Paper-based electroanalytical strip for user-friendly blood glutathione detection

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ABSTRACT
Paper-based devices are always more gaining a relevant position in the field of sensors. The continuous demand for affordable, simple, sustainable, and portable devices, is making paper as the ideal basis towards the realization of analytical tools for the easy self-testing. In this work, we demonstrate, for the first time, the development of a disposable paper-based printed electroanalytical strip for reliable, rapid, and high-throughput detection of glutathione in blood. The detection is based on the thiol-disulfide exchange reaction, which produces a detectable compound easily oxidizable at a Prussian Blue/carbon black nanocomposite involving a favorable low-interference overpotential. This nanocomposite is mixed within a carbon-based conductive ink and successively screen-printed onto a wax-patterned filter paper. The employment of paper provides a reagent-free device, as a consequence of the reagents pre-loading within the testing area. After the experimental conditions have been optimized, glutathione has been detected up to 10 mM, with a detection limit of 60 μM, and a sensitivity of (0.102 ± 0.005) μA/mM. This sensor showed satisfactory repeatability (relative standard deviation equal to 10%, for detection of glutathione 1 mM), especially by considering the hand-made manufacturing process. The “real-world” applicability of this strip has been evaluated by quantifying blood glutathione at physiological levels and by recovery studies achieving satisfactory values.

1. Introduction
Glutathione, a tripeptide formed by cysteine, glutamic acid, and glycine, is the most abundant antioxidant thiol capable to regulate intracellular redox homeostasis. Its reduced form is among the most exploited antioxidant shields in opposing free radicals and toxins [1,2]. Glutathione level within the mammalian cells is greatly significant: in fact, abnormal levels of blood glutathione can be correlated to several diseases including Alzheimer, Parkinson, diabetes, and various cancers [3–6]. Because its monitoring is of undisputed importance for clinical practice, the development of point-of-care device would be very useful to overcome some limitations often bound to time-consuming procedures such as the requirement of laboratory set-up, skilled personnel, and expensive instrumentations. In clinical chemistry, glutathione has been principally measured by using ultraperformance liquid chromatography (UPLC)-mass spectrometry [7], high performance liquid chromatography (HPLC)-electrospray tandem mass spectrometry [8,9], and gas chromatography–mass spectrometry (GC–MS) [10]. Although these techniques are characterized by satisfactory specificity and sensitivity in biological fluids, they are strongly laboratory-dependent. In addition, chromatographic methods usually require sample derivatization. Alternatively, some “lighter” approaches have been proposed for the detection of glutathione, including electrochemiluminescence [11], fluorescence [12], colorimetry [13], electroanalysis [14], and Raman scattering [15]. However, these methods are characterized by certain limitations: external sources (fluorescence), sensitivity (colorimetry), experimental set-up (electrochemiluminescence, Raman scattering).

Among the above-cited approaches, electroanalysis coupled to screen-printed electrodes offers obvious advantages for their miniaturized size and tiny amount of sample required. In fact, only few μL of blood are required to perform the analysis, such as in well-known glucose strips, when compared with mL of human blood specimens usually required for clinical practice. An additional advantage is related to the instruments involved, being the cost and the size of instrument well suitable for cost-effective and on-site analysis. Recently, the research activity in the electrochemical miniaturized devices has highlighted the outstanding features of paper in terms of cost-effectiveness,
customization, and easily disposability, boosting the use of paper in the development of smart electrochemical sensors [16–21]. For instance, the customization of devices can be easily accomplished by using different types of paper (e.g. office paper [22], carbon tape [23], cotton [24,25], filter paper [26]). In case of office-paper, a drop analysis is carried out; instead, in case of filter or chromatography paper, the solution goes through the cellulose network. For microfluidic systems, filter or chromatography paper is usually employed because the porosity of the paper is exploited for moving the solution without the required of any additional pump. In addition, the porous structure allows for loading all the reagents necessary for the specific assay before the measurement, delivering the so-called “reagent-free” devices. The porosity entails also for blocking gross impurities present in the real matrices, avoiding electrode fouling. In the field of paper-based electrochemical sensors, several analytical tools have been reported in literature for the detection of different analytes including chloride [27], nerve agents [28], heavy metals [29,30], bisphenol A [31], lactate [32], and ascorbic acid [33].

In the case of paper-based sensors, at our knowledge, the only one reported for glutathione detection has been developed by Henry group, and it is based on a colorimetric transduction. In detail, they designed an elegant approach for glutathione detection in serum based on the variation of the silver nanoparticle color, due to their aggregation in presence of glutathione, which turned the solution from orange to deep red [34]. However, the colorimetric detection is able to detect glutathione in serum but not in whole blood, being affected by color of/ and the turbidity of the sample. In addition, glutathione is mainly present in erythrocytes at mM level instead of μM level of serum [35], thus the detection of glutathione in whole blood is an important task. To address this issue, glutathione can be electrochemically detected by following its direct oxidation at electrode surface by applying a high overpotential, e.g. 0.8–1 V [36,37]. However, this direct detection allows for poor specificity because real matrices are plenty of species oxidizable at that working potential. Herein, we designed an electrochemical paper-based sensor for detection of glutathione in blood sample exploiting thiol-disulfide exchange reaction to detect glutathione at low applied potential.

Briefly, the process involves the attack by the thiol glutathione (GSH) to the disulfide cysteamine (RSSH), breaking the S−S bond and producing a mixed disulfide (GSSR), with the release of the electrochemically detectable thiol cysteamine (RSH). The cysteamine produced in ratio 1:1 with glutathione, is then detected at a low applied potential thanks to the electrocatalytic properties of Prussian Blue towards the oxidation of cysteamine, accordingly the following reactions [38] (Eqs. (1a)–(1c)):

\[
\text{GSH} + \text{cysteamine (RSSH)} \rightarrow \text{cysteamine (RSH)} + \text{GSSR} \quad (1a)
\]

\[
\text{PB} + \text{RSH} = \text{PW} + \text{RSSH} \quad (1b)
\]

\[
\text{PW} = \text{PB} + e^- \quad (1c)
\]

In detail, after the thiol-disulfide exchange reaction (Eq. (1a)), the cysteamine reacts with PB to give the cysteamine (oxidized form of cysteamine) and Prussian White (reduced form of Prussian Blue) (Eq. (1b)). The reduced form of Prussian Blue (Prussian White) is consequently oxidized at the electrode surface, giving an oxidative current which is proportional to the amount of glutathione present in the analyzed sample. This approach was conceived because Prussian Blue (the electrochemical mediator) is not only capable to measure cysteamine at a working potential much lower than 1 V with reduction of electroactive interferences, but also it overcomes the fouling problem which is a typical drawback of thiol oxidative−based reaction. In order to simplify both the manufacturing and the utilization of this paper-based device, various strategies have been adopted. Prussian Blue nanoparticles, synthesized onto carbon black, have been mixed together with the conductive ink and successively screen-printed onto waxed filter paper. To make the entire system reagent-free, buffered cysteamine has been loaded onto the testing area where the electrodes have been previously printed. Herein, the first electroanalytical paper-based device for blood glutathione detection has been designed and developed. This represents an entirely reagent-free tool for minimally invasive analysis in tiny amount of sample (5 μL). The word “reagent-free” is associated with the measurement at the level of non-specialized users: end-users are only asked to drop a tiny amount of sample onto the testing area where all the reagents have been previously loaded during the manufacture. This user-friendly, low-cost, and portable paper-based tool for blood glutathione self-test has been further improved by the optimization of the analytical parameters (pH, applied potential, cysteamine concentration) and of the blood hemolysis strategies.

2. Experimental

2.1. Apparatus and reagents

Potassium chloride, potassium ferricyanide, ferric chloride, boric acid, glacial acetic acid, ortho-phosphoric acid 85%, potassium dihydrogen phosphate, glutathione, cysteamine, and cysteamine were purchased from Sigma Aldrich. All the solutions were prepared in distilled water. Blood samples were provided by one of the authors, Valeria Manovella. Chronoanapometry measurements were carried out using a portable EmStat® Instrument (PalmSens, Netherlands) connected to a laptop. Blood hemolysis was conducted with ultrasonic bath (LBS2, Falc).

2.2. Fabrication of paper-based strip

To fabricate the paper-based sensor for glutathione detection, four steps (i, ii, iii, iv) were followed:

i) Testing area was designed with a drawing software (Adobe Illustrator) and printed onto filter paper (67 g/m², Cordenons, Italy) by using an office wax printer (ColorQube 8580, Xerox, USA).

ii) Waxed paper was cured in an oven at 100 °C for 2 min, to create specific hydrophobic areas.

iii) Electrodes were manually screen-printed by using a polymeric squeezee and masks. Ag/AgCl ink (Electrodag 6038 SS, Acheson, Italy) was used for the reference electrode, and graphite ink (Electrodag 421, Acheson, Italy) modified with 5% (w/w) Prussian Blue/carbon black powder for the working and counter electrode. 20 min in the oven at 60 °C were enough to cure the inks.

iv) 5 μL of the working solution containing cysteamine dissolved in Britton Robinson buffer were pipetted onto the hydrophilic testing area.

The entire design of the paper-based screen-printed electrode is reported in the supplementary information file (Fig. S1) and the presence of waxed area allows to confine the solution in the delimited electrochemical cell area, avoiding its diffusion towards electric contacts.

2.3. Glutathione detection

The glutathione detection was carried out in amperometric mode by simply dropping 5 μL of sample on sensor testing area, which is the volume to wet the electrochemical cell without any leakage of the sample. The quantification of glutathione is carried out in amperometric mode, by measuring cysteamine at low applied potential (0.3 V vs Ag/AgCl), because cysteamine is the by-product of thiol-disulfide exchange reaction between glutathione and cysteamine (Eqs. (1a)–(1c)).

As reported in Fig. 1, all the reagents needed for the detection of glutathione (i.e. cysteamine 0.05 M in Britton Robinson buffer solution

pH = 9) were pre-loaded in the testing area in order to realize a reagent
free device. After the drying step, the sample was added on the back of
testing area to overcome matrix effect during the glutathione detection
in blood, exploiting the filtering properties of paper. After the thiol-
disulfide exchange reaction, the resulting current (depending on the
glutathione level) was recorded by using a portable potentiostat inter-
faced to a laptop. In detail, the current values were sampled at 100 s to
make the fitting curves. For glutathione detection in real samples, the
blood collection has been carried out by a medical doctor from a col-
league, following Ethical guidelines provided by Occupational health
section, University of Oxford [39]. We supplied Donor Information
Sheet and Donor Consent form to the donor.

3. Results and discussions

3.1. Principle of the glutathione detection by thiol-disulfide exchange
reaction and its optimization

The electrochemical detection of thiols is often limited by the high
overpotential needed to make the reaction happen. Typically, an applied
potential close to 1 V is sufficient to oxidize a lot of compounds at the
electrode surface, decreasing the specificity of the method. To overcome
this problem, we chose another strategy: glutathione participates to the
thiol-disulfide reaction in presence of cystamine. Successively, the by-
produced cysteamine can be easily and selectively detected at the elec-
trode at low applied potential, taking into account the molecules present
in whole blood. In this work, we decided to take advantage of a nano-
composite produced by the combination of Prussian Blue and carbon
black. As already reported in our previous paper, carbon black acts as a
nanosized support for the synthesis of Prussian Blue nanoparticles
starting from its precursors, i.e. potassium ferricyanide and ferric
chloride [40]. Prussian Blue is widely known for its role of artificial
peroxidase towards the reduction of hydrogen peroxide at low interfering
potential [41,42]. Briefly, the by-produced cysteamine (following the
thiol-disulfide reaction between glutathione and cystamine) is oxidized
while Prussian Blue (PB) is converted in its reduced form, Prussian White
(PW). PW is then oxidized back to PB at the screen-printed electrode
(SPE), making it able to electrocatalyze the oxidation of additional cy-
steamine molecules (Eqs. (1a)–(1c)). Experimentally, as a consequence of
cysteamine oxidation, for cyclic voltammetry studies, the PB/PW redox
couple reveals an improvement of the current peak related to the oxi-
dation from PW to PB, coupled to a decrease of the peak related to the
opposite reaction, which converts PB to PW, as shown in Fig. 2A.

To develop a sensitive device, the experimental parameters need to
be optimized, starting from the working conditions of cysteamine de-
tection, which is the thiol-disulfide exchange reaction product. The first
parameter optimized was the potential applied during the chrono-
amperometry measurements. The applied potential should be the
lowest capable to provide the most effective oxidation of cysteamine. A
common issue related to the electrochemical methods is represented by
the possibility to oxidize/reduce other species present in the sample.
The use of PB or other electrochemical mediators such as cobalt pha-
thalocyanine, decreases this chance, especially when compared with
those methods involving carbon-based electrodes [28,43]. As shown in
Fig. 2B, the effect of the applied potential was investigated in the
anodic range comprised between 0.1 and 0.4 V. Although the mean
currents recorded by the application of 0.4 V appears slightly higher in
respect to the others, the application of 0.3 V is consistent with the best
accommodate in terms of sensitivity and repeatability. After the optimi-
ization of the applied potential, the effect of pH was also evaluated,
being the pH affects the oxidation of thiols. As expected, at higher pH a
more sensitive response has been observed because the sulfur moiety of
the cysteamine species becomes deprotonated, rendering the cystee-
amine more easily oxidizable, as in the case of homocysteine [37]. For
further studies, a pH of 9 was chosen as the optimal one, considering
both the current intensity and the repeatability (Fig. 2C). It should be
noted that, even if alkaline environment is required to obtain the op-
timal analytical performance, the end-user is not going to consider this
experimental parameter during measurements, i.e. adjusting pH. In-
deed, the use of porous paper impregnated with all the required

Fig. 1. Schematic representation of the detection mechanism of the paper-based device.
reagents for a certain assay, furnishes a great advantage which relies on the addition of only few μL of the sample, delivering “reagent free” analytical tool. After the pH, last parameter optimized was the cystamine concentration adsorbed on the testing area of the paper-based strip. In presence of a fixed concentration of glutathione, i.e. 5 mM, the level of cystamine was evaluated in the 0.01–1 M range. As reported in Fig. 2D, a concentration of 0.05 M cystamine was effective with the lowest concentration yielding the best compromise in terms of sensitivity and repeatability, thus this value has been selected for the further studies. As shown, an ulterior increase of cystamine did not produce any significant improvement of the signal.

3.2. Glutathione detection in standard solutions

After optimization of applied potential, pH, and cystamine concentration, the paper-based strip was challenged in presence of increasing concentrations of glutathione. To perform the chronamperometric measurements, the testing area was wetted with 5 μL of glutathione solutions prepared in distilled water. Glutathione was detected up to 10 mM as reported in Fig. 3.

The recorded signals increased linearly with the increase of glutathione concentration (0-0.25-0.5-1-2-5-10 mM). With the optimized parameters, the correlation between the anodic current and the concentration of glutathione was described by the following equation: $y = (0.04 \pm 0.02) + (0.102 \pm 0.005)x$ ($R^2 = 0.956$) where $y$ indicates the current in μA and $x$ states the concentration of glutathione in mM. The linear range ($R^2 = 0.956$) is observed in the range up to 10 mM, and the detection limit (LOD), calculated as the signal-to-noise ratio equal to ($S/N = 3$), resulted equal to 60 μM. The relative standard deviation (RSD) calculated for three measurements of 1 mM glutathione was found equal to 10%. This is a strict consequence of the robustness coupled to the strips manufacturing process.

3.3. Storage stability

In order to evaluate the applicability of the developed sensor, a stability study was carried out testing the sensor towards glutathione within 1 month. In detail, sensors were pre-loaded with all the reagents needed for glutathione detection and stored at room condition. The pre-
loaded sensors were tested using a solution of glutathione 3 mM at zero time and after 7, 21, and 30 days. The values obtained are reported in Fig. 4, which are the results of triplicate measurements for each time, demonstrating the valuable storage stability of the sensor at room temperature for at least 1 month.

3.4. Evaluation of the interference

Prior to work with matrix i.e. blood, the detection mechanism should be evaluated in presence of common species that can be founded in this complex media. For instance, in blood, the presence of easily oxidizable compounds such as ascorbic acid, uric acid, and acetaminophen, could be consistent with false positive signals, because these species can be oxidized at the electrode surface in addition to the glutathione. The sensor strip was tested in presence of these interfering species as shown in Fig. 5.

As shown in Fig. 5, the presence of these interfering species does not significantly affect glutathione detection. The concentrations considered for this study have been selected by taking into account their physiological levels in blood at μM level [44–46]. Beside these interfering species, it should be noted that the thiol-disulfide reaction could be sensitive to the presence of other thiols, i.e. cysteine: the involved reaction is not specific for glutathione, but it interests all the thiols. However, as widely reported in literature, the erythrocyte concentration of other thiols is less of around 1% in respect to the glutathione amount [14,35,47,48], avoiding the overestimation of glutathione content in erythrocytes.

It means, accordingly with the analytical performance of the present method (LOD of 60 μM), that the non-glutathione thiols are not capable to affect the signal that arises from glutathione in the mM range.

3.5. Detection of blood glutathione

The ultimate paper-based device was then applied towards the detection of glutathione in blood samples. The challenge in developing a portable test for blood glutathione detection is because this metabolite is almost entirely stored in the erythrocytes. Prior to begin with the real matrix measurements, we evaluated two different easy strategies for treating blood: i) the dilution with distilled water (ratio 1:2 v/v) and ii) a sonication (59 kHz for 10 min) followed by the dilution with distilled water (ratio 1:2 v/v). These treatments have been selected being the dilution and/or sonication usually applied for lysis of erythrocytes [49–51]. Using these treatments we found values of current equal to 110 nA (RSD% = 30) and 145 nA (RSD% = 15), respectively. These results demonstrated that the sonication step improves the repeatability and the release of glutathione by erythrocytes, thus, the treatment that comprises dilution and sonication has been selected for the further studies.

Subsequently, blood was spiked with different amounts of glutathione giving the final concentration in the 1–3 mM range. As reported in Fig. 6, the ultimate paper-based device was used to perform the measurements.

Three levels of glutathione added in blood were analyzed and a good correlation was obtained, demonstrating the suitability of the paper-based strip to detect glutathione in a complex matrix like the blood is. The correlation is described by the following equation, $y = (0.19 \pm 0.02) + (0.119 \pm 0.009)x$, ($R^2 = 0.947$), and a good sensitivity of 0.119 μA/mM is obtained. The repeatability associated to the blood glutathione measurement has been calculated with a RSD equal to 10% for glutathione 1 mM. We would like to stress an important advantage related to the use of paper as the manufacturing substrate for
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**Table 1:** Electrochemical sensors for glutathione detection.

**References**


**4. Conclusions**

Herein, we reported the first example of a paper-based electrochemical strip for glutathione detection. The optimized sensing platform is characterized by a detection limit of 60 μM and a linearity up to 10 mM, using chronoamperometry as technique and +0.3 V vs Ag/AgCl as applied potential. The low applied potential and the overcoming of fouling problem for cysteamine detection (the by-product of the reaction between glutathione and cysteamine) is due to use of ink-nanomodifier constituted of nanoparticles of Prussian Blue grown onto carbon black, demonstrating the suitability of this paper-based sensor for thiol detection (i.e. cysteamine/glutathione). The accuracy of the platform for glutathione detection was demonstrated by analyzing spiked-blood samples. The results achieved demonstrated the effectiveness of this paper-based sensor for affordable, easy-to-operate, and de-centralized detection of glutathione in blood, with the advantage to be a fully agent-free analytical tool. This work represents another evidence of the increasing role of paper in the field of electroanalytical devices and its implementation: step-by-step, coupled to easy customization techniques (screen-printing, wax printing, nanomaterial synthesis) the paper and ink device offer the opportunity for the implementation of paper in the field of sensor is knowledgeably, especially for developing countries where cost-effective and easy to use analytical devices are highly required.

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**Appendix A. Supplementary data**

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.snb.2019.02.082.
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