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*Article*

# Highly Stable Magnetite Modified with Chitosan, Ferrocene and Enzyme for Application in Magneto-Switchable Bioelectrocatalysis

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O direcionamento por campo magnético tem sido aplicado em Bioeletroquímica, principalmente com intuito de transportar enzimas ou mediadores redox imobilizados em partículas de magnetita ($Fe_3O_4$) para a superfície de eletrodos, proporcionando o controle comutável das correntes faradaicas provenientes da biocatálise. Neste trabalho, relata-se um avanço no controle magnético de reações bioeletroquímicas por meio da construção de um sistema que contém simultaneamente partículas magnéticas (para transporte controlado), enzima redox (para biocatálise) e um mediador redox (para mediação da transferência eletrônica). Para isso, sintetizou-se um novo material que consiste em partículas de $Fe_3O_4$ modificadas com ferroceno insolúvel (Fc) e quitosana (Chi) entrecruzada com glicose oxidase (GOx). Quando esse material foi utilizado em suspensão em estudos eletroquímicos, observou-se um aumento de 70% da corrente catalítica de oxidação de glicose quando um campo de 0,24 T foi aplicado perpendicularmente ao plano do eletrodo. Esta é a primeira vez que um controle de processos bioeletrocatalíticos foi relatado utilizando enzima, mediador e magnetita em um sistema único controlado por campo magnético.

Magnetic fields have been used in Bioelectrochemistry to carry enzymes or redox mediators immobilized on magnetite ($Fe_3O_4$) to the electrodes surface, providing a switchable control of faradaic current from biocatalysis. In this work, it is reported an advance in the magnetic control of bioelectrochemical reactions, by construction of a system containing simultaneously a magnetic particle (for controlled driving), an enzyme (for biocatalysis) and a redox mediator (for mediation of electron transfer). The advance was attained by synthesis of a new material ($Fe_3O_4$-Chi-Fc/GOx) that consists of $Fe_3O_4$ particles modified with insoluble ferrocene (Fc) and chitosan (Chi) cross-linked with glucose oxidase (GOx). When this material was used in electrochemical studies, an increase of 70% was observed in the catalytic current of glucose oxidation when 0.24 T was applied perpendicularly to electrode plane. This is the first time that a control of the bioelectrocatalytic process was achieved using enzyme, mediator and magnetite in a unique system switched by a magnetic field.

**Keywords:** magneto-switchable bioelectrocatalysis, magnetite, chitosan, ferrocene, glucose oxidase

## Introduction

The control of bioelectrochemical reactions using a magnetic field was first explored by Willner *et al.*[1] In that study, magnetite particles ($Fe_3O_4$) modified with *N*-(ferrocenylmethyl) aminohexanoic acid were employed to mediate biocatalysis of enzyme glucose oxidase (GOx). In a simple manner, two switchable modes, "switch on" and "switch off", were used in order to induce the electrochemical current from enzymatic reaction. Katz *et al.*[2] have reported the simultaneous control of biocatalytic reactions using two enzymes, GOx and lactate dehydrogenase (LDH). For the latter, PQQ-functionalized magnetic particles were attracted to electrode surface, leading to oxidation of lactate and the blocking the reaction catalyzed by GOx. In another study, Willner *et al.*[3] reported magneto-switchable charge transport and bioelectrocatalysis using maghemite-Au core-shell nanoparticle/polyaniline composites. Dong *et al.*[4] reported the capacity to modulate the electrochemical signal

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without the removal of the $Fe_3O_4$-enzyme biocomposite from the working electrode surface. In this condition, the biocomposite was oriented in alignment with the magnetic field lines resulting in a wire that could be oriented by the angle of magnetic field application. More recently, Liang *et al.*[5] reported a magneto-controlled bioelectrocatalytic system for glucose oxidation, in which ferrocene was grafted to the thiol-terminated $Fe_3O_4$ nanoparticles via UV-induced thiol-ene click reaction.

In general, for magnetic control of bioelectrocatalytic process, some important points should be addressed: (*i*) ferromagnetic particles should exhibit colloidal stability in aqueous suspension, at least, during the time spent for the electrochemical experiments; (*ii*) it is not necessary to obtain magnetite in the form of nanoparticles, because enzyme diameter (e.g., dimeric-GOx) is around of nanometers, and the enzymes should be strongly immobilized on solid surface (magnetic microparticles); (*iii*) a mild microenvironment must be found at the surface of the solid particles on which the enzyme will be immobilized to avoid denaturation and loss of activity. This requirement can be satisfied by the use of appropriate methods for modification of the surface of the particles; (*iv*) the method of enzyme immobilization should guarantee no significant detachment of the protein during the bioelectrochemical experiments; (*v*) the magnetic particle should contain a redox mediator with high electrochemistry stability in order to provide conditions for a fast electron transfer.

As observed in the above mentioned points, several experimental requisites should be addressed to obtain an efficient system for magnetic control of bioelectrocatalytic processes. In the present work, it is reported a simple and effective method to obtain a new material ($Fe_3O_4$-Chi-Fc) from the physical adsorption between the redox mediator ferrocene (Fc) and the surface of $Fe_3O_4$ particles modified natural polymer chitosan (Chi). In order to demonstrate a possible application of $Fe_3O_4$-Chi-Fc, the oxidation of $H_2O_2$ from the glucose oxidation by using $Fe_3O_4$-Chi-Fc/GOx in the presence and absence of a magnetic field was evaluated. The best of our knowledge, this is the first time that an enzymatic reaction is achieved through magnetic particle, redox mediator and enzyme in a single system, and providing a fine control of the bioelectrocatalytic processes.

## Experimental

### Reagents and samples

All solutions were prepared from chemical of high purity and ultra-pure water (Milli-Q, 18.2 MΩ $cm^{-1}$). Ferric chloride 98% (anhydrous $FeCl_3$), ferrous chloride tetrahydrate 98% ($FeCl_2.4H_2O$) and ammonium hydroxide 98% ($NH_4OH$) were purchased from Vetec. Chitosan (Chi) with deacetylation degree of 85% was acquired from the company Polymar Nutrition Science S/A. The redox mediator Fc, acetic acid (glacial) 100%, glutaraldehyde 25% (GA), GOx (from *Aspergillus niger*), bovine serum albumin (BSA) and β-D-glucose, hexane 95% were purchased from Sigma-Aldrich. Sodium phosphate dihydrate ($NaH_2PO_4$) and sodium phosphate monohydrate ($Na_2HPO_4$) used in the preparation of the 0.1 mol $L^{-1}$ phosphate buffer solution (PBS, pH 7.0) were also purchased from Vetec.

### Preparation of $Fe_3O_4$ particles modified with chitosan and ferrocene

$Fe_3O_4$ particles were prepared by co-precipitation method[6] using a mixed aqueous solution of ferric chloride (0.1 mol $L^{-1}$) and tetrahydrous ferrous chloride (0.05 mol $L^{-1}$) in a 2:1 ratio, respectively. An excess of $NH_4OH$ was added until pH 9.0 and a black precipitate was formed. During the addition of reagents, the system was kept in nitrogen atmosphere under magnetic stirring. $Fe_3O_4$ particles in suspension were decanted by magnetic separation and then washed with distilled water until the supernatant presented a pH 7.0. After washing, $Fe_3O_4$ particles were dried at a temperature of 60 °C and macerated to produce a fine powder of homogeneous size. Respecting a stoichiometric ratio of 1:10 ($Fe_3O_4$:Chi), a powder mass of $Fe_3O_4$ particles was weighed and re-dispersed in an acid solution of chitosan (2% acetic acid) 1.0 g $L^{-1}$,[7] producing $Fe_3O_4$-Chi. After 24 h, all $Fe_3O_4$-Chi in suspension was decanted by magnetic separation and then vacuum-dried. Once dry, $Fe_3O_4$-Chi was modified with Fc molecules by physical adsorption, in which a mass of $Fe_3O_4$-Chi (4.0 mg) was added to an excess of 0.1 mol $L^{-1}$ ferrocene dissolved in hexane. The solvent was fully removed by evaporation, producing $Fe_3O_4$-Chi-Fc. The new material obtained was washed with water distilled in order to remove non-adsorbed crystals of Fc and then vacuum-dried at room temperature (25 ºC).

### Preparation of biocomposite $Fe_3O_4$-Chi-Fc/GOx

After the preparation of the $Fe_3O_4$-Chi-Fc, GOx was immobilized by crosslinking with GA (2.5%) in the presence of BSA.[8] For this purpose, 10.0 mg of $Fe_3O_4$-Chi-Fc were dispersed in 2.0 mL of PBS 0.1 mol $L^{-1}$ (pH 7.0) containing BSA (15.0 mg $mL^{-1}$) and GOx (5.0 mg $mL^{-1}$). Then, it was added the solution of 110 µL of GA. This method provides a strong immobilization of GOx on the $Fe_3O_4$-Chi-Fc surface, since the GA acts as a bifunctional cross-linking agent in which bonds can be established between the amino groups present in the structure of the Chi and the mixture BSA/GOx. Before magneto-switchable studies, $Fe_3O_4$-Chi-Fc/GOx was stored for a period of 24 h at 4 °C in order to ensure that all enzymes were bound to the surface of $Fe_3O_4$-Chi-Fc.

### Characterization of $Fe_3O_4$ particles and modified-$Fe_3O_4$

The formation of $Fe_3O_4$ particles in suspension was monitored by UV-Vis spectroscopy using a Varian Cary 50 spectrophotometer. The measurements were performed in a quartz cuvette (1 cm of optical path). The crystal structure of $Fe_3O_4$ particles and modification of $Fe_3O_4$-Chi with Fc were evaluated by X-ray diffractometry (XRD) in a Bruker D8 Focus diffractometer with copper radiation (Cu $K_\alpha$, $\lambda = 0.15406$ nm). The scan angle was understood in a range of $10° \leq 2\theta \leq 70°$ with steps of 0.05°, measured at each 5 s. Fourier transform infrared (FTIR) spectra for powder of $Fe_3O_4$ particles, chitosan and $Fe_3O_4$-Chi were obtained in attenuated total reflectance mode (ATR) on ZnSe crystal using a Varian-600 FTIR spectrometer.

### Magneto-switchable electrochemistry

Chronoamperometric and cyclic voltammetric experiments were carried out in a potentiostat coupled to a system of switchable magnetic field generation, with “switch on” and “switch off” modes, denominated MagnetoElectron (homemade equipment, Figure 1).

The MagnetoElectron equipment was fabricated with magnets of neodymium-iron-boron and all magnets were grouped and positioned perpendicular to the working electrode providing a maximum field of 0.24 T (0.5 cm from the center of the electrochemical cell). The measurement of magnetic field was carried out using a gaussimeter and then used in the electrochemical experiments. For all the electrochemical experiments, a conventional electrochemical cell configuration formed by three electrodes was used: ITO (glass coated by tin oxide doped with indium) electrode was utilized as working electrode (1.0 $cm^2$), platinum as counter electrode (1.5 $cm^3$) and $Ag/AgCl_{sat}$ as reference electrode. For this purpose, a potentiostat/galvanostat μAutolab III was used.

It is noteworthy that during the electrochemical experiments, the two commutation states of the magnetic field modulator (“switch on” and “switch off” modes) were used in two configurations. In the configuration I, the “switch off” mode corresponds only to the suspension electrochemistry (absence of external magnetic), while in the “switch on” mode, all magnets are positioned behind work electrode. Different than what was proposed in the configuration I, the “switch off” mode used in the

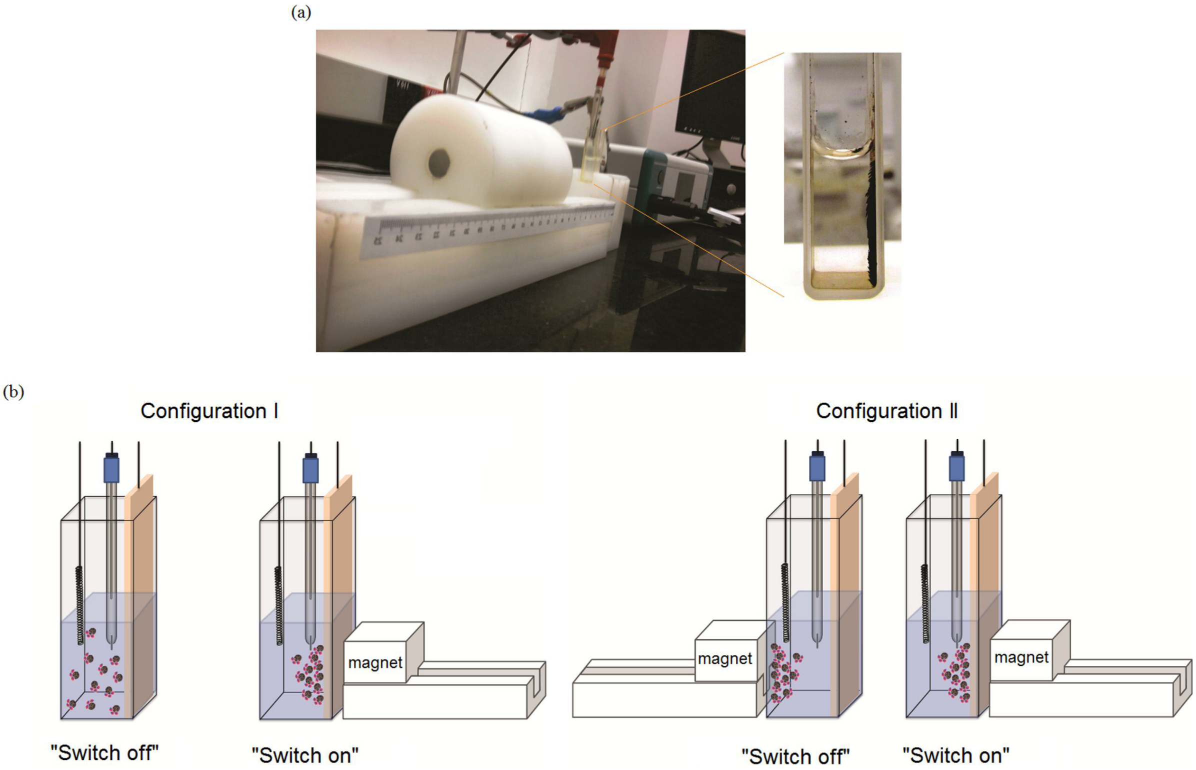

**Figure 1.** (a) Photograph of the electrochemical cell conjugated with a magnetic field modulator (MagnetoElectron). (b) Schematic illustration of magnetic field modulator employed in the configurations I and II.

configuration II was employed when all magnets are positioned in front of the working electrode (inverse position). Thus, the two switched states ("switch on" and "switch off" modes) corresponds to a magnetic field of the same intensity (0.24 T).

## Results and Discussion

### Characterization of $Fe_3O_4$ particles and the $Fe_3O_4$-Chi-Fc

As described in the experimental section, the formation of $Fe_3O_4$ particles occurs according to the following equation:[9]

$$Fe^{2+}_{(aq)} + 2\ Fe^{3+}_{(aq)} + 8\ OH^{-}_{(aq)} \rightarrow Fe_3O_{4(s)} + 4\ H_2O_{(l)} \qquad (1)$$

Electronic spectroscopy (UV-Vis) was used to evaluate the formation of $Fe_3O_4$ particles in suspension. Figure 2a shows the electronic spectra for 3 mmol L$^{-1}$ $FeCl_3$ solution (gray line) and for the suspension of $Fe_3O_4$ particles (black line). $FeCl_3$ solution is characterized by the presence of a band with maximum absorption at 300 nm (overlapped to the absorption band of $FeCl_2$, data not shown) attributed to metal to ligand charge transfer (MLCT) transition in aqua-iron complexes in solution. The disappearance of MLCT after the formation of $Fe_3O_4$ particles results from the removal of the aqua-iron complexes by hydrolysis of ferric ions promoted by $NH_4OH$. The spectrum of $Fe_3O_4$ suspension presents a broad featureless absorption tail typical of transition in the band gap of semiconductor materials.[10] In this condition, the increase of the spectrum baseline is evident due to the light scattering effect[11] generated by colloids present in suspension. Figure 2b shows the diffractograms obtained for $Fe_3O_4$ particles (black line) and ICSD database (card No. 26410) attributed to the magnetite pattern (gray line). Diffraction peaks at 2θ (30.1, 35.5, 43.1, 53.75, 57.2 and 62.85) were consistent with the magnetite pattern, corresponding respectively to the crystal planes (2 2 0), (3 1 1), (4 0 0), (422), (5 1 1) and (4 4 0) of inverse spinel structure.[12]

For magneto-switchable studies, particles with micrometer size are attractive because of the large surface area offered by each particle for the immobilization of large biomolecules. According to Willner and co-workers,[1] Fc-functionalized magnetic microparticles offer a surface coverage of ca. 1000-3000 Fc units *per* particle, available to be used in magneto-switchable bioelectrocatalysis, as it will be described. To obtain the composite for GOx immobilization, the $Fe_3O_4$ particles were modified with Chi and Fc. The former modification consisted in the adsorption of Chi by 24 h on surface of suspended $Fe_3O_4$ particles in 2% acetic acid. The adsorption of Chi was characterized by FTIR in ATR mode. Figure 3a shows the FTIR spectra for $Fe_3O_4$ particles (black line), Chi (light gray line) and the $Fe_3O_4$-Chi (gray line). For the $Fe_3O_4$ particles, the presence of two peaks at 598 and 659 cm$^{-1}$ corresponding to the vibrations of the Fe–O and Fe–O–Fe bonds, respectively, was observed.

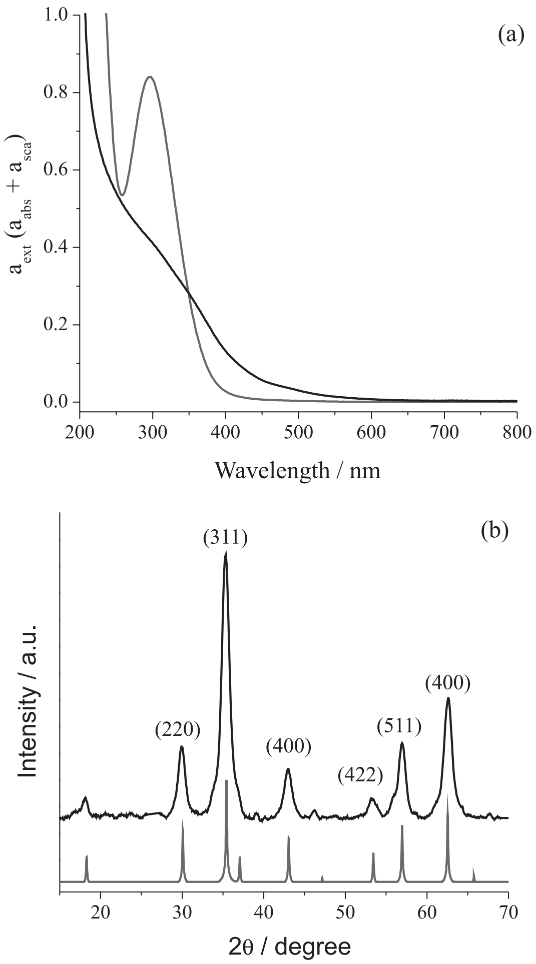

**Figure 2.** (a) UV-Vis spectra of the 3 mmol L$^{-1}$ $FeCl_3$ solution (gray line) and aqueous suspension of $Fe_3O_4$ particles (black line). (b) X-ray diffractograms of the $Fe_3O_4$ particle powder (black line) and ICSD database (card No. 26410) referring to the magnetite pattern (gray line).

For Chi, the presence of peaks at 1650 cm$^{-1}$ (C=O stretch of amide I), 1558 cm$^{-1}$ (C–N stretching of amide II coupled the N–H deformation of primary amine) and 1317 cm$^{-1}$ (amide III) was observed and related to the acetamide groups of chitin deacetylation.[13,14] The $Fe_3O_4$-Chi spectrum shows the characteristic peaks of chitosan and a peak at 660 cm$^{-1}$ typical of magnetite. The interaction Chi with magnetite was evidenced by the shift to a lower wavenumber (1545 cm$^{-1}$) of the peak related to the N–H deformation of primary amine concomitant with the appearance of a peak at 1403 cm$^{-1}$ that is characteristic of this composite.[15,16] These results are consistent with

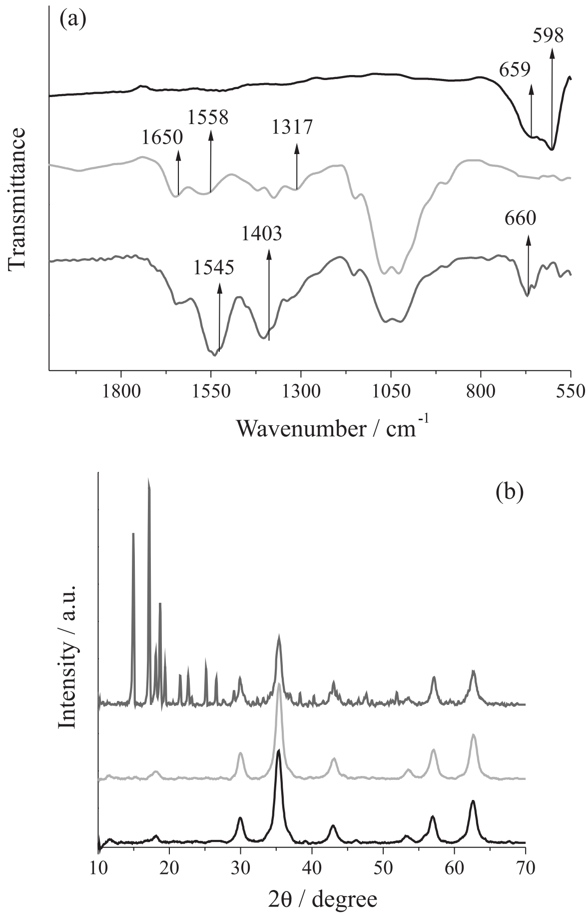

**Figure 3.** (a) FTIR spectra for $Fe_3O_4$ particles (black line), chitosan (light gray line) and $Fe_3O_4$-Chi (gray line). (b) X-ray diffractograms to powders of $Fe_3O_4$ particles (black line), $Fe_3O_4$-Chi (light gray line) e $Fe_3O_4$-Chi-Fc (gray line).

the amino groups present in chitosan as the functional groups responsible for interacting with the surface of $Fe_3O_4$ particles.

$Fe_3O_4$-Chi was then modified with Fc using also physical adsorption process promoted by the solvent evaporation of Fc hexane solution. The obtained material was washed with distilled water several times in order to remove non-adsorbed Fc molecules. This method ensures that $Fe_3O_4$-Chi-Fc contains exclusively molecules of Fc strongly adsorbed on the surface of $Fe_3O_4$-Chi. Figure 3b shows the diffractograms obtained for $Fe_3O_4$ particles (black line), $Fe_3O_4$-Chi (light gray line) and $Fe_3O_4$-Chi-Fc (gray line). The same crystallographic profile was observed for $Fe_3O_4$ particles and $Fe_3O_4$-Chi indicating no change in the crystalline structure after the modification with Chi.[17,18] Furthermore, $Fe_3O_4$-Chi-Fc diffractogram shows peaks related to Fc (2θ from 10 to 20°), indicating that the redox mediator was successfully adsorbed on the surface of $Fe_3O_4$-Chi. To verify whether Fc was effectively adsorbed on the $Fe_3O_4$-Chi, cyclic voltammetric experiments were performed using two suspensions: one containing 10 mg mL$^{-1}$ of $Fe_3O_4$-Chi (without Fc) and the other with 10 mg mL$^{-1}$ of $Fe_3O_4$-Chi-Fc. The suspension containing $Fe_3O_4$-Chi did not show faradaic process. Otherwise, the $Fe_3O_4$-Chi-Fc suspension exhibited two well-defined redox processes.

### Magneto-switchable electrochemistry of $Fe_3O_4$-Chi-Fc

Magneto-switchable voltammetric experiments determined the electrochemical behavior of $Fe_3O_4$-Chi-Fc (10 mg mL$^{-1}$) in the presence and absence of an external magnetic field. For this set of experiments, configuration I was used, in which "switch off" mode corresponds to the electrochemistry of $Fe_3O_4$-Chi-Fc suspension at zero magnetic field. Figures 4a e 4b show the cyclic voltammograms obtained at different scan rates (10-100 mV s$^{-1}$) using "switch off" and "switch on" modes, respectively. At 100 mV s$^{-1}$ scan rate, the "switch on" mode increased 86.2 and 42.3% the current density values (3.64 and 1.63 μA cm$^{-2}$) for the anodic and cathodic currents, respectively. The external magnetic field attracts $Fe_3O_4$-Chi-Fc to the electrode surface in a condition of forced convection that results in a larger amount of $Fe_3O_4$-Chi-Fc deposited onto ITO surface. This leads to higher values of current densities. The optimization of $Fe_3O_4$-Chi-Fc deposition onto ITO surface was achieved by starting the cyclic voltammetry measurement in "switch on" mode one minute after the beginning of magnetic field application. This strategy ensures that all $Fe_3O_4$-Chi-Fc present in the suspension is conducted to be adsorbed on the electrode surface. The application of magnetic field for longer than one minute did not improve the yielding of current density, indicating that this time is enough for saturation of the electrode surface with $Fe_3O_4$-Chi-Fc. Thus, only a short time interval is required for electrochemical measurements in a set of successive magneto-switchable field of $Fe_3O_4$-Chi-Fc.

The quasi-reversible redox processes that are present in the voltammograms can be attributed to Fc, because $Fe_3O_4$ particles and Chi did not show electroactivity at the same potential range. Interestingly, the potential shifts obtained for a "switch on" and "switch off" set of commutative experiments were different. In the "switch on" mode, they were observed with $E_{pc} = -0.16$ V and $E_{pa} = 0.32$ V that results in $\Delta E_{\text{switch on}} = 0.47$ V at 100 mV s$^{-1}$. In the "switch off" mode, they were observed with $E_{pc} = -0.18$ V and $E_{pa} = 0.39$ V, that results in $\Delta E_{\text{switch off}} = 0.57$ V. Considering $\Delta E_{\text{switch on}}$ lower than $\Delta E_{\text{switch off}}$, one can assume that the proximity of the $Fc/Fc^+$ redox pair at the electrode surface affects the electrochemical reversibility and, consequently, the charge-transfer energy. This assumption could be corroborated by comparing the $\Delta E_{\text{switch on}}$ and $\Delta E_{\text{switch off}}$ values obtained at all scan rates, as shown in Figure 4c. In the whole scan rate range used here (from 10 to 100 mV s$^{-1}$), the $\Delta E_{\text{switch on}}$ values were always lower than $\Delta E_{\text{switch off}}$ values. While the result of reversibility affects the kinetic of the redox couple, no catalytic effect was observed for "switch on" case, as shown in Figure 4d.

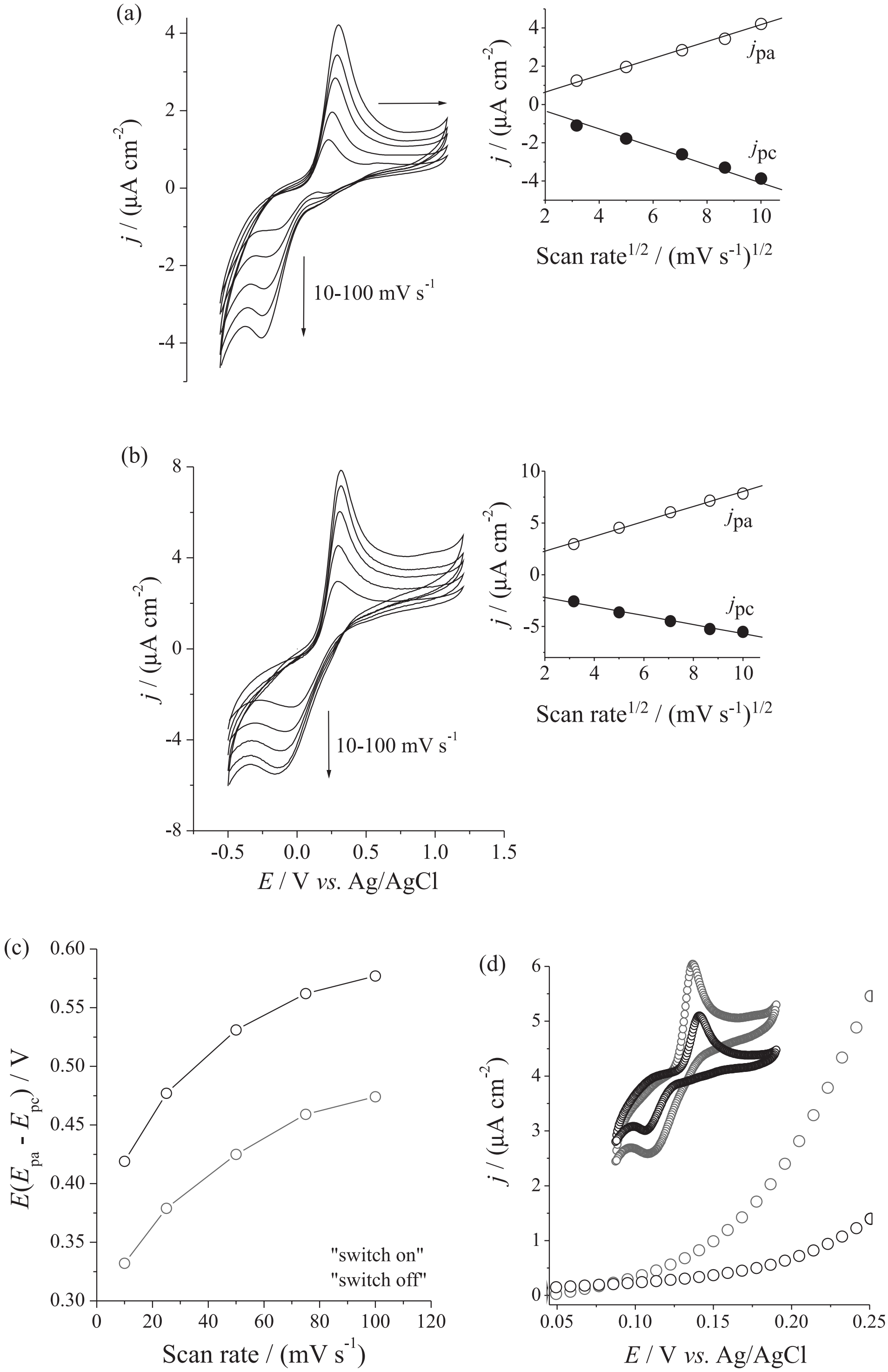

**Figure 4.** Study of scan rate variation (10-100 mV s$^{-1}$) for $Fe_3O_4$-Chi-Fc (10 mg mL$^{-1}$) employing the (a) "switch off" and (b) "switch on" modes (configuration I). (c) Comparison between $\Delta E_{\text{switch on}}$ (gray line) and $\Delta E_{\text{switch off}}$ (black line) for all scan rates (10-100 mV s$^{-1}$). (d) Linear voltammetry referring to the on-set potential of anodic oxidation process of $Fe_3O_4$-Chi-Fc employing "switch on" (gray line) and "switch off" (black line) modes. Inset: cyclic voltammograms of $Fe_3O_4$-Chi-Fc at 100 mV s$^{-1}$ obtained at "switch on" (gray line) and "switch off" modes (black line). Support electrolyte: 0.1 mol L$^{-1}$ phosphate buffer, pH 7.0.

The application of magnetic field ("switch on" mode) affects not only the thermodynamic properties of $Fe_3O_4$-Chi-Fc but also the kinetics of charge transport of Fc. The insets of Figures 4a and 4b show a linear increase of the anodic and cathodic peak currents as function of the square root of the scan rate consistent with electrochemical reactions limited by diffusion. In "switch off" mode, some factors influence the diffusion of the $Fe_3O_4$-Chi-Fc. One effect to be considered is the gravity force acting perpendicularly to the diffusion plane towards the electrode surface (Fick's law). In this regard, precipitation of microparticles was visible only after 15-20 min of suspension at rest. This time is significantly higher than that necessary for a voltammogram measurement and enabled us to disregard the precipitation process for electrochemical results. In the "switch on" mode, with the deposition of $Fe_3O_4$-Chi-Fc on the electrode surface, a linear increase of the faradaic current is expected with increasing of scan rate, typical of a process limited by charge transfer. However, a hyperbolic profile was also observed in the "switch on" mode.

It is possible that the limited diffusional process of redox-modified magnetite adsorbed on the electrode surface may related to the interface structure or/and migration of ions. For instance, considering that after $Fe_3O_4$-Chi-Fc deposition on the electrode surface, there is the formation of an interface resembling a thin film-electrode system, the migration of ions in the internal plane of electrical double layer (inner Helmholtz plane) should occur efficiently in order to maintain the electro-neutrality on the electrode surface after oxidation of Fc with formation of species $Fe_3O_4$-Chi-$Fc^+$. The inner Helmholtz plane is the area where charges not solvated and specifically adsorbed are located and it is known that the adsorption of non-charged species can block the electrode surface and prevent the efficient electron transfer. Thus, it is possible that the non electroactive components of $Fe_3O_4$-Chi-Fc (Chi and $Fe_3O_4$ particles) may be contributing for the impairment of the electron transfer process. This phenomenon can also be discussed in terms of possible deviations of the Cottrell equation for "switch on" mode that describes the change in current in chronoamperometry, as shown in Figure 5.

In this condition, the current measured for $Fe_3O_4$-Chi-Fc depends on the rate at which the electron diffuses to electrode surface. The theoretical electron diffusion constant ($D_{ct}$), was determined for the "film" of $Fe_3O_4$-Chi-Fc deposited on electrode; here, it was considered film-like the $Fe_3O_4$-Chi-Fc deposited in "switch on" mode. The Cottrell equation for an ITO electrode that has planar geometry and boundary conditions in conjunction with Fick's second law of diffusion was considered. The smoothly decaying current-time curve was obtained when plotted according to the Cottrell equation for linear diffusion ($i$ *vs.* $t^{-1/2}$) for 600 s experiment time (equation 2).

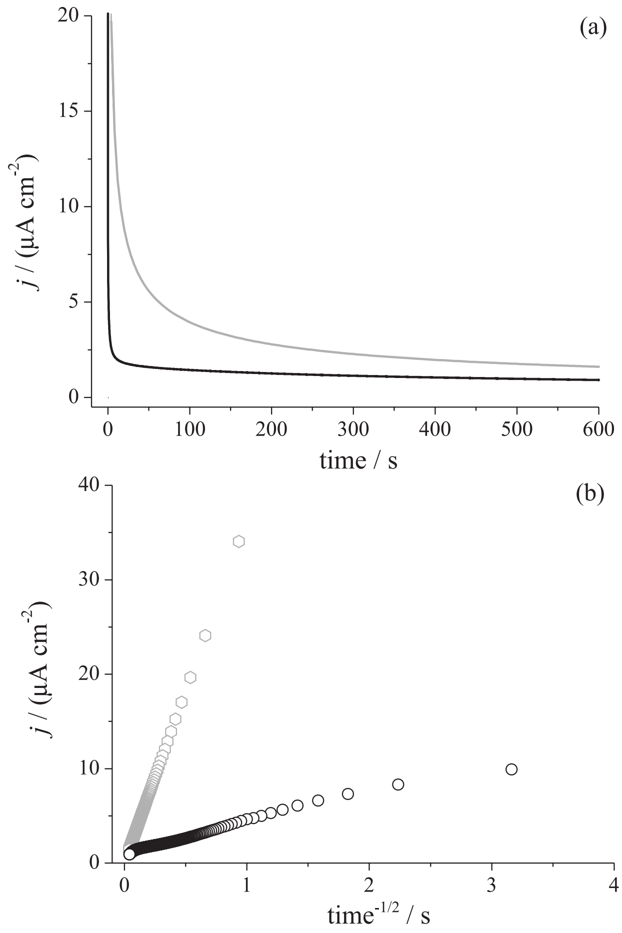

**Figure 5.** (a) Theoretical (black line, using Cottrell equation) and experimental (gray line) chronoamperometry for $Fe_3O_4$-Chi-Fc in "switch on" mode. (b) Theoretical (gray curve) and experimental (black curve) Cottrell plots for $Fe_3O_4$-Chi-Fc in "switch on" mode. Applied potential: 0.34 V.

$$i = \frac{nFAD_{ct}^{1/2}c_i}{\pi^{1/2}t^{1/2}} \qquad (2)$$

In equation 2, $D_{ct}$ is the electron diffusion constant, $c_i$ is the concentration of electroactive ferrocene sites in the "film" ($Fe_3O_4$-Chi-Fc adsorbed), F is the Faraday constant, A is the electrode surface area and t is the time, respectively. Figure 5a shows the simulated (black line) and experimental (gray line) chronoamperometry for "switch on" mode during 600 s. In order to determine the amount of Fc physically adsorbed onto $Fe_3O_4$ particles, the charge (Q = n*e*) was determined from the integration of experimental chronoamperogram, with $\lim_{t\to\infty}$ (t).[19] First, the determination of the adjusted curve leads to the determination of Fc charge and the proportion related to the

$Fe_3O_4$ mass used for the electrochemical experiments. For Fc species was attributed a charge value of 53.4 μC cm$^{-2}$ and an amount of $5.5 \times 10^{-7}$ mol was determined, which corresponds to 0.103 mg of Fc in 10.0 mg of $Fe_3O_4$-Chi-Fc (1.0% of Fc in mass adsorbed on $Fe_3O_4$-Chi). The modeling of the obtained chronoamperometry curves was also used to determine the theoretical value of $D_{ct} = 4.7 \times 10^{-6}$ cm s$^{-1}$; by using equation 2. However, as observed in the chronoamperometric experiments shown in Figure 5a, there is a deviation from theoretical chronoamperogram. The deviation is more clearly seen in the plot *i vs.* $t^{-1/2}$ (Figure 5b) with a rapid growth curve that reaches a finite diffusion up to $t^{-1/2} = 0.2$ s (black curve).

This result can be associated with the concentration-distance profiles of $Fc^+$ sites within $Fe_3O_4$-Chi. The incorporation of $Fe_3O_4$ particles and Chi organic polymer can be the cause of the electron transfer resistance, as observed in previously reported studies using copolymer film at different times during the potential step electrolysis.[20] For the latter, Nakahama and Murray[20] showed the effect of composition of a ferrocene-containing redox polymer on the electrochemistry of its thin film coatings on electrodes. In principle, for $Fe_3O_4$-Chi-Fc/solution interface, the diffusion of electrons should follow infinite diffusion laws, e.g., the Cottrell. At longer times, the "supply" of ferricenium sites becomes limited by the finite thickness of the non-redox species, and consequently the current falls more rapidly than anticipated from equation 2. This is called finite diffusion, and the current-time relation in this regime is ideally represented by an equation identical to that employed for thin-layer electrochemistry.[20] Thus, this result can be consistent with the case of "switch on" mode, with the deposition of $Fe_3O_4$-Chi-Fc on the surface, in which a linear increase of the faradaic current with increasing of scan rate was not observed (Figure 4), indicating a system limited by diffusion process.

### High electrochemical stability of $Fe_3O_4$-Chi-Fc

After evaluating the electrochemical behavior of $Fe_3O_4$-Chi-Fc in the presence and absence of an external magnetic field, by chronoamperometry (configuration II), a more refined control of the redox processes was observed (Figure 6). As illustrated in Figure 1b, "switch off" mode consists in the total removal of redox species from the electrode surface by the application of the magnetic field contrary to the plane of the electrode. For chronoamperometric experiment, a potential of 0.34 V was applied because Fc is in reduced form in $Fe_3O_4$-Chi-Fc. Similarly to the voltammetric experiments, in the "switch on" mode there was an increase in oxidation currents due to $Fe_3O_4$-Chi-Fc deposited onto ITO surface. During 50 s, when the quasi-stationary current was reached, an increase of current density of 1.0 μA cm$^{-2}$ was observed (Figure 6a).

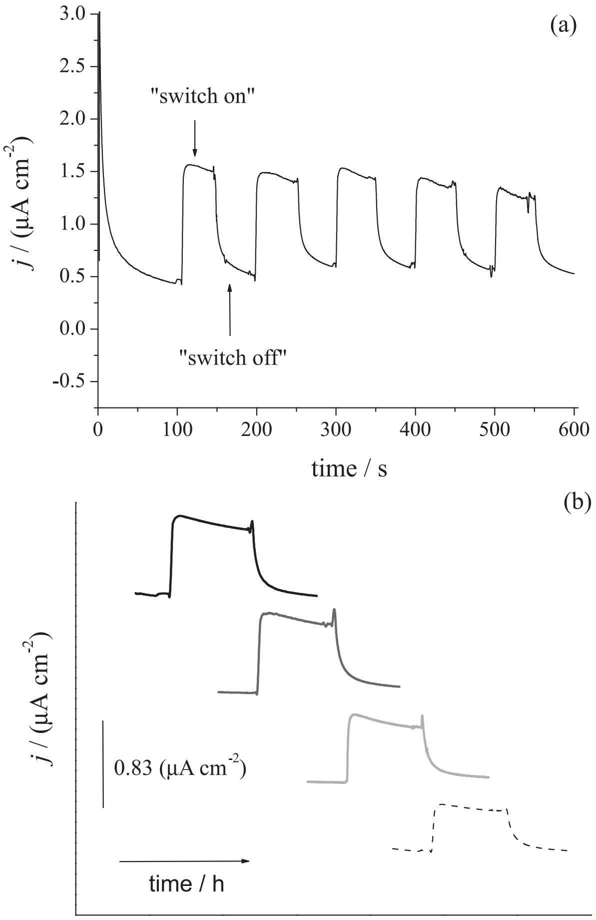

**Figure 6.** (a) Magnetic control of the redox processes of $Fe_3O_4$-Chi-Fc (10 mg mL$^{-1}$) employing the "switch on" and "switch off" modes (configuration II). (b) Electrochemical stability of $Fe_3O_4$-Chi-Fc (10 mg mL$^{-1}$) in several time intervals: 1 h (black line), 5 h (gray line), 12 h (light gray line) and 24 h (dashed line). Applied potential: 0.34 V. Support electrolyte: 0.1 mol L$^{-1}$ phosphate buffer, pH 7.0.

In order to check the electrochemical stability of $Fe_3O_4$-Chi-Fc, the "switch on" and "switch off" modes were applied successively, and no significant loss of current was observed. The same experiments were reproduced more than 10 times with different electrodes and the chronoamperometric currents remained unchanged, a result consistent with high stability and reproducibility of the $Fe_3O_4$-Chi-Fc. In addition to the several cycles of "switch on" and "switch off" modes, the long term stability of this system was examined.

Figure 6b shows chronoamperograms obtained at different intervals, such as 1 h (black line), 5 h (gray line), 12 h (light gray line) and 24 h (dashed line). A stable system was clearly shown with more than 50% of current density values maintained even after several switching cycles collected after 24 h of operation. The decrease in current density was observed only for the chronoamperometric experiments performed in the absence of the enzyme glucose oxidase (GOx). This decrease is related to the consumption of Fc due the application of an oxidation potential on the ITO electrode ($E = 0.34$ V). Thus, at each switching cycle performed, more species are reacting. Also, it is important to mention that the same suspension of $Fe_3O_4$-Chi-Fc (0.103 mg Fc) was used in all electrochemical experiments described in this work, supporting the rationale described above.

### Magneto-switchable bioelectrocatalysis

In order to show an application for $Fe_3O_4$-Chi-Fc, the catalytic activity of GOx when immobilized on $Fe_3O_4$-Chi-Fc was evaluated. For this purpose, $Fe_3O_4$-Chi-Fc/GOx was subjected to magneto-switchable bioelectrocatalysis. GOx is an enzyme-model commonly used in the fabrication of amperometric biosensors and also in bioelectrocatalysis studies.[21-24] Among all studies reported in the literature[1-5] about magneto-switchable bioelectrocatalysis, there is no one describing the immobilization of redox enzymes attached directly to magnetic particles modified with redox mediator. For instance, Willner and co-workers[1-3] reported the use of GOx dispersed in aqueous solution and exclusively the redox mediator was immobilized on surface of $Fe_3O_4$ particles. Dong and co-workers[4] used the GOx immobilized on the surface of $Fe_3O_4$ particles while the mediator molecules are dissolved in the electrolyte.

From the best of our knowledge and differently of all other previous studies, for the first time it was used both GOx and Fc immobilized on the surface of $Fe_3O_4$-Chi particles. The objective here is the construction of a system containing the three components together: magnetic particle, mediator and enzyme. For the latter component, the amino groups of Chi provide a mild environment for enzyme immobilization. Also, as described in experimental section, cross-linking procedure utilizing GA molecules in the presence of BSA provides an effective immobilization of GOx on the $Fe_3O_4$-Chi-Fc surface. The latter can be observed in Figure 7a, in which the system $Fe_3O_4$-Chi-Fc/GOx is easily attracted by the magnetic field. Figure 7b shows the response of $Fe_3O_4$-Chi-Fc/GOx in the absence of glucose (black line) and in the presence of glucose (20 mmol $L^{-1}$) using a magnetic field modulator in "switch off" (light gray line) and "switch on" (gray line) modes (configuration I).

In the absence of glucose, a redox process related to oxi-reduction of Fc mediator was observed. In the presence of glucose, the oxidation current of $H_2O_2$ was observed in the two switchable modes. The oxidation of glucose in the presence of $Fe_3O_4$-Chi-Fc/GOx can be represented by equations 3, 4 and 5.

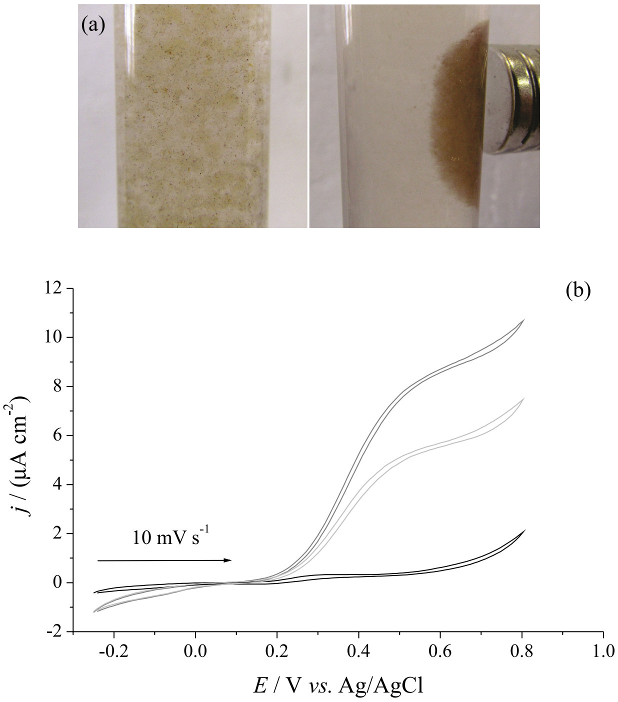

**Figure 7.** (a) Pictures showing the magnetic response of $Fe_3O_4$-Chi-Fc/GOx in aqueous suspension in the presence of a magnet. (b) Cyclic voltammograms of $Fe_3O_4$-Chi-Fc/GOx in the absence of glucose (black line) and in the presence of 20 mmol $L^{-1}$ of glucose in "switch off" (light gray line) and "switch on" (gray line) modes (configuration I). Scan rate: 10 mV $s^{-1}$. Support electrolyte: 0.1 mol $L^{-1}$ phosphate buffer, pH 7.0.

$$\beta\text{-D-glucose} + \text{GOx-FAD} \rightarrow \text{6-D-glucolactone} + \text{GOx-FADH}_2 \quad (3)$$

$$\text{GOx-FADH}_2 + O_2 \rightarrow \text{GOx-FAD} + H_2O_2 \quad (4)$$

$$2Fc \rightarrow 2Fc^+ + 2e^- \quad (5)$$

The oxidation currents attain the maximum around 10.7 μA cm$^{-2}$ at 0.8 V for "switch on" mode, leading to an increase of 3.2 μA cm$^{-2}$ when compared with "switch off" mode. In fact, the current improvement caused by the increased of amount of enzyme on the electrode is very attractive for further studies in biocatalysis.

## Conclusions

A novel material composed by glucose oxidase enzyme, chitosan, ferrocene and magnetite ($Fe_3O_4$-Chi-Fc/GOx) was obtained. $Fe_3O_4$-Chi-Fc showed high stability, retaining 50% of the values of current density after successive cycles of switching run during 24 h. By cyclic voltammetry, it was observed that in the presence of the external magnetic field, the mass transport of $Fe_3O_4$-Chi-Fc is intensified, resulting in the increase of 86.3 and 42.3% for the anodic and cathodic currents, respectively, attributed for the redox couple Fc/Fc$^+$. Also, the redox potential shift is different for the "switch on" and "switch off" commutative experiments. In addition, the kinetics of charge transfer of Fc/Fc$^+$ is also influenced, because the electrochemical reactions are limited by diffusion for both "switch on" and "switch off" modes. This unexpected behavior was attributed to the non-electroactive components (chitosan and iron oxide) of $Fe_3O_4$-Chi-Fc that may contribute to hinder the electron transfer process. For $Fe_3O_4$-Chi-Fc/GOx suspended in 20 mmol L$^{-1}$ of glucose solution, that catalytic current of glucose oxidation was 70% higher for "switch on" than "switch off" mode. The oxidation currents attained the maximum about 10.7 μA cm$^{-2}$ at 0.8 V for "switch on" mode, leading to an increase of 3.2 μA cm$^{-2}$ when compared with "switch off" mode. Based on these results, it is possible to achieve a fine control of the bioelectrocatalysis, because redox mediator and enzyme components are both assembled in a magnetic particle.

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