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

Ran Pang, Mingzhu Li, Chengdong Zhang*

College of Environmental Science and Engineering, Ministry of Education Key Laboratory of Pollution Processes and Environmental Criteria, Tianjin Key Laboratory of Environmental Remediation and Pollution Control, Nankai University, Tianjin 300071, China

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A B S T R A C T

Carbon nanoparticles are promising candidates for enzyme immobilization. We investigated enzyme loading and laccase activity on various carbon nanoparticles, fullerene (C60), multi-walled carbon nanotubes (MWN Ts), oxidized-MWN Ts (O-MWN Ts), and graphene oxide (GO). The loading capacity was highest for O-MWN Ts and lowest for C60. The activity of laccase on various nanomatri ces using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTs) as a substrate decreased in the following order: GO > MWN Ts > O-MWN Ts > C60. 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 10.2% or 12.3%, 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 enantioslectivity. They determined that lipase immobilized onto multi-walled carbon nanotubes (MWN Ts) exhibited exceptionally high activity, whereas lipase immobilized onto oxidized MWN Ts (O-MWN Ts) exhibited low activity with high enantioslectivity.
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 (C60) 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 C60 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 Brunauer–Emmet–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 N2 at 77 K in the relative pressure range of 10⁻⁷–1. The physicochemical properties of the carbon nanoparticles are listed in Table 1.

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 7100g 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 FE, JEOL, Japan).

2.4. Enzyme activity assays

Laccase activity was determined by monitoring the oxidation of ABTs to the cation radical (ABTs⁺) 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⁺ 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
where the turnover number (also termed \( k_{\text{cat}} \)) is defined as the maximum number of molecules of substrate that an enzyme can convert to product per catalytic site per unit of time and can be calculated using the following equation:

\[
k_{\text{cat}} = \frac{V_{\text{max}}}{[E]_{T}}
\]

where \([E]_{T}\) is the total concentration of enzyme (mM).

2.6. Enzymatic degradation of phenolic compounds

Laccase solution (1 mL) containing approximately 0.5 mg of free laccase or 1.5 mg of nanoparticle–laccase conjugate (enzyme loading of 0.5 mg laccase/mg nanoparticle) was added to 20 mL of BPA (10 mg/L) or catechol (10 mg/L) solution. ABTs was added as a mediator at a concentration of 0.5 mM. The mixtures were shaken at 160 rpm at 25°C. An aliquot (1.5 mL) of the reaction was removed at 0 h, 0.25 h, 1, 3 h, 6 h, 9 h, 12 h, 18 h, and 24 h, and 10 μL of concentrated hydrochloric acid was added to terminate the reaction. The samples were centrifuged at 11,100g for 5 min, and the concentrations of BPA and catechol in the supernatants were determined using high-performance liquid chromatography (Alliance 2695, Waters, USA) with a symmetry reversed-phase C18 column (3.5 μm, 4.6 mm × 150 mm) and Waters 2489 UV/visible detector. For BPA analysis, 50 μL of the solution was injected and eluted from the column at a flow rate of 0.8 mL/min using a mobile phase consisting of a 11:9 mixture of acetonitrile:water. BPA was detected at 224 nm. For catechol analysis, the sample was eluted with a 3:7 mixture of acetonitrile:water at a flow rate of 1 mL/min and detected at 206 nm. The reliability of HPLC methods for analysis of bisphenol and catechol were established through its linearity and limits of quantization (LOQs). The validation parameters are provided in Table S1. The chromatograms of the BPA and catechol analyses are shown in Fig. S1.

To detect adsorption of BPA/catechol onto nanoparticle–laccase conjugates 24 h after enzymatic incubation, the mixture was centrifuged at 11,100g for 5 min. The quantity of BPA/catechol in the supernatant was determined to be \( Q_{\text{f}} \). Ethyl acetate (5 mL) was added to the solid precipitate, which was extracted for 15 min with an efficiency of 100 ± 5%. The BPA/catechol in the ethyl acetate extracts was determined by HPLC as \( Q_{\text{c}} \). The initial content of BPA/catechol was \( Q_{0} \); therefore, the content that had been degraded (\( Q_{\text{d}} \)) would be equal to the following: \( Q_{\text{d}} = Q_{0} - Q_{\text{f}} - Q_{\text{c}} \).

2.7. Adsorption of BPA, catechol, and ABTs \(^{+} \cdot \cdot \) on nanoparticle–laccase conjugates

Phosphate buffer (1 mL, 0.1 M) containing 1.5 mg of nanoparticle–laccase conjugate (enzyme loading of 0.5 mg laccase/mg particle) was inactivated by addition of 10 μL of concentrated hydrochloric acid (12 M). Deactivated nanoparticle–laccase conjugates were obtained by centrifugation at 11,100g for 5 min and resuspended in phosphate buffer (1 mL). The deactivated enzyme conjugate solution was then added to 20 mL of substrate solution containing 10 mg/L BPA or catechol or 0.5 mM ABTs because of the undetectable feature of ABTs on UV–vis spectrophotometry. The adsorption of ABTs \(^{+} \cdot \cdot \) onto negatively charged conjugates (both laccase and nanoparticles were negatively charged at pH 7.0) was similar to or higher than that of ABTs because of electrostatic attraction.

2.8. Statistical analysis

Each experiment was performed in triplicate under each tested condition, and the values represent the means ± standard deviation of three experiments. Significant differences (\( P < 0.05 \)) between treatments were determined using one-way ANOVA. All statistical analyses were conducted using Statistical Packages for the Social Sciences (SPSS) Version 19.0.3.

3. Results and discussion

3.1. Adsorption isotherms of laccase on various carbon nanomaterials

The adsorption results of laccase on the four carbon nanomaterials are shown in Fig. 1. The adsorption data were fitted to the Freundlich isotherm, \( q = K_{F}C^{n}_{w} \), where \( q \) (mg/mg) is the equilibrium...
Fig. 1. Adsorption isotherms plotted as immobilized concentration (q) versus the aqueous phase equilibrium concentration (C) of laccase in the presence of 1000 mg/L of various nanoparticles at pH 7.0 in 0.1 M phosphate buffer. Error bars, in some cases smaller than the symbols, represent the standard deviations of three measurements.

Table 2
Summary of fitting parameters of adsorption isotherms.

<table>
<thead>
<tr>
<th>NPs</th>
<th>K_f (mg(^{-a}) mL(^{-1})/mg(^{b}))</th>
<th>n(^a)</th>
<th>R(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C60</td>
<td>11.89 ± 1.64</td>
<td>1.06 ± 0.06</td>
<td>0.99</td>
</tr>
<tr>
<td>GO</td>
<td>10.20 ± 0.11</td>
<td>0.89 ± 0.00</td>
<td>1.00</td>
</tr>
<tr>
<td>MWNTs</td>
<td>4.44 ± 1.54</td>
<td>0.56 ± 0.12</td>
<td>0.94</td>
</tr>
<tr>
<td>O-MWNTs</td>
<td>5.93 ± 0.99</td>
<td>0.63 ± 0.06</td>
<td>0.99</td>
</tr>
</tbody>
</table>

\(^a\) K_f and n were fitting parameters from the Freundlich model.

concentration of laccase on the nanomatrix, C_w (mg/mL) is the equilibrium concentration of laccase in aqueous solution, K_f (mg\(^{-a}\) mL\(^{-1}\)/mg\(^{b}\)) is the Freundlich affinity coefficient, and n (unitless) is the linearity index. The fitting parameters are summarized in Table 2. In general, the Freundlich model provided reasonably good fits to the data, confirming that laccase exhibited non-ideal adsorption on heterogeneous surfaces in addition to multilayer sorption. The relative positions of the isotherms in Fig. 1 indicate that the loading capacity was the highest for O-MWNTs and lowest for C60, and the same trend was observed for the surface area (Table 1). Interestingly, the surface area of O-SWNTs was almost 16 times higher than that of C60 whereas the enzyme loading onto O-SWNTs was only 1.6 times higher than that of C60 at the same initial concentration (0.06 mg/mL). Therefore, in addition to the surface area, other factors may influence the adsorption process.

The differences in adsorption capacity may be caused by different aggregation properties. For instance, carbon nanotubes (CNTs) tend to aggregate as bundles (Fig. 2A and B) because of Van der Waals interactions, the available sorption sites of CNT bundles include the surface area, the interstitial and groove areas formed between the CNTs [22]. GO has a large specific surface area (two accessible sides), and the abundant oxygen-containing surface functionalities (indexed by high oxygen content in Table 1), such as epoxide, hydroxyl, and carboxylic groups [23], and high water solubility increase the dispersibility of GO sheets (Fig. 2C). Nevertheless, Fig. 2D shows that C60 exhibited extensive agglomeration, with the agglomerates ranging from several hundred nm to several μm based on TEM images. Aggregation of spherical C60 resulted in closed interstitial spaces, prohibiting the entrance of the enzyme.

In comparison with MWNTs, because of the introduction of polar carboxylic and hydroxyl groups (indexed by Fourier transform infrared transmission analysis in Fig. S2), O-SWNTs tend to form large agglomerates because of hydrogen bonding interactions (Fig. 2A). This bundling process results in a decrease in the exposed effective surface for adsorption, which explains the slight increase in adsorption capacity observed for O-MWNTs relative to C60. Notably, no clear tubular-like structure was visible in TEM images (Fig. 2A) because O-MWNTs were shortened and thinned as a result of the oxidation treatment [12].

The heterogeneity index (n) for each isotherm also reflected the heterogeneous properties of the adsorption space resulting from aggregation. For C60 agglomerates, the available adsorption space is the external surface, which is relatively homogeneous, and the value of n was thus close to 1.0. For MWNT and O-MWNT aggregates, in addition to the external surface, the interstitial and groove areas are also available for adsorption; thus, the adsorption surface was more heterogeneous and the values of n were much lower than 1.0. Based on the flat surface and good dispensability of GO, the value of n was determined to be 0.89 for the adsorption isotherm.

3.2. Residual activities and kinetic study of the immobilized enzyme

After immobilization, the residual activity of laccase decreased in the following order: GO > O-MWNTs > O-MWNTs > C60. Nearly 65.6% of the activity was retained for GO–laccase conjugates relative to free laccase, whereas only 10.64% of the activity was retained for the C60-immobilized enzyme (Fig. 3). We measured the kinetics to evaluate the affinity of the substrate towards immobilized laccase. Fig. S3 shows the Lineweaver–Burk plots of the immobilized laccase-catalyzed oxidation of the ABTs substrate. The calculated kinetic parameters from Fig. S3 are listed in Table 3.

K_M represents the affinity of the enzyme to its substrate, and when the K_M value is low, the affinity for the substrate is high. The K_M value for C60 immobilized-laccase was almost ten times higher than that of free laccase, whereas the K_M value for GO-immobilized laccase was similar to that of free laccase. The K_M values of nanoparticle–laccase conjugates increased in the following order: C60 > O-MWNTs > MWNTs > GO. In the present study, the K_M values of laccase immobilized to nanomaterials were on the same order of magnitude as those reported for nanoporous gold-immobilized laccase [24].

k_cat corresponds to the oxidation rate, specifically the effective first-order rate constant for the breakdown of the enzyme–substrate complex (catalytic rate constant or turnover number). The k_cat to K_M ratio represents the catalytic efficiency of the enzyme [25]. The k_cat values of laccase immobilized to the four different nanoparticles were lower than those of free laccase, and the catalytic efficiencies were 5.93%, 59.53%, 33.30%, and 26.55% for C60-, GO-, MWNT-, and O-MWNT-immobilized enzymes, respectively, compared to that of free laccase (Table 3).

The increase in K_M values and decrease in k_cat and k_cat/K_M values relative to free laccase were caused by either of the following: (1) conformational changes of the enzyme, resulting in a lower possibility of forming a substrate–enzyme complex, or (2) lower accessibility of the substrate to the active site of the immobilized enzyme [26]. The secondary structure of laccase was first investigated using circular dichroism spectroscopy. Fig. S4 shows that the secondary structures of laccase did not significantly change in the presence of various nanoparticles. Although many studies have reported the structural changes of enzymes during interactions with nanoparticles [14,27], the extent of the structural change may be affected by the nature of the enzyme and nanomaterial, the incubation conditions, and the interactions. Thus, in our study, substrate accessibility is a major concern. Compared to the porous and irregular surface of CNTs and C60 aggregates, the flat surface and two accessible sides of GO, in addition to its high dispersibility, would be favorable for substrate
access. Additionally, substrate accessibility can also be influenced by the orientation of the active site of the enzyme. If the active site is oriented towards a surface that prevents the substrate from accessing the active site, then decreased enzyme activity (reduced rate of product formation) will be observed. Based on the similar $K_M$ values of the GO-immobilized and free enzyme, we can infer that the binding pocket was not perturbed or blocked by GO. Moreover, GO was shown to facilitate electron transfer in metalloprotein-catalyzed oxidative reactions [28]. In general, the observation of the highest catalytic efficiency for laccase immobilized onto GO mainly resulted from the high accessibility of the substrate.

For C60, the $k_{cat}$ value decreased to 50%, whereas the $K_M$ value almost increased 10-fold, which may be the result of crowding. Using the same enzyme loading rate (0.5 mg laccase/mg nanoparticle), protein molecules were attached to the C60 surface in a more crowded manner because of its smaller surface area (less than 10% of that of other carbon nanomaterials). The binding pocket of the enzyme may have been blocked by other enzyme molecules, which would explain the significantly reduced substrate affinity compared to that of other carbon nanomaterials.

### 3.3. Removal of phenolic compounds by immobilized enzymes

The catalytic properties of laccase immobilized onto various carbon nanoparticles were further investigated using BPA and catechol as reducing substrates. The enzymatic degradation is
shown in Fig. S5, and the results from the kinetic models confirmed that the degradation of phenolic compounds obeyed the first-order rate kinetic model based on the high regression coefficient values ($R^2$, Table 4). The degradation rates ($k$) for both BPA and catechol exposed to various nanoparticles decreased in the following order: GO > MWNTs > O-MWNTs > C$_{60}$. These results are consistent with previously reported residual laccase activity with the ABTs substrate. However, to our surprise, even for GO-immobilized laccase, which retained 65.69% activity (Fig. 3) compared to the free enzyme, the kinetic rate was only approximately 10% of that of the free enzyme irrespective of the phenolic compound used as a substrate. The rate ratio $k_{\text{imm}}/k_{\text{free}}$ (effectiveness factor $\eta$) is regarded as the extent of diffusional limitation, where $k_{\text{imm}}$ and $k_{\text{free}}$ are the rates of the reaction catalyzed using the same enzyme concentrations with immobilized and free enzyme under identical conditions [2]. Using BPA as substrate, the $\eta$ values were 0.93%, 10.28%, 9.34%, and 3.73% for C$_{60}$-, GO-, MWNT-, and O-MWNT-immobilized laccase, respectively, which were much lower than those of the ABTs substrate. A similar trend was observed when catechol was used as a substrate. Therefore, diffusional limitation was a significant problem when nanoparticle–laccase conjugates were used to remove phenolic contaminants.

Mass diffusion includes both the external diffusion from the bulk solution to the surface of nanoparticle conjugates and the intraparticle diffusion inside porous supports. The external diffusion may be similar for ABTs and phenolic substrates because of continuous shaking. Intraparticle diffusion may be influenced by the adsorptive forces of solutes of low concentration interacting with carrier materials and porous diffusion. We first investigated the adsorption affinity of various substrates onto nanoparticle–laccase conjugates. Surprisingly, the adsorption of ABTs on GO–laccase conjugates was similar to that of BPA, and the adsorption of ABTs on MWNT–laccase conjugates was even higher than that of BPA (Fig. 4). The adsorption of catechol onto the four nanoparticle–laccase conjugates remained similar. The difference in the affinity of the substrate to the nanomaterial may not be primarily responsible for the significant reduction in the $\eta$ value with phenolic substrates. Therefore, porous aggregates of nanoparticles with high heterogeneity may create a tortuous pathway for the substrate inside the matrix, significantly contributing to the nanoparticle-induced diffusional limitation. This phenomenon is particularly pronounced at low substrate concentrations because porous diffusion can lead to different concentrations inside and outside the matrix.

The slow transfer of active intermediates and release of products may also lead to diffusional limitation. The degradation/removal of phenolic compounds by laccase is described using the following equations:

\[
4(Cu^{2+}) + \text{phenolic compounds} \rightarrow 4(Cu^{2+}) \text{laccase} + \text{radicals} + 4H^+ \quad (6)
\]

\[
4(Cu^{2+}) + O_2 + 4H^+ \rightarrow 4(Cu^{2+}) \text{laccase} + 2H_2O \quad (7)
\]

The phenoxyl radicals attack other phenolic compounds or couple with other phenoxy radicals to form other biodegradation products and/or oligomers [29]. These phenoxy radicals are short-lived and only effective close to the location where they are generated. To react with phenoxy radicals, the mass transfer of phenolic compounds from the bulk solution to the nanomaterial surface will govern the reaction rate (Fig. 5). Other factors, such as product inhibition, which occurs when the insoluble products generated via coupling reactions block the active site of enzyme, may also influence the reaction rate.

In contrast, ABTs is directly oxidized by laccase to the corresponding cation radical ($ABTs^{2+}$), which is detected as the reaction product. Hence, when using ABTs as a substrate, no further chain reactions occur and the diffusional limitation of intermediates is minimized. The ABTs cation radical is highly stable and has been speculated to function as a diffusible oxidant of the enzyme.
[15]. We also tested the assumption that the addition of ABTs as a diffusible mediator accelerates BPA removal as illustrated in Fig. 5; the kinetic parameters are summarized in Table 5, and the degradation data are shown in Fig. 6. As predicted, the values of the effectiveness factor \( \eta \) increased to 1.96%, 22.55%, 11.76%, and 8.82% for C60-, GO-, MWNT- and O-MWNT-immobilized laccase, respectively. In the presence of ABTs, the reaction rate for free laccase slightly decreased, which may have resulted from competition of ABTs with BPA for the active site. Interestingly, although 0.5 mM ABTs was added as a mediator in the presence of 10 mg/L BPA substrate, the reaction rates only increased by approximately 1 to 2-fold. Arboleda showed that the removal efficiency of free laccase increased from approximately 30% to 100% in the presence of 0.01 mM ABTs and 5 mg/L BPA [30]. These results suggest that the carbon nanomatrix may also involve an elector mediator system, such as diffusion limited induction by the adsorption of the ABTs cation radical onto the negatively charged nanoparticle surface, thereby providing a radical scavenger effect [31] for the carbon nanoparticles. Further investigation is required in this area.

Notably, although low reaction rates were detected for nanoparticle–laccase conjugates, the overall removal efficiency was not affected. In our study, after 24 h, the overall transformation efficiencies for BPA (excluding adsorption by matrix) were 97.7% and 98.9% in GO- and MWNT-immobilized systems, respectively (Fig. 6). Almost 100% of catechol was enzymatically transformed in the same two systems. Even for laccase-O-MWNTs, only 26.6% catalytic efficiency was observed in terms of ABTs oxidation, whereas the efficiency was 74.6% and 87.1% overall for transformation of BPA and catechol, respectively, after 24 h. These results suggest that laccase immobilized onto carbon nanomaterials can be active for long periods of time and that the reduced reaction rates did not lead to decreased efficiency when sufficient time was given.

### 4. Conclusion

Carbon nanomaterials have emerged as promising candidates for enzyme immobilization. Our results suggest nanoparticle–mediated effects on substrate accessibility and diffusional limitation need to be carefully considered, which may result in extended reaction times, low efficiency, and high economic cost. In particular, this problem is exacerbated when low concentrations of environmental contaminants are used.

However, nanoparticle-immobilized enzymes continue to be of great scientific and commercial interest because of their wide range of applications resulting from their unique advantages. Therefore, it is necessary to gain insight into the interface between the protein, the nanomaterial, and the bulk substrate solution. By manipulating the particle size, aggregation status, and enzyme density, the wealth of nano-immobilization techniques can be exploited.

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### Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at [http://dx.doi.org/10.1016/j.talanta.2014.07.045](http://dx.doi.org/10.1016/j.talanta.2014.07.045).

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