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Encapsulation of Microperoxidase-8 into MIL-101(Cr/Fe) Nanoparticles: A New Biocatalyst for the Epoxidation of Styrene

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A new biocatalyst MP8@MIL-101(Cr/Fe) was prepared by immobilization of a heme octapeptide, Microperoxidase 8 (MP8) within a mixed metal MOF, MIL-101(Cr/Fe). Both MIL-101(Cr/Fe) and MP8@MIL-101(Cr/Fe) were characterized by PXRD, FTIR spectroscopy and TGA. The catalytic activity of MP8@MIL-101(Cr/Fe) for the oxidation of styrene by H_2O_2 and tBuOOH was then examined under various reaction conditions (nature of the co-solvent and of the oxidant, concentration of the oxidant and of the substrate, time, pH) and compared to that of MP8 alone. Under the best conditions used, MP8@MIL-101(Cr/Fe) was then shown to catalyze the oxidation of styrene about 3 times more efficiently than MP8 alone with approximately 50% selectivity for styrene oxide.

Introduction

In the quest for greener chemistry to replace the often toxic, waste-producing and polluting processes of the chemical industry, nature is often used as a source of inspiration. Enzymes that derive from natural sources have thus appeared as promising tools for eco-compatible cost-effective processes, as they have evolved to be extremely efficient and selective catalysts that operate under ambient temperature, atmospheric pressure and in aqueous media. However, they suffer from two major drawbacks which can adversely affect their application, namely their relatively low stability under operating conditions and their difficult recovery and recyclability.[1–3] Therefore, in the quest for solutions to face these issues, an exponentially growing field of research has been dedicated to the immobilization of enzymes on solid supports. Supports ranging from traditional inorganic and organic supports (sol-gel glasses, biopolymers, synthetic polymers,)[4–8] to a combination of several materials[9–11] were thus developed to provide recyclable heterogeneous catalysts in which enzymes could be protected from harsh operating conditions (T, pH, solvent).

More recently, new hybrid crystalline porous materials built-up from the assembly of inorganic units and polytopic organic ligands, Metal–organic Frameworks (MOFs), have appeared as a promising class of immobilization matrices. These structures combine the advantages of organic and inorganic supports and provide specific interactions that allow the immobilization of enzymes using several techniques.[12–16] For biocatalytic applications, the entrapment of enzymes by adsorption inside the porosity of preformed MOFs has progressively appeared as the preferred technique.[17–19] Indeed, the high surface area and regular porosity of MOFs ensures an homogeneous and well dispersed immobilization of biomolecules whereas 3D confinement enhances their protection and stabilization. MOF nanoparticles that present an interconnected hierarchical porosity have shown very promising biocatalytic properties.[20,21] Indeed, in such materials, the large pores are prone to encapsulate the enzymes, while the small pores allow the diffusion of substrates and analytes. Finally, pending functional groups in MOF frameworks were shown to influence both the stability and the catalytic activity of encapsulated enzymes.[22,23] MOF-enzyme materials have first been studied for the biocatalysis of model reactions, with typical chromogenic substrates such as 3,5-dit-butyl-catechol (DTBC), p-nitrophenyl butyrate (PNPB), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS). Then, more challenging reactions such as for example, oxygen reduction,[24] nerve agent detoxification[25] and tumor specific prodrug activation[26] have been reported.

MOF properties were particularly illustrated in the work we recently reported on the encaging in nanoparticles of MIL-101(Cr) of Microperoxidase 8 (MP8).[27] This mini-enzyme results from the hydrolytic digestion of horse cytochrome c and contains a heme prosthetic group covalently attached to an octapeptide with amino acid residues 14 to 21 of Cyt c (Scheme 1).[28,29] MP8 possesses both a peroxidase- and a Cytochrome P450-like activity that allows the selective oxidation of organic molecules in water, including organosulfur

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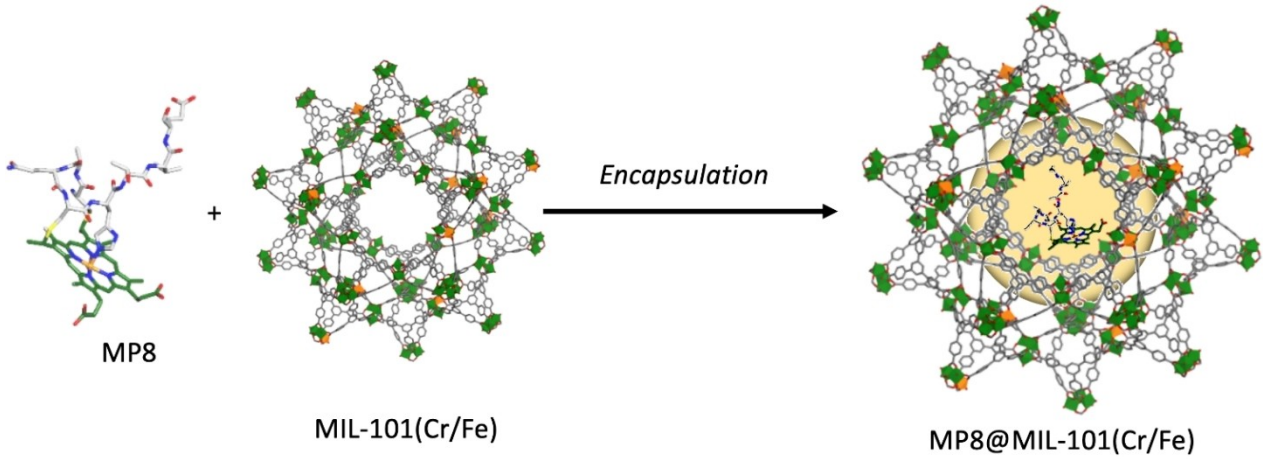

Scheme 1. Molecular structure of MP8, containing an Fe(III)-heme c linked to the amino acid residues 14–21 of Cytochrome c (Structural data were obtained from the structure resolution of PDB·1OCD).[31] Schematic representation of the immobilization process with MIL-101(Cr/Fe).

compounds and alkenes.[28] However, these catalytic activities are highly decreased both at high concentrations of H_2O_2, that cause oxidative destruction of the heme moiety, and under acidic conditions that lead to the protonation of the axial proximal histidine ligand of the iron ion.[29,30] In addition, at high catalyst concentrations, inter-molecular coordination of the N-terminal amino group of the peptide to the iron atom of one molecule as well as π-interactions lead to the formation of low-spin dimers and aggregates.[28] We have recently demonstrated that these drawbacks could be greatly limited by the encapsulation of MP8 into nanoparticles of MIL-101(Cr), a MOF matrix with a high hierarchical interconnected mesoporosity.[27]

The cages of this MOF are compatible with the size of MP8 and part of the porosity remaining is accessible for the diffusion of substrates once the MP8 was immobilized. The obtained MP8@MIL-101(Cr) biocatalyst was reusable and showed long-term stability. It was demonstrated that the MOF matrix could act in synergy with the MP8 and induce a selective oxidation of negatively charged dyes (such as methyl orange) thanks to a charge matching between these dyes and the MOF. It was further demonstrated that nanoparticles of MIL-101(Cr)–NH_2, a MIL-101(Cr) analogue, built up from terephthalate linkers bearing amino groups, could favour the immobilization of higher amounts of MP8, most likely thanks to ionic and H-bond interactions of these amino groups with the four free carboxylic acid groups of MP8.[22] Accordingly, the catalytic activity of MP8@MIL-101(Cr)–NH_2 per material mass was found to be higher than for MP8@MIL-101(Cr) for the selective oxidation of thioanisole derivatives into sulfoxides.

Herein, we investigate the immobilization of MP8 within the mixed metal MIL-101(Cr/Fe). MP8 was immobilised through adsorption within the MOF porosity, following previous studies.[32–34] Moreover, the catalytic activity of MP8 encapsulated in MIL-101(Cr/Fe) was investigated toward a more challenging reaction such as epoxidation of alkenes. This reaction is of high interest as it is a widely used process in the chemical industry, because epoxides are valuable intermediates for laboratory syntheses as well as chemical manufacturing as they can easily be transformed into a large variety of compounds by means of regioselective ring-opening reactions. The two main methods used for obtaining epoxides are homogeneous catalysis, using classically metalloporphyrin catalysts, and biocatalysis, which are in many aspects complementary. Indeed, while enzymes operate under mild conditions with high regioselectivities, synthetic catalysts are more widely applicable and can react with a wider range of substrates but have some disadvantages, such as their possible degradation during the reaction. Concerning biocatalysts, many kinds of peroxidases such as myeloperoxidase (MPO), *Coprinus cinereus* peroxydase (CIP)[35] or chloroperoxidase from *Caldariomyces fumago* (CPO)[36] were described to be able to catalyze the enantioselective epoxidation of styrene by H_2O_2, but with moderate conversions (Resp. 8% for CIP and 40% for CPO).

In the present work, MIL-101(Cr/Fe) was selected as this MOF combines a high porosity suitable for the mass transfer efficiency of reactants and products, the high chemical and thermal stability inherent of MIL-101(Cr) and also coordinatively unsaturated sites (CUS) (Cr^{3+} and Fe^{3+}). We have previously reported that the Lewis acidity property of MIL-101(Cr) can be enhanced by replacing a significant amount of Cr^{3+} by Fe^{3+}.[37] Note that the Lewis acidity of both MIL-101(Cr) and MIL-101(Fe) were previously exploited for the epoxidation of styrene, although these MOFs operate under quite harsh conditions (80 °C, organic solvent…).[38] Here our objective is to design an efficient enzyme/MOF catalyst able to operate under ambient conditions (RT, buffer) as a result of a synergism between the catalytic activity of MP-8 and the Lewis acid property of MIL-101(Cr/Fe).

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Results and Discussion

Synthesis of MP8@MIL-101(Cr/Fe)

To build the material that could be used as catalyst for the epoxidation of styrene, MIL-101(Cr/Fe) was chosen, instead of the previously reported MP8@MIL-101(Cr)-X,[22] for the additional catalytic properties brought by iron.[37] MIL-101(Cr/Fe) was synthetized as described in the experimental section according to a method previously reported by Vallés-García et al.[37] The characterization of MIL-101(Cr/Fe) by combining X-ray powder diffraction (PXRD), Fourier Transform Infrared spectroscopy (FTIR), thermogravimetric analysis (TGA) is fully consistent to that previously reported (Figure 1).[37]

MP8@MIL-101(Cr/Fe) was prepared similarly to the previously reported method by Gkaniatsou et al.[22,27] by simply mixing 1 mL of a 1 mg/mL aqueous solution of MP8 with 1 mL of a 5 mg/mL suspension of MIL-101(Cr/Fe) in ethanol, and the resulting mixture was stirred for 48 h at 37 °C. The samples were washed twice with water, the supernatants were colorless (as confirmed by UV–vis spectroscopy) which indicates that no residual MP8 was present and thus that all of the MP8 was immobilized with the MOF.

Characterization of MP8@MIL-101(Cr/Fe)

MP8@MIL-101(Cr/Fe) was characterized by X-ray powder diffraction (PXRD), Fourier Transform Infrared spectroscopy (FTIR) and thermogravimetric analysis (TGA) and its characteristics were compared to those of the MIL-101(Cr/Fe) MOF (Figure 1).

As indicated by powder X-ray diffraction (PXRD) patterns in Figure 1A, the diffraction peaks for MIL-101(Cr/Fe) are similar to those already reported for MIL-101(Cr/Fe) (4:1).[37] The PXRD pattern of MP8@MIL-101(Cr/Fe) (Figure 1A) is fully consistent with the parental MOF showing that the crystalline structure of MIL-101(Cr/Fe) was preserved after the immobilization of MP8 within its pores. Figure 1B shows the FTIR spectra of free MP8, MIL-101(Cr/Fe) and MP8@MIL-101(Cr/Fe). MIL-101(Cr/Fe) mainly shows bands at 580 cm^{-1} (δ_{Fe-O}) and 772 cm^{-1} (δ_{Cr-O}), characteristic of the iron- and chromium oxide units, and bands characteristic of the terephtalate linkers carboxylate vibrations at 1621 cm^{-1} ($\nu(CO)_{as}$) and 1400 cm^{-1} ($\nu(CO)_s$). The MP8 spectrum mainly shows the characteristic amide I, amide II and amide III vibrations respectively at 1650, 1536 and 1405 cm^{-1}.

The spectrum of the MP8@MIL-101(Cr/Fe) clearly displays the bands at 580, 772 and 1400 cm^{-1} that correspond respectively to the δ_{Fe-O}, δ_{Cr-O} and $\nu(CO)_s$ vibrations already present in the spectrum of MIL-101(Cr/Fe), whereas the characteristic amide I, II and III vibrations of the immobilized MP8 superimpose with the carboxylate $\nu(CO)_{as}$ and $\nu(CO)_s$ vibration bands of the MOF, leading to a broadening of the bands in the 1620–1510 cm^{-1} region for MP8@MIL-101(Cr/Fe). The thermogravimetric analysis of MP8@MIL-101(Cr/Fe) shows a slightly higher decomposition temperature and about 5% higher weight loss when compared to that of MIL-101(Cr/Fe)

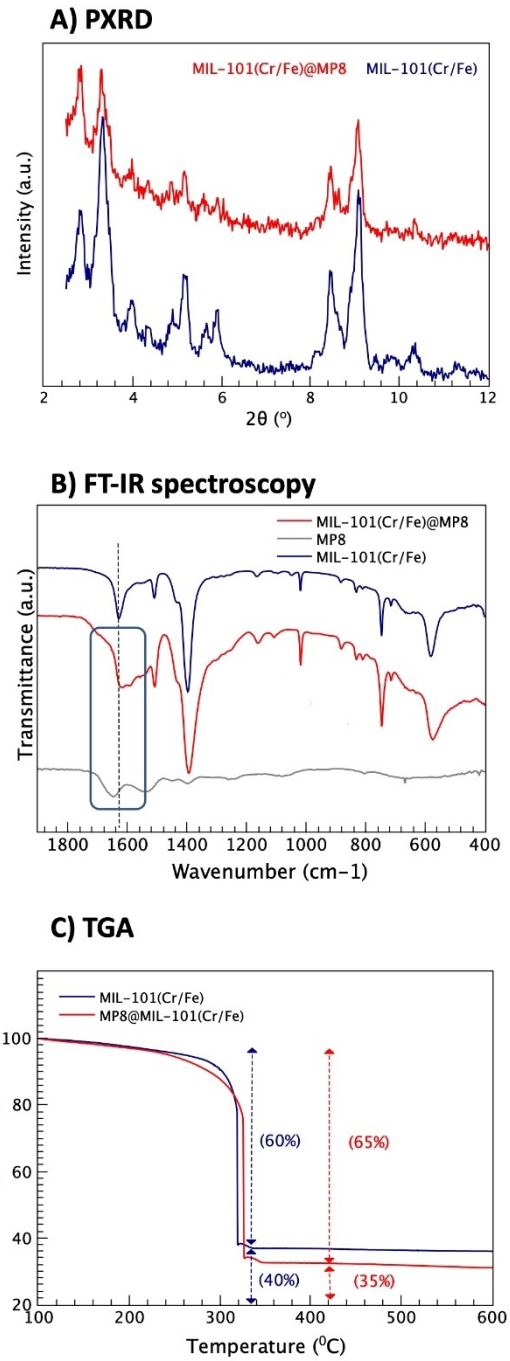

Figure 1. PXRD (A), FTIR (B) and TGA (C) of MIL-101(Cr/Fe) prior and after immobilization of MP8.

(Figure 1C), which is in agreement with the presence of MP8 in the composite.

The morphology of MIL-101(Cr/Fe) and MP8@MIL-101(Cr/Fe) particles were analyzed by SEM and shown in Figure S2. No differences were observed between MIL-101(Cr/Fe) and MP8@MIL-101(Cr/Fe) particles, in agreement with the preservation of MIL-101(Cr/Fe) crystallinity and morphology upon MP8 immobilization and in line with previous studies.

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Evaluation of the catalytic activity of MP8@MIL-101(Cr/Fe)

The catalytic activity of free and immobilized MP8 molecules was first evaluated for the oxidation of styrene in the presence of hydrogen peroxide as oxidant. Initial conditions were inspired from those described by Mashino et al. for the oxidation of styrene by H_2O_2 catalyzed by microperoxidase 11.[39] Thus, the oxidation of 5 mM styrene by 10 mM H_2O_2 was first performed at room temperature in 10 mM HEPES buffer pH 7 containing 30% MeOH(v/v), in the presence of MP8 catalyst (20 μM), as described in the experimental section. After 4 h of reaction, the organic products were then extracted with ethyl acetate and gas chromatography analysis of the organic phase revealed that three products were formed: styrene oxide **1**, phenylacetaldehyde **2** and benzaldehyde **3** (Figure 2).

Thus, the oxidation of styrene by H_2O_2 was performed under the same conditions as those already used with free MP8, but in the presence of 20 μM MP8@MIL-101(Cr/Fe) as catalyst. Under those conditions, the same three products were formed: styrene oxide **1**, phenylacetaldehyde **2** and benzaldehyde **3** (Figure 2). MP8@MIL-101(Cr/Fe) revealed a higher catalytic activity than the free MP8 and could catalyse the oxidation of higher amounts of styrene by respectively a factor of 1.5, showing the protective role of the MOF framework toward the oxidative degradation of MP8. Clearly styrene oxide **1** was the major product, 40 μM being formed with MP8@MIL-101(Cr/Fe) (Figure 2), whereas phenylacetaldehyde **2** was the minor product (7 μM formed with MP8@MIL-101(Cr/Fe)).

Benzaldehyde **3** was also formed as a side product (33 μM). Overall, the MP8@MIL-101(Cr/Fe) appeared to favor the formation of styrene oxide **1**, as shown by **1**/**2** ratio of 5.7 and **1**/**2** + **3** ratio of 1 that were higher than that observed with MP8 as catalyst (**1**/**2** = 1.4, **1**/**2** + **3** = 0.64). In addition, MP8@MIL-101(Cr/Fe) led to a higher yield in oxidation products (80 μM) than MP8 alone (56 μM). The experimental conditions were optimized in order to favor as much as possible the formation of styrene oxide **1**. The variations of several parameters were studied, including reaction time, styrene concentration, nature of organic co-solvent used and finally nature and concentration of the oxidant.

Optimization of reaction conditions

Reaction time: kinetic studies

In order to determine the optimal reaction time, the kinetics of the oxidation of 5 mM styrene by 5 mM H_2O_2 in the presence of 20 μM MP8@MIL-101(Cr/Fe) was examined between 0 and 18 h under the conditions described above. Figure 3 shows the concentration of products obtained as a function of time. It appears that the concentration of styrene oxide **1** increased rapidly until 2 h. of reaction to reach a plateau value of about 40 μM after 4 h. of reaction. In parallel, the concentration of phenylacetaldehyde **2** increased first rapidly until 30 min. and then remained about constant at a 10 μM value, whereas that of benzaldehyde increased rather rapidly during the first 6 h. of reaction and then more slowly to reach 56 μM after 18 h. of reaction.

For sake of comparison, when the same reaction was performed with free MP8 as catalyst, the reaction was finished within 15 min. and the concentrations shown in Figure 2 did not change anymore after longer reaction times. UV–visible analysis of the reaction of MP8 with H_2O_2 under the same reaction conditions showed a rapid disappearance of the characteristic absorption of MP8 at 397 nm that was complete after 15 min. This clearly showed that under those conditions the oxidation of styrene stopped after 15 min. because of the oxidative destruction of the MP8 catalyst by excess H_2O_2.

One of the important effects of the immobilization of MP8 with MIL-101(Cr/Fe) was the protection of MP8 towards

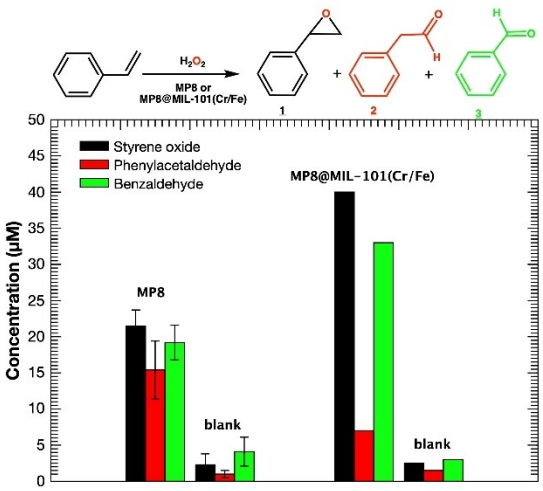

Figure 2. Oxidation of 5 mM styrene by 10 mM H_2O_2 at room temperature in 10 mM HEPES buffer pH 7 containing 30% MeOH(v/v) catalyzed by MP8 or MP8@MIL-101(Cr/Fe) and blank.

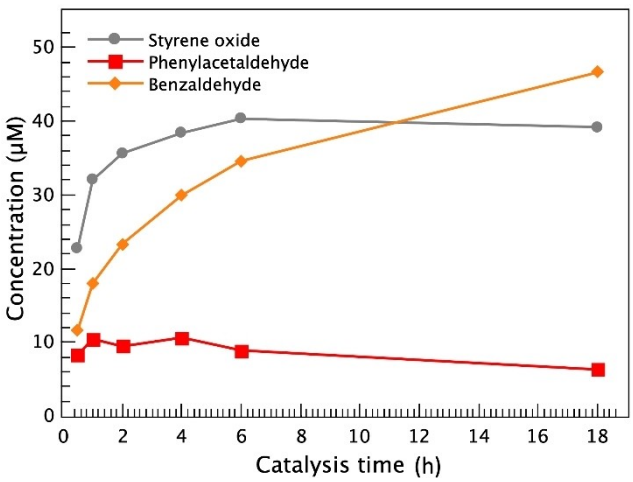

Figure 3. Scheme Caption Kinetics of the oxidation of 5 mM styrene by 5 mM H_2O_2 in the presence of MP8@MIL-101(Cr/Fe) as catalyst in 10 mM HEPES buffer, pH 7, containing 20% v/v MeOH at room temperature: evolution of the concentration of styrene oxide **1**, phenylacetaldehyde **2** and benzaldehyde **3** as a function of time within 18 h. of reaction.

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oxidative degradation by an excess of oxidant that allowed the reaction to proceed for at least 2–4 h. before it stopped, thus leading to an improvement of its yield.

In the following step, the reaction time was fixed at 2 h. since at that time the concentration of styrene oxide **1** obtained was close to its optimal value (36 μM) and the **1**/**2**+**3** ratio was the best (1.1).

Styrene concentration

To determine the optimal concentration of substrate that would lead to the highest concentration of styrene oxide **1** formed, the oxidation of styrene was performed by varying the styrene concentrations from 0.25 to 5 mM. The reaction was performed at RT by using 5 mM H_2O_2 during 2 h, in the presence of either MP8 or MIL-101(Cr/Fe) or MP8@MIL-101(Cr/Fe) as catalyst in 10 mM HEPES buffer (pH 7, containing 20% v/v MeOH). As shown in Figure 4, no styrene oxide was formed in absence of catalyst or in the presence of MIL-101(Cr/Fe). On the contrary, the concentration of styrene oxide obtained after 2 h. of reaction increased with the concentration of styrene to reach respective values of 12±2 μM and 28±1 μM with MP8 alone and MP8@MIL-101(Cr/Fe), which confirmed again that the immobilization of MP8 with MIL-101(Cr/Fe) led to an increase of its catalytic activity.

For further studies the concentration of styrene substrate was thus fixed at 5 mM which corresponds to the optimal amount of styrene oxide.

Nature of organic co-solvent used

The nature of the organic co-solvent used to facilitate the solubilization of the organic substrate into the reaction buffer was also examined. The oxidation of 5 mM styrene by 5 mM H_2O_2 was thus performed at RT in the presence of 20 μM MP8 or MIL-101(Cr/Fe) or MP8@MIL-101(Cr/Fe) as catalyst in 10 mM HEPES buffer (pH 7) containing 20% v/v of co-solvent

Various co-solvents including methanol, isopropanol and 2-methyl-butan-1-ol were used. Under these conditions, methanol clearly appears to be the best co-solvent since, (i) almost no oxidation product is formed both in absence of catalyst and in the presence MIL-101(Cr/Fe) as catalyst, (ii) both the highest total concentration of oxidation products and the highest concentration of styrene oxide are obtained with MP8@MIL-101(Cr/Fe) as catalyst, (iii) the best **1**/**2**+**3** ratio (1.2) is also obtained with MP8@MIL-101(Cr/Fe) as catalyst (Figure 5). With the two other co-solvents, the MP8@MIL-101(Cr/Fe) catalyst was only a slightly better catalyst than MP8 alone as shown both by the ratio of total oxidation products and epoxide (1.2) formed

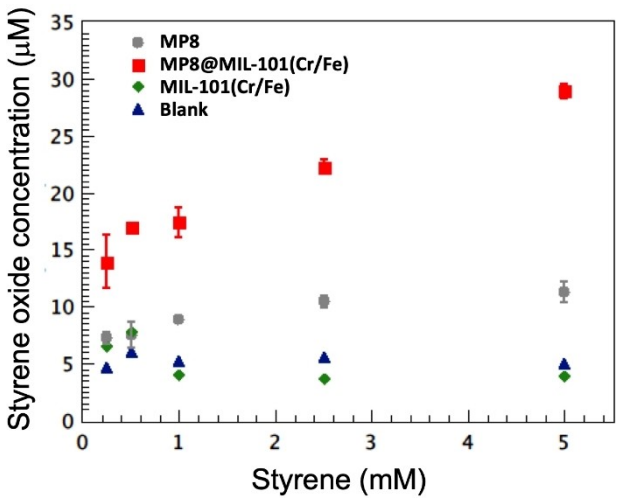

Figure 4. Evolution of the concentration of the styrene oxide formed during the oxidation of increasing concentrations of styrene (0–5 mM) by 5 mM H_2O_2 in the presence of MP8 or MIL-101(Cr/Fe) or MP8@MIL-101(Cr/Fe) as catalyst in 10 mM HEPES buffer, pH 7, containing 20% v/v MeOH at room temperature.

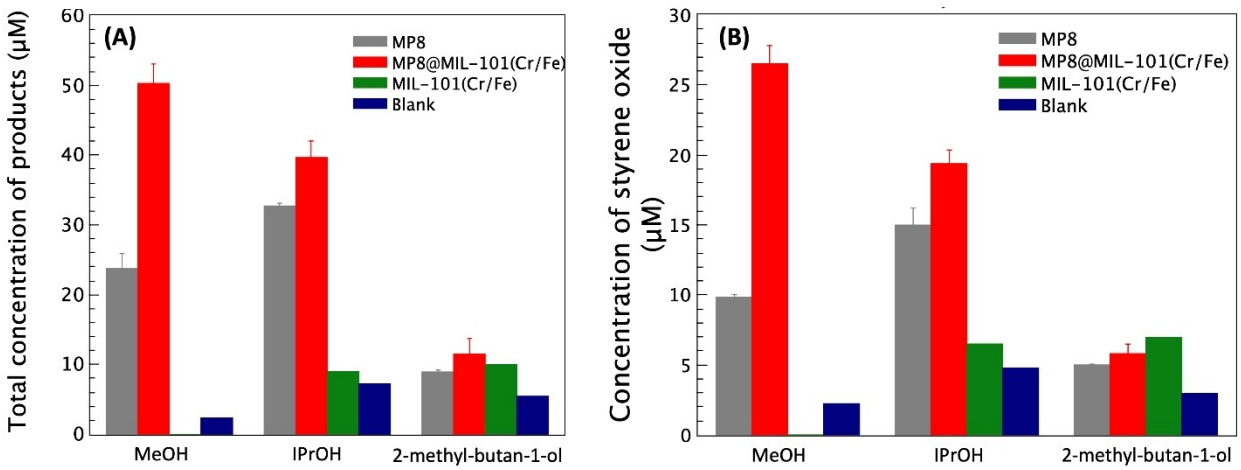

Figure 5. Evolution of the total concentration of products (A) and styrene oxide (B) formed during the oxidation of 5 mM styrene by 5 mM H_2O_2 in the presence of MP8 or MIL-101(Cr/Fe) or MP8@MIL-101(Cr/Fe) as catalyst in 10 mM HEPES buffer, pH 7, containing 20% v/v of methanol, or isopropanol or 2-methyl-butan-1-ol at room temperature.

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respectively formed with both catalysts. Noteworthy, both in isopropanol and 2-methyl-butan-1-ol MIL-101(Cr/Fe) was also found to slightly catalyze the reaction.

Nature and concentration of the oxidant

Two oxidants were tested for the oxidation of styrene catalyzed by MP8 and MP8@MIL-101(Cr/Fe), namely hydrogen peroxide H_2O_2 and ter-Butyl-hydroperoxide tBuOOH. Accordingly, the epoxidation of 5 mM styrene by either H_2O_2 or tBuOOH (at concentrations varying from 1 to 10 mM) was performed in 10 mM HEPES buffer (pH 7) containing 20% v/v of methanol for 2 h at RT in the presence of 20 mM MP8 alone, MIL-101(Cr/Fe) and MP8@MIL-101(Cr/Fe) as catalysts. The obtained results are shown in Figure 6.

With H_2O_2 as oxidant, very low amounts of oxidation product were observed both in the absence of catalyst and in the presence of MIL-101(Cr/Fe) alone as catalyst (4–6 μM) (Figure 6A). On the contrary, both MP8@MIL-101(Cr/Fe) and free MP8 catalyzed the reaction and in both cases the optimal activity was observed for 5 mM H_2O_2. MP8@MIL-101(Cr/Fe) led to both higher concentrations of oxidation products and styrene oxide than free MP8. In addition, a higher percentage of styrene oxide was obtained with MP8@MIL-101(Cr/Fe) than with MP8 alone (Resp. 52 and 43%).

With tBuOOH, low amounts of oxidation product were observed both in the absence of catalyst and in the presence of MIL-101(Cr/Fe) alone as catalyst (6–14 μM) (Figure 6B). As already observed for H_2O_2, MP8@MIL-101(Cr/Fe) and free MP8 also catalyzed the oxidation of styrene by tBuOOH. In the case of MP8@MIL-101(Cr/Fe), the optimal activity was obtained for 5 mM tBuOOH, with a total amount of oxidation products of 80 +8 μM, similar to that obtained with H_2O_2, but with a twice lower relative amount of styrene oxide (25%) than that obtained with H_2O_2 (52%). With free MP8, the total concentration of oxidation products increased linearly with that of MP8 from 0 to 10 mM tBuOOH, but, even for a 10 mM concentration,

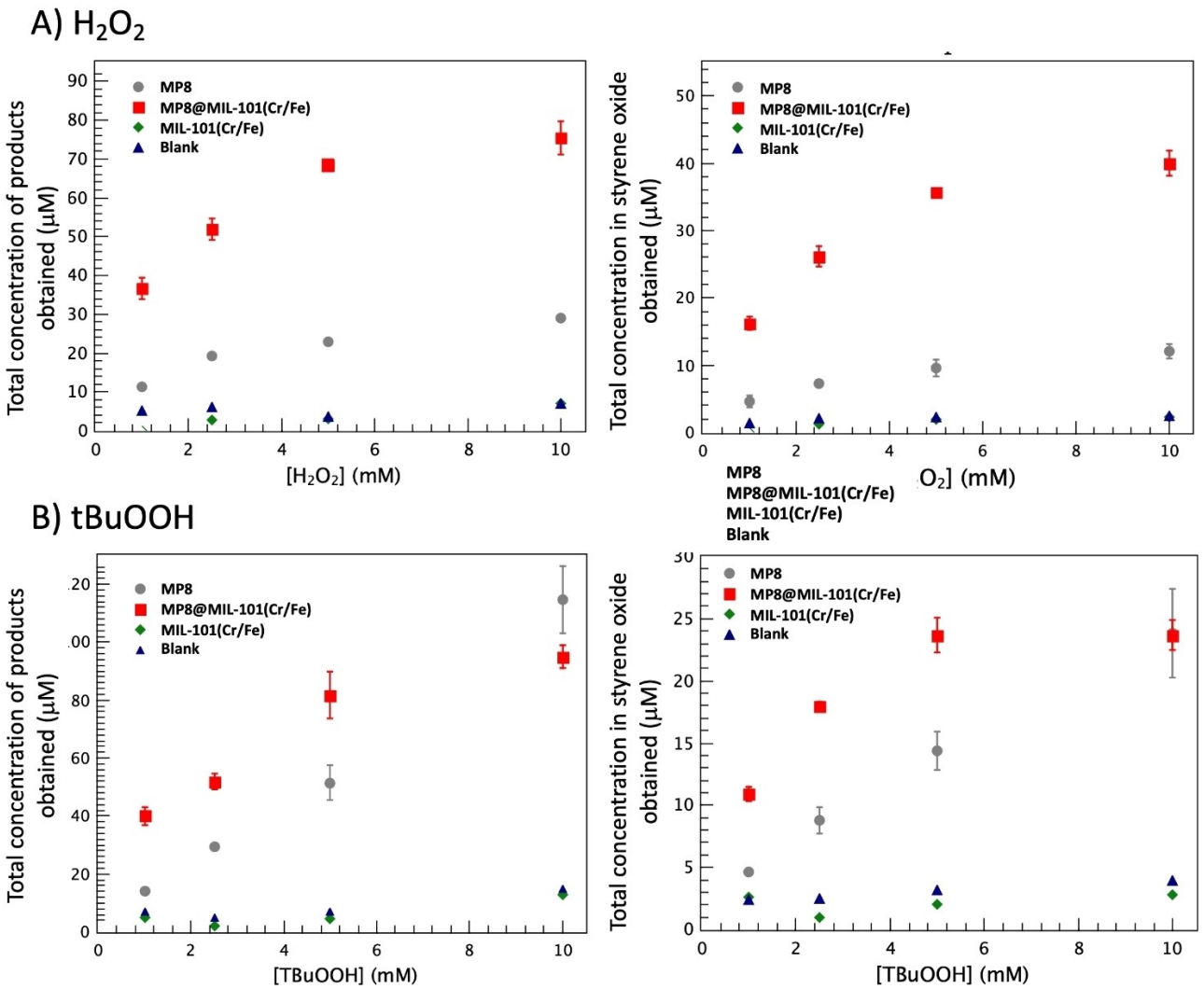

Figure 6. Evolution of the total concentration of products and styrene oxide formed during the oxidation of 5 mM styrene by increasing amounts of H_2O_2 (A) and tBuOOH (B) in the presence of MP8 or MIL-101(Cr/Fe) or MP8@MIL-101(Cr/Fe) as catalyst in 10 mM HEPES buffer, pH 7, containing 20% v/v of methanol.

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both the concentration and relative amount of styrene oxide formed (Resp. 24 μM and about 20%) remained lower than those observed with 5 mM H_2O_2 in the presence of MP8@MIL-101(Cr/Fe) (Resp. 36 μM and about 52%).

Consequently, the highest concentration and relative amount of styrene oxide being obtained with 5 mM H_2O_2 in the presence of 20 mM MP8@MIL-101(Cr/Fe) as catalyst, H_2O_2 appeared as being the best oxidant for the oxidation of styrene into styrene oxide.

Recycling of the catalyst

The biocatalyst was recycled over 3 consecutive catalytic cycles as described in the Supporting Information. As shown in Figure S3, the catalytic activity decreased by a factor 7 after the second cycle and then by a factor 2 after the third cycle, indicating a rather low recyclability of the catalyst. The reasons for such low performance and improving the recyclability of the catalyst are still under investigation. Note that free MP8 cannot be easily separated from the reaction mixture and therefore could not be recycled.

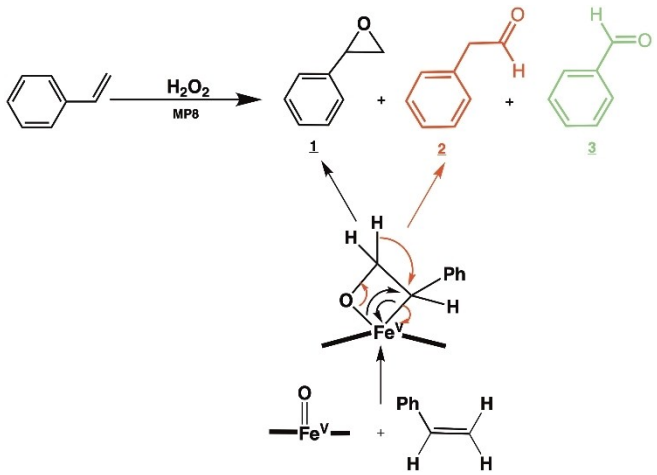

Figure 7. Mechanism of the oxidation of styrene by H_2O_2 catalyzed by hemoproteins[40–42] and iron-porphyrin mimics.[43–45]

Discussion

MP8@MIL-101(Cr/Fe) was prepared and characterized with a combination of characterisation techniques (PXRD, FTIR, TGA), which highlighted the preservation of the crystalline structure of MIL-101(Cr/Fe) and the successful immobilization of MP8.

The catalytic activity of MP8@MIL-101(Cr/Fe) for the oxidation of styrene in aqueous medium was then evaluated and compared to that of free MP8. In all the cases a mixture of three products epoxide **1**, phenylacetaldehyde **2** and benzaldehyde **3** was obtained during this reaction. This is not surprising when considering literature reports concerning the oxidation of styrene by various oxidants catalyzed by hemoproteins[40–42] and model iron porphyrins.[43–45] It has indeed been reported that this reaction passes through a high-valent iron-oxo intermediate species, which after reaction with the double bond can lead either to the epoxide **1** (black arrows in Figure 7) or to phenylacetaldehyde **2** through the so called "NIH Shift" mechanism (Red arrows Figure 7).[40–45]

Obtaining **3** is not surprising either as it is due to the radical oxidative cleavage of the double bond in the presence of O_2 and of an excess of oxidant.[40–45] In agreement with the already reported protective nature of MP8@MIL-101(Cr), MIL-101(Cr/Fe) could efficiently enhance MP8 catalytic activity as, in all the cases, MP8@MIL-101(Cr/Fe) led to higher amounts of oxidation products derived from styrene (**1**, **2**, **3**) and to a better selectivity in favor of epoxide **1**.

Kinetic studies and the optimization of the different parameters of the reaction allowed to determine the best conditions for this reaction. Under those optimal conditions, MP8@MIL-101(Cr/Fe) catalyzed the oxidation of 5 mM styrene by 5 mM H_2O_2 at RT in 10 mM HEPES buffer (pH 7) containing 30% MeOH(v/v) about 3 times more efficiently than MP8 alone with about 50% selectivity for styrene oxide.

Comparison with natural hemes and hemoproteins

This is an interesting result as it was reported that the oxidation of styrene by H_2O_2 catalyzed by microperoxidase (MP-11) led to comparable yields in oxidation products (1-3) but, in this case, the main product obtained was phenylacetaldehyde **2** (**2**/**1** = 2.5), while hemin-Cl did not even catalyze the reaction under the same conditions.[39] When hemoproteins were used as catalysts in the same reaction, different ratios of the three products were obtained as a result of the different nature of their active sites. Chloroperoxidase (CPO)-catalyzed reaction yielded almost the same amount of styrene oxide **1** and phenylacetaldehyde **2**.[42] Besides, the use of hemoglobin or myoglobin as catalysts led to styrene oxide **1** as major product together with the important formation as benzaldehyde **3** product as a second product. Cytochrome P-450 led to styrene oxide **1** as a major product, phenylacetaldehyde **2** being obtained as a minor by-product but using as oxidant either O_2 in the presence of a reductant, NADPH, or iodosobenzene.[42,43]

Comparison with synthetic metalloporphyrins

Finally, synthetic mimics of hemoproteins based on metal complexes of tetraarylporphyrins have been used as efficient catalysts for the oxidation of styrene.[46] In those reactions, styrene oxide **1** as the major product and phenylacetaldehyde **2** as a side product were obtained in high yields and various ratios according to the nature of the porphyrin ligand, but the best oxidants were either organic PhI=O, or inorganic oxidants NaOCl, $KHSO_5$ always in organic solvents.[46] In those systems H_2O_2 was not a very convenient oxidant, as Fenton like reaction, H_2O_2 dismutation and catalase reactions were important

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competitive reactions that limited its use as an oxidant for alkene oxidation, only manganese porphyrins in the presence of imidazole as co-catalyst being an interesting system for the epoxidation of alkenes but in CH_2Cl_2/CH_3CN mixtures as solvent.

Comparison with other materials

The results obtained for the oxidation of styrene by H_2O_2 catalyzed by MP8@MIL-101(Cr/Fe) are compared in table S1 (see Supporting Information) with those reported in the literature with different materials including Fe- and Cr-MIL-101,[38] Alumina,[47] Co–Fe_3O_4,[48] Supported gold nanoparticles,[49] Si/Ti/SiO_2,[50] Titanium silicalite 1.[51] Clearly such a comparison is not easy as the conditions used for MP8@MIL-101(Cr/Fe) are totally different from those used for other materials. Indeed, whereas with our biocatalyst eco-friendly conditions are used, RT, water as solvent, low amounts of reactant (0.005 mmol of H_2O_2 and styrene) and importantly only about 1 mg of catalyst, much harder conditions are used with other materials. The operating temperature is generally between 55 and 107 °C, with a much larger amount of catalyst (between 40 and 400 mg) in an organic solvent (generally acetonitrile) and also with about 1000 times higher amounts of reactants (2.4–40 mmol. H_2O_2 and 1–43.7 mmol. Styrene). Under those conditions, the conversion of styrene is generally higher (6–96.7 %) than that observed with MP8@MIL-101(Cr/Fe) (1.4 %) but noteworthy, the selectivity for the formation of styrene oxide (55 %) in our case is comparable to and even sometimes much higher than those reported in the literature (2.5–86 %).

Conclusion

In the quest for novel eco-compatible processes, a new biomaterial, MP8@MIL-101(Cr/Fe), was synthetized by incorporating a mini enzyme, microperoxidase 8 (MP8), into a protective MOF matrix, MIL-101(Cr/Fe) and was characterized by PXRD, FTIR spectroscopy, SEM, and TGA. This new biomaterial exhibited a high potential as catalyst for the selective oxidation of alkenes to epoxides in water under ambient temperature and pressure, at pH 7, and using as a green oxidant, hydrogen peroxide (H_2O_2), that produces water as a by-product. While higher conversion rates of styrene were already described in the literature with other materials as catalysts, these required harsher conditions involving organic solvents at high temperatures, large quantities of catalyst, oxidant, and substrate. Furthermore, the selectivity of styrene oxide formation with MP8@MIL-101(Cr/Fe) is comparable to and even sometimes much higher than those reported in the literature. Therefore, MP8@MIL-101(Cr/Fe) appears to be a promising alternative catalyst for the epoxidation of alkenes. However, its recyclability needs to be improved. Its catalytic performances compare well with those of already reported microperoxidase 11 and natural hemoproteins, for the oxidation of styrene under biological conditions. Moreover, this paves the way to the catalysis of cascade reactions involving first the oxidation of alkenes into epoxides catalyzed by the MP8 cofactor followed by the reaction of the formed epoxide with for example CO_2[52] or indoles[53] catalyzed thanks to the Lewis Acid properties of the MOF framework.

Experimental Section

Materials and methods: All chemicals were purchased from commercial sources and used without any further purification: Chromium nitrate nonahydrate $Cr(NO_3)_3.9H_2O$ (98.5 %) was from Alfa Aesar, and Iron reduced powder, Tetramethylammonium hydroxide solution (TMAOH, 25 %), Terephtalic acid (98 %), and styrene were all from Sigma Aldrich. Enzymes: Cytochrome c from bovine heart (≥ 95 %), Pepsin and Trypsin were all from Sigma Aldrich.

Synthesis of MOFs: MIL-101(Cr/Fe) was prepared following the method previously reported by Vallés-García et al.[37] Typically, 0.75 mmol of $Cr(NO_3)_3.9\ H_2O$, 0.25 mmol of Fe^0 and 1.5 mmol of terephthalic acid were added in a Teflon reactor, followed by the addition of 10 mL TMAOH (60 mM). The resulting mixture was then heated at 150 °C for 48 h. The resulting solid was recovered by centrifugation (14500 rpm, 20 min) and washed twice with deionized water (35 mL). To remove any iron oxide impurities, density separation was performed and then the solid was washed twice with absolute ethanol (35 mL). The solid was then treated by 35 mL KF aqueous solution (100 mM) under stirring for 1 h. Finally, the solid was washed twice with absolute ethanol and suspended in absolute ethanol. For structural characterizations, the suspension was dried at 100 °C overnight.

Preparation and purification of Microperoxidase 8: The preparation and purification of MP8 was done according to the procedure used by Aron et al.[54] 98 mg of cytochrome c and 4 mg of pepsin were mixed in 5 mL of deionized water. The pH of the solution was adjusted to 2.34 with 1 M HCl before placing it in a water bath at 37 °C. After 1 h, 8 mg of pepsin were added in the media, the pH was adjusted to 2.32 and the mixture was incubated for 4 h. In order to stop the peptic activity of pepsin, the pH was raised to 9.37 by addition of NH_4OH (1 M). At this stage, the main product present in the medium being microperoxidase-11 (MP11), MP8 was subsequently obtained by the digestion of MP11 via the addition of 8 mg of trypsin. After an overnight incubation at 37 °C, MP8 was extracted from the resulting solution by a gel filtration chromatography (biogel P6, 4×100 cm), lyophilized and stored at room temperature. Note that the extinction coefficient $\varepsilon_{396=}1.57\times 10^5\ M^{-1}.cm^{-1}$ was used for the calculation of the enzyme concentration.[54]

Synthesis of MP8@MIL-101(Cr/Fe): MP8@MIL-101(Cr/Fe) was prepared by a similar method as previously reported by Gkaniatsou et al.[22,27] Briefly, 1 mL of a 1 mg/mL aqueous solution of MP8 was mixed with 1 mL of a 5 mg/mL suspension MIL-101(Cr/Fe) in ethanol and kept in an orbital shaking incubator at 37 °C for 48 h. The samples were washed twice with water leading to a colorless supernatant. 1 mL of each supernatant was collected and characterized by UV–visible spectroscopy to determine the residual amount of MP8. The composite was finally suspended in deionized water and stored at 4 °C before further characterizations.

Epoxidation reactions catalyzed by free MP8 and MP8@MIL-101(Cr/Fe): The catalytic activity of free MP8 and immobilized MP8 was assessed towards the epoxidation of styrene in the presence of hydrogen peroxide H_2O_2 or tert-Butyl hydroperoxide (tbuOOH) as oxidant. To optimize the experimental conditions, the variations of several parameters were studied, including the nature and the

concentration of the oxidant, and of the organic co-solvents as well as the styrene concentration.

In a typical experiment, free MP8 or MP8@MIL-101(Cr/Fe) (final MP8 concentration = 20 μM) was mixed with HEPES buffer (pH = 7, 10 mM), 20% v/v organic solvent (MeOH, TFE, IPrOH or Butanol-2) and 5 mM of a styrene in a total volume of 1 mL at room temperature. For free MP8, the solution was homogenized using a vortex mixer followed by the addition of 250 μL of oxidant (H_2O_2 or TbuOOH) and was then incubated at room temperature in an orbital shaker (750 rpm) for 2 h, whereas for MP8@MIL-101(Cr/Fe), the reaction was performed in a closed vial under stirring for 2 h, followed by separation of solid by centrifugation (10000 rpm, 10 min). 100 μL of a benzophenone (10 mM), used as an internal standard, were then added to the solutions. The supernatant was then extracted with 1 mL ethyl acetate and the organic phase was analyzed by gas chromatography: styrene oxide (Retention time, RT = 3.98 min.), benzaldehyde (RT = 2.98 min.) and phenylacetaldehyde (RT = 3.75 min.). All reactions were performed in triplicate and blank reactions were carried out in the absence of catalysts.

Variation of H_2O_2 and tbuOOH concentrations: For the studies at different oxidant concentration, the epoxidation of styrene was performed in the presence of either MP8 alone or MP8@MIL-101(Cr/Fe) under the conditions described above, but with concentrations of H_2O_2 and tbuOOH varying from 1 mM to 10 mM.

Variation of styrene concentration: For these experiments, the conditions were like those described above, except that the oxidant concentration was limited to 2.5 mM and the concentration of styrene varied between 0.25 and 5 mM.

Nature of the organic co-solvent: To study the influence of the nature of the organic co-solvent used to solubilize the styrene substrate, the conditions used were like those described above, except that the oxidant concentration was limited to 2.5 mM in the presence of 20% v/v of either MeOH, TFE, IPrOH or Butanol-2 as co-solvent.

Kinetic studies of the MP8@MIL-101(Cr/Fe)-catalyzed epoxidation of styrene: For the kinetic studies, the oxidation of 5 mM styrene by 5 mM H_2O_2 was performed in 6 separated closed vials containing 1 mL of 10 mM HEPES buffer (pH ^7) in the presence of 20% MeOH as co-solvent and MP8@MIL-101(Cr/Fe) (final MP8 concentration = 20 μM) as catalyst. After reaction times of 30 min, 1 h., 2 h., 4 h., 6 h. and 18 h., the reaction mixture in one vial was analyzed as previously described in the general procedure and the product concentrations were monitored by GC.

Structural characterization: Powder X-ray diffraction patterns were obtained on a Siemens D5000 diffractometer using Cu Kα 1,2 radiation (λ = 1.5406 Å). Infrared spectra were collected with a ThermoScientific Nicolet 6700 FTIR. Thermogravimetric analyses (TGA) were performed on a Mettler Toledo TGA/DSC 1, STAR®System apparatus under O_2 with a heating speed of 4 °C/min. Ultraviolet–visible (UV–vis) spectra were collected on a PerkinElmer LAMBDA 750 UV/Vis/NIR Spectrophotometer. Gas chromatography analyses were performed with a SHIMADZU GC-2014 A, equipped with a Zebron ZB Semi Volatiles column (30 m × 0.25 mm × 0.25 mm).

Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: metal–organic frameworks · enzymes · epoxidation · heme-peptide · oxygen transfer

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