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Direct Electrochemistry of Laccase Immobilized on Au Nanoparticles Encapsulated-Dendrimer Bonded Conducting Polymer: Application for a Catechin Sensor

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The direct electrochemistry of laccase was promoted by Au nanoparticle (AuNP)-encapsulated dendrimers (Den), which was applied for the detection of catechin. To increase the electrical properties, AuNPs were captured in the interiors of the dendrimer (Den-AuNPs) as opposed to attachment at the periphery of dendrimer. To prepare Den-AuNPs, the Au(III) ions were first coordinated in the interior of dendrimer with nitrogen ligands and then reduced to form AuNPs. The size of AuNPs encapsulated within the interior of the dendrimer was determined to be 1.7 ± 0.4 nm. AuNPs-encapsulated dendrimers were then used to covalently immobilize laccase (PDATT/ Den(AuNPs)/laccase) through the formation of amide bonds between carboxylic acid groups of the dendrimer and the amine groups of laccase. Each layer of the PDATT/Den(AuNPs)/laccase probe was characterized using CV, EIS, QCM, XPS, SEM, and TEM. The PDATT/Den(AuNPs)/laccase probe displayed a well-defined direct electron-transfer (DET) process of laccase. The quasi-reversible redox peak of the Cu redox center of the laccase molecule was observed at −0.03/ +0.13 V vs Ag/AgCl, and the electron-transfer rate constant was determined to be 1.28 s^{-1}. A catechin biosensor based on the electrocatalytic process by direct electrochemistry of laccase was developed. The linear range and the detection limit in the catechin analysis were determined to be 0.1−10 and 0.05 ± 0.003 μM, respectively. Interference effects from various phenolic and polyphenolic compounds were also studied, and the general applicability of the biosensor was evaluated by selective analysis of real samples of catechin.

The direct electrochemistry of proteins is an essential process that occurs in biological systems,[1] and the development of electrochemical biosensors that do not require a mediator are based on these electrochemical properties.[2] However, the direct electron transfer (DET) of laccase is generally difficult to observe because the redox-active site tends to be buried within the large protein matrix. Only a few studies have been able to demonstrate the DET of laccase using electrode surface modification with the 3-mercaptopropionic acid self-assembled monolayer[3] or an anionic exchange resin bound to polystyrene. [4] However, no attempt has been made thus far to develop a biosensor that directly utilizes the DET process of laccase. Stable immobilization and favorable orientation of laccase at the electrode surface could provide an opportunity to study the DET process of laccase and to apply a practical biosensing device. Thus, it is necessary to design the stable surface structure of laccase demonstrating the DET process successably to fabricate a third-generation biosensor that is simple in design and results in highly sensitive responses.

Laccase catalyzes the oxidation of the 1,2-dihydroxybenzene group in flavonoids. Of the many flavonoids, catechin is an effective anticancer agent that has been shown to exhibit antimutagenic, antidiabetic, hypocholesterolemic, antibacterial, and anti-inflammatory qualities. [5] Thus, novel electroanalytical methodologies for sensing catechin will be required to study the bioavailability and pharmacokinetics of catechin in human body fluids. Until now, methods often used for chromatographic[6] catechin detection include high-performance liquid chromatography,[7] liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry,[8] and gas chromatography.[9] However, these analytical methods require expensive, bulky, and sophisticated instruments and need to follow a separation process prior to catechin detection. Thus, a simple, sensitive, and stable method is demanded for the detection of catechin. There have been some reports on enzyme-based biosensors for the detection

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of catechin.[10−12] However, these biosensors still suffered from low stability, sensitivity, and specificity. Thus, there is a strong demand for development of a sensitive and stable catechin biosensor.

To increase sensitivity and stability, we have used a gold nanoparticle (AuNP)-containing dendrimer (Den) as the sensor probe. Dendrimers[13,14] are synthetic branched polymers with layered architectures that show promise in several biomedical applications.[15] The unique properties of dendrimers, such as structural homogeneity, integrity, controlled composition, and multiple terminal chain ends available for subsequent conjugation reactions, expand its use as the building block for nanostructures.[16−18] The large numbers of functional surface terminal groups enable synthetic modification of the molecularly ordered nanostructures,[16,19] which might be a promising platform for the fabrication of biosensors with increased sensitivity. However, using only the dendrimer as a probe for electrochemical sensing would not be adequate due to its low conductivity. Thus, the conductivity of the sensor probe using the dendrimer must be increased. To increase the conductivity, dendrimers can be modified with AuNPs. AuNPs are widely used in biotechnology[20] due to their unique physical and chemical properties.[21] Den-AuNP nanocomposites show great promise because the combination of physical and chemical properties of AuNPs and the surface reactivity of dendrimers can be utilized together. Dendrimer-AuNP conjugation is typically done by attaching the AuNPs to the periphery of the dendrimer. Recently, we have immobilized AuNPs on the surface amine groups of poly(amidoamine) (PAMAM) dendrimers, which were used for ultrasensitive DNA and protein detections.[22] However, attachment of AuNPs on the periphery of dendrimers has limited applications because the conductivity of the dendrimer is not significantly increased and the particle size of AuNPs cannot be controlled. On the other hand, encapsulation of AuNPs in the interior of the dendrimer (Den-AuNPs) can increase the conductivity of probes and the size of AuNPs can be controlled by using the dendrimer core as a nanotemplate. However, the application of Den-AuNPs for the study of direct electrochemistry has never been demonstrated nor has the potential use of this nanocomposite as biosensor probe, although the synthesis of AuNPs in the interior of dendimer has been reported.[23] A crucial step in achieving these goals is the stable attachment of Den-AuNPs to an electrode substrate. Of several methods, the attachment of Den-AuNPs through covalent bonding is advantageous over other methods. However, the direct attachment of Den-AuNPs on the electrode surface could prove to be very difficult. To avoid this problem, one could covalently attach Den-AuNPs to a functionalized conducting polymer layer, which can be easily grown on the electrode surface through electropolymerization of the respective monomer. Conducting polymers (CPs)[24] with various functional groups[25,26] have been shown to be potential materials for the stable attachment of dendrimers and for the fabrication of biosensors.[25,27−29] Until now, there have been no reports in DET of laccase at the Den-AuNPs modified electrodes or of its application as a potential biosensor system.

In the present study, to immobilize dendrimer-encapsulated AuNPs effectively to an electrode substrate, we used a functionalized conducting polymer layer that had two amine groups (3′,4′-diamine-2,2′;5′,2″-terthiophene, (PDATT)). The PAMAM dendrimer-encapsulated AuNP was covalently immobilized to the PDATT conducting layer through a covalent bonding between the carboxylic acid-terminated groups of PAMAM dendrimer and the amine groups of PDATT. Laccase was then covalently immobilized to carboxylic acid terminated groups of PAMAM dendrimers. The PDATT/Den(AuNPs)/laccase-modified electrode was used to study DET of immobilized laccase for the fabrication of a catechin biosensor. Various experimental parameters affecting the catechin detection were optimized. In addition, the biosensor was applied to green tea and human urine samples for the detection of catechin and the validity of the sensor was evaluated through comparative analysis with the Folin-Ciocalteu (FC) method.

EXPERIMENTAL SECTION

Reagents. Laccase (EC 1.10.3.2, 50 units mg^{-1}) from *Rhus vernificera*, catechin, malvidin, resorcinol, rutin, gallic acid, quercitin, prodelphinidin, 1-ethyl-3 (3-(dimethylamino)propyl) carbodiimide (EDC), hydrogen tetrachloroaurate ($HAuCl_4$), and dichloromethane (99.8%, anhydrous, sealed under N_2 gas) were purchased from Sigma Co. Laccase was purified as described previously.[3] Tetrabutylammonium perchlorate (TBAP, electrochemical grade) was received from Fluka, purified, and then dried under vacuum at 10^{-5} Torr. A third generation ($G = 3$) poly(amidoamine) dendrimer (diameter, 3.6 nm) with surface terminated succinamic acid groups [PAMAM $(NHCOCH_2CH_2COOH)_{32}$] and sodium borohydride were obtained from Aldrich Co. A terthiophene monomer bearing diamine groups, 3′,4′-diamine-2,2′;5′,2″-terthiophene (DATT), was synthesized according to a previous report.[30] Phosphate-buffered saline solution (PBS) was prepared by modifying 0.1 M disodium hydrogen phosphate (Aldrich) with the admixture of 0.1 M sodium dihydrogen phosphate (Aldrich) with 0.9% sodium chloride. Citrate buffer solution was prepared with 0.1 M citric acid and 0.1 M sodium citrate and was used to prepare the laccase solution. Catechin and other flavonoid stock solutions were prepared in a methanol/water mixture (1:4). The

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real green tea samples were purchased from a local market and used for catechin detection. The tea samples were prepared as follows: ~1.0 g of tea leaves was stepped at 100 °C for 10 min in 20 mL of distilled water. After cooling for 5 min, samples were filtered through a 0.2-μm Millipore membrane filter. Human urine samples were obtained from a laboratory personal. The urine sample was used directly for catechin detection after filtration. All other chemicals were of extrapure analytical grade and used without further purification. All aqueous solutions were prepared in doubly distilled water, which was obtained from a Milli-Q water purifying system (18 M Ω cm).

Instruments. Laccase immobilized Au nanoparticles encapsulated-dendrimer bonded conducting polymer electrode (PDATT/Den(AuNPs)/laccase) (area, 7 mm^2), Ag/AgCl (in saturated KCl), and Pt wire were used as working, reference, and counter electrodes, respectively. Cyclic voltammograms (CVs) and amperograms were recorded using potentiostat/galvanostat, Kosentech model KST-P2. Quartz crystal microbalance (QCM) experiments were performed using a Seiko EG&G model QCA 917 and a PAR model 263A potentiostat/galvanostat. An Au working electrode (area, 0.196 cm^2; 9 MHz; AT-cut quartz crystal) was used for the QCM experiment. XPS experiments were performed using a VG Scientific Escalab 250 XPS spectrometer with a monochromated Al Kα source with charge compensation at KBSI (Busan). TEM images were obtained using a JEOL JEM-2010 electron microscope (Jeol High-Tech Co.) with an acceleration voltage of 200 kV. For obtaining high-resolution−transmission electron microscopic (HRTEM) images of prepared AuNPs encapsulated within dendrimers, the polyDATT/Den (AuNPs)-modified electrode was sonicated for 10 min in ethanol to collect prepared AuNPs from the interiors of the dendrimers. Impedance spectra were recorded with an EG&G PAR 273A potentiostat/galvanostat and a lock-in amplifier (PAR EG&G, model 5210) linked to a personal computer. The frequency was scanned from 100 kHz to 10 Hz at the open circuit voltage, acquiring 5 points/decade. The amplitude of a sinusoidal voltage of 10 mV was used. The Zview2 impedance software was obtained from Scribner Associates Inc.

Preparation of the Laccase Immobilized Sensor Probe. First, a PDATT film was grown on glassy carbon electrodes (GCE) through electropolymerization of the DATT monomer in a 0.1 M TBAP/CH_2Cl_2 solution by cycling the potential three times. After electropolymerization, the PDATT film-coated GCE was washed with CH_2Cl_2 to remove any remaining monomers from the electrode surface. Prior to electropolymerization, GCEs were polished with a 0.05-μm alumina/water slurry on a polishing cloth to a mirror finish, followed by sonicating and rinsing with distilled water. A 5.0 μM PAMAM (G3-$COOH_{32}$) dendrimer solution was mixed with a 10 mM EDC solution by stirring for 6 h to activate surface carboxylic acid groups of the dendrimers. The PDATT film-coated electrode was incubated for 6 h in the EDC treated-dendrimer solution to covalently attach the dendrimer to the PDATT film. The dendrimer attached PDATT-coated electrode was then immersed in a 0.1 M $HAuCl_4$ solution with stirring for 1 h. By this step, Au(III) ions were coordinated to nitrogen ligands in the interior of the dendrimer, which was covalently attached to the PDATT film. A Den-AuNPs attached-PDATT (PDATT/Den (AuNPs) electrode was then obtained by reducing Au(III) ions in the interior of dendrimer with a 1.0 M $NaBH_4$ solution for 20 min. The Au nanoparticles encapsulated-dendrimer bonded PDATT film-modified electrode was washed with distilled water and subsequently incubated for 12 h in a 0.1 M citrate buffer solution containing 50 U/mL purified laccase. The modified electrode was washed with distilled water two times to remove unbound laccase from the dendrimer surface. The schematic representation of the modified electrode is shown in Figure 1.

RESULTS AND DISCUSSION

Characterization of the Laccase Immobilized Probe. The formation of the PDATT film on a GCE was obtained through electropolymerization of a DATT monomer in a 0.1 M TBAP/CH_2Cl_2 solution using a potential cycling method (Figure 2a). The CV recorded during polymerization exhibited two oxidation peaks at +0.75 and +1.35 V versus Ag/AgCl during the first anodic scan, the first peak was due to the oxidation of the amine group to form the imine, and the second peak was due to the oxidation of the monomer to form the polymer.[26] The peak currents decreased as the cycle numbers increased, clearly demonstrating that the polymer film immediately formed after the oxidation of the DATT monomer at +1.35 V. A small and broad reduction peak of the polymer was observed at +0.48 V versus Ag/AgCl during the cathodic scan. The thickness of the polymer film after three potential cycles was ~150 nm, and the amount of PDATT determined by EQCM was 1.35 ± 0.31 μg at a frequency change of ~1.05 kHz. The surface coverage of PDATT was determined to be $(4.8 \pm 0.25) \times 10^{-9}$ mol/cm^2.

AuNPs prepared in the interior of the dendrimer were characterized by CV. Figure 2b shows CVs recorded for PDATT/Den(AuNPs) and PDATT/Den electrodes in a 0.1 M H_2SO_4 solution. The potential was scanned between −0.2 and +1.4 V at a scan rate of 0.1 V/s. An Au reduction peak was observed at +0.85 V versus Ag/AgCl in the case of PDATT/Den(AuNPs) electrode, which was not observed in the case of PDATT/Den, clearly indicating that AuNPs successfully formed in the interior of dendrimers. The third-generation PAMAM dendrimer used in this study has tertiary NH_2 groups inside and surface terminal COOH groups outside of the dendrimer. AuNPs cannot be formed outside of dendrimer particle, because the surface terminal COOH groups in dendrimer have negative charge, which repulse the negative charged AuNPs. On the other hand, AuNPs can be formed inside the dendrimer, because the tertiary amine groups inside the dendrimer can interact strongly with AuNPs. HRTEM images of AuNPs prepared without or with a dendrimer template are shown in Figure 2c and d, respectively. When AuNPs were prepared without a dendrimer template, the size of the AuNPs was much higher (15−20 nm) than those prepared with a dendrimer template. The diameter of the dendrimer was ~3.6 nm (third-generation PAMAM dendrimer), and from the HRTEM image, we confirmed the particle size of the AuNPs prepared with a dendrimer template was 1.7−2.0 nm, which clearly indicates that the AuNPs particle formed inside the dendrimer. The HRTEM image clearly shows that AuNPs were efficiently formed in the interior of the dendrimer without any aggregation. The average size of AuNPs when prepared with a dendrimer template was determined to be 1.7 ± 0.4 nm by HRTEM image.

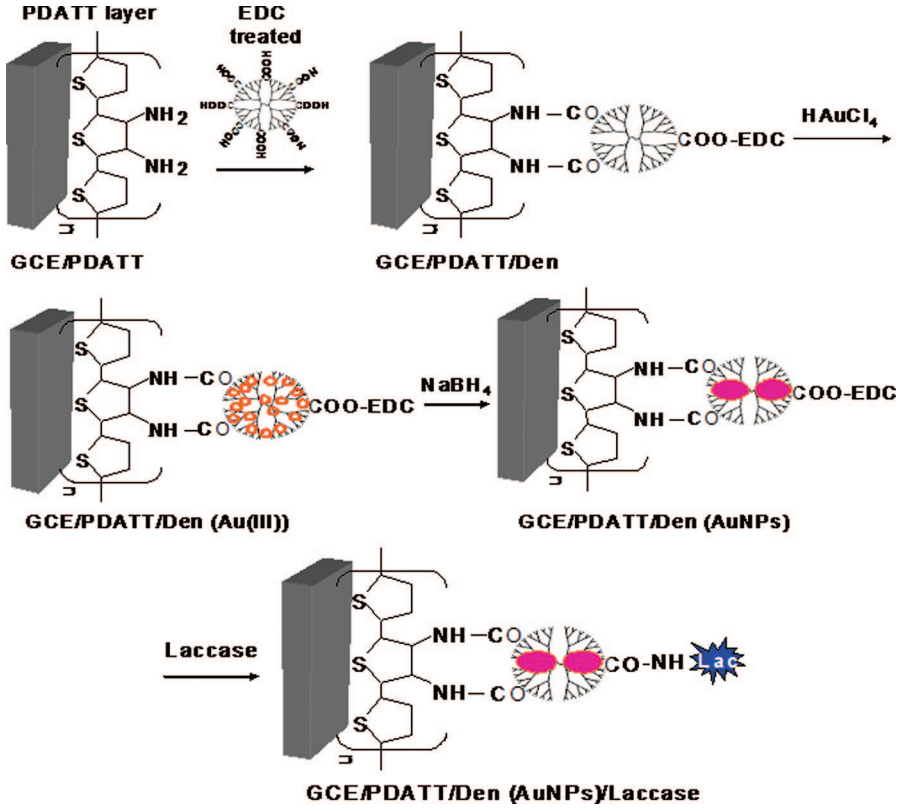

Figure 1. Schematic representation of the fabrication of PDATT/Den(AuNPs)/laccase-modified electrode.

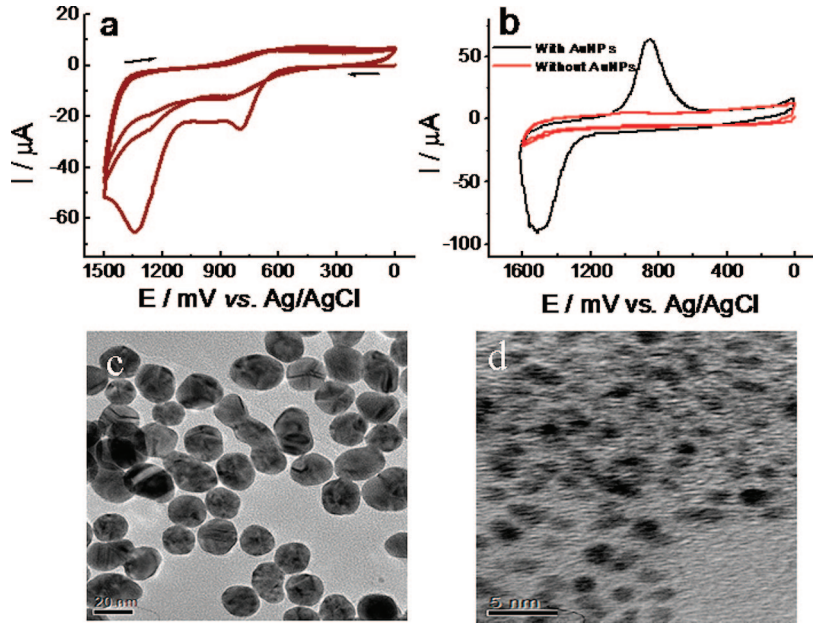

Figure 2. (a) CVs recorded during electropolymerization of 1.0 mM DATT monomer in a 0.1 M TBAP/CH_2Cl_2 solution at the scan rate of 100 mV/s; (b) CVs recorded for a PDATT/Den (AuNPs) (black line) and PDATT/Den (red line) modified electrodes in a 0.1 M H_2SO_4 solution at 0.1 v/s. TEM images obtained for the prepared AuNPs without (c) and with (d) dendrimer as a template.

Electrochemical impedance spectroscopy (EIS)[31] was used to further investigate the impedance changes of the electrode surfaces before and after dendrimers and dendrimer-AuNPs attachment to the PDATT layer. Figure 3 shows (a) Bode and (b) Nyquist plots obtained for PDATT (black, empty square), PDATT/Den (red, filled square), and PDATT/Den(AuNPs) (blue, empty circle) modified electrodes in a 0.01 M PBS solution at pH 7.0. The Bode plot indicated that the impedance value of a PDATT modified electrode increased after immobilization of the dendrimers. The immobilization of the dendrimers also resulted in an

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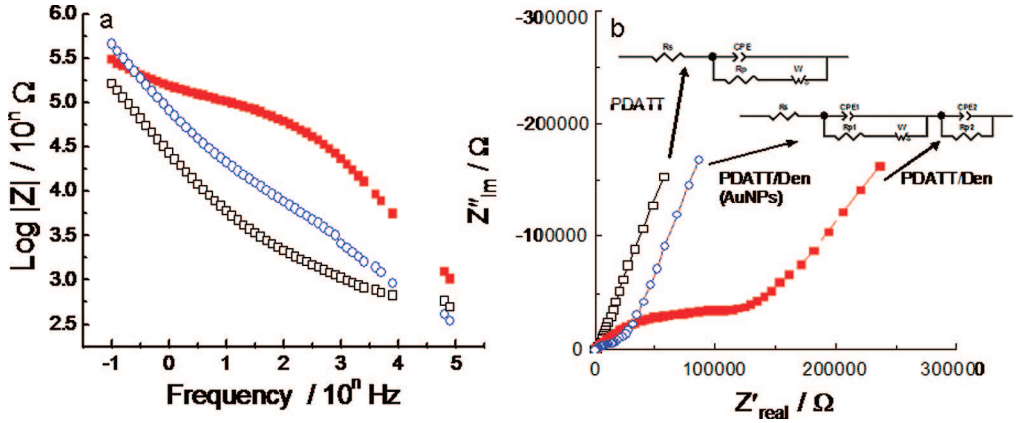

Figure 3. (a) Bode and (b) Nyquist plots obtained for a PDATT (empty square, black), PDATT/Den (filled square, red), and PDATT/Den(AuNPs) (empty circle, blue) probes in a PBS solution. Insets show the equivalent circuits for PDATT and PDATT/Den probes.

Table 1. Fitting Parameters of the Equivalent Circuit Elements Obtained before and after Dendrimer Immobilization onto PDATT and after AuNPs Encapsulation in the Interior of Dendrimer

circuit elements	PDATT film on GCE	after dendrimer immobilization onto PDATT film	after AuNPs encapsulation in Den/PDATT
R_s (Ω cm^2)	27.89	34	43
$Q_{dl}1$ (F cm^{-2} s$^{-(1-\alpha)}$)	2.33×10^{-17}	3.27×10^{-7}	2.21×10^{-15}
R_p1 (Ω cm^2)	8.70	1.05×10^5	1.771
$Q_{dl}2$ (F cm^{-2}s$^{-(1-\alpha)}$)		4.76×10^{-8}	1.16×10^{-6}
R_p2 (Ω cm^2)		28527	7201
α	0.86	0.77	0.79
χ^2	2.6×10^{-4}	1.0×10^{-4}	5.0×10^{-4}

increase of resistance of PDAT, providing further evidence that the impedance value increased after immobilization of the dendrimers. The difference in impedance values (Bode plot) before and after dendrimer immobilization is shown in Figure 3a. After encapsulation of AuNPs in the interior of the dendrimers, the impedance value significantly decreased, implying that AuNPs function as electron-conducting tunnels and facilitate electron transfer. The Nyquist plot (Figure 3b) clearly shows an increase in the charge-transfer resistance due to immobilization of the dendrimers, which then decreased significantly after the encapsulation of AuNPs in the interior of the dendrimers. A simple Randle circuit was utilized to fit the experimental impedance data for PDATT (inset of Figure 3b). However, PDATT/Den and PDATT/Den(AuNPs) probes had to be modeled with a more complicated Randle circuit because the impedance spectra for these probes exhibited two semicircles (Figure 3B). This indicates that the probe was composed of two different layers, PDATT and dendrimers. In the equivalent circuit, R_s represents the solution resistance, R_p1 and R_p2 represent the polarization resistances, W represents the Warburg element, and CPE1 and CPE2 are the constant-phase elements. Values for the parameters of R_s, R_p1, R_p2, CPE1, and CPE2 were obtained by fitting the experimental data to the equivalent circuit using Zview2 impedance software (Table 1). From this analysis, the PDATT modified electrode was shown to exhibit a low R_p value. After immobilizing the dendrimers, the R_p1 and R_p2 values for the PDATT/Den probe were found to be higher than that obtained for the PDATT probe, indicating that the dendrimer layer may be impeding the electron-transfer reaction. However, after encapsulation of the AuNPs, both R_p1 and R_p2 values significantly decreased, indicating that the AuNPs in the interior of the dendrimer made the PDATT/Den probe more conductive, thus facilitating the electron-transfer process. The above results clearly proved that Den-AuNPs were successfully immobilized onto the PDATT probe.

QCM experiments were conducted to determine the amount of dendrimer that immobilize onto PDATT and the amount of laccase that immobilized to PDATT /Den (AuNPs). Figure 4a shows the plots of frequency versus time obtained during immobilization of the dendrimers onto PDATT (dark cyan, triangle). The frequency decreased and reached a steady state after 2 h, indicating that immobilization of the dendrimers was completed within 2 h. After 2 h of immobilization, a 0.73-kHz frequency shift was observed. During immobilization of laccase onto PDATT/Den (AuNPs) (wine, square), the frequency shift also attained a steady state after 2 h, where a 0.55-kHz frequency shift was observed. The amount of dendrimer and laccase immobilized was determined to be 0.80 ± 0.02 and 0.60 ± 0.04 μg, which corresponded to 7.9×10^{-11} and 5.5×10^{-12} mol/cm^2, respectively.

To further characterize these modified surfaces, XPS analysis was performed (Figure 4b). The survey spectrum obtained for the PDATT modified surface only showed C 1s and N 1s peaks. The two C 1s peaks observed at 284.8 and 286.0 eV corresponded to C−H, C−S, or C−C bonds and C−N bonds, respectively. The two N 1s peaks observed at 399.7 and 400.8 eV were due to the presence of $-NH_2$ and for =NH groups, respectively. After immobilization of the dendrimers, the XPS spectrum of PDATT/Den surface showed an additional O 1s peak along with the previously observed C 1s and N 1s peaks. The O 1s peaks observed at 532.3 and 533.2 eV and were due to the presence of the surface carboxylic acid groups of the dendrimer. The N 1s peak at 399.7 eV for $-NH_2$ disappeared, indicating the formation of an amide bonds between the $-NH_2$ groups of the polymer and the COOH groups of the dendrimer. The survey spectrum for the PDATT/Den surface after encapsulation of the AuNPs showed two sharp peaks at 83.50 and 87.3 eV. These peaks corresponded to Au 4f7 and Au 4f5, respectively. In addition, Au 4d5, Au 4d3, and Au 4p3 peaks were also observed at 334.4, 353.3, and 546.4 eV, respectively. Furthermore, the N 1s peak at 400.8 eV (for =NH groups) shifted to a higher energy of 401.9 eV. These results clearly indicate that AuNPs were encapsulated in the interior of the dendrimer. After immobilization of laccase onto PDATT/Den

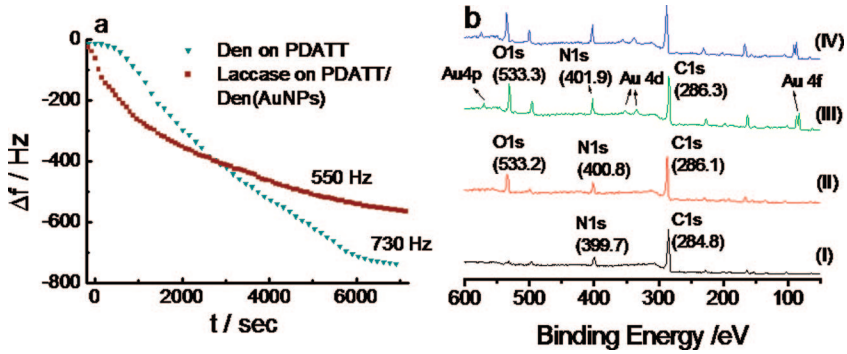

Figure 4. (a) QCM analysis during dendrimer immobilization on the PDATT film (triangle, dark cyan) and laccase immobilization on PDATT/Den (AuNPs) (square, wine) probes. (b) XPS analysis for PDATT (black, (I)), PDATT/Den (red, (II)), PDATT/Den(AuNPs) (green, (III)), and PDATT/Den(AuNPs)/lacacse (blue, IV) modified electrodes.

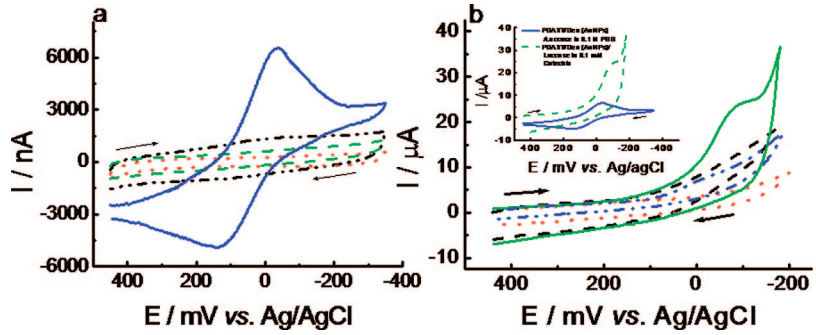

Figure 5. (a) Cyclic voltammograms recorded for a PDATT (red, dotted line), PDATT/Den (green, dashed line), PDATT/Den/laccase (black, dashed dotted line), and PDATT/Den (AuNPs)/laccase (blue, solid line) in 0.1 M PBS of pH 7.0. (b) Cyclic voltammograms recorded in PBS containing 0.1 mM catechin for a PDATT/Den (black, dashed line), PDATT (red, dotted line), PDATT/Den (AuNPs)/laccase (green, solid line), and PDATT/Den/laccase (blue, dashed dotted line) modified electrodes. Inset shows CVs recorded (blue, solid line, in the absence of catechin; and green, dashed line, in the presence of 0.1 mM catechin) for the catalytic current response by DET of laccase.

(AuNPs), the C 1s peak at 286.3 eV in the survey spectrum shifted to a higher energy of 287.5 eV, and the atomic percent of the N 1s peak increased from 5.05 to 9.17, indicating that laccase was immobilized through the formation of amide bond between the $-NH_2$ groups of laccase and the COOH groups of the dendrimer.

Direct Electrochemistry of Immobilized Laccase and Its Catalytic Activity. Figure 5a shows CVs recorded for PDATT (red, dotted line), PDATT/Den (green, dashed line), PDATT/Den/laccase (black, dashed dotted line), and PDATT/Den (AuNPs)/laccase (blue, solid line) in a 0.1 M PBS at pH 7.0. A redox peak was observed at approximately −0.04/+0.14 V versus Ag/AgCl for a PDATT/Den/laccase modified electrode. This redox peak was not observed for PDATT and PDATT/Den modified electrodes. These results indicate that the redox peak comes solely from immobilized laccase onto PDATT/Den, which might have been related to the electron-transfer reaction by copper in laccase. In the case of other small proteins, such as cytochrome *c*, covalent immobilization on only a PTTCA layer also showed the DET process.[32] However, the redox peak of laccase was not observed when it was directly immobilized onto PDATT or PTTCA through covalent bonding, most likely due to improper orientation of the protein. However, in the presence of AuNPs in the interior of the dendrimers, the Cu redox peaks of laccase were observed at −0.03/+0.13 V and the peak currents were found to be significantly higher. This result reveals that dendrimer encapsulated AuNPs can act as an efficient electron promoter for the redox reaction of Cu in laccase. The direct electrochemistry of the immobilized laccase was also examined in the presence or absence of oxygen. The peak current of laccase redox peaks increased significantly in the presence of oxygen without a change in the peak potential. The peak current was about three times higher than that obtained in the absence of oxygen, indicating that the oxygen facilitated the direct electron transfer of laccase. The formal potential of the laccase redox reaction was determined to be +0.05 V from the average peak potentials. However, this value was less positive when compared to that obtained from the DET of laccase from the 3-mercaptopropionic acid self-assembled monolayer.[3] The peak currents were directly proportional to the scan rate up to 0.4 V/s, indicating that the electrode reactions involved in the surface confined process.[33] The peak separation of the redox couple increased with an increase in scan rate up to 0.4 V/s, indicating that the redox reaction was quasi-reversible. The electron-transfer rate constant (K_s) for the redox reaction of laccase was determined using the Laviron equation.[34] The K_s value was determined to be 1.28 s^{-1} at the scan rate of 0.05 V/s. This value of K_s was on same order of magnitude as the value obtained for a self-assembled monolayer modified electrode.[3]

The electrocatalytic behavior of PDATT/Den (AuNPs)/laccase toward catechin detection was studied. Figure 5b shows CVs recorded for a PDATT/Den(AuNPs)/laccase modified electrode in a PBS solution containing 0.1 mM catechin (green, solid line). The modified electrode (PDATT/Den/laccase) even without

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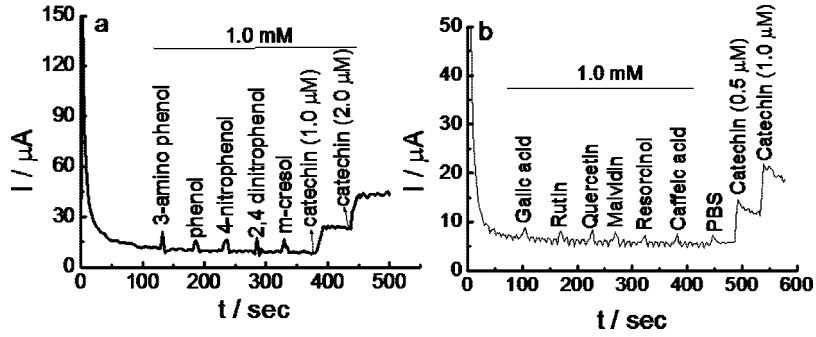

Figure 6. Interference effects from (a) phenolic and (b) polyphenolic compounds.

AuNPs also displayed a small and broad reduction peak for catechin (black, dashed line). The reduction peak at about −0.08 V was proportional to the catechin concentration. In contrast, the PDATT (red, dotted line) and PDATT/Den (blue, dashed dotted line) modified electrodes showed no response in the presence of catechin. The reduction peak at −0. 08 V may have been due to the electrocatalytic reduction of *o*-quinone generated from the oxidation of catechin by DET of laccase. Without the DET of laccase, there was no catalytic reduction current. The cathodic peak was enhanced about five times, which appeared at the similar potential of the reduction peak of DET of laccase, and the anodic peak was found to be decreased, confirming an electrocatalytic process by DET[35] of laccase for catechin detection.

Optimization of Experimental Parameters for Catechin Detection. The experimental parameters for the detection of catechin with a PDATT/Den (AuNPs)/laccase-modified electrode were optimized in terms of pH, temperature, and applied potential. The effect of pH was studied over the pH range of 4.0−8.0 in a PBS buffer solution containing 0.1 mM catechin. The reduction current gradually increased from pH 4.0 to 6.5 and then decreased at pH values higher than 6.5. The maximum reduction current was observed at a pH of 6.5. Thus, 6.5 was chosen as optimum pH, which was a little higher than that determined in a previous study.[12] The effect of temperature on the detection of catechin was studied between 10 and 70 °C. The response was found to be gradually increased as the temperature was increased from 10 to 30 °C, but no additional significant changes were observed between 30 and 55 °C. However, the current response rapidly decreased from 55 to 70 °C due to the deactivation of laccase. Thus, the optimal temperature for the detection of catechin was chosen as 30 °C. In addition, the effect of applied potential on the detection of 0.1 mM catechin was also examined in the chronoamperometric experiment. The current response increased as the applied potential varied between +0.2 and −0.3 V. The maximum response was observed at −0.1 V and the application of more negative potentials up to −0.3 V did not increase the current response. Thus, the PDATT/Den (AuNPs)/laccase modified electrode was polarized at −0.1 V in subsequent amperometric experiments.

Interference Effect. In order to assess the possibility of interference from some other phenolic and polyphenolic compounds, the current responses were measured amperometrically by injecting individual compounds (Figure 6). Common phenolic compounds, such as phenol, *m*-cresol, 3-aminophenol, 4-nitrophenol, and 2, 4-dinitrophenol, did not interfere in catechin detection. This was due to the fact that laccase does not catalyze the oxidation reaction of 3-aminophenol and monophenols. In addition, the fabricated sensor did not respond to other polyphenolic compounds, such as galic acid, rutin, quercitin, malvidin, resorcinol, and caffeic acid even at the 1.0 mM concentration. It is well-known that laccase also oxidized a wide variety of organic substrates including catechol. Thus, some catechol compounds were also examined for interference effect. The electrocatalytic reduction of oxidized catechol compounds by the DET of laccase was observed at the more positive potential of +50 mV. However, electrocatalytic reduction of oxidized catechin compounds was observed at about −100 mV. Thus, at this low potential, catechol did not interfere at the catechin detection at similar concentrations. At 100 times higher concentration of catechol than catechin, it significantly interferes with catechin detection. The catalytic current response of catechin decreased ~19% in the presence of 100 times higher concentration of catechol. However, in biological and plant food samples, compared to catechin, catechol presents at a very low concentration. Thus, the interference from catechol for the catechin detection is not significant in real sample analysis."

Calibration Plot. Chronoamperometric measurements of catechin were carried out with PDATT/Den(AuNPs)/laccase (solid line) and PDATT/Den/laccase (dashed line)-modified electrodes by successive additions of a catechin solution in PBS at pH 6.5 (Figure 7a). The modified electrodes were polarized at −0.1 V. The reduction currents achieved 95% of steady-state currents within 10 s after the catechin solution was introduced. The sensitivity of the PDATT/Den(AuNPs)/laccase electrode was shown to be much higher than that of PDATT/Den/laccase. Figure 7b shows calibration plots for catechin detection. Under the optimized concentration, the steady-state currents revealed a linear relationship with catechin concentrations in the range from 0.1 to 10 μM and 0.5 to 10 μM for PDATT/Den(AuNPs)/laccase and PDATT/Den/laccase-modified electrodes, respectively. These linear dependencies of the catechin concentration yielded regression equations of I_p (μA) = (6.4 ± 0.67) + (9.6 ± 0.13) [C] (μM) and I_p (μA) = (4.2 ± 0.32) + (2.14 ± 0.06) [C] (μM) with the correlation coefficients of 0.997 and 0.996, respectively. The reproducibility expressed in terms of the relative standard deviation (RSD) was ~4.3% at a catechin concentration of 0.5 μM. The detection limit for catechin was determined to be 0.05 ± 0.003 and 0.30 ± 0.05 μM in the presence and absence of AuNPS, respectively, which were based on three measurements of the standard deviation of the blank noise (95% confidence level, k =

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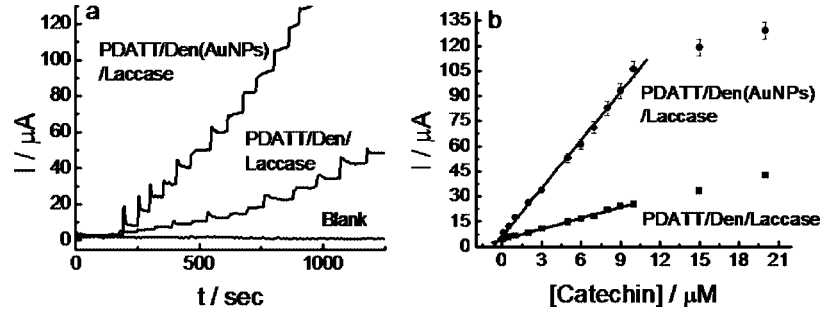

Figure 7. (a) Chronoamperometric measurements with PDATT/Den/laccase and PDATT/Den (AuNPs)/laccase by successive additions of catechin in a PBS solution at pH 7.0 and (b) Calibration plots obtained for PDATT/Den/laccase and PDATT/Den (AuNps)/laccase for catechin detection. Applied potential, −0.1 V.

3, $n = 5$). The values of the detection limit were much lower than previously reported for *Coriolus versicolor*[11] and *Cerrena unicolor*[12] laccase based ampermetric biosensors.

Stability of the Catechin Sensor. The stability of the catechin sensor was determined by measuring the response three times a day for two months. From these experiments, no significant decrease in catechin detection was observed when the sensor was stored in a phosphate buffer solution at 4 °C. These results indicate that the catechin biosensor exhibited not only high sensitivity but also long-term stability. For a period of two months, the biosensor retained more than 92% of its initial response. In addition, the stability of the biosensor to multiple uses was assessed by repetitively using a modified electrode in a 0.1 M phosphate buffer solution. In these experiments, the electrode lost only 4.6% of the initial response in approximately 30 continuous measurements. The superior stability of the catechin biosensor might be ascribed to the stable immobilization of large amounts of laccase on the PDATT/Den-AuNPs electrode.

Real Sample Analysis. To investigate the applicability of the proposed biosensor for a wider range of samples, it was used to detect catechin in commercial green tea samples. We chose green tea as a test sample for catechin determination because green tea is known to contain ample amounts of catechin and has known for its anticancer activity.[36] Green tea samples were twenty-times diluted, and then 1 mL of this solution was added to a 9 mL PBS at pH 7.0. The standard addition method was followed by introducing various concentrations of catechin ranging between 1.0 and 5.0 μM. The catechin concentration in 200 times diluted green tea samples was determined to be 4.3 ± 0.69 μM. This detected amount was comparable to that obtained from the FC method (4.7 ± 0.85 μM). The strong agreement between our method and FC method indicates that the biosensor developed in this study can be used in real green tea samples for accurate catechin detection. The catechin concentration was also determined in human urine samples. However, catechin was not detected in normal human urine samples, therefore spike and recovery tests of catechin were performed. The known concentration of catechin solutions in urine samples was spiked and the calibration method was then used to determine its concentration. For 0.1, 0.5, and 1.0 μM catechin concentration spikes in a urine sample, catechin concentration recovery was between 93 and 105%. The concentration of catechin in the spiked urine samples was also analyzed by the FC method. The result obtained with the proposed catechin biosensor was in good agreement with the result obtained from the FC method (Table 2). The result presented in Table 2 clearly indicates that the proposed catechin biosensor can be used in real biological samples for detecting submicromolar concentration of catechin.

Table 2. Spike and Recovery Results of Catechin in Urine Samples with the Proposed Biosensor and the Folin-Ciocalteu Method

spiked (μM)	recovery by proposed biosensor method (μM)	recovery by Folin-Ciocalteu method (μM)
0.1	0.095 ± 0.003	0.097 ± 0.002
0.5	0.49 ± 0.016	0.495 ± 0.011
1.0	0.98 ± 0.07	0.99 ± 0.04

CONCLUSIONS

The direct electrochemistry of laccase was achieved at the PDATT/Den(AuNPs) probe, which was used for fabrication of a third-generation catechin biosensor. The surface of PDATT/Den(AuNPs) was characterized using CV, EIS, QCM, XPS, SEM, and TEM techniques. The redox process of immobilized laccase was found to be a quasi-reversible process and the standard rate constant of the direct electron transfer of laccase was determined to be 1.28 s^{-1}. The catechin biosensor exhibited a wide linear range (0.1−10 μM), a low detection limit (0.05 ± 0.003 μM), a short response time (less than 10 s). The RSD value was determined to be 4.3% at a catechin concentration of 0.5 μM. In addition, other phenolic and polyphenolic compounds did not interfere with catechin detection. The wider application of the proposed sensor was evaluated by the detection of catechin in real green tea samples, and the amounts detected were comparable to those obtained from the FC method. The above observations strongly suggest that the biosensor developed in this study might be a promising tool for the detection of catechin in food, beverage, and biological samples.

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