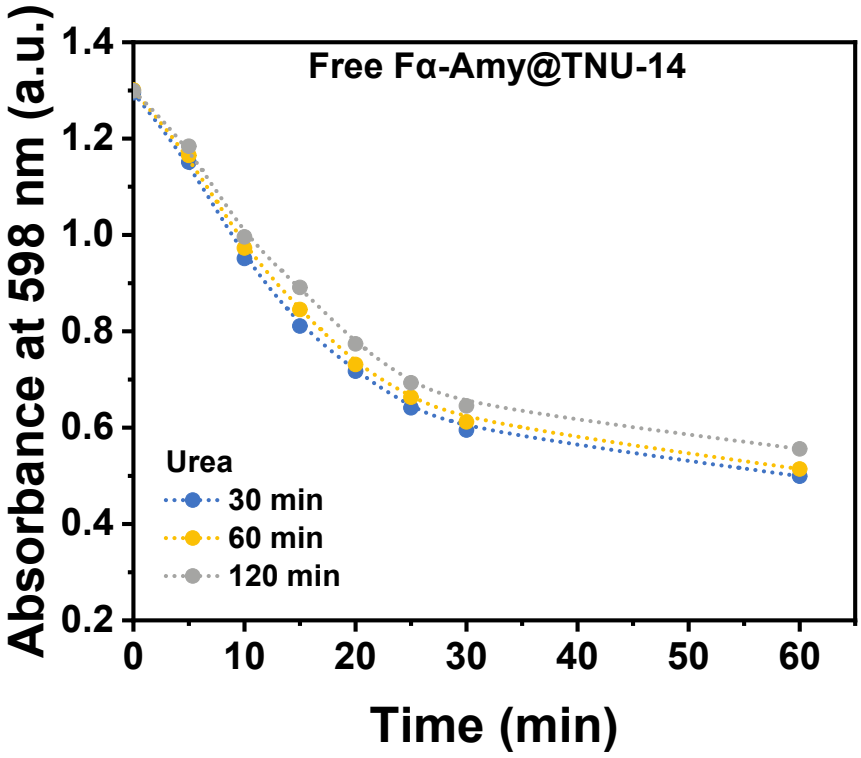


(b)

Fig. S24. Biological activity of (a) free Fα-Amy and (b) Fα-Amy@TNU-14 after proteolytic agent treatment (0.25% trypsin in 0.1 M pH 8 phosphate buffer for 30–120 min).

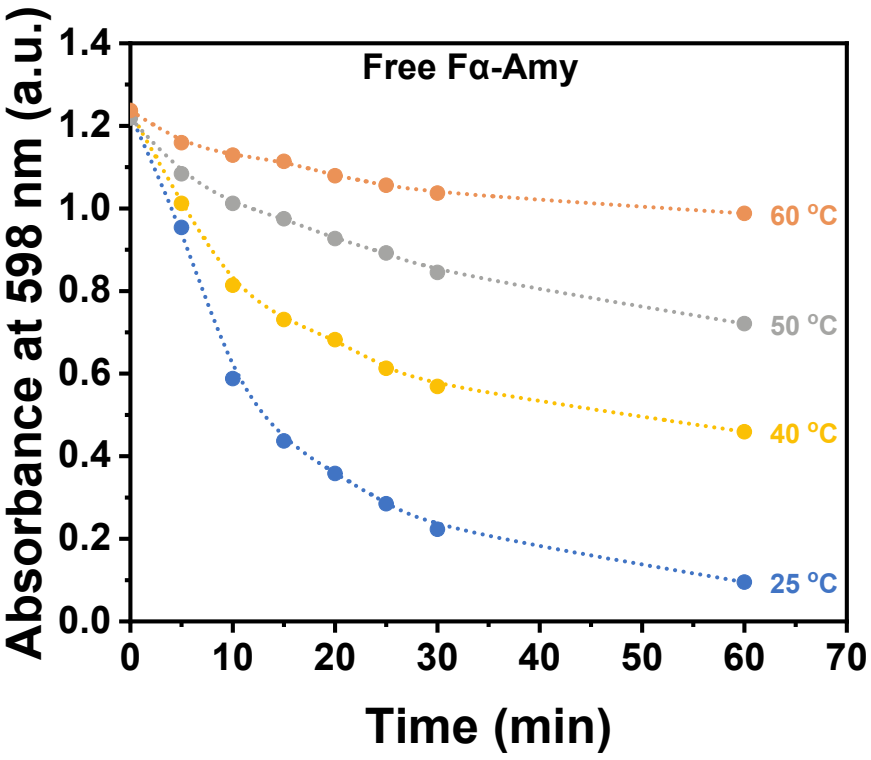


(a)

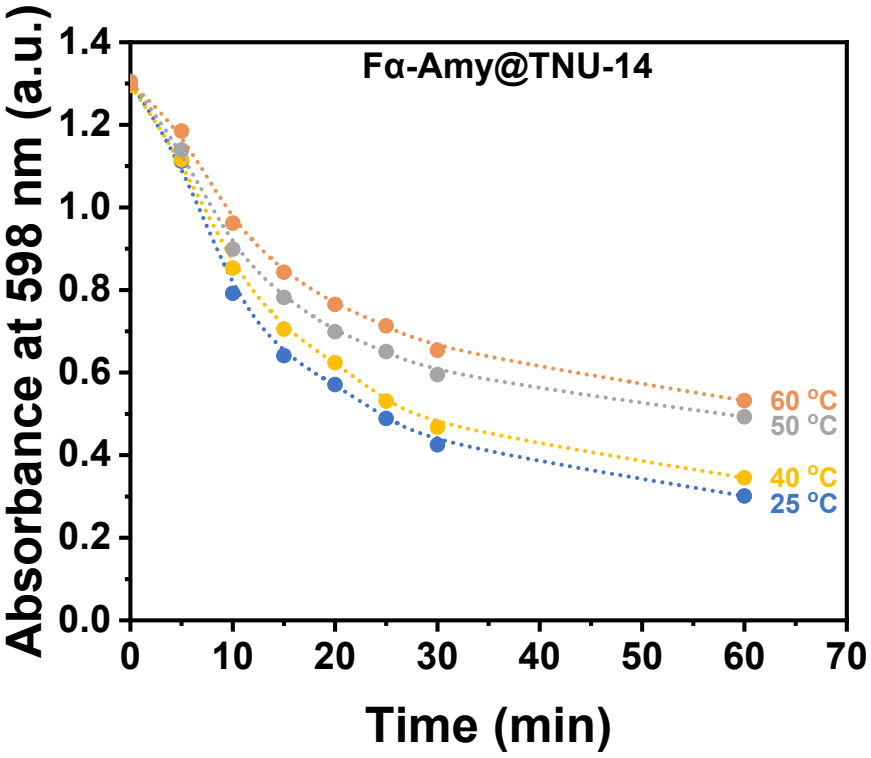


(b)

Fig. S25. Biological activity of (a) free Fα-Amy and (b) Fα-Amy@TNU-14 after treatment with an unfolding agent (urea (6 M) in phosphate buffer (pH = 8, 0.1 M) for 10–120 min).



(a)



(b)

Fig. S26. Biological activity of (a) free Fα-Amy and (b) Fα-Amy@TNU-14 composites in different temperature (25 °C, 40 °C, 50 °C, and 60 °C).

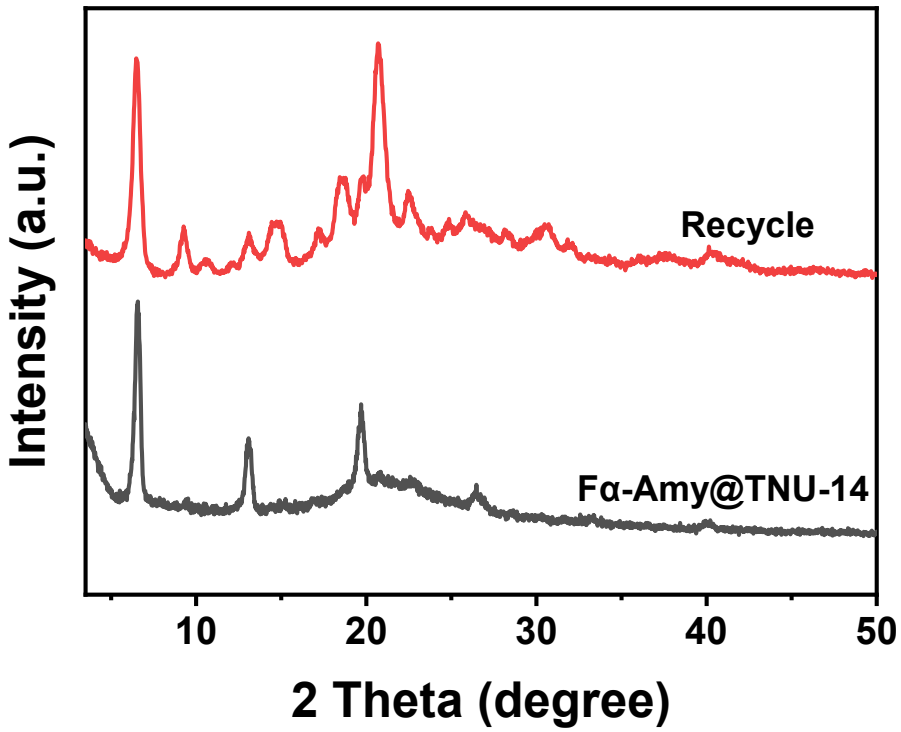


Fig. S27. PXRD of the Fα-Amy@TNU-14 after 10 recycles.

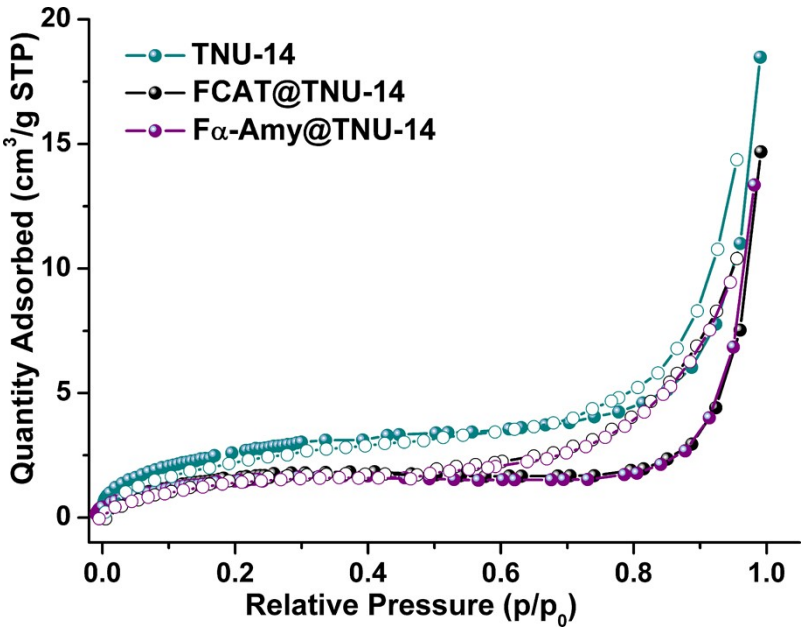


Fig. S28. N_2 sorption isotherms at 77 K of TNU-14, FCAT@TNU-14 and Fα-Amy@TNU-14.