Supplementary Information

Green and Ultrafast One-Pot Mechanochemical Approach for Efficient Biocatalyst Encapsulation in MOFs: Insights from Experiment and Computation

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Experimental section

Materials and chemicals.

All chemicals were obtained commercially and used without additional purification. Zinc oxide nano-sized were purchased from UniRegion Bio-Tech. Imidazole-2-carboxaldehyde (ICA) were purchased from AK Scientific. Xylenol-orange was purchased from Alfa Aesar. Catalase, proteinase K, bovine serum albumin, and D-sorbitol were purchased from Sigma-Aldrich. Hydrogen peroxide (H_2O_2, 30%), hydrochloric acid, and sodium chloride were purchased from SHOWA. Tris, Bradford reagent, coomassie brilliant blue R-250, Acryl/bisTM 40% solution, SDS (20%), ammonium persulfate (APS), and TEMED were from Amresco.

Characterization.

Powder X-ray diffraction (PXRD) analysis of the sample was collected using a D2 PHASER (Bruker Corporation). Scanning electron microscopy (SEM) images were captured using a field emission scanning electron microscope (FE-SEM) Jeol JSM-7000F, operated at an accelerating voltage of 10 kV. OD600 values were recorded using an Ultrospec® 10 cell density meter (Harvard Bioscience, Inc.). N_2 adsorption-desorption isotherms were measured at 77 K on a Micromeritics ASAP 2010 analyzer. The samples were degassed at 105 °C for 24 h before the measurements. Specific surface areas were calculated using the Brunauer-Emmett-Teller (BET) method in the relative pressure range P/P_0 = 0.05-0.30. Pore volumes were obtained from the volumes of N_2 adsorbed at P/P_0 = 0.95 or in the vicinity. Confocal microscopy images were acquired using a TCS-SP5-X AOBS® system (Leica Microsystems). Both enzyme activity and quantification of enzyme loading were examined using an Ocean Optics Maya2000 pro UV/Vis spectrometer equipped with a DH-2000-BAL Deuterium-Tungsten light source.

Synthesis of ZIF-90 by ball-milling.

A mixture of ICA (96.7 mg, 1.0 mmol) and nano-sized (20 nm) ZnO (40.7 mg, 0.5 mmol) with a Zn:ICA molar ratio of 1:2 was placed in a 25 ml Teflon grinding jar. Then, 103 µl of DI water (or Tris buffer 500 mM, pH 7). The mixture was milled using Retsch Mixer Mill MM 400 with 4 zirconia balls (1 cm diameter) at x Hz for y minutes (where x corresponds to 5 Hz, 8 Hz, or 15 Hz, and y values were 10 s, 30 s, 1 min, or 5

min). After ball-milling, 1.5 ml of DI water was added to the grinding jar. The resulting solution was collected after mixing and this process was repeated three times. The obtained solution was centrifuged at 3000 rcf in 5 min, washed with DI water three times, and vacuum dried at room temperature.

Synthesis of Enzyme@ZIF-90 by ball-milling.

The same synthesis procedure was employed, incorporating biocatalyst. A mixture of ICA (96,7 mg,1.0 mmol), nano-sized (20 nm) ZnO (40,7 mg,0.5 mmol), and CAT (or BSA) (10.0 mg) was loaded in a 25 ml Teflon grinding jar, following by the addition of 103 µl buffer solution. The mixture underwent milling using Retsch Mixer Mill MM 400 with 4 zirconia balls (each ball having a diameter in 1 cm and weighing 3.25 g) at x Hz for y minutes (where x corresponds to 5 Hz, 8 Hz, or 15 Hz, and y values were 10 s, 30 s, 1 min, or 5 min). Following ball-milling, 1.5 ml of DI water was added to the grinding jar, and after through mixing, the solution was transferred in a new container. This procedure was repeated three times. The obtained solution was subjected to centrifugation at 3000 rcf in 5 min, washed with DI water three times, and vacuum dried at room temperature.

Preparation of *E. coli*@ZIF-90 by ball-milling.

The same synthesis procedure was employed, incorporating *E. coli* solution. The *E. coli* solution was prepared by centrifuging and washing the raw *E. coli* solution twice with ultrapure water, followed by suspension in Tris buffer (500 mM, pH 7), and adjusting the OD600 to 0.80. A mixture of ICA (96,7 mg,1.0 mmol) and nano-sized (20 nm) ZnO (40,7 mg,0.5 mmol) was loaded in a 25 ml Teflon grinding jar, following by the addition of 103 µl *E. coli* solution. The mixture underwent milling using Retsch Mixer Mill MM 400 with 4 zirconia balls (each ball having a diameter of 1 cm and weighing 3.25g) at 8 Hz for 1 minute. Following ball-milling, 1.5 ml of DI water was added to the grinding jar, and after through mixing, the solution was transferred in a new container. This procedure was repeated three times. The obtained solution was washed three times with deionized water, filtered using a 5 µm cut-off nylon membrane, and then vacuum-dried at room temperature.

Activity of CAT.

The catalytic activity of CAT was assessed through the concentration of H_2O_2 using the FOX assay. The free CAT (0.3 mg) or CAT@ZIF-90 (with an encapsulated enzyme concentration of 0.3 mg) were dispersed in 0.5 mL of Tris buffer solution (100 mM, pH 8.0), and then incubated at 37 °C for 2 hours. After incubation, the samples were added to 0.5 ml hydrogen peroxide substrate solution (0.2 mM). The final concentration of H_2O_2 was monitored by measuring the absorbance of the FOX reagent at 560 nm using an Epoch™ 2 microplate spectrophotometer.

Proteinase K treatment of CAT@ZIF-90.

CAT@ZIF-90, with an encapsulated enzyme concentration of 0.3 mg, along with 0.05 mg of proteinase K, was dispersed in 0.5 mL of Tris buffer solution (100 mM, pH 8.0). After incubation for 2h, the sample was collected by centrifugation and washed 2 times with water. The enzymatic activity was assessed using methods similar to those employed for CAT@ZIF-90.

SDS-PAGE analysis.

Before the protein analysis assay, 2 mg of CAT@ZIF-90 was dissolved in 0.2 M $HCl_{(aq)}$. Following a 5 min incubation, the solution underwent centrifugation (14,000 g) for a brief period. Subsequently, 15 μL of the supernatant was combined with 5 μL of loading buffer (comprising 240 mM Tris–HCl (pH 6.5), 8% SDS, 0.04% (w/v) bromophenol blue, 40% glycerol, and 5% β-mercaptoethanol), and the mixtures were subjected to electrophoresis on a sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) consisting of a 4% polyacrylamide stacking gel and a 12% polyacrylamide resolving gel. Electrophoresis was carried out at 20 mA under reducing conditions (boiled in 95 °C water for 5-10 min). Subsequently, the gel was subjected to Coomassie Blue Fast Staining following the provided instruction manual.

Bradford Assay: protein concentration determination.

In this standard method,[1] the loadings of encapsulated enzymes in ZIF-90 were determined by decomposing the MOFs and reacting with the Bradford reagent. Bradford reagent was purchased from Amresco, and the protocol was given by the supplier.

ZIF-90 Nucleation Simulations.

ZIF-90 nucleation process was simulated in Amber 20 package.[2] Our system's initial setup comprised 50 Zn^{2+} ion, 100 HICA, and 500 water, prepared using the Packmol package,[3] in a 50^3 $Å^3$ simulation box with a 0.4 g/cm^3 density, ensuring a minimum 4.0 Å intermolecular spacing. Force field parameters were assigned with custom-designed force field for Zn^{2+} ion and General Amber Force Field (GAFF)[4] for HICA. The aqueous environment was emulated with the TIP4P-Ew water model.[5] Our simulations employed a 10 Å boundary for both types of interactions and used the leapfrog algorithm, setting a 1 fs time-step, while long-range electrostatic interactions were managed using the Ewald (PME) method. MD simulations were carried out in the canonical ensemble (NVT) ensemble with the Langevin thermostat to maintain the system temperature. After system equilibration at 300 K, the system was compacted by reducing its volume by 0.1 %/ns to a density of 0.5 g/cm^3. Ensuing this, deprotonation of the HICA was initiated, in line with experimental observations. This scheme continued until a final density of 1.4 g/cm^3 was achieved.

CAT@ZIF-90 Nucleation Simulations.

ZIF-90 nucleation process was simulated in Amber 20 package.[2] Our system's initial setup comprised 1 catalase monomer, 1600 Zn^{2+} ion, 3200 HICA, and 16000 water, prepared using the Packmol package,[3] in a 130^3 $Å^3$ simulation box with a 0.4 g/cm^3 density, ensuring a minimum 4.0 Å intermolecular spacing. Force field parameters were assigned with FF14SB[6] for catalase and custom-designed force field for Zn^{2+} ion and General Amber Force Field (GAFF)[4] for HICA. The aqueous environment was emulated with the TIP4P-Ew water model.[5] Our simulations employed a 10 Å boundary for both types of interactions and used the leapfrog algorithm, setting a 1 fs time-step, while long-range electrostatic interactions were managed using the Ewald (PME) method. MD simulations were carried out in the canonical ensemble (NVT) ensemble with the Langevin thermostat to maintain the system temperature. After system equilibration at 300 K, the system was compacted by reducing its volume by 0.1 %/ns to a density of 0.5 g/cm^3. Ensuing this, deprotonation of the HICA was initiated, in line with experimental observations. This scheme continued until a final density of 1.3 g/cm^3 was achieved.

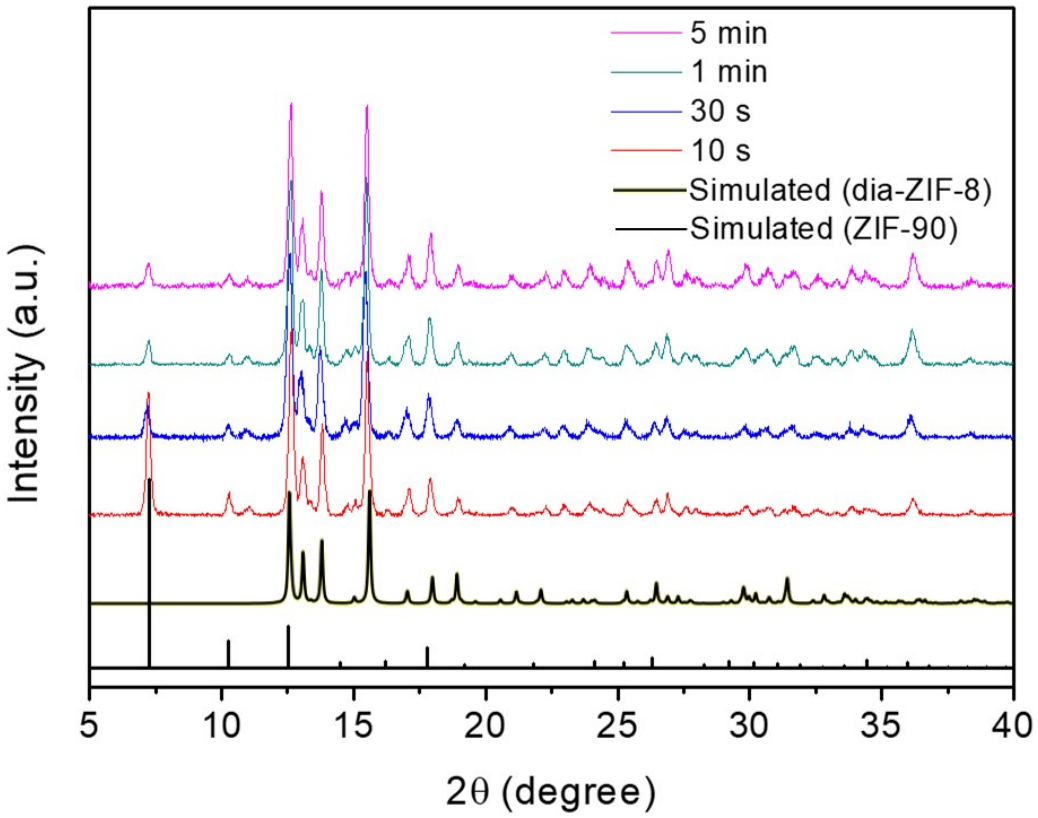

Figure S1. PXRD patterns of the CAT@ZIF-90 samples were obtained using DI water with a milling frequency of 8 Hz and at different reaction times.[7]

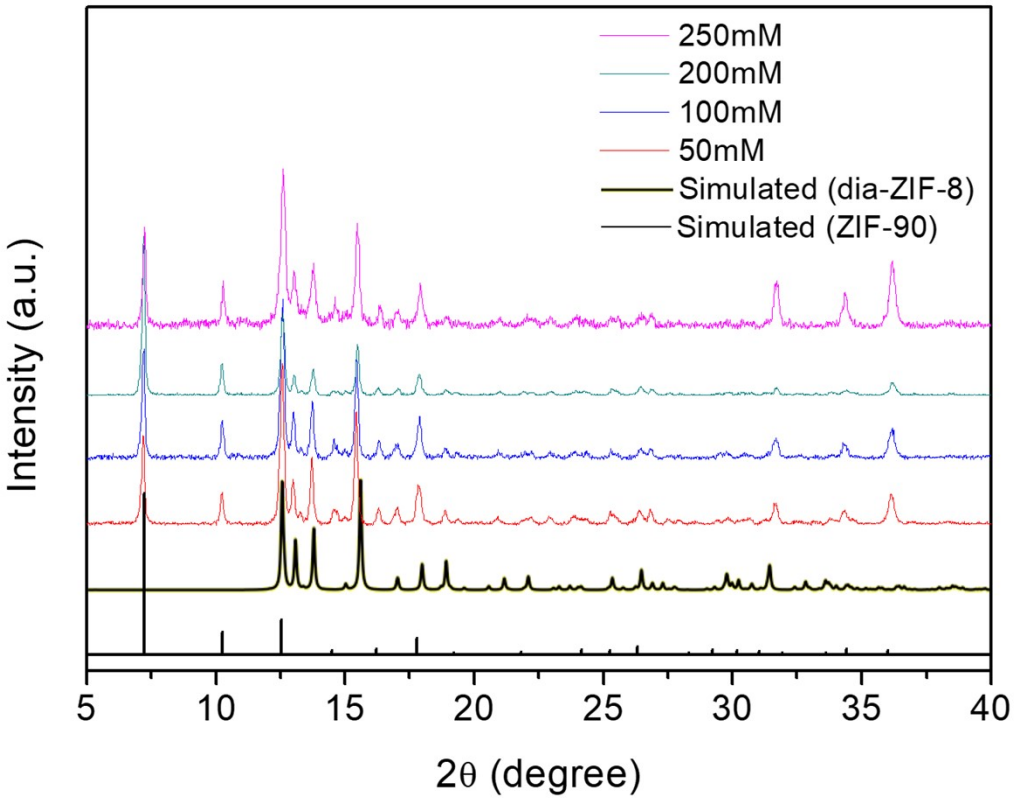

Figure S2. PXRD patterns of the CAT@ZIF-90 were samples obtained using different concentrations Tris buffer solution at pH 7 with a milling frequency of 8 Hz and 10 seconds.[7]

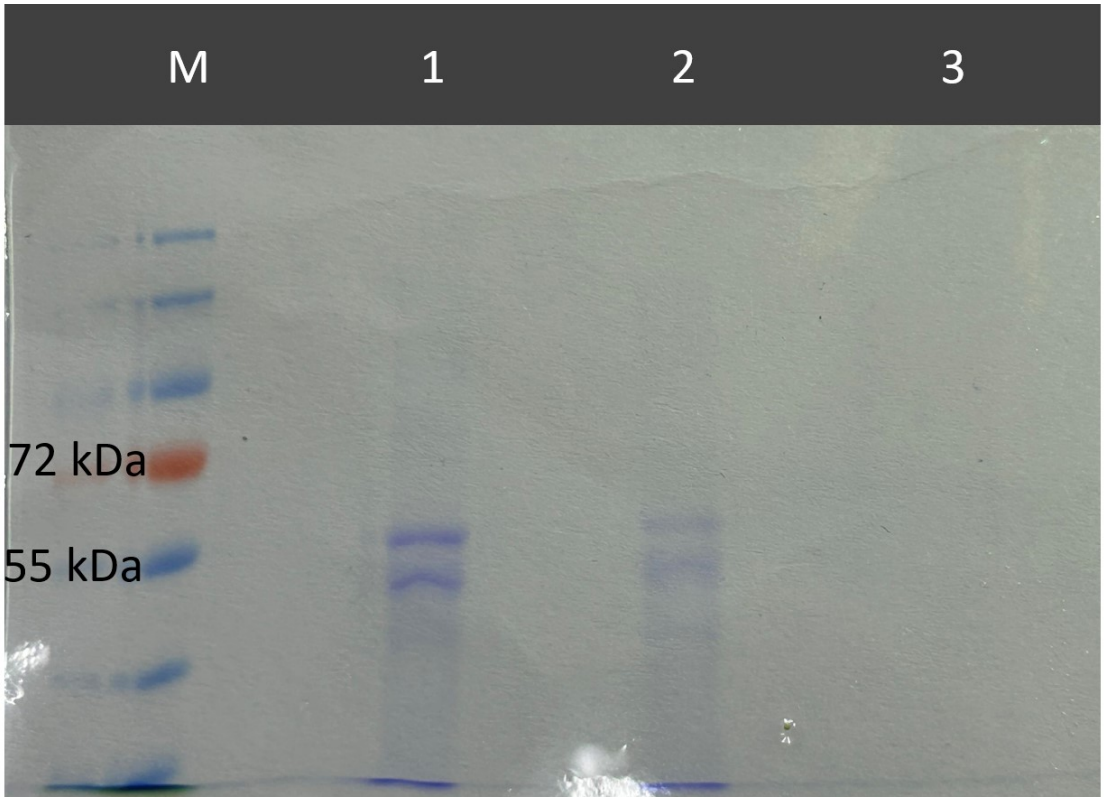

Figure S3. SDS-PAGE gel (M: protein marker, lane 1: free CAT, lane 2: CAT@ZIF-90-Tris-pH7-8Hz-10s, and lane 3: washed CAT-on-ZIF-90-Tris-pH7-8Hz-10s).

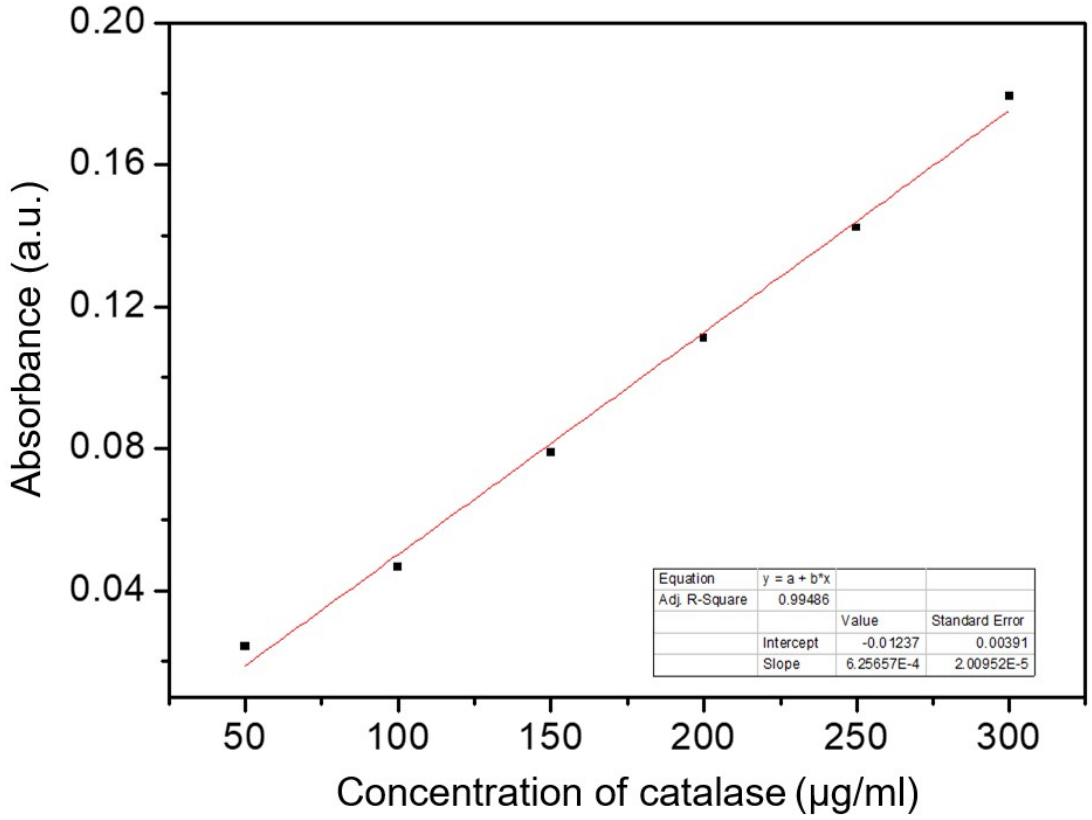

Figure S4. Bradford assay of the catalase concentration. The corresponding standard calibration line of the Bradford assay is shown above.

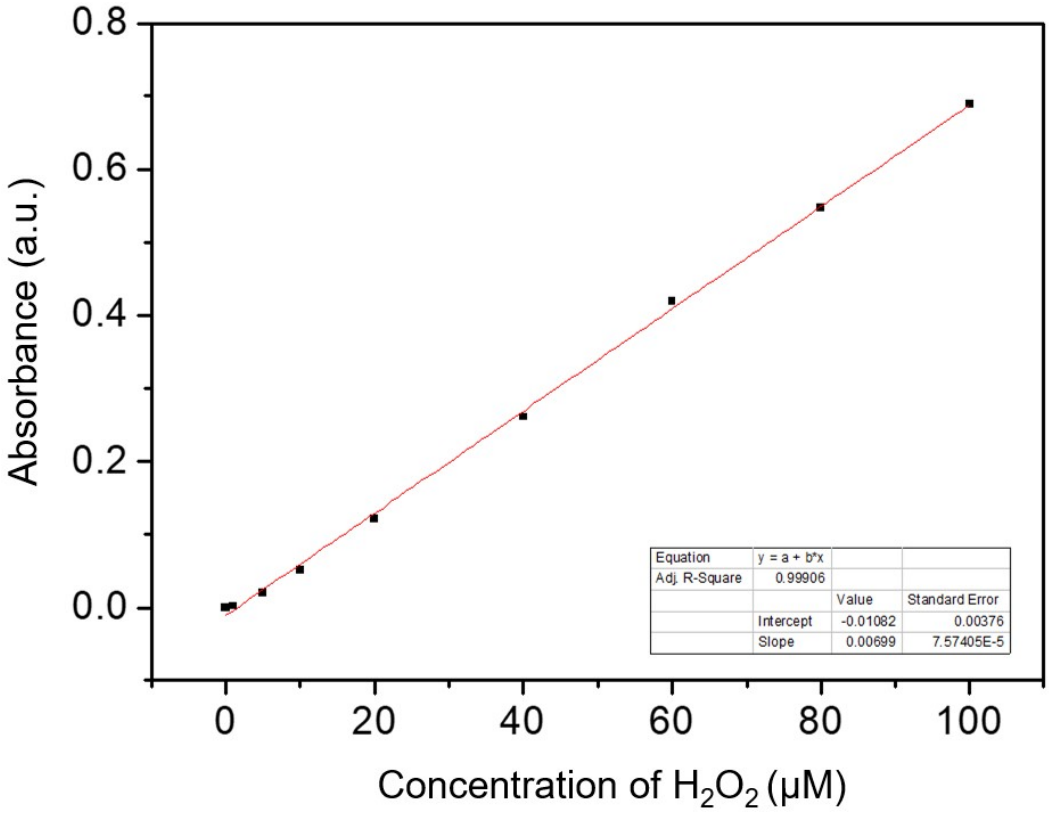

Figure S5. FOX assay of the hydrogen peroxide concentration. The associated calibration line for the FOX assay is presented above.

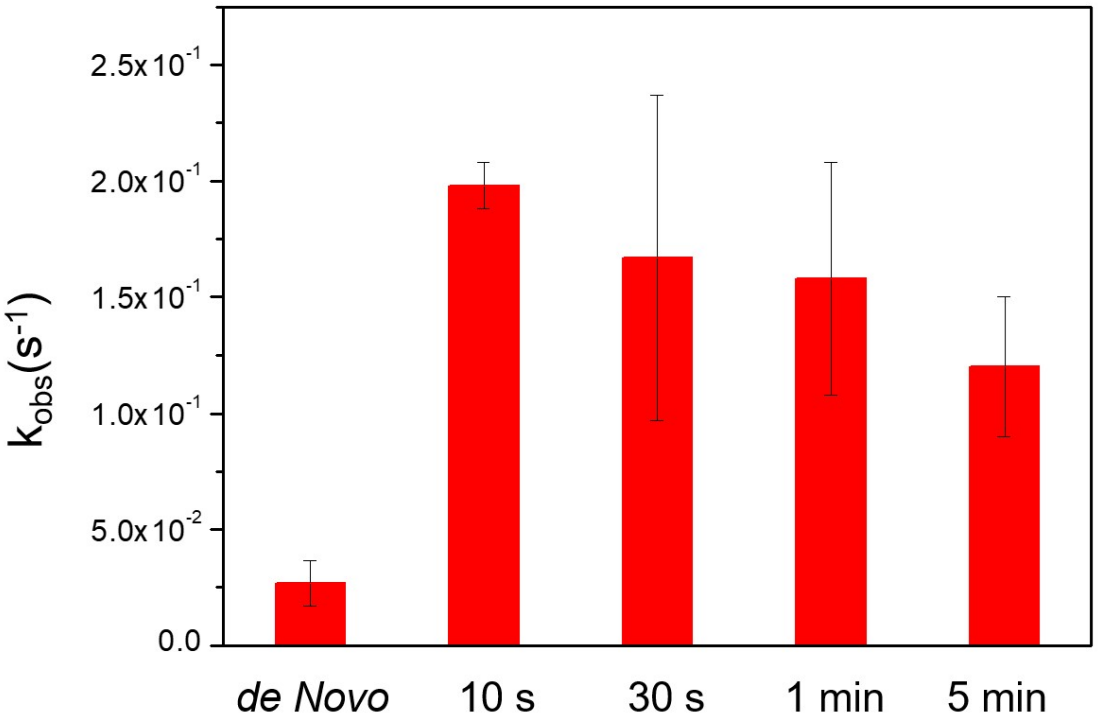

Figure S6. Comparing the retained activity without proteinase K treatment for CAT@ZIF-90 samples obtained through ball-milling at 8 Hz and various reaction times to that of samples synthesized using a *de Novo* water-based approach.[8]

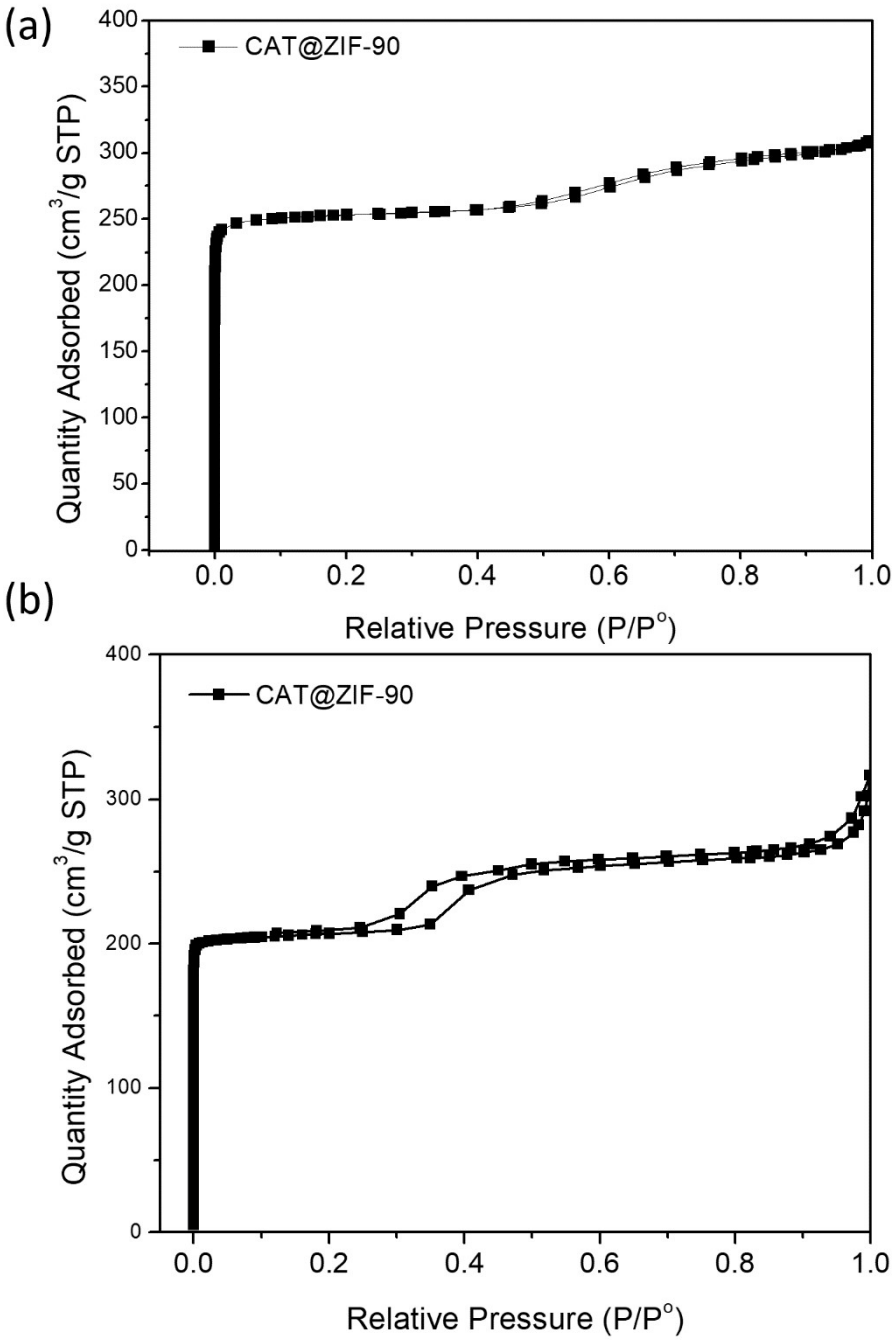

Figure S7. The nitrogen sorption isotherms of CAT@ZIF-90, prepared by (a) water-based and (b) ball-milling approaches.[8]

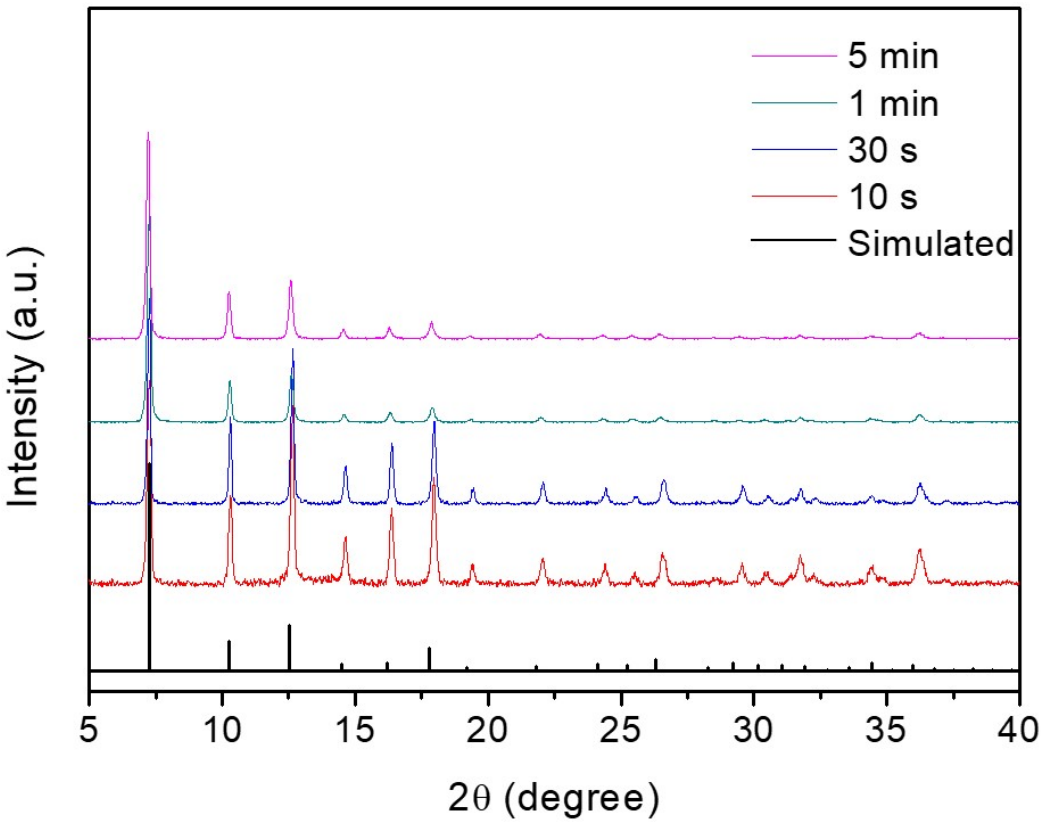

Figure S8. PXRD patterns of the CAT@ZIF-90 samples were obtained using 500 mM Tris buffer solution at pH 7, with a milling frequency of 5 Hz, and at different reaction times.

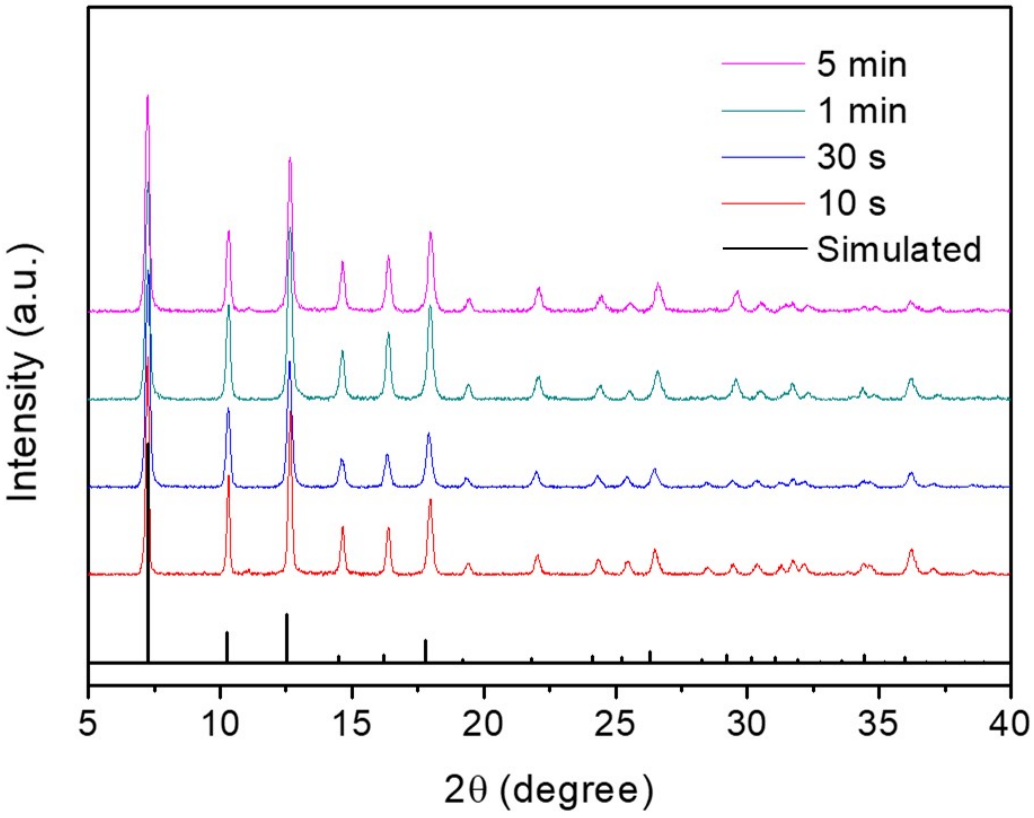

Figure S9. PXRD patterns of the CAT@ZIF-90 were samples obtained using 500 mM Tris buffer solution at pH 7, with a milling frequency of 15 Hz, and at different reaction times.

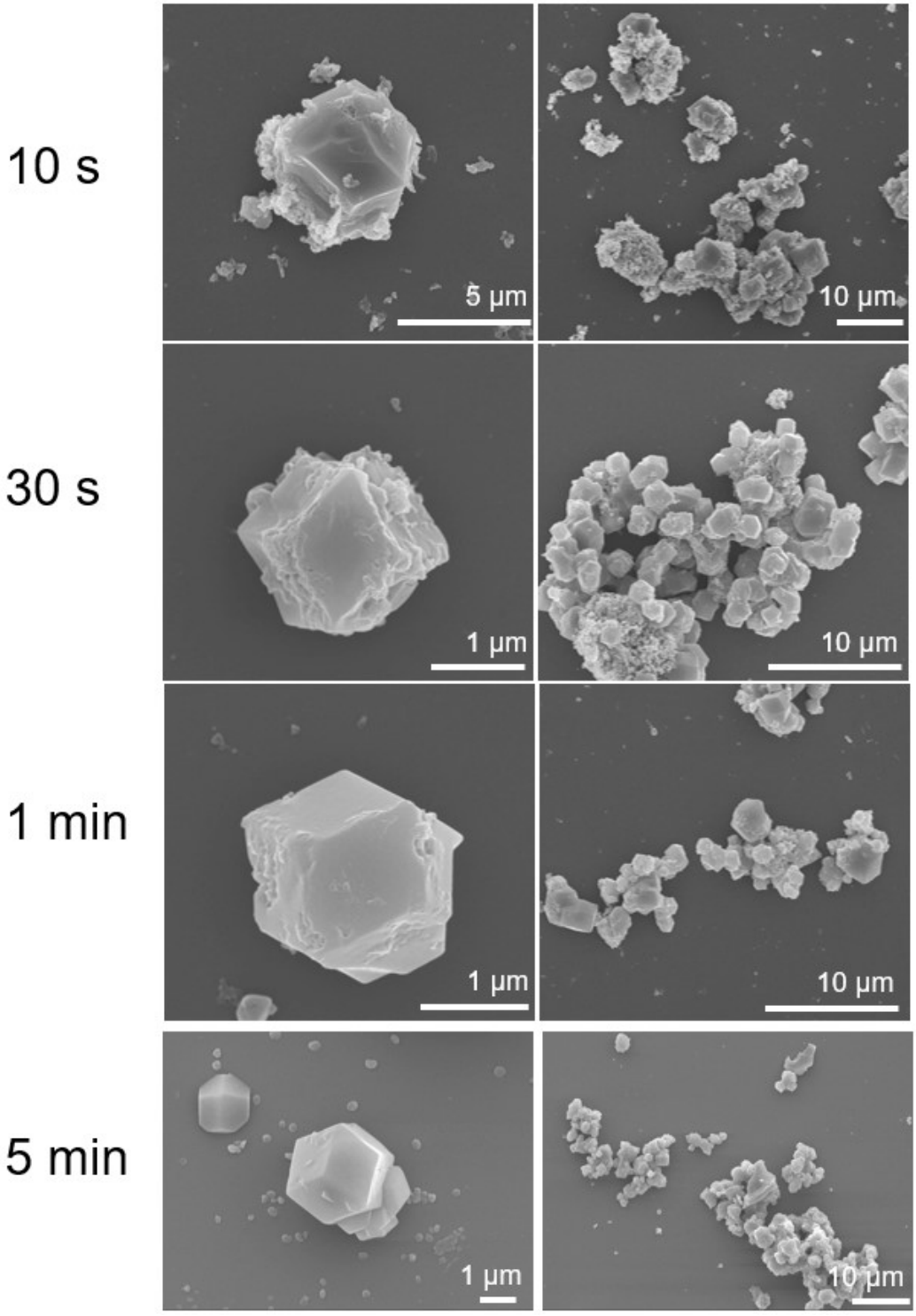

Figure S10. SEM images of the CAT@ZIF-90 samples were obtained using 500 mM Tris buffer solution at pH 7, with a milling frequency of 5 Hz, and at different reaction times.

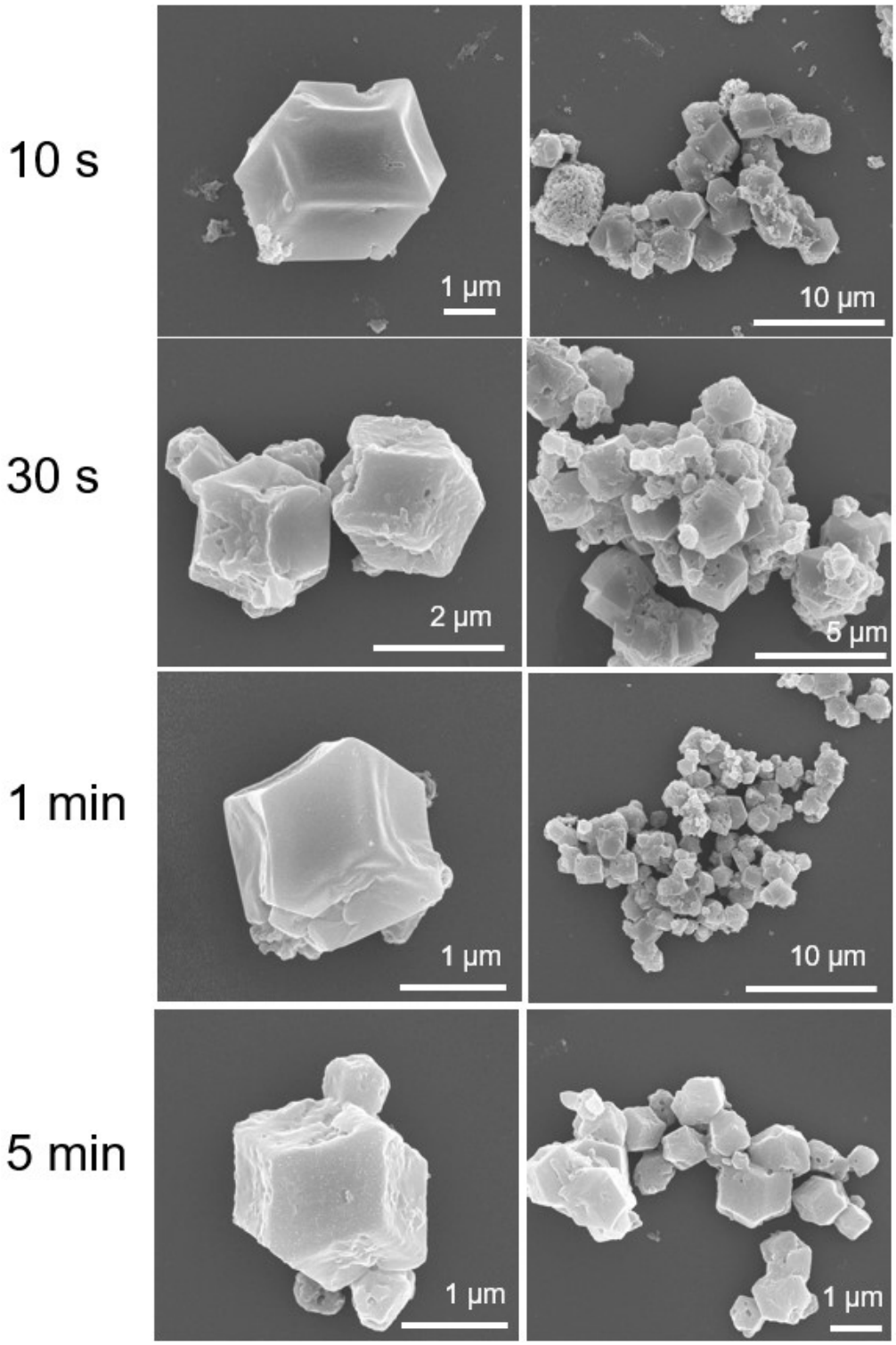

Figure S11. SEM images of the CAT@ZIF-90 samples were obtained using 500 mM Tris buffer solution at pH 7, with a milling frequency of 15 Hz, and at different reaction times.

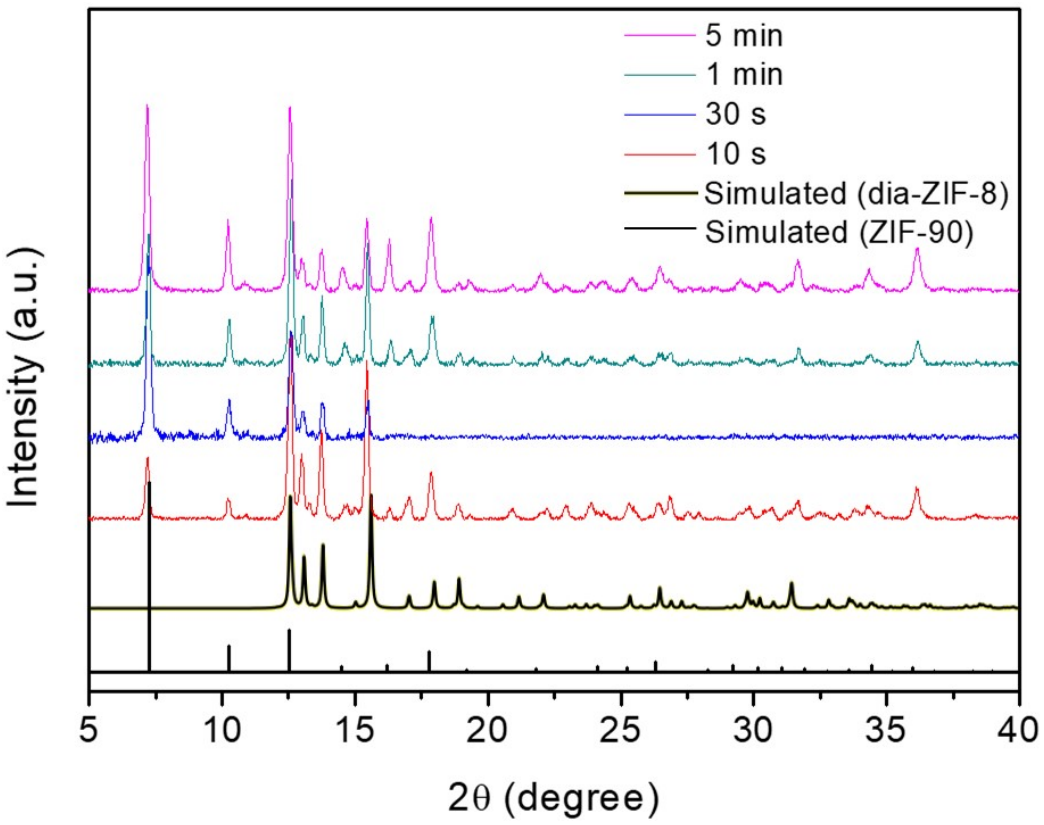

Figure S12. PXRD patterns of the CAT@ZIF-90 samples were obtained using 500 mM HEPES buffer solution at pH 7, with a milling frequency of 8 Hz, and at different reaction times.[7]

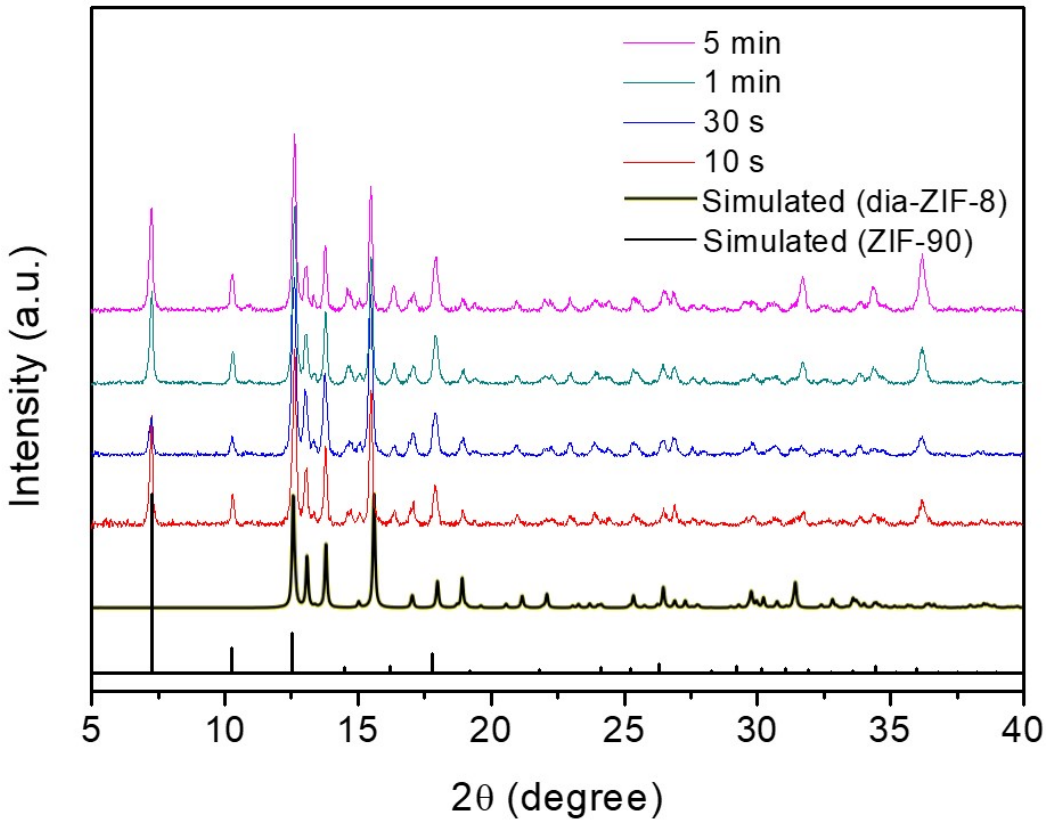

Figure S13. PXRD patterns of the CAT@ZIF-90 samples were obtained using 500 mM MES buffer solution at pH 7, with a milling frequency of 8 Hz, and at different reaction times.[7]

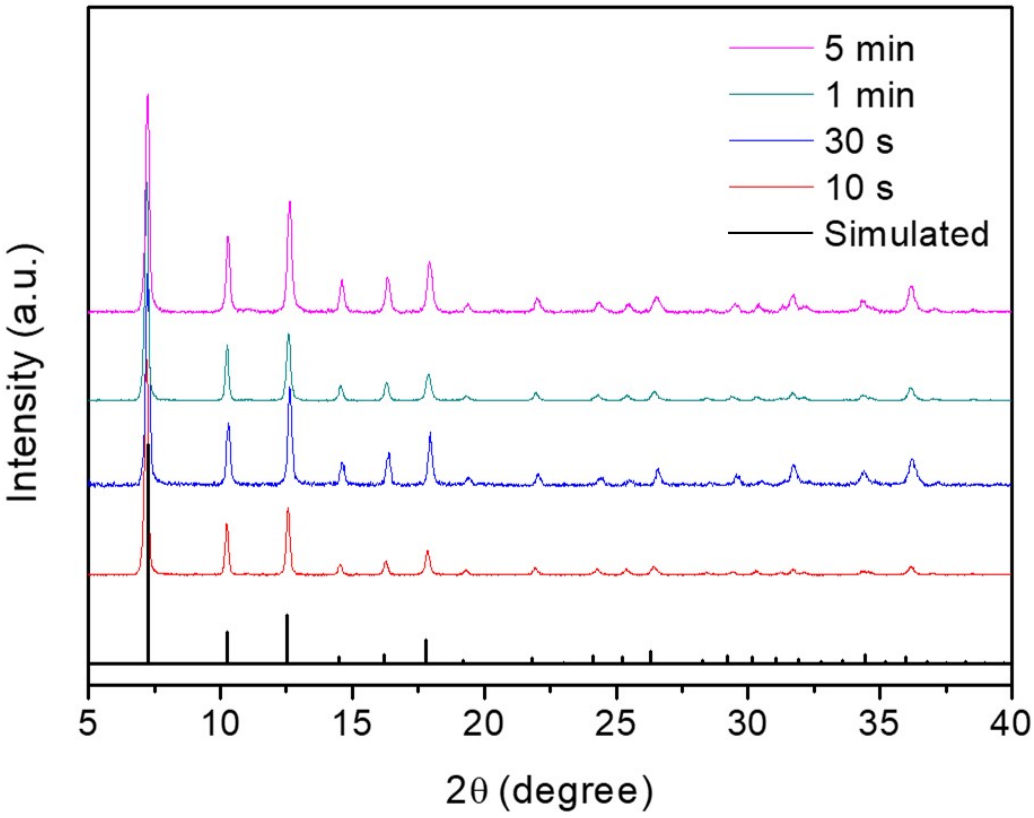

Figure S14. PXRD patterns of the CAT@ZIF-90 samples were obtained using 500 mM Tris buffer solution at pH 8, with a milling frequency of 5 Hz, and at different reaction times.

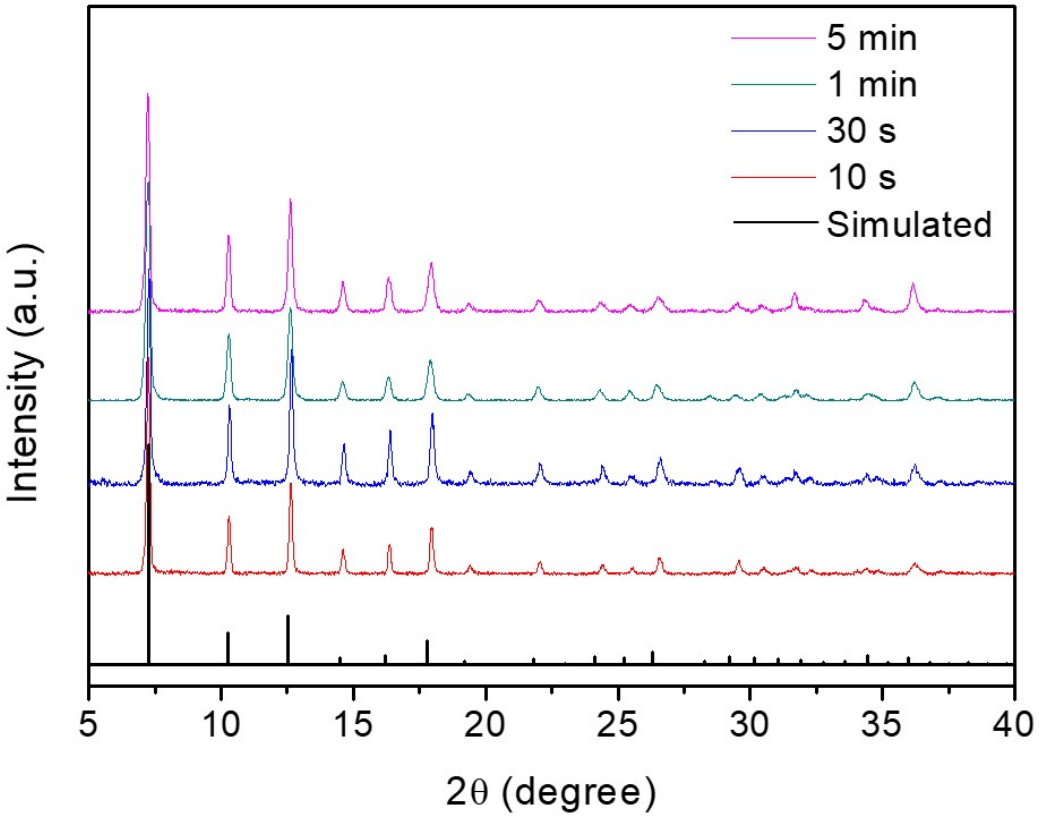

Figure S15. PXRD patterns of the CAT@ZIF-90 samples were obtained using 500 mM Tris buffer solution at pH 8, with a milling frequency of 8 Hz, and at different reaction times.

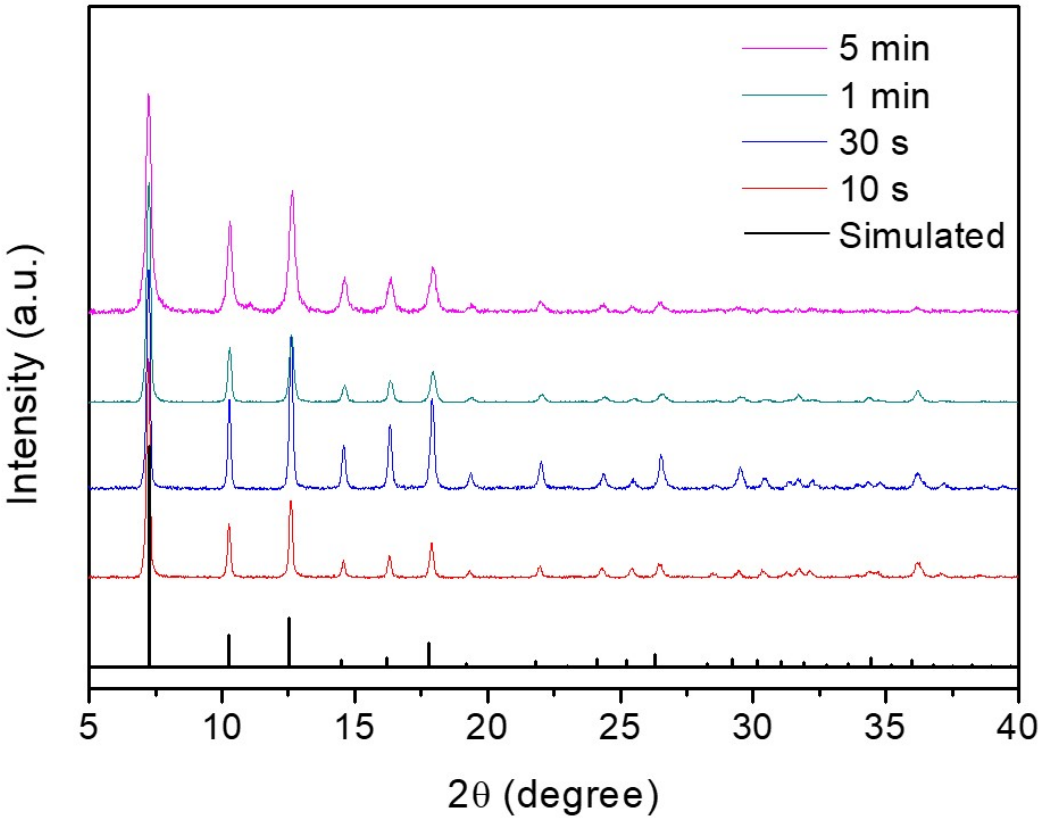

Figure S16. PXRD patterns of the CAT@ZIF-90 samples were obtained using 500 mM Tris buffer solution at pH 8, with a milling frequency of 15 Hz, and at different reaction times.

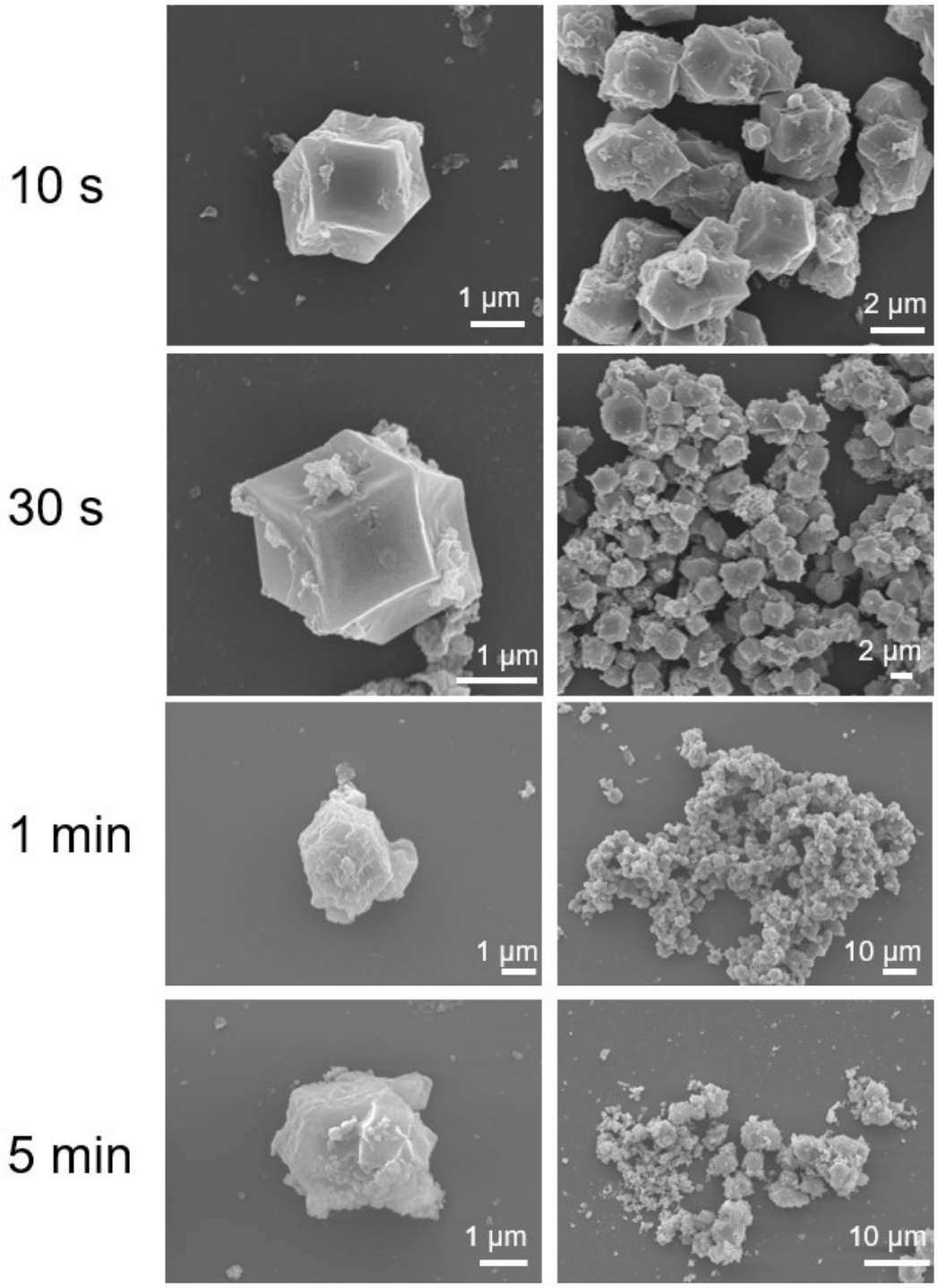

Figure S17. SEM images of the CAT@ZIF-90 samples were obtained using 500 mM Tris buffer solution at pH 8, with a milling frequency of 5 Hz, and at different reaction times.

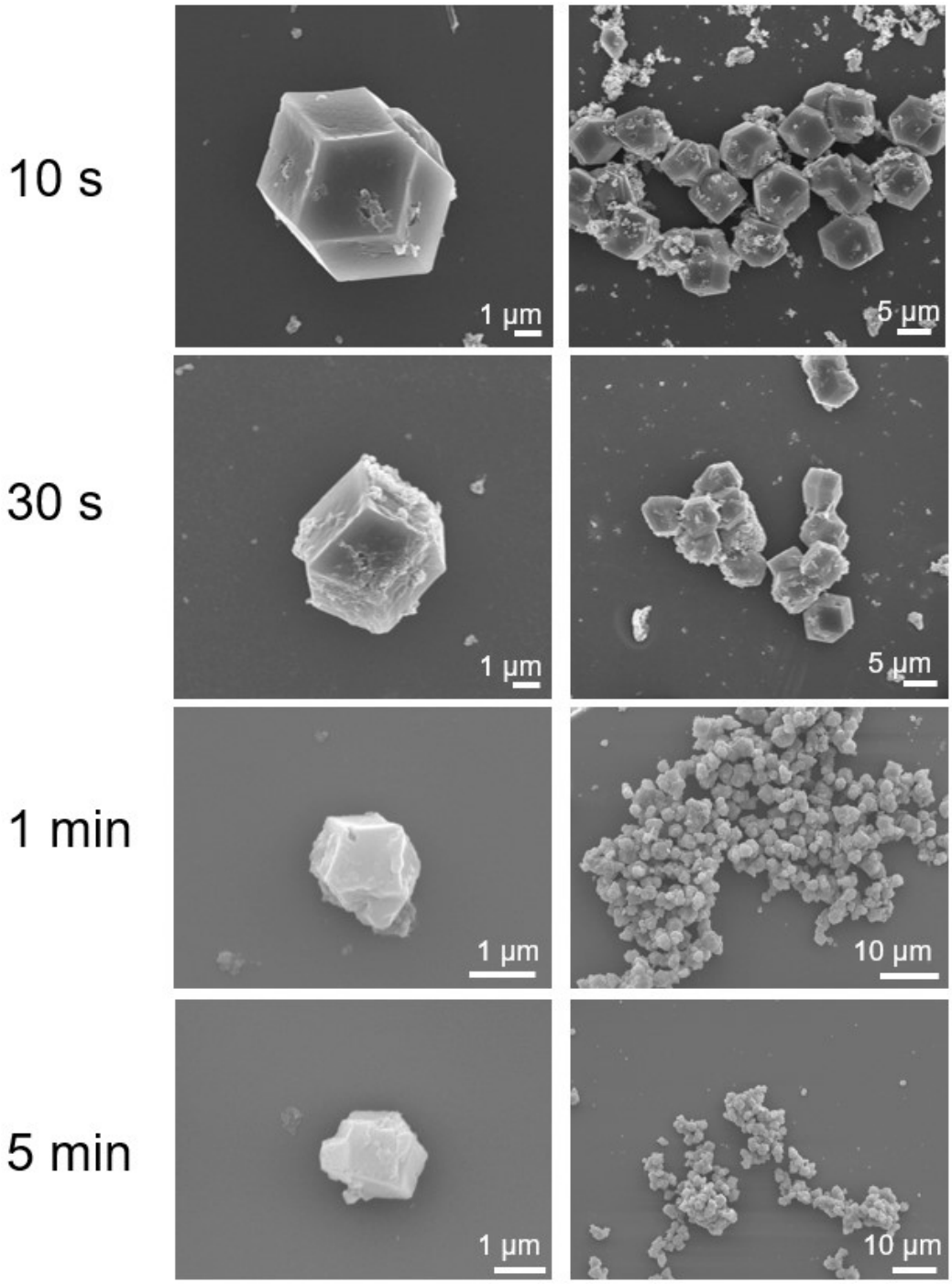

Figure S18. SEM images of the CAT@ZIF-90 samples were obtained using 500 mM Tris buffer solution at pH 8, with a milling frequency of 8 Hz, and at different reaction times.

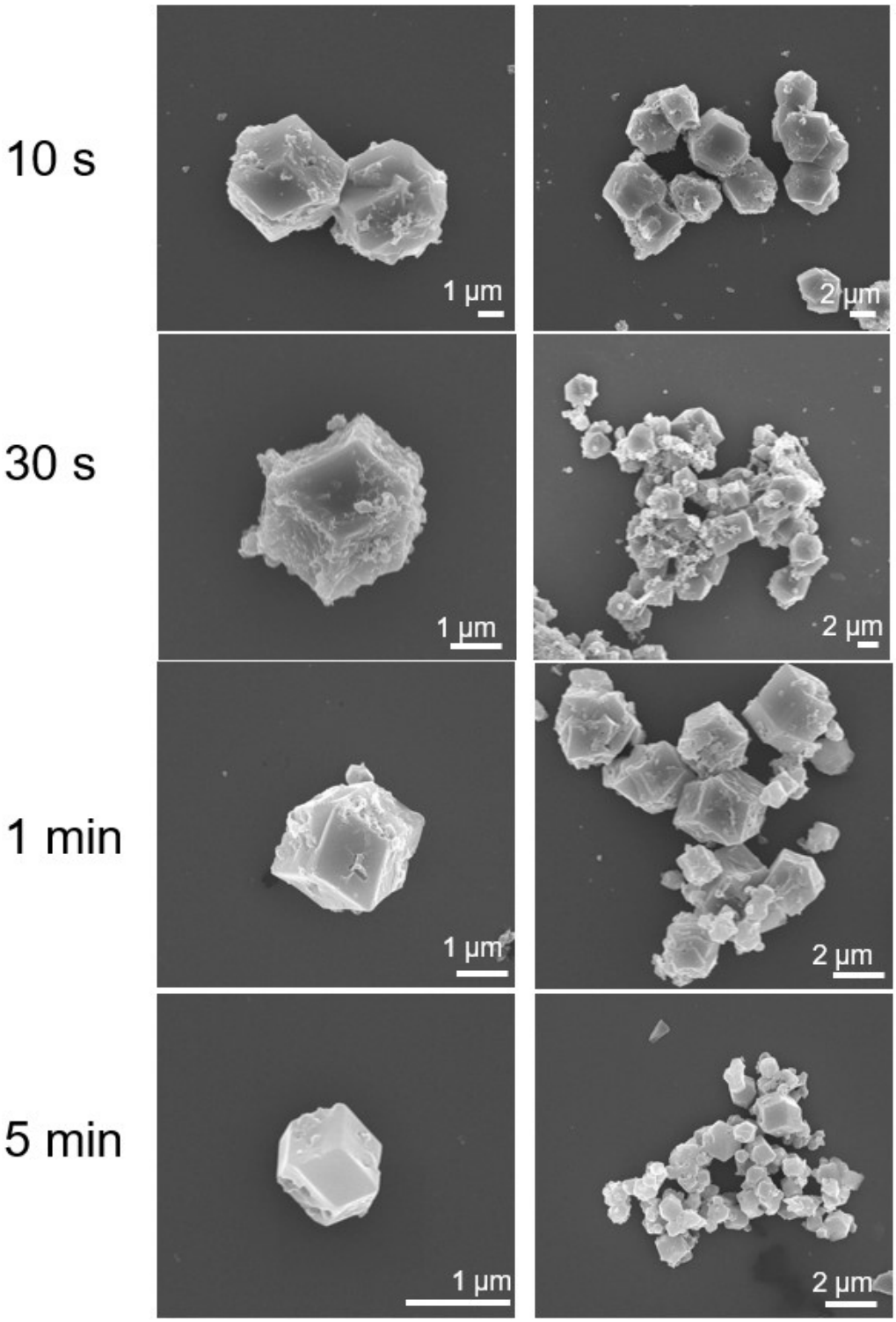

Figure S19. SEM images of the CAT@ZIF-90 samples were obtained using 500 mM Tris buffer solution at pH 8, with a milling frequency of 15 Hz, and at different reaction times.

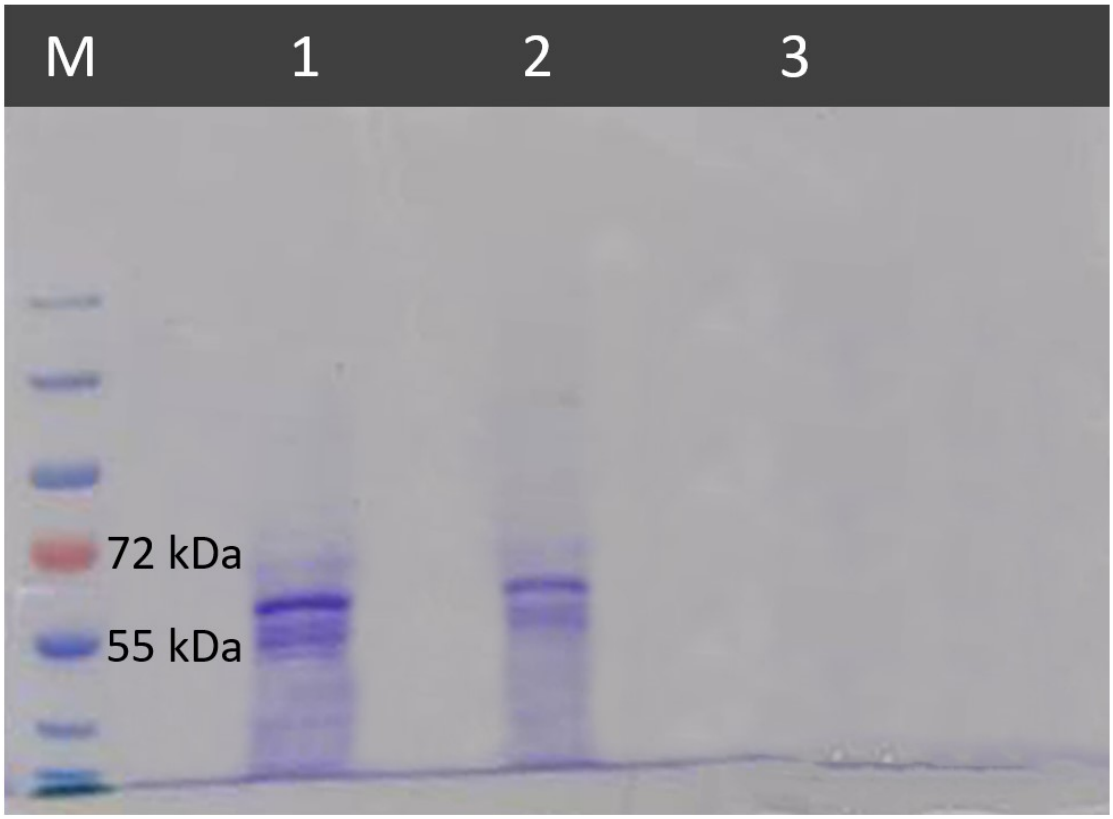

Figure S20. SDS-PAGE gel (M: protein marker, lane 1: free CAT, lane 2: CAT@ZIF-90-Tris-pH8-8Hz-10s, and lane 3: washed CAT-on-ZIF-90-Tris-pH8-8Hz-10s.

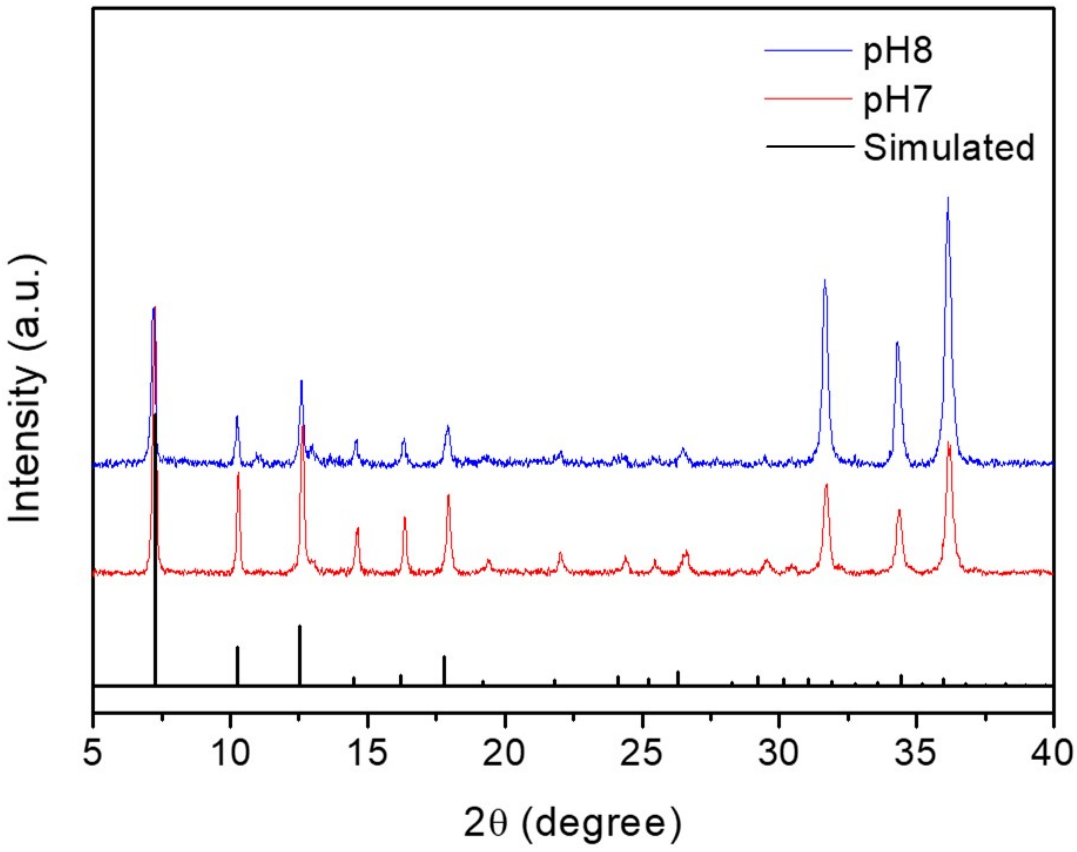

Figure S21. PXRD patterns of the BSA@ZIF-90 samples obtained using 500 mM Tris buffer solution at pH values of 7 and 8, with a milling frequency of 8 Hz and a duration of 10 seconds.

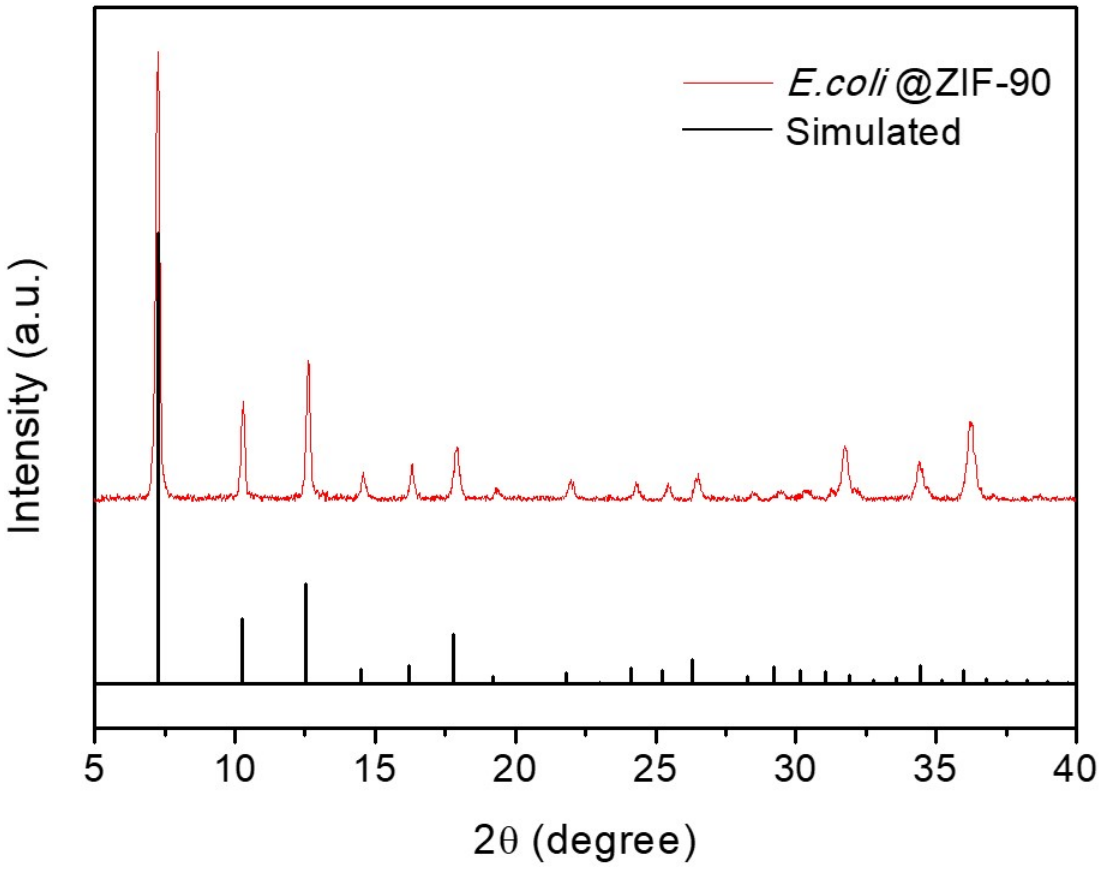

Figure S22. PXRD patterns of the *E.coli*@ZIF-90 sample obtained using 500 mM Tris buffer solution at pH 8, with a milling frequency of 8 Hz and a duration of 1 min.

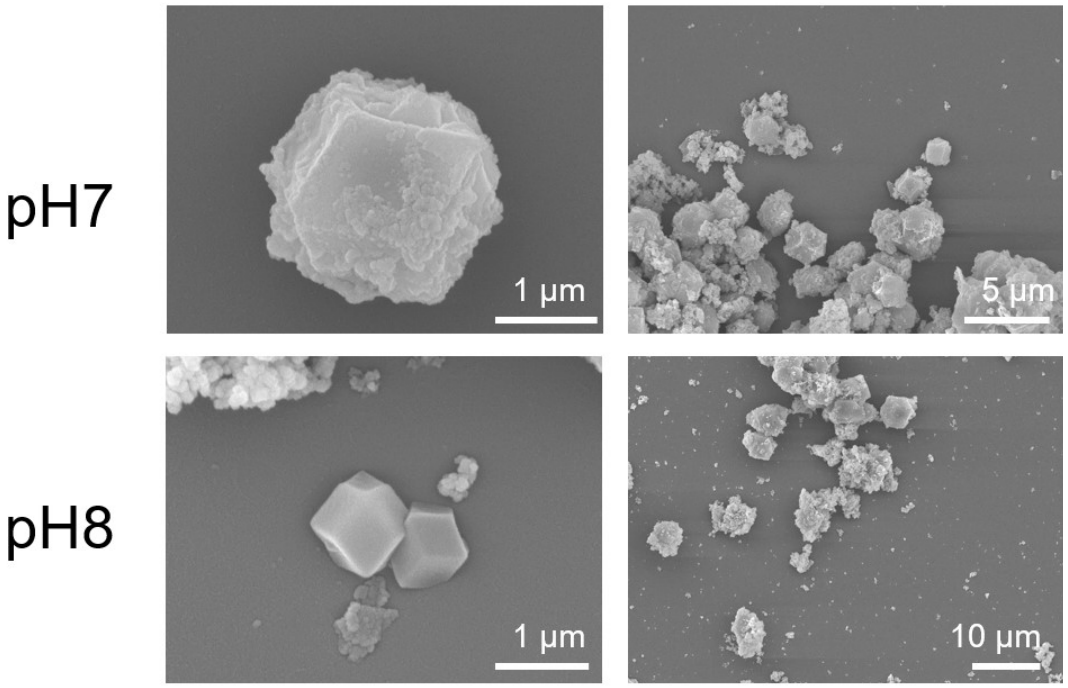

Figure S23. SEM images of the BSA@ZIF-90 samples were obtained using 500 mM Tris buffer solution at pH values of 7 and 8, with a milling frequency of 8 Hz and a duration of 10 s.

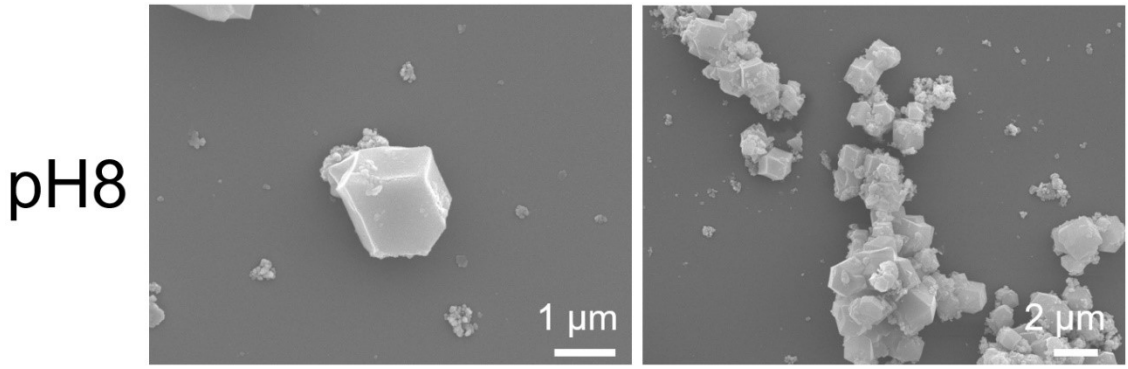

Figure S24. SEM images of the *E.coli*@ZIF-90 samples were obtained using 500 mM Tris buffer solution at pH 8 , with a milling frequency of 8 Hz and a duration of 1 min.

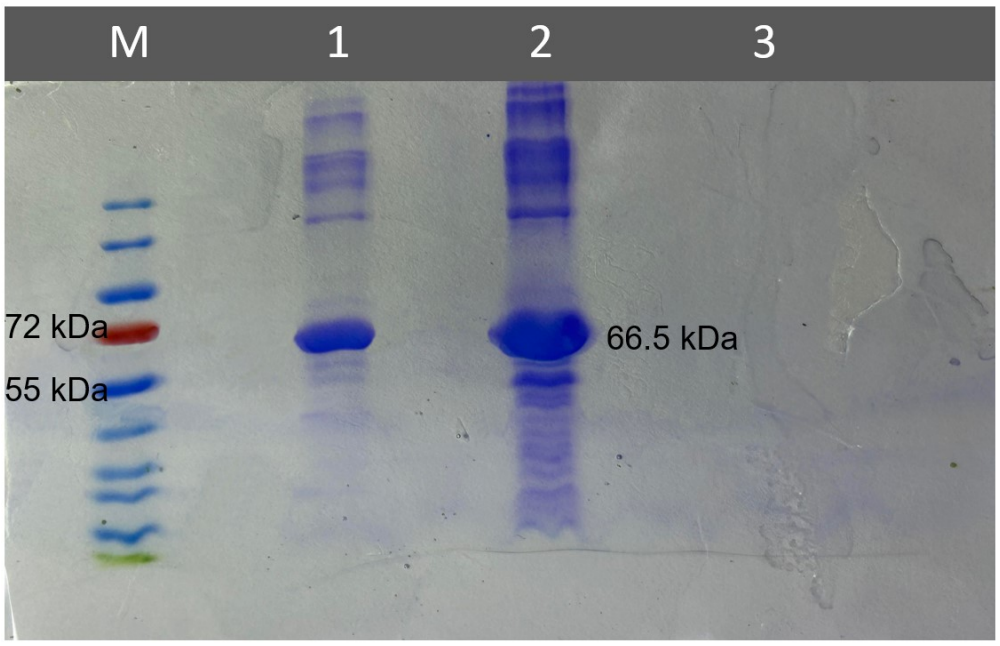

Figure S25. SDS-PAGE gel (M: protein marker, lane 1: free BSA, lane 2: BSA@ZIF-90-Tris-pH7-8Hz-10s, and lane 3: washed BSA-on-ZIF-90-Tris-pH7-8Hz-10s).

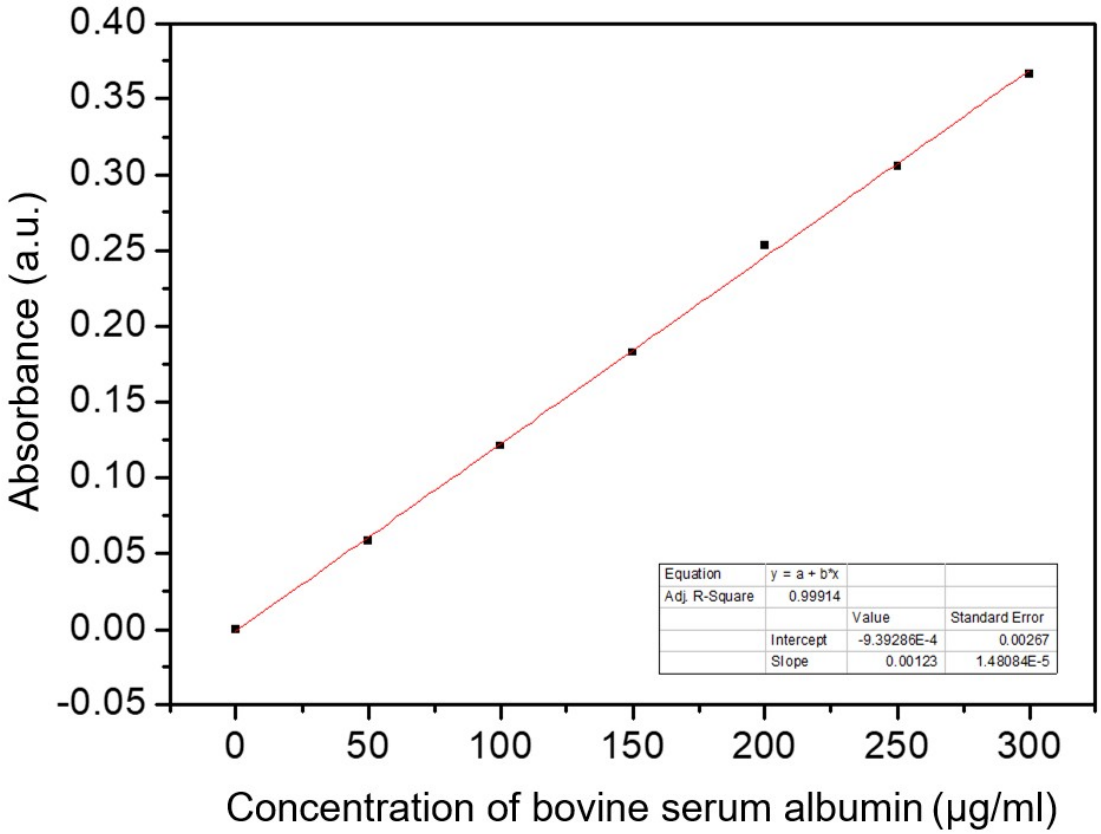

Figure S26. Bradford assay of the bovine serum albumin concentration. The corresponding standard calibration line of the Bradford assay is shown above.

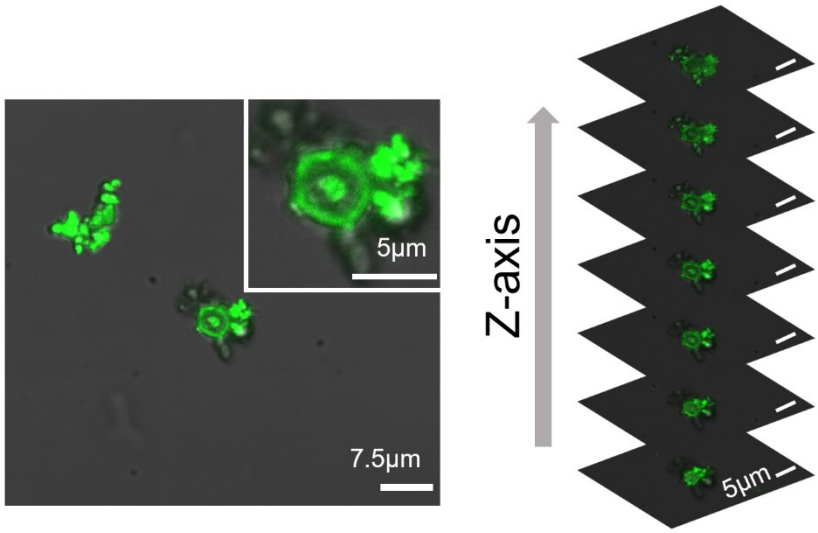

Figure S27. The corresponding cross-sections along the Z-axis of *E. coli*@ZIF-90-Tris-pH7-8Hz-1min obtained with a confocal .

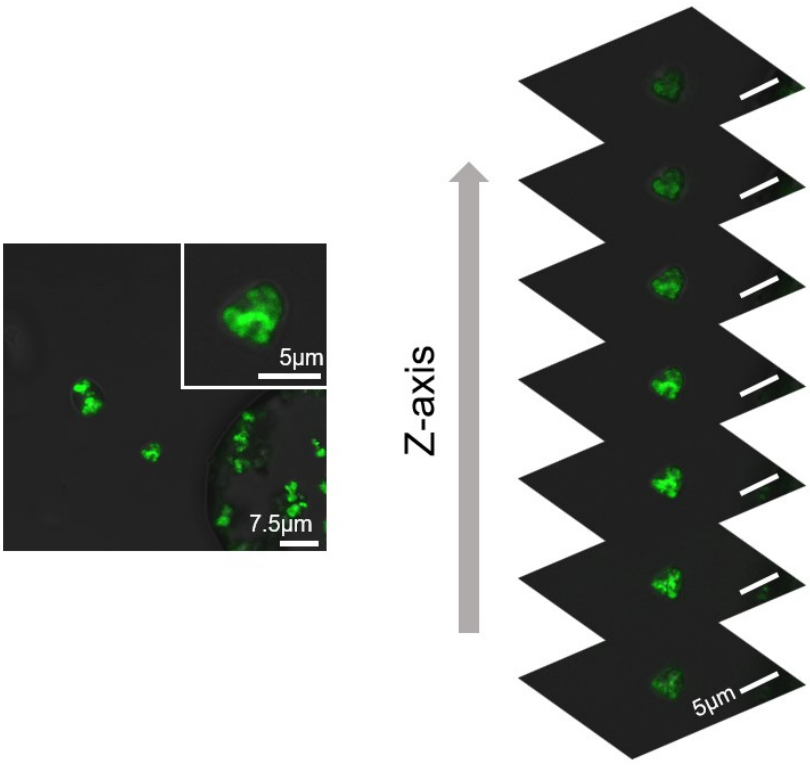

Figure S28. The corresponding cross-sections along the Z-axis of *E. coli*@ZIF-90-Tris-pH8-8Hz-1min obtained with a confocal microscope

Table S1. Kinetic parameters - k_{obs} (s^{-1}) of the CAT@ZIF-90-Tris-pH 7 samples with and without proteinase K at different milling frequencies and durations.

pH 7		10 sec	30 sec	1 min	5 min
5 Hz	With pk	(3.89±0.40)*10^-2	(5.42±0.20)*10^-2	(4.66±0.11)*10^-2	(6.37±0.02)*10^-2
	Without pk	(9.09±0.19)*10^-2	(1.08±0.04)*10^-1	(8.28±0.22)*10^-2	(1.21±0.13)*10^-1
8 Hz	With pk	(9.48±0.04)*10^-2	(1.03±0.01)*10^-1	(9.96±0.08)*10^-2	(8.01±0.19)*10^-2
	Without pk	(1.98±0.01)*10^-1	(1.67±0.07)*10^-1	(1.58±0.05)*10^-1	(1.20±0.03)*10^-1
15 Hz	With pk	(3.60±0.11)*10^-2	(1.69±0.20)*10^-2	(6.73±0.12)*10^-2	(1.28±0.06)*10^-2
	Without pk	(9.22±0.21)*10^-2	(9.22±0.37)*10^-2	(1.21±0.02)*10^-1	(6.05±0.08)*10^-2

Table S2. Kinetic parameters - k_{obs} (s^{-1}) of free CAT and CAT@ZIF-90 samples synthesized using a *de Novo* water-based method, both with and without proteinase K treatment.[8]

	Free catalase	CAT@ZIF-90	CAT@ZIF-90 (with Proteinase K)
$K_{obs}(s^{-1})$	8.97*10^-1	2.68*10^-2	2.46*10^-2

Table S3. Porosity data of CAT@ZIF-90, prepared by (a) water-based [8] and (b) ball-milling approaches, obtained using a Micromeritics ASAP 2010 analyzer.

	Langmuir surface area : S_L (m^2/g)	BET surface area : S_{BET} (m^2/g)	Total pore volume (cm^3/g)	t-plot micropore volume : V_{micro} (cm^3/g)
CAT@ZIF90 (mechanical)	915	865	0.38	0.30
CAT@ZIF-90 (*de Novo*)	1111	843	0.47	0.37

Table S4. Kinetic parameters - k_{obs} (s^{-1}) of the CAT@ZIF-90-Tris-pH 8 samples with and without proteinase K at different milling frequencies and durations.

pH 8		10 sec	30 sec	1 min	5 min
5 Hz	With pk	(9.94±0.35)*10^-2	(1.24±0.01)*10^-1	(8.18±0.09)*10^-2	(9.15±0.10)*10^-2
	Without pk	(2.05±0.08)*10^-1	(2.08±0.08)*10^-1	(1.63±0.01)*10^-1	(1.38±0.04)*10^-1
8 Hz	With pk	(8.28±0.07)*10^-2	(8.31±0.25)*10^-2	(5.77±0.67)*10^-2	(5.33±0.72)*10^-2
	Without pk	(1.36±0.04)*10^-1	(1.54±0.07)*10^-1	(1.26±0.02)*10^-1	(9.14±0.27)*10^-2
15 Hz	With pk	(6.90±0.35)*10^-2	(3.86±0.23)*10^-2	(2.13±0.07)*10^-2	(2.40±0.80)*10^-3
	Without pk	(1.48±0.11)*10^-1	(1.01±0.01)*10^-1	(5.40±0.11)*10^-2	(3.04±0.07)*10^-2

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