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Degradation of phenolic compounds by laccase immobilized on carbon nanomaterials: Diffusional limitation investigation

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ABSTRACT

Carbon nanoparticles are promising candidates for enzyme immobilization. We investigated enzyme loading and laccase activity on various carbon nanoparticles, fullerene (C_{60}), multi-walled carbon nanotubes (MWNTs), oxidized-MWNTs (O-MWNTs), and graphene oxide (GO). The loading capacity was highest for O-MWNTs and lowest for C_{60}. The activity of laccase on various nanomatrices using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTs) as a substrate decreased in the following order: GO > MWNTs > O-MWNTs > C_{60}. We speculated that aggregation of the nanoparticles influenced enzyme loading and activity by reducing the available adsorption space and substrate accessibility. The nanoparticle-immobilized laccase was then used for removal of bisphenol and catechol substrates. Compared to free laccase, the immobilized enzymes had significantly reduced reaction rates. For example, the reaction rate of GO–laccase conjugated with bisphenol or catechol substrates was only 10.28% or 12.33%, respectively, of that of the free enzyme. Considering that there was no obvious structural change observed after enzyme immobilization, nanomatrix-induced diffusional limitation most likely caused the low reaction rates. These results demonstrate that the diffusional limitation induced by the aggregation of carbon nanoparticles cannot be ignored because it can lead to increased reaction times, low efficiency, and high economic costs. Furthermore, this problem is exacerbated when low concentrations of environmental contaminants are used.

1. Introduction

Enzymes catalyze many chemical reactions in living systems under mild conditions. The immobilization of enzymes is a useful tool that can reduce costs by enabling efficient recovery, recyclability, enhanced stability under harsh conditions, and continuous use in enzymatic processes in analytical and medical applications [1,2]. The challenges of using immobilized enzymes are identifying new matrix materials with appropriate structural characteristics, such as morphology and surface functionality, and compositions, in addition to understanding enzyme–matrix interactions to improve the catalytic efficiency [3,4].

Recently, nano-structured materials, such as carbon nanomaterials, nano-sized polymer beads, and metal nanoparticles, have been utilized as immobilization matrices for enzymes. The use of nanoparticles offers many advantages, such as effective enzyme loading, large surface area, and increased mechanical strength [5,6]. Among the nano-structured materials, carbon nanomaterials are the most promising candidates for enzyme immobilization because of their chemical inertness, biocompatibility, and electrical conductivity [7,8]. Ren et al. [9] reported that single-wall carbon nanotubes enhanced the activity of horseradish peroxidase (HRP) by binding to the enzyme close to the active site and participating in the electron transfer process. Graphene oxide (GO)-immobilized HRP showed improved thermal stability and was active over a wide pH range, resulting in higher removal efficiency with several phenolic compounds when compared to soluble HRP [10].

However, the properties of a nanomaterial, such as its surface chemistry, morphology, and size, can influence the adsorption, conformation, and activity of immobilized enzymes. Yang showed [11] that GO greatly enhanced peroxidase activity by unfolding cyt c by electrostatic interactions, whereas reduced GO inhibited cyt c activity via hydrophobic interactions, resulting in decreased substrate accessibility to the heme active site. Boncel et al. [12] demonstrated that different chemical functionalizations of the nano-matrix led to various types of catalytic activity and enantioselectivity. They determined that lipase immobilized onto multi-walled carbon nanotubes (MWNTs) exhibited exceptionally high activity, whereas lipase immobilized onto oxidized MWNTs (O-MWNTs) exhibited low activity with high enantioselectivity.

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However, the effects of the properties of the nanomaterial, nature of the interface between the enzyme and nanomaterial, and nature of the substrate environment on enzyme activity have not been fully elucidated. Many enzymes denature when immobilized on nanostructured surfaces, resulting in lower enzyme activity and weaker substrate binding, and various, sometimes contradictory, mechanisms have been suggested [13,14]. Therefore, more insightful studies are needed to investigate the interactions between enzymes and nanomaterials.

Laccases are extracellular enzymes that catalyze the four one-electron oxidation of electron-rich compounds with a simultaneous four-electron reduction of molecular dioxygen to water [3]. Immobilized laccase has a wide range of commercial applications in the oxidation of dyes and lignins and in ethanol production, waste water treatment, and degradation of toxic polycyclic aromatic hydrocarbons [15]. Laccases have been successfully immobilized onto different nanomaterials, such as a MWNT paste electrode [16], nanocomposites formed by chitosan and carbon nanotubes [17], meso-structured silica materials [18], platinum nanoparticles, and reduced graphene composites deposited onto screen printed electrodes [19]. However, most studies on nanoparticles have focused on the improvement of enzyme activity, loading, and catalytic efficiency rather than reaction rates because nanoparticles offer significantly reduced mass transfer resistance as a result of the shortened diffusional path of substrates compared to large-sized porous materials [20]. However, these studies have not considered that the aggregation of nanoparticles may change their exposure surface, porosity, and stability, leading to altered diffusional paths and substrate accessibility to the immobilized enzyme.

The aim of the present study was to assess laccase immobilized on different carbon nanomaterials, MWNTs, O-MWNTs, GO, and fullerene (C_{60}) as a biocatalyst for the degradation of bisphenol A (BPA) and catechol as model phenolic contaminants. To immobilize the laccase, physical adsorption was used rather than covalent bonding because changes in enzyme structure and activity directly reflect the surface-induced non-specific interactions between the enzyme and nanomatrix. To the best of our knowledge, no reports have systematically evaluated the effects of the carbon nanomaterial on the activity of immobilized laccase and how these interactions affect the degradation of phenolic substrates.

2. Materials and methods

2.1. Materials

Sublimed C_{60} powder (purity > 99.5%) was purchased from SES Research (Houston, TX). MWNTs and O-MWNTs were purchased from Chengdu Organic Chemical Company (Sichuan Province, China). GO (purity > 99%) was purchased from Plannano Technology Company (Tianjin, China). Laccase (EC 1.10.3.2, from *Trametes versicolor*), BPA (purity > 97%), catechol (purity > 99%), and 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTs, purity > 98%) were purchased from Sigma-Aldrich (China). All other chemicals and solvents used were of analytical grade or higher.

The surface elemental compositions of nanoparticles were determined by X-ray photoelectron spectroscopy (PHI-5000 Versa Probe, Japan). The ζ potentials were measured by electrophoretic mobility using a ZetaPALS (Brookhaven Instruments, Holtsville, USA). The Brunauere Emmere Teller (BET) surface areas were measured using an ASAP 2010 Accelerated Surface Area and Poresimetry System (Micromeritics Co., USA), and the surface area was calculated using the multipoint adsorption and desorption data of N_2 at 77 K in the relative pressure range of 10^{-7}–1. The physicochemical properties of the carbon nanoparticles are listed in Table 1.

Table 1
Elemental compositions, zeta potentials, and Brunauer–Emmett–Teller (BET) surface areas of various nanoparticles.

Nanoparticles	Elemental composition[a] C%	O%	Zeta potential[b] (mV)	BET surface area (m^2/g)[c]
C_{60}	98.91	1.09	−12.73	10.96
GO	65.50	32.88	−12.53	145.16
MWNTs	97.40	2.60	−19.05	113.71
O-MWNTs	96.11	3.89	−16.93	165.64

[a] Analyzed by X-ray photoelectron spectroscopy.
[b] Measured by electrophoretic mobility using a zetasizer.
[c] Determined by N_2 adsorption using the BET method.

2.2. Adsorption experiments

Nanoparticles were added to 0.1 M potassium phosphate buffer (pH 7.0) to a final concentration of 1000 mg/L. To increase the dispersion of the nanoparticles, each suspension was ultrasonicated (150 W, 40 kHz) for 6 h. Then, 1 mL of the sonicated sample was dispensed into a microcentrifuge tube and exposed to 50 μL of freshly prepared enzyme solution with final concentrations of 0.5 mg/mL, 1 mg/mL, 1.5 mg/mL, 2.0 mg/mL, and 2.5 mg/mL. The mixture was shaken for 1 h at 160 rpm at room temperature, as adsorption equilibrium is achieved after 30 min of incubation. The samples were subsequently centrifuged at 7100*g* for 5 min, and the supernatants were removed. Three washes with 1 mL of phosphate buffer (0.1 M, pH 7.0) were performed to remove unbound enzyme. All supernatants were analyzed for protein content using the bicinchoninic acid method (BCA) [14]. The amount of enzyme loaded onto the nanoparticles was determined by measuring the difference in the concentration of enzyme in solution before and after exposure to the nanoparticles.

2.3. TEM study

After the adsorption experiments, 1 mg of the nanoparticle–laccase conjugate was resuspended in 1 mL of phosphate buffer and diluted with 50 mL of distilled water. The samples were prepared by air-drying a drop of suspension onto a copper TEM grid (Electron Microscopy Sciences, USA). The morphology of the samples was examined using a JEOL-2010 transmission electron microscope (JEM-2010 FEF, JEOL, Japan).

2.4. Enzyme activity assays

Laccase activity was determined by monitoring the oxidation of ABTs to the cation radical ($ABTs^{+\bullet}$) at 420 nm [21]. The assay mixture contained 0.5 mM ABTs prepared in 0.1 M potassium phosphate buffer at pH 7.0, and the temperature was set at 25 °C; 50 μL of free laccase solution (10 mg/mL) was added to 1 mL of assay mixture in quartz cuvettes and immediately mixed by inversion. The change in absorbance was examined every 5 s over a period of 60 s at 420 nm using a UV–vis spectrometer (TU-1810, Persee Co., China). One unit (U) of laccase activity was defined as the amount of $ABTs^{+\bullet}$ produced by 1 g of enzyme per minute.

To immobilize laccase, 1.5 mg of nanoparticle–laccase conjugate (enzyme loading of 0.5 mg laccase/mg nanoparticle) was immersed into a 1.5 mL assay mixture. After 60 s, the reaction was terminated by the addition of 10 μL of concentrated hydrochloric acid (12 M). Then, the assay mixture was centrifuged at