

RETHINKING THE DITHIOTHREITOL BASED PM OXIDATIVE POTENTIAL:
MEASURING DTT CONSUMPTION VERSUS ROS GENERATION

BY

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THESIS

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Abstract

Air pollution has been proved to be linked with adverse health effect through a large body of studies. This thesis focuses mainly on a major air pollutant, PM 2.5 (particulate matter with aerodynamic diameter less than 2.5 μ m), which has been associated with rising morbidity, increased possibility of obesity, respiratory diseases. The mechanism of how PM2.5 brings impact to human's health has been studied intensively in recent years but the story behind it remains unclear. One hypothesis is that ROS (reactive oxygen species) catalyzed by ambient particulate matter will introduce excessive oxidative stress and further disrupt and destroy the cell. The thesis targeted to measure a specific ROS, hydroxyl radical (\cdot OH) in dithiothreitol (DTT) assay. DTT assay has been a widely-used probe to measure the oxidative potential as it measures the consumption rate of DTT which presumably corresponds to the generation rate of a specific ROS - superoxide radicals ($O_2^{\cdot-}$). However, this conventional assay fails to capture the most damaging ROS - \cdot OH. Rather than taking an indirect measurement of oxidative potential introduced by ROS, this study directly measured the generation rate of \cdot OH. Sodium terephthalate, which is able to oxidize \cdot OH forming 2-hydroxyterephthalic acid (2-OHTA) was chosen as the probe to measure the concentration of generated \cdot OH. 2-OHTA, a fluorescent compound, can be easily measured by a spectrofluorometer at a wavelength of 425nm with the excitation wavelength at 310nm. Several environment-related organic compounds (9,10-phenanthrenequinone, 5-hydroxy-1,4-naphthoquinone, 1,2-naphthoquinone, 1,4-naphthoquinone) were tested to study the pattern of the generation of \cdot OH. Metals such as copper(II), Manganese(II) and iron (II) which exist in the ambient particulate matter abundantly were also examined in this study. Surprisingly, different patterns were observed for both pure compounds and mixtures in DTT oxidation versus \cdot OH generation. The efficiency order of quinones in \cdot OH generation is 5H-1,4NQ>1,2-NQ>PQ>1,4-

NQ, which is different from the efficiency order (PQ>5H-1,4NQ>1,2-NQ>1,4-NQ) in DTT oxidation. Cu(II), which is known to be a dominant metal in DTT oxidation at atmospherically relevant concentration (1 μ M) contributes almost negligibly to the \bullet OH generation. Fe(II), which is mostly inactive in both DTT oxidation and \bullet OH generation, with the presence of the other quinones it showed strong synergistic effect in \bullet OH generation (mixture/sum = 1.6 \pm 0.2, 2.0 \pm 0.3, 1.6 \pm 0.3 and 2.2 \pm 0.3 for PQ, 1,2-NQ, 1,4-NQ and 5H-1,4NQ, respectively). Fenton reaction is responsible for the synergistic effect observed, as superoxide radicals ($O_2^{\bullet-}$) formed by quinones are efficiently converted to \bullet OH by Fe(II) through Fenton reaction. Ten ambient PM samples collected from an urban site were analyzed to investigate the correlation between DTT oxidation and \bullet OH generation and no correlation was observed. The results show that DTT consumption and ROS-generation are two different aspects in DTT assay. Measuring both in the same assay is important to incorporate the synergistic contribution from different aerosol components and thus to provide a more comprehensive picture of the ROS activity of ambient aerosols.

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1. Introduction

Fine particulate matter (PM_{2.5}) have been associated with adverse health endpoints in humans.¹⁻⁴ Since ambient PM composes of many constituents, the toxicity of PM is regarded as a multi-faceted phenomenon.⁵ The toxicity of PM could be induced by the components either individually or synergistically. Most of the recent studies seem to propose the generation of reactive oxygen species (ROS) catalyzed by ambient particulate as the initiating step of the PM toxicity ladder.⁶⁻¹¹ Oxidative stress induced by PM causes disruptions in normal mechanisms of cellular signaling to many pathophysiological conditions in the body such as neurodegenerative and inflammatory diseases.¹²⁻¹⁵

Recognizing the importance of how ROS catalyzed by ambient particulate affect human's health, a variety of chemical probes have been developed to measure the oxidative potential of ambient particles.¹⁶⁻²¹ All these assays simulate the cellular environment to the maximum extent possible in a chemical system. Oxidative stress induced by PM could be measured through a variety of assays either directly or indirectly such as high-performance liquid chromatography,²² spin trapping, electron spin resonance spectroscopy,^{23,24} fluorescence based method^{25,26} or the measurement of rate of loss of antioxidants.^{17,27-29} Since Various particle components participate differentially in the reactions simulated by these assays, there is an inconsistent relationship between the chemical composition and measured assay response. For instance, ascorbate assay appeared to be responsive mostly to Cu^{18,29} while citrate assay is most sensitive to Fe,³⁰ and in glutathione oxidation both Fe and Cu seem to play important roles.³¹

DTT assay has been found to be responsive with a large pool of chemical components such as many aromatic hydrocarbons and two transition metals - Cu and Mn.³² One previous study³³

measured a large number of PM samples in both DTT assay (DTT oxidation rate) and ascorbate assay (ascorbate activity) showed that while DTT activity was associated with three major emission sources, i.e. vehicular emissions, biomass burning and secondary formation, only vehicular emissions (a major source of Cu) was associated with the response of ascorbate assay. Moreover, DTT activity has been shown to be most associated biological endpoints [hemeoxygenase-1 (HO-1) expression, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction activity, fraction of nitric oxide in exhaled breath, and increased relative risk for asthma/wheeze and congestive heart failure] in several toxicological,³⁴ clinical^{8,35} and epidemiological studies.^{7,10} However, DTT assay is not able to capture the generation of hydroxyl radical which is of great importance in ROS cascade. $\cdot\text{OH}$ is the most damaging ROS,³⁶ with an extreme short half-life (approximately 10^{-9} seconds) in vivo.³⁷ In the conventional DTT assay protocol, only the decay rate of DTT is measured. The DTT oxidation rate presumably corresponds to the formation rate of $\text{O}_2^{\cdot-}$, without consideration of the formation of $\cdot\text{OH}$. This could lead to a fatal disadvantage of DTT assay which causes an underestimation of the role of important metals such as Fe in DTT assay which is known to catalyze the ROS generation in biological system.³⁸

This thesis mainly focus on the formation of $\cdot\text{OH}$ in DTT assay to compensate for its limitation. To better understand the mechanisms of ROS generation, several quinones (PQ, 1,4-NQ, 1,2-NQ and 5H-1,4NQ) and metals [Mn(II), Fe(II) and Fe(III)] which are known to be present in the ambient PM were tested for their capability to generate $\cdot\text{OH}$ in DTT assay. The interaction between Fe and quinones was also investigated. To compare the ROS generation and DTT consumption, the rate of DTT oxidation was also measured both from individual compounds and ambient PM corresponds to $\cdot\text{OH}$ formation. The primary objective is to improve the capability of the DTT assay

to measure the ROS generation potential of ambient PM by incorporating the contribution from more PM components.

2. Experimental methods

2.1 Reagents

Dithiothreitol (assay: $\geq 98\%$ TLC), 5,5'-dithiobis (2-nitrobenzoic acid) (assay: $\geq 98\%$), 9,10-Phenanthrenequinone (assay: $\geq 99\%$), 5-hydroxy-1,4-naphthoquinone (assay: 97%), 1,2-naphthoquinone (assay: 97%), 1,4-naphthoquinone (assay: 97%), potassium phosphate dibasic (assay: $\geq 98\%$), potassium phosphate monobasic (assay: $\geq 98\%$), iron(II) sulfate heptahydrate (assay: $\geq 99\%$), copper(II) sulfate pentahydrate (assay: $\geq 98\%$), and Manganese(II) chloride tetrahydrate (assay: $\geq 98\%$) were obtained from Sigma-Aldrich Co. (St.Louis, MO). Disodium terephthalate (assay: 99+%) was bought from Alfa Aesar Co. (Haverhill, MA). All the chemicals bought were of the highest purity available.

2.2 Measurement of $\cdot\text{OH}$

A fluorescence-based approach was used to measure the $\cdot\text{OH}$ generated in the DTT assay. The assay was developed based on previous study from Son et al.³⁹ Four probes [disodium terephthalate (TPT), 3'-p-(aminophenyl) fluorescein, coumarin-3-carboxylic acid and sodium benzoate] for measuring the $\cdot\text{OH}$ catalyzed by ambient PM in the ascorbate assay have been analyzed in his study. Based on several criteria, the TPT was recommended as the most specific, stable and sensitive probe among the four probes.³⁹ The same TPT approach was adopted in this thesis. TPT was added in the reaction vial for capturing the $\cdot\text{OH}$ generated in DTT assay, which forms 2-hydroxyterephthalic acid (2-OHTA). 2-OHTA is a highly fluorescent compound which can be easily measured by spectrofluorometer. The excitation wavelength for 2-OHTA is set at 310nm wavelength and the emission intensity is measured at 425nm. Since the formation of 2-

OHTA is proportional to the generation of $\cdot\text{OH}$, the concentration of 2-OHTA is used to represent the formation of $\cdot\text{OH}$ in all the figures.

2.2.1 Individual compounds or their mixtures

The total reaction volume was 12mL and the reaction was incubated at a temperature of 37°C and pH of 7.4 (maintained by potassium phosphate buffer; 2mL) in a continuously shaken conical centrifuge vial (presterilized polypropylene tubes) using a thermomixer (Eppendorf). 1.2mL of 1mM DTT (final concentration in the reaction vial = 100 μM), 2.4mL of 50mM TPT (final concentration in the reaction vial = 10mM), 2mL potassium phosphate buffer and 6.4mL sample (either individual quinones, Fe, Mn, Cu or their mixtures) constitutes the reaction volume. At designated time intervals (0, 30, 60, 90, 120, 180 minutes for quinones, Fe and their mixtures; 0, 60, 120, 180, 240, 300 minutes for Cu and 0, 60, 120, 180, 240, 300, 360, 420, 480 minutes for Mn), 2mL of the incubating mixture was withdrawn and mixed with 1mL of the 100mM DMSO to quench the reaction. The emission intensity of the fluorescent product 2-OHTA was measured by a Shimadzu spectrofluorophotometer (RF-5301pc). Both of the excitation and emission slit widths were set at 5nm. Each test was accompanied with a blank (Milli-Q water; resistivity = 18.2M Ω cm), analyzed in the same way as sample, by replacing the sample volume with Milli-Q water.

2.2.2 Ambient PM_{2.5} samples

Ambient PM_{2.5} were collected using a High-Volume sampler (HiVol, Thermo Anderson, nondenuded, nominal flow rate 1.13m³min⁻¹, PM_{2.5} impactor) from an urban site. The PM_{2.5}

impactor is located on the roof with a height of approximately 30m from ground level of a parking garage. The parking garage which is adjacent to University Ave. (a major four-lane street in the town), is located in the northern part of University of Illinois Urbana-Champaign (UIUC) campus. The site is about 1km from downtown Champaign and is surrounded by dense housing and business development. Vehicular emission is expected to contribute significantly to the PM collected as slow moving traffic is typical during the rush hours. Besides, business development impacts the PM due to the construction activities. Particles were collected on prebaked (550°C) 8''x10'' quartz filters (Pallflex Tissuquartz, Pall Life Sciences). From June 27, 2016 to August 12, 2016 ten samples along with field blanks were collected, date and time are given in Table 1 (see below), each for an exact duration of 24 hours. After collection the filters were immediately kept in a freezer at temperature of -20°C. Within a week, the sample was analyzed for ROS generation and DTT oxidation.

To measure the $\cdot\text{OH}$ generation from PM samples, twenty punches (each 1'' diameter) were taken from the filter and extracted in 30mL Milli-Q water by sonication for 30 minutes. These extracts were filtered using PTFE syringe filters (0.45 μm pore size; Fisherbrand). 6.4mL of extract was used for measuring the $\cdot\text{OH}$ and the rest for DTT oxidation, in the same manner as pure compounds. Each sample was analyzed with a corresponding blank filter extracted along with the PM sample, and all the data was blank corrected.

Table 1. Details of Ambient PM Samples Collection and Analysis

Sample ID	Start Time	End Time	Analysis Date
1	07/27/2016 12:01pm	07/28/2016 12:01pm	07/29/16
2	07/28/2016 12:50pm	07/29/2016 12:50pm	08/01/16
3	08/01/2016 11:02am	08/02/2016 11:02am	08/03/16
4	08/02/2016 11:50am	08/03/2016 11:50am	08/04/16
5	08/03/2016 12:38pm	08/04/2016 12:38pm	08/06/16
6	08/04/2016 13:38pm	08/05/2016 13:38pm	08/09/16
7	08/08/2016 10:55am	08/09/2016 10:55am	08/10/16
8	08/09/2016 11:49am	08/10/2026 11:49am	08/11/16
9	08/10/2016 12:20pm	08/11/2016 12:20pm	08/12/16
10	08/11/2016 13:05pm	08/12/2016 13:05pm	08/15/16

2.3 DTT consumption rate

The rate of DTT consumption for the samples (pure compounds, their mixtures, and the ambient PM samples) were measured by using an automated instrument developed in the aerosol lab at UIUC and based on the protocol discussed in Fang et al.¹⁹ The instrument adopts a two-step approach. In the first step, a mixture containing PM extract (3.5mL), DTT (0.5mL of 1mM solution, final concentration in the reaction vial = 100 μ M), and potassium phosphate buffer (1mL; pH=7.4) was incubated in a 15-mL conical centrifuge vial, kept in a thermomixer (550rpm and 37°C). In the second step, at specified time points (3, 19, 27 and 35 minutes), an aliquot (40 μ L) of the incubated mixture was withdrawn and mixed with DTNB (0.2mM, 0.5mL), to form 2-nitro-5-thiobenzoic acid (TNB). The light absorption of TNB was measured at 412nm wavelength by a Liquid Waveguide Capillary Cell (LWCC-M-100; World Precision Instruments, Inc., FL, USA), coupled to an online spectrophotometer (Ocean Optics, Inc., Dunedin, FL, USA), which included

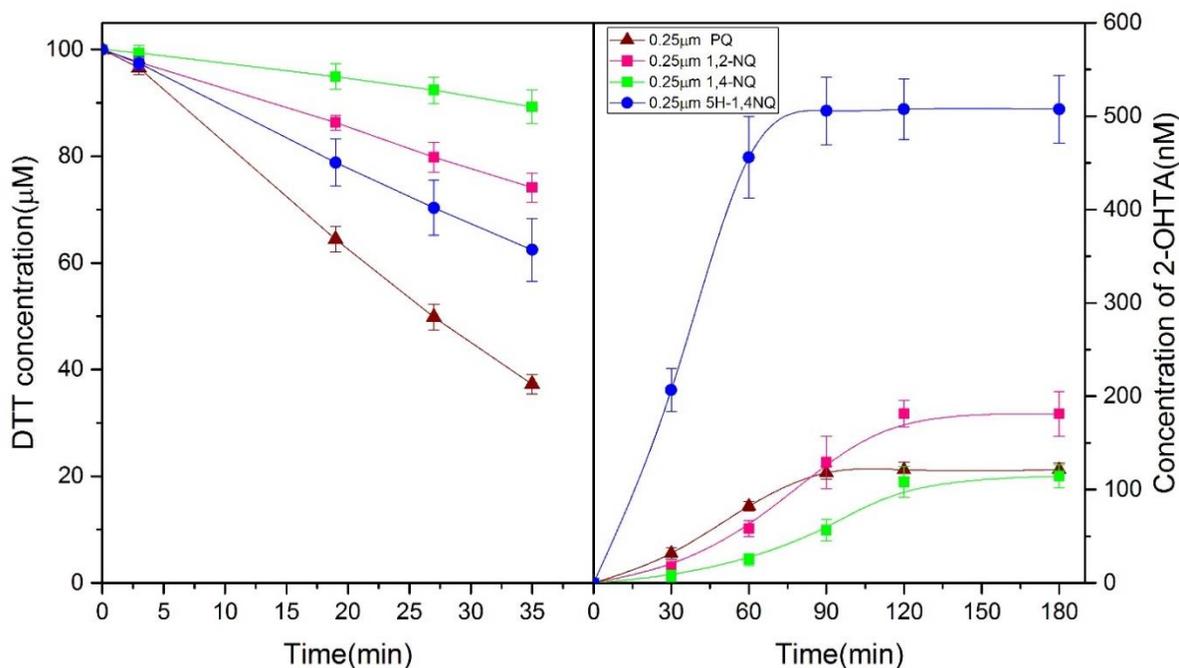
an ultraviolet–visible (UV–Vis) light source (Ocean Optics DH-Mini), and a multi-wavelength light detector (Flame).

3. Results and discussions

3.1 Individual quinones

Quinones and their derivatives are ubiquitous in the ambient atmosphere. Quinone has been approved to be highly efficient in DTT oxidation. Most studies show that primary quinones (contribute < 20%) do not contribute as significant as oxygenated derivative to the water-soluble DTT activity of ambient PM.^{32,40} Several quinones, e.g. 1,2-NQ, 1,4-NQ, PQ, 5,12-Naphthacenequinone (NCQ), Acenaphthequinone (ANQ) and Benzoanthraquinone (BAQ)⁴⁰ have been detected in ambient particles, but only 1,2-NQ, 1,4-NQ, and PQ have significant contribution to DTT activity at their atmospherically relevant concentration.

Figure 1. Pattern of DTT oxidation and $\cdot\text{OH}$ generation (expressed in terms of the concentration of 2-OHTA) from various quinones



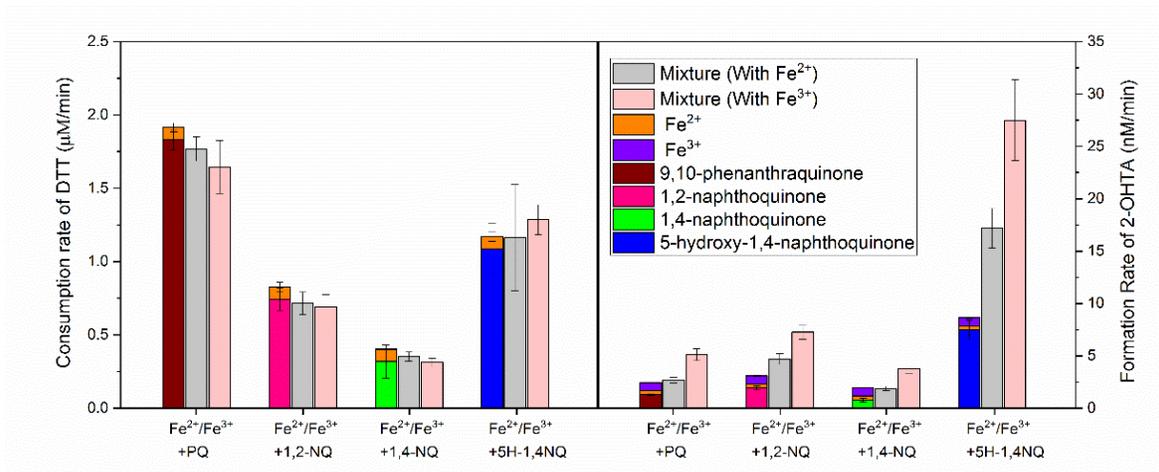
Currently, most of the studies examining the redox capabilities of quinones using DTT assay are focusing on the consumption rate of DTT. Some researches have measured either the rate of formation of $O_2^{\cdot-}$ or hydrogen peroxide (H_2O_2).⁴¹⁻⁴³ However, there is no solid evidence showing the formation pattern of $\cdot OH$ is similar as other reactive oxygen species in DTT assay. Here we measured the rate of generation of $\cdot OH$ by quinones in the DTT assay. We tested on PQ, 1,2-NQ, 1,4-NQ and 5H-1,4NQ as they showed significant contribution to DTT activity in previous studies. As shown in Figure 1, all four quinones are highly efficient in catalyzing the generation of $\cdot OH$ in DTT assay. As a comparison between the pattern of DTT oxidation and $\cdot OH$ formation, the rate of DTT oxidation from these quinones is also shown in Fig 1. For DTT consumption rate, the pattern of all these quinones can be approximated as linear line. However, for three quinones (1,4-NQ, 1,2-NQ and PQ), the rate of $\cdot OH$ formation has an S-shaped curve. The S-shaped pattern is characterized by a slow formation rate at the beginning, followed by a sharp increase in the second phase. It eventually reaches a plateau when the concentration of $\cdot OH$ remain the same. Shang et al.⁴⁴ and Sanchez-Cruz et al.⁴⁵ have proposed the mechanism of metal independent formation of $\cdot OH$ from 1,4-NQ and PQ. Both of the studies suggested the formation of a semiquinone anion radical in the redox cycling of quinone.^{44,45} The semiquinone radical further triggers the reduction of H_2O_2 which is the major mechanism for $\cdot OH$ formation.^{44,45} In the initial phase, the $\cdot OH$ generation rate is comparatively slower possibly due to the relative lower concentrations of both of these intermediates. However, 5H-1,4NQ shows a different pattern from these quinones. The pattern could be characterized as an exponential curve without an apparent lag period in the $\cdot OH$ generation.

Apparently, for the tested four quinones, DTT oxidation and $\cdot OH$ formation do not correspond with each other. PQ is the most efficient one among the four quinones in oxidizing DTT. However,

it does not appear to be the most efficient one in generating $\cdot\text{OH}$. On the other hand, 1-4NQ is the least efficient in oxidizing DTT, although at the initial stage the formation rate of $\cdot\text{OH}$ is slow, it eventually generates almost equal amount of $\cdot\text{OH}$ as PQ. 5H-1,4NQ is the most efficient one in generating $\cdot\text{OH}$, both in terms of the formation rate as well as the total amount.

3.2 Role of iron

Figure 2. Rate of DTT oxidation and $\cdot\text{OH}$ generation (expressed in terms of the concentration of 2-OHTA) from various quinones, Fe (II)/Fe(III) and their binary mixtures (i.e. PQ+Fe, 1,2-NQ+Fe, 1,4-NQ+Fe, and 5H-1,4NQ+Fe)



Although DTT assay has been widely used, the assay has its limitation as it only showed sensitivity to a certain group of compounds. One of the criticisms of DTT assay is its poor sensitivity towards one of the most dominant metals – Fe, present abundantly in ambient PM, and known to catalyze the formation of ROS in biological system.^{46,47} Previous studies conducted using DTT assay have not shown a clear relationship between Fe concentration and PM catalyzed DTT oxidation. This phenomenon was further confirmed by Charrier et al.,³² where they measured the intrinsic potential of pure compounds of Fe [iron(II) sulfate and iron(III) chloride] to oxidize DTT. The studies

showed the contribution from pure compound of Fe [iron(II) sulfate and iron(III) chloride] was less than 5% in the overall DTT activity from ambient PM.³²

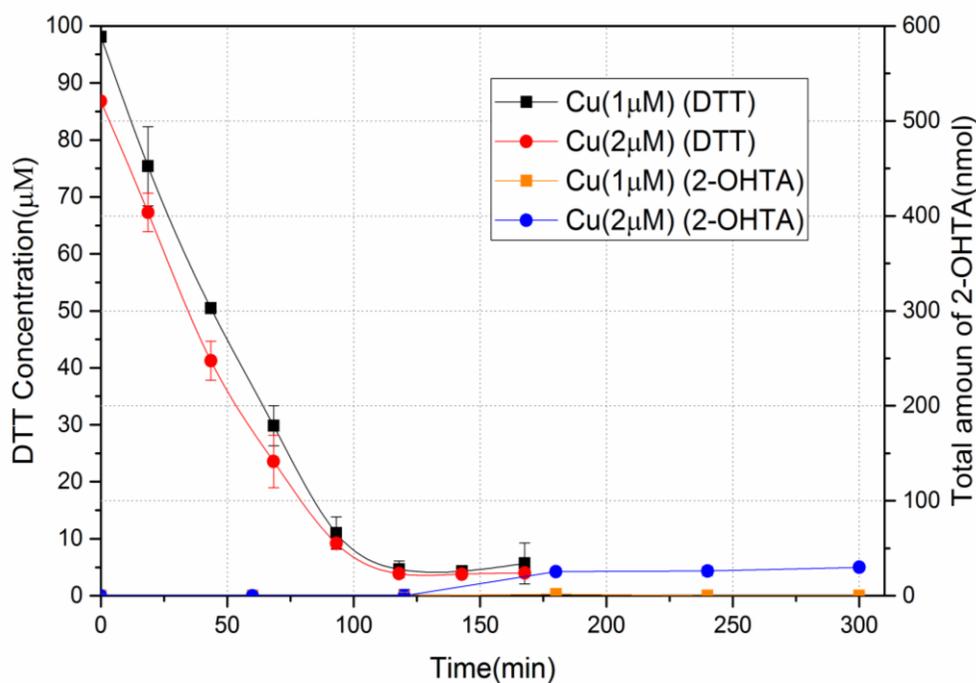
In this study, we measured the DTT activity and $\cdot\text{OH}$ generation from a binary mixture of Fe(II)/Fe(III) and individual quinones. The results approved the capability of the assay of capturing the role of Fe in ROS generation. To assess if the interaction of Fe and quinones has an additive or synergistic/antagonistic effect, the DTT activity and $\cdot\text{OH}$ generation were measured from pure FeSO_4 solution. Although the pattern of $\cdot\text{OH}$ formation from most quinones is not linear, to compare the relative capability of different quinones in catalyzing the formation of $\cdot\text{OH}$, the $\cdot\text{OH}$ formation rate was calculated from the linear (mostly the middle portion of the S-shaped curve) region (Figure 1). These calculated slope for the mixtures and individual compounds are plotted in Figure 2.

Apparently, the intrinsic DTT oxidation potential of Fe(II) is very low which is consistent with the Charreir et al.,³² studies. Even the least DTT active 1,4-NQ is approximately four times of Fe(II) in terms of DTT oxidation potential. Significant additive effect was observed in the mixture of individual quinones and Fe(II) (mixture/sum = 0.92 ± 0.06 , 0.87 ± 0.13 , 0.88 ± 0.27 and 0.99 ± 0.34 for PQ, 1,2-NQ, 1,4-NQ and 5H-1,4NQ, respectively). The $\cdot\text{OH}$ generation from Fe(II) is also very low which is well explained by its incapability to effectively oxidize DTT. However, in contrast to the incapability of Fe(II) both in oxidizing DTT and catalyzing the formation of $\cdot\text{OH}$, the mixture of quinone and Fe(II) is highly efficient in generating $\cdot\text{OH}$. Significant synergistic effect is also observed for most quinones (mixture/sum = 1.6 ± 0.2 , 2.0 ± 0.3 , 1.6 ± 0.3 and 2.2 ± 0.3 for PQ, 1,2-NQ, 1,4-NQ and 5H-1,4NQ, respectively). The process can be characterized by two steps. In the first stage, DTT is oxidized by quinones, generating $\text{O}_2^{\cdot-}$. In the following step, the mixture of Fe and

individual quinones converted superoxide radicals to hydroxyl radicals more efficiently than individual quinones due to Fenton reaction. The synergistic effect observed demonstrates that the assay is able to encompass the role of Fe in measuring the ROS activity of ambient PM based on DTT assay.

3.3 ROS generation from copper

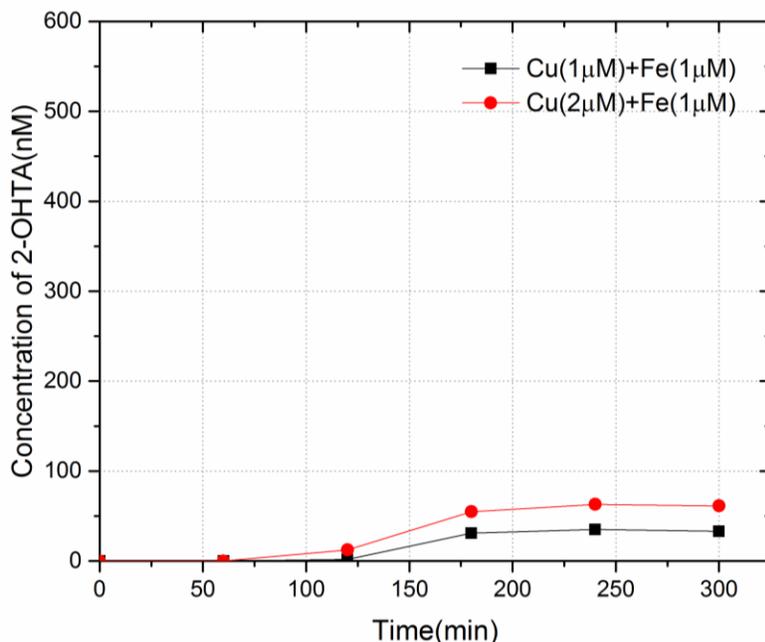
Figure 3. Pattern of DTT oxidation and $\cdot\text{OH}$ generation (expressed in terms of the concentration of 2-OHTA) from Cu (II)



Cu has a relatively high atmospheric concentration and has been proved to be the most active metal in DTT oxidation.^{32,48,49} Previous studies suggest that at San Joaquin Valley of California, Cu could be the most dominant species responsible for ambient PM-catalyzed DTT oxidation.^{32,48,49}

To test if the DTT oxidation catalyzed by Cu(II) corresponds to the ROS generation. The $\cdot\text{OH}$ formation from Cu(II) in DTT assay was measured. In the experiment setup, we set two atmospherically relevant concentrations of Cu(II) (1 and 2 μM). Fig 3 shows the DTT oxidation and $\cdot\text{OH}$ formation catalyzed by Cu(II) in DTT assay. There doesn't seem to exist significant difference between difference concentration of Cu(II) in DTT oxidation rate. This could be explained by previous studies which indicated there was a non-linear relationship dose-response curve for Cu-led DTT oxidation.³² The DTT oxidation rate driven by Cu(II) will get saturated at high concentrations of Cu.³² However, two abnormal patterns emerge in the ROS generation. First, Cu at both of these concentrations is highly inefficient in generating the $\cdot\text{OH}$ in comparison to their efficiency in DTT oxidation. The $\cdot\text{OH}$ generation at 2 μM Cu (II) concentration is only equivalent to 20nM of OHTA in 5 hours, and at 1mM Cu(II) concentration there is almost nothing. However, in comparison, 1,2NQ, which would have a similar DTT oxidation rate at 1/8th of Cu concentration (i.e. 0.25 μM), generates about 180nM OHTA in less than 3 hours. The second abnormal pattern is there existing a long lag period (~2 hours) before $\cdot\text{OH}$ generation begins. To better understand the existence of the lag period, we first tested if $\text{O}_2\cdot^-$ might be generated during the lag phase. The $\cdot\text{OH}$ generation from a binary mixture of Cu(II) and Fe(II) was measured in DTT assay to confirm that. As shown in Figure 4, the addition of Fe(II) has only a marginal effect, confirming that the Cu-led oxidation doesn't lead to any significant ROS generation at atmospherically relevant concentrations of Cu.

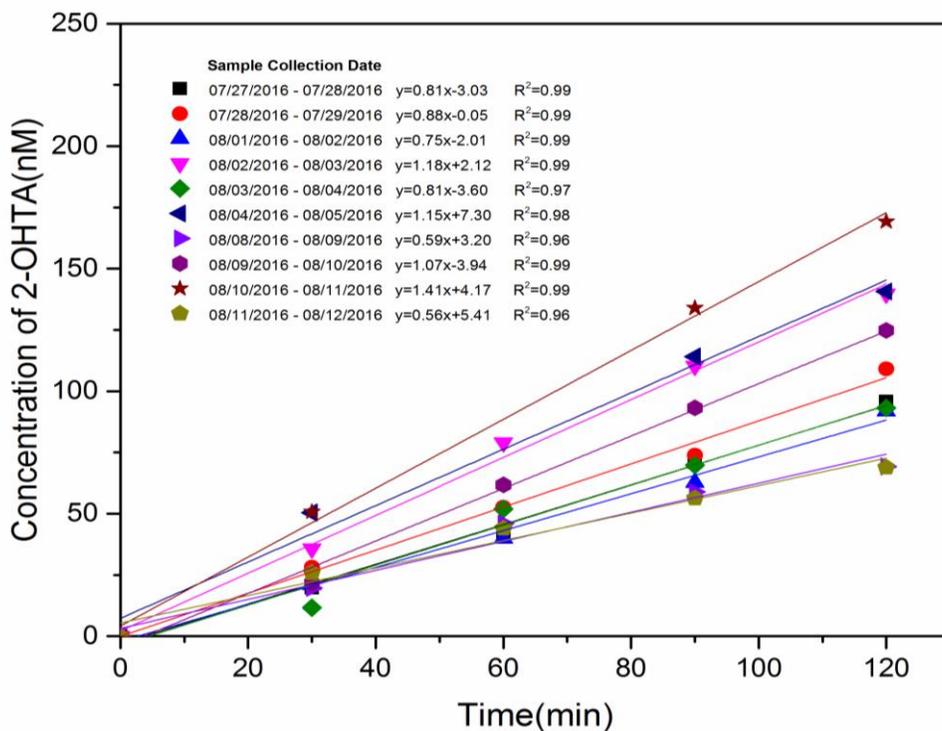
Figure 4. Pattern of $\cdot\text{OH}$ generation (expressed in terms of the concentration of 2-OHTA) from a binary mixture of Cu (II) + Fe (II)



The reason behind the long lag period in ROS generation from Cu is not entirely clear. previous studies by Kachur et al.⁵⁰ have investigated the reaction mechanism of DTT with Cu. The reaction mechanism can be explained by a two-phase DTT oxidation process. In the first step, Cu forms a complex with DTT. Then the complex acts as a catalyst to oxidize free DTT without the generation of $\text{O}_2^{\cdot-}$ or $\cdot\text{OH}$.⁵⁰ The second phase starts when all the free DTT is oxidized.⁵⁰ The complex first disintegrates and then oxidizes the remaining DTT.⁵⁰ During the process the oxygen is reduced to $\text{O}_2^{\cdot-}$, followed by $\cdot\text{OH}$ generation through Fenton-type reaction.⁵⁰

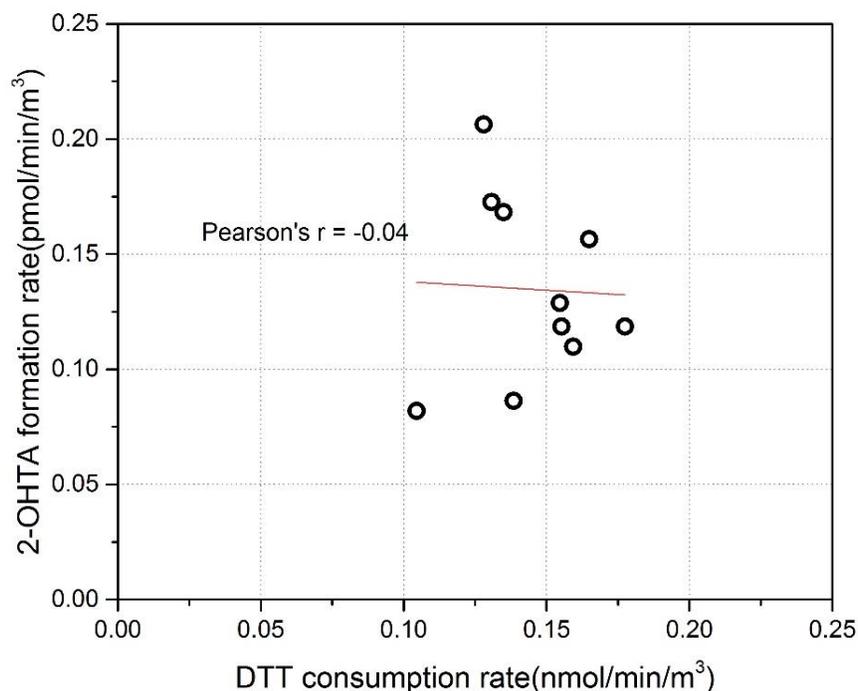
3.4 ROS generation from PM samples

Figure 5. Pattern of •OH generation (expressed in terms of the concentration of 2-OHTA) from PM samples in DTT assay



To better understand the ROS generation pattern from ambient aerosols, ten PM_{2.5} samples were collected from an urban site for analyze. The ROS generation from PM samples were measured in DTT assay. As shown in Figure 5, the pattern of ROS generation from PM samples is highly linear (R^2 for the linear fit = 0.96-0.99) without any lag period. This is different from most of quinones (PQ, 1,2-NQ and 1,4-NQ) which has a S-shaped curve but appears more like 5H-1,4NQ. The linear pattern of ROS makes it easier to compare the ROS generation potential from PM under various environmental settings and seasons.

Figure 6. Rate of DTT oxidation versus •OH generation (expressed in terms of the concentration of 2-OHTA) from PM samples



Since DTT oxidation is a widely used method to estimate the ROS potential from PM, it is critical to test if measuring the •OH generation can provide additional information about the mechanism of ROS generation. To compare the DTT oxidation vs. •OH generation, the same PM extracts were analyzed for the DTT oxidation rates. As shown in Figure 6, the Pearson's r is merely -0.04 which means there is no apparent correlation between DTT oxidation and ROS formation. The rate of •OH generation also has a wider range [Coefficient of Range (CoR=0.5)] than the DTT oxidation (CoR=0.3). These differences are possibly due to the additional compounds participating in generation of •OH than oxidizing the DTT. It suggests by measuring the •OH it might be able to apply the assay to a larger pool of PM components.

3.5 DTT consumption vs. ROS generation

Our results essentially show that DTT consumption and ROS generation are two different aspects of the oxidative properties of ambient PM, and should not be equated. While certain PM components can effectively consume a particular antioxidant, the others could be more efficient in generating the ROS, as demonstrated through the DTT assay in our study. DTT assay is the most commonly employed method for measuring the oxidative potential of ambient PM. The assay has been in use for more than a decade and the rationale is its resemblance with the reaction occurring in cellular mitochondria where nicotinamide adenine dinucleotide phosphate (NADPH) is oxidized to generate $O_2^{\cdot-}$. Therefore, it is generally assumed that the rate of DTT oxidation in presence of ambient PM is proportional to the rate of ROS generation. Our results showing the dissimilar patterns of ROS generation vs. DTT oxidation challenge the generalizability of this assumption. The patterns could be a lot divergent for certain species such as Cu, which despite having a high DTT oxidation potential, leads to very little ROS generation.

Measuring the ROS could be also implied to reassess the role of metals in DTT assay. DTT assay has been proved only to be sensitive to certain metals like Cu and Mn in previous studies. Fe, which is abundant in atmospheric ambient, does not show any ROS potential in conventional DTT assay. However, by measuring the $\cdot OH$ generation, most of the DTT consumed by Cu doesn't lead to any ROS formation (neither $O_2^{\cdot-}$ or $\cdot OH$). At a concentration as high as $2\mu M$, where the DTT oxidation is expected to be dominated by Cu, the contribution in $\cdot OH$ formation is negligible. Mn also has a similar pattern as Cu. At a concentration of $2mM$, while Mn is able to oxidize DTT efficiently, there is no $\cdot OH$ detected in Mn-DTT system even after 8 hours. Consequently, toxicity of Cu and Mn based on their potential to oxidize DTT is misleading. As for Fe, which used to be

considered inactive in the DTT assay, contributes to enhance the $\cdot\text{OH}$ generation in presence of other PM components like quinones. That indicates all metals may have the potential to contribute to ROS formation through different modes directly or indirectly. For other transition metals as V, Cr, Co and Ni,⁵¹ a similar route of $\cdot\text{OH}$ generation might be possible and their contribution in the DTT assay needs to be reinvestigated.

In comparison to DTT consumption, a clear distinction is that ROS generation measurements is effective to capture the synergistic interactions among PM components. Although the lack of synergistic effect in DTT assay is demonstrated only through the interaction of Fe and quinones, research conducted by Charrier et al.³² using binary mixtures of PQ, 1,2-NQ, Fe and Cu also showed either nil or very little interaction among these species in the DTT assay. Besides, studies measuring the ROS formation through other assay such as ascorbate, citrate, glutathione and uric acid, showed strong synergistic interactions among Cu, Fe, PQ and 1,2-NQ. The results in this thesis are in agreement with those studies, show that the capability of ambient PM to generate oxidative stress might be underrepresented by limiting the measurement only to the DTT consumption.

In general, by measuring ROS it has the potential to encompass the role of some metals that are not inactive in conventional DTT assay. Different patterns in DTT consumption and ROS generation could provide a more comprehensive insight into the ROS potential of ambient particulates. Moreover, more comprehensive and systematic studies need to be conducted to narrow-down probable toxic PM components and understanding their synergistic/antagonistic interactions in their ROS potential.

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