X Encontro Brasileiro sobre Adsorção
27 a 30 de Abril de 2014
Guarujá - SP

ADSORPTION BEHAVIOR AND ACTIVITY OF HORSERADISH PEROXIDASE ONTO TITANATE NANOWIRES.

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ABSTRACT: Immobilization of horseradish peroxidase (HRP) onto titanate nanowires (TNW) was investigated through different strategies. TNW were synthesized by hydrothermal method and characterized by Scanning electron microscopy (SEM), X-ray diffraction (XRD), N_2 physisorption (77K) and Fourier transform infrared spectroscopy (FTIR). HRP was stable and active in a wide range of pH with optimal activity at 7.0. The K_m of HRP for 4-aminoantipyrine and H_2O_2 as substrate was 0.77 ±0.25 mM. Immobilization strategies studied were non-specific and covalent coupling through amine groups. The adsorption isotherm had good fit with Langmuir-Freundlich model (LF). The coverage of TNW containing HRP adsorbed by covalent coupling was 1.56 mg_{HRP}/m^2 and residual enzymatic activity around 40%. The enzymatic activity of free HRP and immobilized HRP was monitored as a function of storing time. The results confirm that through covalent binding the enzyme is firmly attached to TNW surface, constituting a very promising platform for a variety of applications such as in biosensing.

KEYWORDS: Horseradish peroxidase, adsorption, titanate nanowires.

1. INTRODUCTION

Adsorption of bioactive materials in solid matrices has drawn increasing interest both in industrial processes and in medical field. Enzymes are natural biocatalysts with applications in numerous areas, and their immobilization has several well-known advantages as multiple uses and, usually, increase of stability (Vianello *et. al*, 2000; Kim *et. al*, 2006; Ansari and Hunsain, 2012)

For application in the biosensing field, the immobilization should provide a biocompatible and inert environment. Besides, enzyme orientation, mobility, stability and biological activity should not be negatively affected by immobilization (Sassolas *et. al*, 2012; Ansari and Hunsain, 2012). In addition, immobilized enzyme should remain firmly bound to the support, especially during the use/application.

Since the performance of the enzyme is highly affected by the immobilization procedure, intensive studies have focused on the development of successful immobilization strategies (Sassolas *et. al*, 2012). Accordingly, the choice of immobilization method and suitable supports are very important.

Nanomaterials can be excellent support for immobilization, because they can provide ideal characteristics for enzyme stability (Feng and Ji, 2011). Some properties of titanate nanostructures make them unique as the large specific area, thermal and chemical stability, optical and electrical properties, biocompatibility and ease of manufacturing (Kassuga *et. al*, 1999).

The use of titanate structures as support for biomolecules immobilization is not well explored in literature yet. Ding et al. (2013) reported the use of titanate nanowires for BSA adsorption. Trypsin adsorption on titanate nanotubes (TNT) was studied by Zakabunin *et. al* (2008), who observed high protein adsorption and stable enzyme activity.

Immobilization of Horseradish peroxidase (HRP) onto TNT by covalent binding preserved the enzyme bioelectrocatalytic activity, appearing as a promise application for biosensing (Sovic *et. al*, 2011). The interest in HRP is based on its versatile nature, finding application in environmental, chemical, pharmaceutical and biotechnological industries (Zhi et. al, 2008).

HRP is a glycoprotein containing heme (iron (III) protoporphyrin IX) as prosthetic group. It requires hydrogen peroxide (H_2O_2) as substrate to achieve different oxidation states (Chattopahyay and Mazumdar, 2000), which constitutes the basis of its use in H_2O_2 detection (Sovic *et. al*, 2011).

This work aims at investigating the adsorption of horseradish peroxidase (HRP) onto titanates nanowires (TNW), comparing two strategies: non-specific adsorption and covalent binding. Adsorption isotherms were obtained and the characterization of the immobilized biocatalyst was carried out through FTIR analysis and specific activity assay.

2. MATERIALS AND METHODS

2.1. Chemicals

Horseradish peroxidase (HRP, MM 44kDa, RZ 3.0) type IV, from Amoracia rusticana, bovine serum albumin (BSA), (3-aminopropyl) trimethoxysilane (APTMS, 97%), hydrogen peroxide (H_2O_2, 30% w/w), 4-aminoantipyrine (Amn, 98%) and phenol (99%) were supplied by Sigma – Aldrich. Glutaraldehyde ($C_5H_8O_2$, 25%), sodium hydroxide (NaOH (PA)), nitric acid (HNO_3, 65% (PA)), dichloromethane (CH_2Cl_2, 99.5%) were purchased from VETEC. All chemicals were analytical grade and used as received.

2.2. Synthesis and characterization of titanate nanowires (TNW)

Titanate nanowires were prepared by hydrothermal synthesis as proposed by Kassuga *et. al* (1999). The synthesized TNW were characterized thorugh X-ray diffraction (XRD, Rigaku Miniflex equipment with Cu K radiation, = 1,5418), Scanning electron microscopy (SEM, FEI Company Quanta 200, Raman spectroscopy (LabRam Horiba HR-800 UV with He-Ne laser) and N_2 physisorption (77 K, ASAP 2020 Micromeritics instrument, with pretreatment under vacuum at 573 K for 12 h).

2.3. Characterization of horseradish peroxidase (HRP)

2.3.1. Enzyme activity: Free HRP activity was determined at different pH using 0.1 M potassium phosphate buffer (PBS). Specific enzymatic activity (U/mg) was determined by UV-spectrophotometry at 510 nm, performed according to the procedure described in Vojinovic (2004).

2.3.2. Determination of rate constants: The consumption of H_2O_2 in the range of concentration from 0.1 – 1 mM was monitored and the K_m and $V_{máx}$ values were determined with Statistica® software by non-linear estimation.

2.4. Adsorption of HRP onto TNW

2.4.1. Non-specific adsorption: 1 mL of a 2 mg/mL HRP solution in 0.1 M PBS was added to 1 mL of TNW dispersion. The mixture was kept under stirring for 24 h at room temperature. The suspension was filtered through a 100 kDa Amicon filter (MILLIPORE) and centrifuged at 4000 rpm for 4 min to separate non-bound HPR.

2.4.2. Chemical adsorption: TNW surface was first functionalized with amino groups, according to Sovic *et. al* (2011), employing ATPMS grafting (Figure 1). Afterwards, the suspension was vacuum-filtered through an 8 pore size filter (Quanty) and washed with dichloromethane. Then, it was dried at 60 °C. 10 mg of TNW/APTMS were dispersed in 500 µL of glutaraldehyde solution (2.5% w/w in 0.1 M PBS pH 7.0) and remained for 1 h under stirring. The mixture was filtered again, followed by repeated washing with 0.1 M PBS pH 7.0. Then, 5 mg of modified TNW was stirred with 1 mL of a 1 mg/mL HRP solution for 2 h. Non-bound HRP was separated using 100 kDa Amicon filter as previously described. The whole procedure was performed at room temperature.

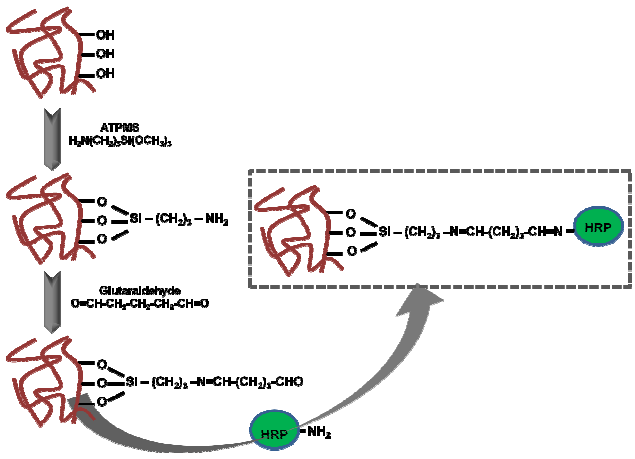

Figure 1. Reaction sequence employed for surface functionalization of TNW and chemical immobilization of HRP through the insertion of amino groups.

2.5. Adsorption isotherms

Time required to achieve adsorption equilibrium was assessed through kinetic study. For this, contact time between enzyme and support was varied from 1h to 24h (for non-specific adsorption) and from 0.5 to 10h (for chemical adsorption).

The amount of HRP adsorbed at each time (Q_e) was determined through the difference between HRP concentration in solution before and after adsorption, as measured by the Bradford method (Bradford, 1976) according to Equation 01:

$$Q_e = \frac{(C_i - C_e)\cdot V}{m} \qquad (01)$$

Where, C_e (mg L^{-1}) is the equilibrium concentration of HRP, C_i is initial concentration of HRP (mg L^{-1}) and m (g) is amount of TNW employed.

Adsorptions isotherms were obtained by varying the adsorbent/adsorbate ratio at 25°C in the range from 1:5 to 1:0.03 (w/w).

2.6. Immobilization efficiency

2.5.1. Enzyme activity and stability: Specific enzymatic activity (U/mg) was determined as described in Item 2.3.1. The activity of the immobilized HRP stored in 0.1 M PBS pH 7.0 at 4 ºC was monitored to evaluate its stability.

2.5.2. FTIR analysis: FTIR spectra were obtained for each step of immobilization strategy by spectrophotometry (Perkin Elmer Spectrum Spectrum 100 FTIR).

3. RESULTS AND DISCUSSION

3.1. TNW characterization

TNW specific surface area was 118 m^2/g, as obtained by the BET method. Figure 2 shows the SEM images of the TNW. Average diameter was around 50 nm. XRD was performed to structural characterization of TNW, as shown in Figure 3, where samples showed the formation of lamellar structure due to the peak at 2 ~ 10° (Morgado *et. al*, 2007). Furthermore, the conversion of TiO_2 anatase phase to titanate was confirmed by the disappearance/change in intensity of the characteristic peaks and the appearance of

peaks 2 = 24.3º, 28.5º, 38.7º and 48.5° that correspond basically with unidimensional trititanates ($Na_xH_{(2-x)}Ti_3O_7$) (Liu *et. al*, 2012; Han *et. al*, 2007; Morgado *et. al*, 2007). The peak at 2 = 24.3º has slightly offset from the peak 25.3º concerning the anatase phase.

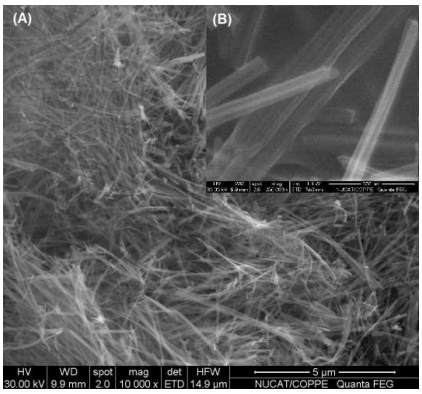

Figure 2. SEM micrography of original TNW with magnification of (A) 10000X and (B) 200000X.

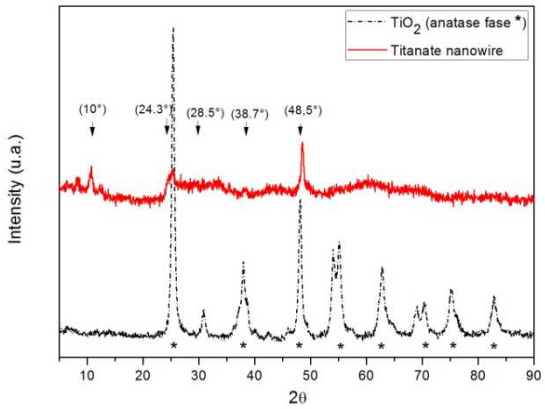

Figure 3. X-ray diffraction patterns of the TNW and the precursor TiO_2.

3.2. HRP characterization

HRP activity was tested in the pH range from 2.0 to 12.0 (Figure 4). The highest activity was observed at pH 7.0. HRP also keeps more than 50% of its activity in the pH range from 4.0 to 10.0, as confirmed by other studies (Mohamed *et al.*, 2011; Chattopadhyay and Mazumdar, 2000), which is very appropriate for industries requiring a broad range of pH activity. At pH 7.0, enzyme specific activity was 969.81±102.25 U/mg.

Figure 5 shows the increase in abs/min as a function of H_2O_2 oncentration. Data were well described by the Michaelis-Menten model using the Statistica® software, as shows the regression coefficient (r^2) of 0,997. The reaction rate increases almost linearly up to a concentration of 0.8 mM, above which tends to stabilize. From these data, kinetic parameters of the catalytic reaction were obtained (Table 1). The apparent K_m value was 0.769 ± 0.247 mM and the literature reported the value of 0.35 mM (Triplett and Mellon, 1992), confirming that H_2O_2 is an excellent substrate for HRP.

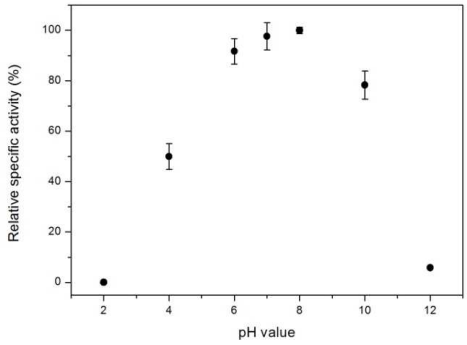

Figure 4. pH optimum of HRP in 0.1 M PBS at room temperature. Each point represents the average of three experiments.

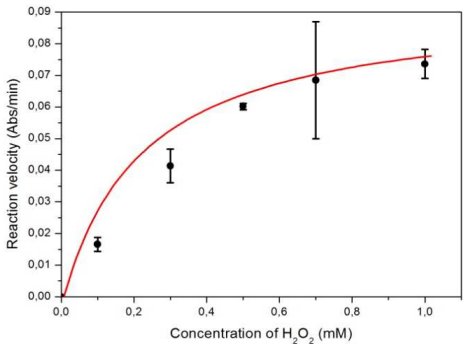

Figure 5. Reaction velocity vs. concentration of H_2O_2 at room temperature. Fitting with Michaelis-Menten model is represented by solid line. Erros bars represent standart deviation for three independent adsorption experiments.

Table 1. Estimated kinetic parameters for HRP.

Parameters	Value
$V_{máx}$ (mM.Min^{-1}/100 µL of solution)	0.11 ±0.02
K_m (mmol L^{-1})	0.7 ±0.25

3.3. Non-specific adsorption of HRP

The effect of contact time on the adsorption of HRP was investigated to determine the time required for achieving adsorption equilibrium. The results are shown in Figure 6.

Adsorption capacities increased sharply up to 1 h of contact. With further increase of time, the adsorption capacity approaches a plateau. Therefore, we chose the contact time of 24 h in all subsequent experiments to ensure adsorption equilibrium. Risio and Yan (2009) and Silva *et al.* (2007) also found 24 h of contact time as enough for HRP adsorption on different surfaces.

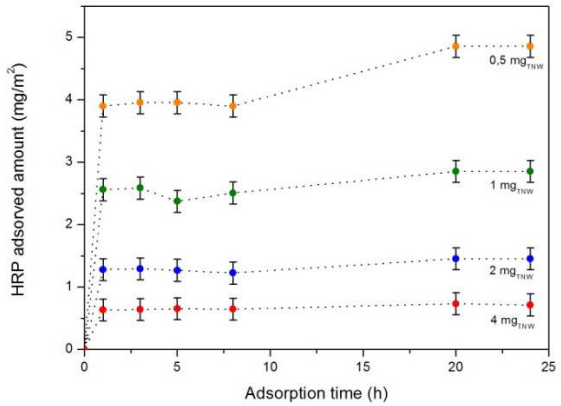

Figure 6. Adsorption of HRP onto TNW samples *vs.* Time at room temperature.

Figure 7 shows the non-specific adsorption isotherm for HRP on TNW. Data were well fitted by the Langmuir-Freundlich model (LF), exhibiting a high correlation coefficient (r^2 = 0,99946). This model is represented by the Equation 02 (Umpleby *et. al.*, 2001; Jeppu and Clement, 2012):

$$Q_e = \frac{Q_m \cdot (K \cdot C_e)^{1/n}}{1 + (K \cdot C_e)^{1/n}} \qquad (02)$$

Where, Q_e is the amount adsorbed at equilibrium (mgHRP/m^2TNW); Q_m is the adsorbed capacity of the system (mg of HRP/m^2TNW); C_{eq} is the aqueous phase concentration at equilibrium (mg/L); K is the affinity constant for adsorption (L/mg); n is the index of heterogeneity.

The fitted values of K and n are summarized in Table 2. In the isotherm, the value of the constant K is related to the affinity between the adsorbate and the adsorbent. In this case, the obtained value indicates a low affinity (Jeppu and Clement, 2012). The *plateau* in adsorption is reach at 15.621 ± 0.232 mgHRP/m^2TNW.

The 1/n parameter is the heterogeneity index, which normally varies from 0 to 1. In contrast, in this work we obtained the value of 1/n > 1. In these cases, some authors attribute this to the phenomenon known as cooperative adsorption, (Fair and Jamieson, 1980; Foo and Hameed, 2010), leading to the formation of several layers.

Specific enzymatic activity after non-specific adsorption was determined. The value found, 27.47 ± 5.44 U/mg, represents a reduction in activity superior to 97% compared to free cells (specific activity of 969.81±102.25 U/mg). Ferapontova and Purganova (2002) reported a loss 70 of % in specific activity for HRP non- specific adsorption onto single-walled carbon nanotubes. As a result of non-specific adsorption, the protein molecule has a high degree of freedom in movement, both rotational and lateral, which can lead to deactivation (Nakata *et. al*, 1996).

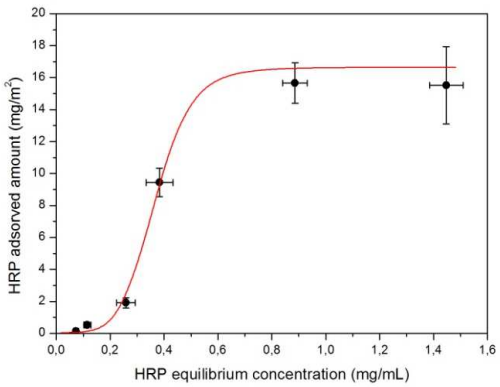

Figure 7. Non-specific adsorption isotherm of HRP at room temperature and the fit by LF model. (solid line). Erros bars represent standard deviation for three independent adsorption experiments.

Table 2. Fitted values of Langmuir-Freundlich parameters for HRP non-specific adsorption onto TNW.

	Q_m mg_{HRP}/m^2_{TNW}	K L/mg_{HRP}	1/n
Estimated value	15.62± 0.45	2.80±0.08	6.06±0.03

3.4. Chemical adsorption of HRP

To immobilize HRP through covalent binding TNW surface was first functionalized with ATPMS. FTIR spectrum in Figure 8 (curve b) shows the appearance the bands at 1223 cm^{-1} and 1510 cm^{-1}, suggesting the presence the NH_2 groups (Sovic *et. al*, 2011, Silverstein and Webster, 2000). The decrease of the bands at 3500 - 3000 cm^{-1} and 1630 cm^{-1} (Figure 5, curve a) is due to the presence

of superficial OH groups in TNW (Vianello *et. al*, 2000). After reaction with glutaraldehyde (Figure 8 curve c), it is observed the disappearance of the band at 1223 cm^{-1}, the decrease of the band at 1510 cm^{-1} and the presence of weak bands between 1700 and 1500 cm^{-1} (C=N base) (Silverstein and Webster, 2000).

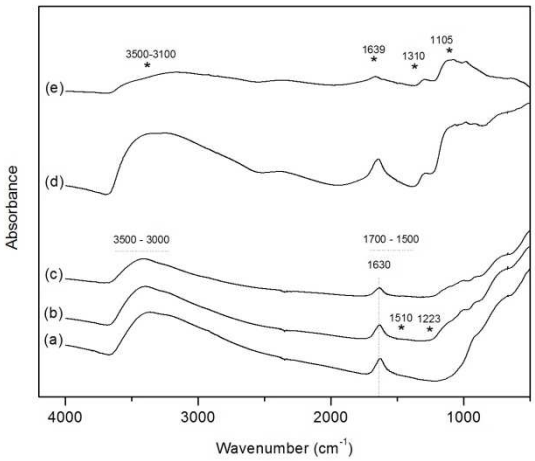

Figure 8 – FTIR spectra of TNW modifications by immobilization strategy covalent: (a) TNW, (b) TNW/ATPMS, (c) TNW/ATPMS/GLUT, (d) TNW/ATPMS/GLUT/HRP and (e) difference between the TNW/ATPMS/GLUT/HRP and TNW/ATPMS/GLUT.

Upon HRP adsorption, spectrum (Figure 8 curve d) shows the peaks at 1639, 1535, 1310 and 1105 cm^{-1} are assigned to the groups –CONH – (amide I), –CN– stretching (amide III), amide II and –CO– bond stretch, respectively (Monier *et. al*, 2010). These peaks related to the presence of the HRP protein (Fig. 8 curve e) can be seen by subtracting curve (c) from curve (d).

After confirming the existence of covalent attachment of HRP onto NTW surface through amine groups, the isotherm for HRP adsorption was obtained for a contact time of 2 h. This time is substantially lower than the time used for non-specific adsorption (24h), as expected, since it consists of a chemical reaction between enzyme and support, giving rise to covalent bound. Figure 9 shows the experimental data and the fit by the LF isotherm model with high correlation coefficient (r^2 = 0.99841). The fitted values of K and n are summarized in Table 3.

The *plateau* is reached at 7.564 ± 2.037 mgHRP/m^2TNW for equilibrium concentrations superior to 0.8 mg mL^{-1} of HRP. In this case, it was also obtained the value of 1/n > 1, which is attributed to cooperative adsorption.

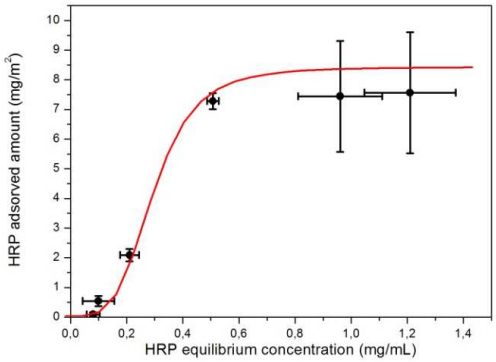

Figure 9. Adsorption isotherm of immobilized HRP onto functionalized TNW for the equilibrium time of 2 h at room temperature and data fitting by LF model (solid line). Erros bars represent standard deviation for three independent adsorption experiments.

Table 3. Fitted values of Langmuir-Freundlich parameters for HRP chemical adsorption onto NTW.

	Q_m mg_{HRP}/m^2_{TNW}	K L/mg_{HRP}	1/n
Estimated value	7.58± 0.4	2.83±0.43	4.26±0.10

It can be observed that the K values for both strategies (non-specific and covalent adsorption) were very similar, indicating equivalent affinity of the enzyme for the substrate. The maximum coverage degree (Qm) was superior for the non-specific immobilization, probably due to multi-layer adsorption.

Chemical adsorption resulted in loss of about 65% in the enzyme activity. The final activity was 344.27±3.90 U/mg. Caramaroni and Fernandes (2004) also observed a loss of 60% of HRP activity after the immobilization. Gómez *et. al* (2006) and Bayramoglu and Arica (2008) reported losses of 22% in HRP activity upon immobilization. This reduction is much lower than that observed for non-specific adsorption. When the protein is chemically adsorbed, its configuration is fixed on surface (Nakata *et. al*, 1996). This remaining catalytic activity is still high and could be explored in the fabrication of biosensors, for instance.

3.5. Enzyme activity stability

HRP stability was evaluated by monitoring the activity as a function of storage time (Figure 10). It is evident that immobilization through covalent binding results in an increase in enzyme stability. For instance, after 160 days of storage, the chemical immobilization results in about 20% of residual activity. On the other hand, in the same period, non-specific adsorption leads to complete loss of enzyme activity, as it is observed with free HRP.

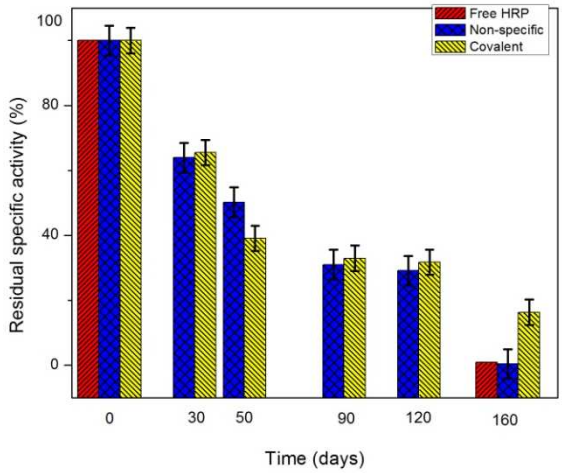

Figure 10. Residual activity of adsorbed HRP molecules by immobilization strategies: non-specific adsorption and covalent. The samples were stored in PBS (pH 7.0) at 4ºC.

4. CONCLUSION

Investigations performed in this study showed that titanate nanowires can be successfully employed for adsorption of the enzyme Horseradish Peroxidase (HRP) by covalent coupling (activation of amine groups through functionalization with ATPMS). The immobilization procedure described resulted in large amount of adsorbed enzyme and low activity losses. By fitting of the experimental adsorption isotherms with Langmuir-Freundlich models it was shown that suggests multilayer adsorption, attributed to the phenomenon of cooperative adsorption. The enzyme is more susceptible to adsorb when adsorbed enzyme molecules are already present at the surface.

HRP residual activity after covalent coupling was superior to 20% even after 160 days, while free HRP become completely inactive in the same period. These results indicate that covalent attachment of HRP to titanate nanowires surface is a very promising support for several biochemical and biotechnological applications.

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6. ACKNOWLEDGMENTS

The authors acknowledge CNPq and CAPES for financial support.