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Immobilization of a Bienzymatic System via Crosslinking to a Metal-Organic Framework

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Abstract: A leading biotechnological advancement in the field of biocatalysis is the immobilization of enzymes on solid supports to create more stable and recyclable systems. Metal-organic frameworks (MOFs) are porous materials that have been explored as solid supports for enzyme immobilization. Composed of organic linkers and inorganic nodes, MOFs feature empty void space with large surface areas and have the ability to be modified post-synthesis. Our target enzyme system for immobilization is glucose oxidase (GOx) and chloroperoxidase (CPO). Glucose oxidase catalyzes the oxidation of glucose and is used for many applications in biosensing, biofuel cells, and food production. Chloroperoxidase is a fungal heme enzyme that catalyzes peroxide-dependent halogenation, oxidation, and hydroxylation. These two enzymes work sequentially in this enzyme system by GOx producing peroxide, which activates CPO that reacts with a suitable substrate. This study focuses on using a zirconium-based MOF, UiO-66-NH_2, to immobilize the enzyme system via crosslinking with the MOF's amine group on the surface of the MOF. This study investigates two different crosslinkers: disuccinimidyl glutarate (DSG) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC)/N-hydroxysuccinidimide (NHS), providing stable crosslinking of the MOF to the enzymes. The two crosslinkers are used to covalently bond CPO and GOx onto UiO-66-NH_2, and a comparison of the recyclability and enzymatic activity of the single immobilization of CPO and the doubly immobilized CPO and GOx is discussed through assays and characterization analyses. The DSG-crosslinked composites displayed enhanced activity relative to the free enzyme, and all crosslinked enzyme/MOF composites demonstrated recyclability, with at least 30% of the activity being retained after four catalytic cycles. The results of this report will aid researchers in utilizing CPO as a biocatalyst that is more active and has greater recyclability.

Keywords: enzyme immobilization; metal-organic frameworks; biocatalysis; crosslinking of enzymes

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1. Introduction

Enzymes have been utilized in biocatalysis due to their ability to achieve high degrees of regio- and stereo-selectivity, as well as their ability to function under mild conditions. This type of catalysis has emerged as a strategy to promote greener chemical syntheses [1]. However, free enzymes are limited in their microenvironment parameters, such as pH, temperature, and solvents [1]. While the application of free enzymes is useful and has been increasingly utilized as a preferred method in some cases, one drawback is the inability to recycle enzymes after catalysis. Research surrounding the enhancement of biocatalytic function has focused on immobilizing enzymes onto solid supports to improve their activity, and recyclability, and increase their durability in different microenvironments [1–3]. The immobilization of enzymes occurs by attaching enzymes to the surface of a material through covalent and non-covalent interactions [1–3] Many reports have described various

methods of immobilizing enzymes on diverse solid supports that exhibit certain desired properties [1,2,4–10]. These methods of immobilization include physical or chemical adsorption, crosslinking, and encapsulation [1–3,11–16]. The immobilization of enzymes by covalently attaching enzymes to solid matrices has been explored by crosslinking enzyme aggregates using precipitating reagents or crosslinkers [1,17]. Crosslinking offers the advantage of producing biocatalytic materials with improved reusability, enhanced stability, and a longer shelf life [1]. Different crosslinker reagents can provide additional specificity in designing biocatalysts for different purposes.

Metal organic frameworks (MOFs) are materials created from a network of organic linkers and inorganic nodes [18]. MOFs exhibit unique physical and chemical properties, such as porosity, tunability, high surface areas, and stabilities that distinguish them from other materials for immobilizing enzymes [11,15,19–24]. The solid support used in this study was UiO-66-NH_2, a zirconium-based MOF that features an amine group on the benzene ring of the organic linker that can serve as a reactive site for creating covalent bonds between an enzyme and a MOF [22,25,26]. In a previous study, we demonstrated that the amine group in UiO-66-NH_2 greatly enhances enzyme activity of the bienzymatic system, horseradish peroxidase and glucose oxidase, after immobilization via physical adsorption due to increased electrostatic and hydrogen bonding interactions at the enzyme/MOF interface [27]. In this study, the amine group served as a covalent bonding site for immobilization via amide bond formation between the enzyme and MOF.

The bienzymatic system chloroperoxidase (CPO)/glucose oxidase (GOx) was chosen to be co-immobilized onto UiO-66-NH_2 via crosslinking. The reaction scheme is shown in Figure 1. Co-immobilization has been shown to enhance biocatalytic activity due to the closer proximity of enzymes and enzyme intermediates producing a cascade of reactions aiding in enzyme activation [28–30]. Many previous reports have used glucose oxidase and other enzymes to generate enzyme-activation molecules, such as hydrogen peroxide [27,30–37]. This study uses the commercially available enzyme GOx to produce peroxide to activate chloroperoxidase (CPO). CPO is a heme-containing protein that is secreted by *Caldariomyces fumago*, a marine fungus, grown in fructose-salts media [38–40]. It is an exceptional protein due to the variety of organic substrates it can accept to perform many different types of oxidation and chlorination reactions [41–56]. It has been used in previous studies for the degradation of organic molecules and synthesis of organic molecules, and it has been shown to be effective when co-immobilized with GOx [6,51,57–76]. This study shows that the immobilization of this bienzymatic system via electrostatic interactions is not reusable and activity is almost eliminated after one cycle, presumably due to the leaching of enzymes [62]. The crosslinking of the enzymes to UiO-66-NH_2 can not only enhance the activity of this bienzymatic system, but also improve the recyclability of its enzymatic function. The results of this report will aid researchers in utilizing CPO as a biocatalyst that is more active and has greater recyclability.

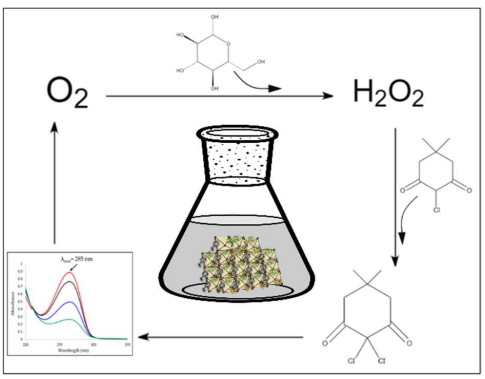

Figure 1. The bienzymatic biocatalytic system of the GOx/CPO cascade reaction of the chlorination of monochlorodimedone, in which GOx utilizes glucose to produce H_2O_2, and CPO uses this H_2O_2 to chlorinate MCD. The decrease in the MCD concentration is monitored by measuring the absorbance at 285 nm.

2. Results

This study focuses on utilizing the amine group of UiO-66-NH_2 as a crosslinking site to study two different crosslinkers, disuccinimidyl glutarate (DSG) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), to immobilize chloroperoxidase (CPO) and glucose oxidase (GOx) on the MOF's surface. The two crosslinkers were chosen based on their relative stabilities in lower-pH environments to create an amide bond between the -NH_2 moiety of the MOF and a carboxylate on the surface of the enzymes. The two crosslinker reagents, DSG and EDC, are amine-reactive, forming an amide bond that is stable under hydrolysis [4,66]. The crosslinker disuccinimidyl glutarate (DSG) is a very common amine-reactive crosslinker used to crosslink peptides, nucleic acids, and proteins with a spacer arm length of 7.7 angstroms [77–79]. The second crosslinker reagent, also known as a coupling reagent, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC)/hydroxysuccinidmide (NHS), is also an amine-reactive agent that activates carboxylic groups and aids in forming amide bonds with amine groups. This coupling reagent has been used in many crosslinking studies involving enzymes and nano materials [80,81]. In this system, the carboxylic groups of the free enzyme will be directly crosslinked with the amine groups of UiO-66-NH_2 to create amide bonds. Crosslinking cooperative enzymes to the same solid support places them in closer spatial proximity, as opposed to being free in solution; therefore, the enzyme activity is expected to be higher for the GOx/CPO bienzymatic system. The scheme for the crosslinking of the enzymes with UiO-66-NH_2 with these amine-reactive reagents is shown in Figure 2.

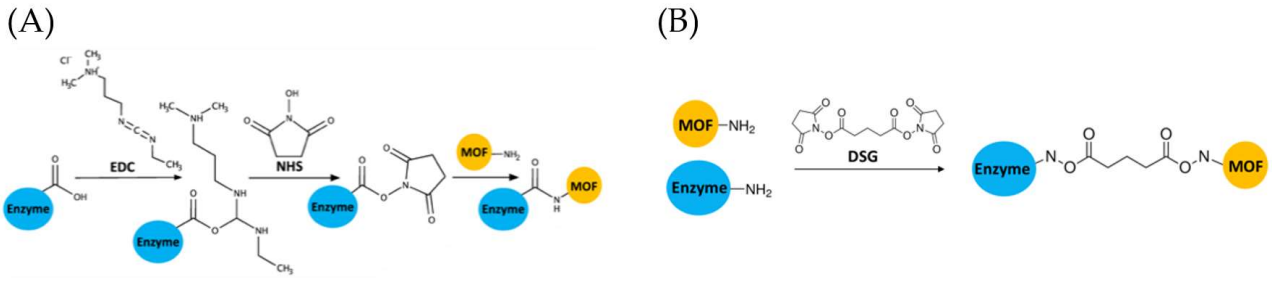

Figure 2. Schematic depicting the reaction of EDC/NHS (**A**) and DSG (**B**), which produces an amide bond between the enzyme and the MOF.

To further help elucidate the stabilities of the crosslinkers, single-enzyme systems were also prepared by immobilizing only CPO on the MOF using each of the crosslinkers. Therefore, a total of four composite materials were investigated: GOx/CPO-DSG@UiO-66-NH_2, GOx/CPO-EDC@UiO-66-NH_2, CPO-DSG@UiO-66-NH_2, and CPO-EDC@UiO-66-NH_2. These were compared with CPO and GOx/CPO immobilized on UiO-66-NH_2 via electrostatic interactions.

Following the immobilization reactions, the XRD pattern for UiO-66-NH_2 was compared with the patterns for CPO-linker@UiO-66-NH_2 composites and bienzymatic GOx/CPO-linker@UiO-66-NH_2 composites for each of the crosslinkers. Figure 3 shows the overlayed PXRD patterns for both crosslinking methods. No changes were observed for any of the crosslinked enzyme/MOF composites, which indicates that the structure of the UiO-66-NH_2 was not affected by the crosslinking reactions.

The chlorination activity was used to determine the effect the linker had on both activity and recyclability of the immobilized enzyme composites. Monochlorodimedone (MCD) is a common substrate used to investigate the halogenation activity of enzymes. Using MCD as the substrate, chlorination by CPO was monitored and resulted in decreased absorbance at 285 nm, indicating the formation of dichlorodimedone. Representative data from this assay are shown in Figure 4. The protein CPO is activated in the presence of peroxide and chloride ions in solution to chlorinate the substrate, MCD. Enzyme activity was determined by measuring the concentration of MCD by monitoring the decrease in absorbance at 285 nm over a 15 min period.

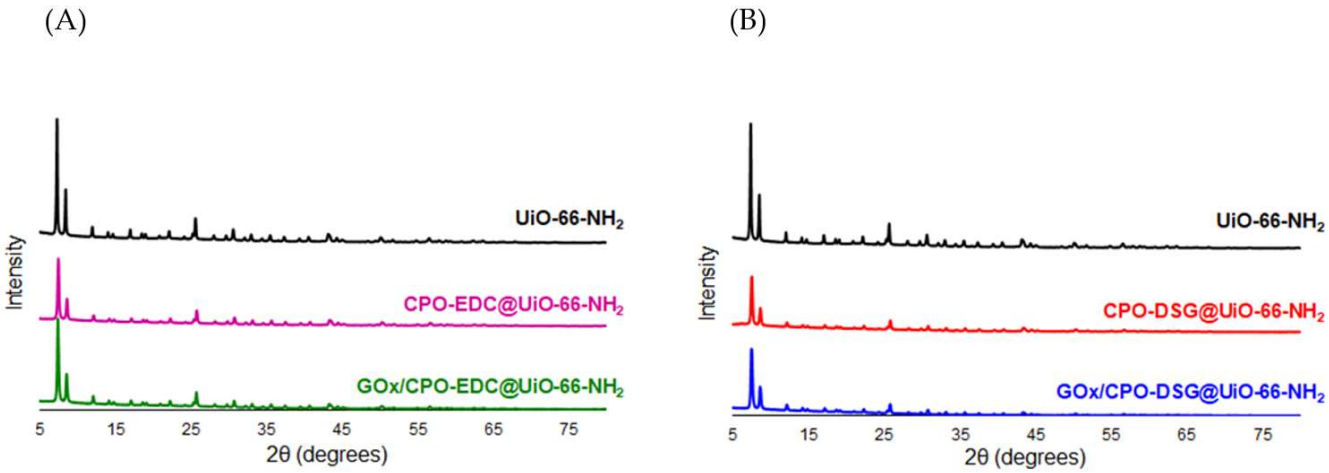

Figure 3. XRD patterns of UiO-66-NH_2, CPO-linker@UiO-66-NH_2, and GOx/CPO-linker@UiO-66-NH_2 for (**A**) EDC and (**B**) DSG crosslinkers.

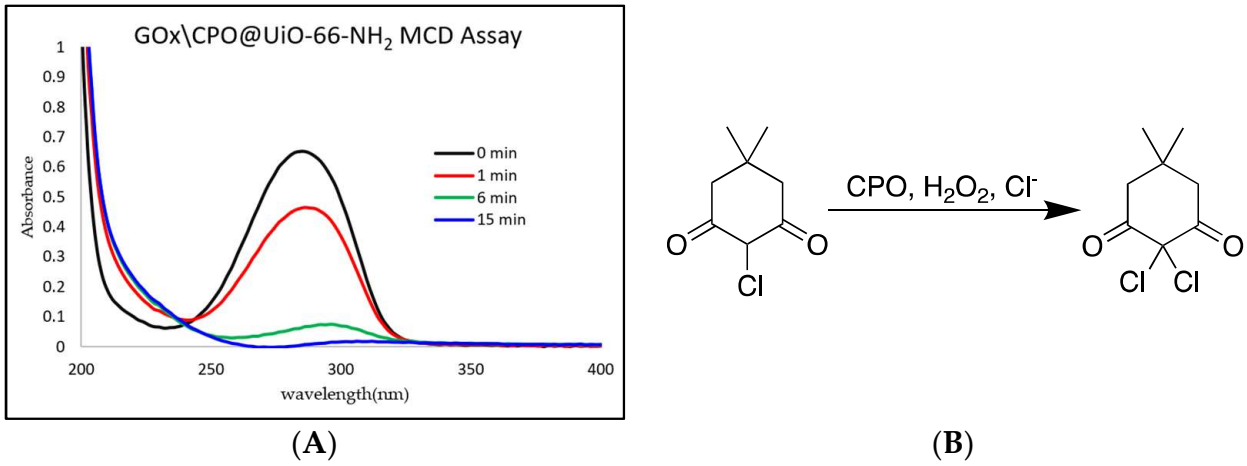

Figure 4. Chlorination of monochlorodimedone assay to assess CPO's chlorination ability. Representative UV/vis data from the assay (**A**) showing the formation of dichlorodimedone (**B**) over time as indicated by the decreasing absorption at 285 nm.

In Figure 5, the activities of single (CPO) and bienzymatic (GOx/CPO) systems immobilized with the two crosslinkers are compared with that of the free enzyme, and the total enzyme loading, which was determined using the Bradford assay, for each composite is reported in Table 1. The loading was consistently higher in the DSG composites compared with their EDC analogues. The bienzymatic system crosslinked with DSG showed enhanced enzyme activity by 54% compared with the free enzyme, showing 4300 U/mg of enzyme activity while the free bienzymatic system showed only 2787 U/mg of enzyme activity. The EDC/NHS-crosslinked composites showed little enzyme activity when compared with the DSG-crosslinked composites. These data suggest that the DSG-crosslinked doubly immobilized system is a good candidate for a biocatalytic system. The enzyme assays were run in triplicate. Catalytic activity is defined in Equation (1).

$$U = \frac{\Delta A \text{min}^{-1}}{1.0^{-4}\text{M}^{-1}\text{cm}^{-1}(1\text{cm})} \quad (1)$$

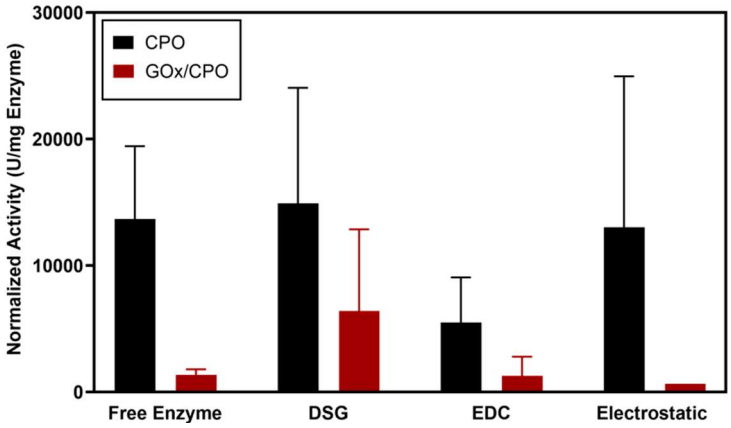

Figure 5. Enzyme activity of the single and doubly immobilized composites comparing the free enzyme, electrostatically immobilized enzyme, and the DSG and EDC cross-linked biocomposites.

Table 1. Enzyme loading of crosslinked composites compared with electrostatic immobilization in μg enzyme/mg MOF as determined by the Bradford assay.

Enzyme@UiO-66-NH_2	Electrostatic (μg/mg)	DSG (μg/mg)	EDC (μg/mg)
GOx/CPO	0.413 ± 0.18	0.458 ± 0.12	0.282 ± 0.17
CPO	0.223 ± 0.22	0.358 ± 0.22	0.179 ± 0.07

To investigate recyclability, each composite was subjected to six sequential cycles of washing and drying, and residual activity was assessed using the MCD assay. The recyclability data are reported in Figure 6. All crosslinked enzyme/MOF composites retained activity across sequential cycles, with all materials retaining at least 30% of their initial activity in the fourth cycle and falling to near zero by the sixth cycle. This is in contrast to the electrostatically adsorbed system, in which no retained activity was observed in the second cycle. This indicates that both DSG and EDC crosslinking is a successful strategy to improve recyclability. The composite with the highest retained activity over four catalytic cycles (> 50%) was the single system crosslinked with DSG, CPO-DSG@UiO-66-NH_2. The crosslinked composites displayed fluctuations in the retained enzyme activity in single systems (Figure 6A) and a constant decrease in the bienzymatic systems (Figure 6B). Note that, in the recyclability studies reported in Figure 6, the enzyme activity was calculated based on the enzyme unit U per mg MOF-enzyme to account for the loss of biocomposite with each filtration and reaction cycle; therefore, any uncertainty in the measured values came from the mass of the biocomposite.

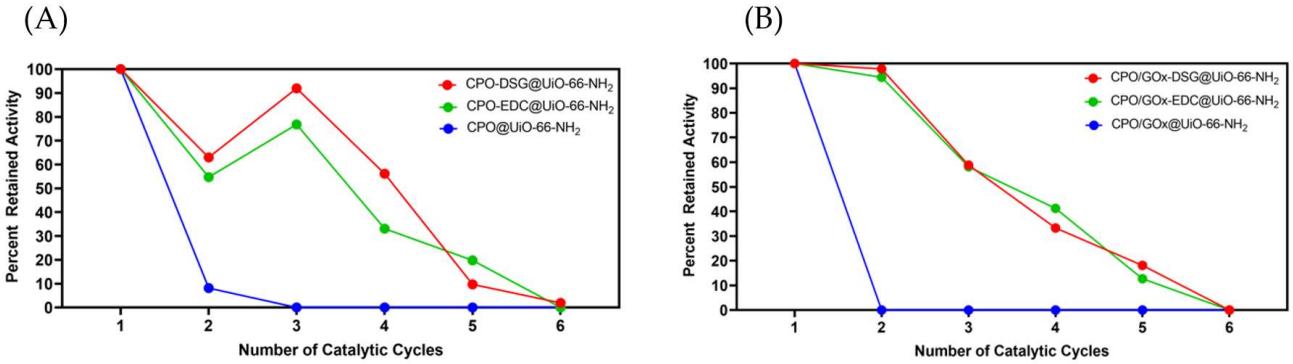

Figure 6. Biocomposites' recyclabilities using DSG (red) and EDC (green) as crosslinkers, and electrostatically immobilized enzymes (blue) showing the percentage of activity retained after the filtering and drying of the biocomposites. Enzymatic activity of the (**A**) single-enzyme system (CPO) and (**B**) bienzymatic system (GOx/CPO).

3. Discussion

Considering the DSG linker, both the single and bienzymatic systems showed higher activity than the free enzymic systems and good recyclability for up to four cycles. Yet, the single-enzyme CPO system performed very well due to the peroxide concentration being constant in solution. In the bienzymatic system (GOx/CPO), the peroxide that was generated from glucose oxidase activated the neighboring chloroperoxidase to chlorinate MCD without an overwhelming peroxide concentration. This proximity was not necessary in the single-enzyme system, where CPO was working on its own with the peroxide available in solution. The EDC/NHS and DSG linkers created amide bonds with the enzymes. The degradation of these amide bonds is an unlikely candidate, since any oxidation of an amine would occur on the UiO-66-NH_2, and the amine-functionalized UiO-66 was stable when soaking in 1 M HCl solution for over 2 h. Although chloroperoxidase is rarely crosslinked with EDC/NHS and most EDC/NHS enzyme crosslinking studies that have been reported had a minimum pH of 4.0, this study required the assay solution to be at a low pH of 2.75 for the reaction to occur [2–10]. This may explain the inability to recycle the biocomposite more than two times.

A decrease in enzyme activity after each cycle was seen in both the single-enzyme and bienzymatic systems in DSG and EDC/NHS. In addition to enzyme leaching, macromolecular crowding could have effects on the proteins' conformation and biochemical rate, resulting in a decrease in enzyme activity. The effects of crowding have been discussed in other literature reports, such as the deacceleration in fast association reactions and weak attractive interaction between crowding agents or crosslinkers and proteins of interest [11–13]. In this report, the higher loading of enzyme/s resulted in higher activity of the enzyme, as measured by the chlorination assay of CPO. While EDC/NHS starts off with considerable enzyme activity and the enzyme's retained activity in each cycle did not fluctuate, it decreased greatly after each run, dropping from 88.5 to 17.5 U/mg MOF-enzyme in the single system and 48.5 to 6.16 U/mg MOF-enzyme after the fifth cycle. The recyclability studies demonstrate the stability between each cycle for the examined crosslinkers in comparison with systems that had been adsorbed electrostatically. While the crosslinkers provided enhanced reactivity and stability for both the single and bienzymatic systems, they were unable to undergo more than four cycles before the activity decreased considerably, presumably due to the leaching of enzymes from MOF substrate into solution.

In this study, enzyme/MOF composites were prepared using two different crosslinkers, DSG and EDC, to immobilize CPO or GOx/CPO on the surface of UiO-66-NH_2. Using this approach, four enzyme/MOF composite materials were prepared: bienzymatic composites GOx/CPO-DSG@UiO-66-NH_2 and GOx/CPO-EDC@UiO-66-NH_2, as well as single-enzyme composites CPO-DSG@UiO-66-NH_2, and CPO-EDC@UiO-66-NH_2. The activity of these systems was measured using the MCD assay. While the EDC-coupled materials showed little activity, both DSG-coupled materials showed enhanced activity over the analogous free enzymes. All four crosslinked enzyme/MOF composites demonstrated enhanced recyclability, with $\geq$ 30% retained activity after four catalytic cycles. This study provides a framework for more investigations into the development of a stable biocomposite utilizing the enzyme CPO for biocatalytic applications.

4. Materials and Methods

4.1. Materials

All reagents were purchased from Sigma Aldrich (St. Louis, MO, USA) and used as supplied. *Caldiormyces fumago* was obtained from ATCC (16373). The Bradford assay materials were supplied by Bio-Rad Laboratories. UiO-66-NH_2 was prepared as previously reported [25].

4.2. Production of CPO

Caldiormyces fumago was grown and cultured using a sample of *C. fumago* purchased from ATCC (16373) in fructose salt media at 24 °C for 7 days [82]. After 7 days, the

biomass was spun down and the resulting media was subjected to two rounds of acetone precipitation (−20 °C) to 40% and 60%. The precipitated protein was spun down, dried, and resuspended in 20 mM MOPSO buffer at pH 6.5. The crude protein solution was run through a G25 Sephadex column and collected and used as prepared.

4.3. Crosslinking

To crosslink chloroperoxidase (CPO) and glucose oxidase (GOx), both enzymes were prepared in separate 20 mM MOPSO solutions at a pH of 6.5. A stock solution prepared from solid GOx from *Aspergillus niger* 145,200 U/g with a concentration of 6 mg/mL was used in all crosslinking reactions. While the concentration of CPO varied due to the different batches of media solution from the grown *C. fumago*, the optimum molar ratio of 1:5 (CPO:Gox) stayed consistent [1]. The concentration of CPO in each batch was calculated by running an MCD assay. All the washing steps were performed using 20 mM MOPSO solution with a pH of 6.5 prepared by diluting solid MOPSO purchased from Sigma Aldrich in nano pure water. In addition, a Bradford assay was performed for every crosslinking method by taking 100 uL of enzyme solution in a 5 mL Bradford reagent 4:1 solution before and after the reaction time. The Bradford assay was performed using Bio-Rad Dye Protein Assay Dye Reagent Concentrate, and a standard concentration calibration curve was generated using the Bio-Rad Lyophilized Bovine γ-Globin. Bradford and MCD assays were performed and quantified spectrophotometrically using a Persee (Auburn, CA, USA) T8-DS Double Beam UV-Vis spectrometer for enzyme loading and enzyme activity, respectively. These crosslinking experiments were run in triplicate.

4.3.1. EDC/NHS

To crosslink using EDC/NHS, the two enzymes were mixed sequentially in solution to have the optimum ratio of 1:5 of glucose oxidase and chloroperoxidase, respectively, and solid EDC and NHS were added to make a final concentration of 10 mM. The chloroperoxidase was crosslinked first since it was not as efficient at loading as the glucose oxidase and the ratio of 1:5 was shown to be the best ratio of the enzyme. Then, 60 mg of MOF was added to the solution and mixed in an ice bath for two hours. The reaction was then quenched with 6 uL of β-mercaptoethanol. The same procedure was repeated to develop the single-enzyme system with only CPO. The resulting composites GOx/CPO-EDC@MOF and CPO-EDC@MOF were washed with MOPSO buffer, vacuum filtered, and desiccated overnight. Solid 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, N-hydroxy succinimide, and β-mercaptoethanol were all purchased from Sigma Aldrich.

4.3.2. DSG

To crosslink using DSG, the enzyme solution was prepared similarly to the previous crosslinking method. After equilibrating to room temperature, 5 mg of solid DSG, was dissolved in DMF to produce a 10 mM solution. An additional 10 mL of 20 mM MOPSO buffer was added to the enzyme solution along with 1.53 mL of the DSG solution and 60 mg of MOF. The reaction solution was mixed in an ice bath for two hours. The reaction was quenched with 1 M Tris buffer with a pH of 7 to obtain a final concentration of 20 to 50 mM of Tris buffer. The same procedure was repeated to develop a single-enzyme system with only CPO. The resulting composites GOx/CPO-DSG@MOF and CPO-DSG@MOF were washed with MOPSO buffer, vacuum filtered, and desiccated overnight.

4.4. Characterization of MOFs and Enzyme/MOF Composites

In order to investigate the effect of crosslinking on the MOF structure, powder X-ray diffraction (PXRD) characterization was performed on UiO-66-NH_2 as well as CPO-linker@UiO-66-NH_2 and GOx/CPO-linker@UiO-66-NH_2 for each of the crosslinkers. PXRD data of the MOF and enzyme/MOF crosslinked composites were collected on a Rigaku (Tokyo, Japan) SmartLab SE diffractometer using a copper anode with Kα1 = 1.54056 Å

and Kα2 = 1.54439 Å fitted with a nickel Kβ filter. The samples were analyzed between 2θ 5–80° with a step size of 0.01 degrees and scan speed of 1 degree/min.

4.5. Enzyme Activity

The crosslinked composites were developed for a bienzymatic biocatalytic system as shown in the reaction scheme in Figure 1. The MCD assay was performed to test the enzyme activity of the two crosslinked composite bienzymatic (GOx/CPO) and single-(CPO) enzyme systems. The reaction mixture consisted of the following reagents in equal volumes: 1 M glucose (for bienzymatic composites), 20 mM H_2O_2 (for single enzyme composites), 20 mM KCl, 300 uM MCD, and 20 mM formic acid buffer with pH 2.75. The initial absorbance of just the reagent mixture was measured; then, the MOF-enzyme composite was added to start the reaction. The absorbance of the mixture was measured by first spinning down the MOF via centrifugation for 30 s and then removing the supernatant to avoid scattering by the MOF at the following time intervals: 3 min, 8 min, and 15 min. This was repeated for the three crosslinked composites, and the enzyme activity was calculated from the decrease in absorbance at 285 nm as the rate of consumption of MCD per minute. Controls of the free enzymes were run in the same manner by duplicating the highest loaded enzyme concentrations from the Bradford assay to be normalized to U per mg of enzyme.

4.6. Recyclability

Recyclability studies were performed by recovering the composite after the first round of the MCD assay. The composite was washed with 20 mM MOPSO buffer at a pH of 6.5, vacuum-filtered, and desiccated overnight. The same assays with the same reagent ratios were run using the same method for 6 cycles while keeping record of the mass of MOF-enzyme composites used for each run to be normalized to the first run by the milligrams of MOF-enzyme composite used.

5. Conclusions

The GOx/CPO system is particularly attractive because it can be used in many applications due to the diverse reactions that chloroperoxidase is able to perform [14–22]. Chloroperoxidase is an extremely versatile enzyme that is known to perform peroxidase, chlorination, and P450-like functions. It is also known to be relatively stable in solution. With this in mind, the goal of this study was to determine if a bienzymatic system of CPO/GOx might make CPO even more stable and reusable than free enzymes in solution. Hydrogen peroxide is a powerful oxidant and can have deleterious effects on enzyme activity, but by coupling the substrate for oxidation that can be channeled directly to the active site of CPO, the problematic effects of utilizing high concentrations of hydrogen peroxide may be avoided. In fact, this was observed for the GOx/CPO-DSG@UiO-66-NH_2 bienzymatic system, which had higher activity than both the CPO free enzyme and CPO-DSG@UiO-66-NH_2.

The recyclability studies focused on the retained activity of chloroperoxidase between cycles and did not quantitate the amount of enzyme leaching, but rather assumed the process of enzyme leaching based on reduced activity. Future studies will investigate enzyme leaching from the solid support and model the effects of macromolecular crowding in the bienzymatic system. Some studies have investigated the potential of strong biocatalysts, such as chloroperoxidase, in synthesizing pharmaceuticals [23,24]. A study used free CPO to synthesize Modafinil due to the enzyme's ability to perform a selective sulfoxidation of the starting material 2-(diphenylmethylthio) acetamide [24]. Modafinil is a drug used to treat narcolepsy as a wake-promoting agent via oral administration [25]. The study tested different parameters at which CPO can give the highest yield, such as pH, running time, and substrate concentrations. Another study showed that crosslinked glucose oxidase and chloroperoxidase on magnetic nanoparticles produced the desired enantiomer of modafinil [23]. Optimizing the recyclability of the enzyme/MOF composites

reported in this study has great potential for further exploring chemical syntheses, providing a framework for more investigations into making a stable biocomposite with CPO for biocatalytic applications.

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Conflicts of Interest: The authors declare no conflict of interest.

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