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An adhesive conducting electrode material based on commercial mesoporous titanium dioxide as a support for horseradish peroxidase for bioelectrochemical applications

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Abstract

An adhesive conducting electrode material containing of graphite, biocompatible ion exchange polymer nafion[®] and commercial mesoporous TiO₂ impregnated with horseradish peroxidase (HRP) is prepared and characterized by amperometric, UV-Vis and N₂ sorption methods. The factors influencing the performance of the resulting biosensor are studied in detail. The optimal electrode material consists of 45% graphite, 50% impregnated HRP-TiO₂ and 5% nafion[®]. The optimum conditions for H₂O₂ reduction are an applied potential of -0.3 V and 0.1 mM hydroquinone. Sensitivity and limit of detection in the optimum conditions are 1 A M⁻¹ cm⁻² and 1 μM correspondingly. The N₂ sorption results show that the pore volume of TiO₂ decreases sharply upon adsorption of HRP. The preparation process of the proposed enzyme electrode is straightforward and potentially can be used for preparation of carbon paste electrodes for bioelectrochemical detections.

Keywords: Mesoporous titania, immobilized enzyme, hydrogen peroxide, composite electrode, Amperometry.

1. Introduction

Mesoporous materials are finding many applications in the fields of science and technology as adsorbents, supports for catalysis and sensing elements [1-3] Owing to large surface area and uniform pore-size distribution [4] that can be tuned to fit dimensions of biomolecules, mesoporous materials have also been applied in biosensors as advanced immobilization matrix impregnated with biomolecules [4-7].

Titania (TiO₂) is a typical inorganic mesoporous material with good biocompatibility, stability and environmental friendliness [8]. Particularly, Y. Wang et al. stabilized gold nano-seeds by TiO₂ colloid using commercial non-porous TiO₂ p25 [9]. The resulted conductive composite was well compatible with HRP and could provide direct electron transfer between the adsorbed enzyme and an electrode. Jiang et al. encapsulated HRP in TiO₂ through phospholipid-templated synthesis [10]. The immobilized HRP showed improved thermal stability and tolerance against extreme pH and inactivating agents.

To date, there are different methods to synthesis TiO₂ such as sol-gel [11], surfactant directing [12], hydrothermal [13] and so on [14-16]. Most of these processes use extremely acidic conditions to form titania sol-gel, which are unsuitable for in-situ enzyme immobilization. Some of these methods need a calcination step at more than 300 °C. For these reasons, there is interest in use of commercial mesoporous TiO₂ with uniform pore size distribution as a support for enzymes for further applications in biosensing. Such solid material impregnated with enzyme can be used as a stable ready-to-use component for producing carbon paste or screen-printed electrodes [17].

Horseradish peroxidase (HRP) is a well-characterized enzyme widely used in bioanalytical applications, for example, as a label in immunoassays or redox enzyme in electrochemical biosensors. The last ones were particularly designed for detection of H₂O₂, phenols and its derivatives [18-20]. Recently, it has been shown that HRP encapsulated in TiO₂ shows improved stability and can be applied for phenolic compounds removal [10]. Electrochemical detection of H₂O₂ was previously demonstrated for HRP immobilized on home-made TiO₂ [21, 22]. The drawback of these approaches is the time consuming preparation step of the sensing material.

In the present work, for the first time, we suggest using commercial mesoporous TiO₂ (Millennium PC500) as an efficient matrix impregnated with a model redox enzyme HRP for applying it as an active component in conductive adhesive electrode material.

2. Material and methods

2.1. Reagents and Solution

Graphite, hydrogen peroxide (H₂O₂), nafion[®] 117 (5% in a mixture of lower aliphatic alcohols and water) and sodium hydroxide were purchased from Sigma-Aldrich. 2-[4-(2-hydroxyethyl)-piperazinyl] ethane sulfonic acid (HEPES) was obtained from VWR,

hydroquinone (HQ) from Acros, TiO_2 (Millennium PC500) from Crystal Global (Fig. S1), Aeroxide[®] TiO_2 P25 from Evonik. Horseradish peroxidase (HRP) (EC 1.11.1.7) was purchased from Calbiochem. The HEPES buffer solution of 10^{-2} M was set to pH 7.0 using NaOH solution. All reagents were used without further purification and all solutions were prepared with deionized water.

2.2. Apparatus

Electrochemical measurements were carried out with the AUTOLAB PGSTAT302N equipped with a Metrohm 628-10 electrode rotator (Metrohm, The Netherlands). A conventional three-electrode electrochemical cell was used with a saturated calomel reference electrode (SCE), a glassy carbon rod electrode as an auxiliary electrode, and the working electrodes were gold rotating disk electrodes (Au) with a diameter of 3 mm. Rotating speed of gold electrode was 2000 rpm in all experiments. All solutions were purged by pure nitrogen for 30 min prior to the experiment and maintained under nitrogen atmosphere during measurements. Electrochemical measurements were performed at room temperature. UV-Vis spectroscopic characterization in liquid state was performed in the range of 190–800 nm using a SYNERGYTM MX (Biotek, USA). UV-Vis Diffuse Reflectance measurements on solid samples were done on an Thermo Electron Evolution spectrometer equipped with an integrating sphere. All of the materials were characterized by nitrogen adsorption-desorption isotherms measured in liquid nitrogen at 77 K on a QUADRASORB SI (Quantachrome Instruments, USA). Samples were measured without degassing as sample preparation in order not to denature the enzyme. As a consequence some of the solvent might remain in the pores, resulting in a slight decrease of the volume of adsorbed nitrogen (Fig. S3 c).

2.3. Horseradish peroxidase immobilization in TiO_2

Prior to use, the TiO_2 was calcined to 450 °C to enlarge its pore size [23]. Surface area of 96.6 m^2/g was found for this material from N_2 sorption experiments (Table S1). When pure TiO_2 is mentioned in the text, this means that no HRP is present. Horseradish peroxidase entrapped in TiO_2 was prepared according to the following procedure: Firstly, 250 mg of mesoporous TiO_2 was added to 3 ml 0.66 mg/mL HRP solution in pH 7.0 buffer. The mixture was stirred for 18 h at room temperature. Then, the suspension was washed with HEPES buffer on a membrane filter (0.45 μm) to remove non-immobilized enzyme and loosely held HRP- TiO_2 . Finally, the HRP- TiO_2 paste was dried in air.

2.4. Preparation of the enzyme electrode

The working Au electrodes were used as a support for HRP-TiO₂ containing matrix. Prior to coating, the electrodes were polished on a polishing cloth (Buehler, Germany) with alumina powder of 1 and 0.05 μm particle size and rinsed thoroughly with distilled water in an ultrasound bath. Then suspension of a composite mixture (7 μl) consisting of graphite, nafion[®] and TiO₂ impregnated with enzyme was dropped on the surface of the electrode and dried at +4 °C. The obtained electrode was marked as Au/Gr/HRP-TiO₂/Nafion electrode. To reveal the role of the porous TiO₂ (Millennium PC500), non-porous commercial TiO₂ P25 powder was mixed with HRP in the same way and the obtained electrode was denoted as Au/Gr/HRP-TiO₂(P25)/Nafion. For comparative studies, Au/HRP-TiO₂/Nafion and Au/Gr/HRP-Gr/Nafion were fabricated with the similar procedures leaving out one of the ingredients.

3. Results and discussion

3.1. Optimization of the experimental parameters

In general, direct oxidation/reduction of HRP at an electrode is prevented due to deep embedding of its redox active center [24]. The long distance between the active site and the electrode surface decelerates the electron transfer rate. As a consequence, HRP-based enzyme-electrodes require a mediator such as hydroquinone (HQ) to shuttle electrons between HRP and an electrode. In our work we used HQ mediated bioelectrocatalysis for detection H₂O₂. First, the experimental parameters were optimized.

3.1.1. Matrix composition

Titania is an inorganic mesoporous material that is aimed at immobilization of biomolecules. To improve electrical characteristics of the matrix, TiO₂ was applied in a mixture with graphite powder as a well conductive supplement. To deposit the graphite-TiO₂ mixture on the electrode we also included in the matrix the ion exchange polymer nafion[®] as an adhesive binder. First, the percentage of nafion[®] was optimized via graphite-nafion[®] mixtures with various percentage ratios. The cyclic voltammograms were recorded between -0.6 and 0.6 V in pure HEPES buffer solution (pH 7.0). In the recorded voltammograms (Fig. S2), when the amount of ion exchange polymer was 2% or less, an unwanted ohmic drop and increase in

capacitance of the electrode appeared. In contrast, 5% weight or more of nafion[®] gave satisfying background and stability of the modified electrodes, the results gave good comparison with other studies [25]. To avoid effects of the polymer on accessibility of biomolecules, the minimal amount of 5% was taken as an optimum, which also provided good characteristics of the three component mixture graphite-TiO₂-nafion[®].

Next, the effect of the graphite to TiO₂ ratio on HQ electrochemistry was studied. Fig. 1 shows the electrochemical behavior of the gold electrodes modified with matrix consisted of different ratios of graphite to TiO₂ in the presence of 1 mM HQ. A redox system related to the presence of HQ is observed. Up to 50% of TiO₂ in the mixture with graphite did not affect noticeably the electrochemical behavior of HQ giving oxidation peak current of ca. 30 μ A.

An increase of TiO₂ content up to 70% resulted in about 30% decrease in HQ currents. It was also clear that TiO₂ without graphite could not provide good electrical conductivity through the layer which resulted in more than one order decrease of oxidation/reduction currents. The mixture 1:1 of graphite to TiO₂ was taken as an optimum since it provided electrical characteristics similar to graphite and contained plenty of mesoporous TiO₂ impregnated with the enzyme. In all further measurements, this ratio was used unless stated otherwise.

3.1.2. Incubation time

To optimize the incubation time, TiO₂ was incubated in 15 μ M HRP solution for different time (1-24 h). Recorded Amperometric curves at applied potential -0.3 V suggest that incubation up to 7 hours resulted in only minor activity of the enzyme immobilized electrodes. Maximal current but, after overnight incubation for 18 hours, the reduction current reached $28.6 \pm 4.0 \mu\text{A}$, which was more than one order higher than after 1 h incubation. This indicates a kinetic (diffusion) delay for enzyme immobilization in the mesoporous TiO₂. Longer incubation (24 h) did not increase the activity of the electrodes. Therefore, the incubation time of 18 h was taken in the subsequent. The supernatant after adsorption for 17 h was clear and colorless (see section 3.3) that suggests adsorption of all protein corresponding 0.8 wt% or $1.8 \cdot 10^{-7}$ mol per g of TiO₂.

3.1.3. Working potential

Sensitivity of the electrodes to H₂O₂ was mainly influenced by the working potential of the amperometric measurements and the concentration of the mediator. The influence of the operating potential on the amperometric response of the Au/Gr/HRP-TiO₂/Nafion electrode

was investigated over a potential range from 0 to -0.8 V in solution containing 0.1 mM HQ and 1 mM H_2O_2 (Fig. 2). The response of the biosensor proportionally increased as the applied potential shifted towards more negative values reaching a plateau at -0.4 V. To keep low background current the potential of -0.3 V was taken for further measurements.

3.1.4. Concentration of the mediator

To optimize the concentration of the mediator, the effect of HQ concentration on the HRP electrode response was studied in the presence of 1 mM H_2O_2 . As shown in Fig. 3, the current response increased with mediator concentration in the range from 0.001 to 0.1 mM and then leveled off similarly to the Michaelis-Menten behavior [26]. The maximal current was $61.0 \pm 11.9 \mu\text{A}$ and a concentration of HQ at half-maximum was around 0.02 mM. To minimize background current in measurements of amperometric responses to H_2O_2 , the concentration of HQ was fixed at 0.1 mM for the following experiments. Though HQ plays a role of the mediator in the present work while H_2O_2 is the analyte of the interest, the same detection principle can be applied for detection of phenolic compounds such as phenol or pentachlorophenol [27]. In that case the analogue of graph in Fig. 3 would be considered as a calibration curve.

3.2. Amperometry detection of H_2O_2

The amperometric response to H_2O_2 at Au/Gr/HRP-TiO₂/Nafion electrodes was studied and compared with other HRP electrodes prepared as described above (see 2.4) to reveal the role of TiO₂. As it is shown in Fig. 4, in all cases the calibration plots exhibit that the current response of the electrodes increases with H_2O_2 concentration. In the same time, the amperometric response of Au/Gr/HRP-TiO₂/Nafion electrode was much higher compared to the other modified electrodes. This evidence confirms that the concept of using a commercial mesoporous TiO₂ as host for HRP is a feasible approach. As a comparison, an electrode modified by non-porous TiO₂ (p25) gave only minor current, which was even lower than that for the electrode modified by graphite with adsorbed HRP. This blank experiment with P25 does indicate that the enzyme attached to outside of the TiO₂ particles (61 m²/g surface area) did not contribute to the catalytic current or was simply removed in washing steps. Assuming the same enzyme activity in all system prepared, one can estimate from the maximal currents that the enzyme loading was at least one order higher in the coating with porous TiO₂ compared to the systems based on non-porous TiO₂ or only graphite.

The concentration-response profile (Fig. 4) for Au/Gr/HRP-TiO₂/Nafion behaved closed to Michaelis-Menten model giving saturation kinetics at concentration higher than 1 mM. The dependence was linear up to 0.4 mM of H₂O₂ with average sensitivity \pm SD (three different electrodes) of $1.09 \pm 0.16 \text{ A M}^{-1} \text{ cm}^{-2}$ and the limit of detection around 1 μM . The long-term stability of the impregnated TiO₂ with HRP was checked to confirm the activity of HRP. The HRP-TiO₂ powder kept its activity after more than two years storage at +4 °C. The freshly prepared powder resulted in the average response to 1 mM H₂O₂ of 28.6 μA with SD of 4.0 μA (n=3) and after two and half years the same material gave 27.6 μA with SD of 1.7 μA (n=3).

3.3. Conformational studies: UV-Vis, UV-DR characterizations and N₂ sorption

UV-Vis spectroscopy was applied to monitor of the enzyme concentration before and after immobilization of HRP on TiO₂ [9]. Fig. 5 displays the spectra of HEPES buffer, HRP in HEPES buffer of pH 7.0 and the supernatant after adsorption of the enzyme into TiO₂. The UV-Vis spectrum of HRP solution gave a typical heme band at 401 nm [9], while there is no absorbance in the supernatant collected after incubation of mesoporous TiO₂ in HRP solution. The data suggest that all enzyme was adsorbed onto TiO₂.

In an additional experiment, UV-DR measurements were done on the powder with incorporated HRP (HRP-TiO₂). As it can be seen in Fig. 6, both HRP and HRP-TiO₂ show the absorption band originating from HRP around 402 nm (heme band). The disappearance of this heme band from the liquid phase and its present in the solid material confirms the presence of HRP on or in the porous TiO₂ material.

To clarify whether HRP actually resided within the TiO₂ pores, pore volume and pore size distribution in the mesoporous TiO₂ were characterized by the nitrogen sorption method [28] before and after HRP loading (Fig. S3). The pore volume of TiO₂ decreased sharply upon adsorption of HRP. This indicates that the mesopore network of TiO₂ is (1) occupied by enzyme molecules and some possible remainder of solvent and (2) the available sites for nitrogen adsorption have been reduced [29].

4. Conclusion

In this study, for the first time, we suggested using commercial mesoporous TiO₂ (Millennium) as a matrix impregnated with HRP as a model redox enzyme. Amperometric, UV-Vis and N₂ sorption studies revealed that the matrix provided a good platform for the

immobilization of HRP. The good biocompatibility of TiO_2 allows HRP retains its active state within the matrix and shows good electrocatalytic response to the reduction of hydrogen peroxide. In addition, our study demonstrates the importance of a mixed matrix electrode where each component adds to the beneficial performance of the electrode. The presence of graphite in the matrix was shown to greatly increase the conductivity of the sensor, while the HRP impregnated TiO_2 causes high affinity to the substrate. This study is expected to provide a great potential for straightforward designing environmental friendly sensing materials that can be used in the construction of other enzyme based biosensors.

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Appendix A. Supplementary information

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Figures Captions

Fig. 1. Cyclic voltammograms of 1 mM HQ in 10^{-2} M HEPES (pH 7.0) at Au/Gr/TiO₂/Nafion electrodes with different ratio of TiO₂ to Gr. Nafion content, 5%. Scan rate, 50 mV s⁻¹.

Fig. 2. Effect of the applied potential on the performance of Au/Gr/HRP-TiO₂/Nafion electrodes. (A) Chronoamperometry in the presence of 1 mM H₂O₂ and 0.1 mM HQ. Numbers denote potentials applied; the asterisk marks the effect of HQ injection. (B) Current-potential profile constructed for a series of the electrodes, each point was measured at least three times. The error bars show the standard deviations.

Fig. 3. Effect of HQ concentration on the current of Au/Gr/HRP-TiO₂/Nafion electrode in the presence of 1 mM H₂O₂. The error bars give standard deviation for series of three different electrodes. The inset shows an amperometry curve recorded for successive addition of HQ, the numbers denote the concentrations of HQ in μ M injected in the cell.

Fig. 4. Chronoamperometry curves (A) and calibration plots (B) for different matrices obtained in presence of 0.1 mM HQ in 10^{-2} M HEPES (pH 7.0). Numbers denote concentration of H₂O₂ injected into the cell. Applied potential, -0.3 V.

Fig. 5. UV-Vis spectra of 0.015 mM HRP solution in 10^{-2} M HEPES buffer (pH 7.0) before (in blue) and after (in red) incubation with TiO₂ for 18 h (250 mg of TiO₂ were added to 3 ml of 0.015 mM HRP) in comparison with pure buffer (in green).

Fig. 6. UV-DR spectra of HRP powder (in blue), TiO₂ impregnated with HRP (in red), blank TiO₂ incubated 18 h in pure buffer (in green). The arrow shows the absorption band of HRP.

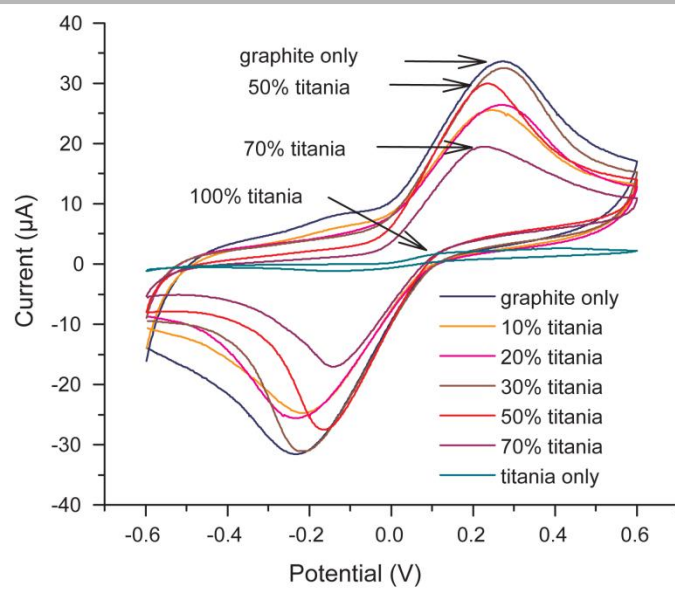


Fig.1

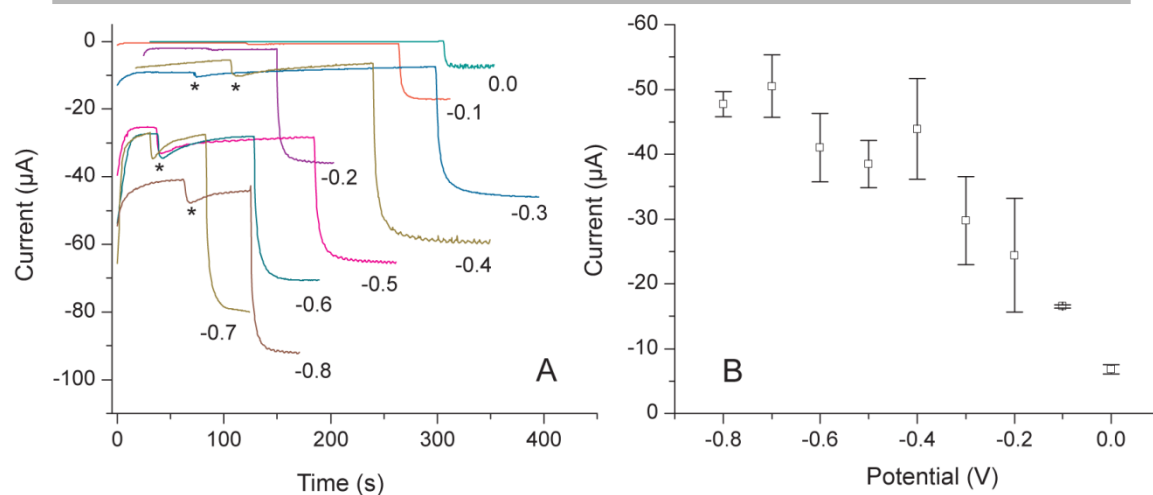


Fig. 2

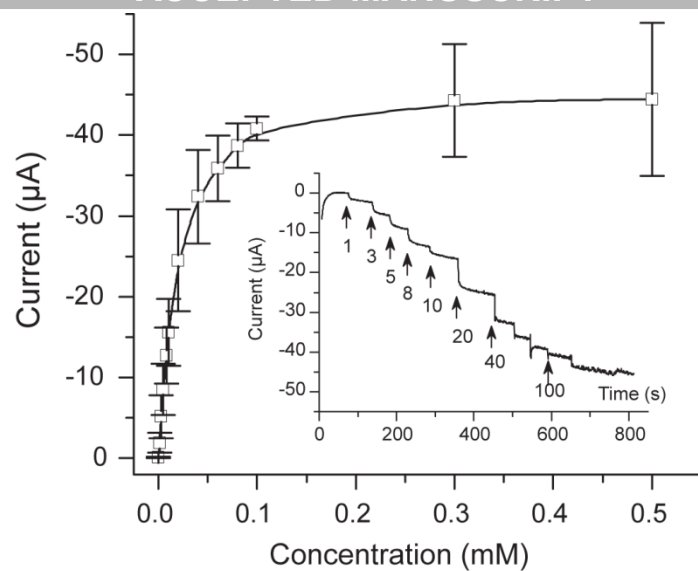


Fig. 3

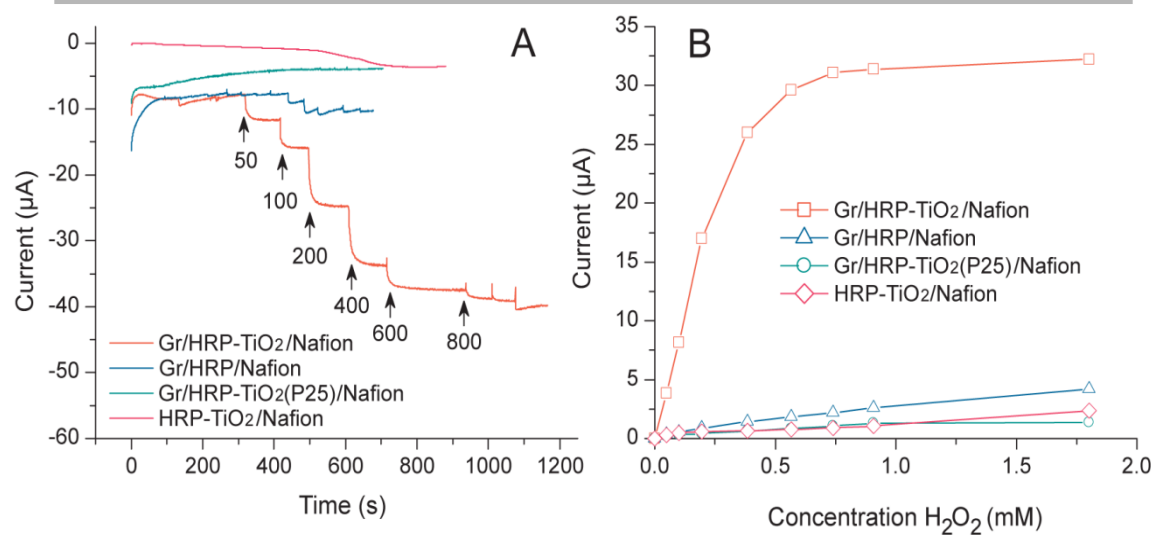


Fig. 4

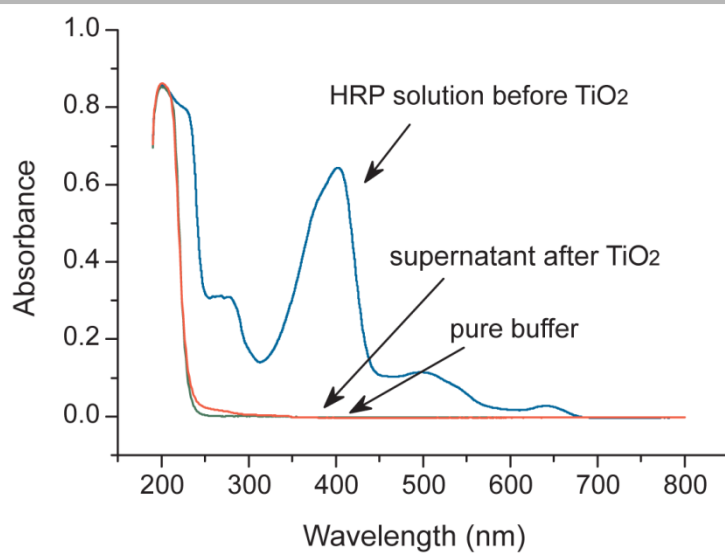


Fig. 5

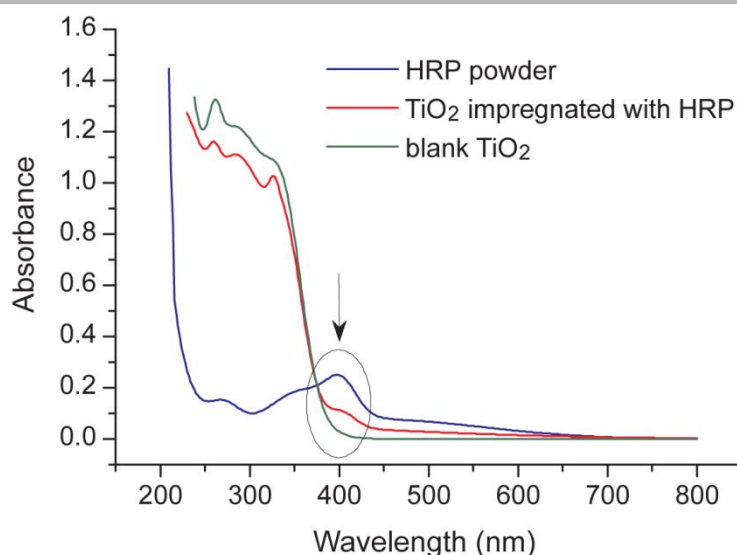


Fig. 6

Highlights

- commercial mesoporous TiO_2 is, for the first time, used as a support for enzymes
- the enzyme impregnated material is a ready-to-use component for biosensing
- characterization of the modified material by EC, UV-VIS, UV-DR, N_2 -sorption and TGA

Commercial mesoporous titanium dioxide as a support for horseradish peroxidase for bioelectrochemical applications

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Commercial mesoporous TiO_2 impregnated with horseradish peroxidase is used as a component of electrode material in H_2O_2 biosensor. Nitrogen sorption and UV-Vis confirm filling of mesopores with enzyme molecules. The benefit of the composite material consisted of graphite, impregnated mesoporous TiO_2 and nafion® is demonstrated.

