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Metal-Organic Framework Disintegrants: Enzyme Preparation Platforms with Boosted Activity

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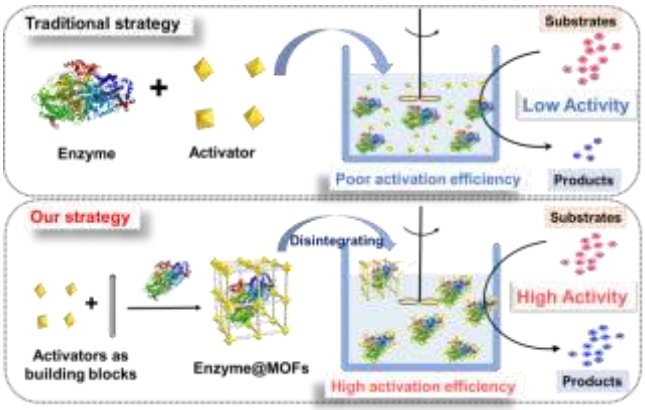
Abstract: High-performance enzyme preparation plays critical roles to realize enzymes' practical applications. We developed a new generation of enzyme formulation platforms using customized enzyme activators (e.g., metal ions) to directly construct metal-organic frameworks (MOFs) as protective carriers of the enzymes. More interestingly, these MOF carriers can also serve as the 'disintegrating agents' that are disintegrable to simultaneously release enzymes and their activators during biocatalysis with boosted activities. For the first time, this highly efficient enzyme preparation platform combines the benefits of enzyme immobilization (enhanced stability, easy operation) and homogeneous biocatalysis (fast diffusion, high activity). Notably, the MOF serves as 'ion pump' that continuously provides metal ion activators who greatly promote the enzymatic activities (up to 251%). Moreover, this MOF-enzyme composite demonstrated an excellent protective effect against various perturbation environments. An in-depth mechanism investigation revealed that the spontaneous activator/enzyme-releasing and ion-pumping effects enable enzymes to sufficiently interact with their activators due to the proximity effects and lead to boost of biocatalytic performance, while directly adding activators into enzyme solution leads to much lower activation efficiency. This study opens up the new avenue for high-performance enzyme preparation, and will significantly promote the broad application of enzymes.

Enzymes with superior activity and unparalleled selectivity are powerful and efficient tools that serve as the "engine" for the development of biomanufacturing and green chemistry.[1,2] However, external perturbations (such as temperature or pH alteration, organic solvents treatment) in their storage and operation process can lead to severe loss of enzymatic activity.[3] Current enzyme preparations usually suffer from deficiencies such as excessive additives, lack of maneuverability or sacrifice of activity, which leads to the instability of quality and complexity in purification that handicaps the industrial application of enzymes.[4-6] Most industrial enzyme preparations only possess a small portion of active enzymes with an excessive amount of additives (e.g., inactive protein, preservatives, salts) to maintain enzyme conformation.[7-9] However, the existence of a large amount of additives can massively influence enzymes' performance.[10] The addition of enzyme activators (e.g. metal ions) can also be ineffective and costly because usually high concentrations of activators are required to take effect due to their low accessibility and weak (or no) interactions with enzymes in biocatalytic systems.[11,12] Moreover, traditional enzyme preparation techniques such as salting out or spray drying often produce enzymes with poor quality. For example, a large amount of ammonium sulfate existing in the salting-out process, as well as high operating temperature of spray drying, can significantly affect enzyme performance.[13] Therefore, the innovation for advanced enzyme preparation is of great significance and urgently desired.

Immobilization of enzymes using solid supports is a popular strategy to stabilize enzymes.[14-16] However, these heterogeneous biocatalytic systems often suffer from the decrease of catalytic efficiency or low enzyme availability due to the diffusion issue caused by the supports. In the past decade, metal-organic frameworks (MOFs) have emerged as a new class of enzyme supports (enzyme@MOFs, @ = encapsulating) which can effectively incorporate and stabilize enzymes,[17-20] due to their adaptable structures, high porosity, tunable pore size, and customizable functionality.[21-24] Interestingly, MOFs can be degradable under mild conditions.[25] Herein, we propose a new enzyme preparation strategy that the protective shell of enzyme (MOFs) can also serve as the 'disintegrating agent' that is disintegrable to release enzymes and their activators when necessary. More interestingly, the MOFs shell can be directly constructed by enzyme activators (e. g. metal ions) that provide a proximity effect for enzymes and their activators. The simultaneously released enzymes and their activators can sufficiently interact and greatly promote the catalytic performance. This multi-functional enzyme formulation platform can combine the benefits of enzyme immobilization (e.g. the enhancement of enzyme stability for storage and operation) and homogeneous biocatalysis (fast diffusion, high activity) for enzyme stabilization and activation. To the best of our knowledge, the development of enzyme-MOF composites as high-performance enzyme formulations has not been reported yet.



Scheme 1. Illumination of MOFs as platforms to construct customized enzyme formulation with boosted activity.

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To demonstrate proof of concept, we developed two new MOF systems that can serve as multifunctional carriers to load and stabilize enzymes. We systematically studied the influence of different metals towards the enzymatic activities after MOF disintegrating, and in-depth investigated the mechanism behind the boosted activity. The promoted biocatalytic activity together with the excellent protection effect, render these enzyme@MOFs systems as a new generation of enzyme preparation platforms (Scheme 1).

A squaric-acid-based MOF ($[M(C_4O_4)(H_2O)_2]_n$, named as **NKMOF-101-M**, NKMOF = Nankai MOF) was developed as the enzyme carrier for enzyme preparation. In the structure of **NKMOF-101-M**, each metal ion is 6-coordinated with six oxygens from four squarate ligands and two water molecules.[26] Each squarate ligand is μ-1,2,3,4-bridging with four metal ions to form a 3-dimensional (3D) **nbo** network. **NKMOF-101-M** possesses many features that well-suited for enzyme formulations: (i) **NKMOF-101-M** can be conveniently prepared in a large scale in aqueous solution at room temperature. The mild synthetic condition could benefit the incorporation of enzymes into the **NKMOF-101-M** platform via an in-situ approach (Figure 1). (ii) **NKMOF-101-M** can be constructed by metal ion activators of enzymes (Zn, Mn, Co, Ni). (iii) **NKMOF-101-M** can be easily disintegrated under mild conditions (e.g., MES buffer, pH = 6.0) to simultaneously release metal ion activators and the incorporated guests. (iv) Variable metal ions (M = Zn, Mn, Co, Ni) can be installed to obtain isostructural **NKMOF-101-M** verified by powder X-ray diffraction (PXRD) (Figure S1) and scanning electron microscope (SEM) data (Figure S2). These features can benefit the systematic study of the influence of metals on enzymatic activity.

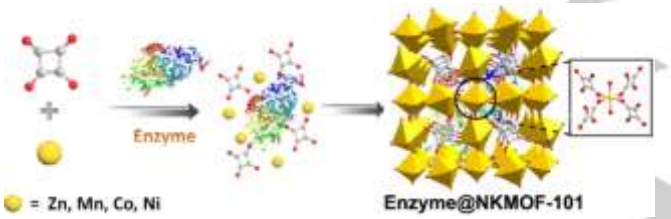

Figure 1. Illustration of enzyme encapsulation process by **NKMOF-101**.

Cytochrome *c* (cyt *c*) is a well-studied model protein that can act as an antioxidative enzyme to remove superoxide (O^{2-}) and hydrogen peroxide (H_2O_2) from mitochondria. And it has been widely used in the pharmaceutical and fine chemical industries.[27] In this study, we selected cyt *c* as a model biomolecule, and directly added them in the synthetic process of **NKMOF-101-M**. PXRD pattern of the harvested **cyt *c*@NKMOF-101-M** composites agree well with those of pristine **NKMOF-101-M** (Figure 2a). SEM images reveal that the as-synthesized **cyt *c*@NKMOF-101-M** composites also display the same morphology as pristine **NKMOF-101-M** (Figure 2c and S3). The loading amount of cyt *c* was determined via a standard curve of UV/Vis through detecting the absorption of cyt *c* at 409 nm (Figure S4). The encapsulation conditions for cyt *c* were optimized based on the loading amount and crystallinity of enzyme@MOFs. Within 10 min, **cyt *c*@NKMOF-101-M** quickly exhibited high encapsulation efficiencies (>90%) and excellent loading capacities (Table S1, 0.346 g/g for **NKMOF-101-Zn**, 0.229 g/g for **NKMOF-101-Mn**, 0.389 g/g for **NKMOF-101-Co**, 0.273 g/g for **NKMOF-101-Ni**).

Various characterization techniques were then used to verify the successful incorporation of cyt *c* in **NKMOF-101-M**. The Fourier transform infrared spectroscopy (FT-IR) data showed a characteristic signal at 1664 cm^{-1} corresponding to the stretching modes of double bonds and carbonyls in cyt *c*, indicative of the successful incorporation of enzymes (Figure 2b). To investigate whether enzymes are adsorbed on the surface of **NKMOF-101-M** or embedded within **NKMOF-101-M**, FITC (fluorescein isothiocyanate)-tagged enzymes and confocal laser scanning microscopy (CLSM) were conducted. CLSM images demonstrated that FITC (fluorescein isothiocyanate)-tagged cyt *c* was mainly embedded in the outer layer of **NKMOF-101-Mn** particles, and embedded in the particle center of **NKMOF-101-Co** and **-Ni**. More interestingly, the 3D CLSM image revealed that FITC-tagged cyt *c* formed a flower-like pattern in **NKMOF-101-Zn**.

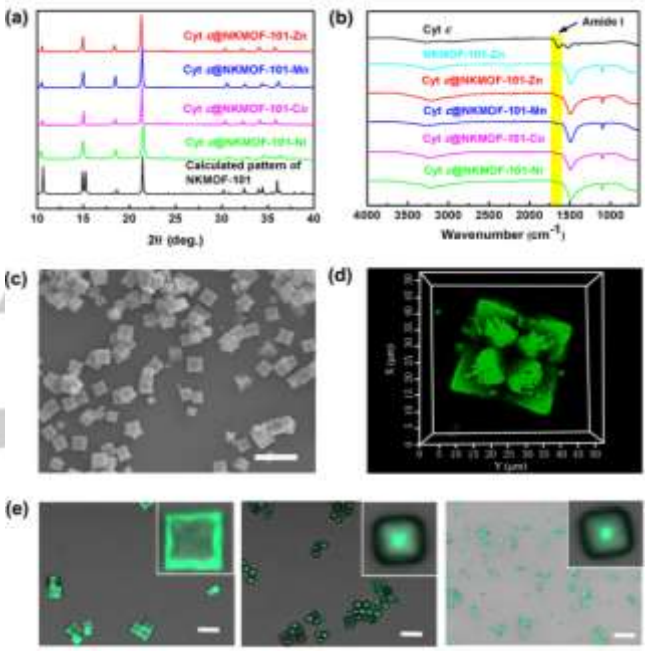

Figure 2. (a) PXRD patterns of **cyt *c*@NKMOF-10-M** composites. (b) FT-IR spectra of **cyt *c* @NKMOF-101-M** composites (amide I mainly from C=O stretching mode). (c) SEM image of **cyt *c*@NKMOF-101-M**. (d) 3D CLSM image of **FITC-cyt *c*@NKMOF-101-Zn**. (e) CLSM image showing the overlay images of **FITC-cyt *c*@NKMOF-101-Mn** (left), **FITC-cyt *c*@NKMOF-101-Co** (middle) and **FITC-cyt *c*@NKMOF-101-Ni** (right). Scale bar 20 μm.

We then evaluated the release efficiency of cyt *c* from **cyt *c*@NKMOF-101-M**, and the results indicated that cyt *c* could be completely released within 30 s from **NKMOF-101-M** under mild conditions (e.g., MES buffer, pH = 6.0) (Figure S5 and Table S2).[28-30] We also calculated and measured the concentration of metal ion and organic linker after the dissolution of MOFs. And low concentrations of metal ion (0.67 μg/mL of Zn^{2+}) and ligand (1.15 μg/mL of squaric acid) were observed, indicating trace amounts of impurities introduced. On the contrary, the well-studied MOF supports, **ZIF-8** and **ZIF-90**, cannot disintegrate under this mild condition. These features, together with the high loading capacity, make **NKMOF-101-M** a highly efficient enzyme preparation platform. To evaluate the catalytic performance, **cyt *c*@NKMOF-101-M** composites were directly added into the reaction system, where **cyt *c*@NKMOF-101-M** can disintegrate to release cyt *c* and metal ions. The catalytic activity was determined using 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonate) (ABTS) (1 mM) and H_2O_2 (10 mM) as the substrates in MES buffer (50 mM, pH 6.0). As shown in Figure 3a, the activities of released cyt *c* were boosted to be 209%, 119%, 152% and 204% for **NKMOF-101-Zn**, **-Mn**, **-Co**, and **-Ni**, respectively

(Figure 3a and 4a), compared with the activity of free enzyme. By contrast, directly adding an equivalent amount of metal ions (e.g., Zn^{2+}), squaric acid, or pure **NKMOF-101-Zn** did not show any activity enhancement compared with free cyt *c*. Pure **NKMOF-101-Zn**, $Zn^{2+,}$ and squarate ligand were found to be inactive towards ABTS (Figure 3b). We also tried to absorb enzymes by **NKMOF-101** directly and observed a negligible amount of enzymes were absorbed (Figure S6) due to the absence of porosity in NKMOFs, which was confirmed by N_2 sorption test (Figure S6). These results further highlighted the advantage of this MOF encapsulating strategy. Further investigation revealed that when metal ions are directly added into the catalytic reaction, a very high concentration of metal ions is required to achieve the same promotion effect as **cyt *c*@NKMOF-101-Zn** composite (Figure S7). However, a high concentration of metal ions will raise the production cost and cause severe pollution issues. These results unveiled that the released metal activators from the MOF matrix dramatically facilitated the promotion of enzymatic activities (Scheme 1), while traditional strategy via directly adding activators lead to nearly no activation.

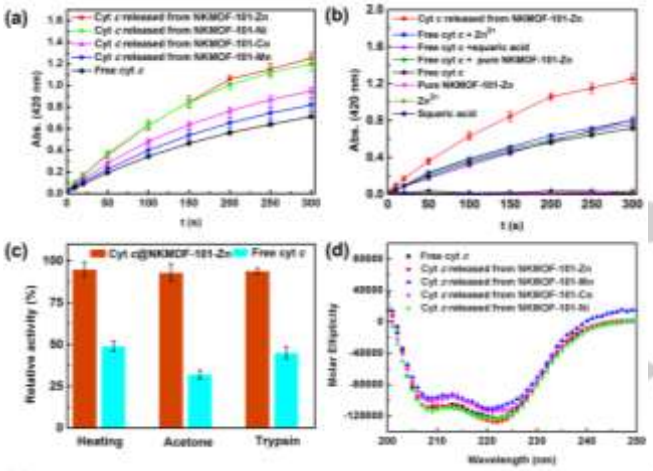

Figure 3. (a) Catalytic curves of free cyt *c* and cyt *c* released from **NKMOF-101**. Test condition: 1 mM ABTS and 10 mM H_2O_2. (b) Catalytic curves of cyt *c* with different treatments. (c) Protective properties of **NKMOF-101-Zn** on the activity of cyt *c* treated with high temperature (80 °C), organic solvent and trypsin (1 mg/mL) for 1 h. (d) CD spectra of free cyt *c* and cyt *c* released from **NKMOF-101-M**.

Maintaining enzymes' activity during the storage and operation process is crucial for enzymes' application. Embracement of enzymes within the confined space created by MOFs can provide an excellent protective effect towards enzymes.[31] Thus, **cyt *c*@NKMOF-101-Zn** with the best catalytic performance was chosen as a representative to investigate the protective effect of **NKMOF-101-M**. As shown in Figure 3c, free cyt *c* lost 51%, 68%, and 55% of its activity after being treated by heating, organic solvent, and trypsin, respectively. By contrast, **cyt *c*@NKMOF-101-Zn** almost entirely maintained the activity after these treatments. Overall, **NKMOF-101-M** can efficiently protect cyt *c* against perturbation conditions without losing their crystallinity (Figure S8). The secondary structure of cyt *c* was also evaluated using circular dichroism (CD) spectra to confirm the protective effect of MOFs. The released cyt *c* was harvested via ultrafiltration with 10 kDa MWCO (molecular weight cut off) devices to remove undesirable impurities (e.g., digested ligands, metal salts). Notably, both free cyt *c* and cyt *c* released from **NKMOF-101-M** indicated a typical α-helix structure.[32] Meanwhile, no difference between released cyt *c* and free cyt *c* was observed, which showed that the in-situ encapsulation process did not affect the conformation of cyt *c* (Figure 3d). These results illustrated that this new enzyme formulation strategy could favorably maintain the original conformation of enzymes and meanwhile provide outstanding protection to enzymes.

In order to evaluate the generality of this enzyme formulation platform, we explored if this strategy can be applied to other MOFs and enzymes. A slight modification of the synthetic condition of **NKMOF-101-M** (i.e. replacing ethanol/water solution with the pure aqueous solution) yielded **NKMOF-102-M** ($[M(C_4O_4)(H_2O)_4]_n$) with a one-dimensional (1D) chain structure (Figure S9). In the structure of **NKMOF-102-M**, each metal ion is 6-coordinated with two oxygens from squarate ligands and four water oxygens. Each squarate ligand is μ-1,3-bridging two metal ions to form 1D chains.[33] Incorporating cyt *c* via the in-situ approach generated needle-like microcrystals of **cyt *c*@NKMOF-102-M** (Figure S10 and S11). PXRD patterns of **cyt *c*@NKMOF-102-M** composites agree well with those of pristine **NKMOF-102-M** (Figure S12 and S13). The loading capacities of cyt *c* were 0.098 g/g, 0.045 g/g, 0.124 g/g, and 0.157 g/g for **NKMOF-102-Zn**, **-Mn**, **-Co** and **-Ni**, respectively (Table S3). CLSM data confirmed the successful incorporation of FITC-labelled cyt *c* in **NKMOF-101-M** (Figure S14). As shown in Figure 4a and Figure S15, the activities of released cyt *c* were boosted up to 251%, 133%, 173% and 171% for **NKMOF-102-Zn**, **-Mn**, **-Co**, and **-Ni**, respectively, compared with the activity of free enzyme. In addition, CD spectra revealed that the released cyt *c* from **NKMOF-102-M** maintained the secondary structure of cyt *c* (Figure S16).

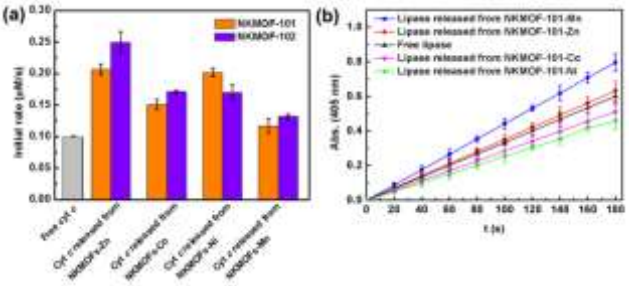

Figure 4. (a) The initial rate of free cyt *c* and cyt *c* released from **NKMOF-101-M** and **NKMOF-102-M**. (b) Catalytic curves of free lipase and lipase released from **NKMOF-101-M**. Test condition: 1 mM *p*-NPA.

We also chose an important industrial enzyme, lipase, as a representative further to evaluate the generality of this enzyme preparation strategy, because lipase is widely used in various fields, including food and cosmetic industries.[34,35] As revealed by PXRD, FT-IR, SEM, and CLSM data (Figure S17-S20), lipase can be efficiently encapsulated into **NKMOF-101-M** to yield **lipase@NKMOF-101-M** composites. The loading amount of lipase was determined via a standard Bradford assay method (Figure S21). **NKMOF-101-M** (M = Zn, Mn, Co, Ni) can quickly encapsulate lipase within 10 min and possess high loading capacities (0.122 g/g, 0.097 g/g, 0.115 g/g and 0.168 g/g for **NKMOF-101-Zn**, **-Mn**, **-Co** and **-Ni**, respectively) (Table S4). The catalytic activity of **lipase@NKMOF-101-M** was determined by an assay using 4-nitrophenyl acetate (*p*-NPA) (1 mM) as the substrate in HEPES buffer (50 mM, pH = 6.5). As shown in Figure 4b, the activity of lipase released from **lipase@NKMOF-101-Mn** and **lipase@NKMOF-101-Zn** were boosted to be 133% and 110%, respectively, compared to that of the free lipase. On the contrary, **lipase@NKMOF-102-Co** and **lipase@NKMOF-**

102-Ni showed 15% and 24% decrease in activity, respectively. These results demonstrate that different metal ions showed different impacts on the activity of enzymes. Thus, NKMOFs can act as customizable platforms for different enzymes via tuning the metal species to achieve the best catalytic performance. In addition, CD spectra revealed that the released lipase from **NKMOF-101-M** also maintained the secondary structure of lipase (Figure S22).[36] This enzyme preparation strategy has, therefore, proven to be a facile and versatile platform to fabricate enzyme preparations with enhanced stability and boosted activity for biocatalytic applications.

In order to unveil the mechanism behind the dramatic increase of enzymatic activity, **cyt *c*@NKMOF-101-Zn** was selected as a representative subject for further investigation. After collecting the released cyt *c* from **cyt *c*@NKMOF-101-Zn** via ultrafiltration, we first washed with a large amount of water to remove the excess free Zn, and then used energy dispersive X-ray spectroscopy (EDX) to characterize the recovered cyt *c*. EDX data clearly showed Zn existed in the recovered cyt *c*, indicating the binding of Zn^{2+} with cyt *c* (Figure S23). Moreover, we found those Zn residues can be gradually removed from the recovered cyt *c* via multiple washing for a long time, indicative of weak interaction between Zn^{2+} and cyt *c*. This result further proved that the boosted enzymatic activity is originated from the sufficient interaction between metal activators and enzymes during the disintegrating of enzyme@MOFs. We also used inductively coupled plasma-optical emission spectrometry (ICP-OES) to track the MOF dissolution process, and the observed metal releasing kinetics revealed that NKMOFs could be fully dissolved within ~30 s. This result implied the process of disintegrating MOF scaffold probably created a transient high local concentration of metal ions around the enzymes, which facilitated the association of metal activators towards the enzymes for enhanced catalytic performance. We also tracked enzymatic kinetics of cyt *c* in **NKMOF-101-Zn** using Michaelis-Menten model and compared it with its free counterparts. The results revealed that **cyt *c*@NKMOF-101-Zn** showed a K_m of 0.16 mM and a k_{cat} of 3.65 s^{-1}, while free cyt *c* possesses K_m and k_{cat} of 0.27 mM and 1.44 s^{-1}, respectively (Table 1). The decreased K_m compared with free enzyme implies that cyt *c* in **NKMOF-101-Zn** has a higher affinity towards substrates. The increased substrate affinity and k_{cat} can probably be attributed to the activation effect of Zn^{2+} from **NKMOF-101-Zn** system, which is consistent with literature results[37,38] and our observation. On the basis of these results, we proposed that the boosted activity of enzymes in NKMOFs can be attributed to the activators (metal ions) from the matrix that can weakly interact and promote the affinity of enzymes and their substrate. Literature reports have also revealed that metal ions can boost the activity of enzymes through coordinating to the active-site residues.[39-40] This promotion effect together with the advantages of the enzyme immobilization systems (e.g., proximity effect)[38] contribute to the outstanding biocatalytic performance of enzyme@NKMOF platforms.

Table 1. Comparison of kinetic parameters for free cyt *c* and recovered cyt *c* from **NKMOF-101-Zn**.

Catalysts	K_m (mM)	k_{cat} (s^{-1})	k_{cat} / K_m ($s^{-1}mM^{-1}$)
Cyt *c*	0.27	1.44	5.33
Recovered Cyt *c* from **NKMOF-101-Zn**	0.16	3.65	22.82

In conclusion, we have successfully created a new enzyme preparation platform that can in-situ assemble enzymes and their activators (e.g. metal ions) into one system (i.e. MOFs). The formed enzyme@MOFs composites can directly perform as 'disintegrating agents' to spontaneously release enzymes and their activators to boost the catalytic reaction. To demonstrate the proof of concept, we developed two new MOF platforms (**NKMOF-101-M** and **NKMOF-102-M**) based on a squarate ligand and various metal activators (e.g., M = Zn, Mn, Co, Ni). Our results demonstrated this novel preparation platform can favorably maintain the original conformation of enzymes and provide outstanding protection towards enzymes. More importantly, we found that these enzyme@NKMOFs platforms are easily disintegrated under mild condition (e.g. in buffer solution at room temperature) to simultaneously release enzymes and their activators, thereafter the enzymatic activity was dramatically boosted up to 251% compared with free enzymes. Moreover, this novel platform can be fine-tailored for different enzymes via tuning the metal species to achieve the optimal catalytic performance. An in-depth mechanism investigation revealed that enzyme@NKMOFs served as activator ion pumps during the disintegrating process of MOF shells, which can continuously supply metal ion activators to sufficiently interact with enzymes and promote the affinity of enzymes and their substrate due to the proximity effect. Ongoing work in our group will focus on promoting this green and inexpensive enzyme preparation for practical applications. This study will broaden the application scopes of MOFs and opens up the new avenue for high-performance enzyme preparation.

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Keywords: metal-organic frameworks • enzyme preparation • disintegrants • boosted activity • enzyme immobilization

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Research Articles

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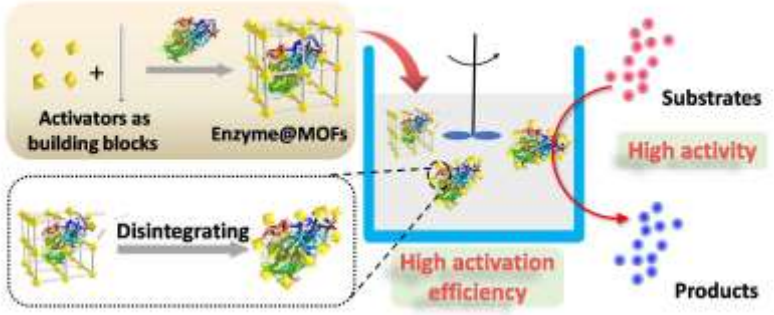
Hongde An, Jie Song, Ting Wang, Nannan Xiao, Zhenjie Zhang, Peng Cheng, Shengqian Ma, He Huang, Yao Chen*.

Metal-Organic Framework Disintegrants: Enzyme Preparation Platforms with Boosted Activity



We have created a new generation of enzyme formulation using MOFs as carriers for boosted enzymatic activity and enhanced stability.