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Electrochemical Dithiothreitol Assay for Large-Scale Particulate Matter Studies

Kathleen E. Berg¹, Laurelle R. Turner², Megan L. Benka-Coker³, Sarah Rajkumar³, Bonnie N. Young³, Jennifer L. Peel³, Maggie L. Clark³, John Volckens^{3,4}, Charles S. Henry^{1,2,*}

¹Dept of Chemistry, Colorado State University

²Dept of Chemical & Biological Engineering, Colorado State University

³Dept of Environmental and Radiological Health Sciences, Colorado State University

⁴Dept of Mechanical Engineering, Colorado State University

Abstract

Particulate matter (PM) air pollution is associated with human morbidity and mortality. Measuring PM oxidative potential has been shown to provide a predictive measurement between PM exposure and adverse health impacts. The dithiothreitol (DTT) assay is commonly used to measure the oxidative potential of PM_{2.5} (PM less than 2.5 μm aerodynamic diameter). In the common, kinetic form of this assay, the decay of DTT is quantified over time (indirectly) using 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent) via UV/vis absorbance spectroscopy. The loss of DTT can also be quantified directly using electrochemical detection. The objectives of this work were (1) to evaluate the electrochemical assay, using commercially available equipment, relative to the UV/vis absorbance assay, and (2) to apply the electrochemical method to a large (>100) number of PM_{2.5} aerosol filter samples. Also presented here is the comparison an end-point assay to the kinetic assay, in an attempt to reduce the time, labor, and materials necessary to quantify PM oxidative potential. The end-point, electrochemical assay gave comparable results to the UV/vis absorbance assay for PM filter sample analysis. Finally, high filter mass loadings (higher than about 0.5 μg PM per mm^2 filter) lead to sub-optimal DTT assay performance, which suggests future studies should limit particle mass loadings on filters.

1. Introduction

Human exposure to particulate matter (PM) air pollution has been linked to adverse health effects and millions of premature deaths worldwide each year (Cohen et al. 2017; Kioumourtzoglou et al. 2016; Morakinyo et al. 2016). The toxicological mechanism(s) of PM are still being elucidated, but one common hypothesis is that PM generates reactive oxygen species (ROS) leading to increased oxidative stress and inflammation in the body (Alfadda and Sallam 2012; Anderson, Thundiyil and Stolbach 2012; De Vizcaya-Ruiz et al. 2006). PM oxidative potential, the PM's ability to oxidize target molecules, is arguably a more biologically relevant measurement of PM toxicity as opposed to PM mass alone (Borm

*Corresponding author: chuck.henry@colostate.edu, 1872 Campus Delivery, Department of Chemistry, Colorado State University, Fort Collins, CO 80523.

et al. 2007; Brook et al. 2010; Cho et al. 2005; Janssen et al. 2014). PM oxidative potential is hypothesized to be more relevant to human health because PM composition is not uniform, and each PM component has different reactivities with biological systems (Charrier and Anastasio 2012; Janssen et al. 2014).

The oxidative potential of a PM sample can be measured through antioxidant loss monitoring (Cho et al. 2005; Janssen et al. 2014). Crobeddu *et al.* (2017) argue that ascorbic acid and glutathione depletion assays show the best correlation to intracellular ROS. Other studies, however, have shown a lack of association between ascorbate and health outcomes, likely due to ascorbate's high reactivity in the presence of Cu(II) (Fang et al. 2015a; Vidrio, Jung and Anastasio 2008). Glutathione depletion is also sensitive to Cu and Fe (Godri et al. 2011). Another drawback of antioxidant depletion assays is the time required for sample incubation, which decreases the sample throughput. The glutathione depletion assay takes approximately four hours (Mudway et al. 2004), and the ascorbic acid assay takes one to two hours (Fang et al. 2015a; Janssen et al. 2014; Yang et al. 2014) but is often run simultaneously with glutathione for four hours (Ayres et al. 2008; Mudway et al. 2004). A more thorough comparison of PM oxidative potential measurements can be found elsewhere (Wang et al. 2018).

Dithiothreitol (DTT), a reducing agent known to react with a large number of chemicals relative to other antioxidants, is frequently used for *in vitro* quantification of PM oxidative potential (Charrier and Anastasio 2012; Charrier et al. 2016; Cho et al. 2005; Fang et al. 2015b; Xiong et al. 2017). In the DTT assay, PM oxidizes DTT to the disulfide, and the remaining DTT is quantified over time. DTT reactivity with PM has been correlated with relevant biomarkers of inflammation and oxidative stress in human studies (Delfino et al. 2013) and *in vivo* assays (Crobeddu et al. 2017; Li et al. 2003). DTT concentration is commonly quantified with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent), which reacts with DTT to form 2-nitro-5-thiobenzoic acid (TNB), a chromophore that can be quantified with 412 nm light. DTT concentration can also be directly measured via electrochemistry (Sameenoi et al. 2011). The electrochemical detection method yields comparable results as UV/vis detection but eliminates additional reagents and reduces PM sample mass requirements (Koehler et al. 2014; Sameenoi et al. 2012). While there are advantages to the electrochemical detection of DTT, the assay described by Sameenoi *et al.* is limited to those who have electrode and microfluidic device fabrication equipment, which involves a large, upfront investment of equipment and labor.

We present here a modified version of the electrochemical DTT assay developed by Sameenoi *et al.* using commercially available equipment. This new DTT assay uses a commercially available wall-jet flow cell with replaceable electrodes. While an end-point assay format can be used with UV/vis or electrochemical detection, it has not been applied to either DTT detection method to our knowledge. Kinetic and end-point assay formats were compared and gave comparable results. The single (post-initial) time point further simplifies the assay by reducing labor and consumable goods. The DTT reactivity of water-soluble extracts from PM_{2.5} filter samples collected during field studies in Honduras, separated into personal and area/indoor exposure, was also tested against the UV/vis assay developed by Charrier and coworkers (Charrier et al. 2016), modified from the original published UV/vis

assay developed by Cho and coworkers (Cho et al. 2005). The Charrier *et al.* assay modifications are the addition of 50:50 trifluoroethanol (TFE):water, before DTT addition, to the PM solution and the Chelex treatment of phosphate buffer to remove transition metals (where Cho *et al.* used EDTA). Unlike EDTA, the Chelex is filtered out prior to the DTT assay and will not interfere with the assay results. Here, unlike many previous assays, including those developed by Cho *et al.* and Charrier *et al.*, we do not use a chemical quenching agent, further reducing consumables. This study evaluates the combined effectiveness (accuracy, ease of use, reduced cost, and reduced assay time) of the electrochemical and end-point DTT assay on a large-scale study of aerosol filter samples, applicable to users without access to custom electrochemical methods.

2. Methods

2.1 DTT Assay

Electrochemical experiments were performed using a CHI 812 potentiostat (CH Instruments, Austin, TX). Purified (18.2 MΩ cm) water was used for all experiments (MilliPore Milli-Q system, Billerica, MA, USA). Dithiothreitol (DTT), 2,2,2-trifluoroethanol (TFE), and Chelex® 100 mesh were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium phosphate monobasic and sodium phosphate dibasic were purchased from Fisher Scientific (Pittsburg, PA, USA). 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent) was purchased from Pierce Chemical (Dallas, TX, USA).

Filter samples were handled with plastic forceps and ceramic scissors to minimize possible metal contamination (Charrier and Anastasio 2012). Prior results from Charrier *et al.* have suggested that targeting 10 µg/mL PM in the DTT assay gives the most consistent results (Charrier et al. 2016). Unless otherwise stated, the massed filter samples were cut to achieve the target concentration of 10 µg/mL PM using between 0.5 and 1 mL total volume of fresh 75 µM DTT in 0.1 M Chelex-treated sodium phosphate buffer (pH 7.4). The filters were initially wetted with 15 µL of 50:50 TFE:water (v:v) in a 1.5 mL centrifuge tube (Eppendorf, Hamburg, Germany). Samples were incubated in the DTT solution at 37 °C in a water bath for 30 min. The reaction was then effectively halted in a 5 ± 3 °C water bath for five min (Sameenoi et al. 2012). Sample aliquots were intermittently injected into the wall-jet flow cell (FLWCL, DropSens, Spain) at 85 µL/min. The working electrode (DropSens 410 SPE, DropSens, Spain) was held at +0.3 V vs. Ag/AgCl. A calibration curve was generated for each electrode used prior to running the assay. The relative value of initial peak currents were measured and adjusted to the lab blank filter reactivity rate, divided by the summation of the incubation and cooling time (35 min). For the UV/vis assay, 400 µL of 75 µM DTNB was added to discrete 100 µL DTT aliquots over a 45-min period (approximately 0, 15, 30, and 45 min) and analyzed immediately at 412 nm (Agilent 8453, Santa Clara, CA, USA) with a molar absorptivity of 14 150 M⁻¹ cm⁻² used for concentration calculations.

2.2 Filter Sampling

Air sampling filters (n=105 personal and n=106 area samples) were collected from March 3 through April 30, 2015 as part of a Honduras cookstove study as previously reported (Rajkumar et al. 2018). One blank filter was collected every two weeks. PM_{2.5} was sampled

onto 37-mm PTFE-coated glass fiber filters (Fiberfilm™ T60A20, Pall Corporation, KY, USA) using Triplex Cyclones (BGI, Mesa Labs, Butler NJ, USA) and AirChek XR5000 pumps (SKC Inc., Eighty Four, PA, USA) operating at 1.5 L/min for 24 hrs. The filters were equilibrated for at least 24 hrs and then pre-weighed to the nearest microgram at Colorado State University (CSU) using a microbalance (Mettler-Toledo microbalance model MX5, Columbus, OH, USA). After collection of the PM_{2.5} sample, filters were stored at −22 °C and then transported to CSU, equilibrated, and post-weighed. The filters were then stored in a −80 °C freezer until tested. The whole filter particle mass is the difference in masses before and after collection, accounting for the mean change in field blanks. Personal filters were collected near the participant's breathing zone; area filters were collected 76–127 cm from the stovetop in the participants' kitchens. Each household had one personal and one area exposure filter; therefore the two data sets from personal and area exposure are not independent from each other.

3. Results and Discussion

3.1 Assay Validation

The electrochemical DTT assay has been reported as an accurate, precise, and simple assay that also uses less consumable materials than the UV/vis absorbance assay by eliminating DTNB addition and lowering the DTT solution volumes (Koehler et al. 2014; Sameenoi et al. 2012). The first assay steps (extracting the filter, adding DTT, and removing aliquots) are comparable between the absorbance and electrochemical assays (Figure 1). The hydrophobic filter is initially wetted with a TFE/water solution and then extracted into a phosphate buffer solution of DTT (Vidrio et al. 2009). The timing of the removal of aliquots is discussed below. However, with the electrochemical system, flow injection analysis is used and the aliquot is directly injected into the detector system in a similar manner as described by Sameenoi *et al.* (2012). The direct DTT injection replaces indirect detection with the DTNB chromophore, eliminating the need for extra reagents, decreasing assay costs, and removing the added labor and uncertainty associated with indirect detection. For the electrochemical system presented here, only commercially available equipment, a DropSens poly(methyl methacrylate) (PMMA) wall-jet flow cell with DropSens screen-printed carbon electrodes with a built-in reference electrode, was used to validate the electrochemical assay. While we opted to use DropSens components, there are numerous commercially available potentiostats, electrodes, and flow cells that could achieve the same results. Our system has a limit of detection (LOD), calculated as three times the standard deviation of the blank, of 8.45 μM DTT. The current signal was $0.0137 \pm 0.0006 \mu\text{A}/\mu\text{M DTT}$ (n=24). Additionally, one person can analyze five samples per hour using the electrochemical flow cell system, which is a higher rate than the approximate one sample per hour with either the two-person manual or semi-automated absorbance assays reported previously (Fang et al. 2015b).

Besides the electrochemical assay presented here eliminating the time, labor, and consumables associated with preparing, adding, and mixing a quenching and developing reagent, we have combined it here with an end-point assay to further reduce the time and labor. End-point assays are commonly used to simplify kinetic analyses (Boozer et al. 2006; Daniel et al. 2009; Giuliano, Haskins and Taylor 2003; Karadag, Ozcelik and Saner 2009).

The original assay continually removes aliquots from the DTT chemical reaction, which leads to additional labor, time, and materials (Cho et al. 2005). In the example of the semi-automated system developed by Fang *et al.* (Fang et al. 2015b), there would still be a benefit from eliminating the reagents and measuring fewer time points because the reagent solutions would not have to be purchased or prepared, and assay time would be reduced from about 60 to 30 minutes per sample, potentially leading to higher sample throughput at a lower cost. To help reduce time and labor requirements that limit large-scale aerosol studies, we evaluated the ability to reduce the number of time points needed for the DTT reaction rate calculation. We compared removing multiple aliquots over time to using one, post-initial, time point, also referred to as an end-point assay. The end-point assay principle can be applied to both the electrochemical and UV/vis assays due to the linear reactivity of DTT used in the DTT assay. With the linear reactivity of DTT and having a proper calibration curve for accurate and precise measurements (Noblitt et al. 2016), the DTT rate can be accurately calculated from two time points. Replicates at each time point are still needed to decrease the uncertainty. The DTT reactivity of the same filter was used at two different PM concentrations, 5 and 10 $\mu\text{g/mL}$, with the multiple aliquot (kinetic) and the end-point assays. As seen in Figure 2, measured DTT consumption rates between the end-point and kinetic assays fall along the same line (for a given PM concentration). The DTT reactivity of both PM filter pieces at 5 $\mu\text{g/mL}$ was 509 ± 9 ($n=12$ for each assay type) nM DTT min^{-1} , and both 10 $\mu\text{g/mL}$ PM DTT reactivities were 740 ± 20 ($n=9$ for each assay type) nM DTT min^{-1} . The end-point assay was therefore used during the rest of this study.

3.2 Evaluation of Mass-Normalized Bias

Potential DTT reactivity bias in a mass-normalized PM system has recently been reported (Charrier et al. 2016). Charrier *et al.* show that DTT reactivity is not always linear with extracted PM concentration, most likely due to high Cu concentration in the samples, as Cu(II) has a non-linear reactivity with DTT, yielding higher DTT reactivity at lower PM concentrations when it is PM-mass normalized. Charrier *et al.* state the need for 10 $\mu\text{g/mL}$ extracted PM concentration to be compared between studies to reduce the mass-reactivity bias. The linearity of several samples was tested for bias with the mass-normalized response (Figure 3). Three of the four filter samples show no mass-normalized (i.e., the 95% confidence interval about the slope contains zero). One filter, F031, had a significant negative slope with a linear fit (-0.004 ± 0.001 , $R^2=0.88$) but has an improved coefficient of determination with a non-linear fit. A power function fit yields a coefficient of determination of 0.95. This is likely due to a higher Cu concentration in the filter sample, yielding a non-linear mass-normalized response, as seen previously (Charrier et al. 2016). To help alleviate the potential bias in the large-scale study, a PM concentration of 10 $\mu\text{g/mL}$ was used for evaluation of real filter samples, as suggested by Charrier *et al.*

3.3 Evaluation of Honduras Cookstove Study $\text{PM}_{2.5}$ Filter Samples

The electrochemical and absorbance assays have previously been shown to give similar results (Sameenoi et al. 2012). Here, the modified electrochemical DTT assay was also compared against the UV/vis assay with real aerosol filter samples using Deming regression analysis (Cornbleet and Gochman 1979), which incorporates the error of both methods into the fit. The same filters were used for comparison, and the extraction technique was the

same for both assays. The Deming regression best fit results in a line with a slope of 1.05 and an intercept of 0.119 (Figure 4), and the correlation coefficient of 0.79. The standard deviation values of the two assays should not be directly compared as the electrochemical assay utilized lab blanks (filters that were not exposed to the field), and the absorbance assay utilized field blanks, as either can be used with the DTT assay.

After comparing the modified electrochemical DTT assay to the UV/Vis assay, the practicality of using the electrochemical assay with a large sample size was addressed. In this study, we tested 211 filters collected in Honduras as part of an epidemiology cookstove project (Rajkumar et al. 2018). The filter samples were separated into area and personal exposures. The average area exposure DTT reactivity rate ($\text{nmol DTT min}^{-1} \text{ m}^{-3}$) was 9.38 with a reactivity range between 0.460 and 31.8. Personal exposures showed an average DTT reactivity rate ($\text{nmol DTT min}^{-1} \text{ m}^{-3}$) of 5.90 with a reactivity range between 1.03 and 18.4.

Even though the same PM concentration was used, there was a limitation found within this filter sample set. It has been assumed that the DTT reactivity is unaffected by the collected filter's whole filter particle mass (WFPM, the filter's change in mass during collection), and to our knowledge, this assumption has not been tested. With the assumption that the total collected PM mass does not affect the DTT assay results, as long as the extracted PM concentration is consistent, a linear relationship would be expected between the WFPM-normalized reactivity (DTT reactivity multiplied by WFPM) relative to the WFPM of the filters, with the resulting slope being equivalent to the average DTT reactivity of the samples. However, a strong linear relationship is not seen with the filters tested here (Figure 5) as the coefficients of variation, R^2 , are 0.33 and 0.27 for the area and personal sample sets, respectively. With the area exposure filter samples (Figure 5a), a deviation from linearity is clearly seen above about 0.5 mg WFPM. The non-linearity above 0.5 mg could be explained by the large mass loadings on the filter hindering mass transport of the PM into the bulk solution and/or inaccessibility of the DTT to PM buried on the filter, causing lower reactivity per PM mass than expected. This limit to mass loadings could also simply be a limitation of the extraction method used here. The same deviation from linearity is not clearly seen with the personal exposure filters (Figure 5b) as the PM mass loadings do not exceed 0.8 mg and less than 10% of the filter mass loadings are greater than 0.5 mg. The filter type and the PM source, as the samples tested here were driven by wood burning, might also have an influence on the value where the deviation from linearity is seen. Future work is required to confirm if the deviation from linearity at high WFPM is from the mass transport or the extraction method used here. If it is a mass transport problem, future PM studies should be cognizant of the mass loadings onto the filter and the bias it introduces into the DTT assay. If the extraction method presented here is used, then mass loadings should remain smaller than about 0.5 mg per 37 mm filter, equivalent to $0.5 \mu\text{g PM per mm}^2$ of filter, but future work will solidify this cautionary statement. If another extraction method is used, then the linearity of WFPM-normalized reactivity should still be tested in the range of filter mass loadings to confirm no bias with high WFPM.

4. Conclusion

In this work, we developed an electrochemical system to directly measure DTT using commercially available electrodes in a wall-jet flow cell. Our system was validated through comparison to the UV/vis assay, and this work is the first time, to our knowledge, that the electrochemical DTT assay has been applied to a large sample size (211 samples). The time and labor was reduced by using an end-point assay instead of the kinetic assay. The presented end-point assay can be used in the future during a UV/vis detection DTT assay. The assay showed deviation from expected linearity between filter PM_{2.5} mass and the WFPM-normalized DTT reactivity with high mass loadings likely due to a slow mass transport or the inability of DTT to react with PM components. Future studies will need to address the observed lower reactivity at high mass loadings by limiting mass loadings onto filters. Future work will need to test if the linear deviation is from the PM source, filter type, extraction method, or another factor. The end-point electrochemical assay presented here increases the sample throughput to five samples per hour, but future work should seek to improve sample throughput further.

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Figure 1:

Schematic of the filter extraction and electrochemical assay. A filter piece is cut and massed, extracted into TFE/water, then DTT in phosphate buffer is added for a known PM concentration. The solution is incubated at 37 °C and then thermally quenched in 5 °C fridge. Aliquots are removed and analyzed with flow injection chronoamperometry. Example data shown where the peak current is decreasing over time, with replicates.

Figure 2:

Comparison of DTT loss with the end-point and kinetic assays for 2 different PM concentrations: 5 $\mu\text{g/mL}$ (orange circles and navy triangles, respectively, $509 \pm 9 \text{ nM min}^{-1}$ loss rate) and 10 $\mu\text{g/mL}$ (red squares and blue diamonds, respectively, $740 \pm 20 \text{ nM min}^{-1}$ loss rate). Error bars represent the standard deviation of three replicates.

Figure 3:

Determining bias in mass-normalized response with four random filter samples (F013, F031, F096, H125) at various PM concentrations. Filters F013, F096, and H125 have no significant slope at a 95% confidence level. Filter F031 has a power fit of $y = 0.2403x^{-0.694}$ with R^2 of 0.95. Error bars represent standard deviation of three replicates.

Figure 4:

Comparison of filter DTT reactivity of the absorbance assay with electrochemical assay using Deming regression (dashed line is the best fit from the regression analysis, $y=1.05x+0.119$) of 19 filter samples. $R^2=0.63$. Error bars represent standard deviation of three replicates of three different filter pieces.

Figure 5:

Comparison of normalized reactivity vs. whole filter particle mass in a) 106 area and b) 105 personal filter samples. Normalized reactivity is the DTT reactivity rate (nmol DTT min⁻¹ mg⁻¹ PM m⁻³ air) multiplied by the whole filter particle mass (mg PM). The units have been simplified in regards to grams of PM. Error bars represent three replicates of three filter sections.