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Biocatalytic Modification of Naturally Occurring Iron Porphyrin

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Hematin, a hydroxyferriprotoporphyrin, is the stable, oxidized form of heme. Heme has been reported to be the active catalytic center of naturally occurring peroxidases such as horseradish peroxidase (HRP). While there have been reports on the use of hematin as a catalyst for oxidative polymerization reactions, these reactions could be carried out only at high pH conditions due to limited aqueous solubility of hematin at lower pH conditions. We report here the biocatalytic modification of hematin using a lipase, Novozyme-435. Hematin has been modified by tethering monomethoxy polyethylene glycol (mPEG) chains which provide aqueous solubility over a fairly wide range of pH conditions. This pegylated Hematin (PEG-Hematin) is synthesized via a one-step solventless reaction and the products formed can be isolated with minimal purification. The PEG-Hematin synthesized serves as a robust alternative to HRP for the polymerization of aniline and phenol.

Keywords: biomimetic catalysts, green chemistry, hematin, lipases, enzymatic polymerization.

1. Introduction

The past few decades have seen an upsurge in the use of enzymes for accomplishing certain unique transformations and polymerization reactions under benign conditions. Enzyme catalysis provides a wide range of possibilities for the synthesis of a variety of advanced functional materials. Enzyme catalyzed reactions can also be carried out in solventless conditions or in aqueous/organic media rendering, them amenable to a variety of systems including biological entities. Enzymes are also known to exhibit high reaction selectivity, chemoselectivity, substrate selectivity, regio-, and enantioselectivity.

Hydrolases are a class of enzymes used widely in biocatalysis and known to catalyze the hydrolytic cleavage of C–O and C–N bonds (1). Lipase B from *Candida Antarctica* (CAL-B), belonging to the class of hydrolases, is one of the most extensively used enzymes in organic syntheses (2). In particular, lipases have been known to perform enantioselective hydrolytic reactions and catalyze the formation of a wide range of ester and amide bonds (3).

The extensive industrial applications of conducting polymers in electrochromic devices (4), light-emitting dioides (5) and lightweight batteries (6) have provided an impetus towards the development of new electronic and photonic materials. With the ultimate goal of increasing the commercial viability of this class of materials, a considerable amount of research has been devoted to exploring new methods and catalysts to augment processability and environmental compatibility of these polymers. The search for methodologies for eco-friendly syntheses was initiated with the concept of enzyme catalyzed polymerization.

Oxidoreductases such as Horseradish Peroxidase (HRP), obtained from natural and renewable sources, have been known to catalyze the oxidative coupling/polymerization of aniline (7) and phenol (8) based monomers under benign conditions in aqueous and mixed solvent systems (9). These biocatalytic reactions can be carried out in the presence of polyelectrolyte templates or in organized media such as micelles (10). However, the wide use of HRP has been restricted owing to its low stability/activity in highly acidic conditions as well as its high cost.

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The important roles played by porphyrins in nature and their possible applications in catalysis have been the subject of research over the last few decades (11,12). Porphyrin complexes might thus, prove to be of significant importance for providing useful insights into possible reactions of catalytic complexes such as peroxidases and cytochrome-P450, in which the porphyrins play the crucial role. Hematin, a constituent of the active site of several oxidoreductases such as *cytochrome c* has been used as a catalyst for the polymerization of phenols (13). Hematin is a hydroxyferriprotoporphyrin with Fe^{3+} in a high spin state. The mechanism for the hematin catalyzed polymerization has been proposed and the intermediate states involved are believed to be similar to the intermediate states observed in the mechanism of HRP-catalyzed polymerization.

Unlike peroxidases, hematin does not contain the protein environment; hence it is stable in organic solvents as well as high pH conditions. Hematin is thus a promising and inexpensive alternative to peroxidases. Hematin, however, is water soluble only at very high pH and thus is not effective, as is, for the low pH conditions required for the oxidative polymerization of several monomers.

The goal of this paper is to modify hematin to render it soluble and stable in aqueous media and develop an effective and economic renewable catalyst for the oxidative polymerization. Novozyme-435 (*Candida antarctica* lipase B), obtained from the fermentation of a genetically modified fungus has been used as a catalyst. Novozyme-435 is commercially available, active towards a fairly wide range of substrates and can catalyze esterification as well as amidation reactions in organic solvents or under solventless conditions. In addition, these reactions are typically carried out under conditions of mild temperature and pressure.

2. Experimental

2.1. *Materials*

Hematin, aniline monomer (purity 99.5%) and hydrogen peroxide (30 wt%) were purchased from Aldrich Chemicals Inc., Milwaukee, WI, and were used as received. The hydrogen peroxide was diluted to 0.3% (in deionized water), and this solution was used for polymerization. Poly(ethylene glycol monomethyl ether) ($M_n \sim 5{,}000$) was purchased from PolySciences, Inc. Novozyme-435, an immobilized enzyme, was a gift from Novozymes Inc., Denmark, and was dried over P_2O_5 under vacuum prior to use. Spectra-pore dialysis membrane was purchased from VWR Scientific. All other chemicals were of reagent grade or better.

2.2. *Synthesis of PEG-hematin*

Hematin (1 gm, 1.57 mmol) and mono-methoxy poly (ethylene glycol) [mPEG] (4.74 mmol) were placed in a round-bottom flask and dried under vacuum at 90°C. The enzyme (10% by weight with respect to reactants) was added to the reaction mixture and the reaction flask was placed in a constant temperature oil bath maintained at 90°C under vacuum. The reaction was allowed to proceed for 48 h, after which it was quenched by adding mildly acidic water (pH 6). The enzyme was filtered off and any unreacted monomer under vacuum. The filtrate was dialyzed using membrane (molecular weight cut-off 6000) to remove unreacted mPEG. After the completion of dialysis, the product (Figure 1) was obtained as a fluffy solid by freeze-drying.

2.3. *Characterization*

UV-visible near-IR spectra were obtained using a Perkin-Elmer Lambda 9 spectrophotometer. Thermogravimetric analysis (TGA) was performed using a TA Instrument, Hi-Res 2950 thermogravimetric analyzer. The TGA of all samples were carried out in air. GPC analysis was carried out on an Agilent GPC with RI detector using DMF as a solvent and 0.05% Lithium Bromide. GPC analysis was done on a DMF column calibrated with Poly (ethylene glycol). The sample solutions were filtered through 0.4 μm Millipore filters, and 20 μL of solution was loaded into the column. 8 mg/ml of the mPEG-Hematin was dissolved in DMF and a flow rate 1 ml/min was used.

3. Results and discussion

As stated earlier, hematin is soluble in water only at a high pH and thus is not as effective in the neutral/low pH aqueous conditions required for the template assisted enzymatic approach. Hence, it would be advantageous to structurally modify these iron containing porphyrins and obtain water-soluble derivatives. Our goal was to esterify the iron porphyrin using a benign one-step synthesis. While there have been reports on the modification of porphyrins as catalysts for oxidative polymerization, these involve time consuming multiple steps and purification (14).

Roy et al. (15) have reported the esterification of hematin in dimethyl formamide (DMF) with poly(ethylene glycol) [PEG] chains in the presence of activators such as N,N′-carbonyldiimidazole and 1,8 diazabicyclo[5.4.0]undec-7-ene (DBU). However, the synthetic route has certain drawbacks associated with it. The reaction is carried out in DMF in which both hematin and PEG-Hematin are soluble. This makes the work-up and purification a tedious process. Further, since the reaction is performed at room temperature, the porphyrin can exist in a substantially aggregated form. These drawbacks made it compelling to design a more facile route to synthesize this novel catalyst.

Esterification of hematin [Hem] with monomethoxy poly(ethylene glycol) [mPEG] was performed at 80–85°C for 48 h under solventless conditions (Figure 1). Use of mPEG rather than a difunctionalized PEG offers better control over the functionalization (and potentially avoids

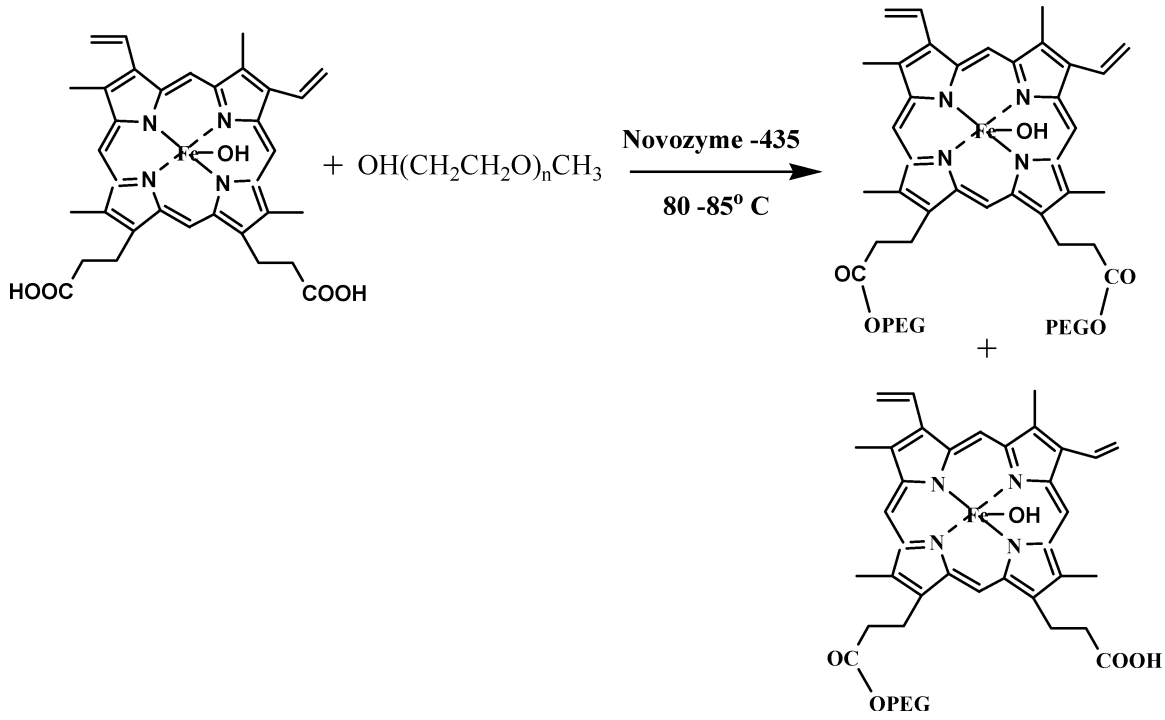

Fig. 1. Scheme for the esterification of hematin with poly (ethylene glycol).

looping). After the reaction is complete, the reaction mixture was poured into slightly acidic water at pH 6. Hematin is insoluble in water at neutral and lower pH and hence dissolves out and was filtered along with the catalyst. The unreacted mPEG was then removed by dialysis. The molecular weight of the mPEG was varied and the effect of the mPEG chain length on the esterification was evaluated. A series of mPEG(s) were selected with molecular weights ranging from 350-5,000 Da and the esterification reactions were carried out using the procedure described above.

3.1. *Estimation of hematin*

Since the catalytic center in the PEG-Hematin is hematin, it is desirable to know the actual percent of hematin present in the pegylated Hematin. Hematin exhibits a prominent

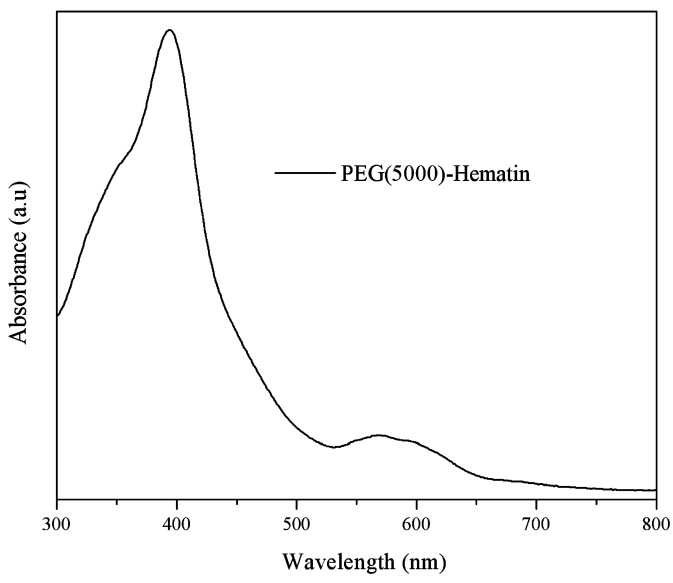

Fig. 2. UV-Visible spectrum for mPEG-5000 Hematin.

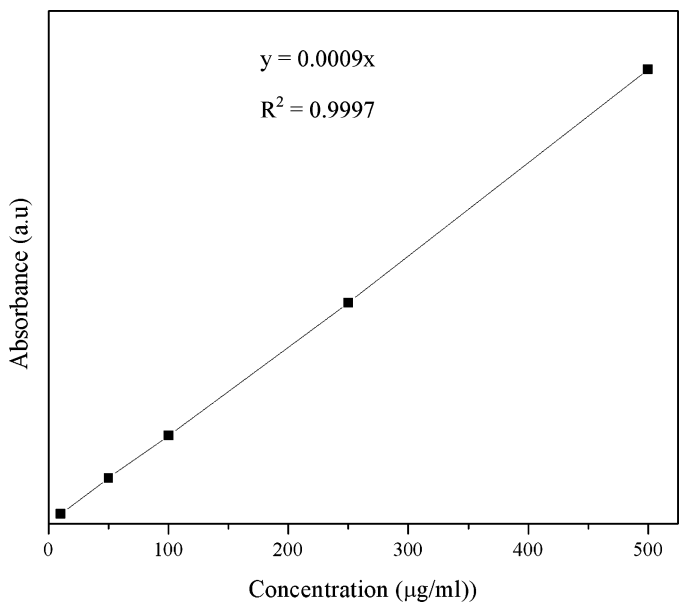

Fig. 3. Calibration curve for hematin at different concentrations.

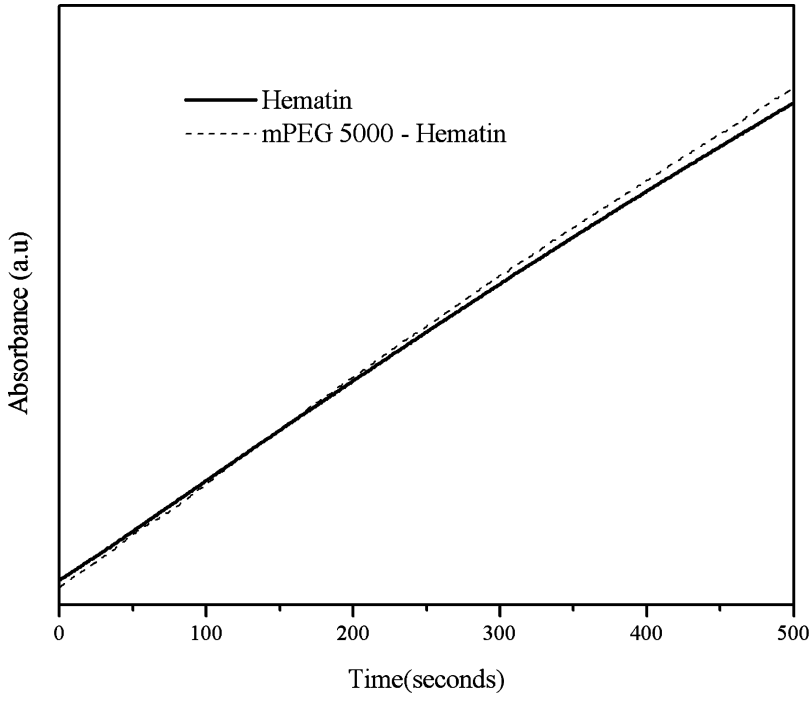

Fig. 4. Catalytic activity of hematin and mPEG- Hematin.

absorption peak at 395 nm (16) and hence, UV-Visible spectroscopy was used to determine the extent of functionalization of the pegylated hematin(s). Figure 2 depicts the UV-Visible spectrum for hematin functionalized with mPEG-5000. The UV-Visible spectra of hematin dissolved in DMF were first recorded at different concentrations and a calibration graph constructed (Figure 3). The maximum amount of hematin that can be present in functionalized form in the PEG-hematin was calculated based on the molecular weights of hematin (633 a.m.u) and PEG. When an mPEG of molecular weight 5000 is used and assuming that the hematin is functionalized at both ends, the maximum percent of hematin that can be present is: 633/10633 $*$ 100 = 5.95%. Since the reaction mixture is poured into slightly acidic water, the unreacted hematin precipitates out. The percentage of hematin in mPEG-Hematin was then calculated using the calibration curve of hematin and found to be 1.25% in the case mPEG 5000. This implies an extent of functionalization of around 21%, not taking into account the presence of possible unreacted mPEG in the final product. With decrease in molecular weight of the PEG chains, the esterification reaction did not proceed efficiently. We found that the extent of functionalization decreases with decrease in the molecular weight of the mPEG chains used. At the current time, the dependence of the esterification reaction on the molecular weight of the PEG is being investigated. We believe that a longer mPEG chain (higher molecular weight PEG) tethered to the hematin helps solubilizing the hematin to a better extent. In addition, longer PEG chains have been shown to prevent the aggregation of porphyrin units (17). Hence, with an increase in the mPEG chain length, aggregation of the individual units of porphyrin is perhaps minimized. Since the hematin functionalized with mPEG 5000 shows the maximum extent of functionalization, complete characterization of this ester has been carried out.

GPC analysis on the hematin functionalized with MPEG 5000 indicated peaks at 10,887 Da and peaks at around 5700 Da, which shows that there was still some mono-functionalized hematin remaining in the reaction mixture. We believe that the effectiveness of these compounds as biomimetic catalysts will not be significantly compromised by the presence of both the mono- and bi-functionalized hematin compounds. The infrared spectrum of hematin has been reported to exhibit absorption at 1718 cm^{-1} which are attributed to the =C=O stretch of the acid group in hematin. This peak splits into a doublet and moves to 1708 and 1702 cm^{-1} in the mPEG- Hematin indicating the conversion of the acid (hematin) to the ester.

NMR studies on the pegylated hematin were not conclusive. NMR spectrum of the porphyrin molecule however, could not be obtained due to the high paramagnetic resonance field of the Fe (III) present.

3.2. *Catalytic Activity of Pegylated Hematin*

The catalytic activity of the pegylated hematin was analyzed using the peroxidase assay typically used to ascertain the activity of enzymes such as HRP. Since the catalytic center in HRP is an iron containing porphyrin, the peroxidase assay was adopted for comparing the activity of hematin and mPEG-Hematin. The peroxidase assay

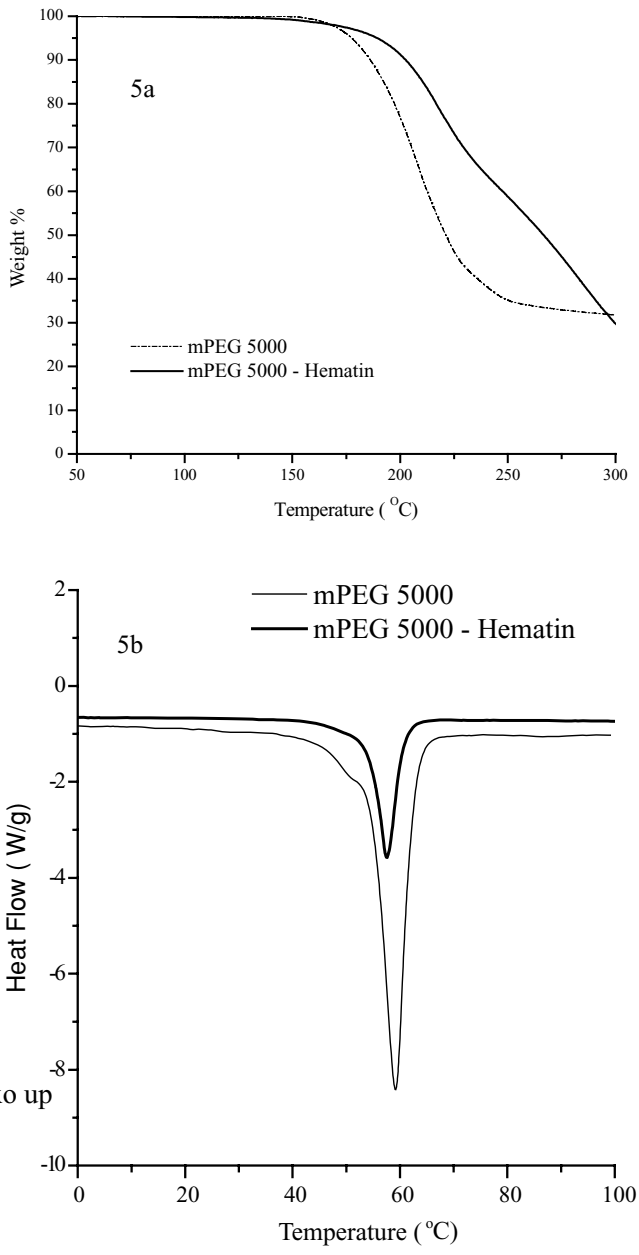

Fig. 5. Thermal analysis (a) TGA analysis of mPEG-Hematin, (b) DSC.

measures the efficiency of the catalyst in the oxidative transformation of pyrogallol to purpurogallin. Concentrations of hematin and mPEG-Hematin were chosen such that both compounds have similar optical densities. As seen in Figure 4, the catalytic activity of hematin remains unchanged on modification with poly (ethylene glycol). This affirms the fact that the catalytic center remains intact upon enzymatic modification.

3.3. *Thermal Analysis of mPEG-Hematin*

The TGA thermogram for mPEG-Hematin is provided in Figure 5(a). The mPEG-Hematin is more stable than the corresponding mPEG from which it is derived. The DSC curves for mPEG-hematin and corresponding mPEG are provided in Figure 5(b). As observed, the melting point for the mPEG-Hematin is shifted to lower temperatures (approximately 4°C shift). This behavior may be explained by a small molecule such as hematin acting as a plasticizer and lowering the melting point of the pegylated hematin.

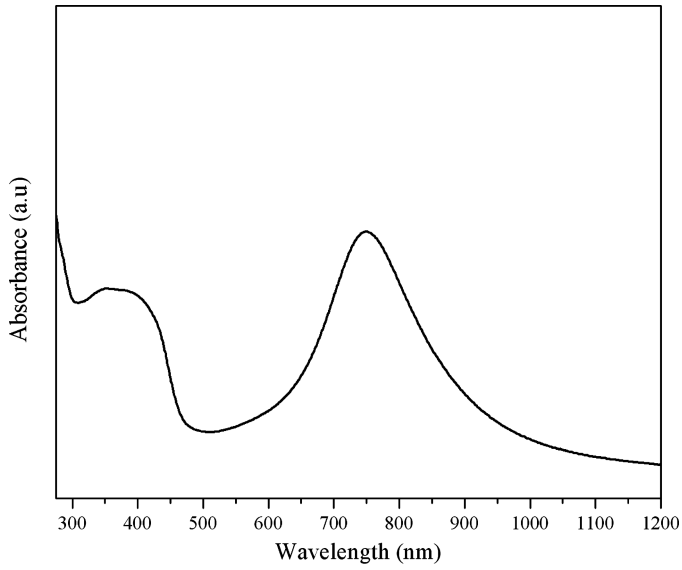

Fig. 6. Polymerization of aniline using mPEG-Hematin.

3.4. *mPEG-Hematin Catalyzed Polymerization of Aniline*

The enzymatically synthesized mPEG-Hematin was also used to catalyze the polymerization of aniline in the presence of sulphonated polystyrene (SPS). The polyaniline/SPS complex exhibits polaron bands at 400 nm and 750 nm regions and longer wavelength absorption beyond 1100 nm (Figure 6). This is consistent with earlier reports which have observed similar features for the polyaniline/SPS complex (7,18). Further, the longer wavelength absorption has also been observed in the chemically synthesized polyaniline and has been interpreted in terms of the presence of the polymer chain with extended conjugation (19). These results indicate that the enzymatically synthesized PEG-Hem serves as an efficient biomimetic catalyst for the polymerization of aniline.

4. Conclusions

In summary, a novel biocatalytic esterification route has been developed for the modification of a naturally occurring iron porphyrin, hematin yielding a more soluble and effective biomimetic catalyst. The esterification is a simple one-step process catalyzed by the lipase, Novozyme-435. The product can be obtained with minimal purification

and is water-soluble over a fairly wide range of pH. GPC results confirm the functionalization of hematin. Initial results suggest that the pegylated hematin also serves as a robust catalyst for the polymerization of monomers like aniline and phenol. It is envisioned that this catalyst can be used as a viable and economical alternative to naturally occurring enzymes like HRP.

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