Talanta 119 (2014) 240–247

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Determination of thiol functional groups on bacteria and natural organic matter in environmental systems

Balaji Rao [a], Carolyne Simpson [b], Hui Lin [a], Liyuan Liang [a], Baohua Gu [a,*]

[a] Environmental Sciences Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831, United States
[b] University of Tennessee, Knoxville, Department of Chemistry, Knoxville, TN 37996, United States

ARTICLE INFO

Article history:
Received 9 August 2013
Received in revised form
31 October 2013
Accepted 2 November 2013
Available online 9 November 2013

Keywords:
Thiols
Mercury
Fluorescence spectroscopy
Titration
NOM

ABSTRACT

Organic thiols (R–SH) are known to react and form complexes with some toxic soft metals such as mercury (Hg) in both biotic and abiotic systems. However, a clear understanding of these interactions is currently limited because quantifying thiols in environmental matrices is difficult due to their low abundance, susceptibility to oxidation, and measurement interference by non-thiol compounds in samples. Here, we report a fluorescence-labeling method using a maleimide containing probe, ThioGlo-1 (TG-1), to determine total thiols directly on bacterial cells and natural organic matter (NOM). We systematically evaluated the optimal thiol labeling conditions and interference from organic compounds such as disulfide, methionine, thiourea, and amine, and inorganic ions such as Na^+, K^+, Ca^{2+}, Fe^{2+}, Cl^-, SO_4^{2-}, HCO_3^-, and SCN^-, and found that the method is highly sensitive and selective. Only relatively high levels of sulfide (S^{2-}) and sulfite (SO_3^{2-}) significantly interfere with the thiol analysis. The method was successful in determining thiols in a bacterium *Geobacter sulfurreducens* PCA and its mutants in a phosphate buffered saline solution. The measured value of $\sim 2.1 \times 10^4$ thiols $cell^{-1}$ (or ~ 0.07 μmol g^{-1} wet cells) is in good agreement with that observed during reactions between Hg and PCA cells. Using the standard addition, we determined the total thiols of two reference NOM samples, the reduced Elliot soil humic acid and Suwanee River NOM, to be 3.6 and 0.7 μmol g^{-1}, respectively, consistent with those obtained based on their reactions with Hg.

1. Introduction

Organic thiols (R–SH) are a class of reduced sulfur compounds that occur in soil, fresh and marine water systems. They are typically found in association with organic materials in the natural environment [1–4]. Thiols (e.g., cysteine and glutathione) of biological origin are known to participate in reactions such as oxidative stress mitigation, metal uptake and detoxification, and bio-molecular activation of microorganisms [5,6]. Thiols along with other reactive functional groups (e.g., carboxylates, quinone and semiquinones, etc.) can govern the fate and speciation of certain metal ions in the environment [4,7,8]. For instance, reactions of inorganic mercury (Hg) species with natural organic matter (NOM) result in both reduction of Hg(II) and oxidation of Hg(0), depending on the redox state and Hg/NOM ratios [7,8]. These studies suggest the involvement of two competing mechanisms: reduction by semiquinones and complexation by thiol-induced oxidation. Similar behaviors of reduction, oxidation, and surface binding of Hg have been observed on Hg-methylating bacteria including *Geobacter sulfurreducens* and *Desulfovibrio desulfuricans* ND132 [9,10]. However, despite their importance, techniques for direct quantification of thiols on bacterial cells and NOM are not readily available [11]. As a key aspect of studying the processes that regulate the fate and transport of such metal ions as Hg, a robust and sensitive analytical approach for quantifying the organic thiols on NOM and bacteria is needed.

Current techniques for thiol measurements include electrochemistry, mass spectroscopy, X-ray absorption spectroscopy (XAS), and chemical derivatization for light based spectroscopic analysis [12–16]. However, direct analysis of thiols in environmental and biological matrices faces significant challenges due to their low abundance, susceptibility to chemical and photochemical oxidation, and inherent absence of distinguishable spectral characteristics. The XAS techniques suffer from low sensitivity with thiol detection limits on the order of micromolar (μM) to millimolar (mM) levels [14]. Although electrochemical techniques are reported to detect nanomolar levels of pure thiol compounds [12,17], they are yet to be demonstrated for direct analysis of thiols on NOM and bacterial cells. Recently, chemical labeling agents have been used to selectively react with thiols to enhance their light absorption or fluorescence emission resulting in sensitivity up to femtomolar levels [16,18]. However, these analyses are often

* Corresponding author. Tel.: +1 865 574 7286.
E-mail address: gub1@ornl.gov (B. Gu).

0039-9140/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.talanta.2013.11.004

performed in laboratory prepared solutions and involve considerable sample preparation, chemical separation and detection by high performance liquid chromatography [19], thus making them unsuitable for direct quantification of thiols on intact bacteria in the culture media. Furthermore, they usually require a prior knowledge of the nature of thiols in the samples, which presents a problem for measurement of samples such as NOM where information regarding the specific type of thiols is unknown [20].

A different approach to minimize sample alteration has been recently reported for the measurement of unknown thiols using a fluorescence reagent, monobromo(trimethylammonio)-bimane (qBBr), that belongs to a class of bimane compounds [11]. This technique was applied to measure thiols on a Gram positive bacterium, *Bacillus subtilis*, and natural water sample containing NOM. The thiol concentration was quantified by performing fluorescence titration of the sample with incremental amounts of qBBr at levels below and above the total thiols present. The method utilized deionized water as the clean background solution for sample processing and analysis to minimize matrix interferences [11], but the effectiveness of this method in complex matrices, such as phosphate buffered saline (PBS) solutions that are needed to maintain the structure and viability of bacteria cells, was not evaluated. As the authors noted, Gram-negative bacteria, such as *G. sulfurreducens* and *Shewanella oneidensis*, are prone to lysis in deionized water because they have softer cell envelopes than *B. subtilis* [11]. Currently, no effective methodologies are available to quantify reactive thiols on these organisms, which have limited our ability to assess their reaction processes that affect the transformation and transport of metal ions like Hg.

Here, we present an improved measurement technique for thiols in complex environmental matrices using a fluorescence labeling reagent that consists of a thiol-reactive maleimide group, known as ThioGlo-1 (TG-1) (3H-Naphthol[2,1–b]pyran-s-carboxylic acid) [21,22]. TG-1 reacts with R–SH compounds quantitatively through Michael addition mechanism (1:1 on a molar basis). TG-1 has been used for determining thiols in biological proteins and tissues [21,23] but never used for direct quantification of thiols on bacteria and NOM. TG-1 is very sensitive to thiol-containing compounds and exhibits high fluorescence quantum yield after reaction with thiols [22,24]. We report systematic evaluation of the labeling conditions and optimization to minimize potential interferences for thiol determination with a high selectivity and sensitivity. We applied the optimized conditions for the measurement of low micromolar concentrations of thiols on a Gram negative methylating bacterium *G. sulfurreducens* PCA and its mutants in the culture solutions, as well as on two NOM samples.

2. Experimental

2.1. Bacteria and NOM sample preparation

Details of the preparation of bacterial samples have been reported elsewhere [9,10]. In brief, *Geobacter sulfurreducens* PCA and two of its mutant strains were harvested from the growth media in the late exponential growth phase, pelletized by centrifugation (1500*g* for 10 min) in an anaerobic chamber, and the supernatant then discarded. The pelletized cells were re-suspended and washed 3 times in de-aerated PBS solution consisting of 0.14 M sodium chloride (NaCl), 3 mM potassium chloride (KCl), 10 mM disodium phosphate (Na_2HPO_4), and 2 mM potassium phosphate (KH_2PO_4) with pH adjusted to 6.6. The PBS was chosen to mimic the background matrix used for bacterial Hg methylation studies [9,10] and to maintain the structure and viability of bacteria cells. The washed cell suspension was immediately used for TG-1 labeling and analysis. A portion of the suspended bacteria was used for cell number density measurement by analyzing the optical density (OD) at 600 nm, which was further validated by direct cell counting using a hemocytometer under a microscope [9,10]. Thiol measurements were then performed at different cell densities (from 8×10^{12} to 3×10^{13} cells L^{-1}) using both the wild-type (WT) and the mutant strains in PBS. The cell number densities before labeling and after fluorescence measurement were mostly identical, suggesting that no significant cell lysis occurred during analysis.

Two NOM samples, Elliot soil humic acid (HA) and Suwannee River NOM (SR-NOM), were obtained from the International Humic Substance Society (IHSS). These NOM samples were chemically reduced with hydrogen (H_2) in the presence of palladium (Pd) catalyst (5% Pd on alumina powder, 1 g L^{-1}) and stored under anoxic conditions, as described previously [7]. The NOM stock solutions (1 g L^{-1}) were diluted to a final concentration of 50 and 100 mg L^{-1} for HA and SR-NOM, respectively, in PBS before analysis.

2.2. Chemical reagents and standards

TG-1 with a purity $>99.0\%$ was obtained from EMD Millipore chemicals (San Diego, CA) and used without further purification. A stock solution was prepared by directly dissolving the salt in 100% dimethyl sulfoxide (DMSO) and stored at 4 °C. TG-1 working standards (25, 50 or 100 μM) were prepared in acetonitrile from the stock on the day of sample analysis. Stock solutions (10 mM) of organic thiols and non-thiols were prepared in deionized Milli-Q water (>18 MΩ cm). They include L-cysteine (CYS; $>99\%$) from Acros Organics, glutathione (GSH) from Fisher BioReagent, thiosalicylic acid (TS; 97%), 4-mercaptobenzoic acid (4-MB; $>99\%$), L-cystine (CYI; $>98\%$), 4-aminobenzoic acid (4-AB; 99%), 4-hydoxybenzoic acids (4-HB; $>99\%$), thiourea (TU; $>99\%$), and L-methionine (MTI; $>98\%$) from Sigma-Aldrich.

To study the effects of common cations in the thiol measurement, the stock solutions of iron (Fe^{2+}) (18 mM) and calcium (Ca^{2+}) (25 mM) were prepared in deionized water from ferrous sulfate ($FeSO_4\cdot7H_2O$, 99.5%, Avantor Performance Materials) and calcium chloride (anhydrous $CaCl_2$, 96%, EM Science), respectively. Copper (Cu^{2+}) stock solution (16 mM) was prepared from a reference standard (Baker Analyzed Reagent, $>99.9\%$ purity). A metal chelating agent EDTA (ethylenediaminetetraacetic acid potassium salt, Reagent grade) was used to evaluate its ability to mitigate the effect of metal ions during analysis. Potential interference of sulfur-containing compounds was determined in the presence of sulfide (Na_2S, anhydrous salt, 98%, Sigma-Aldrich), thiocyanate (KSCN, 99%, EM Science), 9,10-anthraquinone-2,6-disulfonic acid (AQDS, 98%, Sigma-Aldrich), and sulfite (Na_2SO_3, $>98\%$, ACS reagent). These compounds were freshly prepared in PBS at 10 mM. Sulfite appeared unstable in PBS so that 1 mM EDTA was added to ensure its stability [25].

2.3. Fluorescence spectroscopic measurement

All fluorescence labeling experiments with TG-1 was performed in dark by mixing 1–3 mL of the sample with 0–0.05 mL of the TG-1 working standards at varying concentrations. Unless otherwise specified, all titrations, including studies of the effect of interfering ionic species, were performed in the PBS at pH 6.6 (used for bacteria samples). The buffer was found to be sufficient in maintaining its pH during all titrations. Reference thiols, bacteria, and NOM samples were allowed to react with TG-1 for ~2 h at room temperature, and this reaction time was sufficient to obtain stable fluorescence emission for all samples. Fluorescence spectra were recorded on a Fluorolog fluorescence spectrophotometer equipped with both excitation and emission monochrometers (Johin-Yvon SPEX Instruments,

New Jersey). All measurements were performed in either 1 or 3 mL capacity clear quartz cuvettes. A 450-W Xenon arc lamp was used as the excitation source. An excitation wavelength (λ_{ex}) of 379 nm was used for all samples, and the peak emission intensity ($\lambda_{em,pk}$) at ~513 nm was used for plotting the titration curves and subsequently determining the total thiol concentrations.

3. Results and discussion

3.1. Detection of reference thiols

Labeling of standard reference thiols such as cysteine (CYS), glutathione (GSH), and thiosalicylic acid (TS) with TG-1 gave intense fluorescence compared to the background (GSH without TG-1 or TG-1 alone in PBS) (Fig. 1a). However, the fluorescence intensity varied considerably with different thiols even at identical concentrations. This property makes direct quantification from total fluorescence values problematic for samples containing unknown or multiple thiol mixtures. To overcome this problem, we performed fluorescence titration with TG-1 at varying concentrations covering values from less than to greater than the sample thiol concentration ([R–SH]). In theory, titration of a sample with incremental amounts of TG-1 should result in a distinct inflection in the emission intensity precisely at the point corresponding to the total thiols present in the sample. A plot of fluorescence intensity obtained by the titration of 1 μM CYS, and separately 1 μM GSH, with TG-1 shows a steep increase in emission intensity in the region where [TG-1] < [R–SH] followed by a much slower increase at [TG-1] > [R–SH] (Fig. 1b). Linear curve fitting using the least squares method for the two regions

Table 1
Measurement of total thiols in model thiol compounds (GSH, CYS, TS), a methylating bacterium *Geobacter sulfurreducens* PCA (PCA WT) and its mutants (*ΔomcBESTZ* and *ΔhgcAB*), and NOM samples (HA and SR-NOM). Numbers in parenthesis represent one standard error.

Standards/ samples[a]	Description	Total R–SH concentration (μM)		Number of trials
		Expected	Measured	
GSH	Glutathione	1	1.03 (0.05)	3
CYS	Cysteine	1	0.99 (0.02)	2
CYS+GSH	Cysteine+glutathione (1:1)	1	1.07	1
TS	Thiosalicylate	0.5	0.51	1
PCA WT	*G. sulfurreducens*-PCA (10^{13} cells L^{-1})	–	0.34 (0.05)	4
ΔomcBESTZ	PCA mutant (10^{13} cells L^{-1})	–	0.74 (0.08)	2
ΔhgcAB	PCA mutant (10^{13} cells L^{-1})	–	0.11 (0.01)	2
HA	50 mg L^{-1}	–	0.18 (0.02)[b]	2
SR-NOM	100 mg L^{-1}	–	0.07 (0.01)[b]	2

[a] All measurements performed in phosphate buffered saline (PBS) at pH = 6.6.
[b] Analysis performed by standard addition with GSH at concentrations from 0.2 to 0.5 μM.

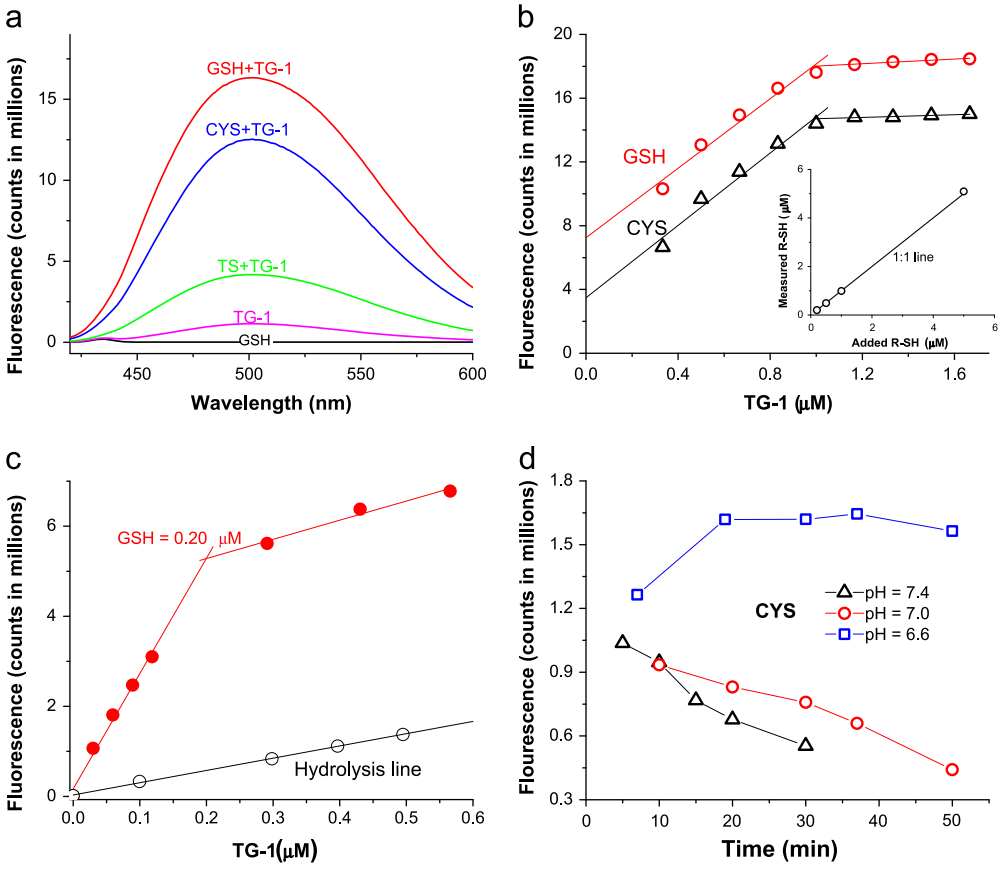

Fig. 1. (a) Comparisons of fluorescence emission spectra of 1 μM glutathione (GSH), cysteine (CYS), and thiosalicylate (TS) after TG-1 labeling in PBS; spectra of GSH without labeling and TG-1 (2 μM) were used as controls. (b) TG-1 titration of 1 μM GSH and CYS in PBS, showing an inflection point corresponding to the measured thiol concentration of ~0.99 μM. The inset shows the standard calibration of GSH titration at varying concentrations. (c) TG-1 titration of GSH at a lower concentration (0.2 μM). The slope at [TG-1] > [R–SH] is attributed to the background fluorescence produced by the hydrolysis of TG-1 in water. (d) TG-1 titration of CYS (0.05 μM) at varying pH conditions showing CYS-TG-1 conjugate in PBS unstable at pH > 7. All fluorescence measurements were performed at the excitation and emission wavelengths of 379 and 513 nm, respectively.

results in an interception of the two lines essentially at 0.99 μM (TG-1 as *X*-axis) with an accuracy of ~99% for both CYS and GSH. Even though GSH labeling with TG-1 exhibited higher fluorescence intensity than that of CYS, the inflection point occurred at the same total thiol concentration, independent of the chemical structure or nature of thiols involved (e.g. CYS, GSH, TS, etc., Table 1). The technique was demonstrated to give excellent accuracy and linearity for thiol determination using GSH as an example (Fig. 1b inset) and can thus be used to quantify total thiols in unknown samples. The ratio of the slopes obtained at [TG-1] < [R–SH] to the slope corresponding to [TG-1] > [R–SH] is progressively greater with higher concentrations of thiol present (Fig. 1b and c and Fig. S1). However, at lower thiol concentrations the slope ratio becomes smaller and makes it difficult to obtain an inflection point. For example, at [R–SH] < 0.2 μM the slope ratio is less than 5 (Fig. 1c). The slope corresponding to [TG-1] > [R–SH] is largely attributed to the background fluorescence produced by the hydrolysis of TG-1 in water [24].

We also note that, for cysteine, higher pH (7.0 and 7.4) resulted in decreasing fluorescence intensity over time following the addition of TG-1 (at time 0) (Fig. 1d). This phenomenon was found to be unique for cysteine since other thiol compounds did not exhibit similar behavior. A previous study based on reaction of cysteine with *N*-(1-pyrene)maleimide has attributed this behavior to the intramolecular aminolysis of succimido rings formed in the adducts [26]. For this reason, all analyses reported in this study were performed either in PBS or bicarbonate buffer solutions at pH 6.6 to ensure stable fluorescence obtained for all samples.

3.2. Selectivity, interferences, and optimization

One significant challenge in analyzing environmental samples such as bacteria and NOM is potential interferences resulting from other organic and inorganic compounds, including metal ions, which may react with TG-1 or affect its fluorescence yield. Accordingly a high selectivity is paramount for the method to be applicable for these analyses. We first evaluated the potential interferences resulting from non-thiol sulfur compounds including cystine (CYI), methionine (MTI), and thiourea (TU), and from carboxyl and hydroxyl compounds such as 4-aminobenzoic acid (4-AB) and 4-hydroxybenzoic acid (4-HB). Results indicate that these non-thiol compounds did not display significant fluorescence in comparison with those of thiols (CYS, GSH, TS, and 4-MB), and thus confirm the selectivity of TG-1 for thiol analysis (Fig. 2a). Furthermore, the addition of these non-thiol compounds (MT, 4-AB, 4-HB, and TU) at three orders of magnitude higher concentration (1000 μM) than that of GSH (0.5 μM) did not affect the titration results (Fig. 2a inset); the measured GSH concentration was 0.49 ± 0.01 μM, demonstrating excellent measurement accuracy. The absence of interference by the carboxyl, hydroxyl, amine, and S-containing organic compounds at millimolar concentrations

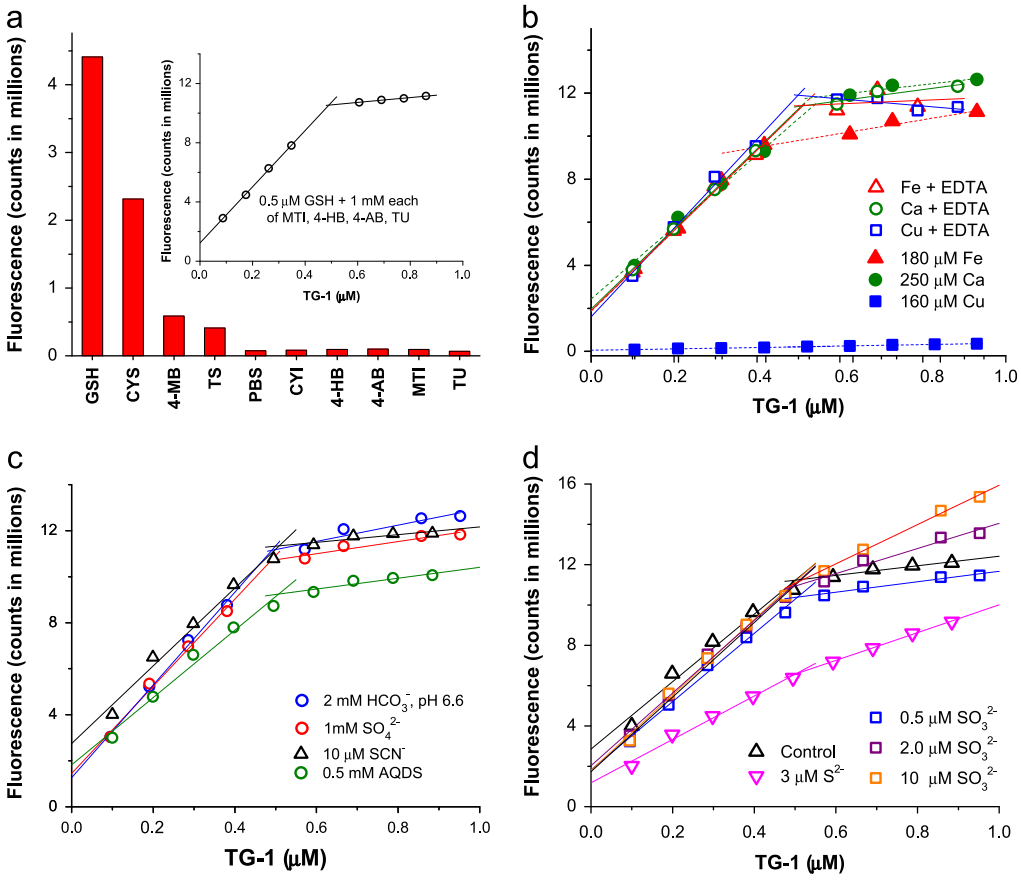

Fig. 2. (a) Comparisons of fluorescence intensities of TG-1 labeled thiols (0.1 μM each of GSH, CYS, 4-mercaptobenzoite (4-MB), and TS) and non-thiol organic compounds (0.1 μM) including L-cystine (CYI), 4-hydoxybenzoic acids (4-HB), 4-aminobenzoic acid (4-AB), L-methionine (MTI), and thiourea (TU) in PBS. The inset shows that no interferences occurred during titration of GSH (0.5 μM) in the presence of 1 mM each of 4-HB, 4-AB, MTI, and TU. (b) Titration of GSH in the presence of interfering cations Fe^{2+}, Ca^{2+} and Cu^{2+} at 180, 250 and 160 μM, respectively, with or without added EDTA (10 mM). (c) Titration of GSH directly in 2 mM bicarbonate solution at pH 6.6 (no PBS) and in the presence of 1 mM SO_4^{2-}, or 10 μM SCN^-, or 0.5 mM AQDS in PBS. (d) Titration of GSH in the presence of sulfide (S_2^-) (3 μM) and sulfite (SO_3^{2-}) at 0.5, 2, and 10 μM.

is important as these compounds are typically more predominant than thiols in bacteria and NOM samples [4].

We also evaluated potential interferences from metal ions including Ca^{2+}, Fe^{2+}, and Cu^{2+}, which are often present in natural waters. The test was done by the titration of 0.5 μM GSH in the presence of orders of magnitude higher concentrations of metal ions including Fe^{2+}, Ca^{2+}, and Cu^{2+} at 180, 250, and 160 μM, respectively. Among the tested metal ions, Cu^{2+} (160 μM) severely interfered in GSH analysis, causing a >99% decrease in the fluorescence intensity throughout the titration and a loss of the inflection point (Fig. 2b). Ferrous iron (180 μM) and Ca^{2+} (250 μM) did not cause a similar decrease in fluorescence intensity (Fig. 2b). The obtained inflection point in the presence of Fe^{2+} decreased slightly and was about 75% of the expected thiol value (0.38 μM). The metal interference problem, however, can be alleviated by the addition of 10 mM EDTA, a common chelating agent for metal ions. This resulted in nearly complete recovery of emission intensities even in the presence of 160 μM Cu^{2+} with a measured accuracy within ±5% (Fig. 2b). The presence of high concentrations of Na^+ (140 mM) and K^+ (5 mM) in the background PBS solution did not show any effect on the analysis.

In addition to the metal ions, we evaluated the potential interference from thiocyanate (SCN^-), sulfate (SO_4^{2-}), and AQDS in PBS, as well as direct titration in the bicarbonate buffer solution (2 mM HCO_3^- in 10 mM NaCl at pH 6.6) (no PBS) (Fig. 2c). Sulfate (1 mM) and thiocyanate (10 μM) did not show any appreciable interference in the analysis. AQDS (0.5 mM), added to mimic the effects of background chromophoric species in NOM samples, resulted in slightly lower emission intensities. However, the inflection point is still obtained at 0.5 μM with a near 100% accuracy (Fig. 2c). Direct titration of GSH in the bicarbonate buffer did not alter the measured thiol value either (Fig. 2c). Since natural waters are usually buffered by bicarbonate and carbonate, our results indicate that the method can be readily utilized for thiol measurements in natural waters with a simple pH adjustment to the optimum value of 6.6.

Sulfite (SO_3^{2-}) and sulfide (S^{2-}) are also commonly observed in natural water and sediments, typically produced by sulfate reducing bacteria under anoxic environments. Similarly to experiments described above we performed the titration of 0.5 μM GSH and evaluated the potential interference from these anions at varying concentrations (Fig. 2d). Sulfite at equal molar GSH concentration (0.5 μM) did not exhibit significant interference in the titration. However, at higher concentrations of SO_3^{2-} (≥2 μM) the relative slope of the fluorescence intensity at [TG-1] > [GSH] increased considerably, and with ≥10 μM SO_3^{2-} the inflection point became indistinguishable. Similarly the presence of S^{2-} (3 μM) resulted in an adverse effect on GSH measurement (Fig. 3d). The absence of inflection point, along with increasing emission intensity with TG-1 concentration, indicates that sulfides and sulfites can compete with thiols for reaction with TG-1 and can thus present a significant issue using this methodology. A recent study has also shown that sulfite can interfere protein thiol analysis using TG-1 reagent [24]. Our results nevertheless demonstrate that, with the exception of sulfite, sulfide and copper ions, the method is robust for quantitative analysis of thiols even in the presence of orders of magnitude higher concentrations of metal cations, anions, the amino, carboxylic, and other S-containing organic compounds.

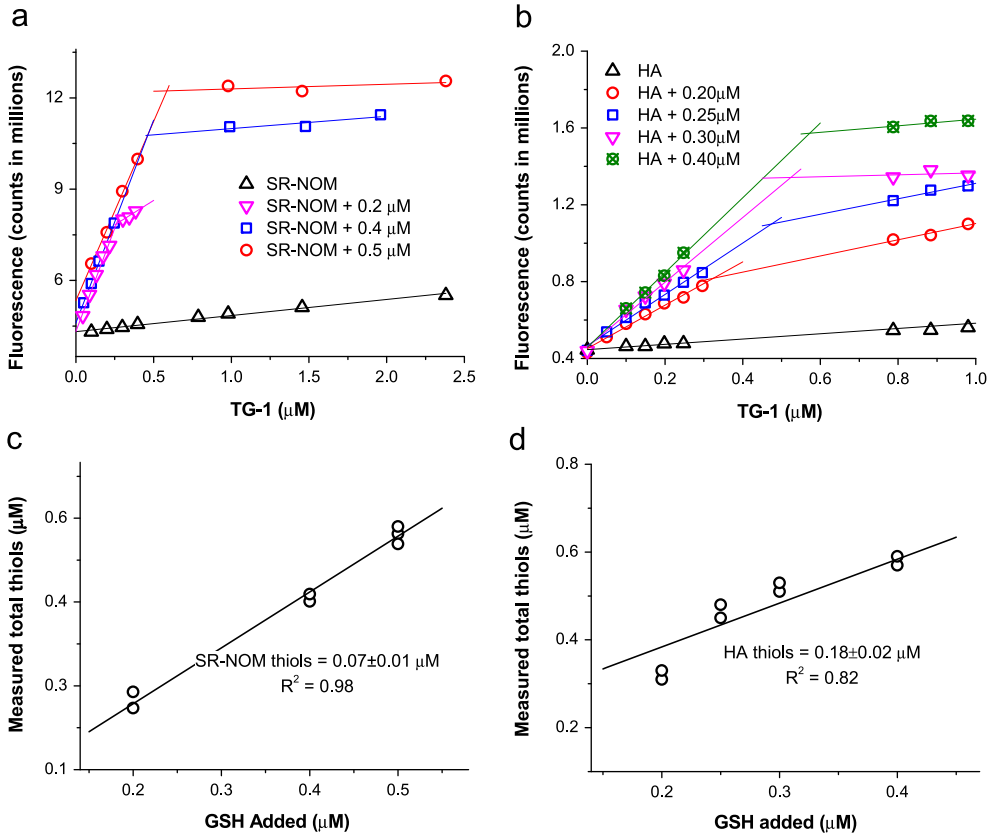

Fig. 3. TG-1 titration for total thiol determination in (a) reduced Suwannee River natural organic matter (SR-NOM) (100 mg L^{-1}) and (b) soil humic acid (HA) (50 mg L^{-1}). (c) and (d) are the corresponding standard addition plots of SR-NOM and HA with GSH (from 0.2 to 0.5 μM). The intercept is used to determine the total thiols in SR-NOM and HA.

3.3. Detection of thiols in NOM

We subsequently applied the titration method to determine the total thiols in naturally-occurring NOM samples including SR-NOM and soil HA (Fig. 3). These two NOM samples were chemically reduced and previously used in reactions with Hg under anoxic conditions [7,8,27]. Samples were prepared and titrated with TG-1 similarly as described above. An increase in the fluorescence intensity of both SR-NOM and HA was observed with increasing [TG-1] (Fig. 3a and b), but the slope appeared comparable to that of the TG-1 hydrolysis, indicating that the total thiol concentration in these samples is very low (<0.2 μM). NOM itself emits intense fluorescence due to its conjugated aromatic structural properties [28,29]. Increasing NOM concentrations thus simultaneously increase the inherent NOM fluorescence, which interferes with thiol measurements through titration. To overcome these problems, both the HA and SR-NOM samples were spiked with known amounts of glutathione (0.2–0.5 μM) followed by titration with TG-1 (Fig. 3a and b). The measured thiols in the spiked NOM samples were plotted against the concentration of added GSH and the intercept obtained by the linear regression was taken as the total thiol of the NOM (Fig. 3c and d). The calculated average thiol concentration in the reduced SR-NOM (100 mg L^{-1}) was 0.07 ± 0.01 μM (Fig. 3c). Similarly, we obtained an average thiol concentration in the HA sample at 0.18 ± 0.02 μM (with 50 mg L^{-1} HA) (Fig. 3d). This translates to a thiol content of 0.7 ± 0.1 and 3.6 ± 0.4 μmol g^{-1} for the SR-NOM and HA, respectively. The thiol value for HA is in a good agreement with the reported –SH value estimated based on its specific binding with Hg of the same HA (3.5–7.0 μmol g^{-1}) [8]. Similarly a thiol value of 4–5 μmol g^{-1} was reported for NOM samples isolated from different sources based on their reactions with Hg using equilibrium dialysis ligand-exchange (EDLE) techniques [30,31]. Other studies provided estimated thiols in NOM at 1.5–30 μmol g^{-1} [14,20,32,33] by assuming that NOM contains a total S of 0.44%, and 1–20% of the S is present as thiols [27]. If we assume an average thiol content of 1–5 μmol g^{-1} dissolved NOM, this translates to a detection limit of about 20–100 mg L^{-1} NOM (or ~10–50 mg L^{-1} dissolved organic C). Since thiols are unstable under oxidizing conditions, we caution that the measured thiol content in NOM can vary significantly depending on the source and oxidation state of NOM [8].

3.4. Detection of thiols on bacterial cells

We further tested the TG-1 titration method to determine total thiols on a bacterium *G. sulfurreducens* PCA, which has been widely studied for its roles in mercury methylation and reduction processes in the environment [34,35]. This was done first by suspending the wild-type PCA cells at 0.9×10^{13} cells L^{-1} in PBS in a series of vials, to which different concentrations of TG-1 were added. Similar to those observed with reference thiols (Fig. 1b), a plot of fluorescence intensity with TG-1 (Fig. 4a) showed a steep increase in emission intensity at low TG-1 concentration followed by a decreased slope at higher concentrations. This resulted in a distinct inflection point, where the total thiol concentration on PCA cells was determined to be 0.28 ± 0.03 μM (at 0.9×10^{13} cells L^{-1}). To ensure the measurement accuracy, the same titration experiments were performed by the standard addition method using 0.2 μM GSH in a series of the suspended PCA cells. In this case, the measured total thiol

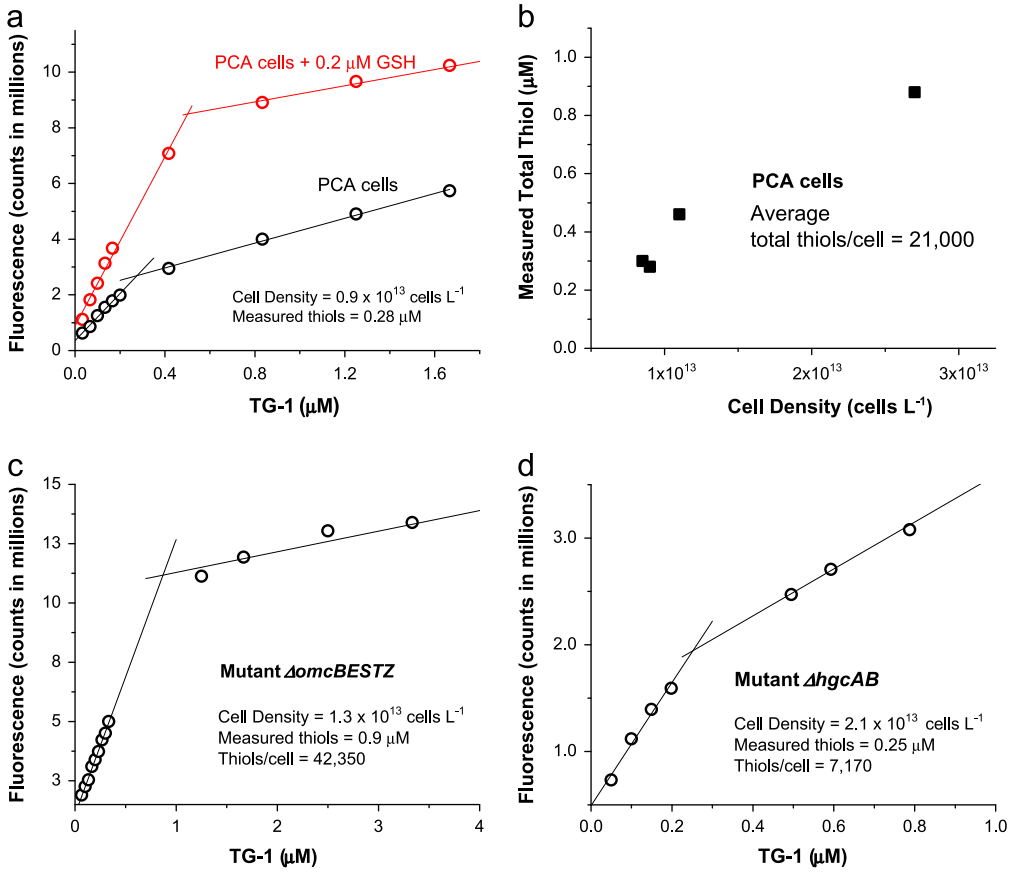

Fig. 4. (a) Determination of total thiols on *Geobacter sulfurreducens* PCA cells in PBS by both direct titration and standard addition with GSH (0.2 μM). (b) Total thiols on PCA cells determined by direct titration at varying cell concentrations (from 8×10^{12} to 3×10^{13} cell L^{-1}). (c) and (d) titration of two mutant strains of PCA, Δ*omcBESTZ* and Δ*hgcAB*, in PBS.

concentration was 0.49 ± 0.05 μM (Fig. 4a), which is approximately equal to the sum of GSH (0.2 μM) added and that present on PCA cells (0.28 μM). These results clearly demonstrate an excellent analytical accuracy for determining thiols on bacterial cells and a good recovery by the standard addition (105%). We next assessed the method robustness through measurements of total thiols at three different cell concentrations (ranging from 0.8×10^{13} to 3×10^{13} cells L^{-1}). Results showed a near linear relationship between the measured total thiols and the cell concentration (Fig. 4b), with an estimated average thiol concentration of 0.34 ± 0.05 μM at 10^{13} cells L^{-1}. This thiol concentration translates to $\sim 2.1 \times 10^4$ thiols $cell^{-1}$ or ~ 0.07 μmol g^{-1} wet cells. Using qBBr titration technique, Joe-Wang et al. [11] reported a total thiol concentration of ~ 25 μmol g^{-1} (wet cells) on an aerobic Gram-positive bacterium *B. subtilis*. This concentration is nearly 3 orders of magnitude higher than the value we measured on PCA cells. A direct comparison of these values may not be appropriate since bacteria species are different. However, our measured thiol content (2.1×10^4 thiols $cell^{-1}$) is in a good agreement with the value (1.1×10^4 thiols $cell^{-1}$) estimated indirectly during reactions between *G. sulfurreducens* PCA and Hg^{2+} [9]. Hu et al. recently reported that Hg(II) reduction increases with increasing cell concentrations initially, reaches a maximum, and then decreases with further increasing cells in the system [9]. This is because *G. sulfurreducens* PCA is known to reduce metal ions such as Hg^{2+} [34,36], but strong binding between Hg^{2+} and cell thiolates inhibits Hg reduction [9,37]. A nearly complete inhibition of Hg(II) reduction occurred at the cell concentration of $\sim 5 \times 10^{12}$ cells L^{-1}, which is equivalent to $\sim 1.1 \times 10^4$ thiolate binding sites per cell that were present to complex all the Hg (50 nM) [9].

To further demonstrate the applicability of this methodology, we analyzed the total thiol concentration in two mutant strains of *G. sulfurreducens* PCA, Δ*omcBESTZ* deficient in outer membrane *c*-type cytochrome genes [38] and Δ*hgcAB* deficient in mercury methylating genes [39]. The measured average thiol concentrations for Δ*omcBESTZ* and Δ*hgcAB* mutants were 0.74 ± 0.08 and 0.11 ± 0.01 μmoles at 10^{13} cells L^{-1}, respectively (Fig. 4c and d). The corresponding thiol number density per unit cell was calculated to be $\sim 4.5 \times 10^4$ and 0.7×10^4 thiols $cell^{-1}$ for Δ*omcBESTZ* and Δ*hgcAB*, respectively. The lower thiol density in strain Δ*hgcAB* than the wild type may be related to changes of the bacteria physiological properties by producing less thiols on the surface since the methylating genes are deleted [39]. Our preliminary results also indicate that the decrease in thiol content in strain Δ*hgcAB* led to an increased reduction rate of Hg(II), consistent with the notion that thiols form complexes and inhibit Hg reduction by cells [37]. The removal of c-type cytochromes however resulted in a higher thiol concentration in Δ*omcBESTZ* than the wild type and thus a decreased reduction rate of Hg.

4. Conclusions

In summary, we report the development of an improved method for determining total thiols on both intact bacteria cells and environmental NOM samples. The measured thiol values on *Geobacter sulfurreducens* cells and reduced soil HA are in good agreement with those values estimated based on reactions with Hg^{2+}, which form complexes stoichiometrically with thiols in both biotic and abiotic systems [2,4]. The primary advantage of this technique compared to a previous titration method using qBBr is its applicability in buffer or culture solutions that are necessary to maintain intact bacterial cells. Also, the method is specific to organic thiols, although relatively high levels of S^{2-} and SO_3^{2-} (> 2 μM) could significantly interfere with the analysis. Copper ions also interfere with the analysis, but this problem can be resolved by the addition of metal chelators such as EDTA. The successful determination of thiols in *Geobacter sulfurredences* PCA and its mutants shows that our method may be applied to other organisms with little or no sample preparation. The specificity and versatility of the methodology are especially important as it could help in providing insights into the mechanism of complex interactions between metals and thiols on bacteria. We recommend that the standard addition be used to avoid complex matrix interferences and to achieve a detection limit of thiols at low micromolar levels in environmental samples such as bacteria and NOM.

Acknowledgements

We thank D. R. Lovley and colleagues at the University of Massachusetts, Amherst, for providing the Δ*omcBESTZ* mutant, Richard Hurt Jr. and Dwayne Elias of ORNL for providing the Δ*hgcAB* mutant, and Xiangping Yin for technical assistance. This research was sponsored by the Office of Biological and Environmental Research, Office of Science, US Department of Energy (DOE) as part of the Mercury Science Focus Area (SFA) Program at ORNL, which is managed by UT-Battelle LLC for the DOE under Contract DE-AC05-00OR22725.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.11.004.

References

[1] A. Vairavamurthy, K. Mopper, Anal. Chim. Acta 236 (1990) 363–370.
[2] U. Skyllberg, A. Drott, Environ. Sci. Technol. 44 (2010) 1254–1259.
[3] R. Al-Farawati, C.M. van den Berg, Environ. Sci. Technol. 35 (2001) 1902–1911.
[4] M. Ravichandran, Chemosphere 55 (2004) 319–331.
[5] D.A. Dickinson, H.J. Forman, Biochem. Pharmacol. 64 (2002) 1019–1026.
[6] M. Soszyński, G. Bartosz, Free Rad. Biol. Med. 23 (1997) 463–469.
[7] W. Zheng, L. Liang, B. Gu, Environ. Sci. Technol. 46 (2011) 292–299.
[8] B. Gu, Y. Bian, C.L. Miller, W. Dong, X. Jiang, L. Liang, Proc. Natl. Acad. Sci. USA 108 (2011) 1479–1483.
[9] H. Hu, H. Lin, W. Zheng, B. Rao, X.B. Feng, L. Liang, D.A. Elias, B. Gu, Environ. Sci. Technol. 47 (2013) 10922–10930.
[10] H. Hu, H. Lin, W. Zheng, S.J. Tomanicek, A. Johs, X.B. Feng, D.A. Elias, L. Liang, B. Gu, Nature Geosci. 6 (2013) 751–754.
[11] C. Joe-Wong, E. Shoenfelt, E.J. Hauser, N. Crompton, S.C. Myneni, Environ. Sci. Technol. 46 (2012) 9854–9861.
[12] J.C. Harfield, C. Batchelor-McAuley, R.G. Compton, Analyst 137 (2012) 2285–2296.
[13] M. Man, R. Bryant, Anal. Biochem. 57 (1974) 429–431.
[14] A. Manceau, K.L. Nagy, Geochim. Cosmochim. Acta 99 (2012) 206–223.
[15] K. Kusmierek, G. Chwatko, R. Glowacki, P. Kubalczyk, E. Bald, J. Chromat. B 879 (2011) 1290–1307.
[16] X. Chen, Y. Zhou, X. Peng, J. Yoon, Chem. Soc. Rev. 39 (2010) 2120–2135.
[17] L.M. Laglera, A. Tovar-Sánchez, Talanta 89 (2012) 496–504.
[18] B. Pernet-Coudrier, M. Waeles, M. Filella, F. Quentel, R.D. Riso, Sci. Total Environ. 463–464 (2013) 997–1005.
[19] R.A. Winters, J. Zukowski, N. Ercal, R.H. Matthews, D.R. Spitz, Anal. Biochem. 227 (1995) 14–21.
[20] U. Skyllberg, P.R. Bloom, J. Qian, C.-M. Lin, W.F. Bleam, Environ. Sci. Technol. 40 (2006) 4174–4180.
[21] S.K. Wright, R.E. Viola, Anal. Biochem. 265 (1998) 8–14.
[22] M.E. Langmuir, J.-R. Yang, A.M. Moussa, R. Laura, K.A. LeCompte, Tetrahedron Lett. 36 (1995) 3989–3992.
[23] N. Ercal, P. Yang, N. Aykin, J. Chromatogr. B 753 (2001) 287–292.
[24] S. Hoff, F.H. Larsen, M.L. Andersen, M.N. Lund, Analyst 138 (2013) 2096–2103.
[25] R.E. Humphrey, M.H. Ward, W. Hinze, Anal. Chem. 42 (1970) 698–702.
[26] C.-W. Wu, L.R. Yarbrough, F.Y. Wu, Biochemistry 15 (1976) 2863–2868.
[27] W. Dong, L. Liang, S. Brooks, G. Southworth, B. Gu, Environ. Chem. 7 (2010) 94–102.
[28] J. Chen, B. Gu, E.J. LeBoeuf, H. Pan, S. Dai, Chemosphere 48 (2002) 59–68.
[29] J. Chen, E.J. LeBoeuf, S. Dai, B. Gu, Chemosphere 50 (2003) 639–647.
[30] W. Dong, Y. Bian, L. Liang, B. Gu, Environ. Sci. Technol. 45 (2011) 3576–3583.
[31] M. Haitzer, G.R. Aiken, J.N. Ryan, Environ. Sci. Technol. 37 (2003) 2436–2441.
[32] K. Xia, U.L. Skyllberg, W.F. Bleam, P.R. Bloom, E.A. Nater, P.A. Helmke, Environ. Sci. Technol. 33 (1999) 257–261.
[33] M. Haitzer, G.R. Aiken, J.N. Ryan, Environ. Sci. Technol. 36 (2002) 3564–3570.

[34] H.A. Wiatrowski, P.M. Ward, T. Barkay, Environ. Sci. Technol. 40 (2006) 6690–6696.
[35] E.J. Kerin, C.C. Gilmour, E. Roden, M. Suzuki, J. Coates, R. Mason, Appl. Environ. Microbiol. 72 (2006) 7919–7921.
[36] D.R. Lovley, S.J. Giovannoni, D.C. White, J.E. Champine, E.J.P. Phillips, Y.A. Gorby, S. Goodwin, Arch. Microbiol. 159 (1993) 336–344.
[37] B. Mishra, E.J. O'Loughlin, M.I. Boyanov, K.M. Kemner, Environ. Sci. Technol. 45 (2011) 9597–9603.
[38] J.W. Voordeckers, B.-C. Kim, M. Izallalen, D.R. Lovley, Appl. Environ. Microbiol. 76 (2010) 2371–2375.
[39] J.M. Parks, A. Johs, M. Podar, R. Bridou, R.A. Hurt, S.D. Smith, S.J. Tomanicek, Y. Qian, S.D. Brown, C.C. Brandt, Science 339 (2013) 1332–1335.