Metal–Organic Frameworks

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

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Abstract: *An enzyme formulation using customized enzyme activators (metal ions) to directly construct metal–organic frameworks (MOFs) as enzyme protective carriers is presented. These MOF carriers can also serve as the disintegrating agents to simultaneously release enzymes and their activators during biocatalysis with boosted activities. This highly efficient enzyme preparation combines enzyme immobilization (enhanced stability, easy operation) and homogeneous biocatalysis (fast diffusion, high activity). The MOF serves as an ion pump that continuously provides metal ion activators that greatly promote the enzymatic activities (up to 251 %). This MOF–enzyme composite demonstrated an excellent protective effect against various perturbation environments. A mechanistic investigation revealed that the spontaneous activator/enzyme release and ion pumping enable enzymes to sufficiently interact with their activators owing to the proximity effects, leading to a boost in biocatalytic performance.*

Introduction

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 solvent 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 (for example, inactive protein, preservatives, salts) to maintain enzyme conformation.[7–9] However, the existence of a large amount of additives can massively influence enzyme performance.[10] The addition of enzyme activators (such as metal ions) can also be ineffective and costly because usually high concentrations of activators are required to take effect owing 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] owing 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 (such as 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 (for example, 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

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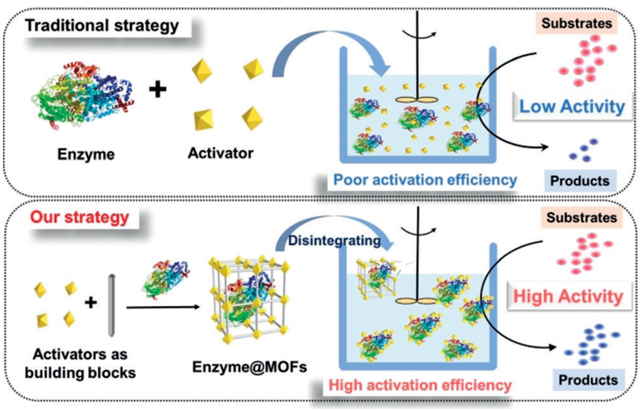
high-performance enzyme formulations has not yet been reported.

Results and Discussion

To demonstrate the 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 investigated the mechanism behind the boosted activity in depth. The promoted biocatalytic activity together with the excellent protection effect render these enzyme@MOFs systems 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 oxygen atoms 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: 1) NKMOF-101-M can be conveniently prepared in a large scale in aqueous solution at room temperature, and the mild synthetic conditions could benefit the incorporation of enzymes into the NKMOF-101-M platform via an in situ approach (Figure 1); 2) NKMOF-101-M can be constructed by metal ion activators of enzymes (Zn, Mn, Co, Ni); 3) NKMOF-101-M can be easily disintegrated under mild conditions (for example, MES buffer, pH 6.0) to simultaneously release metal ion activators and the incorporated guests; 4) various metal ions (M = Zn, Mn, Co, Ni) can be installed to obtain isostructural NKMOF-101-M verified by powder X-ray diffraction (PXRD) (Supporting Information, Figure S1) and scanning electron microscope (SEM) data (Supporting Information, Figure S2). These features can benefit the systematic study of the influence of metals on enzymatic activity.

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; Supporting Information, Figure 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 (Supporting Information, 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 (Supporting Information, Table S1, 0.346 $g\,g^{-1}$ for NKMOF-101-



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

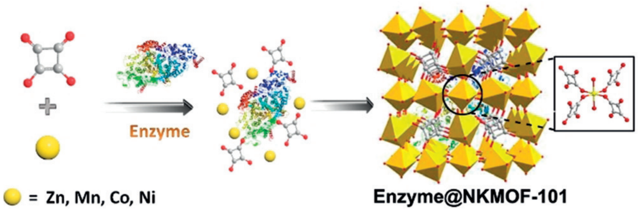


Figure 1. Illustration of the enzyme encapsulation process by NKMOF-101.

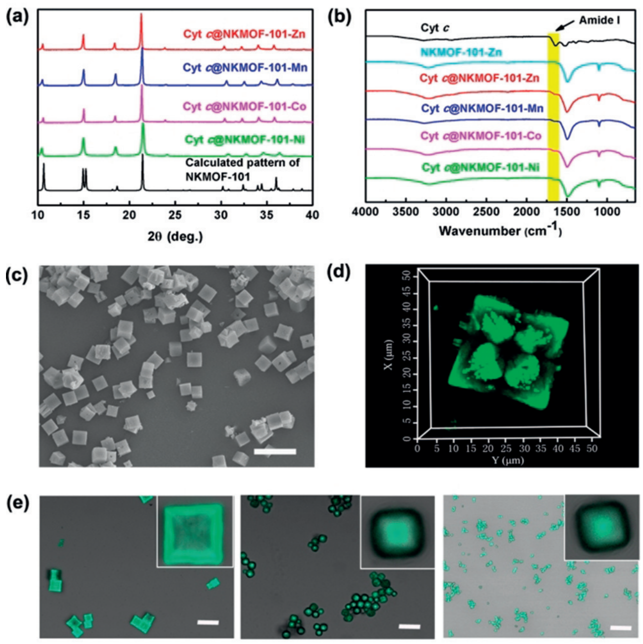


Figure 2. a) PXRD patterns of cyt *c*@NKMOF-10-M composites. b) FTIR spectra of cyt *c* @NKMOF-101-M composites (amide I mainly from C=O stretching mode). c) SEM image of cyt *c*@NKMOF-101-M. d) 3D CLSM image of FITC-cyt *c*@NKMOF-101-Zn. e) CLSM image showing the overlay images of FITC-cyt *c*@NKMOF-101-Mn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

Zn, 0.229 $g\,g^{-1}$ for NKMOF-101-Mn, 0.389 $g\,g^{-1}$ for NKMOF-101-Co, and 0.273 $g\,g^{-1}$ 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 (FTIR) 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.

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 (for example, MES buffer, pH 6.0; Supporting Information, 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. Low concentrations of metal ion (0.67 $\mu g\,mL^{-1}$ of Zn^{2+}) and ligand (1.15 $\mu g\,mL^{-1}$ 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 (Figures 3a and 4a), compared with the activity of free enzyme. In contrast, directly adding an equivalent amount of metal ions (such as 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 (Supporting Information, Figure S6) owing to the absence of porosity in NKMOFs, which was confirmed by N_2 sorption test (Supporting Information, 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 (Supporting Information, Figure S7). However, a high concentration of metal ions will raise the production cost and cause severe pollution issues.

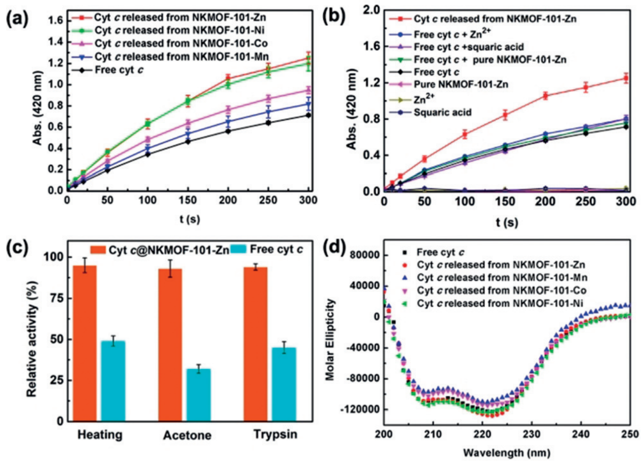


Figure 3. a) Catalytic curves of free cyt *c* and cyt *c* released from NKMOF-101. Test conditions: 1 mM ABTS and 10 mM H_2O_2. b) Catalytic curves of cyt *c* with different treatments. c) Protective properties of NKMOF-101-Zn on the activity of cyt *c* treated with high temperature (80 °C), organic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

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.

Maintaining enzyme activity during the storage and operation process is crucial for the application of enzymes. 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 (Supporting Information, 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 (for example, 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.

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 (that is, 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 (Supporting Information, 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 (Supporting Information, Figures S10 and S11). PXRD patterns of cyt *c*@NKMOF-102-M composites agree well with those of pristine NKMOF-102-M (Supporting Information, Figures S12 and S13). The loading capacities of cyt *c* were 0.098 $g\,g^{-1}$, 0.045 $g\,g^{-1}$, 0.124 $g\,g^{-1}$, and 0.157 $g\,g^{-1}$ for NKMOF-102-Zn, -Mn, -Co and -Ni, respectively (Supporting Information, Table S3). CLSM data confirmed the successful incorporation of FITC-labeled cyt *c* in NKMOF-102-M (Supporting Information, 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. Furthermore, CD spectra revealed that the released cyt *c* from NKMOF-102-M maintained the secondary structure of cyt *c* (Supporting Information, Figure S16).

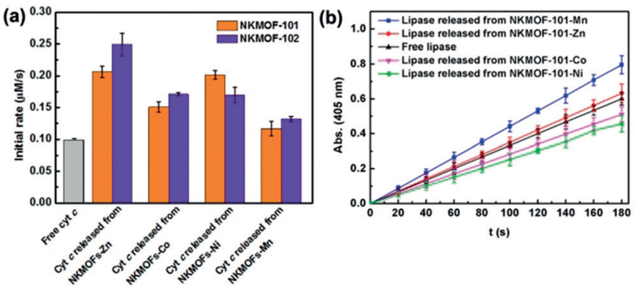


Figure 4. a) The initial rate of free cyt *c* and cyt *c* released from NKMOF-101-M and NKMOF-102-M. b) Catalytic curves of free lipase and lipase released from NKMOF-101-M. Test conditions: 1 mM *p*-NPA.

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, FTIR, SEM, and CLSM data (Supporting Information, Figures 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 (Supporting Information, Figure S21). NKMOF-101-M (M = Zn, Mn, Co, Ni) can quickly encapsulate lipase within 10 min and possess high loading capacities (0.122 $g\,g^{-1}$, 0.097 $g\,g^{-1}$, 0.115 $g\,g^{-1}$ and 0.168 $g\,g^{-1}$ for NKMOF-101-Zn, -Mn, -Co, and -Ni, respectively; Supporting Information, 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-101-Co and lipase@NKMOF-101-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. Furthermore, CD spectra revealed that the released lipase from NKMOF-101-M also maintained the secondary structure of lipase (Supporting Information, 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.

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* (Supporting Information, 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 confirmed 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 about 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 previous results[37,38] and our observation.

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

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 (such as the proximity effect)[38] contribute to the outstanding biocatalytic performance of enzyme@NKMOF platforms.

Conclusion

We have successfully created a new enzyme preparation platform that can assemble enzymes and their activators (metal ions) in situ into one system (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 (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 (such as 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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Conflict of interest

The authors declare no conflict of interest.

Keywords: boosted activity · disintegrants · enzyme immobilization · enzyme preparation · metal–organic frameworks

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