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Immobilization of laccase in a sponge-like hydrogel for enhanced durability in enzymatic degradation of dye pollutants

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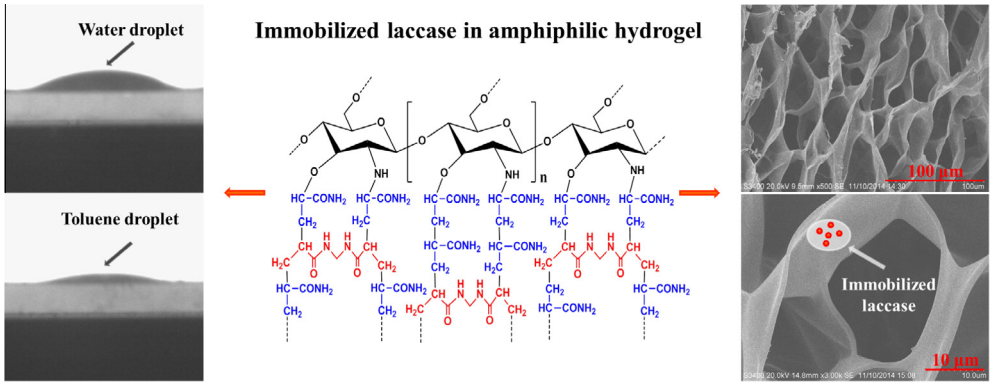
GRAPHICAL ABSTRACT



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ABSTRACT

A highly stable and efficient biocatalyst was fabricated by encapsulating *Trametes versicolor* laccase within a chitosan grafted polyacrylamide hydrogel (denoted as Lac-PAM-CTS). Scanning electron microscopy and nitrogen adsorption–desorption tests demonstrated that channels of diameter of 10–20 μm were regularly distributed throughout the sponge-like Lac-PAM-CTS. Besides, there were massive mesopores and macropores in the lamellar walls of the hydrogel. Such a network structure reduced the diffusion resistance of the hydrogel to the target substrates. The recovered activity of the obtained Lac-PAM-CTS was 40.8%. As compared to free laccase, the Lac-PAM-CTS showed enhanced thermal and chemical stability. The positive surface charge of the Lac-PAM-CTS endowed it with a pre-enrichment effect in the treatment of anionic dyes. In a continuous six-cycle batch decoloration of Malachite Green, the Lac-PAM-CTS showed much better durability than the free laccase. The results here suggest that sponge-like hydrogel is a good supporting matrix for laccase.

1. Introduction

Laccase (benzenediol oxygen oxidoreductase, EC 1.10.3.2) is an efficient and environmentally friendly biocatalyst, which is produced by numerous fungi, plants and bacteria [1]. It is effective in oxidation of various organic pollutants with the concomitant reduction of oxygen to water without the need of high temperature, pressure, and harsh chemical environments [2,3]. Laccase has shown an excellent application prospect in the treatment of dyeing effluents [4]. However, even with these advantages, the poor reusability, low operational stability and high production

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costs of free laccase restrict its wide applications. Immobilization of laccase on insoluble supports is a useful strategy to optimize their operational performance in large-scale industrial applications [3–5].

The recent approaches for immobilization of enzyme can be categorized into three types: non-covalent adsorption, covalent attachment, and entrapment. Adsorption is easy in operation, but the bonding of the biocatalysts to the surface of the supports is relatively weak, leading to the leaching of enzyme. Covalent attachment normally leads to an improvement of the enzyme stability, but the immobilized enzyme inevitably undergoes partial deactivation due to the conformational restrictions [6]. Entrapment is the physical confinement of the guest enzyme into a host matrix (mainly polymeric networks or microcapsules). This immobilization method is applied widely because of the several merits: mild immobilization condition, limited leaching and denaturation, and high immobilization efficiency [7]. The entrapment of laccase in polymeric hydrogels presents remarkable advantages: improved resistance to thermal and chemical inactivation, remarkable storage and operational stability [8]. However, in the synthesis of hydrogels, the use of cross-linking agents/initiators or the change of temperature/pH subject the enzyme to less-than-ideal conditions that may result in partial or total loss of its catalytic activity [9]. Therefore, the selection of gel components is a key problem in the immobilization of enzyme into hydrogels.

Polyacrylamide (PAM) has been widely studied as an embedding matrix for enzymes because of the favorable mechanical stability and rigidity, environmental friendliness, and low cost [4,10]. In a new variation of traditional entrapment, soluble prepolymers (e.g., poly(vinyl alcohol), polyetherimide, polyvinyl pyrrolidone) or prepolymers bearing active functional groups (e.g., chitosan (CTS)) are used partly instead of the polymeric monomers [11]. Grafting is an attractive approach to impart a variety of functional groups to a prepolymer, wherein monomers are covalently bonded onto the polymer chain [12]. Graft copolymers usually present high mechanical property and good stability by combining the advantages of both the prepolymer and the newly synthesized one [13]. Besides, the properties of the resultant copolymers could be easily regulated by introducing prepolymers with particular functional groups. For example, hydrogel of CTS and acrylic copolymer with different swelling and diffusion characteristics were synthesized by varying the concentrations of CTS, initiator and cross-linker [14]. High hydrophilicity of hydrogel could be achieved by choosing prepolymers with polyelectrolytic nature or having abundant functional groups such as amine, carboxylic acid, hydroxyl, and sulfonic acid groups in the polymer chain [15]. CTS is a biodegradable and inexpensive material with excellent biocompatibility, high protein affinity, abundant reactive functional groups, and good regenerability [16]. Over the last decade, CTS-based materials have been extensively examined and a number of potential products have been developed for wastewater treatment [3,17,18]. The grafting of molecules onto CTS is of great importance to develop new materials with combined properties of both the grafted molecules and the natural polymers. Hydrogels made from CTS are appealing for biological applications because of their high water content and good biocompatibility [19].

Graft copolymers are suitable for the immobilization of various biological molecules (proteins, enzymes, antibodies) by encapsulation into the hydrogel matrix [4]. The entrapment immobilization of enzymes in hydrogels could result in high protein loadings up to 96.5% as well as remarkable enzyme stability and reusability [20,21]. However, discussion about the recovered activity was limited in most of the reports on immobilized enzymes. One intrinsic drawback of the classic entrapment method is that only a small amount of entrapped enzymes are catalytically active. Some of the enzymes within the matrix might be deactivated during the entrapment process while some other entrapped enzymes are physically inaccessible to the substrate molecules, even though they still possess the native structures. The retention and expression of enzyme activity are closely related to the particle size, porosity, and polarity of the supporting matrix [9,22], which are all key parameters in determining the accessibility of the immobilized enzymes to substrates. Therefore, the optimization of the network structure of hydrogels is a key issue in the immobilization of enzymes.

An amphiphilic PEG-g-F68 hydrogel was prepared with polyethylene oxide polypropylene oxide–polyethylene oxide block-copolymers (e.g. PEO80-PPO27-PEO80, F68) as mediators in laccase-induced graft polymerization of diacrylic derivate of polyethylene glycols (PEG) [9]. The specific features of hydrophilic and hydrophobic domains are important factors that govern the adsorption performance against a particular solute, because they can interact effectively with water through the hydrophilic domains and extract and retain any organic substance dissolved in water through the hydrophobic domains. The PEG-g-F68 hydrogel exhibited a macroporous structure in which the immobilized laccase preserved almost total activity (ca. 90%). A synergetic effect on pollutant adsorption was observed between the macroporous structure and the amphiphilic domains in the graft copolymer [9]. The above results suggest that hydrogels with both amphiphilic property and sponge-like structures are excellent candidates for enzyme immobilization.

In the present work, CTS and acrylamide were selected as the building blocks to fabricate hydrogels for immobilization of laccase because of the aforementioned merits of them. The immobilization was conducted by adding dissolved laccase into the mixture of CTS, acrylamide, and N,N′-methylene-bis-acylamide (MBA, used as a cross-linker) for grafting copolymerization under mild conditions. One of the main objectives is to optimize the network structure of the hydrogel for a better diffusion of the substrate molecules to the active centers of the immobilized laccase. Decoloration of Acid Orange 7 (AO7, a representative of azo dyes) and Malachite Green (MG, a widely used and high aquatic toxic triarylmethane dye) by the resultant hydrogel-entrapped laccase was employed to evaluate its stability and efficiency for potential applications.

2. Materials and methods

2.1. Materials

AO7, MG, sodium acetate (NaAc), CTS (80–95% deacetylation), potassium persulfate ($K_2S_2O_8$), toluene, citric acid monohydrate ($C_6H_8O_7{\cdot}H_2O$) and disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4{\cdot}12H_2O$) were all of analytical grade and were purchased from Shanghai Reagent Station, China. Other chemicals, including *Trametes versicolor* laccase with a nominal activity of $\geqslant$0.50 U/mg (lot result: 0.92 U/mg), 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), acetic acid (HAc), methanol, acrylamide and MBA were purchased from Sigma–Aldrich and were used as received. Deionized water was used for preparation of all aqueous solutions.

2.2. Fabrication of hydrogel-entrapped laccase

CTS, acrylamide, MBA, and $K_2S_2O_8$ were all crucial factors that govern the structure of the resultant hydrogels. The effects of these factors on the efficacy of immobilization were evaluated by using an L_9 $(3)^4$ orthogonal array design matrix. As shown in Table S1 in the supplementary materials, according to the recovered