

Natural Molybdenite- and Tyrosinase-Based Amperometric Catechol Biosensor Using Acridine Orange as a Glue, Anchor, and Stabilizer for the Adsorbed Tyrosinase

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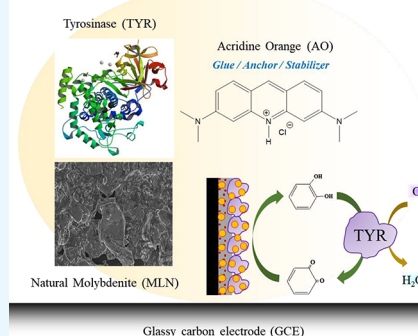
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ABSTRACT: To develop a natural mineral-based electrochemical enzyme biosensor, natural molybdenite (MLN), tyrosinase (TYR), and acridine orange (AO) were coadsorbed onto a glassy carbon electrode (GCE). The developed TYR/AO/MLN-GCE-based amperometric TYR biosensor exhibited excellent performance for highly sensitive determination of catechol (linear range, 0.1–80 μM ; sensitivity, 0.0315 $\mu\text{A}/\mu\text{M}$; LOD, 0.029 μM ; response time, <4 s) with good reproducibility and good operational and storage stabilities. The electrochemical impedance spectroscopy (EIS) and quartz crystal microbalance with dissipation (QCM-D) revealed interesting roles of AO: (1) an efficient glue for enhancing the amount of the adsorbed TYR on the MLN-GCE, (2) an anchor for efficient orientation of the adsorbed TYR on the MLN-GCE, and (3) a stabilizer providing a suitable microenvironment for the adsorbed TYR on the MLN-GCE surface. This physical adsorption-based AO-coupled enzyme-modification strategy onto natural MLN would be a versatile strategy to develop cost-effective and environment-friendly natural mineral-based electrochemical biosensors and bioelectronic devices.

Natural mineral-based electrochemical enzyme biosensor



INTRODUCTION

Molybdenum disulfide (MoS_2) is a layered two-dimensional (2D) nanomaterial with unique optical, electrical, and mechanical properties and has been applied to various devices (e.g., nanoelectronics, optoelectronics, and sensors).^{1–3} Among them, the combination of biomolecules and MoS_2 is attractive, because we can expect the synergy effect of excellent biospecificity of biomolecules and unique properties of MoS_2 . Up to now, various biomolecules/ MoS_2 hybrid modified electrodes have been designed to develop MoS_2 -based electrochemical enzyme biosensors. For example, glucose oxidase,^{4–6} horseradish peroxidase,⁷ cholesterol oxidase,^{8,9} acetylcholinesterase,¹⁰ hemoglobin,¹¹ and myoglobin¹² have been employed for this purpose. However, most of these MoS_2 -based biosensors are made from chemosynthetic pure MoS_2 .

Compared to the synthetic pure MoS_2 , natural molybdenite (MLN) from natural resources is cost-effective and has a high yield. In some cases, natural MLN possesses comparable (and/or more excellent) advantages to (over) the synthetic pure MoS_2 and is successfully applied for the development of excellent electrocatalysts for hydrogen evolution¹³ and anodes of lithium-ion batteries.¹⁴ Thus, we can expect that the combination of natural MLN and enzyme provides a new

biosensor platform using cost-effective and environmental-friendly natural resources.

Tyrosinase (TYR: monophenol; *o*-diphenol: oxygen oxidoreductase) catalyzes the oxidative conversion of a wide variety of mono- and diphenol compounds to corresponding *o*-quinones.¹⁵ As an attractive application of TYR for bioanalytical fields, the TYR-modified electrodes can be used for the determination of not only substrates (i.e., toxic phenol compounds)^{16–21} but also inhibitors such as respiratory toxins and pesticides.^{22–24} In addition, TYR is applicable to a bi-enzyme recycling-based sensitive sensing system.²⁵ Furthermore, TYR can be used as an indicator enzyme for electrochemical immunosensing²⁶ and electrochemical determination of coliforms.²⁷ Thus, we believe that TYR is one of the attractive biocatalysts to develop natural MLN-based novel enzyme sensors. However, there are a few reports on TYR/

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MLN-based electrochemical biosensors from our best knowledge.

In general, to immobilize enzymes on the matrix surface, (1) covalent linkage to the matrix via functional groups, (2) cross-linking using bi-functional coupling reagents, (3) entrapment in a hydrogel and smart polymer, and (4) physical adsorption onto the matrix are possible. Among them, physical adsorption is the simplest and can be done under mild conditions. However, upon the adsorption, enzymes often suffer from significant loss in activity due to unfolding processes on the surface.^{28–30}

In a previous study, we reported that the glucose oxidase (GOx) adsorbed on the carbon felt (CF) showed almost no apparent bioelectrocatalytic activity mainly due to an unfavorable conformational change and/or unfavorable enzyme orientation on the CF surface. However, when the GOx was adsorbed from the mixed aqueous solutions with phenothiazine dye (methylene blue), the adsorbed GOx showed sufficient bioelectrocatalytic activity.³¹ We concluded that MB plays an interesting role as a stabilizer to depress the surface-induced denaturation of the adsorbed GOx.²⁸ Furthermore, we reported the signal enhancement effect of acridine orange (AO) on TYR-immobilized CF-based flow amperometric biosensors.^{32,33} In these TYR/CF-hybrid systems, (i) AO prevented the surface-induced denaturation of adsorbed TYR on the highly hydrophobic CF surface.²⁹ (ii) AO enhanced the catalytic activity of TYR (both free TYR in the solution phase and the covalently immobilized TYR on the CF).³³

In this study, natural MLN, TYR, and acridine orange (AO) were coadsorbed onto a glassy carbon electrode (GCE) to develop a natural mineral/TYR-based electrochemical enzyme sensor. We evaluated the sensor performance by using catechol as a model analyte and found three interesting roles of AO: (1) an efficient glue for enhancing the amount of the adsorbed TYR on the MLN-GCE, (2) an anchor for efficient orientation of the adsorbed TYR on the MLN-GCE, and (3) a stabilizer providing a suitable microenvironment for the adsorbed TYR on the MLN-GCE surface. This physical adsorption-based AO-coupled enzyme-modification strategy would be a new protocol to develop simple and cost-effective enzyme sensors using environment-friendly natural minerals.

RESULTS AND DISCUSSION

Characterization of MLN and the Sensor Surfaces.

The components and phase characteristics of natural MLN were evaluated by using XRD (Figure 1). The three diffraction peaks at $2\theta = 14.4^\circ$, 44.1° , and 60.1° correspond to the (002), (006), and (110) planes of hexagonal MoS_2 (2H- MoS_2 , JCPDS no. 37-1492), respectively.³⁴ The XRF data revealed that the purity of MLN is about 86.5%, and the main impurities of MLN are SiO_2 , FeS_2 , and ZnS (Table 1).

The surface morphologies of the MLN-GCE, TYR/MLN-GCE, and TYR/AO/MLN-GCE were measured by FE-SEM (Figure 2). The layered structure of MLN, which is the typical characteristic of the layered MoS_2 , can be easily observed in Figure 2A. Thus, we can expect the characteristics based on the 2D structure of MoS_2 even for this natural MLN. In the case of the TYR/MLN-GCE (Figure 2B), the uneven parts seem to increase, which implies the modification of TYR on MLN. Especially, the invisible MoS_2 layered structure and thicker membrane-like morphology of the TYR/AO/MLN-GCE

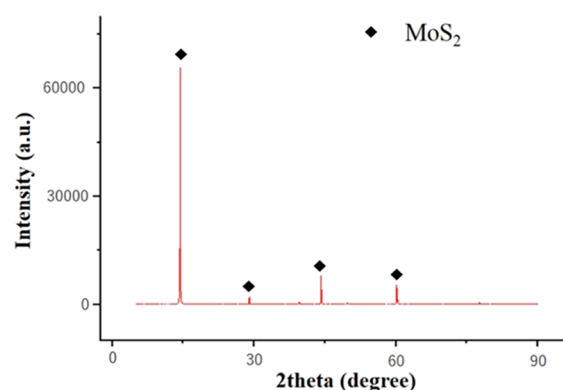


Figure 1. X-ray diffraction of natural MLN.

Table 1. XRF Analysis of MLN Powder

component	MoS_2	SiO_2	FeS_2	ZnS	other impurities
weight (%)	86.5	5.2	3.3	1.9	3.1

(Figure 2C) indicate the higher amount of immobilized TYR on the MLN surface with the aid of AO.

Raman spectroscopy has been widely applied to characterize the structural and physical properties of 2D-layered materials, such as graphene and MoS_2 .^{34,35} The shift in the peak position, the changes in the linewidth, and the Raman ratio can be used to analyze and evaluate the extrinsic interaction.³⁴ As displayed in Figure 3A, natural MLN (black) shows an E'_{2g} peak at 368.5 cm^{-1} and A_{1g} peak at 395.5 cm^{-1} with a peak separation (Δk) of 27 cm^{-1} , indicating that MLN has a 2H- MoS_2 crystal structure.¹⁴ As compared with MLN (black), the TYR-adsorbed MLN (TYR/MLN: blue) and TYR- and AO-coadsorbed MLN (TYR/AO/MLN: red) showed a blue shift in both E'_{2g} and A_{1g} peaks, in the order of 6 and 10 cm^{-1} , respectively. These results suggest that the adsorbed TYR on MLN leads to vibration hardening of the Mo–S bonding. The TYR/AO/MLN (red) shows a wider linewidth as compared with the TYR/MLN (blue) and MLN (black). The change in linewidth reflects the presence of varying force constants associated with structural changes between the inner and outer layers of the materials.³⁵ Thus, these results suggest that adsorbed AO and/or the adsorbed AO/TYR complex on MLN cause some kind of disorder factor influencing the crystal structure of MoS_2 . The Raman peak ratio is known to reflect the degree of crystallinity.¹⁴ As shown in Figure 3B, the Raman ratio (I_E/I_A) was decreased by the adsorption of TYR on MLN, and this tendency was more significant for the TYR/AO/MLN. Overall, from this Raman analysis, it can be speculated that the adsorbed TYR layers of the TYR/MLN and TYR/AO/MLN show different physico-chemical properties.

The Bioelectrocatalytic Activity of the TYR Adsorbed on the GCE Surface. As a measurement principle of this TYR-modified electrode, TYR catalyzes the oxidation of catechol to *o*-quinone in the presence of molecular oxygen, and the produced *o*-quinone is electrochemically reduced back to catechol and then enzymatically re-oxidized repeatedly (Figure 4B, inset). As a result, the sensor signal (the reduction current) is amplified due to the redox recycling driven by the enzymatic oxidation and the electrochemical reduction, which allows highly sensitive determination of catechol.^{16–18} Therefore, the response of the TYR-modified electrode would be

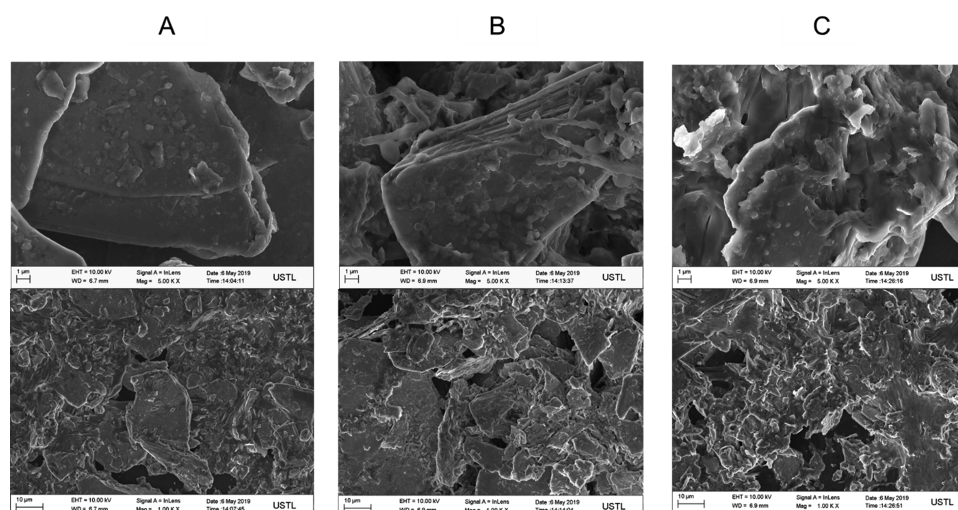


Figure 2. SEM images of (A) the MLN-GCE, (B) TYR/MLN-GCE, and (C) TYR/AO/MLN-GCE.

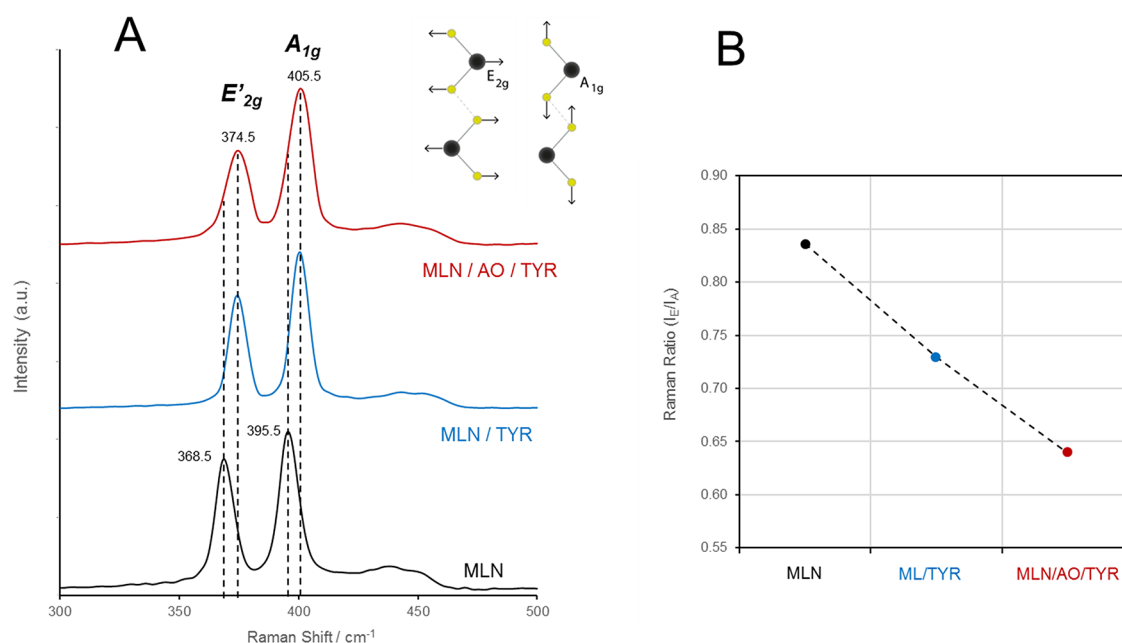


Figure 3. (A) Raman spectra of the MLN-GCE (black), MLN/TYR-GCE (blue), and MLN/AO/TYR-GCE (red). Inset: atomic displacement of two Raman active modes of bulk MoS₂ (A_{1g} and E_{2g}). Molybdenum atoms are in black and sulfur atoms are in yellow. (B) Calculated Raman ratio (E'_{2g}/A_{1g}) from the spectra in panel (A).

influenced by the following factors: (1) the bioelectrocatalytic activity of the TYR adsorbed on the MLN-GCE, (2) the diffusion of the substrate and product across the adsorbed layer, and (3) the electron transfer properties of the MLN-GCE. From this viewpoint, to confirm the effect of the MLN and AO adsorbed on the GCE on the electrochemical properties of GCE, we measured the cyclic voltammograms of the MLN-GCE, AO-GCE, and bare-GCE in catechol-dissolved electrolyte buffer solution. Figure 4A shows the cyclic voltammetry (CV) responses of the MLN-GCE, AO-GCE, and bare-GCE in air-saturated 0.1 M PBS containing 0.1 mM catechol. As compared to the bare-GCE, the AO-GCE tends to facilitate the electrochemical reduction of dissolved oxygen. However, concerning the electrochemical redox reaction of the catechol/*o*-quinone system, all electrodes showed almost the same responses. These results indicate that MLN possesses

excellent electrochemical property and does not show negative effects on the present system.

Figure 4B compares the CV curves obtained in air-saturated PBS containing 0.1 mM catechol at four TYR-modified electrodes. If TYR retains enough activity, then the reduction current of *o*-quinone should increase with the significant decrease in the oxidation current of catechol, resulting in an asymmetric-shaped voltammogram. Meanwhile, if TYR loses its activity on the electrode surface, then the voltammograms would be symmetric based on the reversible electrochemical redox reactions of the catechol/*o*-quinone system. As can be seen in the CV curve, the shape of the CV curve of the TYR-GCE (green) is almost symmetric, indicating that the activity of the TYR adsorbed on the bare-GCE (without MLN and AO) is very weak. In contrast, the shape of CV curves of the other three electrodes was asymmetric (i.e., the decrease in the oxidation current and the increase in the reduction current).

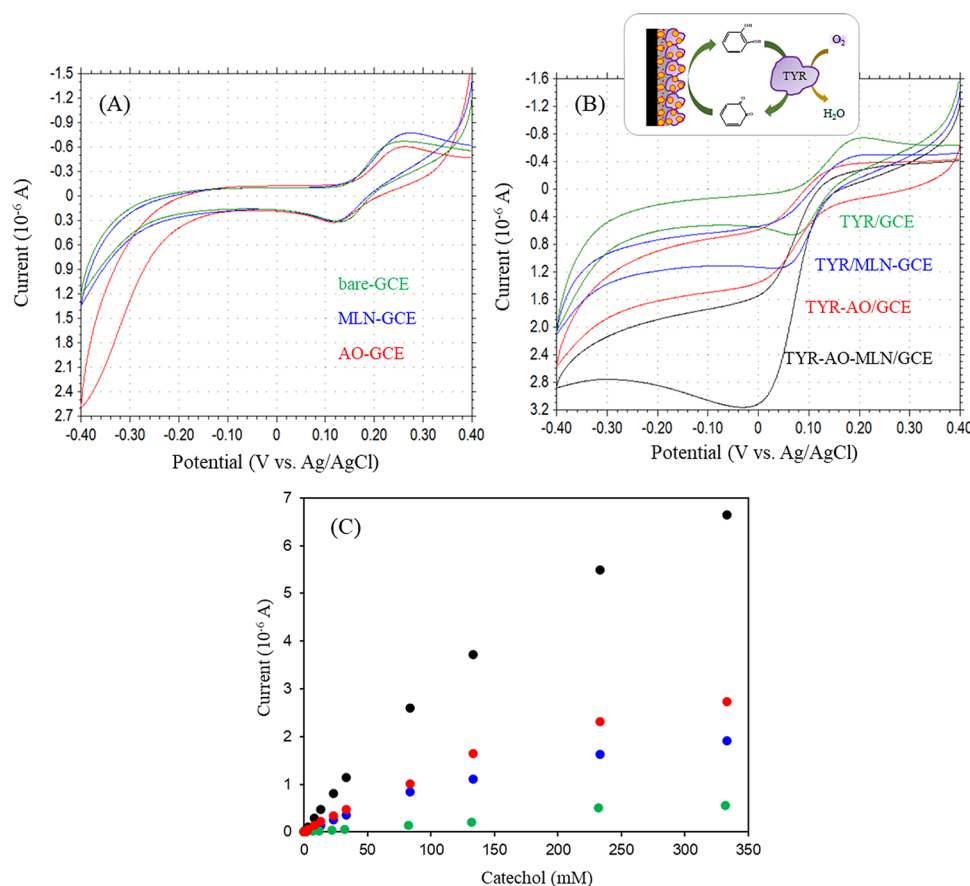


Figure 4. (A) Cyclic voltammograms of the AO-GCE (red), MLN-GCE (blue), and bare-GCE (green) in air-saturated 0.1 M PBS (pH 7.0) containing 0.1 mM catechol. The potential scan rate is 5 mV/s, and the starting potential is +0.4 V vs Ag/AgCl. (B) Cyclic voltammograms of the TYR/AO/MLN-GCE (black), TYR/AO-GCE (red), TYR/MLN-GCE (blue), and TYR-GCE (green) in air-saturated 0.1 M PBS (pH 7.0) containing 0.1 mM catechol. The measurement conditions are the same as in panel (A). The inset is the measurement principle of this sensor. (C) Relationship between the catechol concentration and the steady-state cathodic current response by four TYR-modified GCEs obtained by constant-potential amperometry at an applied potential of -0.05 V vs Ag/AgCl.

Among these three electrodes, the TYR/AO/MLN-GCE (black) showed the largest reduction current with almost complete disappearance of the oxidation current, which is the typical catalytic signal for the oxidation of catechol via TYR on the electrode.^{16,17} Meanwhile, the TYR/AO-GCE (red) and TYR/MLN-GCE (blue) showed smaller reduction current with a slightly asymmetric CV shape. These results suggest that both MLN and AO are essential to obtain larger bioelectrocatalytic current via TYR adsorbed on the GCE surface. In addition, the most notable features of this system are the synergy effects of AO and MLN to obtain a larger bioelectrocatalytic response.

To compare the sensor response more clearly, next, we measured constant potential amperometry at detecting the potential of -0.05 V vs Ag/AgCl. We selected this potential based on the CV curve (Figure 4B), and at this potential, we can reduce the background current arising from the direct electrochemical reduction of dissolved oxygen in air-saturated PBS. After the background current had reached a low level (10^{-8} A level), the standard solutions of catechol were added every 50 s into the stirring PBS to obtain amperometric $i-t$ curve responses. The current responses were rapid and the current reached another steady state in less than 4 s after the sample additions. Figure 4C illustrates the relationship between the catechol concentrations and the steady-state cathodic current responses obtained by four electrodes. The

ratio of the magnitude of the current responses to 330 μ M catechol of the TYR/AO/MLN-GCE (black), TYR/AO-GCE (red), TYR/MLN-GCE (blue), and TYR-GCE (green) was ca. 12:4.8:3.5:1. These results indicate that the coadsorption of AO with MLN is much effective to obtain a higher bioelectrocatalytic response for catechol via the adsorbed TYR on the GCE surface.

Evaluation of the Sensor Surface by Electrochemical Impedance Spectroscopy (EIS). EIS is a useful tool to evaluate interfacial properties of surface-modified electrodes.³⁶ The charge transfer resistance (R_{ct}), which can be quantified based on the diameter of the semicircular part of the Nyquist plot, is a useful parameter for evaluating the interfacial properties of the adsorbed protein layer on the electrode surface.³⁶ This evaluation is based on the following assumptions: (1) the electro-active species can directly diffuse to the bare spots on the electrode through pores and defects of the adsorbed layer, and (2) the electro-active species can permeate through the adsorbed layer and react on the electrode surface. Figure 5 represents the Nyquist plots of various GCEs obtained by using $[\text{Fe}(\text{CN})_6]^{4-/3-}$ as an electrochemical redox probe. The TYR/MLN-GCE (blue) and TYR-GCE (green) showed much larger R_{ct} values as compared with the MLN-GCE (brown) and bare-GCE (black), respectively. Thus, it is clear that TYR was surely adsorbed on bare-GCE and MLN, even though the responses

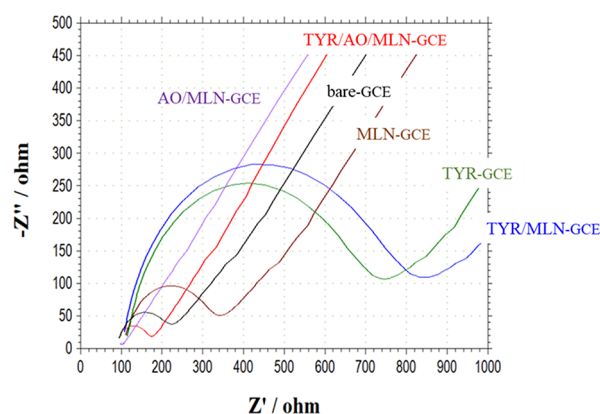


Figure 5. EIS Nyquist plots of the bare-GCE (black), MLN-GCE (brown), AO/MLN-GCE (purple), TYR-GCE (green), TYR/MLN-GCE (blue), and TYR/AO/MLN-GCE (red). The electrolyte is N_2 -saturated 0.1 M PBS (pH 7.0) containing 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$. The potential was set to the formal potential of the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox system. The amplitude is 0.005 V. The frequency is from 0.1 Hz to 10 kHz. Concentrations of each species in adsorption solution: $[\text{MLN}] = 10 \text{ mg/mL}$ (dispersion); $[\text{AO}] = 0.5 \text{ mM}$; $[\text{TYR}] = 0.25 \text{ mg/mL}$.

of the TYR-GCE and TYR/MLN-GCE were much smaller than that of the TYR/AO/MLN-GCE (see Figure 4A,B). On the other hand, differing from the prediction, the R_{ct} of the TYR/AO/MLN-GCE (red) was smaller than those of the bare-GCE (black) and MLN-GCE (brown). At pH 7.0, AO ($\text{pK}_a \approx 10.4$) exists in cationic form. Thus, it can be considered that the electrostatic interaction between the negatively charged $[\text{Fe}(\text{CN})_6]^{3-/4-}$ and cationic AO would facilitate the electron transfer of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ and/or permeation of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ across the adsorbed TYR/AO layer, resulting in a smaller R_{ct} . This prediction is also supported by the fact that the AO/MLN-GCE (purple) showed the smallest R_{ct} .

In general, proteins tend to lose their native conformations upon adsorption on the surface, especially on the hydrophobic surface.^{28–30} Based on the molecular dynamic (MD) simulation and the water contact angle experiment, a MoS_2 nanosheet has a hydrophobic and low-friction surface.³⁷ Furthermore, the structure and conformation of proteins are changed upon adsorption on MoS_2 .³⁸ Separately, upon the adsorption onto a hydrophobic graphite surface, larger “soft

proteins” such as bovine serum albumin loses their secondary structure almost completely.³⁹ The TYR used here is a tetrameric protein with a molecular mass of 120 kDa, composed of two subunits of $\sim 43 \text{ kDa}$ (H subunit) and two subunits of $\sim 14 \text{ kDa}$ (L subunit).⁴⁰

Consequently, based on the observation of the CV and the constant-potential amperometry (see Figure 4) and EIS (Figure 5), the following predictions might be possible: (i) the adsorbed TYR on the bare-GCE loses most of its catalytic activity, probably due to the unfavorable change in conformation and/or unfavorable adsorbed orientation, and (ii) the adsorbed TYR on MLN also loses its catalytic activity, but the magnitude of this negative effect is not so much severe compared to direct adsorption onto the bare-GCE. In contrast, when AO was used as a coadsorbent for TYR and MLN on the GCE surface, the adsorbed TYR showed much excellent bioelectrocatalytic activity (Figure 4B,C). Thus, most remarkable results emerged from above observations. Is that why the existence of AO during the TYR adsorption enhances the bioelectrocatalytic current response of the TYR/AO/MLN-GCE?

Adsorption Behavior of TYR Evaluated by Quartz Crystal Microbalance with Dissipation (QCM-D). QCM-D is a useful tool for monitoring the mass of adsorbed species via change in the resonance frequency, ΔF , and for getting information about the viscoelasticity of the adsorbed layer by measuring the dissipation factor, D .^{41,42} For the adsorbed proteins, QCM-D provides not only the mass but also the thickness, the conformational change of the proteins, and the level of hydration of the films of biomolecules.^{43,44}

From this viewpoint, we studied the effect of AO on the adsorption of TYR to MoS_2 with the use of QCM-D equipped with a MoS_2 sensor tip. As shown in Figure 6A, for the adsorption of TYR alone, based on the adsorption and desorption curve, the net ΔF was ca. 6.5 Hz, which corresponds to the mass of the adsorbed TYR. Meanwhile, for the adsorption of the TYR/AO mixture (Figure 6B), the time to reach a plateau seems to be longer, and the ΔF was ca. 16 Hz, which is ca. 2.5 times larger than the case of TYR alone. Even considering the molecular weight of AO and TYR, more than two times larger ΔF suggests that the presence of AO in TYR adsorption solution enhances the mass of the adsorbed

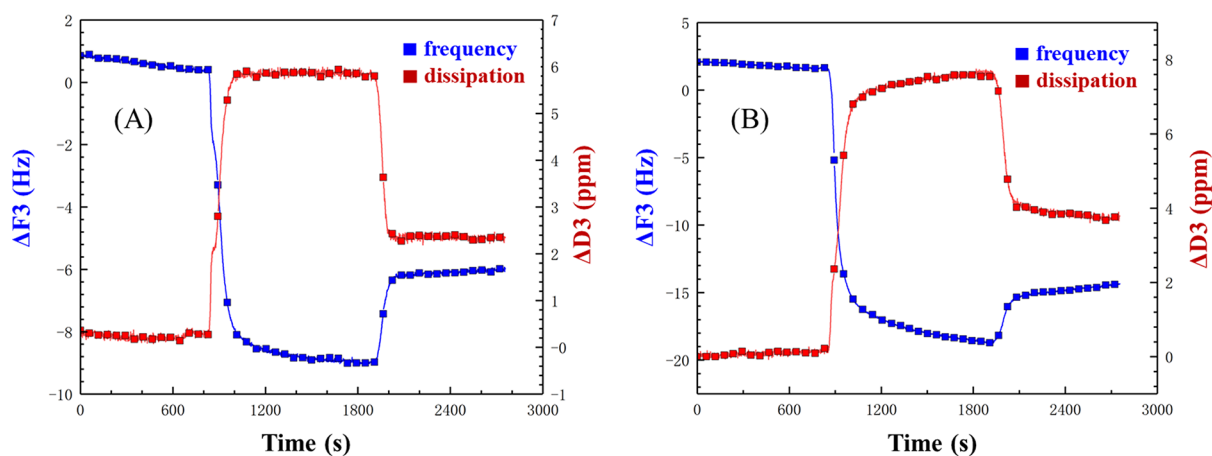


Figure 6. QCM-D responses for the adsorption of (A) TYR alone and (B) TYR/AO mixture on the MoS_2 surface in 0.1 M PBS (pH 7.0). Changes in frequency and dissipation at 15 MHz. The experiment was conducted at 25 °C ($[\text{TYR}] = 0.25 \text{ mg/mL}$; $[\text{AO}] = 0.5 \text{ mM}$). The carrier and sample flow rate was 100 $\mu\text{L/min}$.

TYR layer. At pH 7.0, AO ($pK_a \approx 10.4$) exists in cationic form, and TYR ($pI \approx 4.3$) has a net negative charge.⁴⁵ A maximum protein adsorption is often found at pH values close to pI of the protein, because electrostatic repulsion of neighboring proteins is reduced at pI .^{43,44} TYR from *Agaricus bisporus* mushroom that is used in this study contains two H subunits of ~ 392 residues and two L subunits of ~ 150 residues.⁴⁰ According to the amino acid sequence information in this literature, the H subunit contains 41 positively charged residues (Arg, 10; His, 13; Lys, 18) and 48 negatively charged residues (Asp, 26; Glu, 22). The L subunit contains 13 positively charged residues (Arg, 6; His, 1; Lys, 6) and 14 negatively charged residues (Asp, 8; Glu, 6). Although all charged amino acids do not necessarily exist on the protein surface, the TYR surface charge contribution is as follows: 108 of positively charged amino acid residues and 124 of negatively charged residues at pH 7.0. Thus, if the binding of cationic AO to the anionic amino acid residues leads to a change in pI of the AO-TYR complex, then it would influence the mass of the adsorbed TYR. Indeed, it is known that AO binds to some enzymes and influence their activities.^{46,47}

By the way, not only the amount but also the orientation of enzymes at the interface also influences the response of enzyme electrodes.⁴⁸ Aromatic dyes readily adsorb on the hydrophobic surface. It has been reported that phenothiazine dyes (toluidine blue O) do act as an anchor for efficient orientation of hydrogenase at the graphite electrode.⁴⁹ From the above experimental results and the literature's information, we predict that the adsorbed AO on the surface and/or bound AO to TYR would have the following possible roles: (1) an efficient glue for enhancing the amount of the adsorbed TYR on MoS_2 and (2) an anchor for efficient orientation of the adsorbed TYR on MoS_2 .

As described above, the change in energy dissipation (ΔD) of QCM-D provides useful information (e.g., thickness, conformation, and hydration state) about the adsorbed protein layer. The adsorption/desorption kinetic curves of ΔD indicate that the ΔD value for the TYR/AO mixture ($\Delta D = 3.9$ ppm) was ca. 1.7 times larger than that for TYR alone ($\Delta D = 2.4$ ppm). These results suggest that the thickness of the TYR/AO layer is ca. 1.7 times thicker than that of the TYR layer. Furthermore, the $\Delta D/\Delta F$ value for the TYR/AO layer was 370×10^{-9} , and that for the TYR layer was 240×10^{-9} . A smaller $\Delta D/\Delta F$ value for the TYR layer suggests that TYR is more rigidly attached to MoS_2 . This prediction supports the result of EIS: i.e., the TYR/MLN-adsorbed surface showed a larger R_{ct} of EIS than the TYR/AO/MLN-adsorbed surface (see Figure 5). Therefore, it is reasonable to assume that the TYR adsorbed on MoS_2 (or MLN) might lose its activity due to a surface-induced conformational change (e.g., spreading on the surface), leading to a thinner, rigidly bound (compact), and less hydrated layer. In contrast, in the presence of AO, the adsorbed TYR forms a thicker, less compact, and more hydrated layer, which is supported by larger ΔD and $\Delta D/\Delta F$ values and a smaller R_{ct} value of EIS. These results lead us to predict the third role of AO: AO may do act as a stabilizer, providing a more flexible and suitable microenvironment for the TYR layer on the MLN-GCE to keep its native activity.

Optimization and Analytical Characteristics of the TYR/AO/MLN-GCE-Based Sensor. Experimental variables, which can affect the sensor performance, were investigated based on the steady-state amperometric response toward $10 \mu M$ catechol. For the adsorption parameters, the MLN

concentration, TYR concentration, and adsorption time were optimized (Figure 7A–C). As a result, 10 mg/mL MLN, 0.25

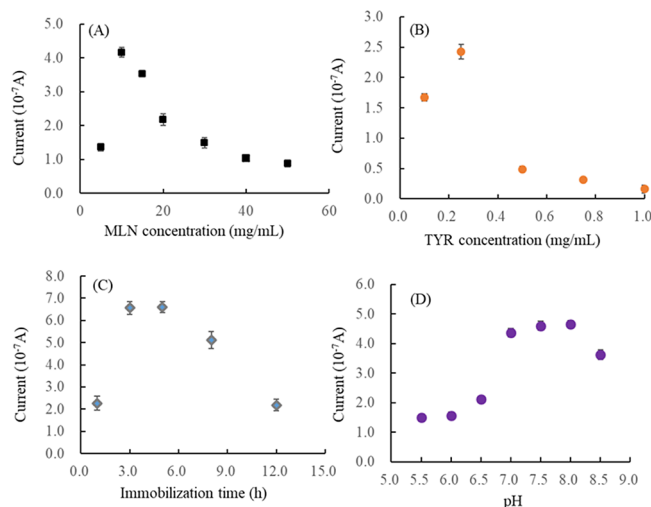


Figure 7. Effect of (A) the MLN and (B) the TYR concentrations in adsorption solution and (C) the immobilization time for adsorption and (D) electrolyte pH on the amperometric current response for $10 \mu M$ catechol. [MLN] = 10 mg/mL (for panels B–D); [TYR] = 0.25 mg/mL (for panels A, C, and D); electrolyte pH = 7.0 (for panels A–C). The applied potential is -0.05 V vs Ag/AgCl.

mg/mL TYR, and 3 h adsorption time were chosen as the optimum for the following experiments. For the measurement condition, the effect of the electrolyte pH was investigated (Figure 7D). Differing from ordinal TYR-based biosensors with optimum pH at around 6.5 to 7.0 ,^{16–20} this TYR/AO/MLN-GCE-based TYR sensor showed the largest responses under the weak alkaline condition around 8.0 . Some kind of interaction between the natural MLN and TYR may influence its characteristic change.

We subsequently evaluated the analytical properties of the TYR/AO/MLN-GCE as an amperometric catechol biosensor. Figure 8 displays the typical current–time curve of the present biosensor on the successive additions of various concentrations of catechol in air-saturated PBS (pH 7.0) at an applied potential of -0.05 V vs Ag/AgCl. The steady-state cathodic

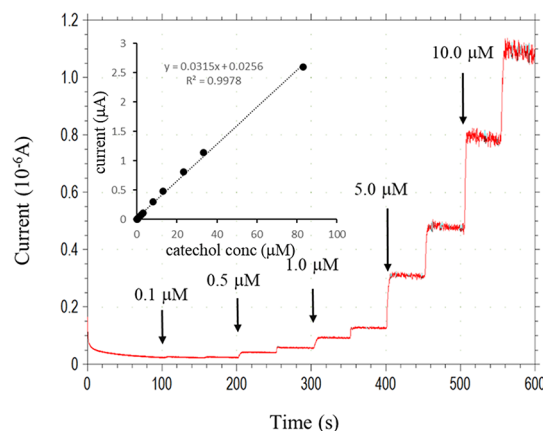


Figure 8. Amperometric current–time response curves of different concentrations of catechol obtained by the TYR/AO/MLN-GCE biosensor in 0.1 M air-saturated PBS (pH 7.0). The inset is the calibration curve of the TYR/AO/MLN-GCE biosensor.

background current changed rapidly upon the addition of catechol and reached another steady-state current within 4 s (to the 95% steady-state current). These results imply that diffusion of catechol, *o*-quinone, H⁺, and O₂ surrounding the adsorbed TYR on the MLN-GCE is relatively smooth, which contribute a rapid response for catechol. The present biosensor showed a linear range of catechol from 0.1 to 80 μM and the regression equation $I = 0.0315c + 0.0256$, with a coefficient of 0.9978. Although we can see the response to 0.1 μM catechol, the estimated limit of detection (LOD) using the sensitivity was 0.029 μM with a signal-to-noise ratio of 3 (noise level, 3 nA). Table 2 summarizes the performances of the reported

Table 2. Comparison of the TYR-Based Electrochemical Catechol Biosensors^a

TYR biosensor	linear range (μM)	LOD (μM)	ref
TYR/Au/GO-SPE	0.083–23	0.082	16
TYR/AuNPs/DHP-GCE	2.5–95	0.17	17
TYR/GO-GCE	0.05–50	0.03	18
TYR/BBND	5.0–120	3.28	19
TYR/ND-PS	5.0–740	0.9	20
TYR/PPy-GCE	0.002–100	0.002	21
TYR/AO/MLN-GCE	0.1–80	0.029	this study

^aGO, graphene oxide; SPE, screen-printed electrode; AuNPs, gold nanoparticles; DHP, dihexadecylphosphate; BBND, boron-doped nanocrystalline diamond; ND-PS, nanodiamonds–potato starch; PPy, polypyrrole.

TYR-based catechol biosensors compared with the present TYR/AO/MLN-GCE biosensor. Although the sensitivity of this sensor is not necessarily superior, this mineral-MLN-AO-based sensor shows comparable performance as compared to other fascinating nanomaterial-based systems. The effect of possible interferents on the response toward catechol was examined. After the steady-state current for 10 μM catechol had been obtained, 100 μM D-glucose, ascorbic acid, uric acid, creatinine, and various metal ions (i.e., Ca²⁺, Ba²⁺, Pb²⁺, Ni²⁺, Co²⁺, Cr³⁺, Ag⁺, and Fe³⁺) were added consecutively. These substances showed no negative interference toward the response of the sensor. The operational stability of the biosensor was assessed by measuring the successive addition of 1 μM catechol. After 10 additions, the electrolyte was replaced with fresh PBS, and the same measurements were repeated five times. The relative standard deviation (RSD) of the 50 successive measurements was 4.8%, indicating that this TYR/AO/MLN-GCE-based sensor has good operational stability. The reproducibility of the sensor preparation was checked by measuring 10 μM catechol with different sensors. The RSD of five different sensors was 4.5%, indicating the feasibility of this method. The stability toward long-term storage was examined. The biosensor retained 75% of the initial response after 2 weeks of storage under the dry state at 4 °C in a refrigerator. The responses to other TYR substrates (i.e., other catechol derivatives such as catecholamines and monophenol compounds) are now underway in our laboratory.

CONCLUSIONS

We have proposed a simple and versatile protocol to fabricate the natural MLN-based electrochemical enzyme biosensor by using AO as an effective glue, anchor, and stabilizer for the adsorbed TYR on the MLN-modified GCE. If this strategy can be adopted to other enzymes and proteins, then this physical

adsorption-based AO-coupled protein-modification strategy would be expected as one of the versatile tools to develop various bioelectronic devices using other various fascinating materials.

EXPERIMENTAL SECTION

Chemicals and Solutions. Tyrosinase (EC 1.14.18.1, 2687 units/mg, lot no. T3824) from mushroom, catechol, and acridine orange hydrochloride (AO) were purchased from Sigma-Aldrich (Shanghai, China). Natural molybdenite (MLN) was obtained from Yichun Luming Mining Co. Ltd. (Yichun, China).

Phosphate buffer solution (PBS, pH 7.0) was prepared using 0.1 M K₂HPO₄ and 0.1 M KH₂PO₄. Various concentrations of standard solutions of catechol were prepared with 0.1 M PBS daily and stored in amber bottles. All solutions were prepared using Milli-Q water (MING-CHE 24UV, Shenyang, China).

Apparatus. X-ray diffraction (XRD) measurements were performed with a Bruker D8 Advance diffractometer. X-ray fluorescence (XRF) experiments were carried out with an S8TLGER (Bruker). The morphologies of the modified electrode surfaces were observed using field emission scanning electron microscopy (FE-SEM, SIGMA-HD, ZEISS, Germany). Raman spectroscopy was performed using a laser micro-Raman spectrometer (JASCO NRS-4100, Japan) with an excitation wavelength of 532.0 nm laser with a working distance on a ×100 lens. The Raman spectra of MLN, MLN/TYR, and MLN/AO/TYR were recorded by depositing the samples on a GCE rod (3 mm in diameter and 5 mm in length). All electrochemical measurements such as cyclic voltammetry (CV), constant-potential amperometry, and electrochemical impedance spectroscopy (EIS) were performed with a CHI 660E workstation (Shanghai Chenhua, China). Quartz crystal microbalance with dissipation (QCM-D) measurements were performed with a Q-Sense analyzer (Biolin Scientific) equipped with a MoS₂-coated sensor tip.

Sensor Fabrication. Prior to surface modification, a glassy carbon electrode (GCE; 3.0 mm in diameter) was polished with 1.0, 0.3, and 0.05 μm α-alumina slurries to make a shiny surface. The cleaned GCE was rinsed and sonicated with distilled water and ethanol. The MLN suspension (10.0 mg/mL), AO, and TYR solutions were prepared using 0.1 M PBS (pH 7.0). The mixture (30 μL) of MLN, AO (0.5 mM), and TYR (0.25 mg/mL) was dropped onto the GCE surface and stood for 1 h at room temperature to adsorb MLN, TYR, and AO. Before measurements, the electrode was carefully rinsed with 0.1 M PBS. To evaluate the effectiveness of MLN and AO for the bioelectrocatalytic activity via the adsorbed TYR on the GCE, we prepared various electrodes: (i) only TYR was adsorbed on the GCE (TYR-GCE), (ii) TYR and MLN were coadsorbed on the GCE (TYR/MLN-GCE), (iii) TYR and AO were coadsorbed on the GCE (TYR/AO-GCE), (iv) TYR, MLN, and AO were coadsorbed on the GCE (TYR/AO/MLN-GCE), (v) only MLN was adsorbed on the GCE (MLN-GCE), (vi) only AO was adsorbed on the GCE (AO-GCE), and (vii) MLN and AO were coadsorbed on the GCE (AO/MLN-GCE).

Electrochemical Measurements. Electrochemical measurements (cyclic voltammetry, constant-potential amperometry, and electrochemical impedance spectroscopy) were performed with a conventional three-electrode system using 0.1 M PBS (pH 7.0) as an electrolyte at ambient temperature (20 °C). The TYR-modified GCE, Pt wire, and Ag/AgCl (sat.

KCl) were used as working, counter, and reference electrodes, respectively. CV for the evaluation of bioelectrocatalytic activity of the adsorbed TYR on the GCE was performed in air-saturated 0.1 M PBS (pH 7.0) containing 0.1 mM catechol over the potential range of -0.4 to $+0.4$ V vs Ag/AgCl (starting potential was $+0.4$ V) with a potential scan rate of 5 mV/s. Constant-potential amperometry was done at an applied potential of -0.05 V vs Ag/AgCl with continuous stirring of air-saturated 0.1 M PBS with a stirring bar (15 mm in diameter). After the steady-state background current had been obtained, a 50–100 μ L aliquot of a substrate standard solution was added to 15 mL of electrolyte PBS, and the current change was recorded. To compare the interfacial properties of the TYR/AO/MLN-GCE, TYR/MLN-GCE, TYR-GCE, MLN-GCE, AO/MLN-GCE, and bare-GCE surface, electrochemical impedance spectroscopy (EIS) was measured in deoxygenated 0.1 M PBS containing 5 mM $\text{K}_3\text{Fe}(\text{CN})_6$ and $\text{K}_4\text{Fe}(\text{CN})_6$. To remove the dissolved oxygen, pure nitrogen gas was bubbled into PBS for at least 20 min, and nitrogen gas was kept flowing over the solution during the electrochemical measurements. The applied potential was set at the formal potential of the $[\text{Fe}(\text{CN})_6]^{4-/3-}$ redox system (i.e., 0.238 V vs Ag/AgCl at pH 7.0). The frequency was from 0.1 Hz to 10 kHz.

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Notes

The authors declare no competing financial interest.

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