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Fabricating Covalent Organic Framework Capsules with Commodious Microenvironment for Enzymes

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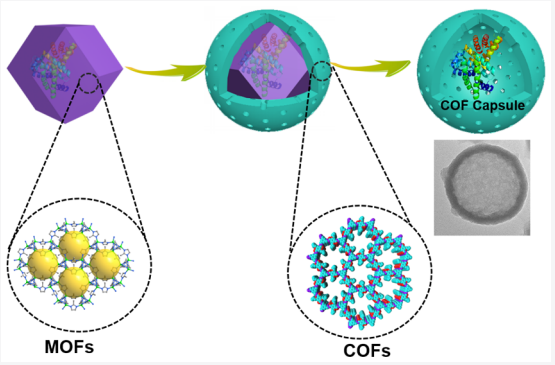
ABSTRACT: Enzyme immobilization has been demonstrated to be a favorable protocol to promote industrialization of biomacromolecules. Despite tremendous efforts to develop new strategies and materials to realize this process, maintaining enzyme activity is still a formidable challenge. Herein we created a sacrificial templating method, using metal−organic frameworks (MOFs) as sacrificial templates to construct hollow covalent organic framework (COF) capsules for enzyme encapsulation. This strategy can provide a capacious microenvironment to unleash enzyme molecules. The improved conformational freedom of enzymes, enhanced mass transfer, and protective effect against the external environment ultimately boosted the enzymatic activities. We also found that this strategy possesses high versatility that is suitable for diverse biomacromolecules, MOF templates, and COF capsules. Moreover, the dimensions, pore sizes, and shell thickness of COF capsules can be conveniently tuned, allowing for customizing bioreactors for specific functions. For example, coencapsulation of different enzymes with synergistic functions were successfully demonstrated using this bioreactor platform. This study not only opens up a new avenue to overcome the present limitations of enzymatic immobilization in porous matrixes but also provides new opportunities for construction of biomicrodevices or artificial organelles based on crystalline porous materials.



INTRODUCTION

As natural catalysts, enzymes possess fascinating characteristics, such as high efficiency and regio- and stereoselectivity, allowing for continual advances in biotechnological applications.[1,2] However, the intrinsically fragile nature of enzymes greatly impedes their industrialization. Enzyme immobilization has become a favorable strategy with increased stability and reusability, easy operation, energy conservation, and continuous-flow production, prompting the application of enzymes.[3−11] Despite these merits, most confined enzymatic systems still confront great challenges. For instance, tightly packed enzymes within host materials typically lack conformational freedom that may greatly affect molecular recognition.[12,13] Mass transfer of substrates or products can also be a limiting factor, and thus internally adsorbed enzymes may display substantially reduced activity.[14,15] Moreover, traditional incorporation methods such as adsorption often suffer from low loading or high leaching due to weak interaction or pore size mismatching.[16] Exploration of new platforms to overcome these challenges for optimal enzyme performance in confined space is, therefore, in urgent demand.

Compartmentalization of biomacromolecules is a common phenomenon in living systems.[17,18] The eukaryotic cell is a prototypical example whereby the unique biochemical microenvironment allows the spatial control of various functions. Such enclosed units confer physical protection against external disturbance, while maintaining a certain degree of freedom for the internal contents.[19,20] Inspired by nature, the design and construction of biomimetic "smart capsules" can be a promising approach to overcome the challenges of confined enzymatic systems.[21−23] Organic polymer and inorganic nanoparticles are materials commonly used to prepare capsules through various approaches.[18,24,25] However, they may suffer from irregular structure or lack of chemical functionality.[26,27] Covalent organic frameworks (COFs) are an emerging class of crystalline porous materials with high and well-defined porosity, high stability, and readily engineered functionality, allowing them to be excellent hosts for enzyme encapsulation.[28−31] However, the harsh synthetic conditions required in the fabrication of COFs make it impractical to directly make COF capsules encapsulating biomacromolecules through a

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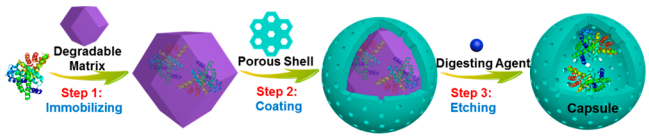
"one-pot" strategy.[32−34] The sister material of COFs, metal−organic frameworks (MOFs), can in situ encapsulate biomacromolecules to form biomacromolecule@MOF (@ = encapsulating) with high loading and good protection.[35−38] However, this often results in a penalty to lose the conformational freedom of enzymes, leading to a detrimental effect on enzyme activity. Many reports including our recently work have proved that biomacromolecule@MOF can be easily digested to release biomacromolecules without losing their bioactivities which render MOFs as perfect sacrificial templates to make COF capsules.[37] Combining the benefits of both COFs and MOFs to form "smart capsules" may open up new opportunities to greatly enhance the performance of confined enzymes: (i) the MOF encapsulation can protect enzymes from harsh synthetic conditions, such as those amenable to COF formation, allowing for COFs to directly coat enzyme@MOF particles; (ii) the high porosity of COF shells then allows for subsequent digestion of MOF cores while facilitating mass transfer of substrates, resulting in active, conformationally unhindered enzymes within a protective COF capsule.

Herein we created a versatile approach to fabricate biomimetic "smart COF capsules" for the encapsulation of active biomacromolecules. Biomacromolecules are first encapsulated within digestible MOFs via in situ encapsulation to form biomacromolecules@MOF which can protect biomacromolecules and act as templates for the growth of biomacromolecule@MOF@COF core−shell structures. Finally, the MOF core was etched away to liberate the biomacromolecules within the hollow COF capsule (Scheme 1). Such a commodious microenvironment in the formed

Scheme 1. Stepwise Approach To Construct Biomacromolecule@capsule



biomacromolecule@COF offers protective effects while maintaining the conformational freedom of biomacromolecules and facilitating the mass transfer of substrates/products, which is essential for the high activity or synergistic effect of different enzymes. This study opens up a new avenue to overcome the present limitations of enzymatic immobilization in porous matrixes.

RESULTS AND DISCUSSION

Materials Preparation and Characterizations. A supporting matrix is required for COF capsule formation to protect enzymes against harsh synthetic conditions and to act as a template. ZIF-90 has been previously shown to efficiently load various biologically active macromolecules (e.g., enzymes) and protect them against harsh conditions while also being feasibly digested under mildly acidic conditions.[35−37] Thus, ZIF-90 is a perfect candidate to demonstrate the concept due to the presence of unreacted aldehyde groups in the ligand 2-imidazolecarbaldehyde (ICA), which could be anticipated in the amine−aldehyde condensation reaction involved in a subsequent COF formation and therefore allows the MOF particles as templates in the formation of COF shells (Figure 1). To maintain the activity of biomacromolecules during the

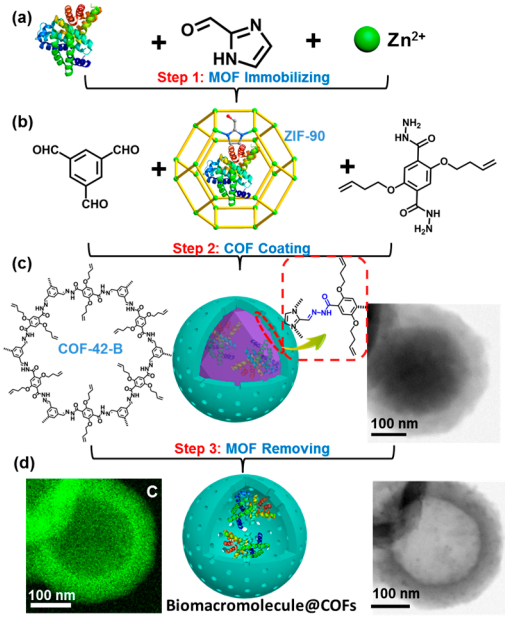


Figure 1. Synthetic route to biomacromolecule@COF capsules. (a) Immobilizing biomacromolecules in ZIF-90 via an in situ encapsulation method. (b) A one-pot reaction of ZIF-90 with COF monomers. (c) Core−shell structure of biomacromolecule@ZIF-90@COF-42-B (middle), structure of COF-42-B (left), a TEM image of the core−shell structure (right). (d) Capsule structure of biomacromolecule@COF-42-B (middle), EDS carbon distribution (left) and a TEM image (right) of biomacromolecule@COF-42-B capsule.

synthesis process, an acid-stable COF that can be synthesized under mild conditions is then required for the construction of capsules, for which COF-42 was chosen.[39,40] COF-42 was synthesized via the room temperature condensation of 1,3,5-triformylbenzene (TB) and 2,5-bis(ethoxy)-terephthalohydrazide (BETH) under acetic acid catalysis. However, because protic acids could decompose the enzyme@MOF complexes, a MOF-compatible catalyst, $Sc(OTf)_3$,[41] was used instead of acetic acid. Various structural analogues of the BETH monomer were screened to find one that produced a crystalline COF-42 product (Table S1, Figures S1 and S2). It was found that only 2,5-bis(butenyloxy)-terephthalohydrazide (BBTH) succeeded to afford a crystalline COF-42 analogue (named as COF-42-B) which was stable under acid conditions (e.g., pH = 5, phosphate buffer (PB) solution, 50 mM) (Figures S3 and S4).

To verify the construction strategy of COF capsules, BSA (bovine serum albumin) was chosen as a model biomacromolecule on the basis of its low cost and high solubility. BSA@ZIF-90 was first prepared via the in situ encapsulation method according to the literature.[36] The encapsulation efficiency of BSA was measured to be as high as 99%, using a standard Bradford Assay, and the loading percentage was calculated to be 0.22 g BSA per gram of ZIF-90 (Figure S5). Subsequently, the as-prepared BSA@ZIF-90 was mixed with TB, BBTH, and $Sc(OTf)_3$ in 1,4-dioxane/mesitylene (3:1 v/v) for 30 min. PXRD patterns and TEM images showed a combination of two crystalline phases (i.e., ZIF-90 and COF-42-B) (Figures 2 and S6), indicating the existence of BSA@ZIF-90@COF-42-B. FT-IR results further verified the successful encapsulation of BSA (Figure S7). Our previous result has demonstrated that ZIF-90 can be digested in aqueous solution with pH < 7.[37] We then

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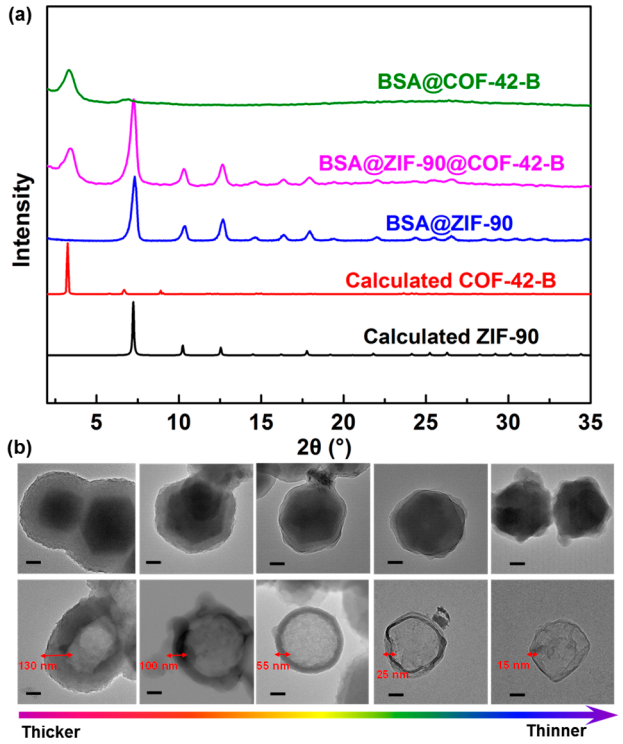

Figure 2. Characterizations of BSA@COF-42-B capsule. (a) PXRD patterns of products in different synthesis stages. BSA@ZIF-90 (blue), BSA@ZIF-90@COF-42-B (purple), BSA@COF-42-B (green), calculated pattern of ZIF-90 (black), and COF-42-B (red). (b) TEM images showing BSA@ZIF-90@COF-42-B precursors (upper) and BSA@COF-42-B capsules (below) with different shell thicknesses tuned via adding different amounts of BSA@ZIF-90 (from left to right, 6.0 mg, 9.0 mg, 12.0 mg, 15.0 mg, and 18.0 mg). Scale bar: 100 nm.

exposed the as-prepared BSA@ZIF-90@COF-42-B to a PB solution (pH = 5, 50 mM). The progress of the digesting reaction was monitored by TEM, which showed a clear transformation from the core−shell structure to a hollow capsule within 1 h. Tracking characteristic PXRD peaks of ZIF-90 afforded a result consistent with TEM data (Figures S8 and S9). Energy-dispersive spectrometer (EDS) element mapping further demonstrated the successful construction of COF-42-B capsules (Figure 1d). Porosity of these materials was found to be retained during the synthetic procedure (Figure S10). N_2 sorption data collected at 77 K revealed the Langmuir surface areas of 1390 m^2/g, 610 m^2/g, 436 m^2/g, and 835 m^2/g, BET surface areas of 723 m^2/g, 367 m^2/g, 235 m^2/g, and 530 m^2/g, pore size distribution centered around 0.9, 1.8, 1.2, and 1.8 nm for BSA@ZIF-90, COF-42-B, BSA@ZIF-90@COF-42-B, and BSA@COF-42-B, respectively. Moreover, it was found that the thickness of the COF-42-B shell could be readily adjusted by altering the amount of added BSA@ZIF-90. Upon increasing the amount of BSA@ZIF-90, the PXRD reflection peaks assigned to ZIF-90 exhibited increased intensity relative to the intensity of the COF-42-B peaks (Figure S11), and TEM images showed the COF shell varying from 130 to 15 nm (Figure 2b). A control experiment was further performed to test if BSA can be directly incorporated into COF-42-B via the traditional pore adsorption method. The attempt failed because the channel size of COF-42-B was much smaller than the molecule size of BSA (Figure S12).

Exploration of the Generality of Capsule Fabrication Strategy. To explore the generality of this synthetic strategy, we examined the use of ZPF-2 (ZPF = zeolitic pyrimidine framework) as the sacrificial template, instead of ZIF-90. ZPF-2 with a sod topology was synthesized from $Zn(NO_3)_2{\cdot}6H_2O$ and 2-hydroxy-5-fluoropyrimidine (Figure S13) in aqueous solution at room temperature.[42] Herein, for the first time, we demonstrated that ZPF-2 could efficiently incorporate biomacromolecules via in situ encapsulation method. As revealed by TEM, PXRD, and FT-IR (Figures S14−S16), ZPF-2 can efficiently encapsulate BSA and serve as a sacrificial template to yield hollow BSA@COF-42-B capsules. Additionally, we found that the digesting rate of ZPF-2 was faster than that of ZIF-90 and that the MOF cores were completely removed in 10 min (Figure S17). Moreover, we found that ZIF-8 could also be applied to fabricate COF capsules via an approach similar to that for ZIF-90 and ZPF-2 (Figures S18−S20). However, only a relatively small amount of ZIF-8 could be incorporated within COF-42-B because increased amounts of ZIF-8 inhibited the formation of COF-42-B. We also explored whether other COF materials could be used to make COF capsules. A hollow COF capsule containing BSA was successfully fabricated with COF-43-B (Figures S21 and S22).[39] This stepwise strategy has, therefore, proven to be an adaptable method to fabricate biomacromolecule-containing hollow COF capsules that could foreseeably be modified for use in highly specific applications.

Biocatalysis Application of COF Capsules. On the basis of the successful encapsulation of BSA in COF capsules, we further explored whether enzymes can be trapped within COF capsules. Catalase (CAT) is a well-known multimeric protein with four subunits, which can catalyze the breakdown of hydrogen peroxide (H_2O_2) to generate O_2 and H_2O, protecting cells from the oxidative damage caused by hydrogen peroxide.[43] To immobilize CAT within COF capsules, ZPF-2 was selected as the model sacrificial MOF. EDS mapping was performed to confirm the successful encapsulation of CAT within the capsules. As shown in Figure 3, Fe, the characteristic element present in CAT, was uniformly dispersed in all samples, indicative of the successful encapsulation of CAT. Additionally, confocal images further verified the uniform

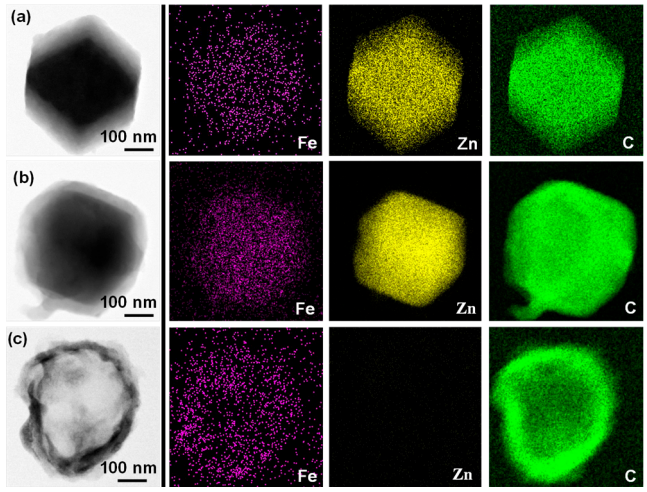

Figure 3. Bright-field images (left) and EDS elemental mapping (right) of Fe, Zn, and C for (a) CAT@ZPF-2, (b) CAT@ZPF-2@COF-42-B, and (c) CAT@COF-42-B.

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dispersion of CAT in all samples (Figure S23), and circular dichroism (CD) and UV−vis spectra confirmed that the structure of CAT was retained during this synthesis process (Figure S24).

Factors such as particle size and capsule shell thickness were also investigated to optimize the catalytic activity of this novel enzyme-incorporated system. A series of CAT-loaded ZPF-2 crystals with particle sizes ranging from 100 to 8000 nm were synthesized (Figure S25). TEM images were used to confirm particle size uniformity for each sample (Figure S26a). Catalytic activity toward H_2O_2 was evaluated via the FOX method (Figures S27 and S28). With the same amount of enzymes, CAT@ZPF-2 achieved the highest K_{obs} of 0.0473 s^{-1} with a particle size of ∼1500 nm. COF capsule shell thickness was also tuned by varying the amount of added CAT@ZPF-2 core (with particle size of ∼1500 nm). The results reveal that the activity of enzyme@COF-42-B capsules obeyed a Gaussian distribution when shell thickness was modified (Figure S29), and the best performance was achieved with a shell thickness of ∼60 nm (K_{obs} = 0.0743 s^{-1}) (the loading capabilities were 1.66 g/g). Generally, the thicker the capsule shell, the higher the mass transfer resistance. Besides thickness, capsule size of COF-42-B also plays a crucial role in the activity of encapsulated enzymes. Thereafter, we prepared a series of COF capsules in the range of 350−1500 nm, with a capsule thickness of ∼60 nm (Figure S26b−g) and found that capsules of ∼1500 nm exhibited the best performance (Figure S30). Thus, optimized COF capsules of ∼1500 nm size and ∼60 nm thickness were used for further study.

The biocatalytic assay revealed that CAT@ZPF-2@COF-42-B before MOF etching presented enzyme activity 49% lower than that of CAT@ZPF-2, which should be ascribed to the high mass transfer resistance in CAT@ZPF-2@COF-42-B. After etching treatment, the generated CAT@COF-42-B capsule demonstrated a dramatic 58% improvement of activity compared to CAT@ZPF-2, which could be 210% when compared to CAT@ZPF-2@COF-42-B (Figure 4a). Negligible leaching of CAT was detected during the etching process (Figure S31). A further comparison of the kinetics of CAT@ZPF-2 and CAT@COF-42-B revealed that their V_{max} and K_M were 2.73 μM/s and 0.0625 mM, and 3.98 μM/s and 0.0531 mM, respectively (Figure S32). Although their activities are lower than free CAT (Figure S33), the results show significantly boosted biocatalytic activity for CAT@COF-42-B capsule structure after the removal of MOFs, indicating that unleashing CAT molecules from the tightly packed MOF composite to the commodious microenvironment of COF capsules is beneficial for enzymatic performance. Additionally, we also investigated the CAT-loaded COF-43-B capsules which showed a trend similar to that for COF-42-B (Figure S34). After optimization, the activity of CAT@COF-43-B exceeded CAT@COF-42-B and showed an increase up to 81% compared with that of CAT@ZPF-2 (Figure 4b) (loading capabilities were 1.49 g/g). This result is probably due to the larger pore size of COF-43-B (3.2 nm) compared with COF-42-B (1.9 nm) (Figure S35) that facilitate the mass transport of substrates and products. These results indicate that the synthetic strategy can be applied as a versatile and tunable approach for on-demand preparation of a highly efficient bioreactor based on COF capsules.

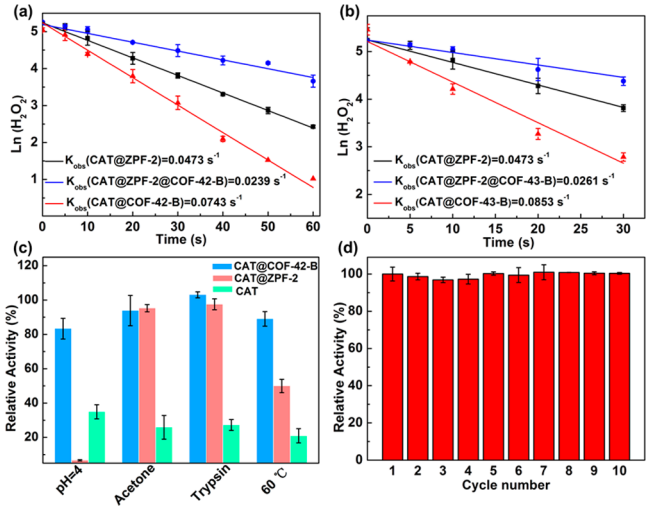

Figure 4. Biological activity of CAT@COF capsules. (a) Kinetics of degradation of H_2O_2 for CAT@ZPF-2 (black), CAT@ZPF-2@COF-42-B (blue), and CAT@COF-42-B (red). (b) Kinetics of degradation of H_2O_2 for CAT@ZPF-2 (black), CAT@ZPF-2@COF-43-B (blue), and CAT@ COF-43-B (red). (c) Activity percentage of CAT, CAT@ZPF-2, and CAT@COF-42-B after treatment under various harsh conditions. (d) Recycling experiments of CAT@COF-42-B.

Exploration of Protection Effect and Reusability of COF Capsules. The protection effect of the formed COF capsules was examined under various perturbation conditions, such as acid, proteases, acetone, and high temperature (Figure 4c). CAT@COF-42-B prepared via CAT@ZPF-2 was selected as a representative. First, we assessed its acid tolerance in disodium hydrogen phosphate−citric acid buffer (100 mM, pH = 4) for 30 min. CAT@COF-42-B was seen to retain 85% of its original activity while free CAT retained only 35%, and CAT@ZPF-2 lost almost all activity (>99%) due to the leaching of CAT that resulted from dissolution of ZPF-2 under the acidic condition (Figure S36a). COF-42-B was shown to protect CAT with 95% of original activity retained after exposure to acetone for 60 min. In contrast, free CAT retained only 25% of activity. A similar result was observed after exposure to protease trypsin (6 mg/mL) for 60 min: CAT@COF-42-B retained ∼100% of its activity, while free CAT retained only 25% of activity. Next, the influence of heating was evaluated. After treatment under 60 °C for 10 min, free CAT and CAT@ZPF-2 retained only 20% and 50% of original activity, respectively, while CAT@COF-42-B retained 88% of original enzymatic activity. These results revealed that CAT@COF-42-B capsules demonstrated a superior protective effect that surpassed that of its counterparts such as free CAT and CAT@ZPF-2. Furthermore, we investigated the catalytic durability and stability of CAT@COF-42-B, which is an important criterion for heterogeneous biocatalysts. CAT@COF-42-B could be readily recovered without significant loss of activity for at least 10 cycles (Figure 4d), and negligible leakage was detected during recycling experiments (Figure S36b). In comparison, traditional porous materials such as SBA-15 and active carbon were used to immobilize CAT via the conventional adsorption method. Their loading capabilities were demonstrated to be 0.21 g/g, and 0.09 g/g, respectively (Figures S37 and S38). The formed CAT composites exhibited much lower enzyme loading or activity compared with the CAT@COF capsule (∼1.6 g/g, Figure S39) and exhibited no recyclability (Figure S40).

Cascade Reactions Conducted in COF Capsule Systems. In practical applications, two or more enzymes are

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often required to cooperate with each other for advanced functions.[44] Fabricating multienzyme architectures has garnered increasing attention with great potential in the field of biocatalysis and advanced microdevices, such as "artificial organelles".[27,45,46] For example, GOx can spontaneously oxidize glucose into gluconic acid, meanwhile producing H_2O_2 which can serve as a substrate of CAT. Combining these two enzymes represents a model cascade reaction of a biologically relevant system.[47] To prove the concept of cascade reactions in COF capsules, GOx was first encapsulated in COF-42-B capsules such as CAT. We found that GOx@COF-42-B using ZPF-2 as a sacrificial template exhibited an improved activity (~170%) compared to GOx@ZPF-2 (Figure 5a). We coencapsulated GOx and CAT into COF-42-B and

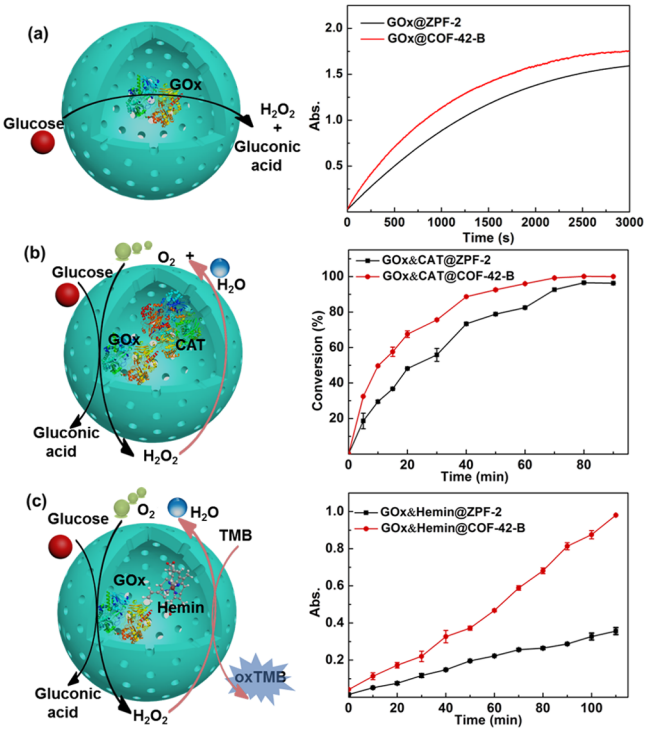

Figure 5. Catalytic performance of various systems. (a) GOx@ZPF-2 and GOx@COF-42-B activity using a colorimetric assay based on the oxidation of ABTS by a Cyt *c* coupled system (λ = 660 nm). (b) GOx and CAT@ZPF-2 and GOx and CAT@COF-42-B activity based on the kinetic consumption of glucose. (c) GOx and Hemin@ZPF-2 and GOx and Hemin@COF-42-B activity using a colorimetric assay.

examined their cascade reaction effect. The cascade catalytic efficiency was evaluated by the detection of glucose consumption using the 3,5-dinitrosalicylic acid (DNS) method (Figure S41).[48] Compared to GOx@ZPF-2, GOx and CAT@ZPF-2 exhibited a much higher glucose consumption rate attributed to the participation of CAT. As shown in Figure 5b, GOx and CAT@COF-42-B composite produced a 130% consumption rate of glucose compared to its counterpart (GOx and CAT@ZPF-2). These results demonstrated that the synergistic effect between the two enzymes was significantly enhanced after being unleashed from ZPF-2 to the commodious COF capsule. To further prove the concept of cascade reactions in the COF capsule, another multienzyme bioreactor based on GOx and Hemin@COF-42-B was evaluated spectroscopically using 3,3′,5,5′-tetramethylbenzidine (TMB) and glucose as substrates, accompanied by a colorimetric change detectable at 650 nm (Figure 5c). The results revealed that GOx and Hemin@COF-42-B showed an enhanced activity of 180% compared to its counterpart (GOx and Hemin@ZPF-2) and further validated that the COF capsule can provide freedom to multiple enzymes for better cascade performance, which implies potential applications in the fabrication of bioreactors or advance microdevices.

CONCLUSION

In summary, we created a facile three-step approach to fabricate novel COF capsules to encapsulate various biomacromolecules which cannot be easily incorporated by COFs via traditional approaches (i.e., adsorption method). First, biomacromolecules are first encapsulated within digestible MOFs via in situ encapsulation to form biomacromolecule@MOF systems. This composite can further act as templates for the growth of biomacromolecule@MOF@COF core−shell structures. Finally, the MOF cores were etched away to unleash the tied biomacromolecules within the hollow COF capsule. This facile, scalable, and effective strategy afforded high-performance COF bioreactors, which can maintain enzyme conformational freedom, stabilize the enzyme, and meanwhile allow effective diffusion of the substrates and products. The COF capsules can provide capacious internal environments for the optimal performance of the encapsulated biomacromolecules with good protection effect against external perturbations. The encapsulated enzymes in COF capsules exhibited excellent reusability which surpassed that of traditional porous materials such as SBA-15. In addition, this synthetic platform exhibits high versatility and tunability: different sacrificial MOF templates (e.g., ZIF-90, ZIF-8, and ZPF-2) and COF shells (COF-42-B and COF-43-B) as well as various enzymes (e.g., CAT, GOx) were used in this study; various factors such as the COF shell thickness and pore size could be fine-tuned to optimize the performance of the bioreactors. We also demonstrated that multiple enzymes could be easily coencapsulated within one COF capsule using this synthetic strategy and act as advanced bioreactors capable of efficient cascade reactions. Such diverse advances may inform the further understanding of enzyme behavior in both in vivo and in vitro systems and expand the possibilities for the construction of microdevices, artificial organelles, and even artificial cells.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.0c00285.

Detailed experimental procedures, materials, FT-IR, ^{1}H NMR, PXRD, TEM, etc. (PDF)

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Notes

The authors declare no competing financial interest.

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