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One-Step Chemiluminescent Assay for Hydrogen Peroxide Analysis in Water

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Abstract: The detection of hydrogen peroxide is of great importance in the environmental field. For this, a homogeneous technique has been developed here for sensitive and rapid quantification of hydrogen peroxide. In this technique, hemoglobin was used as a bioreceptor, where heme groups acted as electroactive centers to catalyze hydrogen peroxide reduction. The chemiluminescence reagent luminol is also a peroxidase substrate and can be oxidized by hemoglobin—thus generating a CL signal. The principle of the designed biosensor was based on the competition between hydrogen peroxide and luminol towards hemoglobin. Under optimized conditions, the chemiluminescent signal decreased with increasing hemoglobin concentrations within the linear range of 0.5 to 12 mM, with a correlation coefficient R^2 of 0.99762. The limit of detection was calculated to be as low as 0.308 mM. The selectivity of the biosensor was successfully demonstrated against different interferents. The developed strategy provides a one step, simple, and low-cost bioanalytical method which can be applied for the monitoring of other peroxidase substrates.

Keywords: chemiluminescence; hydrogen peroxide; hemoglobin; one-step analysis; environmental monitoring

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1. Introduction

In recent decades, biosensors have emerged as valuable tools in various fields: healthcare, environmental monitoring, food safety, agriculture, and biosecurity. They are considered miniature analytical devices that have the ability to detect and quantify specific biological or chemical substances via a biological recognition element coupled with advanced sensing technologies. Additionally, compared to complex analysis methods, biosensor technology is considered a simple, rapid, and cost-effective tool with high potential performance [1]. This technology is able to provide real-time, accurate, and sensitive detection for a wide range of analytes ranging from small molecules to proteins. In medicine, biosensors enable the early detection of diseases by detecting biomarkers, the facilitating of appropriate interventions, and improved treatment effects. In parallel, for environmental monitoring, biosensors provide a cost-effective and efficient platform for the detection and quantification of pollutants in air, water, and soil. By utilizing specific enzymes, antibodies, or microorganisms, biosensors can detect contaminants such as heavy metals, pesticides, and toxins, enabling the rapid identification and remediation of polluted sites [2–4]. For this, biosensors have been the subject of numerous research works that have aimed to develop diagnostic devices with attractive performance levels [5]. Recently, the use of proteins as elements of bio-recognition has reduced the cost of biosensors compared to biological molecules such as antibodies and enzymes [6].

Hydrogen peroxide (H_2O_2) is a strong oxidizing agent, considered one of the most reactive oxygen species, that is widely used in several areas of human activity (healthcare, antiseptic, antimicrobial, and chemical industries) [7]. It is considered a crucial intermediate in various biological and environmental processes [8]. Naturally, H_2O_2 exists in

living organisms as a reactive metabolic product of oxygen produced by mitochondria, the endoplasmic reticulum, and different enzymes. It plays a critical role in several biological processes including cell signaling, the immune response, and oxidative stress [9]. The normal values of H_2O_2 concentrations in the different body fluids can differ. For example, in blood plasma, the normal range is estimated to be between 1 and 5 μM, with increased concentrations (up to 50 μM) observed in many chronic and infectious diseases [10]. Abnormal levels of H_2O_2 can induce several diseases such as cancer, cardiovascular disorders, and neurodegenerative diseases [11,12]. At the cellular level, the accumulation of hydrogen peroxide can trigger inflammation and lead to apoptosis, and when its concentration in the blood reaches toxic levels, it can cause bioenergetic system failure and certain pathological disorders [13]. Moreover, H_2O_2 is a product of several enzymatic reactions and can be used as a biomarker for detecting various biological condition disorders [14]. It is also implicated in metabolic pathways that utilize oxidases, peroxidases, cyclooxygenase, lipoxygenase, myeloperoxidase, catalase, and other enzymes [15]. Hydrogen peroxide is also involved in environmental processes such as water treatment, wastewater management, and soil remediation [16]. Hence, monitoring H_2O_2 levels in these systems is essential for assessing the effectiveness of treatment methods, ensuring compliance with regulatory standards, and evaluating the environmental impact of various activities. In parallel, since H_2O_2 is a highly reactive and potentially hazardous chemical, its quantification in laboratories, industrial facilities, and storage areas helps ensure safety and prevent incidents associated with its mishandling or leakage [16]. Therefore, hydrogen peroxide is considered an important analyte to monitor due to its significance in clinical diagnosis and its importance in environmental monitoring and industrial systems. Several analytical techniques have been used for H_2O_2 analysis, including high-performance liquid chromatography, fluorimetry, and spectrometry [17]. However, these techniques require complex processes and sophisticated equipment [18]. In addition, many biosensors have been developed for H_2O_2 detection, but most of them are based on the use of natural enzymes, which is laborious, time consuming, and expensive—limiting their widespread application [19]. Therefore, the development of simple, rapid, nonenzymatic biosensors is highly recommended to overcome these limitations.

Horseradish peroxidase (HRP)-based biosensors have emerged as a prominent and widely used platform for hydrogen peroxide detection [20–22]. HRP—a heme-containing enzyme—offers the ability to catalyze the oxidation of H_2O_2, making it an ideal candidate for biosensing applications [23,24]. The enzymatic reaction between HRP and H_2O_2 results in the production of a measurable (colorimetric, fluorescent, etc.) compound, enabling the quantification and analysis of hydrogen peroxide concentrations [25,26]. HRP can be modified and integrated into different detection platforms such as electrochemical, optical, and immunoassay-based systems, allowing for versatile and adaptable applications [27,28]. Despite their widespread use, HRP-based biosensors for hydrogen peroxide (H_2O_2) detection suffer from certain limitations [29]. The high cost associated with HRP will restrict its accessibility and negatively affect the feasibility of large-scale production [30]. Additionally, the susceptibility of HRP to be inhibited or interfered with various substances commonly found in complex sample matrices limit the use of HRP-based biosensors. This limitation presents challenges in real-world applications, where samples may contain interfering substances that will negatively affect the reliability and accuracy of H_2O_2 detection [31]. Overcoming these interferences often requires additional sample preparation steps or complex calibration procedures, further complicating the analytical process. Furthermore, the reaction rate between HRP and H_2O_2 may not be always optimal, leading to either slow response times or erroneous results. All these barriers may hinder real-time monitoring applications, limiting the utility of HRP-based biosensors [32,33]. Additionally, it is worth noting that HRP-based biosensors have been extensively studied and optimized over the years, leading to improvements in their performance and reliability [34–36]. However, the aforementioned limitations still persist and create challenges in the biosensors' practical applications.

Hemoglobin (Hb) is a protein composed of four polypeptides chains (two-α and two-β), each containing one electroactive iron heme group. It is found in human erythrocytes (red blood cells) with a molar mass of approximately 67,000 g/mol [37,38]. Since each heme can bind to an oxygen molecule, Hb tetramers can bind up to four oxygen molecules. Several studies have focused on understanding the electrochemical behavior of heme proteins to investigate their properties and biological activities. Hb is principally used as an ideal model for the study of electron transfer reactions of heme [39,40]. Even though Hb is not considered as a biological electron transfer carrier, it has demonstrated its enzymatic-like catalytic activity. Moreover, since Hb exhibits good electrocatalytic activity for the reduction of different peroxidase substrates, it can replace peroxidase in the fabrication of non-enzymatic biosensors [41,42]. For this, Hb has been utilized as an HRP substitute for H_2O_2 detection due to its similar structure to peroxides, excellent stability, reasonable cost, and well-documented structure [43,44].

Hb is commonly used as a bioreceptor to develop H_2O_2 biosensors, as it can catalyze the reduction of H_2O_2, and chemically re-oxidized [44,45]. However, most of reported Hb based biosensors for H_2O_2 detection use electrochemical detection [46–48]. This latter method suffers from a slow electron transfer and a high overpotential, which can negatively influence the sensing performance and lead to a slow response time [49]. In addition, Hb immobilization has faced certain limitations and challenges. The stability of the immobilized Hb may be negatively affected, leading to reduced sensitivity and accuracy [50,51]. In addition, electrochemical Hb-based biosensors rely on the direct electron transfer between the immobilized Hb and the electrode for hydrogen detection. However, other electroactive molecules present in the sample—such as ascorbic acid, uric acid, or certain drugs—can compete for the electrode surface, leading to non-specific responses and false readings. This interference issue challenges the selectivity and specificity of the biosensors, and can lead to additional steps such as sample pre-treatment or advanced signal processing algorithms to reduce these interferences [52–54]. Moreover, the immobilization process can be complex and require optimization for achieving efficient Hb attachment and preserving its catalytic activity [55]. Various immobilization techniques—such as physical adsorption, covalent binding, or entrapment—have been explored [56]. However, finding the optimal immobilization strategy that ensures both high activity retention and stability remains a big challenge [57,58]. Furthermore, the limited reproducibility of the immobilization process can restrict its practical applications. Achieving appropriate and strong immobilization of Hb on multiple electrodes or in large-scale manufacturing can be challenging [59,60]. In addition, heterogeneous sensing assays are time consuming, laborious, expensive, and require multiple steps of modification and coating procedures [61,62]. For this, significant efforts have been made to principally overcome these limitations, increasing the electron transfer rate, decreasing the overpotential, and providing a suitable microenvironment to enhance electrocatalytic H_2O_2 detection [46,48,53]. Recently, homogenous assays have presented high potential due to their simplicity, ease of fabrication, and high throughput [63]. In this context, optical biosensors have attracted more attention due to their simplicity and homogeneous detection. These homogenous assays based on fluorescence, chemiluminescence, or bioluminescence do not require complicated probe immobilization or laborious procedures—thus allowing a one-step, rapid, and direct detection of analytes [64,65].

Optical detection methods are principally based on the use of light for sensing and analysis procedures. Optical detection techniques offer numerous advantages, including high sensitivity, rapid analysis, and a non-destructive nature. Chemiluminescence is a simple detection method based on the emission of light produced by a chemical reaction in the presence of chemiluminescence reagents (luminol, iso-luminol, etc.) and without the use of an external light source [66,67]. The low-cost luminol–H_2O_2 chemiluminescent (CL) reaction is the most commonly used system in CL analysis; it produces a strong CL reaction at a wavelength of 425 nm, under appropriate experimental conditions [68]. CL reactions have a wide range of analytical applications in various fields (environmental monitoring systems, food analysis, and diagnosis of disease) [69,70]. The wide use of this

method is principally attributed to its high sensitivity, rapidity, important signal-to-noise ratio, and wide dynamic range. Moreover, the absence of the excitation light source makes this technique simple and cost-effective compared to optical methods [71,72]. Furthermore, it is noteworthy to mention that in the literature, there are only a few studies that use chemiluminescence detection based on hemoglobin for H_2O_2 detection [73]. Based on these advantages, CL reactions have been used, in this work, to develop a non-enzymatic biosensor for H_2O_2 detection, using Hb as a bioreceptor.

In the present study, Hb has been used as a bioreceptor to develop a H_2O_2 biosensor. The originality of this work lies in the competition of the target and luminol solution toward Hb, which showed a high peroxidase-like activity for H_2O_2. The detection principle is mainly based on chemiluminescence signal diminution in the presence of H_2O_2. The H_2O_2 reacts with the Hb, which is essential for the luminol reaction—thus leading to a lower chemiluminescence signal. Additionally, since the reaction of Hb with H_2O_2 and luminol is not time-consuming, the developed assay is considered a rapid one-step bioanalytical platform for hydrogen peroxide detection.

2. Materials and Methods

2.1. Reagents and Apparatus

Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) was purchased from Bio-Rad (Hercules, CA, USA) Hemoglobin from bovine blood, hydrogen peroxide, glucose, fructose, galactose, phenol, dopamine, ascorbic acid, sodium phosphate dibasic (Na_2HPO_4), and sodium phosphate monobasic (NaH_2PO_4) were purchased from Sigma−Aldrich (Saint-Louis, MO, USA). Phosphate buffer solution (PBS 0.01 M) was prepared using Na_2HPO_4 and NaH_2PO_4. Hb solution (3 μg $mL^{-1)}$ was prepared using PBS buffer (0.01 M, pH 7.4). All luminescence measurements were recorded at 425 nm using a microplate reader (PerkinElmer EnSpire, Waltham, MA, USA), employing a 96-well microplate.

2.2. Preparation of the Chemiluminescent Biosensor for the Detection of H_2O_2

A 96-well microplate was used to perform the developed assay. For that, 25 μL of Hb solution (3 μg mL^{-1}) was added to each well, followed by 25 μL of different concentrations of H_2O_2. After incubation, 25 μL of luminol solution was also added. Then, the chemiluminescence intensity was measured at an emission wavelength of 425 nm; all the experiments were repeated 3 times under the optimized detection conditions.

2.3. Interference Studies

In order to assess the selectivity of the proposed technique for H_2O_2 detection, some interfering species (Ascorbic Acid, Galactose, Glucose, Fructose, Dopamine and Phenol) were tested. This assay was conducted under the same procedure as for H_2O_2 detection. In brief, 25 μL of each interferent (0.1 mM) was incubated with 25 μL of Hb solution (3 μg mL^{-1}) in each well of the microplate. Then, 25 μL of luminol solution was added, and the luminescence intensity was measured at 425 nm.

2.4. Hydrogen Peroxide Detection in Real Samples

In order to confirm the response of our system in real samples, Hb solution (3 μg mL^{-1}) was incubated with water samples spiked with known concentrations of H_2O_2 (0.5, 2, 4, 12 mM). The chemiluminescence detection of H_2O_2 was performed using the same procedure mentioned above.

3. Results and Discussion

3.1. Principle of the Developed Assay

In this study, Hb, containing four iron heme groups, acted as a catalyst for our target (H_2O_2) and for luminol in the CL reaction. This chemiluminescence reagent is also a peroxidase substrate and can be oxidized by hemoglobin to generate a signal [74,75]. For this, the designed biosensor was based on the competition between hydrogen peroxide

and luminol towards hemoglobin (Figure 1). Two control groups were tested to confirm the feasibility of the developed assay. In the presence of luminol alone, the obtained chemiluminescence signal was minimal and negligible, because luminol by itself does not have the ability to produce significant chemiluminescence. However, in the presence of Hb, the obtained chemiluminescence signal was very high, which indicates the interaction between Hb and luminol.

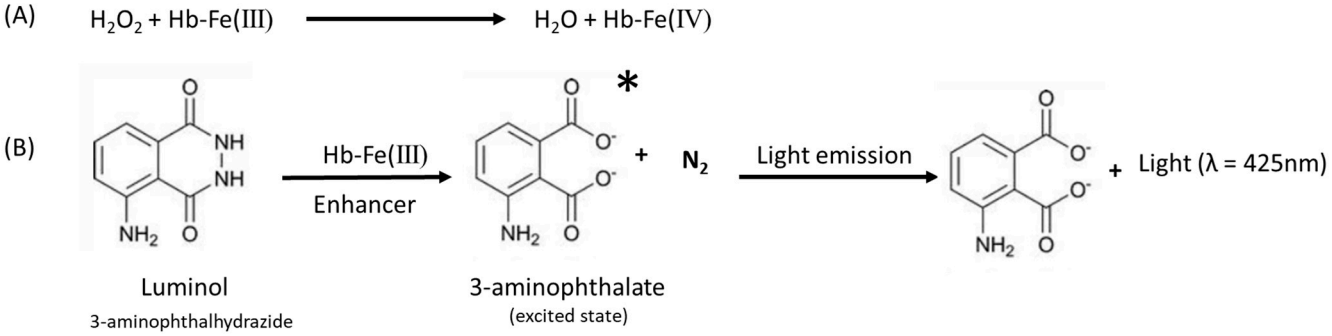

Figure 1. (**A**): The reaction of hydrogen peroxide with hemoglobin. (**B**): Chemiluminescence oxidation of luminol by hemoglobin. * shows the excited state of the molecule [76,77].

In the absence of the target, luminol will be oxidized by the total amount of Hb present, resulting in a strong chemiluminescence signal. However, in the presence of H_2O_2, the latter will be oxidized by hemoglobin—thus leaving a reduced number of active sites for the chemiluminescence reaction and a weaker signal. Hence, the CL signal of the Luminol/H_2O_2 system decreased when the Hb reacted with the target (H_2O_2) and switched to its oxidized form (Figure 2). Based on this principle, hydrogen peroxide can be easily quantified by measuring the chemiluminescence signal generated by the Hemoglobin/luminol reaction. Given its simplicity, the proposed bioassay can be performed in a few minutes in homogeneous conditions, requiring only one step.

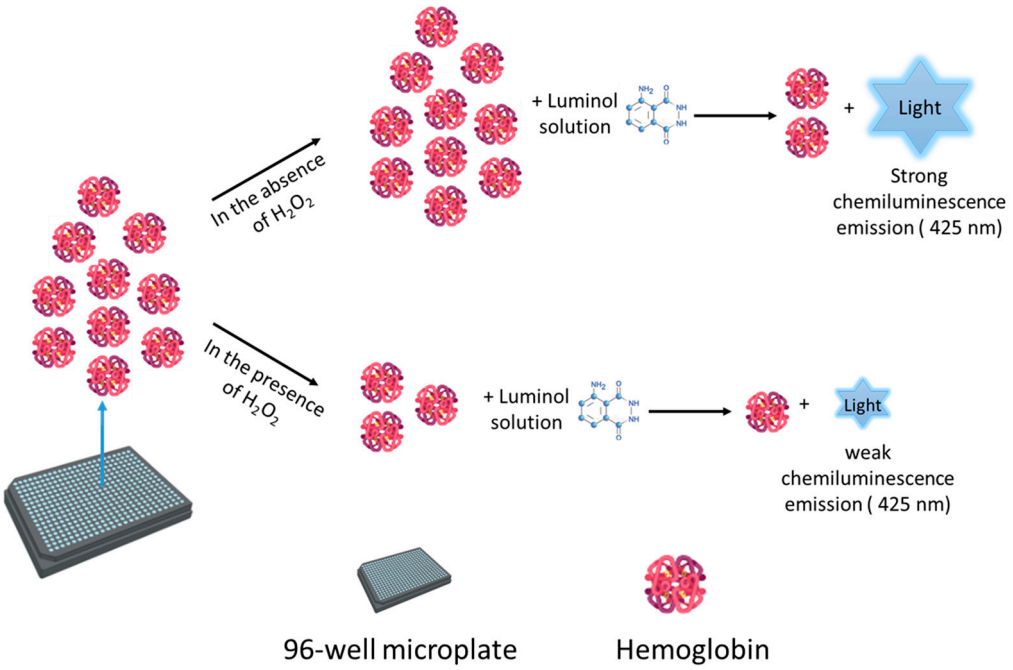

Figure 2. Schematic illustration of the sensing strategy of the developed label-free homogenous chemiluminescent assay for hydrogen peroxide detection.

3.2. Optimization of Incubation Time

The reaction time for the H_2O_2 and hemoglobin has an important effect on the chemiluminescence intensity. Therefore, the effect of the incubation time was investigated by incubating the Hb solution (3 μg mL^{-1}) with H_2O_2 (12 mM) for different periods of time (15, 20, 30, 40 min). After each incubation time, luminol solution was added and the CL intensity was measured at 425 nm. The obtained results show that the difference in the chemiluminescence signal (I_0 (Signal in the absence of Hb) − I_1 (Signal in the presence of Hb)) reached a plateau after 20 min of incubation. After this, the signal started to decrease to almost 60,000 after 40 min. Therefore, 20 min was selected as the optimal time for the H_2O_2–Hb reaction.

3.3. Detection of Hydrogen Peroxide

The analytical performance of the proposed chemiluminescent assay was assessed under the optimized detection conditions (incubation time: 20 min, Hb concentration: 3 μg mL^{-1}). In this study, chemiluminescence detection was based on the reaction between the Hb heme groups and the H_2O_2, in the presence of luminol solution as a CL reagent. Figure 3 shows that the chemiluminescence signal decreased gradually with increases in H_2O_2 concentration, which indicates that the CL intensity was strongly dependent on the H_2O_2 concentration. The decrease in the chemiluminescence intensity was mainly due to the competition between the H_2O_2 and luminol solution towards hemoglobin. The calibration curve presenting the chemiluminescence intensity as a function of H_2O_2 concentrations is illustrated in Figure 3.

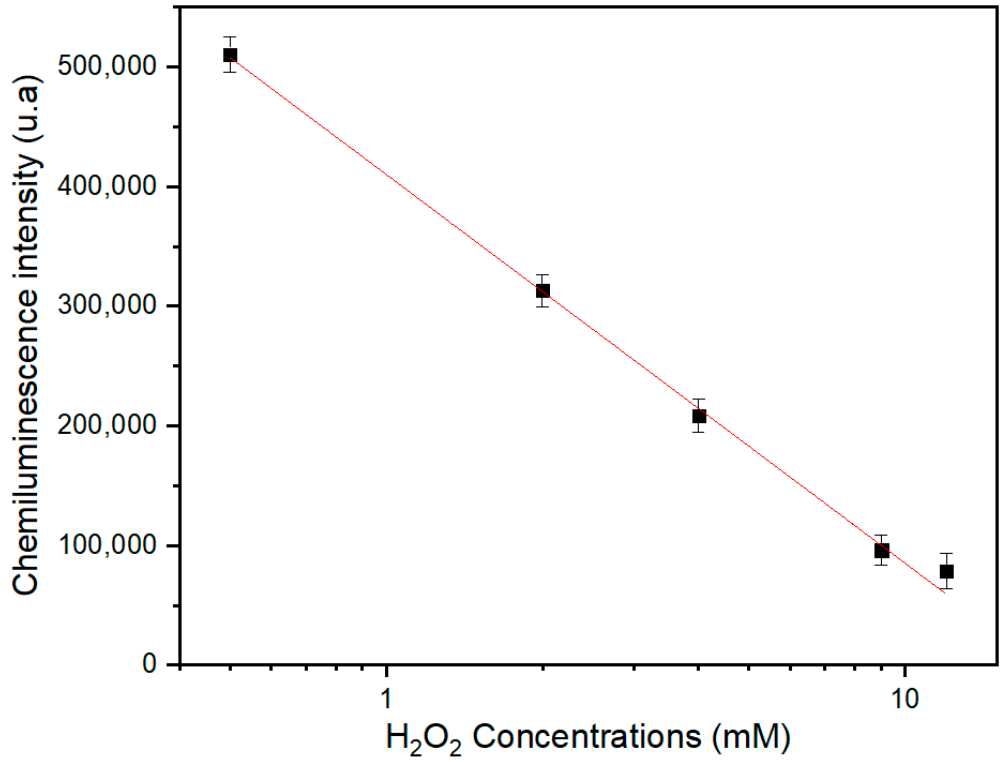

Figure 3. Calibration curve of the developed label-free chemiluminescent assay for H_2O_2 concentrations ranging from 0.5 to 12 mM at 42 nm. n = 3.

The results show that the chemiluminescence response was linearly proportional to the concentration of H_2O_2 in the range of 0.5–12 mM. The regression equation was Y= −324,967.5X + 410,043.5 (Y: chemiluminescence intensity, X: log H_2O_2 concentration), with a coefficient of determination (R^2) of 0.99762. The relative standard deviation (RSD) of H_2O_2 detection in the linear range was less than 4% (n = 3), demonstrating the good reproducibility of the developed assay. The limit of detection was calculated to be 0.308 mM using 3σ/S, where S is the slope and σ is standard deviation of the blank.

It is very important to note that most of hydrogen peroxide biosensors reported in the literature are based on electrochemical detection, using Hb as a bioreceptor [40,47,53]. However, the active sites of Hb are deeply buried in the polypeptide chain structure. In addition, since most of the developed biosensors involve chemical modifications and the use of mediators and nanomaterials, Hb electrocatalytic activity will be negatively affected after its immobilization [78–81]. For example, Mashitah M. Yusoff and co-workers co-immobilized Hb (2 mg mL^{-1}) with Tin (IV) oxide ($SnO_{2)}$-polyaniline (PANI) nanofibers on a glassy carbon electrode surface for hydrogen peroxide detection. Despite the obtained LOD, the use of SnO_2-PANI composite for Hb immobilization affects its catalytic activity and makes this platform laborious and time-consuming [82]. Moslem Mansour Lakouraj and his colleagues also developed an electrochemical biosensor based on the immobilization of hemoglobin (Hb) onto the surface of a glassy carbon electrode (GCE), modified with a nanocomposite made from polypyrrole@poly (styrene-alt-maleic anhydride) grafted with 4-aminobenzenesulfonate. The developed platform was highly sensitive, but it required different immobilization steps for the Hb, which hindered its electrocatalytic activity [83]. In addition, Ali Saad Elewi et al. immobilized Hb (6 mg mL^{-1}) on a screen-printed carbon electrode modified with gold nanoparticles for hydrogen peroxide detection [53]. Moreover, Mehdi Baghayeri et al. immobilized Hb (20 mg mL^{-1}) onto a Palladium@Fe_3O_4-MWCNT nanocomposite modified glassy carbon electrode [84]. Likewise, Wei Sun and his co-workers successfully synthesized an FeS@ molybdenum disulfide (MoS_2) nanocomposite to immobilize Hb (15 mg mL^{-1}) for Hydrogen peroxide detection [85]. All of these electrochemical biosensors required the synthesis of nanocomposites/nanomaterials, involving sophisticated designs for the multi-step operations and required high temperatures, which is time consuming. While most of the developed Hb-based biosensors present a low LOD, only a few can maintain the intrinsic activity of immobilized Hb. Moreover, compared to electrochemical sensors that require heterogenous detection methods, optical sensing platforms are principally based on homogenous assays that avoid immobilization steps, as well as the coating and washing steps required in the majority of heterogeneous assays [53,82,86].

To overcome these limitations, we have developed a one-step homogenous assay. The platform is rapid, cost effective, and maintains the electrocatalytic activity of Hb, as it does not require any immobilization or complicated steps. Moreover, compared to the already developed biosensors that use high concentrations of Hb (>2 mg mL^{-1}), this work involves only the use of 3 μg mL^{-1} of Hb, which is economic. To the best of our knowledge, this is the first report describing a simple, label-free, homogenous assay based on Hb as a bioreceptor for the chemiluminescence detection of H_2O_2.

Overall, the development of Hb-based H_2O_2 biosensors using the chemiluminescence detection method represents a significant breakthrough in the field of biosensing. On the other hand, the use of Hb as an enzyme substitute offers cost-effectiveness and stability. This combination of factors makes these biosensors highly attractive for various applications in different fields.

3.4. Selectivity and Reproducibility of the Developed H_2O_2 Biosensor

Selectivity is considered one of the key features in the development of a good sensing platform, as the presence of many interferents in real samples will affect the accuracy of the detection system. For this, the selectivity of the proposed homogenous assay was investigated using different potential interfering species, including glucose, fructose, galac-

tose, dopamine, phenol, and ascorbic acid. For this interference study, a concentration of 2 mM was chosen for H_2O_2 and the interferents. As can be observed in Figure 4, the obtained results show that, in the presence of interferents, the difference in the signal intensity ((I_0 (Signal in the absence of analyte) − I_1 (Signal in the presence of analyte)) was very low—indicating the reaction of the total amount of Hb with the luminol solution. However, in the presence of the hydrogen peroxide, the difference in the signal was highly significant—confirming the specific interaction of Hb toward H_2O_2. These results confirm the high selectivity of our biosensor for H_2O_2 detection. This latter can be attributed to the high catalytic activity of Hb toward the target, indicating its potential application in complex matrices.

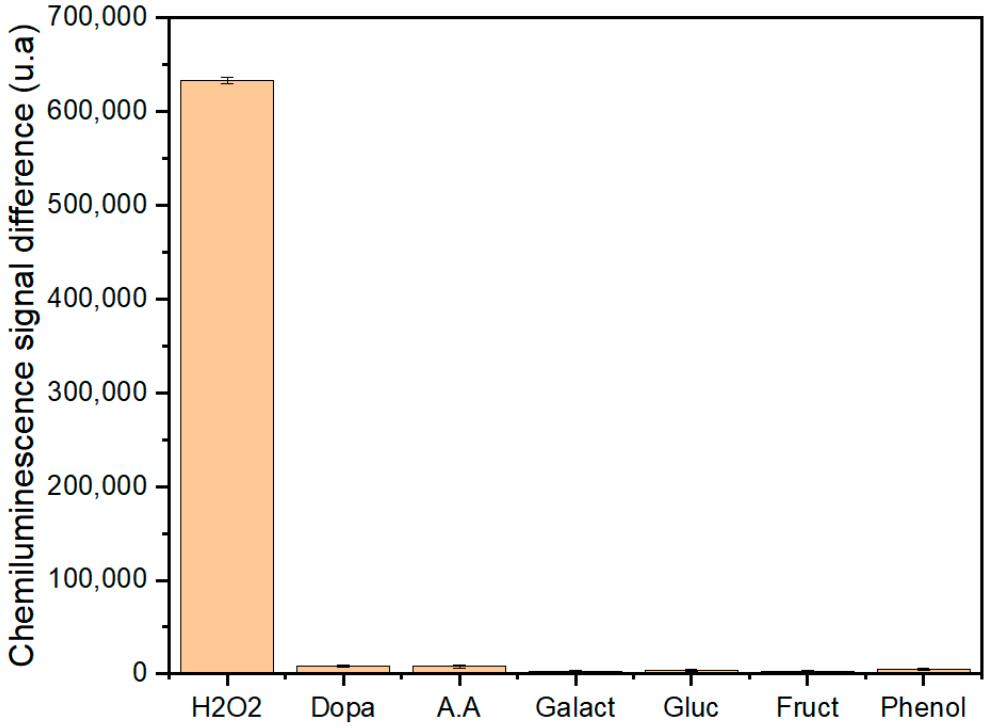

Figure 4. Chemiluminescence response of H_2O_2, and some interfering species (2 mM); glucose (Gluc), fructose (Fruct), galactose (Galact), dopamine (DOPA), phenol, and ascorbic acid (A.A). Reaction conditions: Hb 3 μg mL^{-1}, λ = 425 nm, the chemiluminescence signal difference = I_0 − I_1 (I_0 (Signal in the absence of the analyte), I_1 (Signal in the presence of the analyte). *n* = 3.

Besides the good performance of the homogenous assay, reproducibility is one of the key issues in developing a reliable sensor. For this purpose, four wells of the microplate were prepared separately to measure the same concentration of H_2O_2. After this, the chemiluminescence signal measurements of the prepared wells were recorded. The results showed that the difference between the signals was not significant, with an RSD of 3.1%. These results confirmed that the developed platform is selective and highly reproducible.

3.5. Real Sample Analysis

In order to validate the performance of the developed platform, water samples spiked with known concentrations of H_2O_2 (0.5, 2, 4, 12 mM) were prepared. As shown in Table 1, recovery rates ranging from 90% to 133% were obtained, with good RSDs in the range of 0.29–2.36%. These satisfactory experimental results indicate the good accuracy and applicability of this homogenous assay for H_2O_2 detection and environmental monitoring.

Table 1. Determination and recovery results of H_2O_2 in water samples (n = 3).

Sample	H_2O_2 Concentration (mM)	Concentration Found (mM)	Recovery Percentage %	RSD% (n = 3)
Water	0.5	0.45	90	0.97
	2	2.67	133.5	2.36
	4	4.81	120.25	1.43
	12	12.36	103	0.29

4. Conclusions

In conclusion, while HRP-based biosensors have proven valuable in H_2O_2 detection, they also suffer from certain limitations. Their high cost, limited stability, and susceptibility to interference are significant problems that hinder their widespread application. Therefore, in this work, a homogenous chemiluminescent assay was developed for hydrogen peroxide detection using Hb as a bioreceptor. The development of hydrogen peroxide biosensors based on hemoglobin utilizing the chemiluminescence detection method holds great promise for numerous applications. The chemiluminescence detection method employed for this platform provides several benefits, including its simplicity, rapidity, excellent selectivity, and wide dynamic range. In addition, the use of Hb as a substitute for HRP offers several advantages—most notably its cost-effectiveness and stability in solution. Moreover, Hb exhibits remarkable enzyme-like catalytic activity, and extensive research has documented its bio-electrocatalytic ability for reducing H_2O_2—thus making it an excellent candidate for the development of H_2O_2 biosensors. This article has highlighted the significant advancements in this field and emphasized the potential of Hb as a valuable alternative to the commonly used enzyme horseradish peroxidase (HRP) in the fabrication of H_2O_2 biosensors. Since H_2O_2 and luminol reagent are peroxidase substrates, their competition towards Hb was used as the principle of detection in this technique. The proposed biosensor showed good sensing performance for nonenzymatic H_2O_2 detection in the range of 0.5–12 mM, with high selectivity and reproducibility. The applicability of the developed device was assessed in the presence of a real water sample. This method offers a reliable approach for detecting H_2O_2 with a wide range of potential applications, such as clinical diagnostics, environmental monitoring, and food safety.

The development of Hb-based H_2O_2 biosensors may lead to further research and innovation. By optimizing the Hb concentrations, exploring novel chemiluminescent substrates, and enhancing the overall biosensor performance, researchers can continue to refine and advance this technology. It is expected that Hb-based H_2O_2 biosensors will continue to evolve, leading to improved performance, increased reliability, and expanded application possibilities. The future holds great promise for the development and utilization of these biosensors, enabling the overcoming of critical challenges and contributing to advancements in healthcare and environmental monitoring.

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