Supporting information

Alternative protein encapsulation with MOFs: overcoming the elusive mineralization of HKUST-1 in water

■ EXPERIMENTAL SECTION

Materials and reagents. All chemicals are commercially available and used as received. Benzene 1,3,5-tricarboxilic acid (BTC) was purchased from Alfa Aesar (USA). Copper chloride dihydrate ($CuCl_2{\cdot}2H_2O$) was purchased from PanReac AppliChem (Germany). Trichloroacetic acid (TCA), copper (II) acetate and sodium acetate were purchased from Thermo Scientific (USA). Sodium hydroxide (NaOH), Casein (Cas), Azocasein, Subtilisin Calsberg (SubC), bovine serum albumin (BSA), bovine heart cytochrome c (CytC) and lyophilized *Micrococcus lysodeikticus* ATCC No. 4698 were purchased from Sigma-Aldrich (USA). Hen egg white lysozyme (Ly), Trypsin (Try) and 3,3',5,5'-Tetramethylbenzidine, free base (TMB) were purchased from GolBio (USA). Tris(hydroxymethyl)aminomethane (Tris base) was purchased from Santa Cruz Biotechnology (USA). Hydrochloric acid (HCl) 37% and Abs. Ethanol (EtOH) from Scharlab (Spain). Milli-Q water was obtained from a Millipore Milli-Q system.

Instrumentation and physico-chemical characterization. Particle size measured as hydrodynamic diameter was collected on a Zetasizer Ultra equipment operating at 25 °C, equipped with a red (633 nm) laser and avalanche photodiode detector (Malvern, UK). All aqueous dispersions were prepared by ultrasonication in a water bath. Polystyrene cuvettes (DTS0012) were used for size measurement and Folded Capillary Zeta Cell (DTS1070) was used for measurement of ζ potential. A UV-vis microplate spectrophotometer Multiskan Sky (Thermo Scientific) was employed for UV-vis measurements. FT-IR was performed on an ALPHA II spectrometer (Bruker) in the range 400 – 4000 cm^{-1} using the ATR accessory with a diamond window. XRPD patterns were obtained using an X-ray diffractometer (PANalytical Empyrean) with copper as a radiation source (Cu-Kα 1.5418 Å) operating at 40 mA and 45 kV and equipped with an X'Celerator detector. Measurements were collected on quartz capillaries or in a high throughput screening (HTS) platform. TGA profiles were collected using a TGA 550 (TA instruments) at temperatures from 25 to 800 °C under N_2. The heating rate was stablished in high resolution mode (HR), starting by 10 °C/min and decelerating when significant weight variation is measured. Transmission electron microscopy (TEM) images were obtained with JEOL JEM 1010 100Kv (JEOL Ltd., Japan). N_2 isotherms were measured with a TRISTAR-2 apparatus (Micromeritics) at 77K. Before the measurement, samples were degassed at 100 °C for 1 hour in vacuum. Surface area was calculated using the Brunauer-Emmett-Teller (BET)[1] equation from the adsorption curve.

The encapsulation efficiency was assessed by determining the difference in protein concentration in the supernatant by BCA protein assay (Thermo Scientific, Pierce BCA Protein Assay Kit). The method

combines the reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium (biuret reaction) with the colorimetric detection of purple-colored complex formed by chelation of the cuprous cation with bicinchoninic acid (BCA) in a 1:2 ratio.[2] Aliquots of the supernatant (1 mL) were recovered by micropipette. Measurements were performed in microplate following product instructions. All the experiments were performed in triplicates.

Synthesis of Ho and transformation into HKUST-1 (HKo): In a typical synthesis, 525 mg of H_3BTC (2.5 mmol) and 1.5 mL of NaOH 5M (7.5 mmol) were mixed and dissolved in ~ 30 mL of Mili-Q water. After sonication, the pH of the solution was adjusted to 7.0 with diluted HCl and adjusted to 50 mL (final concentration: Na_3BTC 50 mM). The metal solution was prepared with 425.5 mg of $CuCl_2{\cdot}2H_2O$ (2.5 mmol) and adjusted to 50 mL (final concentration: $CuCl_2$ 50 mM). Both solutions were loaded in 50 mL syringes with perfusion lines and put on a perfusor (B. Braun Space, Germany). In a glass vial, 1 mL of MiliQ water and stirred at 1200 rpm at room temperature (RT), and 10 mL of both ligand and metal solution were added simultaneously at 120 mL/h (total addition time: 5min). Considering the line dead space volume (~3.15 mL) the volume of solutions prepared was enough to 4 reactions. Immediately after the addition, a blue precipitate was formed and was left stirring for 1h. The final pH was around 5.6. Then, the solid was recovered by centrifugation (5000 rpm 10 min) and the supernatant discarded. The solid obtained was washed three times with MiliQ water H_2O and recovered by centrifugation. Then, the sample **Ho** was left to dry on air or stored in water as a suspension.

For the transformation into HKUST-1 (**HKo**), the as synthesized solid from the synthesis was firstly washed with abs. EtOH, centrifuged, and left immersed overnight in 20 mL solutions of 25 mM acetic acid (HAc), 25 mM hydrochloric acid (HCl) or 8.3 mM H_3BTC in ethanol. H_3BTC was selected as final acid for the transformations. After that, the resulting solid was recovered again and washed for third time with EtOH. The sample was then dried under vacuum or stored as a suspension in EtOH.

Synthesis of H1 and transformation into HKUST-1 (HK1): A synthetic procedure similar to Ho was employed for obtaining H1, but a different pH was used instead. The ligand solution was prepared identically, and metal solution was prepared with 425.5 mg of $CuCl_2{\cdot}2H_2O$ (2.5 mmol) and 0.5 mL of HCl 5M (2.5 mmol) and adjusted to 50 mL (final concentration: $CuCl_2$ 50 mM, HCl 50 mM). Same conditions were employed for the perfusor-assisted synthesis. The final pH was around 4.4. Then, the solid was recovered by centrifugation (5000 rpm 10 min) and the supernatant discarded. The solid obtained was washed three times with ddH_2O and recovered by centrifugation. Then, the sample **H1** was left to dry on air or stored in water as a suspension.

For the transformation into HKUST-1 (**HK1**), the as synthesized solid from the synthesis was firstly washed with abs. EtOH, centrifuged, and left immersed overnight in ethanol. After that, it was recovered again and washed for third time with EtOH. The sample was then dried under vacuum or stored as a suspension in EtOH.

Interconversion of Ho and H1: Ho solid can be converted into **H1** upon overnight incubation of the as synthesized solid in 20 mL of 8.3 mM H_3BTC in water. Then, the solid was recovered by centrifugation, washed three times with Milli-Q water and left to dry on air.

H1 transformation into **Ho** was carried out by overnight incubation of the as synthesized solid in 20 mL of 25 mM NaOH in water. The solid was recovered by centrifugation, washed three times with Milli-Q water and left to dry on air.

Protein encapsulation: Different synthetic procedures have been explored. In a first attempt, same solutions were prepared as in Ho synthesis, but only the $CuCl_2$ solution was loaded into a syringe. In a vial, 10 mL of the Na_3BTC solution was added with 10 mg of the corresponding protein under stirring. On top of this, 10 mL of the $CuCl_2$ solution was added with perfusor at 120 mL/h. After complete addition, the resulting material was collected by centrifugation and washed three times with Milli-Q water.

The final synthesis for the encapsulation of proteins were carried out with the same procedures described previously for both Ho and H1, but 1 mL of 10 mg/mL protein solution was added in the glass vial instead of Milli-Q water. The obtained solid was washed as indicated for Ho and H1, and transformation into HKo and HK1 was done in the same way.

Encapsulation efficiency of Lysozyme in Ho as a function of the initial amount of protein and BTC: A series of encapsulation synthesis were performed varying the amount of lysozyme (a high pI protein) and changing the amount of Na_3BTC solution added before the synthesis. In a general procedure, 1 mL of 50 mM $CuCl_2$ and varying volumes of 50 mM Na_3BTC (pH 7.0) were simultaneously added in 3 minutes (20 mL/h) with a perfusor on top of 0.1 mL of lysozyme solution with different protein concentrations (10, 20, 30, 40 and 50 mg/mL). In all the synthesis, the Cu:BTC stoichiometry was fixed to 1:1, but a varying percentage of the 1 mL of Na_3BTC solution (0, 25, 50, 75, 100%) was added before starting the addition of $CuCl_2$ plus the other percentage of Na_3BTC.

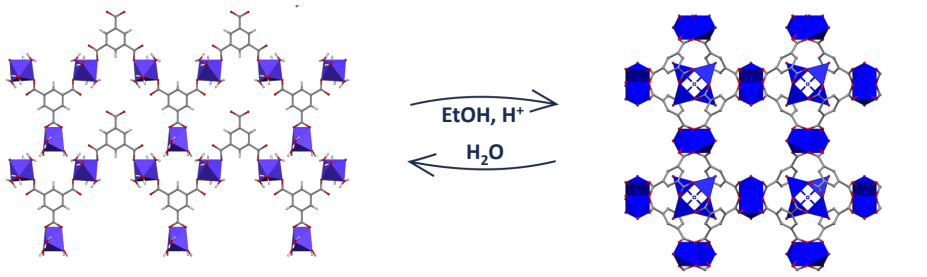
This set up allows the screening of diverse conditions of protein to MOF percentages (10, 20, 30, 40, 50 %, considering that around 10 mg of MOF are formed under this synthetic conditions) and different initial abundance of Na_3BTC. Encapsulation efficiency was measured from the quantification of the supernatant.

Cytochrome C peroxidase activity assays: Kinetic assays were preformed employing a microplate reader. On a microplate well, 10 µl of sample was added, followed by the addition of 250 µL of 50 mM acetate buffer (pH 5.0), 532 µM of TMB and different concentrations of H_2O_2 substrate (98, 49, 19.6, 9.8, 4.9, 2.45, 1.225, 0.49 and 0.0 mM). In all samples, the concentrations were adjusted to match 9 µg/mL of CytC and 100 µg/mL of MOF. When $Cu(OAc)_2$ was employed, 0.5 mM solutions were used in samples. The assays were performed at room temperature (~25°C). Absorption at 652 nm was measured every 15 s for 20 minutes. For the calculation of the concentration of product, the extinction coefficient ($\varepsilon = 3.9 \cdot 10^4\ M^{-1} \cdot cm^{-1}$)[3] was employed. Initial rate values were taken and employed for Michaelis-Menten fitting.[4]

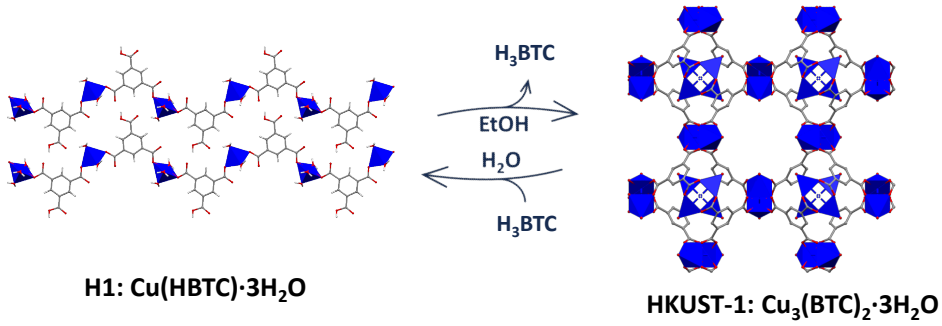
Lysolytic assays: The activity was measured with a microplate reader by following the lysis of a suspension of lyophilized bacteria.[5] A suspension of *Micrococcus lysodeikticus* (0.45 mg/mL) in phosphate buffer (100 mM, pH 6,25) was prepared. Prior to assays, samples were diluted 1:1 in 100 mM EDTA pH 8.0 to degrade the MOF and ensure the release of the enzyme. On a microplate well, 10 µl of sample were added, followed by 250 µL of the suspension. The assay was carried at 25 °C, and absorbance at 450 nm was measured every 15 s for 20 minutes. Activity units were defined as a decrease of 0.001 units in absorbance per minute (AU = $\Delta Abs \cdot min^{-1}$). Lysozyme concentration was quantified in samples and employed to obtain the activity per unit of enzyme (AU/mg). This data was employed for the comparison of samples.

Proteolytic assays: Protease activity was measured spectrophotometrically by the azocasein hydrolysis method [6–9]. Prior to assays, samples were diluted 1:1 in 100 mM EDTA pH 8.0 to degrade the MOF and ensure the release of the enzyme. 150 µL of sample were added to 150 µL of 100 mM Tris-HCl buffer pH 7.5 in a 1.5 mL centrifuge tube. 300 µL of 1 % (w/v) azocasein dissolved in the Tris-HCl buffer were added, and the reaction mixture was incubated at 40 °C for 10 min in a dry block heater. The reaction was stopped by adding 600 µL of 10 % (w/v) trichloroacetic acid (TCA). This was followed by centrifugation at 13.400 rpm for 5 min. 800 µL of the supernatant was collected and neutralized by adding 200 µL of 1.8 N NaOH. The absorbance at 440 nm was measured with a microplate reader (250 µL/well of neutralized supernatant was employed). Activity units were defined as an increase of 0.001 units of absorbance per minute (AU = $\Delta Abs \cdot min^{-1}$). Trypsin and Subtilisin C concentration was quantified in samples and employed to obtain the activity per unit of enzyme (AU/mg). This data was employed for the comparison of samples.

In samples of azocasein@HKUST-1, similar procedure was applied, but 100 µL of 100 µg/mL Trypsin or Subtilisin C were employed as sample and 500 µL of 100 µg/mL of azocasein (in free form or encapsulated at 10% loading). When MOF solution was employed, 1 mg/mL was applied. The mixture was heated at 40 °C for 10 minutes in a dry block heater. From this point, same procedure was applied as before.



Scheme 1: Schematic representation of the Cu-BTC dense phase (**Ho**) transformation into HKUST (**HKo**).



Scheme 2: Schematic representation of the Cu-BTC dense phase (**H1**) transformation into HKUST (**HK1**).

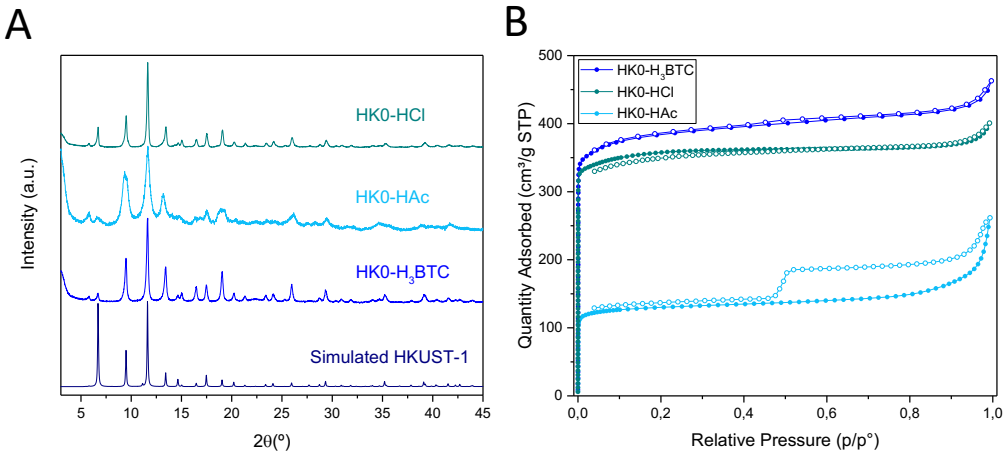


Figure S1: (a) XRPD of the HKo phases obtained upon immersion of Ho on acidified ethanol with trimesic acid (H_3BTC), HCl or acetic acid (HAc). (b) N_2 sorption of HKo phases obtained with the different acids.

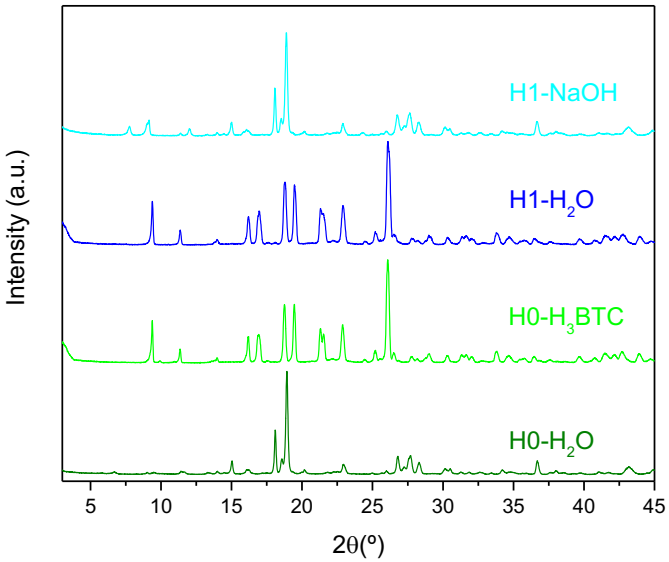


Figure S2: XRPD patterns of **Ho** and **H1** obtained at different pH in water and their corresponding interconversions after incubation for 24 h in 8.7 mM H_3BTC (Ho —> H1) and 8.7 mM NaOH (H1 —> Ho). In both cases, the amount of Cu^{2+} corresponds to 25 mM.

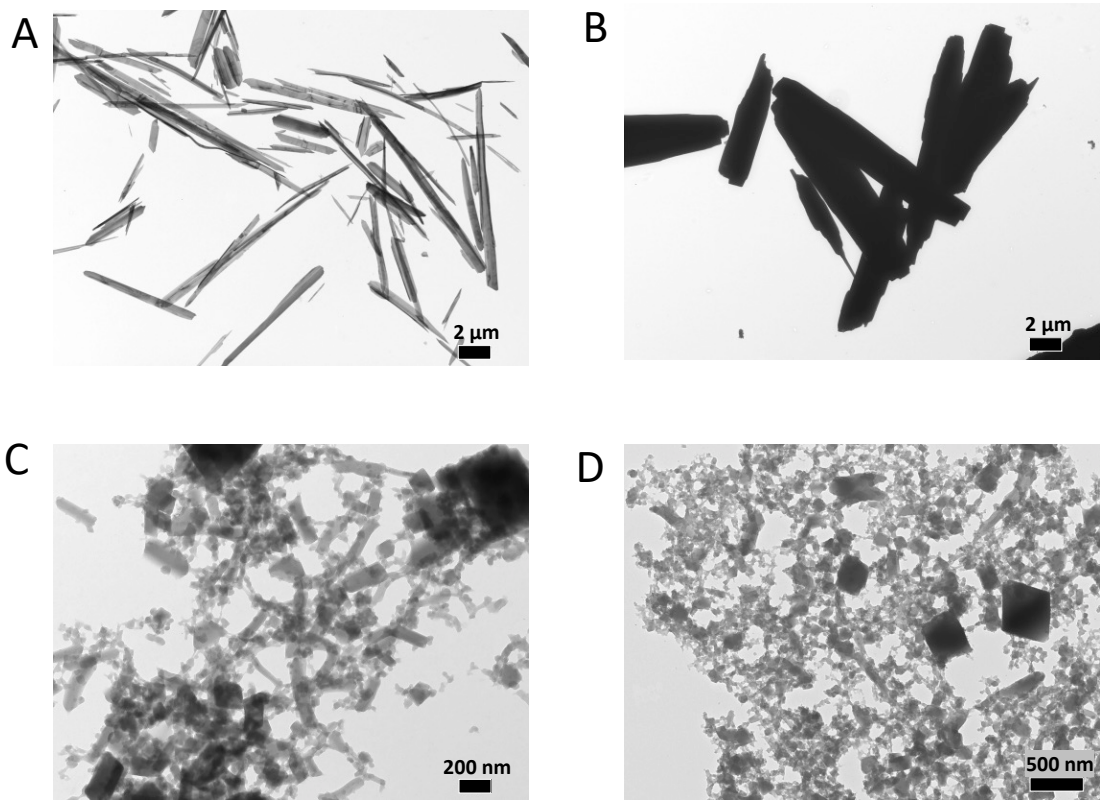


Figure S3: TEM images of (a) Ho, (b) H1, (c) HKo and (d) HK1. Scale indicated in images.

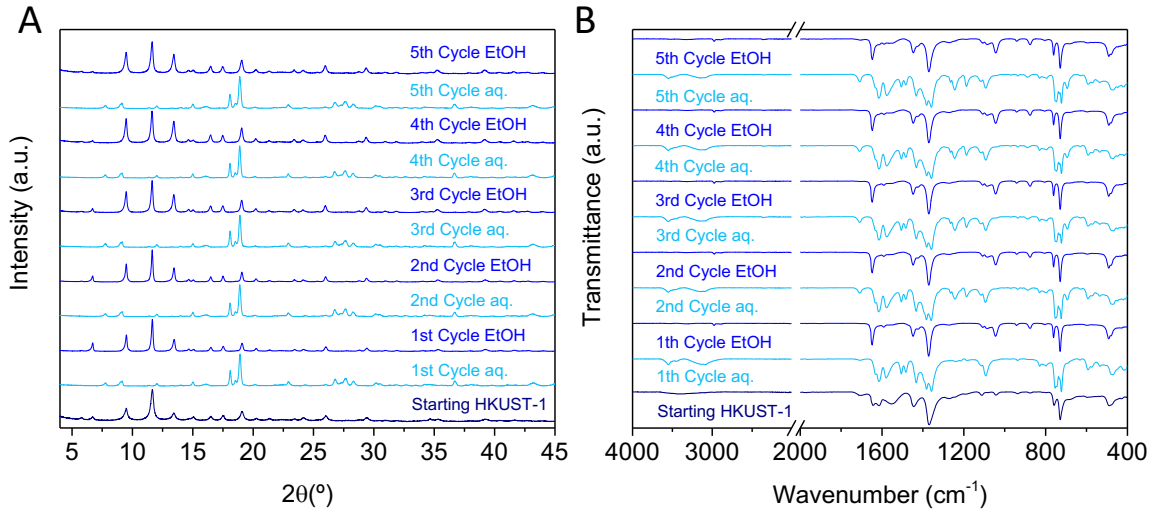


Figure S4: (a) XRPD patterns and (b) ATR-FTIR spectra of HKo sample after successive degradation in H_2O and reconstruction in 8.7 mM H_3BTC ethanol solution.

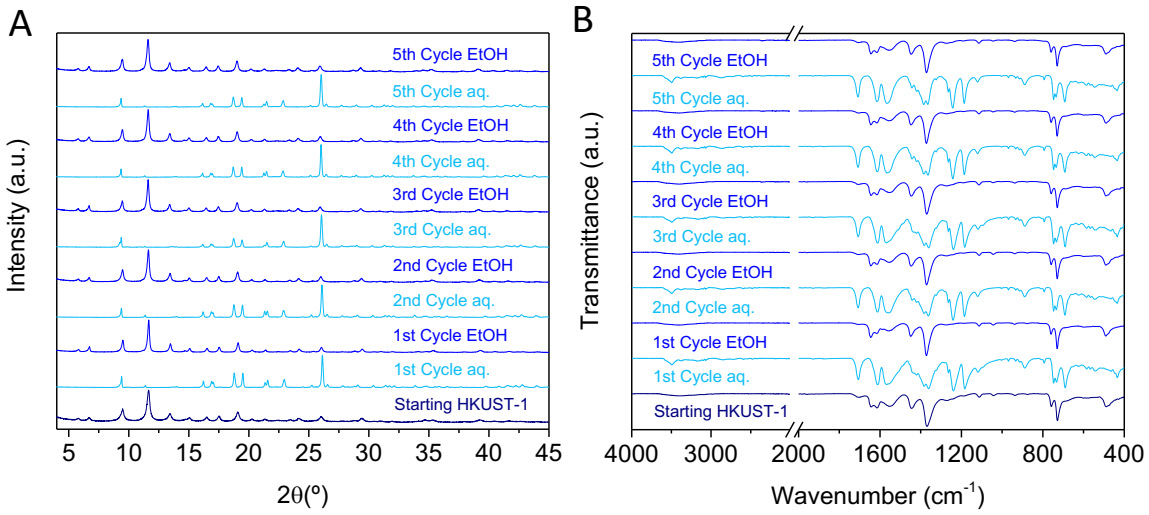


Figure S5: (a) XRPD patterns and (b) ATR-FTIR spectra of HK1 sample after successive degradation in 8.7 mM H_3BTC aqueous solution and reconstruction in absolute ethanol.

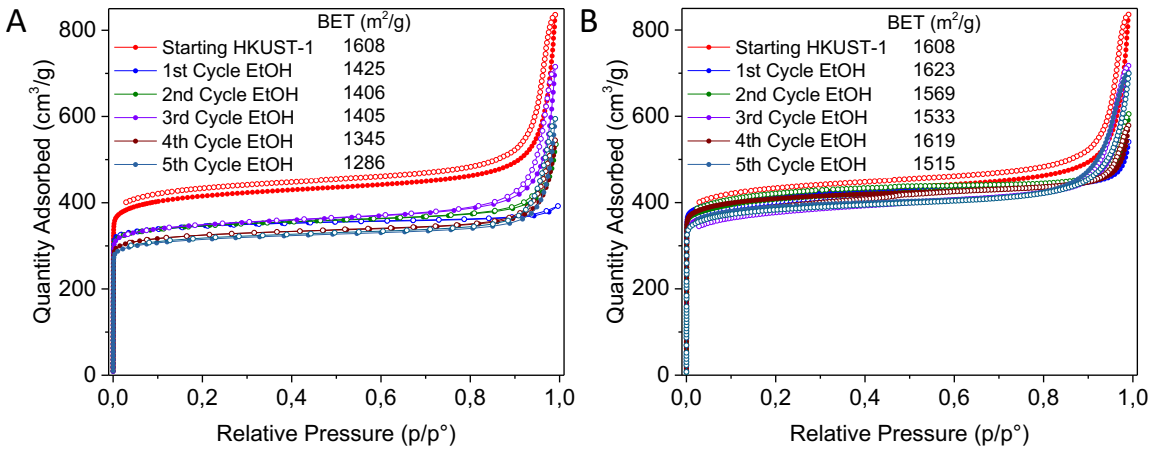


Figure S6: (a) N_2 sorption isotherms of HKo sample after successive degradation in H_2O and reconstruction in 8.7 mM H_3BTC ethanol solution (b) N_2 sorption isotherms of HK1 sample after successive degradation in 8.7 mM H_3BTC aqueous solution and reconstruction in absolute ethanol. BET values are indicated along with the legend.

Table S1: Isoelectric point (pI) of the proteins employed in this work.

	Isoelectric point (pI)	Reference
Cas	4.6	[10]
BSA	5.4	[11]
SubC	9.4	[12]
CytC	10.25	[13]
Try	10.5	[14]
Ly	11.35	[15]

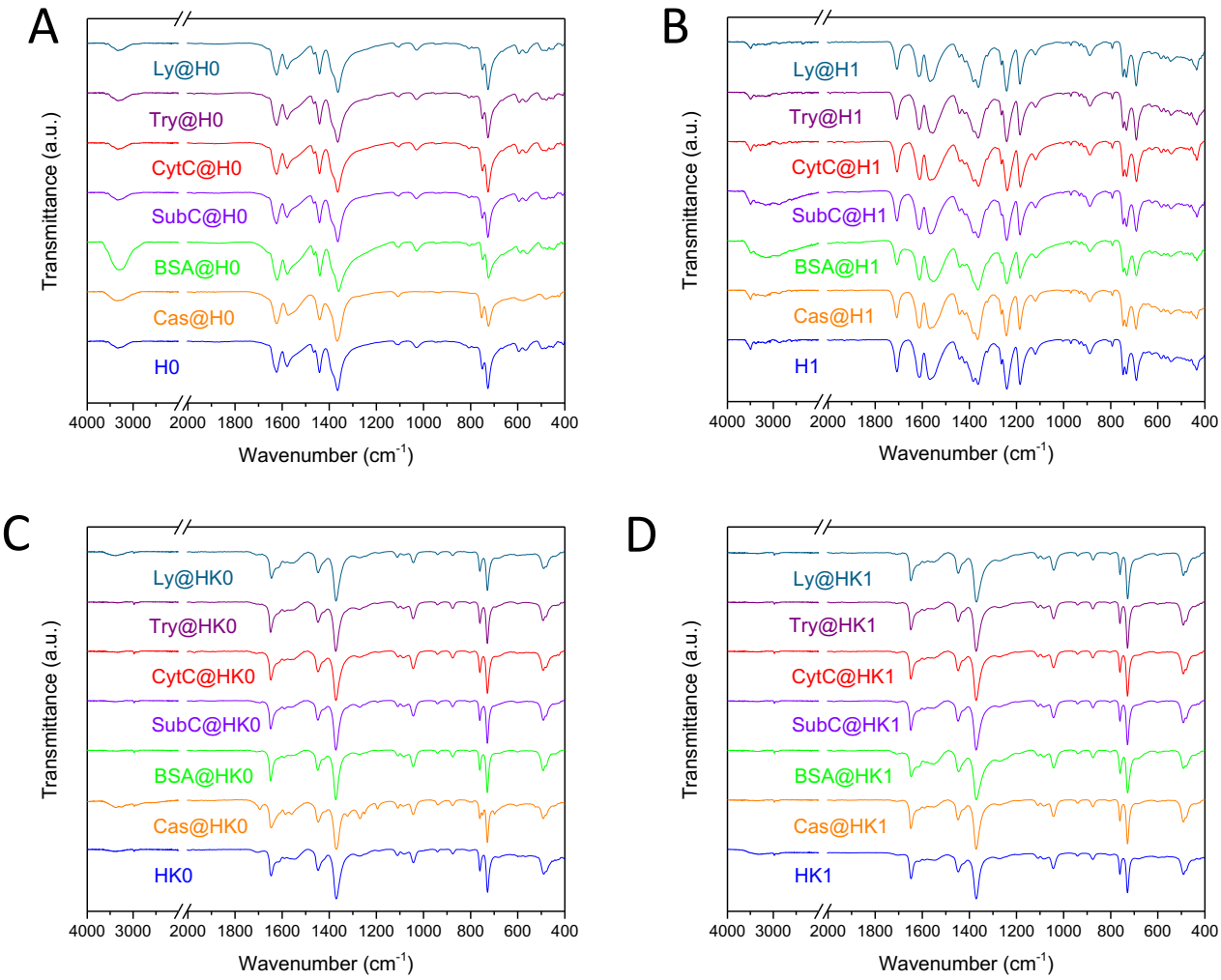


Figure S7: ATR-FTIR spectra of the different (a) protein@Ho, (b) protein@H1, (c) protein@HKo and (d) protein@HK1 materials. The overlapping of the amide I and II bands from proteins with the carboxylate bands at 1614, 1564 and 1360 cm^{-1} hinders their detection by this technique.

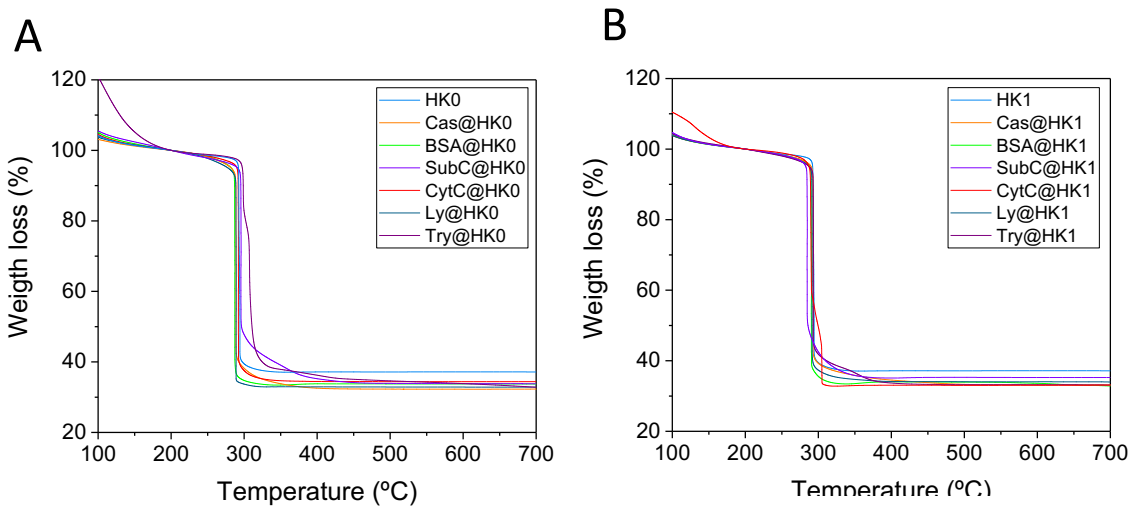


Figure S8: (a) TGA profile curves of HK0 and protein@HK0 composites and (b) HK1 and protein@HK1 composites.

$$\% \, Protein = \frac{(Weigth\ reference)_{700°C} - (Weigth\ sample)_{700°C}}{(Weigth\ reference)_{700°C}} \cdot 100 \quad \textbf{(1)}$$

Table S2: Inorganic residue of HKo and protein@HKo composites after normalization to the weight at 200 °C. The protein content was calculated with the formula (1). HKUST-1 theoretical inorganic residue is calculated assuming complete evacuation of the solvent from molecular formula: $Cu_3(C_9H_3O_6)_2$.

	Inorganic (%)	**Protein (%)**
HKUST-1 (theoretical)	39.5	-
HKo	37.2	-
Cas@HKo	32.3	13.2
BSA@HKo	33.7	9.2
SubC@HKo	33.8	9.0
CytC@HKo	34.4	7.4
Try@HKo	32.8	11.7
Ly@HKo	32.9	11.6

Table S3: Inorganic residue of HK1 and protein@HK1 composites after normalization to the weight at 200 °C. The protein content was calculated with the formula (1). HKUST-1 theoretical inorganic residue is calculated assuming complete evacuation of the solvent from molecular formula: $Cu_3(C_9H_3O_6)_2$.

	Inorganic (%)	**Protein (%)**
HKUST-1 (theoretical)	39.5	-
HK1	37.2	-
Cas@HK1	33.1	10.8
BSA@HK1	32.8	11.6
SubC@HK1	35.2	5.1
CytC@HK1	33.0	11.0
Try@HK1	33.1	10.8
Ly@HK1	34.0	8.5

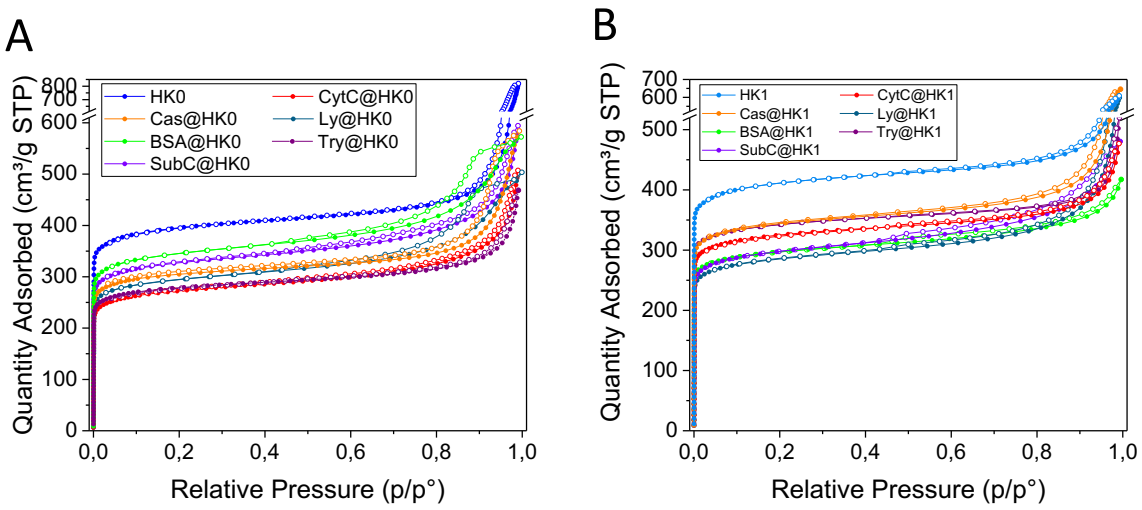


Figure S9: (a) N_2 sorption isotherms of HKo and protein@HKo composites and (b) HK1 and protein@HK1 composites.

Table S4: BET values calculated form the N_2 isotherms of HKo and HK1 samples.

	BET values ($m^2 \cdot g^{-1}$)	
	Sample group	
Protein encapsulated	**HKo**	**HK1**
Ø	1529	1611
Cas	1221	1358
BSA	1351	1174
SubC	1291	1176
CytC	1120	1317
Ly	1194	1153
Try	1115	1323

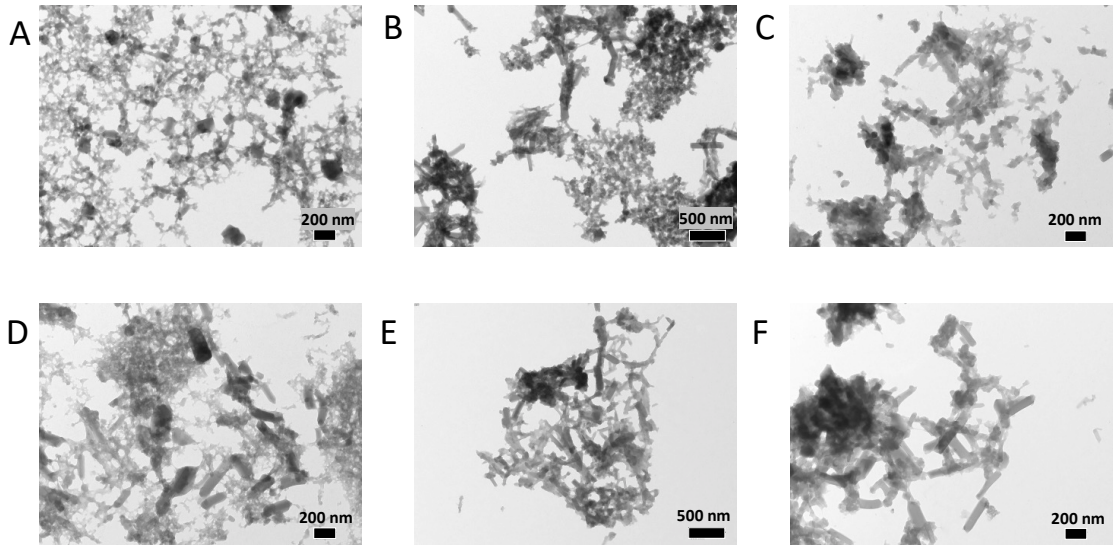


Figure S10: TEM images of (a) Cas@HKo, (b) BSA@HKo, (c) SubC@HKo, (d) CytC@HKo, (e) Try@HKo and (f) Ly@HKo. Scale indicated in images.

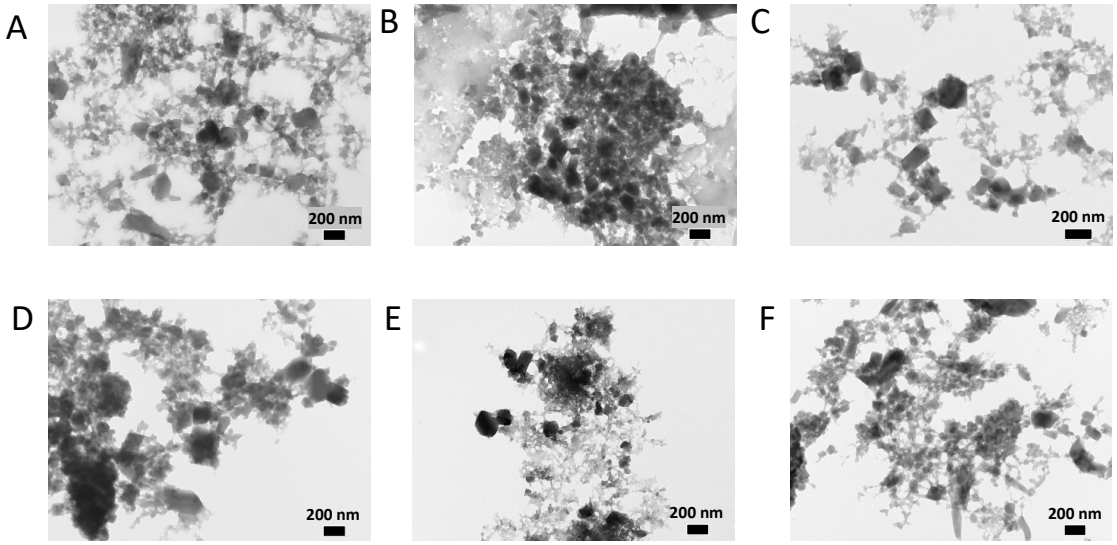


Figure S11: TEM images of (a) Cas@HK1, (b) BSA@HK1, (c) SubC@HK1, (d) CytC@HK1, (e) Try@HK1 and (f) Ly@HK1. Scale indicated in images.

Table S5: Kinetic parameters on the peroxidase activity of CytC in the H1 and HK1 systems.

	Vmax (nmol·s^{-1})	Km (mM)
CytC	324.8	48.5
H1	159.3	49.2
H1 + CytC	337.1	42.4
CytC@H1	94.7	23.9
HK1	169.8	38.1
HK1 + CytC	806.8	28.4
CytC@HK1	837.5	47.8

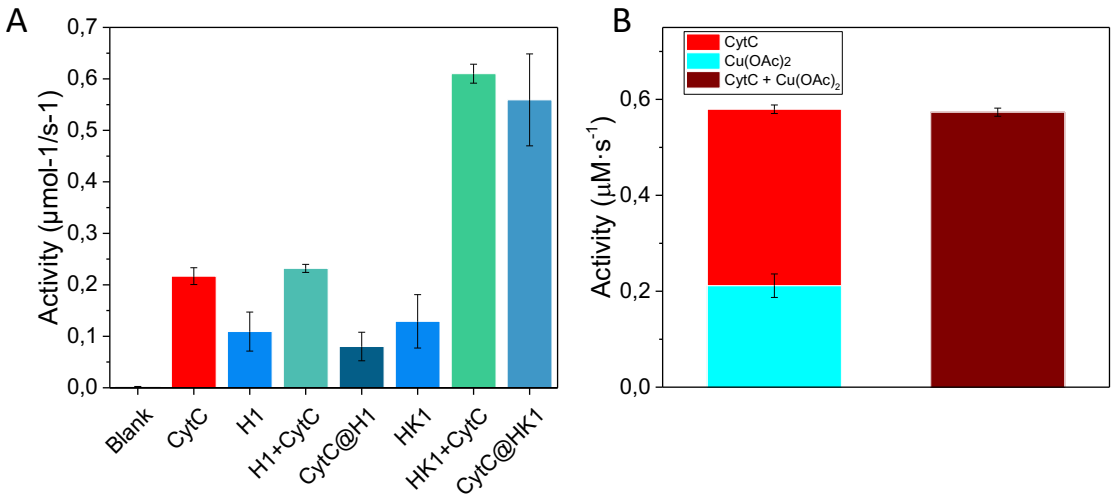


Figure S12: (a) Comparison of the peroxidase activity of the CytC@H1 and CytC@HK1 biocomposites as compared to the free CytC, empty H1 and KH1, and related physical mixtures. (b) Comparison of the sum of the peroxidase activity of free CytC and $Cu(OAc)_2$ with the peroxidase activity of CytC+$Cu(OAc)_2$ physical mixture. Precise amounts of protein and MOFs were added in each case.

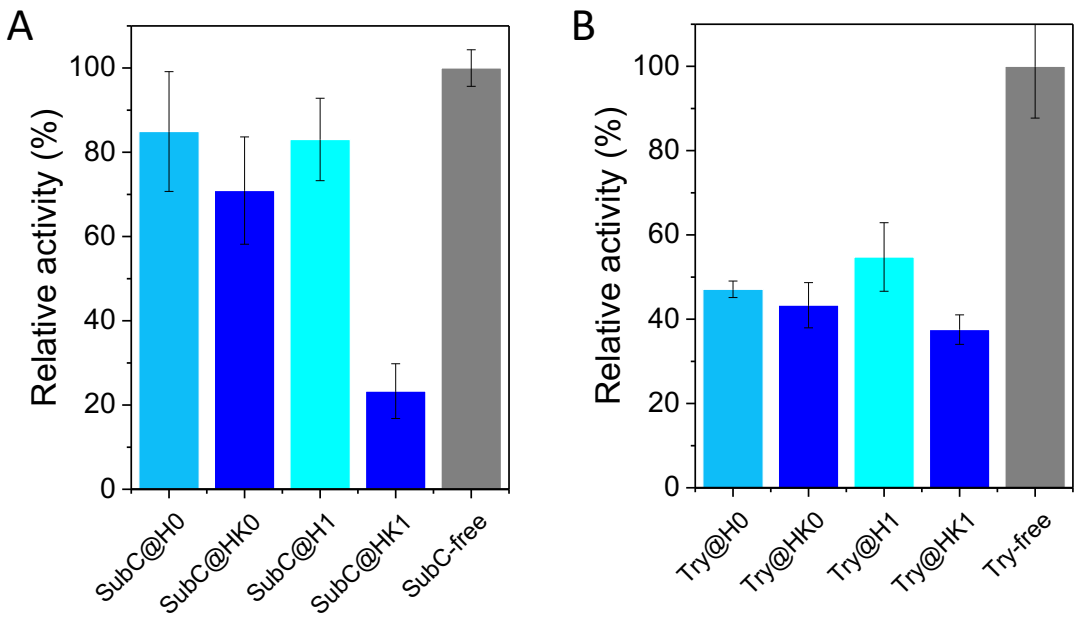


Figure S13: Proteolytic activity retained of (a) SubC and (b) Trypsin encapsulated by different phases after EDTA exposure.

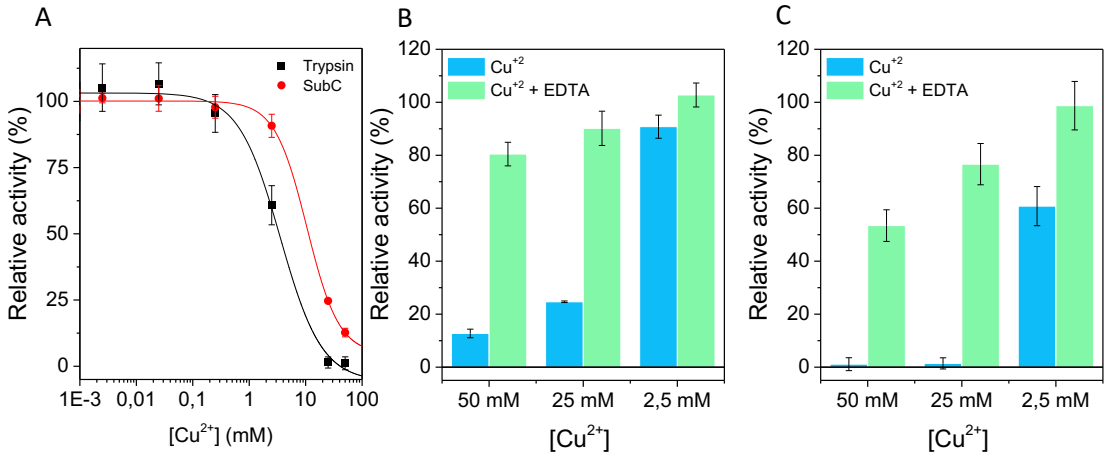


Figure S14: (a) Dose-response curve of SubC and Try against Cu^{2+}. Activity of (b) SubC and (c) Try after exposure to different concentrations of Cu^{2+} and incubation of the same samples with the chelating agent EDTA.

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