A Paper-Based Bi-Enzymatic Sensor for Chemiresistive Glucose Detection

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Abstract

At-home testing of glucose levels is crucial for safe monitoring of a variety of diseases, such as pancreatitis, psoriasis, cirrhosis, acute myocardial infarction, and, of course, diabetes. Current blood tests are invasive, leading to the research of alternative biofluids such as saliva, tears, and sweat. Nevertheless, such fluids are limited in quantity and contains various interfering molecules, posing stringent technological requirements. In this study, a simple paper-based glucose sensor is designed by using the conductive polymer poly(3,4-ethylene-dioxythiophene) polystyrene sulfonate (PEDOT:PSS) and a bi-enzymatic solution of Horseradish Peroxidase (HRP) and Glucose Oxidase (GOx). Unlike most electrochemical glucose sensors, this sensor employs a chemiresistive mechanism, allowing simple configurations, low costs, and easy signal measurements. The designed sensor demonstrates a working range suitable for various biofluid analysis, e.g., saliva, with a limit of detection of 1.1 μM and a linear detection range of 102 − 104 μM while only requiring 40 μL sample volume. Detection in whole artificial saliva is also carried out to demonstrate the sensor applicability. The limited analyte volume required, and the suitable detection range and limit of detection achieved by this sensor make it an excellent candidate for developing a non-invasive, at home glucose meter.

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Abstract

At-home testing of glucose levels is crucial for safe monitoring of a variety of diseases, such as pancreatitis, psoriasis, cirrhosis, acute myocardial infarction, and, of course, diabetes. Current blood tests are invasive, leading to the research of alternative biofluids such as saliva, tears, and sweat. Nevertheless, such fluids are limited in quantity and contains various interfering molecules, posing stringent technological requirements. In this study, a simple paper-based glucose sensor is designed by using the conductive polymer poly(3,4-ethylene-dioxythiophene) polystyrene sulfonate (PEDOT:PSS) and a bi-enzymatic solution of Horseradish Peroxidase (HRP) and Glucose Oxidase (GOx). Unlike most electrochemical glucose sensors, this sensor employs a chemiresistive mechanism, allowing simple configurations, low costs, and easy signal measurements. The designed sensor demonstrates a working range suitable for various biofluid analysis, *e.g.*, saliva, with a limit of detection of 1.1 µM and a linear detection range of $10^2 – 10^4$ µM while only requiring 40 µL

sample volume. Detection in whole artificial saliva is also carried out to demonstrate the sensor applicability. The limited analyte volume required, and the suitable detection range and limit of detection achieved by this sensor make it an excellent candidate for developing a non-invasive, at home glucose meter.

1. Introduction

Glucose monitoring and measurement is essential for healthcare purposes, as abnormal glucose levels have been correlated to a variety of diseases, including diabetes, pancreatitis, acute myocardial infarction, cirrhosis, preeclampsia, lung cancer, and others.[1] In particular, diabetes mellitus is a chronic condition characterised by high blood glucose levels due to defects in the production or action of insulin, a hormone produced by the pancreas that regulates glucose metabolism. When insulin is insufficient or ineffective, glucose accumulates in the blood, leading to chronic hyperglycaemia.[2] Early detection and screening of individuals at risk is crucial for diabetes management and prevention, while patients already affected need to assess their glucose level multiple times a day.[3] Hence, accurate monitoring of blood glucose levels is crucial for diabetes management, preventing long-term complications, such as cardiovascular diseases and kidney damages.[4]

Clinically, glycaemia testing is widely addressed by invasive blood collection methods, which are often associated with pain and inconvenience.[5] This is particularly challenging for individuals requiring frequent glucose monitoring, such as patients with diabetes. The need for frequent monitoring led a to the development of Point-of-Care Testing (POCT) techniques and devices, which facilitate rapid and convenient at-home glucose testing.[6] Commercially available solutions, such as finger-prick tests and glucose monitoring patches, however, are still invasive. The same goes for recent developments in patches making use of microneedles and iontophoresis to measure interstitial fluid glucose levels, which are still minimally invasive.[7] Hence, research endeavours recently focused on alternative biofluids such as saliva,[5,6] and sweat,[7,8] , offering non-invasive solutions for glucose monitoring. The advantages of a non-invasive and portable device for glucose monitoring would be many-fold: (i) reduction of potential infections and irritations from needles and microneedles, (ii) reduced discomfort for patients, and (iii) increased compliance toward testing. In particular, salivary glucose level showed promising for diabetes screening and management, with many studies reporting a correlation with blood glucose levels.[9] Nevertheless, the development of a POC test to analyse such biofluids faces multiple challenges: (i) the quantity of glucose is smaller

than the one in blood (*e.g.*, in saliva it is $2 \times 10^2 - 8 \times 10^3$ µM compared to $4 \times 10^3 - 2 \times 10^4$ µM in blood)[9]; (ii) saliva, sweat, and tears production is dependent on hydration and other factors; (iii) the volume of such biofluids available for analysis at each one time is small, requiring a sensor able to function with very low sample volumes; (iv) the presence of proteins often hinders detection via biofouling, requiring sample pretreatment which are not always suitable for POC settings; and (v) the cost needs to be affordable for the average user. In this regard, amperometric sensors, whether enzymatic or non-enzymatic,[5] are the most used for glucose detection, exhibit high sensitivity and selectivity, while they typically require expensive electrode materials, complex fabrication process, and a relatively big sample volume (unless a certain degree of miniaturization is carried out).[10] Chemiresistive sensors, instead, provide several advantages, including simple configuration, low costs, low sample volume required, and easy signal measurement,[11] and they are, for these reasons, particularly attractive for developing low-cost and user-friendly POCT devices.

In our previous works, an enzymatic sensor was developed for the detection of hydrogen peroxide by using a single enzyme, horseradish peroxidase (HRP), on both paper-based [10] and hydrogel-based [12] substrates when combined with conductive polymer poly(3,4–ethylenedioxythiophene):polystyrene sulfonate PEDOT:PSS, highlighting the potential of chemiresistive enzyme-based detection methods. The possibility of adding a second enzyme to detect more complex molecules was also evaluated.[13]

Following these results, this work aims to study the possibility of developing a low-cost paper-based chemiresistive sensors for glucose detection, built upon the previous system via the addition of glucose oxidase (GOx). The sensor sensitivity was evaluated using a low amount of sample (*i.e.*, 40 µL), and its selectivity and storage stability were also investigated. To demonstrate the applicability of the sensor, tests with artificial saliva were also carried out. To the best of our knowledge, this is the first time a chemiresistive biosensor employing PEDOT:PSS has been used to detect glucose in artificial saliva.

2. Material and methods

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2.1. Materials

High-conductivity grade aqueous solution 1.1 wt% of poly(3,4-ethylene-dioxythiophene) polystyrene sulfonate (PEDOT:PSS), both acidic and neutral, horseradish peroxidase (HRP) ($\geq$ 250 units g^{-1}), glucose oxidase (GOx) from *Aspergillus niger* (1.58 x 10^5 units g^{-1}), D-(+)-Trehalose dihydrate, α-Amylase from *Bacillus licheniformis* (500-1500 units mg^{-1}), uric acid (> 99%), lactic acid ($\geq$ 85%), hydrocortisone (or cortisol), cholesterol, urea, Bovine Serum Albumin, Nafion perfluorinated resin solution (20 wt% in lower aliphatic alcohols and water), and artificial saliva, were purchased from Sigma-Aldrich (Australia). D(+)-glucose was purchased from Merck KGaA (Germany). Sucrose was purchased from Ajax Finechem (Australia). Citric acid anhydrous was purchased from Analytical Reagent (Australia). Carbon dispersion paste was purchased from Dycotec Materials (United Kingdom). Milli-Q water and phosphate buffer saline (PBS) were used as solvents.

2.2. Sensor fabrication

The sensor fabrication protocol was similar to the one reported in our previous study with some modifications [10]. Briefly, PEDOT:PSS was printed through a 0.15-mm internal diameter needle on Whatman paper 1 (Sigma-Aldrich, Australia) using an EnvisionTEC 3-D Bioplotter (Germany). The printing parameters were optimized to 20°C, 0.130 mm of needle offset, 0.3 bar, and 25 mm/s. The printed samples were left to dry in the air for 2 h. Afterwards, carbon paste edges were printed through a 0.25-mm internal diameter needle. The printing parameters were optimized to 25°C, 0.130 mm of needle offset, 3.0 bar, and 10 mm s^{-1}. The printed samples were left to dry in the air for 3 h. The final sensor dimensions were 2.8 cm x 0.5 cm, while the carbon paste edges were 2 cm distant.

The two enzymes, HRP and GOx, were dissolved in PBS at different concentrations. Then, 70 μL of the enzymatic solution was drop-casted on each sensor and left to dry for two hours.

2.3. Chemiresistive testing and storage

The electrical resistance of the sensors was measured before and after adding the additive and letting the sensor dry for 1 h by using a potentiostat (8846A, Fluke, USA). The sensing performance was

quantified by calculating the percentual difference between the initial and final resistance as shown in Equation (1):

$$\Delta R \ (\%) = \frac{R_f - R_0}{R_0} \times 100\% \,, \tag{1}$$

where R_0 and R_f are the initial and final resistance, respectively.

Sensors were also enclosed in aluminium foil pouches with humidity absorber bags, stored at 4°C and tested for up to 14 days to address their longevity. Before the beginning of the storage test, some of the sensors were freeze-dried at - 76 °C for 24 h (Epsilon 2-4 LSCplus Scitek freeze dryer).

2.4. UV-visible spectroscopy

GOx or a mixture of HRP and GOx were dissolved in PBS. The concentrations of GOx and HRP adopted were 20 and 5 mg mL^{-1}, respectively. The enzymatic solution was mixed with PEDOT:PSS with a ratio of 1 to 2. Then, 100 μL of water or an aqueous solution of glucose 10 mM was added to 1 mL of the previous solution.

The UV-vis absorption spectra of the formed solutions diluted at 1:20 and 1:40 were measured using a SpectraMax M3 (Molecular Devices, USA). Two tests were addressed: the first in the range 350-1000 nm with a step of 10 nm and the second in 600-950 nm with a step of 5 nm.

2.5. Characterization

Prior to scanning electron microscopy, the paper-based samples were sputter coated using a plasma coater to deposit a 16 nm thick gold/palladium layer. Then, scanning electron microscopy unit (Phenom™ XL G2 Desktop SEM, Thermo Fisher Scientific) with a back scatter electron detector (15kV) was used to study the microstructure of the samples.

2.6. Artificial saliva and Nafion

Artificial saliva samples with different concentrations of glucose, ranging from 0 to 1000 mM, were tested with the designed sensor. To simulate real-life conditions, various molecules, such as cholesterol, uric acid, lactic acid, sucrose, cortisol, and α-amylase were added in different concentrations to artificial saliva (**Table S1**).

2.7.   Data analysis

Each datapoint has been repeated at least three times and results were expressed as *mean ± standard deviation*. One-way ANOVA followed by Tukey comparison was performed by using GraphPad Prism (GraphPad Software, Massachusetts, USA).

3.   Results and discussion

3.1.   Sensing mechanism

In our previous study, it was demonstrated that the change in conformation between benzoid (more coiled hence less conductive) and quinoid (more linear hence more conductive) is the governing factor for the mechanism of PEDOT:PSS for detection of analytes [10,14]. Here, the feasibility of utilising the bienzymatic system comprised of PEDOT:PSS, HRP, and GOx for detecting glucose, was examined by measuring the PEDOT:PSS/HRP/GOx absorption spectrum at 850 nm before and after the glucose addition. As was anticipated, we observed a decreased in absorption near 850 nm due to a redox interaction between PEDOT:PSS and HRP/GOx in the presence of 10 mM glucose (**Figure 1A**, **1B**, and **1C**). It is important to note that conformational change in PEDOT:PSS is governed by the presence of HRP due to electron transfer mechanism on its heme group, while the glucose catalytic oxidation still occur in the presence of GOx. As pH has a significant effect on PEDOT:PSS chemiresistive properties and enzyme activity, all the experiments were conducted at pH 7, while it was not possible to replicate these results using acidic PEDOT:PSS, which has a pH of about 2 (**Figure S1**). Indeed, GOx stability, hence activity, decreases at pH lower than 3-3.5.[12,14]

The UV absorbance of PEDOT:PSS solution in the presence of HRP, GOx, and glucose decreased, which was attributed to the interaction between enzymes and PEDOT:PSS. As schematically shown in **Figure 1D** for the mechanism of catalytic reaction, GOx catalyses the transformation of glucose to D-glucono-δ-lactone, consuming molecular oxygen and releasing H_2O_2.[15] Electron transfer from GOx to water happens through the GOx co-enzyme flavin adenine dinucleotide (FAD)[5]. The produced hydrogen peroxide is then a substrate for the reaction catalysed by HRP, which results in the production of a water molecule.[16] This reaction is accompanied by the electron transfer, which

has previously been demonstrated to be able to induce structural changes in PEDOT, causing a variation in its conductivity.[10] The change in conductivity is used as a detection signal, as it is proportional to the amount of hydrogen peroxide consumed by HRP.
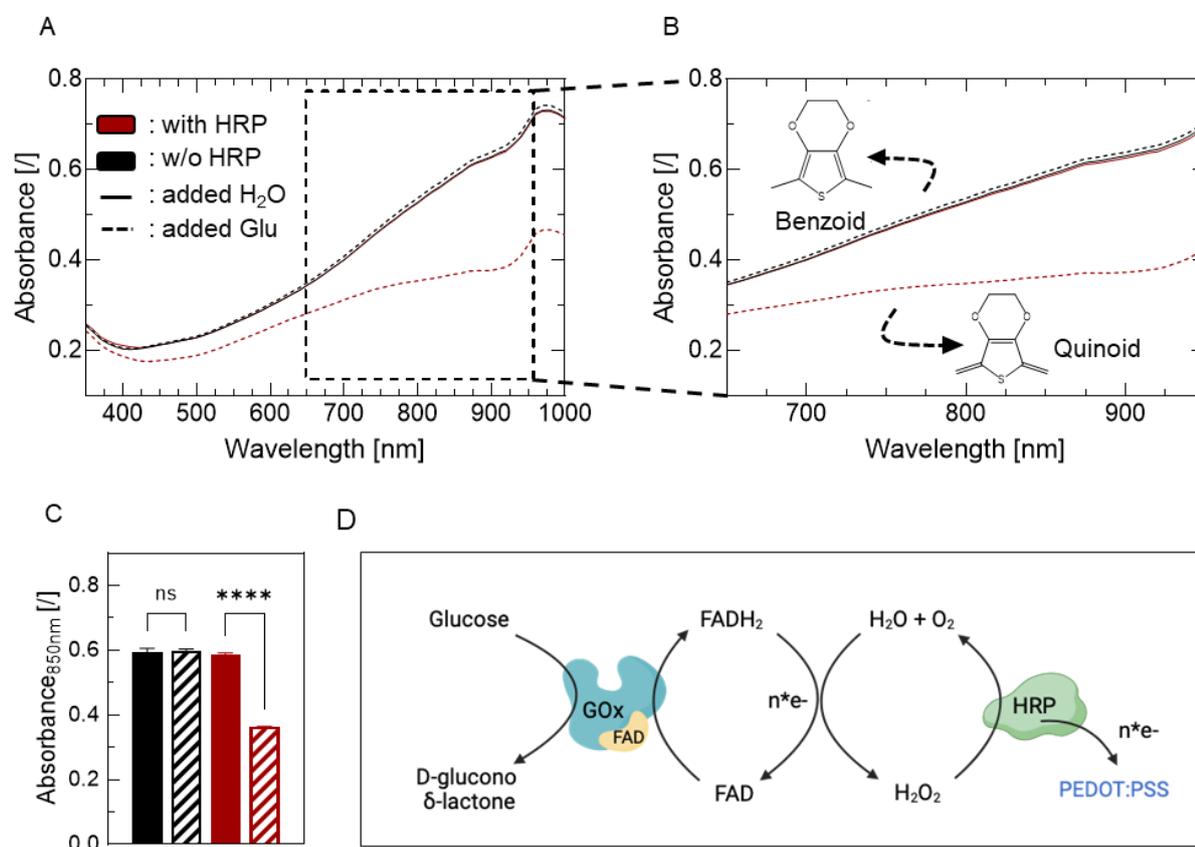

Figure 1. UV/Vis absorption spectra of neutral PEDOT:PSS/GOx (black lines) and PEDOT:PSS/HRP/GOx (red lines) after addition of water (solid lines) or glucose (Glu – dashed lines) in the range between A) 350 nm and 1000 nm and B) 650 nm and 950 nm. C) Value of absorbance at 850 nm for PEDOT:PSS/GOx (black) and PEDOT:PSS/HRP/GOx (red) after the addition of water (solid bars) or glucose (patterned bars). Note the significant variation ($p < 0.0001$) of absorbance when both enzymes are present. D) Proposed detection mechanism.

3.2. Glucose detection

To verify the validity of the proposed detection mechanism and assess its practicality, we fabricated a paper-based biosensor using ink-jet printing technology. This sensor consisted of PEDOT:PSS, HRP, and GOx as shown in **Figure 2A**. PEDOT:PSS was initially printed onto a paper substrate, then dried and decorated with both HRP and GOx (unless specified) via drop casting. Two carbon ink strips were also printed at the edge of each sensor sample, to enhance the contact with the potentiostat. For the optimal signal acquisition, we determined the amounts of HRP and GOx of 5 mg mL^{-1} and

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10 mg mL^{-1}, respectively (**Figure S2**). It is important to note that in the absence of the enzymes, we did not observe any conductivity variation at different glucose concentrations (**Figure S3**).

The designed sensor was sensitive to glucose as the electrical resistance was significantly changed across a concentration range spanning from 100 µM to 10 mM. As demonstrated in **Figure 2B**, a semilogarithmic linear trend was observed at both acidic and neutral pH levels, correlating resistance changes with varying glucose concentration within the range examined. However, as a result of increasing the resistance at lower pH the calibration curve was shifted up and the linear range was also changed at neutral pH from 100 µM (ΔR ≈ - 10%) to 10 mM (ΔR ≈ - 30%), while for acidic PEDOT:PSS was varied from 500 µM (ΔR ≈ - 5%) to 10 mM (ΔR ≈ - 15%). These differences may be attributed to the presence of stabilisers in the conductive polymer solutions, pH effect and potential variation in benzoid and quinoid ratios in PEDOT:PSS at these two pH levels.[17] Nevertheless, the PEDOT:PSS/HRP/GOx sensor produced under neutral pH exhibited a superior performance, detecting lower glucose concentrations with a more pronounced decrease in resistance (signal). The limit of detection (LOD), calculated using the formula $LOD = 3.3\,{}^{\sigma}/_{S}$ (where σ is the standard deviation and S is the slope of the calibration curve), is equal to 1.11 µM.
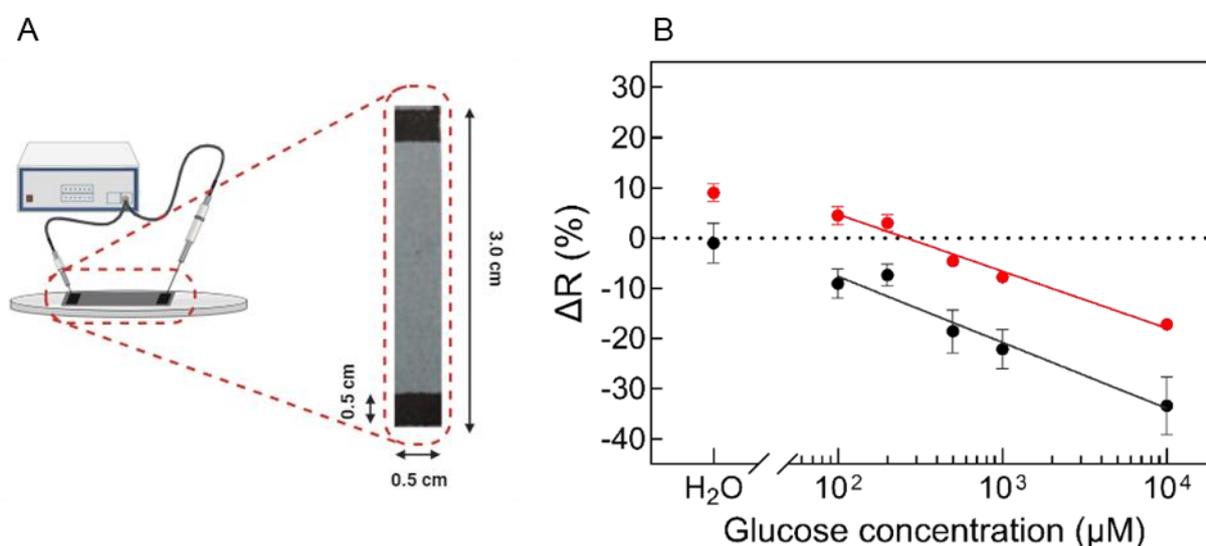
Figure 2. A) Schematic of sensor developed. B) The variation of PEDOT:PSS/ HRP (5 mg mL^{-1}) and GOx (10 mg mL^{-1}) resistance vs glucose concentration in acidic (red) and neutral (black) conditions. The interpolations line plotted are ΔR = -(11.345 ± 1.125) log[Glu] + 27.435 ± 3.185 (R^2 = 0.9566)

and ΔR = -(13.14 ± 3.06) log[Glu] + 18.63 ± 9.23 (R^2 = 0.8383) for acidic and neutral PEDOT:PSS, respectively. The errors in the interpolation equation are 95% confidence intervals.

3.3. Selectivity and storage

In the sensor design, selectivity plays a pivotal role. While enzymes inherently exhibit selectivity due to their active sites, it is essential to consider that certain molecules can interact with or deactivate enzymes, potentially affecting the sensor's response. Furthermore, certain molecules have been known to directly impact PEDOT:PSS conductivity, such as acids.[18,19] The sensitivity of the PEDOT:PSS/HRP/GOx sensor was tested against other common interfering molecules such as other sugars, organic acids, proteins and other metabolites like urea and cholesterol. Where relevant, the concentration of such molecules tested was the same as the concentration they are commonly found in saliva (see **Table S1**), to simulate the environment found in the biofluid. Interestingly, glucose concentration decreased the PEDOT:PSS/HRP/GOx sensor resistance, whereas all other compounds resulted in an increase in resistance (**Figure 3A**). Sugars (*i.e.*, trehalose, sucrose, and fructose) induce a relatively negligible increase in resistance when compared to other metabolites, such as urea and cholesterol, certain acids, like uric acid and lactic acid, and proteins such as cortisol and α-amylase. It is hypothesized that these molecules intercalate between PEDOT:PSS chains, thereby hindering electron transfer, hence reducing electrical conductivity. This hypothesis is supported by the observation that the most electrically neutral molecules, *namely* cholesterol and proteins, cause the highest increase in resistance. For acids, the change in resistance is likely due to pH variations, with resistance increasing inversely with the acidity of the molecules, ranging from uric acid to citric acid [20]. When all the interfering molecules are simultaneously introduced to the sensor, the resulting increase in resistance is similar to the average increase caused by each individual molecule. However, the addition of glucose results in a smaller proportional increase in resistance relative to the amount of glucose added (**Figure S4**). By establishing the total increase in resistance in the absence of glucose as the new baseline, the sensor's response to glucose addition mirrors the response observed in the absence of interferents, with 1 mM of glucose causing a decrease of 35.5% in resistance (**Figure 3B**).

These finding suggest that a dynamic calibration system or a passive-active system with dual sensors may be necessary for practical applications, given the variability in the level of these molecules among individual and across different biofluids.

Stability and shelf life are another important parameter that should be considered for designing enzymatic biosensors. Enzymes often have a limited shelf life, but various techniques like immobilization and encapsulation have been employed to improve their stability. Additionally, stabilizers such as sugars and polyols, particularly disaccharides like trehalose, sucrose, maltose, and lactose, are used to protect enzymes from denaturation during the drying or lyophilization processes. In this study, we observed that freeze drying prior to storage significantly increased the sensor stability. Specifically, under the storage conditions of low humidity and 4°C, the sensor's stability enhanced from three days to 10 days as depicted in **Figure 3C**. The effect of freeze-drying lies in its effect on the water activity within the PEDOT:PSS/HRP/GOx samples, which subsequently influences enzyme activity and, consequently, the sensor's response.[21] Therefore, as a practical consideration, after sensor preparation, it can be reliably used for up to ten days if stored in the refrigerator. For future production scalability, it can be considered maintaining the enzymes as a kit, allowing their addition to the PEDOT:PSS conductive polymer prior to incorporating analyte samples, considering that lyophilized enzymes may be stored at -20°C for up to 5 years.[22] Alternatively, addition of stabilizing agents, chemical immobilization of the enzymes, or storage at lower temperatures may be considered. For instance, certain studies have demonstrated to be able to stabilize GOx and maintain its activity for periods spanning between 2 and 8 weeks.[23]
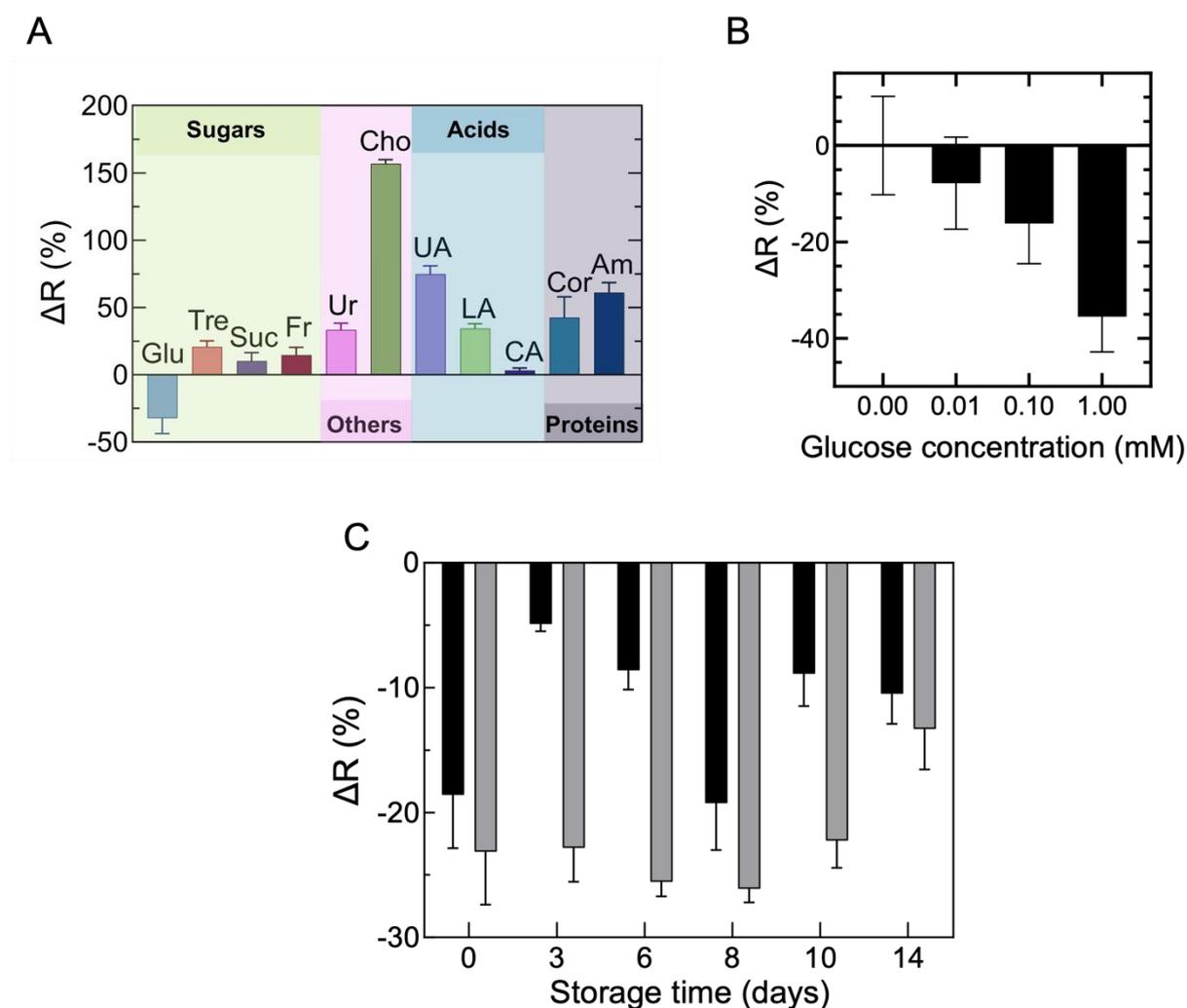
Figure 3. A) Response of the sensor to sugars (Glu: glucose, Tre: trehalose, Suc: sucrose, Fr: fructose), other metabolites (Ur: urea, Cho: cholesterol), organic acids (UA: uric acid, LA: lactic acid, CA: citric acid), and proteins (Cor: cortisol, Am: α-amylase). The concentration of all chemicals added is reported in **Table S1**. B) Response of the sensor to the mixture of interferents tested singularly in panel A (0.00 mM), and the mixture plus glucose at different concentrations. Note that the response without glucose was used as baseline (i.e., shifted to result in a null signal). C) Response of the PEDOT:PSS/HRP/GOx sensor, freeze-dried (grey) and not (black), to the addition of 500 µM of glucose after different days of storage.

3.4. *Glucose detection in artificial saliva*

The effect of artificial saliva (A.S.) on the response of the PEDOT:PSS/HRP/GOx sensor was examined. Exposing glucose in artificial saliva rather than water decreased the response of the sensor at all glucose concentrations of about 10%, resulting in an upward shift of the calibration curve. This is most likely due to the effect of various salts present in artificial saliva that comprised of sodium

chloride (NaCl), potassium monobasic (KH$_2$PO$_4$), potassium chloride (KCl), potassium thiocyanate (C$_2$H$_4$KN$_3$OS), and urea. As the salts in the PBS solution (*i.e.*, sodium chloride, potassium chloride, and sodium and potassium phosphates) did not cause a denaturation of the enzyme, such increase is possibly due to urea, which can cause an increase in resistance of the system (Figure 3A) and denaturation of GOx, or potassium thiocyanate, as some salts have been demonstrated to denature both HRP and GOx.[24] This effect, similarly to what done with the effect of interferents, was removed by readjusting the baseline to the signal obtained by the addition of A.S. without glucose. The resulting signal is similar to the one obtained in water, albeit ~5% smaller on average (**Figure 4A**). However, actual saliva is more complex and contains other compounds such as proteins and other metabolites that may result in biofouling and interference for measurement, as previously discussed.[25,26] For instance, saliva contains around 1 mg mL^{-1} of proteins, including amylase, lysozyme, and albumin, which may hinder detection.[27,28]

To verify the potential applicability of the sensor in human saliva, A.S. was spiked with uric acid (0.06 mM), lactic acid (1.8 mM), sucrose (1 mM), cortisol (0.74 mM), cholesterol (50 mg mL^{-1}), and α-amylase (0.38 mg mL^{-1}) before assessing the sensor response. Upon adjustment of the baseline, the effect of the interferents is evident, as the signal produced by the PEDOT:PSS/HRP/GOx sensor drastically decreases compared with the one produced in their absence (Figure 4A). It is unclear whether such an evident reduction is due to the salts, the interferents, or a combination of both. This reduction in signal is even more evident when comparing the calibration curves of the sensor exposed to different fluids, *i.e.,* water, water with interferents, A.S., and A.S. with interferents (**Figure 4B**). The addition of interferents had a negligible impact on the slope of the sensor when the fluid in which glucose was dissolved was water (Figure 4B left, slope decreased from 13.14 ± 3.06 to 11.73 ± 3.75). Similarly, the addition of salts (*i.e.*, the passage from water to A.S.) also did not change the slope significantly (solid lines in Figure 4B, slope went from 13.14 ± 3.06 to 15.02 ± 5.16). Conversely, the addition of interferents to A.S. drastically reduced the slope of the calibration curve (Figure 4B right, from 15.02 ± 5.16 to 3.55 ± 0.70). It is then probable there is a combined effect of salts with

other molecules. Nevertheless, the sensor was still able to differentiate between different glucose concentrations, albeit not as accurately as before, without requiring any pre-treatment of saliva. This would prove a notable advantage in POCT application, with most sensors for salivary glucose detection requiring centrifugation or filtration of saliva.[9,29,30]
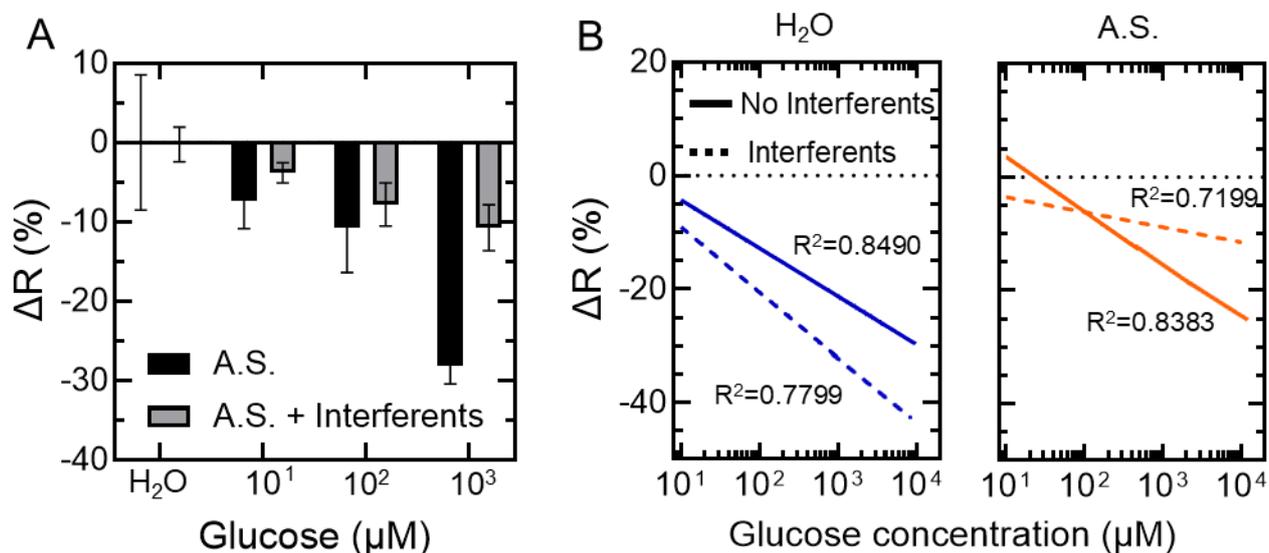
Figure 4. A) Resistance variation change in percentage of sensor exposed to artificial saliva (A.S.) and A.S. loaded with interferents. The resistance variation has been shifted based on the baseline (i.e., the resistance variation in the absence of glucose) B) Variation of the calibration curve of the PEDOT:PSS/HRP/GOx sensor due to the addition of interferents in water (left, blue curves) and A.S. (right, orange curves). Note how the slope does not significantly change upon addition of interferents in water, but drastically flattens in A.S.. The equations of the calibration curves depicted in Figure 4B are: $-(13.14 \pm 3.06)\log[\text{Glu}] + 18.63 \pm 9.23$ (solid blue line, H_2O without interferents); $-(11.73 \pm 3.75)\log[\text{Glu}] - 31.85 \pm 6.98$ (dashed blue line, H_2O with interferents); $-(15.02 \pm 5.16)\log[\text{Glu}] + 28.12 \pm 10.71$ (solid orange line, A.S. without interferents); $-(3.55 \pm 0.70)\log[\text{Glu}] - 10.96 \pm 1.43$ (dashed orange line, A.S. with interferents). The baseline of both calibration curves with interferents has been adjusted.

3.5. Comparison with other salivary glucose sensors and future perspectives

In development of amperometric sensor, chemiresistive sensors for glucose detection come with the advantages of offering simpler designs, not requiring particularly complex fabrication methods, and a lower cost [12]. This advantages often come at the cost of sensitivity and detection range, which are usually lower than the amperometric counterpart.[5]

The PEDOT:PSS/HRP/GOx sensor developed in this study demonstrated comparable performance with other chemiresistive sensors reported in the literature (**Table S2**), albeit being the only one for which selectivity and detection range are suitable for salivary glucose quantification, as all other sensor either lack in sensitivity or specificity, regardless of the use of nanostructures or complex design and manufacturing processes.

When compared to other salivary glucose sensors, which are all amperometric (**Table 1**), the PEDOT:PSS/HRP/GOx sensor present three main advantages. First, artificial saliva was tested without any need for pre-processing or dilution in this study. Avoiding the need of filtration, centrifugation, and other pretreatments simplifies its application, reducing cost and improving user-friendliness. Second, most of the amperometric sensor reported do not report the sample volume required for analysis, while only highlighting how an electrolyte solution is used to carry out the electrochemical analysis. Volumes of analysis in the millilitre range are not suitable for the quantification of salivary glucose, as humans produce only 0.1-0.2 ml/min of saliva on average,[31] drastically limiting its availability. As such, a volume sample of 40 µL, such as in the case of this study, is more appropriate for such applications. Finally, amperometric sensors require a battery, a three-electrode system, and often employ complicated designs and fabrication methods. Contrarily, chemiresistive sensors, as in the case of the PEDOT:PSS/HRP/GOx sensor, can function even with intermittent voltage or passive power systems, such as an NFC, and exhibit a simpler fabrication and design. Hence, the sensor herein developed demonstrate greater potential for a POCT single-use quantification of salivary glucose for applications such as cardiometabolic diseases screening.

Future endeavours include the test using human saliva samples, to validate the result obtained and evaluate the necessity for re-calibration of the sensor, and study on the potential reduction of the response time, which at the current stage is 60 minutes and precludes application such as diabetes monitoring for glucometer replacement. At the same time, the addition of a filter layer on top of the sensor to reduce the combined effect of salts and interferents will be investigated.

Table 1. Salivary glucose sensors.

Transduction	Receptor	Materials	LOD [µM]	Linear detection range [µM]	Storage	Selectivity	Simulated environment test	Electrolyte	Sample volume	Ref
Amperometric	Non-enzymatic	CuO-NA/Cu	0.1	1 - 6 × 10³	30 days	AA, Da, Ml, Su, Ga, Ma, Lc, Fr, Am	Human saliva (centrifuged and supernatant mixed with NaOH)	HCl or NaOH	N/A	[32]
		Cu-NPG/SPE	0.13	1 - 1.5 × 10³	N/A	AA, UA, NaCl, KCl, Ga, Fr, Su	Synthetic saliva	H₂SO₄ or synthetic saliva	N/A	[33]
		Au HC/Cu₃O₄ needles	20	20 - 100 2 × 10³ - 10 × 10³	N/A	AA, UA, Da, cortisol	Synthetic saliva	KOH	N/A	[34]
		CuO NR – SnOₓ/Nafion/GCE	3.08	3.08 – 6 × 10³	30 days	Da, LA, AA, NaCl	Human saliva (diluted in NaOH)	NaOH	N/A	[35]
		Ni foam – CoO NN	0.55	0.5 – 2.252 × 10³	21 days	NaCl, KCl, AA< Da, UA, I-glutamine, LA	Human saliva (spiked and diluted in NaOH solution)	NaOH	50 mL	[36]
		CoNi-N@GaN-3S	0.06	0.06 – 10 10 – 6 × 10³	30 days	Ga, NaCl, Urea, UA, Da, LA, AA, paracetamol, estradiol, estriol	Human saliva (filtered, centrifuged, treated with NaOH powder, and spiked)	NaOH	20 mL	[37]
Amperometric, impedimetric, and chemiresistive	Non-enzymatic, MIP	AAM-NNMBA/Au-SPE	2.8	2.8 - 280	3 months	Su, Lc	Human saliva (centrifuged)	Ferri/ferrocyanide	N/A	[30]
Amperometric	Enzymatic	GOx/GLU/AuNPs/GO(or W₂S)/PEDOT:PSS/ITO	2.33 1	3.8 – 373.33 0.74 – 440.67	N/A	AA, UA, Su	Human saliva (diluted in PBS and filtered)	PBS	N/A	[38]
		FTO-CNTs/PEI/GOx	70	70 – 700	14 days	AA, Da, UA	Artificial saliva	PBS or KCl	N/A	[39]
Chemiresistive	Bi-enzymatic	PEDOT:PSS/HRP/GOx	1.1	100 – 10× 10³	10 days	Trehalose, Su, Fr, Urea, Cholesterol, UA, LA, CA, Cortisol, Am, Albumin	Artificial saliva	N/A	40 µL	This work

AA: ascorbic acid; AAM: acrylamide; Am: amylase; CNT: carbon nanotube; Da: dopamine; Fr: fructose; FTO: fluorine-doped tin oxide; Ga: galactose; GCE: glassy carbon electrode; GLU: glutaraldehyde; HC: honeycomb; ITO: indium tin oxide; LA: lactic acid; Lc: lactose; Ma: mannose; Ml: maltose; MIP: molecularly imprinted polymers; NA: nanoarray; NNMBA: N, N'-methylene bis-acrylamide; NPG: nanoporous gold; NP: nanoparticle; NR: nano rod; PEI: polyethylenimine; SPE: screen printed electrode; Su: sucrose; UA: Uric Acid.

4. Conclusion

Herein, detection of glucose in the range between 100 µM and 10 mM was achieved combining conductive polymer PEDOT:PSS to a bienzymatic system consisting of HRP and GOx. The stability and selectivity of the sensor were also tested, with shelf life being as long as 10 days and PEDOT:PSS/HRP/GOx able to differentiate between many molecules commonly found in some biofluids, and especially saliva, including organic acids, other sugars, other metabolites, and proteins. Due to the suitable detection range and low LOD achieved by the sensor, future applications in the quantification of glucose in saliva for the screening of certain diseases is possible and will be further investigated. Regardless of the need of recalibration, stemmed by the combined effect of salts and proteins, commonly found in most biofluids, the sensor exhibited a sensing performance comparable or better than other chemiresistive glucose sensors in the literature. Additionally, the low-cost of fabrication as well as the simplicity of chemiresistive measurement setup make this sensor competitive against other types of sensors and traditional ways of glucose analysis.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Conflict of Interest

Authors declare no conflict of interest.

CRediT authorship contribution statement

Riccardo Zulli: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review and editing; **Jacopo Giaretta**: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review and editing; **Theja Prabhakar**: Investigation, Writing – review and editing; **Ronil J. Rath**: Conceptualization, Visualization, Writing – review and editing; **Suvan Shrestha**: Data curation, Formal analysis, Investigation, Writing – review and editing; **Sara Spilimbergo**: Supervision, Visualization, Writing – review and editing; **Syamak Farajikhah**: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – review and editing; **Fariba Dehghani**: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Validation, Visualization, Writing – review and editing.

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Table of content

Riccardo Zulli, Jacopo Giaretta, Theja Prabhakar, Ronil J. Rath, Suvan Shrestha, Sara Spilimbergo, Syamak Farajikhah[*], Fariba Dehghani[*]

A Paper-Based Bi-Enzymatic Sensor for Chemiresistive Glucose Detection

This paper presents the development of a paper-based glucose sensor utilizing poly(3,4-ethylene-dioxythiophene) polystyrene sulfonate (PEDOT:PSS) and a bi-enzymatic system with Horseradish Peroxidase (HRP) and Glucose Oxidase (GOx). The chemiresistive sensor is optimized for detecting glucose in biofluids such as saliva, requiring only 40 µL of sample volume and exhibiting a detection limit of 1.1 µM with a linear range of 10^2–10^4 µM.