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Use of zein microspheres to anchor carbon black and hemoglobin in electrochemical biosensors to detect hydrogen peroxide in cosmetic products, food and biological fluids

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ABSTRACT

Hemoglobin-containing electrochemical biosensors are useful for detecting hydrogen peroxide through oxidation of the iron ion, but high efficiency can only be reached with appropriate immobilization strategies for hemoglobin. In this work, we combined zein from corn seed with carbon black to immobilize hemoglobin, as proof of concept, and form an electroactive film that could determine hydrogen peroxide within the concentration range from 4.9×10^{-6} to 3.9×10^{-4} mol L^{-1}, and limit of detection of 4.0×10^{-6} mol L^{-1}, using differential pulse voltammetry. The biosensor could also detect hydrogen peroxide in commercial samples of oxygenated water, synthetic serum (physiological and glycoside) and milk. The high performance is ascribed to the large surface area and conductive nature of the porous film that had carbon black and hemoglobin anchored on zein microspheres,

according to scanning and transmission electron microscopies. It is significant that a protein from renewable sources zein combined with a low-cost carbon material (carbon black) serves as matrix for immobilization of biomolecules. It is significant that a protein from renewable sources combined with a low-cost carbon material serves as matrix for immobilization of biomolecules.

Keywords

Biosensor; Zein; Hemoglobin; Carbon black; Hydrogen peroxide

1. Introduction

Electrochemical biosensors have been used for a variety of purposes, mostly owing to their versatility, potential low cost and high performance [1]. Key issues in reaching high performance include a matrix onto which the active layer is to be immobilized and the efficiency in charge transfer to the electrode. Nanomaterials are normally employed to improve conductivity and increase the surface area in electrochemical biosensors [2-11], while biopolymers afford an ideal surface to immobilize biomolecules [12]. Indeed, natural polymers such as chitosan [13], silk fibroin [14] and potato starch [15] have been used. In this work, we exploit the possibility of utilizing zein in a special architecture to fabricate hemoglobin (HB)-containing electrochemical biosensors. The choice of zein (ZE) was motivated by two main reasons. Being extracted from natural sources and stable in aqueous solutions, zein is expected to create a friendly environment for immobilizing biomolecules. In addition, it is a low cost material from renewable sources [16, 17], and promising for biocompatible wearable sensors and biosensors for applications in sports, health monitoring and cosmetics [18, 19]. Zein is a prolamin protein found in corn endosperm tissues, comprising 80% of the amount of proteins in corn seeds [20, 21]. It may be solvent extracted from corn gluten [20, 22], and used in fibers, adhesives, coatings, ceramics,

paints, cosmetics, food packaging, and textiles [23, 24]. Zein has been also employed in electrochemical sensors [25].

The choice of a suitable matrix is crucial for electrochemical biosensors containing hemoglobin (HB), which are based on the facile oxidation of iron in the heme group induced by hydrogen peroxide (H_2O_2) [26]. HB is a metalloprotein comprising four globin chains coordinated to the heme group with an iron ion bound to a porphyrin [27], which is essential to carry oxygen and carbon dioxide through the circulatory system in the human organism [22, 27]. The architectures of reported HB-containing biosensors to detect H_2O_2 include graphene/Fe_3O_4 nanocomposite [28] and titanium dioxide nanotubes anchored on a chitosan film [29]. Detection of the powerful oxidizing agent H_2O_2, on the other hand, is a primary goal in many electrochemical studies because H_2O_2 is present in the human body, being a by-product of enzymatic action of oxidases [30, 31]. H_2O_2 is an important analyte since it may be used to determine glucose [32], and in the diagnosis of such diseases as Parkinson's, cancer, stroke, atherosclerosis and Alzheimer's disease [33, 34]. Detection is also relevant owing to its use in the food, pharmaceutical, textile, and pulp and paper industries [35].

The direct anchoring of HB on electrode surfaces is unlikely to yield high-performance biosensors since HB is not conducting and may be (at least) partially denatured [36]. One has therefore to resort to strategies that accompanied recent advances in biosensor technology and instrumentation, which involve nanomaterials and biopolymers [37]. In the design of a biosensor to detect H_2O_2 with a HB-containing biosensor, we opted to combine zein with a low-cost carbonaceous material, namely carbon black (CB). CB is a low-cost, amorphous carbon alloy [38], [39], used as pigment for paints and in the manufacture of conducting polymers and polymer composites, in

lithium batteries and fuel cells [40] [41]. It consists of spherical carbon particles with diameters ranging from 3 to 100 nm [42], which form fused aggregates smaller than 1000 nm [43]. CB has high conductivity owing to its sp^2 carbon-carbon bonds, being thus suitable for biosensors [44, 45]. Among many examples, CB has been utilized in biosensors to detect pesticides, glucose, drugs, heavy metals, phenols, nitrites and nitrates, and hormones [43, 46-52].

The results presented in this paper are ample demonstration of the adequacy of the materials chosen. Using scanning electron microscopy (SEM) and transmission electron microscopy (TEM), we were able to observe formation of ZE microspheres on which HB and CB could be anchored to form a conducting film to be deposited on glassy carbon electrodes (GCE). With differential pulse voltammetry, we show that the biosensor made with GCE coated by ZE, HB and CB is able to detect H_2O_2 in commercial samples, synthetic serum (physiological and glycoside) and milk.

2. Experimental details

Human hemoglobin (HB) and the other chemicals were purchased from Sigma-Aldrich, Dynamics and/or Fluka, while CB VXC72R was supplied by Cabot Corporation. All solutions were prepared with ultrapure water (Megapurity – resistivity ≥ 18.0 MΩ cm). The measurements using HB were carried out with deoxygenation using high-purity nitrogen for 10 min. A 0.2 mol L^{-1} phosphate buffer solution was used as support electrolyte.

A potentiostat/galvanostat (AutoLabPGSTAT101 Methrom) controlled with Nova 2.0 software was employed with a conventional three-electrode electrochemical cell containing a glassy carbon electrode (GCE: 3 mm diameter) as working electrode, a

platinum electrode as auxiliary electrode and Ag/AgCl in KCl (3.0 mol L^{-1}) as reference electrode. Parameters such as supporting electrolyte pH, scan rate (*v*), amplitude (*a*) and modulation time for the DPV technique were studied and the values selected are shown in Table S1. The optimized parameters were used to obtain the analytical curves. Electrochemical impedance spectroscopy (EIS) results were acquired with a FRA2 software in a frequency range from 0.1 Hz to 100 kHz, amplitude of 10 mV and under open circuit potential (OCP) with 0.1 mol L^{-1} KCl solution containing $5x10^{-3}$ mol L^{-1} $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$. The Nyquist plots were analyzed with a Randles modified equivalent circuit $[R_s(CPE[R_{ct}Z_W])]$, where R_s is the solution resistance, R_{ct} is the charge transfer resistance, Z_W is the Warburg impedance and CPE is a constant phase element.

UV–vis absorption spectra were recorded with a Hitachi UV-Vis spectrometer using quartz cells with 10 mm of optical path at a resolution of 1.0 nm and a wavelength of 250 to 1000 nm. The Zetasizer ZS 90 spectrometer (Malvern instruments) was used to measure the Zeta potential in 10 μg mL^{-1} dispersions of ZE, CB-ZE, HB-ZE and HB-CB-ZE sonicated for 30 min without controlling the pH. A Tensor II (Bruker) spectrophotometer with a resolution of 4 cm^{-1} in the absorbance mode in the 500 to 4000 cm^{-1} range was used in FTIR analyses. The samples were prepared in KBr pellets (Sigma-Aldrich) containing 1% by weight of each compound. SEM images were obtained using a LEO-440 (Zeiss-Leica) microscope operating at 20 kV. TEM images were acquired with a FEI TECNAI G2 F20 high-resolution transmission electron microscope (HR-TEM) at 200 kV, with samples deposited on carbon-coated copper grids.

ZE dispersion was prepared following the methodology by Sun with a few modifications [20]. 0.1% (w/v) of ZE was added in 50 mL of 70% (v/v) ethanol, treated in a water bath for 45 min at 85 ºC with subsequent cooling. The resulting dispersion was

dropped into 60 mL of ultrapure water under vigorous magnetic stirring at a rate of 10 mL/min. The dispersion was heated for 15 min at 50°C to remove the remaining ethanol. A dispersion containing 2 mL of ZE and 1 mg of CB was homogenized in ultrasonic bath for 30 min. Nitrogen (N_2) was bubbled for 10 min in this dispersion to remove dissolved oxygen. 5.0 mg of HB were added to the mixture and the system was closed and left under magnetic stirring for 30 min. The GCE surface was modified by casting 3 μL of HB/CB/ZE dispersion with subsequent drying for 1 h at room temperature. Figure 1 shows a scheme for electrode preparation, namely HB-CB-ZE/GCE.

Commercial samples of oxygenated water containing 10, 30 and 40 volumes were diluted in ultrapure water at the final concentration of 10^{-2} mol L^{-1}. Synthetic serum (physiological and glycoside) was prepared by fortification with a final concentration of 10^{-2} mol L^{-1}. The milk sample was prepared adding 0.01 mL of tween 20 stabilizer and 2 g of skimmed milk in 100 mL of 0.1 mol L^{-1} phosphate buffer saline (pH = 7.4) solution. This solution was homogenized and fortified to reach 10^{-2} mol L^{-1} H_2O_2. All samples were obtained in the local market in Araras city (São Paulo/Brazil).

3. Results and discussion

3.1. Structural and morphological features of HB-CB-ZE films

The micrograph in Fig. 2A shows spherical ZE particles with heterogeneous sizes dispersed on the GCE surface. ZE spheres form an interconnected layer with HB, causing considerable aggregation as shown in Fig. 2B, and film porosity increased with incorporation of carbon black, according to Fig. 2C. The TEM images in Fig. 2D and 2E

show ZE particles ranging from 1 to 4 μm and CB with 500 nm, whereas HB interconnects the particles, thus yielding a porous, electroactive film.

The dispersion with HB-CB-ZE had the lowest zeta potential in Fig. 3A because addition of CB or HB in ZE decreases the repulsion between the particles in solution, thus decreasing stability. These features lead to a film with interconnected aggregates, consistent with the MEV data in Fig. 2C, and to an increased surface area that enhances electrochemical performance. The spectra of ZE and CB-ZE in Fig. 3B exhibit a small band at 265 nm owing to ZE [53] and no absorption bands in the visible region (350 to 700 nm). CB does not absorb in this wavelength region. The spectra for HB-ZE and HB-CB-ZE display a Soret absorption band at 400 nm corresponding to HB heme group [54]. This means that ZE was not denatured, otherwise the Soret absorption band would be displaced or could not be observed [55]. Figure 3C shows the FTIR spectra for all the materials in KBr pellets, with band assignment being given in Table S2. The spectra for CB-ZE (d), HB-ZE (e) and HB-CB-ZE (f) display bands of the individual components ZE (a), CB (b) and HB (c), with insignificant shifts. The exception is the band at 2400 cm^{-1} in (b) which is suppressed in the other spectra probably due to the interaction of the amine group of the proteins with CB. It is worth mentioning that the spectrum for HB-CB-ZE (f) contains the typical HB bands, and therefore the molecular-level interactions did not cause denaturing of HB. We should expect that this does not occur in the cast films either.

3.2. Electrochemical features of the biosensor and H_2O_2 detection

Figure 4A shows cyclic voltammograms for bare GCE, ZE/GCE and CB-ZE/GCE with formal potentials (ΔE_P) of 200 mV and 100 mV for bare GCE and CB-ZE/GCE, respectively. ΔI_P for CB-ZE/GCE was higher than for bare GCE due to the increased electroactive area and conductivity afforded by CB. The absence of reduction and oxidation peaks for ZE/GCE indicates electrode blocking due to the non-electroactive protein. The CV for scan rates between 10 and 500 mV s^{-1} in Fig. S1 indicated that the electrochemical reaction is diffusion controlled for GCE and CB-ZE/GCE, as the peak current (I_P) (anodic) varied with the square root of the scan rate. The larger slope of 5.9×10^{-5} for CB-ZE/GCE, compared to 4.8×10^{-5} for bare GCE, confirms the faster electrochemical kinetics and electrocatalytic activity promoted by nanomaterials with large surface areas.

The electroactive area was 0.06 and 0.08 cm^2 for GCE and CB-ZE/GCE, respectively, as calculated using the Randles-Sevcik equation $I_p = 2.69\times10^5 ACD^{1/2}n^{3/2}v^{1/2}$, where I_p is the anodic or cathodic peak current (A), A is the electroactive area (cm^2), C (mol L^{-1}) is the probe concentration, D is the diffusion coefficient (7.6×10^{-6} cm^2 s^{-1}) , n is the number of electrons transferred in the redox process, and v is the scan rate (V s^{-1}). The charge-transfer resistance (R_{ct}) inferred from Fig. 4B was 0.5, 70, 0.03 and 4.4 kΩ for GCE, ZE/GCE, CB-ZE/GCE and HB-CB-ZE/GCE, respectively. The higher R_{ct} for ZE/GCE indicates that the long chains of ZE block the electrode whereas CB promotes charge transfer due to its conductivity, consistent with the results in Fig. 4A. Moreover, an even higher R_{ct} (4.4 kΩ) was observed for the HB-CB-ZE/GCE biosensor since HB was successfully immobilized on the surface.

The cathodic current peaks taken from the voltammetric profiles in Fig. S2A increased linearly with the scan rate from 50 to 500 mV s^{-1}, as depicted in Fig. S2B. The

values of α and k_s calculated using Laviron equation [56] for irreversible systems are 1.06 and 30 s^{-1}, respectively. They are higher than in other studies [57, 58] owing to an enhanced electron transfer between HB and the electrode surface. These electrochemical features make the biosensor promising for detecting peroxide. The CV in Fig. 4C for HB-CB-ZE/GCE biosensor had a well-defined reduction peak at -0.38 V, which can be assigned to reduction of Fe^{3+} to Fe^{2+} in the heme group of HB.

The H_2O_2 electroreduction with a higher cathodic peak current in the presence of 6.5×10^{-4} mol L^{-1} H_2O_2 in Fig. S3 confirms that HB-CB-ZE/GCE is able to detect peroxide. While Fe^{3+} of HB heme group is electrochemically reduced to Fe^{2+}, H_2O_2 is reduced to oxygen and water as summarized in the equation $2Fe^{2+} + H_2O_2 \leftrightarrow 2Fe^{3+} + ½\ O_2 + H_2O$ [59]. H_2O_2 detection using DP voltammograms is shown in Fig. 4D for concentrations ranging from 4.9×10^{-6} to 3.9×10^{-4} mol L^{-1}. The cathodic peak current increased linearly with H_2O_2 concentration, as illustrated in the inset of Fig. 4D with a linear regression $I(\mu A) = 3.9\times10^{-7} + 5.3\times10^{-3}\ C_{H2O2}$ and correlation coefficient of 0.992. The limit of detection (LOD), defined as 3×Sb/m, where Sb is the standard deviation of the analytical curve and m is the slope of the linear regression, was 4.0×10^{-6} mol L^{-1}.

The HB-CB-ZE/GCE biosensor was applied to detect H_2O_2 in commercial oxygenated water samples containing 10, 30 and 40 volumes, in synthetic serum (physiological and glycoside) and milk. Table 1 lists the H_2O_2 concentration added and detected with a maximum variation of 19%, which is acceptable for this kind of sample.

The reproducibility of the fabrication procedure was evaluated using five different HB-CB-ZE/GCE sensing layers in which a relative standard deviation (RSD) of 2.78% was obtained for detecting 9.9×10^{-5} mol L^{-1} H_2O_2. An RSD of 1.85% was calculated for five successive measurements using the same sensing layer, suggesting good repeatability and reproducibility of the HB-CB-ZE/GCE platform. The selectivity of this biosensor was confirmed with commercial samples of H_2O_2, milk and synthetic serum (physiological and glycoside). First, an aliquot of commercial samples was added to the electrochemical cell (final concentration of 4.9×10^{-6} mol L^{-1} H_2O_2) and DPV was performed. An aliquot of H_2O_2 was added for a final concentration of 4.9×10^{-6} mol L^{-1} in milk and synthetic serum samples. The commercial samples of H_2O_2 were fortified with EDTA, while milk and synthetic serum (physiological and glycoside) samples were fortified with sodium nitrate, calcium chloride, tin chloride and glucose. The concentration of interfering species was 24.5, 49 and 4900 µmol L^{-1}. The results showed maximum interference ranging from 15% to 1.3% (Table S3), and therefore HB-CB-ZE/GCE is found selective for H_2O_2.

HB-CB-ZE/GCE exhibited a similar or even better performance in terms of LOD and linear range for H_2O_2 detection compared to sensing layers using HB listed in Table 2. Wang and collaborators [28] reported a lower LOD, but their biosensor made with graphite oxide and Fe_3O_4 nanoparticles is more expensive and more time-consuming to produce. We highlight the feasibility and easy fabrication of the biosensor for detecting peroxide in different kinds of samples.

4. Conclusion

A biosensor has been successfully developed using a stable, porous and conductive sensing layer made with ZE microspheres, CB and HB as electrode material for detecting H_2O_2. The morphological features showed a rough surface with spherical agglomerates, thus making the HB-CB-ZE film to have an increased surface area compared to films from the separate components. According to TEM and SEM images, the electroactive film consists of aggregated HB and CB anchored on ZE microspheres. The biosensor was applied to detect H_2O_2 using differential pulse voltammetry for different types of samples. For standard H_2O_2 solutions, the electroanalytical performance was comparable to most HB-containing electrochemical biosensors in the literature. Furthermore, the biosensor could detect H_2O_2 in real samples of food (milk), physiological serum and in commercially-available of oxygenated water at different H_2O_2 concentrations. Demonstration of the potential use of the biosensor also came from systematic studies to verify reproducibility, stability and robustness against possible interferents. The use of ZE, in particular, may be extended to other matrices in biosensors as their microspheres should anchor other nanomaterials and biomolecules.

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Figure 1 - (1) ZE dispersion preparation, (a) heat treatment of ZE in ethanol at 85 °C for 30 min, (b) Anti-solvent technique in water. (2) Fabrication of HB-CB-ZE/GCE biosensor: (a) Preparation of CB-ZE dispersion, (b) Preparation of HB-CB-ZE dispersion, (c) Casting HB-CB-ZE on GCE.

Figure 2 – SEM images of (A) ZE, (B) HB-ZE, (C) HB-CB-ZE. TEM images of HB-CB-ZE (D and E).

Figure 3 - (A) Zeta potential for ZE, CB-ZE, HB-ZE and HB-CB-ZE. (B) UV-Vis spectra for ZE, CB-ZE, HB-ZE and HB-CB-ZE. (C) FTIR spectra for (a) ZE, (b) CB, (c) HB, (d) CB-ZE, (e) HB-ZE and (f) HB-CB-ZE.

Figure 4 - (A) CV for bare GCE (black), ZE/GCE (blue) and CB-ZE/GCE (red) in 1.0×10^{-3} mol L^{-1} $[Fe(CN)_6]^{3-/4-}$ and 0.1 mol L^{-1} KCl solution at scan rate 100 mV s^{-1}. **(B)** Nyquist plots for bare GCE, ZE/GCE, CB-ZE/GCE and HB-CB-ZE/GCE. **(C)** CV for HB-ZE/GCE, HB-CB-ZE/GCE and CB-ZE/GCE in 0.2 mol L^{-1} phosphate buffer (pH 7.0) at scan rate 50 mV s^{-1} (without peroxide). **(D)** DPV for HB-CB-ZE/GCE in the presence of H_2O_2 at concentrations of (1) 0; (2) 4.9×10^{-6} mol L^{-1}; (3) 4.9×10^{-5} mol L^{-1}; (4) 9.9×10^{-5} mol L^{-1}; (5) 1.9×10^{-4} mol L^{-1}; (6) 2.9×10^{-4} mol L^{-1} and (7) 3.9×10^{-4} mol L^{-1} in 0.2 mol L^{-1} phosphate buffer (pH 5.5); v = 25 mV s^{-1}, a = 85 mV, modulation time = 10 ms. *Inset*: Analytical curve for H_2O_2 detection with HB-CB-ZE/GCE.

Table 1. Recovery values for H_2O_2 in commercial samples of oxygenated water, milk, and serum samples.

Sample	Added H_2O_2 (mol L^{-1})	HB-CB-ZE/GCE (mol L^{-1})	Recovered (%)
Oxygenated Water (10 Vol.)	1.9×10^{-4}	1.8×10^{-4}	97.0
	9.9×10^{-5}	1.1×10^{-4}	110
	9.9×10^{-5}	1.0×10^{-4}	108
Oxygenated Water (30 Vol.)	4.9×10^{-6}	4.4×10^{-6}	91.0
	9.9×10^{-5}	9.7×10^{-4}	98.0
	1.9×10^{-4}	1.9×10^{-4}	102
Oxygenated Water (40 Vol.)	4.9×10^{-5}	5.8×10^{-5}	119
	9.9×10^{-5}	1.1×10^{-4}	112
	1.9×10^{-4}	1.8×10^{-4}	96.0
Synthetic serum (physiological)	9.9×10^{-5}	1.1×10^{-5}	112
	1.9×10^{-4}	1.9×10^{-4}	101
	1.9×10^{-4}	1.8×10^{-4}	96.8
Synthetic serum (glycoside)	4.9×10^{-5}	4.0×10^{-5}	82.0
	4.9×10^{-5}	4.9×10^{-5}	101
	9.9×10^{-5}	9.4×10^{-5}	95.1
Milk	4.9×10^{-5}	4.0×10^{-5}	82.0
	1.9×10^{-5}	2.1×10^{-5}	109
	1.9×10^{-4}	1.6×10^{-4}	84.0

Table 2 – Electroanalytical performance using sensing layers with HB for detecting H_2O_2.

Biosensor	Linear range (mol L^{-1})	LOD (mol L^{-1})	Reference
HB-graphene-Fe_3O_4/GCE*	2.3×10^{-6} – 9.6×10^{-3}	1.1×10^{-6}	[19]
HB-CHT/nano-$CaCO_3$/GCE*	3.7×10^{-5} – 8.3×10^{-4}	8.3×10^{-6}	[51]
HB-CNTs*	2.1×10^{-4} – 9.0×10^{-4}	9.0×10^{-6}	[52]
HB-AgNPs-PAMAM/GCE*	6.0×10^{-6} – 9.1×10^{-5}	4.9×10^{-6}	[18]
HB-PIT@Fe_3O_4/GCE*	2.0×10^{-6} – 3.5×10^{-4}	5.4×10^{-7}	[53]
HB-CB-ZE/GCE	4.9×10^{-6} – 3.9×10^{-4}	4.0×10^{-6}	This work

* HB-graphene-Fe_3O_4/GCE: hemoglobin, graphene and iron (III) oxide on a vitreous carbon electrode; HB-CHT/nano-$CaCO_3$/GCE: hemoglobin, chitosan and calcium carbonate nanoparticles on a vitreous carbon electrode; HB-CNTs: hemoglobin and carbon nanotube on glassy carbon electrode; HB-AgNPs-PAMAM/GCE: hemoglobin, silver nanoparticles and poly(amidoamine) dendrimer on a vitreous carbon electrode; HB-PIT@ Fe_3O_4/GCE: hemoglobin, poly(indole co-thiophene) and iron (III) oxide on glassy carbon electrode.

Highlights

A new conductive film is proposed by using zein microspheres

The new biosensor containing zein, carbon black and hemoglobin was applied for H2O2 determination

The proposed biosensor is easy to prepare and presents low cost

The new thin film could be extended for other electrochemical sensing and biosensing

*Graphical Abstract (for review)