A novel amperometric inhibition biosensor based on HRP and gold sononanoparticles immobilised onto Sonogel-Carbon electrode for the determination of sulphides

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ABSTRACT

A novel inhibition biosensor used for the detection of sulphides (Na\textsubscript{2}S) has been developed. The biosensor is based on the immobilisation of horseradish peroxidase (HRP) on the Sonogel-Carbon (SNGC) electrode using glutaraldehyde, Poly(4-vinylpyridine) and gold sononanoparticles (AuSNPs). The Poly(4-vinylpyridine) was used due to its high affinity for sulphide anions, while the presence of gold sononanoparticles enhances the electron transfer reaction and improves the analytical performance of the biosensor. The amperometric measurements were performed at an applied potential of −0.15 V vs. Ag/AgCl in 50 mM sodium acetate buffer solution pH = 6.0. The apparent kinetic parameters (\(K_{\text{mapp}}\), \(V_{\text{max}}\)) of immobilised HRP were calculated in the absence of inhibitor (sulphide) using caffeic acid as substrate. Under the optimal experimental conditions, the determination of sulphide can be achieved in a dynamic range of 0.4–2.8 µM with a low limit of detection of 0.15 µM. The electrochemical impedance spectroscopy (EIS) was also used to characterise the interactions of substrate and inhibitor with the enzyme-modified electrode. The developed biosensor exhibited high sensitivity, selectivity and stability, and can be successfully applied to the detection of sulphide in water.

1. Introduction

Sulphides are widely present in natural water samples and usually represent the total of hydrogen sulphide (H\textsubscript{2}S), bisulphide (HS\textsuperscript{−}) and sulphide (S\textsubscript{2}−) ions which remain in equilibrium in aqueous solution. The hydrogen sulphide, which is soluble in water, is
extremely toxic to aquatic animals, and most authorities suggest that no more than 0.002 mg/L of sulphide must be present in natural waters [1]. The long-term action of sulphide in low concentrations causes chronic diseases of respiratory tract, blood, eyes, skin and digestive disturbance. Headaches, vertigo, impaired hearing and vascular and autonomic dysfunction also occur [2]. Sulphides can be generated in several ways such as bacterial reduction of naturally occurring sulphates in seawater or by rotting vegetation, and industrial waste discharge [3]. The determination of sulphides has great importance due to its high toxicity and also its serious threat for the environment and human life [4]; hence a reliable analytical technique is required for recognising this compound at low levels. Accordingly, many methods for monitoring sulphide have been developed, such as potentiometric [5,6], polarographic [7–9], chromatographic [10,11], spectrophotometric [12–14], chemiluminescence [15], voltammetric [16–18] and amperometric [19,20]. Some of these methods are time consuming due to the sample pretreatment and most of them cannot monitor trace amounts of sulphides.

The quantitative determination of toxic compounds at low concentration using useful monitoring devices is often required and of great importance. Therefore, biosensors provide a more effective on-site control of water treatment processes. In addition, biosensors show important advantages: simplicity of analysis, low cost, high sensitivity and short response time [21]. Inhibition-based biosensors have been developed for specific determination of sulphides with simplicity, specificity and accuracy [22–24]. Yang et al. [23] have developed an amperometric horseradish peroxidase (HRP) inhibition biosensor for the determination of sulphides by using cysteamine to form a versatile self-assembled monolayer (SAM) tightly attached to the gold electrode surface. Recently, other biosensor has been developed for sulphide detection by immobilising Coprinus cinereus peroxidase (CIP) on the surface of screen-printed electrode (SPE) with a detection limit of 0.3 µM [24].

The Poly(4-vinylpyridine) (PVPy) polymer was found to provide an effective matrix for adsorbing negatively charged sulphide ions (S$_2^-$) by complexing them to its aromatic nitrogen [25,26]. In order to enhance the sensitivity of the biosensor, we also used gold sononanoparticles (AuSNPs) that increase the active surface and promote the electron transfer between the electrode and the active sites of the enzyme [27,28]. In our previous work, it has been reported that the performance of Sonogel-Carbon electrode (SNGCE) in response to cyanide is largely promoted by addition of a small amount of AuSNPs [29].

In the present work, a new amperometric biosensor based on the cross-linking of HRP with glutaraldehyde (GA), polyvinyl polymer and AuSNPs at the SNGCE surface has been developed for the detection of sulphide ions. The experimental conditions influencing the analytical performance of HRP biosensor were optimised. The modified SNGCE exhibited attractive performances for determination of sulphides, such as sensitivity and fast amperometric response, as well as good selectivity and stability.

2. Experimental

2.1. Reagents and instrumentation

Sodium acetate buffer solution (50 mM, pH = 6.0) was prepared from sodium acetate and acetic acid (Riedel-de-Haën). The sodium acetate buffer was used as supporting
electrolyte for all the electrochemical measurements. HRP (E.C. 1.11.1.7, 274 U/mg), PVPy (molecular weight: 160,000), GA (25% (v/v) in water), sodium sulphide (≥98% Na$_2$S 9H$_2$O), lead(II) nitrate (Pb(NO$_3$)$_2$) and bovine serum albumin (BSA) were purchased from Sigma. Methyltrimethoxysilane (MTMOS) was from Merck (Darmstadt, Germany), caffeic acid from Fluka and hydrogen peroxide 30% (w/w) from Riedel-de-Haën. All reagents were of analytical grade and used as received without further purification.

The synthesis of the Sonogel-Carbon (SNGC) materials as well as the ultrasonic synthesis of AuSNPs were carried out by sonicating with a high-power ultrasonic generator, SONICATOR 3000, from MISONIX (MISONIX, Inc. Farmingdale, NY, USA) (equipped with a 13 mm titanium tip), that provides a maximum power of 600 W. Graphite powder natural, high purity 200-mesh, 99.999% (metal basis), was from Alfa Aesar (Johnson Matthey GmbH, Germany). Glass capillary tubes, i.d. 1.15 mm (A = 0.0415 cm$^2$), were used as the bodies for the composite electrodes.

2.2. Synthesis of gold sononanoparticles (AuSNPs)

AuSNPs were synthesised by following the method described previously [30]. First, KAuCl$_4$ aqueous solution was sonicated for 1.5 min with a high-power ultrasound generator. Then, dihydrate sodium citrate aqueous solution was added; subsequently the colour of the solution turns grey immediately. After 4 min of sonication, the colour of the solution changes into dark red wine, indicating the formation of AuSNPs. The total time of synthesis is 5.5 min. A complete structural characterisation by transmission electron microscopy (TEM), X-ray diffraction (XRD) and ultraviolet–visible (UV–Vis) spectroscopy of these AuSNPs was previously reported and illustrated in Figures S1, S2, S3 and S4 (Supplementary material) [30].

2.3. Preparation of Sonogel-Carbon electrode

The SNGCE was fabricated as described in our previous papers [31,32]. A mixture of 500 µL of MTMOS and 100 µL of 0.1 M HCl was prepared and insonated for 10 s. Then, 0.5 g of graphite powder was added to the obtained sonosol, and adequately dispersed. After several minutes, the resulting material acquires enough consistency to fill the glass capillary tubes. About 24 h later, the surface of the electrodes could be polished, before modification, with emery paper No. 1200 (Struers) to remove extra composite material, wiped gently with weighing paper, thoroughly washed with distilled water, and allowed to dry at room temperature. Finally, a copper wire was inserted as the electrical contact into the electrodes, being ready to be used.

2.4. Enzyme immobilisation method on the SNGC surface

The biosensor developed in this study was obtained by the immobilisation of HRP enzyme, AuSNPs and PVPy via adsorption onto the SNGC surface. A mixture of HRP with AuSNPs, PVPy and GA was prepared. It was composed of 5 µL of PVPy (0.5%), 17 µL of HRP solution (1 mg mL$^{-1}$ of HRP dissolved in 5.4 µL of distilled water and 11.6 µL of AuSNPs), 25 µL of AuSNPs and 5 µL of GA (0.5% in water). Then 4.1 µL of this mixture was dispersed over the SNGC electrode surface and was dried at room temperature for 1 h.
2.5. **Amperometric measurements**

Amperometric measurements were performed in an electrochemical cell containing 10 mL of 50 mM stirred sodium acetate buffer (pH = 6.0) at room temperature and at a fixed applied potential of −0.15 V vs. Ag/AgCl, by using a PalmSens potentiostat interfaced to a computer. A conventional three-electrode system comprising a SNGCE (1.15 mm diameter) as the working electrode, Ag/AgCl as the reference electrode, and a stainless-steel rod as the auxiliary electrode was used. The amperometric detection of sulphide ions by the (HRP + AuSNPs)/SNGCE biosensor was investigated by sequentially increasing the concentration of Na₂S.

2.6. **Procedure for enzyme inhibition measurements**

The inhibition measurements started when the modified SNGCE was incubated into a cell containing 10 mL acetate buffer solution (pH = 6.0) and 0.1 mM H₂O₂. Then, a given concentration (10 µM) of caffeic acid (substrate) was added to record a steady-state current (I₀). After adding a known inhibitor (sulphide) concentration in solution, the decreased current (I₁) was recorded, which was directly proportional to the final concentration of sulphide in solution. Under inhibition effect of sulphide, the current (I₁) is smaller than current (I₀), and the degree of inhibition (I(%) (%) was evaluated according to the following Equation (1) [33]:

\[
I(\%) = \left(\frac{I₀ - I₁}{I₀}\right) \times 100
\]

where I₀ and I₁ are the current values recorded before and after inhibition, respectively.

The possible operation scheme for a biosensor based on the inhibition of HRP enzyme in the presence of the substrates is illustrated in **Scheme 1**.

2.7. **Impedance spectroscopy measurement**

Impedance measurements were carried out using an impedance analyser (Model: Versa STAT 3) at a fixed potential of −0.15 V. The impedance frequency range was 100 mHz–100 kHz with signal amplitude of 10 mV. The experiments were measured at room temperature and the electrochemical impedance spectroscopy (EIS) measurements were stable throughout the scanning process. The measurements were carried out in the absence of a redox probe, in 50 mM acetate buffer solution at room temperature. The pH was kept constant throughout all the measurements (pH 6.0). All connections were made with coaxial cables and the measurements were performed in the dark inside a Faraday cage.

3. **Results and discussion**

3.1 **Amperometric response to caffeic acid**

HRP utilises hydrogen peroxide to oxidise aromatic phenols and phenolic acids such as hydroquinone, catechol and caffeic acid. In this study, the caffeic acid was chosen as substrate, owing to its electrochemical reversibility and its high affinity to peroxidase.
enzyme (HRP) [29,34]. Amperometric measurements were performed in a stirred cell containing 0.1 mM H₂O₂ and 50 mM acetate buffer solution (pH = 6.0) at the working potential of −0.15 V vs. Ag/AgCl using modified SNGCE. The fixed applied potential was chosen in agreement with a previous optimisation [23,29], in which it was demonstrated that the optimal potential is −0.15 V.

The typical calibration curve for a modified SNGCE biosensor and the corresponding amperogram obtained by successive additions of caffeic acid are shown in Figure 1. A linear response was obtained between 3 µM and 13 µM with a correlation coefficient of 0.977. The detection limit (LOD) was calculated as 35 nM based on a signal to noise ratio of 3; with a sensitivity of 1.2 A M⁻¹ cm⁻². The apparent Michaelis constant ($K_{\text{Mapp}} = 6$ µM), and the saturation value of the cathode current ($I_{\text{max}} = 1.9 \times 10^{-4}$ A cm⁻²), were determined in the absence of inhibitor (Na₂S) from caffeic acid (substrate) calibration curves (Figure 1 inset).

3.2 Optimisation of experimental conditions

3.2.1 Effect of buffer pH

In the present work, 0.7 µM of Na₂S was used as inhibitor for the determination of the optimal working pH and the response at various pH values between 4 and 8 was studied. It can be observed from the Figure S5 (Supplementary material) that a maximum inhibition degree (%) was obtained at pH 6.0 for amperometric measurements. Then the activity slowly decreases with increasing pH up to 8.0. Thus, pH 6.0 was used in all further experiments.

3.2.2 Optimisation of the immobilisation method

The enzyme immobilisation method by cross-linking requires proteins, such enzyme and BSA, and a co-reticulation agent, namely, GA. High amount of BSA is usually employed in order to obtain membrane easily handled. In this work, BSA is replaced by PVPy, since this later polymer would accumulate the $S^{2−}$ anions. PVPy is known to form thin film that adheres strongly on the surface of the electrode, reason why it is widely used in sensor applications [35–37]. Furthermore, since PVPy is charged positively, it can easily interact with ions charged negatively, such as sulphides.

The immobilisation using PVPy with and without AuSNPs was compared to the one using BSA in order to evaluate the best response towards substrate (caffeic acid) and
inhibitor (Na₂S). Indeed, the apparent Michaelis–Menten constant values ($K_{\text{Mapp}}$) were 6 µM and 2.5 µM of caffeic acid for the (HRP + PVPy + AuSNPs)/SNGCE and (HRP + BSA + AuSNPs)/SNGCE, respectively (results not shown). Figure 2 shows that the use of PVPy improves the response towards sulphide in term of sensitivity, especially when using PVPy with AuSNPs (curve a). This biosensor (HRP + PVPy + AuSNPs)/SNGCE showed a higher current change in comparison with (HRP + BSA + AuSNPs)/SNGCE (curve b) and (HRP + BSA)/SNGCE (curve c) biosensors. The experimental results showed a notable difference in $I_{10}$ and $I_{50}$ towards sulphides anions for the three sensors, where $I_{10}$ and $I_{50}$ are the concentrations of the inhibitor corresponding to 10% and 50% of the inhibition signal, respectively. Indeed, when using the (HRP + BSA + AuSNPs)/SNGCE biosensor, the $I_{10}$ and $I_{50}$ were 1.8 µM and 3.8 µM, respectively, which were much higher than the values ($I_{10} = 0.18$ µM and $I_{50} = 0.76$ µM) obtained when using (HRP + PVPy + AuSNPs)/SNGCE. Hence, PVPy was chosen for further experiments.

**3.2.3 Effect of substrate concentration**

The effect of substrates (H₂O₂ and caffeic acid) concentrations on the inhibition of sulphides by the HRP electrode was evaluated. It was observed that at low concentration of substrate (0.05 mM of H₂O₂ and 5 µM of caffeic acid), the current response generated by the proposed biosensor was so small that the sulphide inhibition was not detectable (Figure S6. Supplementary material). Nevertheless, at too high concentrations of substrates, the active sites of the enzyme may be saturated by the substrate and insensitive to sulphide in the case of competitive inhibition. Consequently, concentrations of
0.1 mM of H$_2$O$_2$ and 10 µM of caffeic acid were chosen as the optimal concentrations for further sulphide amperometric measurements.

### 3.3 Na$_2$S measurements

#### 3.3.1 Amperometric measurements

Sulphides as HRP inhibitors have been long investigated [38,39]. Zhao et al. [38] reported that sulphides were able to interact and directly attack the haem protein by reducing iron centres of porphyrin systems to the corresponding ferrous-sulphide complexes. This causes much more severe inactivation of HRP, and therefore inhibits the reduced signal of the substrate [38]. Thus, the (HRP + PVPy + AuSNPs)/SNGCE can be used to monitor the trace of sulphides via inhibition.

Amperometric responses of (HRP + PVPy + AuSNPs)/SNGCE biosensor over the concentration range of 0.4–2.8 µM of Na$_2$S are shown in Figure 3(a). A linear response was obtained between 0.4 and 1.2 µM of Na$_2$S with a correlation coefficient of 0.983 (Figure 3(b)). Indeed, it has been demonstrated that there is a linear relationship between the degree of inhibition and the inhibitor concentration, with the slope equal to $I_{50}$ [40]. The amperometric measurements were conducted under the optimised experimental conditions. The electrode responded to the increment of sulphide concentration over a specified range. A plot of the biosensor inhibition percentage as a function of the inhibitor (Na$_2$S) concentration is shown in Figure 3(b), and the inhibition parameters related to this curve are presented in Table 1. The $I_{10}$ was found to be
0.18 µM which was much lower than for the (HRP + BSA + AuSNPs)/SNGCE developed in our previous work \cite{29}, and also much lower than others modified electrodes, such as CIP/chitosan/SPE, HRP–SAM–Au and Con A/HRP bilayers \cite{22–24}.

### Table 1. Principal analytical data referred to the calibration curves for the amperometric response of (HRP + PVPy + AuSNPs)/SNGCE biosensor and comparison with other sulphides inhibition biosensors reported in the literature.

| Biosensor                       | LOD (µM) | $I_{10}$ (µM) | $I_{50}$ (µM) | Ref |
|---------------------------------|----------|---------------|---------------|-----|
| CIP/chitosan/SPE                | 0.3      | 1.2*          | 9.0           | \cite{38} |
| HRP–SAM–Au                     | 0.3      | 0.5*          | 5.0           | \cite{23} |
| Con A/HRP bilayers             | 0.05     | 1.2*          | 22            | \cite{22} |
| (HRP + BSA + AuSNPs)/SNGCE     | NS       | 1.8           | 3.8           | \cite{22} |
| (HRP + PVPy + AuSNPs)/SNGCE    | 0.15     | 0.18          | 0.76          | This work |

CIP: *Coprinus cinereus* peroxidase; SPE: screen-printed electrode; HRP: horseradish peroxidase; SAM: self-assembled monolayer; Au: gold electrode; Con A: concanavalin A; BSA: bovine serum albumin, AuSNPs: gold sononanoparticles; SNGCE: Sonogel-Carbon electrode; PVPy: poly(4-vinylpyridine); NS—not specified.

*Calculated from the calibration curves of the reported references.

3.3.2 Electrochemical impedance spectroscopy of the inhibition biosensor

EIS was employed to characterise the (HRP + PVPy + AuSNPs)/SNGCE biosensor in 50 mM of sodium acetate buffer pH = 6.0, in the presence and in the absence of substrates (H$_2$O$_2$ and caffeic acid) and inhibitor (Na$_2$S). Different sets of impedance spectra corresponding to the conditions used in the amperometric experiments were obtained: Those spectra are (i) spectra recorded in buffer, (ii) after addition of the two substrates (H$_2$O$_2$ 0.1 mM and caffeic acid 10 µM) and, also (iii) after adding known concentrations of inhibitor (Na$_2$S). Data fittings and simulations were performed using Z-view software. All electrochemical impedance spectra were fitted by using the same equivalent circuit model, which can be described as cell resistance, $R_s$, in series with the parallel combination of a double layer capacitance, $C_{dl}$, and a charge transfer resistance, $R_{ct}$. The Nyquist diagrams are illustrated in Figure 4 and the value of each electrical
element in the equivalent circuit is shown in Table 2. The value of the electron transfer resistance, \( R_{ct} \), is the most important parameter, which reflects the electron transfer kinetics in the surface of the electrode. The capacitance values did not change significantly when adding substrates or inhibitor. As observed in Figure 4, the Nyquist plot of the \((\text{HRP} + \text{PVPy} + \text{AuSNPs})/\text{SNGCE}\) biosensor in 50 mM acetate buffer pH = 6.0, with \( \text{H}_2\text{O}_2 \) and caffeic acid (CA) (curve a), as a function of the inhibitor (\( \text{Na}_2\text{S} \)) concentration: 1.2 \( \mu \text{M} \) (curve b), 2.4 \( \mu \text{M} \) (curve c) and 7.2 \( \mu \text{M} \) (curve d).

**Figure 4.** Complex plane impedance spectra for \((\text{HRP} + \text{PVPy} + \text{AuSNPs})/\text{SNGCE}\) biosensor in 50 mM acetate buffer pH = 6.0, with \( \text{H}_2\text{O}_2 \) and caffeic acid (CA) (curve a), as a function of the inhibitor (\( \text{Na}_2\text{S} \)) concentration: 1.2 \( \mu \text{M} \) (curve b), 2.4 \( \mu \text{M} \) (curve c) and 7.2 \( \mu \text{M} \) (curve d).

**Table 2.** Parameter of the equivalent fitting circuit parameters for \((\text{HRP} + \text{PVPy} + \text{AuSNPs})/\text{SNGCE}\) biosensor in 50 mM acetate buffer, pH = 6.0.

| Electrode               | Analyte                      | \( R_{ct} \) (M\( \Omega \)) | \( C \) (pF cm\(^{-2}\)) |
|------------------------|------------------------------|-------------------------------|--------------------------|
| \((\text{HRP} + \text{PVPy} + \text{AuSNPs})/\text{SNGCE}\) | Acetate buffer               | 1.17                          | 0.46                     |
|                         | \( \text{H}_2\text{O}_2 \) (0.1 mM) + CA (10 \( \mu \text{M} \)) | 0.269                         | 0.53                     |
|                         | \( \text{Na}_2\text{S} \) (1.2 \( \mu \text{M} \))          | 0.893                         | 0.52                     |
|                         | \( \text{Na}_2\text{S} \) (2.4 \( \mu \text{M} \))          | 0.982                         | 0.54                     |
|                         | \( \text{Na}_2\text{S} \) (7.2 \( \mu \text{M} \))          | 1.23                          | 0.50                     |

The results from impedance spectroscopy measurements show that the interaction of sulphide ions with HRP leads to an increase of the charge transfer resistance. These results confirm the data obtained using amperometric measurements.
3.4 Determination of the type of enzyme inhibition

To estimate the inhibition mechanism, a plot of % degree of inhibition versus Na₂S can be constructed at two different substrate concentrations, as described by Amine et al [40]. Figure 5 shows the effect of two different caffeic acid concentrations on the I₅₀ obtained by using (HRP + PVPy + AuSNPs)/SNGCE biosensor. The inhibition curve is shifted to the right suggesting a competitive inhibition. Indeed, the shift of the inhibition curve to the left should be observed in the case of uncompetitive inhibition, while the inhibition curve remains unvaried in the case of non-competitive inhibition [40]. From Table 1, it can be also seen that, the I₅₀ values of sulphide, measured at 10 µM and 50 µM of caffeic acid by using the (HRP + PVP + AuSNPs)/SNGCE biosensor, are dependent on the substrate concentration. The I₅₀ increased significantly with the caffeic acid concentration; this rising of the I₅₀ values indicates a decrease in the enzyme/substrate interaction. Hence, it can be concluded that sulphides inhibits HRP activity in a competitive way. This was confirmed by the fact that the degree of inhibition decrease when increasing the substrate concentration (caffeic acid), since it competes with the inhibitor [40]. The similar behaviour was noticed in our previous work [29] while Zhang et al. [41] reported that sulphides act as an anticompetitive inhibitor.

![Figure 5](image_url)

Figure 5. The percentage of inhibition as a function of the concentration of the inhibitor at the (HRP + PVPy + AuSNPs)/SNGCE in presence of 10 µM (curve a) and 50 µM (curve b) of caffeic acid. Experimental conditions: electrolyte, 50 mM of sodium acetate buffer pH = 6.0 in the presence of 0.1 mM of H₂O₂; applied potential, −0.15 V vs. Ag/AgCl.
3.5 Selectivity

It is well known that sulphide and cyanide have an inhibitory effect on HRP enzyme; accordingly, interference between these two anions has been widely reported [22–24,29]. Indeed, cyanide could be eliminated from the solution containing sulphide and cyanide prior to any measurement. Cyanide removal process consists of two reaction tanks. In the first one, cyanide is oxidised to cyanate by the addition of chlorine. Caustic NaOH is added to keep the pH within the range of 9.5–10. In the second tank, conditions are adjusted to oxidise cyanate to carbon dioxide and nitrogen by introduction of additional chlorine. Again, NaOH is added to the second tank to maintain the pH at 8 [42].

In this study we propose an indirect selective determination of sulphides in a mixture of sulphide and cyanide which can be performed in two steps by using lead nitrate Pb(NO$_3$)$_2$: (i) performing an amperometric measurement of sulphide and cyanide; and (ii) removing sulphide by precipitation with lead nitrate Pb(NO$_3$)$_2$. Thus, if the analysed sample contained cyanide, an inhibition signal will remain after addition of lead nitrate; otherwise, no inhibition signal would be noticed even in the presence of sulphide. Figure 6(a,b) illustrate the response towards sulphide before and after adding Pb(NO$_3$)$_2$.

3.6 Real simple analysis

To apply the enzyme-inhibition sensor to determine sulphide ions in tap water, known concentrations of Na$_2$S (0.18 µM and 0.76 µM) were added separately to two distinct tap water samples from two different locations.

Enzyme inhibition was investigated by using amperometric measurements at (HRP + PVPy + AuSNPs)/SNGCE. The average sulphide recovery rates were estimated to be 92 ± 3% by comparing the sulphide ions concentrations recovered from the tap water with the respective concentrations of the added standard solution.

![Figure 6. The Na$_2$S detection at (HRP + PVPy + AuSNPs)/SNGCE biosensor in absence (a), and in presence of 100 µM Pb(NO$_3$)$_2$ (b). Experimental conditions: electrolyte, 50 mM sodium acetate buffer pH = 6.0 with 0.1 mM H$_2$O$_2$ and 10 µM caffeic acid; applied potential, −0.15 V vs. Ag/AgCl.](image-url)
3.7. Stability of the electrodes

Commonly used amperometric biosensors have disadvantages, such as enzyme leakage and denaturation of the enzyme on the electrode surface, which affect the lifetime of enzyme modified electrodes [43].

On one hand, the stability of the developed electrode was evaluated by monitoring the response currents with 10 µM of caffeic acid over a 30-day period. The response was constant for the first 15 days. After 30 days, the response of the biosensor decreased to 95.1% of its original response. On the other hand, the storage stability was tested by incubating the electrodes in six different solutions (acetate buffer or acetate buffer containing trehalose 2%, gelatin 2%, sucrose 10%, BSA 5% or dextran 2%). Then the amperometric responses were compared with freshly prepared electrodes.

The relative biosensor response to 10 µM caffeic acid as a function of storage time is shown in Figure 7. Each point corresponds to an average of about two measurements, and they were plotted as a percentage value relative to the original response observed the first day. Measurements were carried out at three different periods of storage (after 1, 2 and 4 weeks of storage at room temperature). It was observed that 77% and 40% of activity was preserved after storing the biosensor during 4 weeks in buffer solution containing trehalose and gelatin, respectively, while an important loss (~97%) of the enzyme activity was noticed for the electrodes stored in acetate buffer, sucrose, BSA and dextran.

Figure 7. Effects of storage at room temperature on the residual activity at the (HRP + PVPy + AuSNPs)/SNGCE-modified biosensor after incubation in solutions containing different stabilisers: acetate buffer (●) or acetate buffer containing trehalose 2% (♦), gelatin 2% (○), sucrose 10% (□), BSA 5% (△) or dextran 2% (★) for 1, 2 and 4 weeks. Experimental conditions for establishing the enzymatic residual activity: 50 mM sodium acetate buffer pH = 6.0 with 0.1 mM H₂O₂ and 10 µM caffeic acid; applied potential, −0.15 V vs. Ag/AgCl.
4. Conclusion

An amperometric inhibition biosensor based on cross-linking of HRP mixed with AuSNPs and PVPy on SNGCE surface was satisfactorily prepared with the aim of determining sulphide. Under the optimal experimental conditions (0.1 mM H$_2$O$_2$, 50 mM acetate buffer solution (pH = 6.0), at the potential applied of −0.15 V vs. Ag/AgCl), the sulphide determination is achieved in the dynamic range of 0.4–2.8 µM with a low limit of detection (0.15 µM). Lower values of $I_{10} = 0.18$ µM and $I_{50} = 0.76$ µM were obtained by using (HRP + PVPy + AuSNPs)/SNGCE biosensor compared to $I_{10} = 1.8$ µM and $I_{50} = 3.80$ µM attained at (HRP + BSA + AuSNPs)/SNGCE biosensor. The $I_{10}$ and $I_{50}$ values achieved in this work were also lower than $I_{10} = 1.2$ and $I_{50} = 9.0$ µM achieved at CIP/chitosan/SPE, and than $I_{10} = 0.5$ and $I_{50} = 5.0$ µM attained at HRP-SAM-Au, showing the high sensitivity of the proposed biosensor. It has also been demonstrated that PVPy and AuSNPs enhance dramatically the inhibition response in terms of sensitivity. EIS measurements have served to corroborate amperometric results: the HRP inhibition due to the decrease of the electron transfer in the presence of sulphide ions. Moreover, the kinetic analyses showed that the inhibition of sulphides on the HRP activity was competitive. Finally, good selectivity and stability studies are also reported. From all the results obtained, the biosensor described here showed much better performance than other biosensors reported in the literature and can be used as a promising approach for detecting sulphide.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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