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A simple assay for glutathione in whole blood

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A method for detecting glutathione selectively in whole blood deposited on filter paper is described. GSH is fractionated from proteins, hemoglobin and other potentially interfering components and determined using a resorufin-acrylate fluorescent probe. The relative standard deviation is lower than 5% (n = 5). Recoveries of GSH from whole blood are between 94% and 108.6%.

Glutathione (GSH) is the major intracellular thiol. It plays a significant role in regulating the redox status of cells[1, 2] and in gene expression, cell proliferation and apoptosis.[3, 4] Cellular GSH concentrations range from 1–10 mM whereas plasma levels range from 1–6 μM.[4-6] Diminished levels of GSH are characteristic of chronic diseases, collectively the leading cause of mortality in the world.[7] They include heart disease, stroke, cancer, chronic respiratory diseases and diabetes. In 2009, Atkuri and co-workers reported GSH deficiency as a hallmark of mitochondrial disorders.[8] Mitochondrial disorders can cause organ failure, seizures, stroke-like episodes and premature death. Depleted intracellular GSH levels have also been reported in various neurodegenerative disorders such as Alzheimer's and Parkinson's disease.[9, 10] Elevated GSH levels generally increase antioxidant capacity and resistance to oxidative stress, and are observed in many types of cancer cells.[2] Higher levels of GSH have been reported in a number of different human cancer tissues including, bone marrow, breast, colon and lung.[11-13] While GSH is important in the detoxification of carcinogens, its elevated state in cancerous cells and solid tumors makes them more resistant to chemo- and radiotherapy.[14]

Current clinical laboratory methods for GSH determination include chromatographic separations followed by electrochemical detection, derivatization for spectrophotometric detection, or mass spectrometry.[15] Most fluorescent agents that are used in the derivatization step are not specific for GSH and react with other structurally similar, competing thiols such as cysteine (Cys) and homocysteine (Hcy). Enzymatic and immunoassays are also used to determine GSH but have the disadvantage of using fragile biological materials, are labor intensive and require skilled handling and specialized storage.[16, 17] Various useful probes for GSH detection have been recently reported. However, many of them are not selective for GSH in the presence of related analytes.[18, 19] There are relatively few indicators that achieve good selectivity for GSH over other biological thiols.[20] Examples include a monochlorinated BODIPY-based ratiometric fluorescent sensor developed by Niu and co-workers,[21] a *bis*-spiropyran ligand developed for cellular imaging,[22] a quantum-dot-based off-on fluorescent probe[23] and cyanine-based fluorescent probes reported by Yin *et al*.[24] Our group reported a highly selective resorufin acrylate-based probe **1** (Fig. 1) for the determination of GSH in deproteinized human blood plasma.[25, 26]

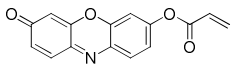

Fig. 1 Structure of the resorufin acrylate-based probe **1**.

While promising, the utility of the recently reported probes as part of a minimally-invasive blood spot technique has not yet been demonstrated. A small volume of blood dries quickly on paper as cells rupture, emptying their GSH stores. Whole blood GSH measurements generally minimize variability caused by multiple sample preparation steps.[27] Moreover, in many diseases, GSH plasma and whole blood levels are not correlated.[27-30] Therefore, there is a need to develop simple methods that can detect and quantify GSH in whole blood.

About three decades ago, extreme sample dilution (*e.g.* 5000-fold) was used to minimize hemoglobin (Hb) interference in the determination of GSH in dried blood spots (DBS) on filter paper.[31] Hb content would be reduced to less than 30 μg/mL reducing absorbance near 415 nm to about 0.25 in a standard 1 cm cuvette. Apart from dilution, Hb can be removed using a commercial product, HemogloBind™, which can isolate and remove up to 90% of blood Hb.[32] However, this product is expensive, still requires some dilution

(500-fold) and even with 10% Hb remaining after HemogloBind™ processing, there is still significant spectral overlap from Hb.

A relatively simple method for the detection and quantitation of GSH in whole blood deposited and dried on filter paper without interference from Hb is described herein. It involves extraction, reduction (with immobilized tris(2-carboxyethyl)phosphine, TCEP), deproteinization and concomitant fractionation of GSH using size exclusion chromatography (Fig. 2). In the process, Hb is completely removed and GSH can be detected using **1**.

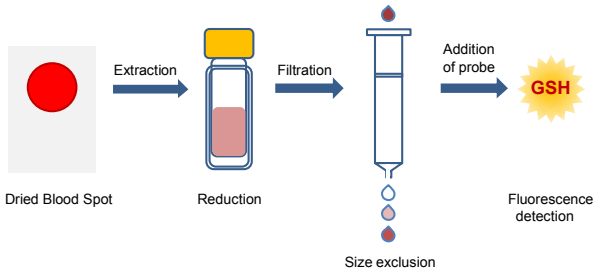

Fig. 2 Procedure for the detection and quantification of GSH on DBS using probe **1**. Blood (30 µL) is spotted on filter paper and dried for 24 h. The dried blood is extracted into buffer (0.6 mL, 50 mM phosphate pH 7.4) and treated with TCEP gel (1:1 v/v) at rt for 1 h, with gentle shaking. Separation of the reduced plasma from the gel is achieved by filtration using a 0.45 µm PVDF filter vial. The reduced sample is passed through a PD MiniTrap™ G-25 Sephadex™ column and six fractions of 0.3 mL are collected. Acrylate probe **1** (2.5 µM) is added to the fractionated solutions in 2.0 mM CTAB media at pH 7.4 (phosphate buffer, 50 mM). Spectra (UV-Vis and fuorescence) were collected immediately upon addition of the probe.

Upon passing the extracted GSH from the DBS through a PD MiniTrap™ G-25 Sephadex™ column, all proteins eluted in the first three fractions (see supporting information and Fig. 2). This was confirmed by examining the UV-Vis absorption spectra of the fractions (Fig. 3). The peaks at 400, and 500-600 nm, which are related to Hb, are present in fractions F1-F3 but absent in fractions F4-F6.

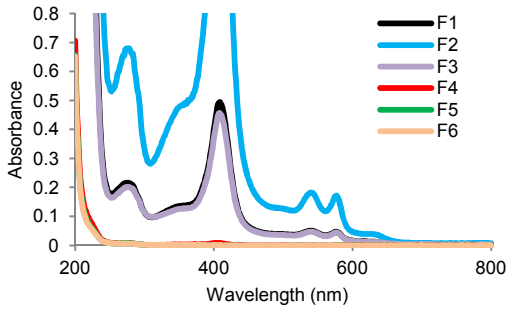

Fig. 3 Absorption spectra of DBS fractions F1-F6 in 50 mM phosphate buffer at pH 7.4. Fractions were collected and analyzed immediately.

Fractions F4-F6 were tested for the presence of GSH using **1** in the presence of CTAB (cetyltrimethylammonium bromide). Upon addition of **1** (2.5 µM) to the fractions in 2.0 mM CTAB media at pH 7.4 (50 mM phosphate buffer), significant fluorescence responses were observed in fractions F4 and F5 indicating the presence of native GSH in blood (Fig. 4). The high responses in fractions F1-F3 are a result of the reaction of the probe interaction with proteins. Fraction F6 behaved in the same manner as the blank. To ascertain that fractions F4 and F5 indeed contained GSH, we spotted and processed a solution containing only GSH exactly as described above, and collected all six fractions. Upon addition of the probe and CTAB, only fractions F4 and F5 developed a pink color and a significant fluorescence response. The spectra from fractions F1-F3, where proteins elute, were the same as the blank (Fig. S1, ESI†).

To investigate the effectiveness of this gel filtration method in deproteinizing dried blood, another control experiment was performed using a standard solution of only human serum albumin (HSA) at its whole blood concentrations (60 mg/mL). As expected, no spectral responses were observed in fractions F4-F6, confirming the absence of HSA in these fractions (Fig. S2, ESI†). Moreover, to rule out interference from Hb in GSH-containing fractions (F4 and F5), control experiments were conducted using commercial Hb (20 g/dL).

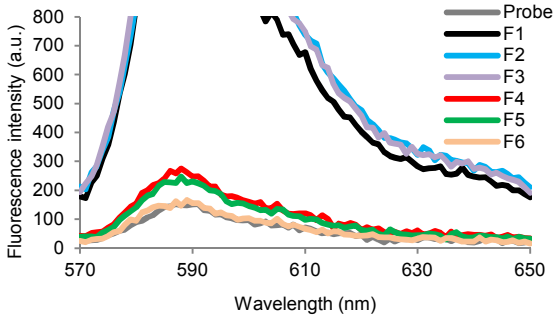

Fig. 4 Fluorescence emission spectra (λex = 565 nm) of **1** (2.5 µM) in solutions of DBS fractions (F1-F6) and 2.0 mM CTAB media buffered at pH 7.4 (phosphate buffer, 50 mM). Spectra were taken immediately upon addition of the probe.

As shown in Fig. 5, Hb eluted in the protein-containing fractions (F1-F3). No absorption or fluorescence responses due to Hb were observed in the remaining fractions (Fig. S3, ESI†). Taken together, the control experiments effectively confirm that the fluorescence responses observed in fractions F4 and F5 are due to native GSH and not residual proteins.

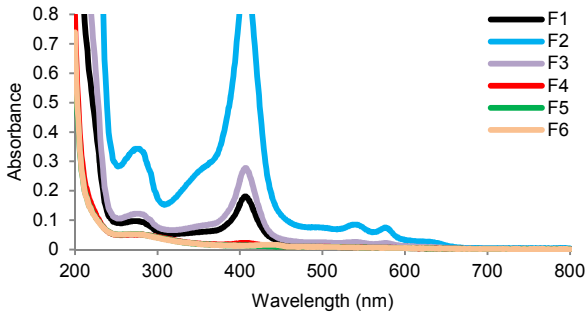

Fig. 5 Absorption spectra of Hb fractions in phosphate buffer at pH 7.4. Fractions were collected and analyzed immediately.

The method sensitivity was optimized by increasing the blood spot drying time from 3 to 24 h (Fig. 6). After processing the 24 h sample, the GSH signal was increased significantly relative to the 3 h sample upon addition of the probe and CTAB. This was attributed to more complete GSH release from erythrocytes.

Standard addition recoveries of spiked GSH were between 94.0% and 108.6%, with suitable precision (Table 1). These results demonstrate the potential applicability of the method in the quantitative detection of GSH in human blood.

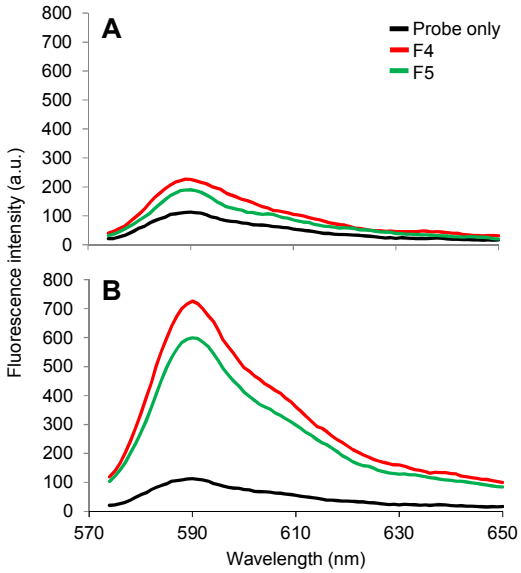

Fig. 6 Fluorescence emission spectra (λex = 565 nm) of **1** (2.5 μM) in solutions of DBS fractions (F4 and F5) from blood dried at different times and 2.0 mM CTAB at pH 7.4 (phosphate buffer, 50 mM). Spectra were taken immediately upon addition of the probe. (A) blood dried for 3 h (B) blood dried for 24 h.

Table 1. Determination of GSH in dried blood samples (n = 5).

GSH added (mM)	GSH measured (mM)	Recovery (%)	RSD (%)
0.0	1.35	-	3.12
0.5	1.90	108.6	4.90
1.0	2.32	96.4	3.24
2.0	3.23	94.0	4.10

The fluorescence response of **1**-CTAB towards fractionated GSH increased linearly upon the incremental addition of GSH to the blood samples prior to spotting over a physiological relevant range (0-2 mM) (Fig. 7).

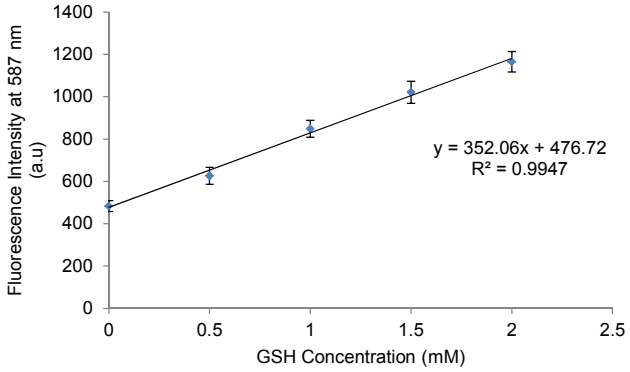

Fig. 7 Spectral response of probe **1** towards DBS spiked with increasing concentrations of GSH. All solutions contained **1** (2.5 μM), 2.0 mM CTAB media and 50 mM phosphate buffer (pH 7.4). Data represents the average of five independent experiments done on different days using fresh DBS samples.

The concentration of GSH in a DBS of commercial pig blood was determined by standard addition and was found to be 1.35 ± 0.16 mM, a value that falls within the range of reported intracellular GSH levels (1-10 mM).[4, 5]

In conclusion, a new procedure whereby GSH can be efficiently fractionated from blood components has been successfully applied to DBS processing. In addition to other proteins, this method facilitates the removal of Hb, allowing a wider selection of optical probes to determine GSH in biological media. The deproteinization is carried out via filtration, a more benign method as compared to the use of acids and organic solvents.[33, 34] The procedure additionally does not require any centrifugation steps. Due to the simplicity and user-friendliness of this technique it has high potential utility for minimally-invasive GSH monitoring, and allows for analysis of samples mailed to centralized labs.

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Notes and references

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†Electronic Supplementary Information (ESI) available: Detailed experimental procedures and additional spectral data. See DOI: 10.1039/c000000x/

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