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Chapter

Development of an Ethanol Biosensor Based on Silver Nanoparticles/Polyaniline/Graphite/Epoxy Composite for Friendly Analytical Application

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Abstract

Electrochemical biosensors for measuring ethanol were developed in this study. Silver nanoparticles were incorporated to increase sensibility. Firstly, the transducer element was prepared with silver nanoparticles/polyaniline/graphite/epoxy composite (AgNPs/PANI/GEC) where the enzymes *alcohol oxidase* (AOD) and *horseradish peroxidase* (HRP) were immobilized by adsorption. The composition of the immobilized solution was indicated by an experimental design (85% of the bi-enzymatic solution, 10% of albumin and 5% of glutaraldehyde). The immobilization method adopted in this study showed to be highly reproducible. The values of variance and standard deviation were low (0.003 and 0.053, respectively—means of three electrodes). The linear range was 0–30 g/L ($R^2 = 0.983$) and the sensitivity 0.004 mA L/g. A second biosensor was made with a transducer prepared with AgNPs/PANI/GEC. A chitosan film was applied over the adsorbed enzymatic solution to avoid desorption of the immobilized enzymes. The AgNPs/PANI/GEC electrodes were characterized using cycle voltammetry and the composite surface by scanning electron microscopy. The calibration for ethanol samples with this second composite fitted in a range of 0.0–0.35 g/L ($R^2 = 0.984$). Square rate voltammetry was the electrochemical analytical method used to obtain the amperometric calibration curves.

Keywords: alcohol oxidase, horseradish peroxidase, polyaniline, silver nanoparticles, electrochemical biosensor

1. Introduction

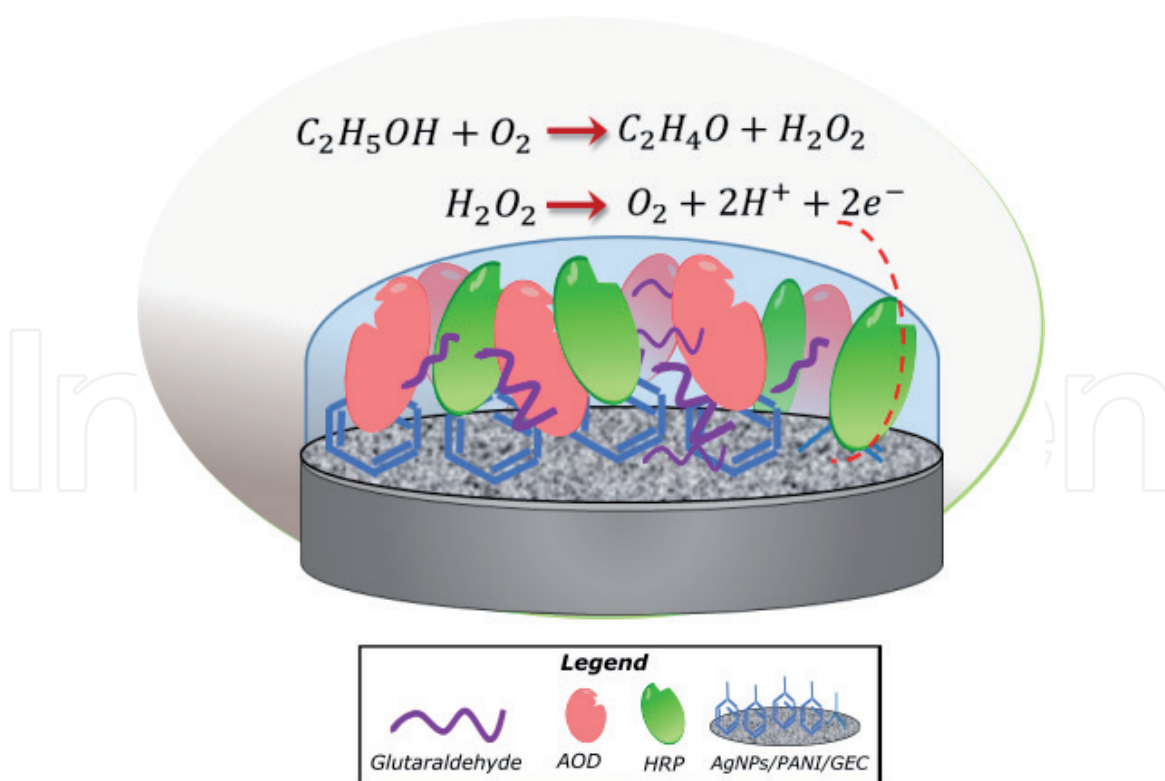
Biosensors with electrochemical transducers are attractive from a commercial point of view due to its low expense and simplicity of construction and because it is easy to employ in a broad range of applications and portable [1, 2]. The voltammetric and amperometric technique analyses are a full implementation to measure low levels of concentration. Additionally, as an electrochemical surface method, it has the

advantage of generating a response signal working with small sample volumes, in real time. The working electrode can be prepared by surface immobilization of one or more enzymes, which are involved in recognition of the analyte. The enzymes could be regarded as the critical component of the electrode since they are related to the selectivity of the sensor to catalyze the formation of the electroactive detection product [3]. Additionally, the enzymes are ideal biological recognition elements in the construction of biosensors, because of their high specificity, which enables the development of analytical methods with high accuracy [1]. Biosensor technologies are recognized as an emerging science to produce analytical devices that can help to detect specific compounds in complex mixtures such as liquid waste residues and blood serum [4, 5]. Nowadays, ethanol analysis is fundamental for criminal justice systems, in clinical and toxicological diagnostic analyses such as blood, serum and urine analysis, monitoring medical conditions of HIV patients, as well as public safety issues regarding the pilots and drivers. In the food and beverage industries, the determination of alcohol content is critical for the control of the fermentation process and product quality. Besides these applications, determination of ethanol is also important in agricultural, biofuel and environmental analyses [6–8]. *Alcohol oxidase* (AOD) allows the qualitative and quantitative determination of ethanol or methanol, removal of alcohol or aldehyde and hydrogen peroxide production and removal of oxygen [9]. *Horseradish peroxidase* (HRP) uses the haem group and hydrogen peroxide substrate to oxidize a variety of organic and inorganic compounds [10]. The enzymatic mechanisms of these enzymes together facilitate the detection of AOD substrates by electrochemical and spectroscopic methods such as ethanol. The co-immobilization of the enzymes holds the potential to increase the selectivity and amplify the sensitivity of the biosensor improving the potential for quantitative ethanol detection [11, 12]. Polyaniline (PANI) has attracted the attention of the scientific community in the last two decades. PANI, a family of conductive semi-flexible polymers in a green protonated emeraldine form had high electrical conductivity and low production cost. PANI has been explored for various applications, including those in biosensors due to some useful features such as redox conductivity and polyelectrolyte characteristics, high surface area, chemical characteristics, long-term environmental stability and tunable properties enhancing the electron transfer flux ability and also the reversibility of the electrochemical response signal [12–14]. In the previous work, the electroconductivity of PANI-GEC was reported and verified that PANI is an attractive polymer compound to be applied in sensor interface transducer biosensors. A conductivity of 28 $\mu\text{S}/\text{cm}$ for 30 w/w% PANI-GEC was measured and cyclic voltammograms for 10 mM potassium ferricyanide obtained working with a scan rate of 100 mV s^{-1} . The composite can act as an effective mediator in the transference of electrons in redox or enzymatic reactions [14]. With the use of nanomaterials, greater sensitivity and attachment of enzymes are achieved due to their high surface area as well as the physical, chemical and electronic properties. The literature reported that nanomaterial application had attracted much attention regarding the development of high-performance electrochemical biosensors [13]. The preparation of chemically modified electrodes with silver nanoparticles (AgNPs) has been applied to amplify the electrochemical response signal. Since silver is four times cheaper than gold and shows excellent catalytic activity and good electrical/heat conductivity, its application is very favorable in electroanalysis acting as a pre-concentrator of species of interest and/or mediating redox reactions [15]. The literature also reported an increase of 9.71% in the anodic peak current and 32.35% for the cathode peak current in the presence of AgNPs. The authors observed an increase in the reversibility of the voltammetric response signal in the composite based on AgNPs/PANI/GEC and the ratio of the anodic (I_{pa}) and cathodic (I_{pc}) peak currents $|I_{pa}/I_{pc}| = 1.07$ at 40 mV s^{-1} . Nevertheless, the ratio was of the order of 1.28 for composite without AgNPs [16]. From a point of view of analytical instruments,

biosensors are used also for quality control, because they have important technical characteristics, such as low response time, high selectivity, stability under the conditions of the analysis, and reproducibility of the measurements. So, ethanol as a hydrous biofuel (Brazil, 5.3 volume % of water) or in the anhydrous form mixed in blends with gasoline (USA—10%, 15% and 85% of anhydrous ethanol, western Europe—5% of anhydrous ethanol and Brazil—27% of anhydrous ethanol) have being used in regular cars and flex fuel vehicles. Methods for monitoring the percentage of ethanol in the mixtures (product quality control) or in case of spilling of gasohol blends need to be develop. Many efforts for different potentiometric, amperometric, and spectrophotometric biosensors have been developed for ethanol analyses, with microorganisms like *Gluconobacter oxydans*, *Saccharomyces ellipsoideus*, or enzymes as alcohol dehydrogenase or alcohol oxidase were just reported in the literature [17]. In Brazil, there is a rigorous program for a strict control of the physicochemical characteristics of the gasohol blend and hydrated fuel alcohol to prevent adulteration or environmental contamination. Nowadays, the 4.0 industry claims for more robust and sensitive instruments for long-distance transmission and data transfer systems to an analytical central station in monitoring and process control program. Only a few biosensors are commercially available at present for analysis control and the integration of nanomaterials composites within these enzymatic biosensors brings new strategies for enhancing their analytical performances [16, 17]. The high ethanol solubility turns the assessment and analytical methods limited. Many road or pipeline accidents can spill fuel blends into the environment. Significant environmental impacts related to ethanol spills have been to surface water and fishes were killed several days after as a result of oxygen depletion. Spilled ethanol from the surface through soil to groundwater contamination is also of concern, and anaerobic biodegradation of ethanol in groundwater results in the production of methane [18]. The development of more selective and integrated systems for application in fast and high accuracy analysis needs further innovation and research investments. The enzyme immobilization methods represent an important step for the new technologies applied in bioinstrumentation techniques. The incorporation of silver nanoparticles in two different composites and the electrochemical response signals generated from each biosensor were investigated. An experimental design was used and statistic analysis to define the best condition for low cost enzymatic immobilization method. The AgNPs/PANI/GEC biosensor with AOD and HRP immobilized enzymes was firstly prepared to detect ethanol. A second composite with only AgNPs/GEC was prepared and the immobilized AOD and HRP adsorbed enzymes covered with a chitosan film. Voltammetry/amperometric techniques were applied to characterize the electrochemical transduction systems. Calibration curves were obtained for each composite electrode biosensor in order to evaluate the ethanol analytical ranges and detection levels.

2. Electrode construction and characterization

The composite used in the manufacture of the electrode for the biosensor comprised 40% of PANI, 35% epoxy and 25% enriched with graphite AgNPs as described in [16]. The mixture was inserted into the empty end of the Teflon support (rod of 50 mm length × 7 mm in outer diameter, recessed 3 mm diameter × 3 mm deep). The electrodes containing the composites were maintained in an incubator at 30°C for 24 h, and then, the end polished with 1200 mesh sandpaper. In this work two strategies of enzyme immobilization were studied, one by adsorption on the surface composite and a second way covering the bi-enzymatic solution with a film of chitosan polymer. **Figure 1** shows an illustration of the composite surface with

**Figure 1.**

Schematic diagram of the surface composite with the immobilized AOD and HRP enzymes and the reaction electrode sequence.

enzymes immobilized by adsorption without the chitosan film addition, with the two sequential enzymatic reactions occurring. The ethanol is firstly oxidized to acet-aldehyde, and hydrogen peroxide is also produced by the action of the immobilized AOD. After that, the hydrogen peroxide is decomposed in oxygen and water by the immobilized HRP. The oxi-reduction reaction generates electrons by the hydrogen peroxide hydrolysis, and the mechanism could be expressed as follows [19]:



The chitosan film was prepared by dripping a chitosan solution onto the immo-bilized enzymes adsorbed on the composite surface. In order to prepare the chitosan solution, 2.0 g was dissolved with 100 ml of acetic acid 0.5% (v/v) and the result-ing solution rested without mixing for 2 hours and, in sequence, mixed for 6 h at 1000 rpm. A vacuum filtration pump was used to remove the chitosan particles that did not dissolved completely, and pH was adjusted to 4.9 with acetic acid [20]. The prepared electrodes with chitosan film were stored at room temperature for 6 h and after at 8°C. The reagents used in this research were polyaniline (emeraldine salt), epoxy resin DER 332, glutaraldehyde and enzyme *alcohol oxidase* EC 1.1.3.13 (obtained from *Pichia pastoris*) purchased from Sigma-Aldrich; sodium citrate, 4-aminoantipy-rine, hydrogen peroxide, phenol and ethanol 95% P.A. (analytical grade) from Vetec; silver nitrate (Synth); and enzyme *horseradish peroxidase* (EC 1.11.1.7) from Toyobo, Brazil. The lyophilized enzyme was suspended in phosphate buffer pH 7.0, filtered on

qualitative-grade filter paper (INLAB) and placed on Spectrum[®] dialysis membrane (12,000–14,000 kDa) at 8°C immersed in deionized water that was exchanged every 12 hours for 24 h. The purified enzyme was kept in a freezer. Graphite powder (Fluka) was used in the preparation of composites. Sodium phosphate reagent and albumin used in immobilization experiments purchased from Sigma-Aldrich. The composite based in graphite was enriched with a colloidal dispersion of AgNPs synthesized by the Turkevich method [21]. The distribution of AgNPs was characterized by UV–vis-visible spectrophotometer (Shimadzu, model UV—1800 120 V) reading the absorbance at a scanning wavelength between 250 and 700 nm [22, 23]. Atomic force microscopy (AFM) images were taken to identify the AgNPs in the freshly prepared colloidal suspension using an atomic force microscopic Solver NEXT, NT-MDT Integration Solutions for Nanotechnology, SPM Controller (P9 XPM Systems Digital Control Platform), model BL900 [23]. Tyndall effect or the scattering of light by colloidal particles in suspension was observed for recently obtained silver nanoparticles in water using a laser pointer [21]. Scanning electron microscopy (SEM) images were also taken with a scanning electron microscopy—EDS: BAL-TEC SCD 005. Square wave voltammetry was the electrochemical analysis method applied to the generated response signal characterization for different ethanol solution concentrations. The parameters used during the tests with square wave voltammetry were 10 mL of the electrolyte solution, frequency 25 Hz scan rate of 40 mV/s potential between 0.2 and 0.75 V. A potentiostat (Autolab, PGSTAT12 model), reference electrode Ag/AgCl (ALS model RE-012167 1B) and counter electrode platinum wire were used. D-optimal mixture design was used to evaluate the influence of variables on the parameters of the immobilization method based on optimal composition proposed in the literature [24, 25]. The experimental design of the mixture was prepared with the following restrictions: glutaraldehyde volume ranging from 1 to 10%, albumin volume of 0.5–10% and volume of enzyme solution ranging from 80 to 98.5%. The resulting peak current (mA), generated by the formulations tested, was the response signal adopted to evaluate the performance of electrochemical bi-enzymatic biosensors. The mixture response surfaces and principal component analysis calculations were performed using the Statistica 8.0 software. The components used in the manufacture of the lock solution were 2.5% (v/v) glutaraldehyde, albumin 1% (w/v) and an enzymatic solution composed of AOD (286 U) and HRP (2640 U). **Table 1** shows the proposed compositions generated by the mixture experimental design software.

From the proportions suggested by the statistical analysis, 10 µL of immobilization solution was deposited on the electrode surface which remained stored at 4°C for 12 h. Peak currents obtained by square wave voltammetry using parameters were described in Section 2.4. An electrolytic cell containing 10 mL of sodium phosphate buffer (pH 7.0) with the addition of 0.5 mL of 95% ethanol solution was used. Calibration of the AgNPs/PANI/GEC biosensor was performed under optimized conditions at 0.4 V versus AgCl in 0.1 M sodium phosphate buffer solution (pH 7.0).

Experiment	Bi-enzymatic solution (%)	Glutaraldehyde (%)	Albumin (%)
1	80.00	10.0	10.0
2	98.50	01.0	0.50
3	89.00	01.0	10.0
4	89.50	10.0	0.50
5	89.25	5.50	5.25

Table 1.
Percent composition proposed by the design experiments.

10 μL of an immobilization solution, whose experimental design estimates provide the greatest resulting peak current within the planned boundaries, was deposited on the composite electrode surface, which remained stored at 4°C for 12 h. For the construction of the standard curve, aliquots of 95% ethanol PA (concentration: 789 g/L) were added to obtain different concentrations of ethanol. The resulting peak currents were obtained by square wave voltammetry carried out in triplicate. The investigation of repeatability of the biosensor was performed using 9.85 μL of sodium phosphate buffer solution (pH 7.0) with the addition of 0.15 μL of 95% ethanol PA (concentration: 789 g/L) making a concentration of 12.8 g/L of ethanol.

2.1 AgNPs colloidal dispersion characterization

The absorption spectrum of AgNPs colloidal dispersion is shown in **Figure 2(A)**, which exhibits an absorption band at approximately 400 nm, consistent with the results reported in the literature [21, 22]; typical absorption band is in the region of 350–450 nm, confirming that their synthesis was successful. Such bands are unique physical properties of these nanoparticles. When an external magnetic field, such as light, is applied to metal, the conduction electrons move in tandem to provide a distributed load disturbance known as plasma, located close to the metal surface [21]. A relationship between the color of the colloidal silver nanoparticles with the diameter (6–28 nm for yellow color) and the shape was just demonstrated in other researches [23, 24]. **Figure 2(B)** shows the atomic force microscopy measurements of the colloidal AgNPs solution, to show the surface topography of the as-formed silver nanoparticles is homogeneous and uniformly modified on the colloidal solution to obtain a surface-based nanocomposite system like published in previous studies [23–28].

The surface of the graphite/AgNPs composite was prepared by mixing 150 mg of graphite powder with 50 mL of the nanosilver colloidal suspension. A dry composite was obtained after 12 h at 100°C for complete water evaporation [16]. The enriched graphite with the silver nanoparticles, after homogenization, was characterized by MEV images. **Figure 3** shows the rugged surface of the composite used to immobilize the bi-enzymatic solution.

The characterization of the graphite/AgNPs composite surface by SEM generated an irregular and rough surface (image magnification of 2500 times **Figure 3(A)**) that was better seen when amplified at 6,500 times as shown in **Figure 3(B)**. The corresponding selected area energy dispersive spectrometer (EDS) analysis was defined by the yellow circle in **Figure 3(B)**. **Table 2** shows the percentage values (wt/wt%) of the elements in the AgNPs/GEC sample characterized by EDS.

The method used in this work generated a very low level of silver nanoparticles in the composite mixture, but even a few quantities of dispersed silver nanoparticles on graphite power had an important effect on the voltammetric response signal as reported previously in the literature [16] for a AgNPs/PANI/GEC. The authors observed that the AgNPs insertion resulted in an increase of both generated current peaks, anodic and cathodic, for the electrodes prepared with 25% graphite/AgNPs, 40% of PANI and 35% of epoxy resin. The system showed reversibility character, demonstrating that the graphite mixture, PANI, epoxy resin and AgNPs increase the electroconductivity of the electrode.

2.2 Enzyme solution composition statistical analysis

Table 3 shows the averages of the resultant peak currents according to each enzyme immobilization solution.

Figure 4 shows the three-dimensional response surface and the corresponding contour plot obtained by the statistical experimental design. The high, intermediate

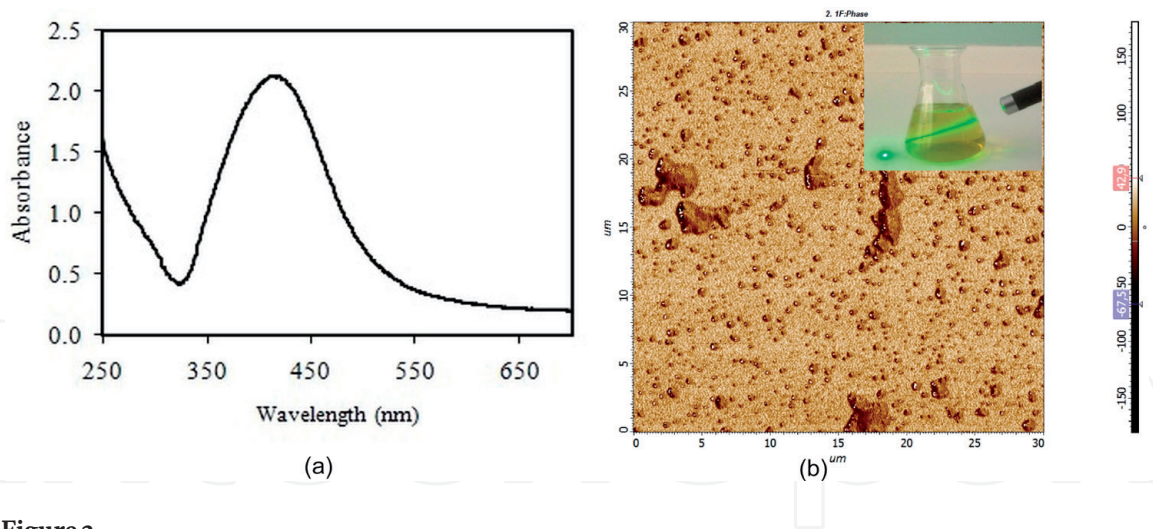


Figure 2.
(A) Absorption spectrum of the AgNPs colloidal dispersion with a maximum at 400 nm. (B) AFM 2D image of silver nanoparticle colloid on the surface composite sensor (scan scale: 30 μm \times 30 μm ; height scale: 5.0 nm). Top window in (B): Tyndall effect for colloidal AgNPs suspension.

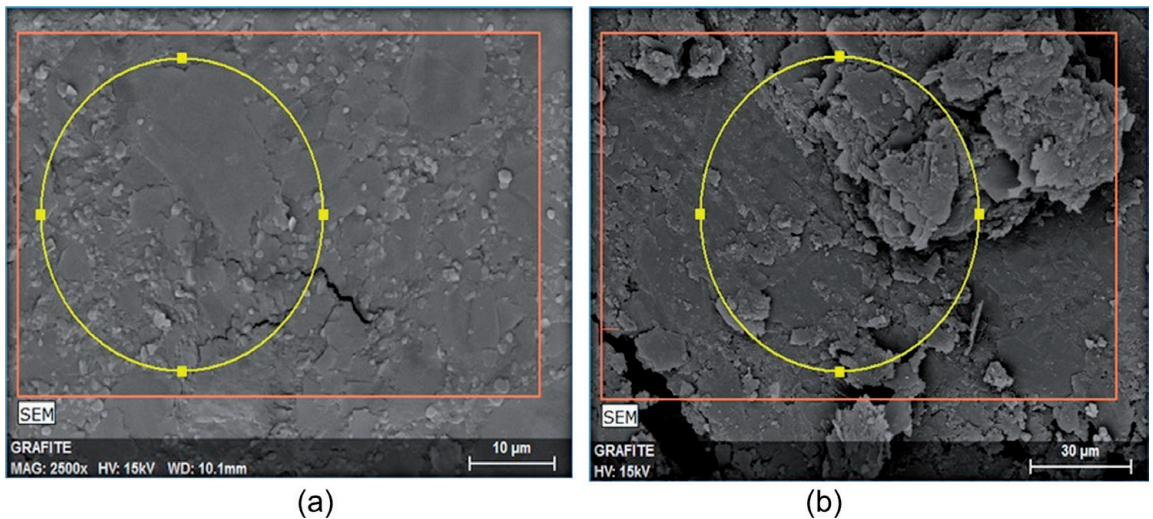


Figure 3.
Surface scanning microscopy photograph of the graphite/AgNPs composite: (A) 2,500 \times and (B) 6,500 \times .

Element	Weight (%)
Carbon	83.96
Oxygen	15.62
Silver	0.42
Total	100

Table 2.
Composite composition by EDS analysis.

and low bands of the resulting peak current are produced in the region from green to red depending on the intensity of the generated peak current. For the range of the three pseudo-components in the mixture (enzyme solution, glutaraldehyde, and albumin) investigated in this work, a two-dimensional triangle plot can represent the contours as shown in **Figure 4(A)**. The generated contour lines are based on the constraints of the experimental planning 80–98.5% for the bi-enzymatic solution, 1–10% for glutaraldehyde (2.5%) and 0.5–10% for albumin solution (1% w/v). **Figure 4(B)** shows a

Experiment	I_{p_c} (mA)	Variance	SD ^a
1	0.015	9.33×10^{-06}	3.06×10^{-03}
2	0.155	3.10×10^{-05}	5.57×10^{-03}
3	0.143	6.16×10^{-04}	2.48×10^{-02}
4	0.181	2.86×10^{-04}	1.69×10^{-02}
5	0.134	4.93×10^{-05}	7.02×10^{-03}

^aStandard deviation.

Table 3.
Amperometric peaks current means.

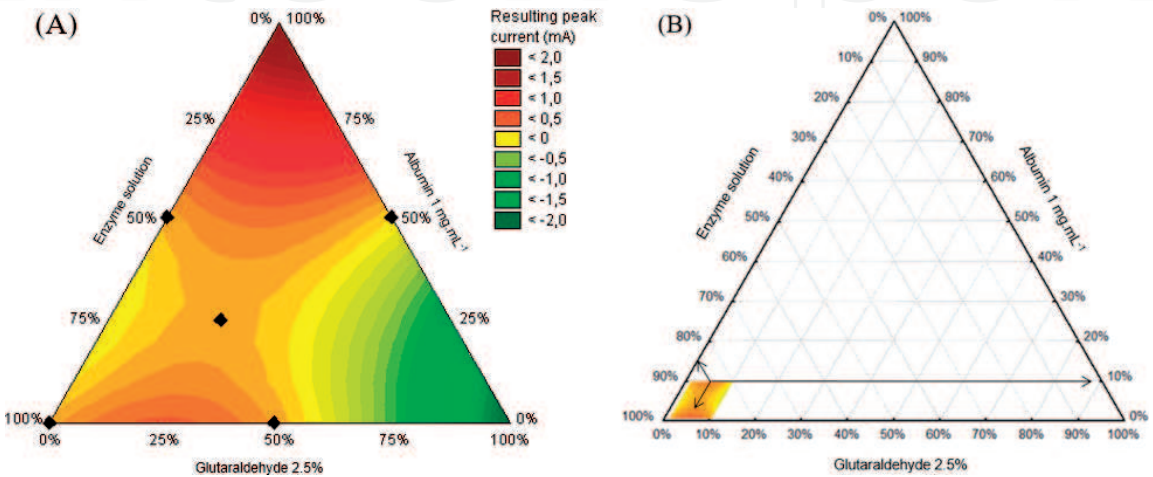


Figure 4.
(A) Level curves for the resulting peak current produced by the electrode with immobilized enzymes according to the pseudo-components of the mixture. (B) Ternary diagram restriction for the resulting peak current produced by the electrode with immobilized enzymes depending on the components, the enzyme solution, albumin and glutaraldehyde.

ternary constraint diagram used to define the best percent composition of the pseudo-component mixture suitable to achieve the highest peak current.

Figure 4(B) shows the planning area is surrounded by a diamond, and the two regions that provide the largest resulting peak currents appear in darker orange. The correct reading of the percentage of each pseudo-component for the mixture is highlighted with arrows, which indicate the direction side of each percentage in the ternary constraint diagram. Two dark orange areas were found in the investigated planning region. The first region indicates a resultant high-peak current supplied by an electrode with immobilized enzymes containing a solution composed of 85% enzyme solution, 10% albumin and 5% glutaraldehyde. The second region indicates a resultant high-peak current delivered by an electrode with immobilized enzyme and volume solution containing 94.5% of the bi-enzymatic solution, 0.5% albumin and 5% glutaraldehyde. The composition of the immobilization solution selected for this study was that of the first region (85% enzyme solution), a lower enzyme content and high-peak current. **Table 4** shows the regression coefficients of pseudo-components used to identify the relevant components and their interactions.

These results indicate that the enzyme solution factor is statistically significant for a confidence interval (CI) of 95% since their level p-value is less than 0.05. Glutaraldehyde, albumin and the interaction of second-order AB (enzyme solution interacting with glutaraldehyde) are marginally significant for the same confidence interval, for $0.05 < p \text{ level} < 0.10$. The second-order interaction AC (enzyme

Factor	Coefficient	p (CI: 95%) ^a
(A) Enzymatic solution	0.0001873	0.0000315
(B) Glutaraldehyde 2.5% (%)	−0.0044553	0.0982712
(C) Albumin 1 (% w/v)	0.0042506	0.0962228
AB	0.0091370	0.0862246
AC	−0.0083963	0.1053623

^aCI: confidence interval.

Table 4.
 Regression coefficients of pseudo-components and statistical significance.

solution interacting with albumin) is not statistically significant at the same confidence interval level and $p > 0.10$.

2.3 Calibration curve for the AgNPs/PANI/graphite biosensor

The construction of the calibration curve plotted in **Figure 5(B)** was obtained from the data shown in **Table 5**. These results show the values of peak currents (mA) obtained with the ethanol concentrations utilized in this study (0–30 g/L) are shown in **Figure 5(A)**. An increase of the response signal, proportional to the level of the ethanol concentration in the sample, was observed. The linearity was fitted by Eq. (4):

$$I(\text{mA}) = 0.004\text{Ethanol}(\text{g/L}) + 0.118 \tag{4}$$

with a coefficient correlation R^2 of 0.983. The sensitivity expressed by the angular coefficient of the linear adjustment is equal to 0.004 (mA L/g). In **Table 5** the variance and standard deviation values are shown for a mean of three new biosensors recently prepared for the measurements of each ethanol concentration solution.

The standard curve shows a positive linearity confirming the oxidation of ethanol by AOD. Ethanol oxidation occurs on the biosensor surface resulting in a stream of electrons at this location. Within the established ethanol concentration range applied in this study, an increase in the ethanol concentration into the electrolytic cell will generate a greater flow of electrons confirming the results reported previously for bi-enzymatic biosensors [29–32].

The limit of detection (LOD) was calculated by Eq. (5) and the limit of quantification (LOQ) by Eq. (6) [33]:

$$LOD = \frac{3S_b}{a} \tag{5}$$

$$LOQ = \frac{10S_b}{a} \tag{6}$$

in which S_b is the standard deviation of the measurements in white and a the sensitivity of technique. For the AgNPs/PANI/GEC biosensor, the LOD and LOQ calculated values were 3.54 g/L and 11.8 g/L, respectively.

The repeatability was determined by repetitive measures (five) by square wave voltammograms and refers to the agreement between successive measurements of the same sample for each working electrode [33]. The reproducibility was described as the agreement between results (signals) obtained with the same method with three recently prepared biosensors (B1, B2 and B3) [34]. For five sequential analyses, the relative square deviation values (RSD) were 1.54 (B1), 1.01 (B2) and 2.11% (B3)

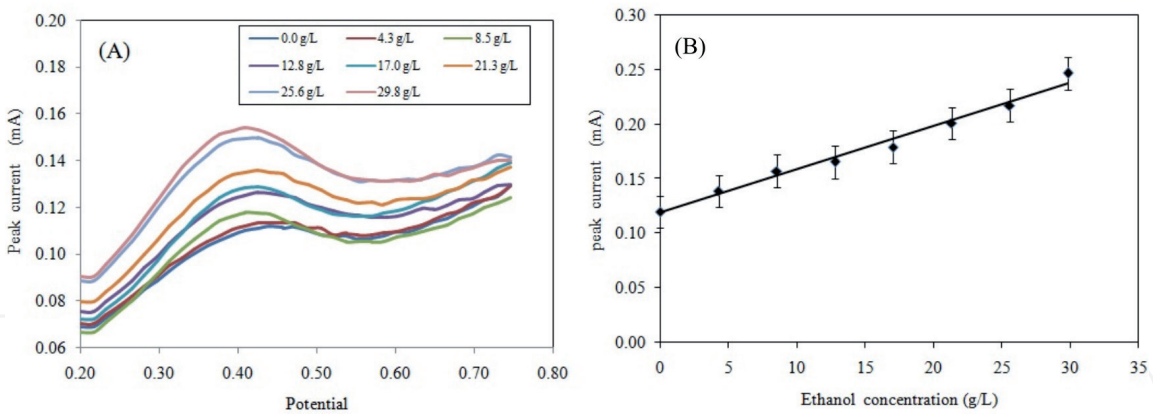


Figure 5.
(A) Peak current generated by square wave voltammetry (mean values of triplicate biosensors measurements).
(B) Calibration curve for the bi-enzymatic ethanol biosensor.

Ethanol ^a (g/L)	Current ^b (mA)	Variance	SD ^c
0.0	0.120	2.23×10^{-05}	4.73×10^{-03}
4.3	0.139	6.33×10^{-06}	2.52×10^{-03}
8.5	0.157	4.00×10^{-06}	2.00×10^{-03}
12.8	0.165	4.33×10^{-06}	2.08×10^{-03}
17.0	0.179	7.00×10^{-06}	2.65×10^{-03}
21.3	0.201	3.90×10^{-05}	6.24×10^{-03}
25.6	0.217	6.33×10^{-06}	2.52×10^{-03}
29.8	0.247	2.03×10^{-05}	4.51×10^{-03}

^aDiluted ethanol concentration with sodium phosphate buffer pH 7.0.
^bMean of three measurements.
^cStandard deviation.

Table 5.
Ethanol biosensor amperometric responses.

for each tested biosensor. The reproducibility values are shown in **Table 6** as the media value from measurements determined with three different biosensors recently built. The variance, standard deviation and RSD values are also shown in **Table 6**.

The results were considered satisfactory as variance and standard deviation values were around 10^{-3} and 10^{-2} , respectively, for the analytical determinations carried out with standard ethanol solution and three freshly prepared working bio-electrodes. To investigate a biosensor with higher sensitivity and accuracy and lower cost production, a composite prepared only with graphite and silver nanoparticles was also studied. The new working electrodes received a film of chitosan covering the immobilized enzymes that were adsorbed on the recently prepared composite. The response time for the square wave analysis was 37 s.

2.4 Biosensor prepared with graphite/AgNPs

2.4.1 Composite electrochemical characterization

Firstly, cyclic voltammetry was run in order to evaluate the reversibility of the electrochemical response signal for the AgNPs/GEC used to build the working electrode. The generated voltammetry curves were investigated for different voltage velocity (10–100 mV/s), and the profiles were shown in **Figure 6(A)** for electrodes

Analyses	Ethanol ^a (g/L)	Variance ^b	SD ^c	RSD ^d (%)
1	15.32	0.003	0.053	0.35
2	15.24	0.006	0.076	0.50
3	15.11	0.003	0.059	0.39
4	14.86	0.015	0.121	0.81
5	14.79	0.017	0.130	0.88

^aMedia value from measures determined with three different biosensors recently built.
^bVariance values.
^cStandard deviation.
^dRelative standard deviation.

Table 6.
Variation of ethanol concentration for the reproducibility.

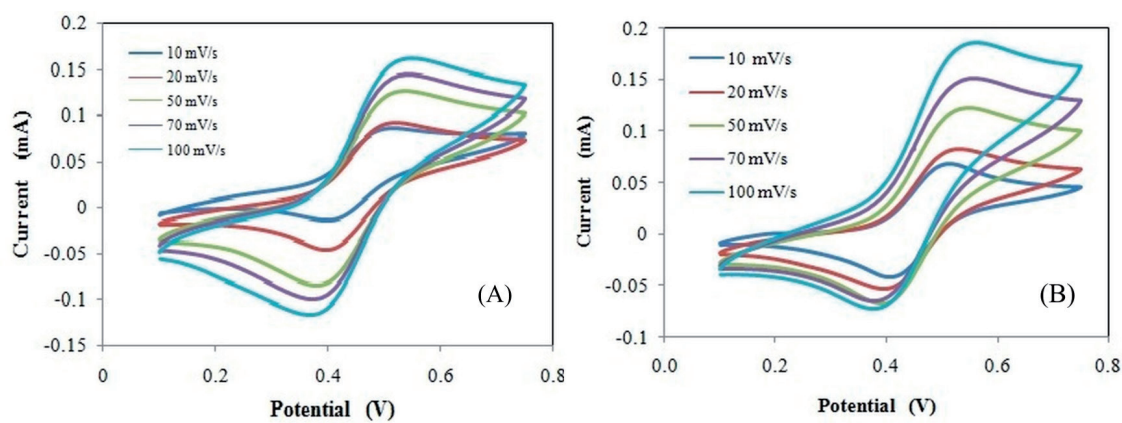


Figure 6.
Cyclic voltammetry for sensors electrodes in $K_3[Fe(CN)_6]/KCl$ pH 7.0 solution. Scanning run of the applied voltage velocity (10, 20, 50, 70, 100 mV/s). (A) Freshly prepared composite electrodes and (B) after 70 days.

built with freshly prepared composite. Electrodes prepared 70 days before were also tested and the cyclic response signals plotted in **Figure 6(B)**.

According to the Randles-Sevcik equation (Eq. (7)) for cycle voltammetry analysis, a linear correlation can exist between cathode or anode peak current, and the velocities square root can be applied during the scanning experiments (**Figure 7**) [35]:

$$I_p = 0.4463nF\sqrt{\frac{nFD}{RT}} AC\sqrt{v} \tag{7}$$

where I_p is the peak current, n is the number of electrons, F is the Faraday constant, T is the temperature in Kelvin, R is the gas constant, A is the surface area of the working electrode, D is the diffusion coefficient of the electroactive species, C is the bulk concentration of the electroactive species and v is the scan rate of voltammograms. Thus, the diffusion coefficients for ferrocene and ferricenium at 298 K are calculated from the slope of the plot of I_p versus \sqrt{v} .

The linear correlations are shown in **Table 7** for the electrodes prepared with graphite/epoxy composite (A), with AgNPs/GEC stored for 70 days (B) and with AgNPs/GEC recently prepared (C). The linearity (R^2) for both types of current I_{pc} and I_{pa} is close to 1.00 ($R^2 = 0.997\text{--}0.998$) suggesting that the system features a diffusion mass transfer.

The $|I_{pc}/I_{pa}|$ ratio values are shown in **Table 8**, where (I_{pc}) is the reverse current and (I_{pa}) the input current for the three electrode conditions studied and calculated for each velocity rate applied during the scanning experiments.

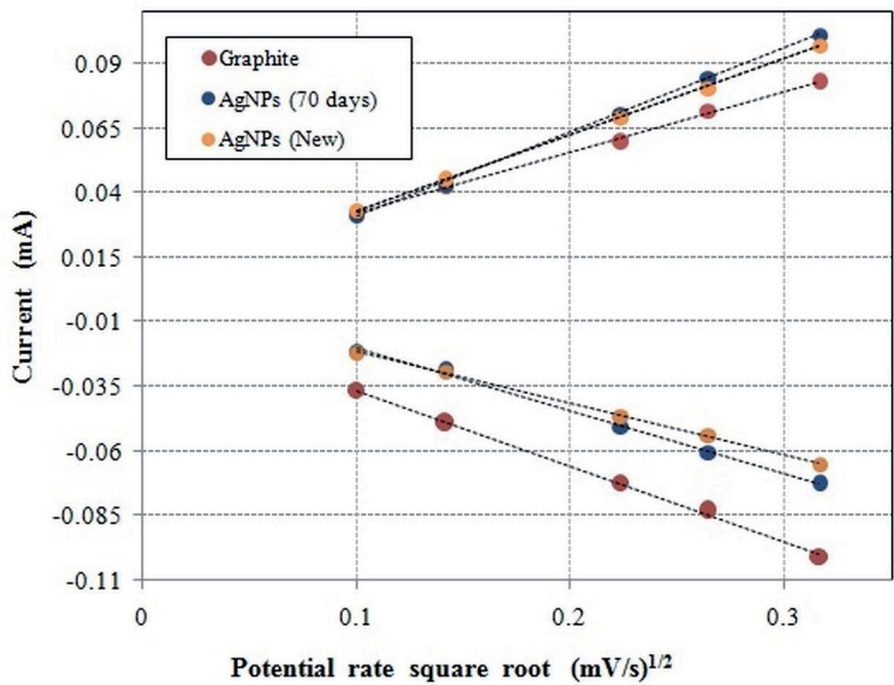


Figure 7. Linear correlations for cathode and anode peaks current with the velocity square roots ($K_3[Fe(CN)_6]/KCl$ 10 ml solution, pH 7.0).

	Anode	R ²	Cathode	R ²
A	$I = 0.294(v)^{1/2} + 0.003$	0.999	$I = -0.200(v)^{1/2} - 0.001$	0.998
B	$I = 0.325(v)^{1/2} + 0.001$	0.999	$I = -0.243(v)^{1/2} - 0.004$	0.998
C	$I = 0.235(v)^{1/2} + 0.008$	0.997	$I = -0.291(v)^{1/2} - 0.007$	0.998

Table 7. Linear correlations for current and scanning velocity square rate.

ν (mV/s)	I_{pc}/I_{pa}		
	A	B	C
10	1.041	1.148	1.155
20	1.141	1.207	1.145
50	1.165	1.131	1.215
70	1.239	1.147	1.152
100	1.234	1.145	1.217

Table 8. Average values of cathode and anode peak current ratios obtained for three electrodes of the same characteristics and method of preparation showed in item 2.4.1.

The reversibility characteristic of a voltammetric cycle is verified when the $|I_{pc}/I_{pa}|$ ratio is equal to 1.0. For the three different composites investigated in this study, the reversibility was observed when low voltammetric rates (mV/s) were applied to the voltammetric cycle analysis. The graphite-epoxy composite electrodes recently built showed a reversible performance ($|I_{pa}/I_{pc}| = 1.041$) for a scanning rate of 10 mV/s. For the AgNPs/GEC electrodes stored during 70 days at 8°C, working with 50 mV/s, a quasi-reversible system was observed ($|I_{pa}/I_{pc}| = 1.131$). The biosensor

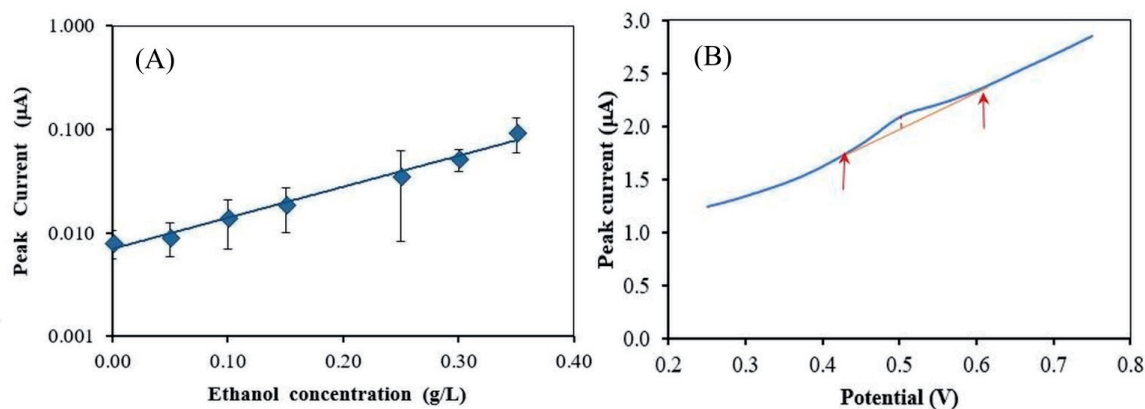


Figure 8. Ethanol electrochemical calibration (A). Screen printed data for the electrochemical analysis of a square wave voltammetry curve for a sample prepared with 0.35 mL of ethanol and 9.65 mL of phosphate buffer (B).

built with AgNPs/GEC recently prepared also showed a quasi-reversible ($|I_{pa}/I_{pc}| = 1.145$) behaviour for 20 mV/s. These results are similar to those obtained by other authors [1, 3, 9], but with biosensor complex designs, compared to ours.

2.4.2 Calibration curve for the graphite/AgNPs biosensor

Square wave voltammetry was applied to generate the resulting peaks for standard ethanol solutions tested for the new calibration curve. The square wave analyses were conducted with 20 Hz, amplitude of 20 mV and step potential of 1 mV from 250 to 750 mV. The response time chitosan ethanol bi-enzymatic AgNPs/GEC biosensor to 0.35 g/L was 208 s. The working electrodes were prepared (triplicates) and used to analyze ethanol in a range of 0.0–0.35 g/L in phosphate buffer pH 7.0. The intensity of peak current (I) generated for each measurement was determined and media of the resulting values correlated with respective ethanol concentrations. The obtained data were well fitted ($R^2 = 0.984$) as an exponential function: $I(\mu A) = 0.007e^{6.899[\text{Ethanol, g/L}]}$ shown in **Figure 8(A)**. A curve for an electrochemical analysis sample obtained with the software General Purpose Electrochemical System (GPES) 4.9.005 version, Metrohm, Utrecht, Holland, is showed in **Figure 8(B)**.

The LOD for the AgNPs/GEC biosensor was 3.48×10^{-3} and 0.0116 g/L the value for LOQ. The smaller square wave electrochemical response signals obtained were due to the chitosan applied over the bi-enzymatic solution adsorbed by the composite. Mass transfer restriction effects could reduce the intensity of the amperometric signal. Comparing the amperometric response signals generated by the different composites proposed in this work, the range of analysis measure and the nature of the sample may be considered before the choice of the biosensor working electrode. A dilution is frequently necessary to fit the analyte sample concentration into the linear range sensibility of the developed biosensor. The reduction of waste discarded after each analysis contributed to the reduction of waste disposal in the environment since low sample volumes are used for the electrochemical analysis. This advantage encourages researchers in maintaining their efforts in developing continuously new composites and devices to improve the analytical proceedings [36].

3. Conclusions

Ethanol was estimated by electrochemical methods based on AOD- and HRP-modified electrodes with simple assembly and operation since it does not require the addition of a cofactor other than O_2 . The mixture experimental design was efficient in

determining the quantities of each component of the enzyme immobilization solution, avoiding spending time and the use of many expensive reagents to obtain the desired results. The graphite powder-silver nanoparticle-polyaniline was used as conducting composite for the working electrode, and the composite surface roughness was adequate to immobilize the enzymes AOD and HRP (solution composed of 85% bi-enzymatic solution, 10% albumin and 5% glutaraldehyde). The biosensor showed response signal linearity in the concentration range from 0 to 30 g/L ($R^2 = 0.983$) and sensitivity of 0.004 mA L/g, and LOD and LOQ were 3.54 and 11.8 g/L, respectively. These results indicate a robust bioelectrode suitable for analyses of contents up to 30 g/L of ethanol in samples. The repeatability and reproducibility of the biosensor were considered satisfactory since the variance and standard deviation showed low values. Relative standard deviation values were also low, below 2.2 and 0.9% for repeatability and reproducibility, respectively. The biosensor prepared with the AgNPs/GEC, working with the better bi-enzymatic solution composition, detected very low ethanol concentrations in a range of 0–0.35 g/L ($R^2 = 0.984$), a higher sensitivity of 6.899 $\mu\text{A.L/g}$, LOD of 3.48×10^{-3} g/L and LOQ of 0.0116 g/L. Two AgNPs composites are investigated in this work and characterized to develop bi-enzymatic biosensor for ethanol analysis. The ranges of ethanol solutions and the analytical performances were also investigated. Low levels of ethanol in standard samples could be detected with the AgNPs/GEC bi-enzymatic biosensor. The AgNPs/PANI/GEC biosensor was well fitted for high ethanol content in standard samples.

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