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Integration of a precolumn fluorogenic reaction, separation, and detection of reduced glutathione

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Abstract

Reduced glutathione (GSH) has been determined by fluorescence detection after derivatization together with a variety of separations. The reactions between GSH and fluorescent reagents usually carry out during the sample pretreatment and require minutes to hours for complete reactions. For continuous monitoring of GSH, it would be very convenient to have an integrated microdevice that could perform online precolumn derivatization, separation, and detection. Heretofore, thiol-specific fluorogenic reagents require fairly long reaction times, preventing effective online precolumn derivatization. We demonstrate here that the fluorogenic, thiol-specific reagent, ThioGlo-1, reacts rapidly enough for efficient precolumn derivatization. The second order rate constant for the reaction of GSH and reagent (pH 7.5, room temperature) is $2.1 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$. The microchip integrates this precolumn derivatization, continuous flow gated sampling, separation and detection on a single device. We have validated this device for monitoring GSH concentration continuously by studying the kinetics of glutathione reductase (EC 1.8.1.7), an enzyme that catalyzes the reduction of oxidized glutathione (GSSG) to GSH in the presence of β -NADPH (β -Nicotinamide adenine dinucleotide phosphate, reduced form) as a reducing cofactor. During the experiment, GSH being generated in the enzymatic reaction was labeled with ThioGlo-1 as it passed through a mixing channel on the microfluidic chip. Derivatization reaction products were introduced into the analysis channel every 10 s using flow gated injections of 0.1 s. Baseline separation of the internal standard, ThioGlo-1, and the fluorescently labeled GSH was successfully achieved within 4.5 s in a 9 mm separation channel. Relative standard deviations of the peak area, peak height, and full width at half maximum (FWHM) for the internal standard were 2.5%, 2.0%, 1.0%, respectively with migration time reproducibility for the internal standard of less than 0.1% RSD in any experiment. The GSH concentration and mass detection limit were 4.2 nM and $\sim 10^{-18}$ mol, respectively. The Michaelis constants (K_m) for GSSG and β -NADPH were found to be $40 \pm 11 \text{ }\mu\text{M}$ and $4.4 \pm 0.6 \text{ }\mu\text{M}$, respectively, comparable with those obtained from UV/Vis spectrophotometric measurements. These results show that this system is capable of integrating derivatization, injection, separation and detection for continuous GSH determinations.

The ubiquitous tripeptide, reduced glutathione (GSH) acts as an anti-oxidant in cells.¹ It is particularly important for its ability to react with reactive oxygen species (ROS) directly or indirectly via enzymatically catalyzed reactions making it a principal component of an organism's antioxidant response.² Compared with other organs, brain is especially vulnerable to ROS because it is rich in lipids with unsaturated fatty acids.^{3, 4} Typically, the product of the reactions of GSH with ROS is oxidized glutathione, GSSG, which is reduced to GSH by the NADPH-dependent enzyme glutathione reductase. Alterations in this energy (NADPH)-redox (GSH/GSSG) "axis" occur in aging, and are thought to be responsible for the progress

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of neurodegenerative diseases.^{5, 6} Astrocytes play a role in assisting neurons by augmenting their GSH concentration. Efflux rates of GSH from astrocytes through hemichannels can be gated by changes in the concentration of extracellular Ca^{2+} ,⁷ and hypoxia-ischemia.⁸ Thus, there is an interest in continuous monitoring of GSH, and related thiols in the extracellular space of tissues, including brain with good temporal resolution.^{2, 7, 9} Both electrochemical^{10, 11} and fluorescence detection^{12, 13} of GSH and other thiols has been used with high performance liquid chromatography and capillary electrophoresis. Determination of GSH and related thiols on microfluidic chips has been performed using UV,¹⁴ and electrochemical detection.¹⁵ Laser induced fluorescence (LIF) detection^{16–18} on microfluidic chips used offline derivatization of GSH and other thiols. Despite the need for the derivatization, LIF is widely applied for its robust nature and consistent calibration compared to electrochemical detection. Also, labeling of the thiol group reduces its reactivity during the analysis, protecting it from degradation.¹⁹ Qin²⁰ and Ling²¹ *et al.* used 2,3-naphthalene dicarboxaldehyde (NDA) for on-column derivatization of GSH by simply including the fluorescent tag in the electrophoresis buffer. However, as the derivatization process was carried out simultaneously with separation, this approach is not optimal. The goal of this work is to improve the analysis platform for thiols, including the speed of the separation, the detection limit of the determination, using a derivatizing reagent that is thiol-specific, and doing continuous analysis.

The published reaction times for fluorogenic labeling of thiols are fairly long, which commonly range from several minutes to an hour.^{22–26} Some of them even require heating for a fast and complete derivatization.^{23, 26} We and others have used a commercial fluorescent tag, ThioGlo-1²⁷ (N-(2-carbomethoxy-9-methoxy-3-oxo-3H-naphtho [2,1-b] pyran-10-yl) maleimide) for labeling thiols in intact cells,^{28–30} cell homogenates or supernants.^{29, 31} Others have shown the potential of ThioGlo reagents for labeling thiols before HPLC analysis.³² These derivatizations were performed offline with a 20-minute reaction time. In this work, we determined the reaction rate at pH 7.5 for the derivatization of GSH by ThioGlo-1 and demonstrated the feasibility of using ThioGlo-1 for online derivatization of GSH.

Glutathione reductase is a flavoprotein that catalyzes the β -NADPH-dependent reduction of GSSG to GSH and maintains a suitable intracellular thiol/disulfide redox potential for an organism.^{33, 34} Maintaining an adequate level of reduced cellular GSH over GSSG is especially important for cells to fight against oxidative stresses and xenobiotics.^{35, 36} The kinetics and mechanism of the glutathione reductase-catalyzed reactions have been studied.^{33, 34, 37, 38} The most widely used tool to analyze the kinetics and mechanism of glutathione reductase is UV/Vis absorbance as described by Racker,³⁹ which relies on the decrease in the absorbance at 340 nm of NADPH. Transferring the enzyme reaction from the traditional cuvette or well plate to microchannels with LIF detection not only reduces the consumption of samples and reagents, but also has much higher sensitivity compared with that of UV/Vis absorbance detection. Meanwhile, interferences caused by other chemicals that coexist with the target compound, such as enzyme, by product, unreacted substrates can be eliminated from the detection by separation, which ensures more reliable results.

In this work we used the reaction catalyzed by glutathione reductase as a model system to validate the online derivatization, multiple injection, separation and detection of GSH with good temporal resolution on an integrated microfluidic platform. GSH concentration in the enzyme reaction mixture was measured using an online precolumn derivatization and a 4.5 s electrophoretic separation. In any given experiment, the reproducibility of the migration times of the internal standard was excellent, less than 0.1% relative standard deviation. The kinetic constants obtained for glutathione reductase are comparable with those obtained through traditional methods.

EXPERIMENTAL SECTION

Chemicals and Reagents

ThioGlo-1 was purchased from Calbiochem (San Diego, CA). Proflavine hemisulfate salt hydrate (proflavine), sodium hydroxide, tris(hydroxymethyl) aminomethane, hydrochloric acid, glutathione reductase from baker's yeast (suspended in 3.6 M ammonium sulfate, 168 units/mg protein, containing 0.1 mM dithiothreitol, pH 7.0), dimethyl sulfoxide (DMSO), GSH, GSSG disodium salt, β -nicotinamide adenine dinucleotide phosphate, tetrasodium salt hydrate, and 1,6-diaminohexane were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO) and used without purification.

Stock solutions of glutathione reductase, GSSG and β -NADPH were prepared using 20 mM Tris-HCl buffer (pH 7.5). The concentrations of these solutions were 3.33 units/mL, 13.2 mM, and 2.41 mM, respectively. The stock solutions of proflavine and ThioGlo-1 were prepared using Milli-Q water (Millipore Co., Billerica, MA) and DMSO with the final concentrations of 4.74 mM and 2.5 mM, respectively. Stock solutions were aliquoted into small vials and stored at -20°C and diluted serially to the desired concentrations on the day of experiment. All working solutions were kept on ice during the experiment when not in use. The running buffer for microfluidic chip electrophoresis was 20 mM Tris-HCl buffer (pH 7.5) with 0.1 mM 1, 6-diaminohexane, unless otherwise specified. All buffers were prepared using Milli-Q water, filtered through a nylon 0.22 μm filter and degassed by ultrasonication prior to use.

Design and Fabrication of the Microfluidic Chip

The microfluidic chip was fabricated using traditional lithography techniques. Briefly, borofloat glass (1.1 mm) coated with chromium and AZ 1500 photoresist (Telic, Valencia, CA) was exposed to UV light through a negative film mask (Pixels, Charlottesville, VA) containing the design of the channels to be etched. Following development and removal of the underlying chrome, the glass was etched in HF solution containing nitric acid ($\text{HF}/\text{HNO}_3/\text{H}_2\text{O}$:20/14/66) to a depth of 20 μm . The remaining photoresist and chromium was then removed and the glass cleaned and dried. Uncoated borofloat cover glass was drilled using 1.1 mm diameter diamond tipped drill bits (Kingsley North Inc., Norway, MI) to provide access holes at the end of each channel. Cover plates were cleaned and aligned with the etched glass plate and the two pressed together to form an initial bond. The device was permanently bonded by thermal annealing at 675°C for 3.5 hours. The layout of the microfluidic chip and the voltage scheme are shown in Figure 1. The channels were determined to be $\sim 30\text{ }\mu\text{m}$ wide at the bottom and $\sim 70\text{ }\mu\text{m}$ wide at the top, with a depth of $\sim 20\text{ }\mu\text{m}$. The total length of the precolumn reaction channel was $\sim 46.3\text{ mm}$. The detection point (DP) was located 9 mm from the injection cross. Platinum electrodes were placed into all reservoirs to serve as electrical contacts to the high voltage power supply. During each run, the electrodes in reagent reservoir and sample reservoir were kept at ground, while the electrode in waste reservoir was connected to a negative high voltage power supply. Sample injection was by a gated approach, which has advantages in shortening the analysis cycle, enhancing the temporal resolution and reducing contamination between reservoirs.^{40, 41} The electrode in buffer reservoir could be switched between “ground” and “float” to achieve the flow gated sampling. In the “sampling” stage, the electrode in the buffer reservoir was set to “float” for a very short period allowing a small segment of the reaction mixture to be directed into the separation channel. In the “separation” step, this electrode was switched back to ground, resulting in electrophoretic separation of the components in the injected aliquot. These steps were computer controlled and repeated automatically to achieve continuous sampling and analysis.

Instrumentation

The home-made confocal LIF detector used in this system consisted of a DPSS cw laser (CrystaLaser, Reno, NV) operating at 403.1 nm. The dichroic and the emission filters were a 435DRLP and a 3RD470–520 band pass filter, respectively (Omega Optical, Inc, Brattleboro, VT). The signal was detected by a head-on metal package PMT module H5784-01 (Hamamatsu Photonics, Bridgewater, NJ). A program written locally in LabView 8.2 (National Instruments, Austin, TX) was used to control simultaneously the high voltage power supply (built locally) for the microfluidic chip, the flow gated sampling, the working voltage of the photomultiplier tube and the signal collection. A SpectraMax M2 from Molecular Devices (Sunnyvale, CA) was used in the ThioGlo-1/GSH kinetic experiments. The reactions of GSH and ThioGlo-1 were carried out in 20 mM Tris buffer (pH 7.5) and 25°C. The excitation and emission wavelengths were 403 nm and 495 nm, respectively.

Enzyme Kinetics Experiments

Before each run, mixtures of β -NADPH and GSSG at the desired concentrations were prepared using 20 mM Tris-HCl buffer (pH 7.5) and transferred into the sample reservoir. Derivatizing reagent, which contains 50 μ M ThioGlo-1 and 1.9 μ M proflavine as internal standard, was prepared using 20 mM Tris-HCl buffer (pH 7.5) with 0.1 mM 1,6-diaminohexane then added into reagent reservoir. Buffer reservoir and waste reservoir were both filled with running buffer. The enzyme catalyzed reaction was initiated by the addition of 10 μ L of glutathione reductase solution into the substrate mixture in sample reservoir to achieve a final concentration of 7.93×10^{-4} unit/mL. The final volume of solution in each reservoir was 200 μ L. Immediately after addition of GR, -6000 V was applied to waste reservoir, while the reagent and sample reservoirs were kept at ground and the buffer reservoir was switched between two states, “ground” and “float”. Typically, the sampling time was 0.1 s and the separation time was 9.9 s unless otherwise stated. There were usually 40–50 repeat injections for each sample per run. Before changing to the next sample, the channels were rinsed and reconditioned in sequence with 0.1 M NaOH and running buffer each for 5 min by applying -6000 V at the waste reservoir and keeping all other reservoirs at ground. All experiments were carried out at room temperature.

RESULTS AND DISCUSSION

Derivatization of GSH with ThioGlo-1

The kinetics of the reaction between GSH and ThioGlo-1 (see Fig. S1) were first measured in a spectrofluorometer to test the reagent's suitability for online precolumn derivatization. When the initial concentrations of GSH and ThioGlo-1 are 5 μ M and 25 μ M, respectively, it takes about 14 s to generate a fluorescent signal equivalent to 95% of the signal intensity at equilibrium (see Fig. S2). Based on this fact, it is reasonable to assume that as long as the online derivatization reaction lasts longer than 14 s, the reaction will be nearly complete.

The average fluorescence intensity of the product at equilibrium with different initial concentrations of GSH but the same initial concentration of ThioGlo-1 is linear when ThioGlo-1 is in excess (see Fig. S3). The relationship between the fluorescence intensity, I (RFU) and the initial GSH concentration, G_0 (M) can be mathematically described by the nonlinear equation derived from the definition of equilibrium constant of the reaction between GSH and ThioGlo-1 with three coefficients, equilibrium constant, K (M^{-1}), fluorescence coefficient, α (M/RFU) and the initial concentration of ThioGlo-1, T_0 (M). (Equation 1) A nonlinear fit shows that the equilibrium constant of this reaction is on the order of $10^8 M^{-1}$ (a more accurate assessment is not possible because the reaction is virtually quantitative at all of the concentrations used).

$$K = \frac{I \cdot \alpha}{(G_0 - I \cdot \alpha) \cdot (T_0 - I \cdot \alpha)} \quad (1)$$

Based on this approximate equilibrium constant, it is reasonable to assume that the rate of the reverse reaction is much slower than the rate of the forward reaction when the GSH and reagent concentrations are in the range studied (low micromolar) (Equation 2). Under this assumption, a linear relationship can be derived between the fluorescence intensity of the product and the reaction time as indicated in Equation 3, with the rate constant of the forward reaction k_f as the slope of the line.

$$\frac{\ln \frac{T_0 - I \cdot \alpha}{G_0 - I \cdot \alpha} - \ln \frac{T_0}{G_0}}{(T_0 - G_0)} = k_1 \cdot t \quad (3)$$

The value of k_f determined from a global fitting of seventeen sets of data is $2.1 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, with a standard deviation of $2.2 \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$ (see S4). Based on the above results, k_{-f} can also be estimated, which is on the order of 10^{-4} s^{-1} . Although the reaction between thiols and maleimide is reversible,⁴² under our experimental conditions, the reverse reaction does not factor into the detection. Therefore, ThioGlo-1 is suitable for online precolumn derivatization of GSH and quantitative analysis.

Microfluidic Capillary Electrophoresis

Utilizing flow gated injection,⁴³ the microfluidic chip operates in a continuous sampling/separation mode, which allows continuous monitoring of the changing concentration of the analyte GSH. Another advantage of flow gated injections is that the analyte, reagent, and buffer reservoirs are all kept isolated throughout the experiment. Therefore dilution or contamination of analytes by the other reagents and buffers is reduced compared to a pinched injection, which enables more repeatable and reliable results. Although the flow gated injector is time dependent and electrophoretically biased,^{44, 45} it is possible to use an internal standard to eliminate these problems. The sample volume loaded by the flow gated valve is determined by both the short period of time when the potential at buffer reservoir is floating and the flow rate of the sample streams. The operation of the high voltage relay was controlled by the onboard clock on the data acquisition device, which provided precision in the floating time. However, the flow rate of the sample streams can be affected by changes in the zeta potential, the conditions in the microfluidic channels, differences in the relative liquid levels between buffer reservoir and waste reservoir, and fluctuations of the pH in each reservoir during continuous analysis. To mitigate any effect of the variability in the injection volume, an internal standard, proflavine, was added to the reagent reservoir. Proflavine has an excitation maximum of 445 nm and emission maximum of 511 nm at neutral pH, both of which are suitable for our LIF detector.⁴⁶ Moreover, it is positively charged under the separation conditions and should migrate before the analyte to avoid additional analysis time. Figure 2 (A) shows the separation of proflavine (internal standard, IS), unreacted ThioGlo-1 (dye) and the ThioGlo-1 labeled GSH in the microfluidic chip using 20 mM Tris-HCl buffer (pH 7.5) as running buffer. The sampling time was 0.1 s and the voltage applied at the waste reservoir was -6000 V.

Although the resolution of the IS and Dye peaks reaches 4.0 in Figure 2 (A), the IS peak is tailing. This may be caused by electrostatic interactions between the cationic proflavine and the negatively charged inner wall of the channel. Supporting this idea, when proflavine was used, the channels became clogged, even if the microfluidic chip went through a conditioning step between runs. Proflavine is relatively insoluble in water, and adsorbed proflavine may nucleate precipitation. The addition of aliphatic diamines, especially 1,4-diaminobutane, to the running buffer to neutralize the negative charges on the wall has been investigated by several groups.^{47, 48} For these experiments, 1, 6-diaminohexane was chosen since it is less toxic than other diamines with shorter aliphatic chains. While it will compete for surface adsorption sites with proflavine, it will also change the zeta potential at the microchannel wall. Figure 2 shows a decrease in the velocities of proflavine (IS), unreacted ThioGlo-1 (dye), and ThioGlo-1-GSH adduct (GSH*) as the concentration of 1, 6-diaminohexane increases, evidence of a reduced zeta potential and electroosmotic flow rate. Based on these results, a concentration of 0.1 mM 1, 6-diaminohexane was chosen for further testing based on the separation time needed for the analysis.

To test the effect of 1, 6-diaminohexane in the reaction channel, electropherograms were obtained with and without 0.1 mM 1, 6-diaminohexane in the reagent reservoir during continuous analysis of GSH produced in the sample reservoir by the enzyme reaction. It is clear from Figure 3 that the repeatability is significantly improved from using the additive. Figure 3 (C) shows the fluctuation in the FWHM of the GSH-ThioGlo-1 reaction product (GSH*) peaks in both cases. The RSD of the FWHM for GSH* peaks was 2.2% when 0.1 mM 1, 6-diaminohexane was included in reagent reservoir. However, this value increased to 12.8% when 1, 6-diaminohexane was excluded from reagent reservoir. Adding 1, 6-diaminohexane to both the derivatizing reagent and running buffer was helpful for improving the resolution of the separation, reducing the adsorption of proflavine and stabilizing the quantitative measurements, especially in continuous sampling. To avoid any interference with the enzyme reaction, 1, 6-diaminohexane was not added to sample reservoir.

Applying the above optimized conditions for the separation, the concentration and mass limit of detection for GSH were determined to be 4.2 nM and 10^{-18} mol using ThioGlo-1 as the derivatizing reagent. The repeatability of the continuous injections was evaluated by measuring the peak parameters for the internal standard. The relative standard deviation of the peak area, peak height, FWHM and migration time for the internal standard were found to be 2.5%, 2.0%, 1.0% and less than 0.1%, respectively.

We briefly examined the applicability of the system to thiols that do not have the correct structure to react rapidly with NDA as GSH and γ -GluCys do. The thiols CG and C, as well as γ -GluCys, have similar sensitivities to GSH. Thus, the Thio-Glo-1 reagent is applicable to a wider scope of thiols than is NDA.

Enzyme Kinetic Study

glutathione reductase is a well studied flavoenzyme, which catalyzes β -NADPH-dependent reduction of GSSG to maintain a healthy cellular ratio of [GSH]/[GSSG] as shown by the equation below.^{33, 49}

Numerous studies have demonstrated that in the absence of product inhibition, the double reciprocal plot (Lineweaver-Burk plot) of the reaction rate against one of the two substrates, the other being held constant and saturating, will give a straight line.^{38, 49-53} Thus if we obtain two linear Lineweaver-Burk plots, each of which yields a correct Michaelis constant for the

non-saturating substrate, then we know that the derivatization, injection, separation and detection of GSH on this microfluidic platform are reliable.

We utilized the microfluidic chip, and the conditions just developed, to measure the initial forward rate as a function of concentration of one substrate while the concentration of the other substrate was kept at a saturating concentration. As the initial rate was to be measured, inhibition caused by the product was assumed to be negligible. The reaction rate in this case depends only on the concentration of the enzyme–substrate complex, assumed to be nearly constant, therefore; a linear conversion of the substrate to product is predicted. Figure 4(A) shows the series of electropherograms obtained for a rate measurement at one substrate concentration; in this particular measurement the concentration of GSSG was saturating.⁵² The electropherograms for all of the injections in this experiment are plotted in parallel in Figure 4 (B).

The electropherogram in Figure 4 (A) can be divided into three regions based on the detected peaks. In the first region (0–40 s), no peaks are observed. This is the time needed by the compound with the highest mobility to pass through the reaction and separation channels before being detected. In the second region, located between 40 and 130 s, the IS peaks come out steadily and the dye peaks start to arrive and gradually become stable. No GSH* peak is detected in this region. GSH* peaks begin to be detected in the third region beginning at approximately 130 s. Calculations based on Ohm's and Kirchoff's laws show that it takes about 106 s for GSH to traverse the reaction channel, i.e., the reaction time is about 106 s. Based on the rate constant determined above, the ThioGlo-1 concentration in the reaction channel, and assuming a pseudo-first order reaction (ThioGlo-1 in excess) the time required to achieve 95% conversion of GSH to GSH* would be 5 s. The time for a complete mixing of dye and GSH in aqueous solution is estimated to be ~2 s, based on the diffusion coefficient of GSH, $D = 6.7 \times 10^{-10} \text{ m}^2/\text{s}$,⁵⁴ and the channel width, 50 μm . Thus, conversion of GSH to GSH* is virtually complete. Comparison of relative peak areas for the online reaction and offline reaction (5 minutes) shows that the reaction is, in fact, complete. For evaluation of enzyme kinetics, data points were collected once the later peaks demonstrate the expected constant signal (ThioGlo-1 and IS) and a linear increases in the height and area of the GSH* peak due to the enzyme reaction was observed. Based on this reasoning, the first several peaks of GSH* are excluded in the calculation of the initial forward rate.

For the enzyme kinetic studies, the concentration of glutathione reductase was fixed at $7.93 \times 10^{-4} \text{ unit/mL}$; this concentration was chosen to allow the steady state phase of the enzyme catalyzed reaction to last long enough to collect sufficient data while also giving a detectable change in GSH. The appropriate forms of the rate equations for the initial rates are^{37, 55}

$$v_i = \frac{V_{\max} [A] [B]}{K_{mB} [A] + K_{mA} [B] + [A] [B]} \quad (5)$$

$$\frac{1}{v_i} = \frac{K_{mB}}{V_{\max}} \frac{1}{[B]} + \frac{1}{V_{\max}} \left(1 + \frac{K_{mA}}{[A]} \right) \quad (6)$$

In these equations, v_i is the initial forward rate obtained under different substrate B concentrations, $[B]$. $[A]$ is the fixed concentration of substrate A. K_{mA} and K_{mB} are the Michaelis constant of substrate A and B, respectively, and $V_{\max}$ is the maximum rate. If substrate A is kept at saturating concentration, $[A]$ would be much large than K_{mA} . Therefore, the term $K_{mA}/[A]$ can be neglected and Equation (6) would be further simplified to

$$\frac{1}{v_i} = \frac{K_{mB}}{V_{max}} \frac{1}{[B]} + \frac{1}{V_{max}} \quad (7)$$

A Lineweaver-Burk plot based on Equation (7) is shown in Figure 5. The relationship between the initial rate of the forward reaction of glutathione reductase and the concentration of β -NADPH (1–100 μ M) at a saturating concentration of GSSG (1000 μ M) is demonstrated in Figure 5(A). The corresponding plot showing the initial rate versus the concentration of GSSG (10–1000 μ M) at a saturating concentration of β -NADPH (100 μ M) is shown in Figure 5(B). The initial concentrations of both GSSG and β -NADPH were chosen to be evenly distributed in the inverse plot. The initial forward rates were obtained by performing linear regression of the GSH concentration *vs.* time. The apparent K_m of β -NADPH and GSSG for glutathione reductase obtained from the Lineweaver-Burk plots were $4.4 \pm 0.6 \mu$ M and $40 \pm 11 \mu$ M, respectively. V_{max} values calculated from each plot were close to each other; it was 11.0 ± 1.3 nM/s when the concentration of GSSG was fixed and 10.8 ± 2.6 nM/s when the concentration of β -NADPH was fixed.

The K_m values of GSSG and β -NADPH for glutathione reductase (baker's yeast) measured by Massey *et al.* were 55 μ M and 3.8 μ M, respectively.³³ Mavis *et al.* reported similar K_m values of 61 μ M and 7.6 μ M for GSSG and β -NADPH.⁵⁶ The apparent K_m values of GSSG and β -NADPH we obtained using an integrated microfluidic method for evaluation of glutathione reductase kinetics are comparable with these values.

CONCLUSIONS

We have demonstrated the use of ThioGlo-1 as an online derivatizing reagent for quantitative determinations of GSH. The reagent derivatizes GSH more rapidly than had been thought to be the case. This knowledge was then utilized to develop an integrated microfluidic method for evaluation of enzyme kinetics employing on-chip precolumn derivatization. For the gated separation analysis, proflavine as an internal standard minimized variable injection volume effects without added analysis time and 1, 6-diaminohexane addition resulted in stabilization of the peak parameters from GSH, especially during continuous analysis. The sensitivity of the determinations is excellent with a GSH concentration and mass detection limits of 4.2 nM and 10^{-18} mol. The combination of the simple microfluidic chip with well-chosen chemistry has led to an integrated system capable of continuous monitoring of GSH concentrations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Layout of the microfluidic chip used for enzyme analysis. RR, SR, BR, and WR represent reagent reservoir, sample reservoir, buffer reservoir and waste reservoir, respectively.

Figure 2.

Effect of 1, 6-diaminohexane in running buffer on the separation of proflavine (IS), unreacted ThioGlo-1 (dye), and ThioGlo-1-GSH adduct (GSH*). The sample reservoir was filled with 3 μ M GSH in 20 mM Tris-HCl buffer (pH 7.5) and the reagent reservoir was filled with 50 μ M ThioGlo-1 and 1.9 μ M proflavine in the same Tris-HCl buffer. The concentration of 1, 6-diaminohexane in the running buffer in buffer reservoir and waste reservoir varied from (A) 0 mM; (B) 0.1 mM; (C) 0.5 mM; (D) 1.0 mM. Sampling and separation times were 0.1 s and 9.9 s, respectively. Potential applied at the waste reservoir was -6000 V.

Figure 3.

Effect of 1, 6-diaminohexane in the reagent reservoir on the enzyme kinetic analysis. (A) No 1, 6-diaminohexane; (B) 0.1 mM 1, 6-diaminohexane; (C) FWHM of the GSH* peaks in both experiments. The sample reservoir contained 25 μ M GSSG, 50 μ M β -NADPH. The reagent reservoir contained 300 μ M ThioGlo-1 and 9.5 μ M proflavine. Experiments were started once glutathione reductase was mixed with the substrate solution at a final concentration of 5.29×10^{-3} unit/mL in the sample reservoir. Sampling and separation times were 0.1 s and 9.9 s, respectively. Potential applied at the waste reservoir was -6000 V.

Figure 4.

(A) Series of electropherograms obtained on a microfluidic chip with continuous flow gated injection. The concentrations of GSSG and β -NADPH were 1000 μ M and 100 μ M, respectively. The inset magnifies one set of peaks from an injection made between 300 and 309 s. The small valley, GI, around 300.5 s resulted from the flow gated injection. 'IS', 'Dye' and 'GSH*' represent proflavine, unreacted ThioGlo-1 and ThioGlo-1-GSH adduct, respectively. (B) Reconstructed plot of PMT reading vs time for all injections in this series.

Figure 5.

(A) Lineweaver-Burk plot showing the initial rate of the forward reaction of glutathione reductase against concentration of β-NADPH at 1000 μM GSSG. (B) Lineweaver-Burk plot showing the initial rate of the forward reaction of glutathione reductase against concentration of GSSG at 100 μM β-NADPH. Error bars are $\pm\sigma$ (n=14).