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Review

Use of Dithiothreitol Assay to Evaluate the Oxidative Potential of Atmospheric Aerosols

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Abstract: Oxidative potential (OP) has been proposed as a useful descriptor for the ability of particulate matter (PM) to generate reactive oxygen species (ROS) and consequently induce oxidative stress in biological systems, which has been recognized as one of the most important mechanisms responsible for PM toxicity. The dithiothreitol (DTT) assay is one of the most frequently used techniques to quantify OP because it is low-cost, easy-to-operate, and has high repeatability. With two thiol groups, DTT has been used as a surrogate of biological sulfurs that can be oxidized when exposed to ROS. Within the DTT measurement matrix, OP is defined as the DTT consumption rate. Often, the DTT consumption can be attributed to the presence of transition metals and quinones in PM as they can catalyze the oxidation of DTT through catalytic redox reactions. However, the DTT consumption by non-catalytic PM components has not been fully investigated. In addition, weak correlations between DTT consumption, ROS generation, and cellular responses have been observed in several studies, which also reveal the knowledge gaps between DTT-based OP measurements and their implication on health effects. In this review, we critically assessed the current challenges and limitations of DTT measurement, highlighted the understudied DTT consumption mechanisms, elaborated the necessity to understand both PM-bound and PM-induced ROS, and concluded with research needs to bridge the existing knowledge gaps.

Keywords: DTT; oxidative potential; reactive oxygen species; particulate matter

1. Introduction

Particulate matter (PM) emitted from various sources (e.g., vehicle emissions and industry emission, wildfire, biogenic sources, and volcano eruption, Figure 1) has been associated with millions of premature deaths, cardiovascular and respiratory morbidity worldwide each year [1–6]. A common hypothesis of its toxicological mechanism is through the generation of reactive oxygen species (ROS) that could interact with reduced biomolecules (e.g., NADPH and glutathione (GSH)) and subsequently induce oxidative stress in biosystems [1,7–10]. As a measure of the capacity of PM to oxidize target molecules, oxidative potential (OP) has been proposed to denote the intrinsic ability of PM to generate ROS [11–14]. Over the years, multiple cellular and acellular assays have been developed to quantify the OP to predict the potential toxicity of PM (Figure 1) [15–17]. Among them, the acellular dithiothreitol (DTT, $HSCH_2(CH(OH))_2CH_2SH$) assay is one of the most frequently used methods, which can be easily conducted on a laboratory bench scale, providing a fast output under an easily-controlled environment [13,18].

Dithiothreitol (DTT), known as Cleland's reagent, has been used as a protective reagent to reduce disulfide bridges in proteins and prevent dimerization of sulfur atoms of thiolated DNA for its low redox potential (−0.33 V at PH = 7) since the 1960s [19,20]. In 2002, Kumagai et al. found that quinones in diesel exhaust particles were capable of promoting the generation of ROS in biological systems and reacted rapidly with proximal protein thiols [21]. In 2005, Cho et al. first introduced the DTT-based chemical assay to quantitatively measure the OP of PM [13]. Since then, the DTT assay has been broadly used to assess the OP of various aerosol systems, including primary emitted particles, secondary aerosols, chamber generated aerosols, and field collected aerosols [1,16,22,23].

Generally, the term of OP^{DTT} implies the chemical reactivity and potential toxicity of PM constituents in regard to their oxidative properties when considering PM exposure and the associated health effects [18,24]. While PM is currently regulated by mass concentrations to protect environmental and public health, particle size, number concentrations, and chemical composition are also imperative matrices that are directly linked to the adverse health outcomes [11,23,25,26]. For example, quinones and transition metals have been recognized as major contributors to the OP^{DTT} of PM. Nevertheless, a large knowledge gap remains between DTT consumption by these contributing catalytic species and the resulting toxicity. In addition, the apportionment of PM components responsible for DTT consumption has not been fully elucidated due to the complex nature of PM compositions and potential interactions among PM components. Thus, conflicting OP^{DTT} results and inconclusive correlations between OP^{DTT} and biological responses from cellular assays have been reported in various studies, which can be in part attributed to the non-standardized assay design, as well as the lack of consideration for cellular mechanisms in response to PM exposure. Here, we reviewed the current research findings on the assay design for DTT measurement, correlations between DTT assay results and biological responses, the caveats of non-standardized DTT assay protocols, the understudied chemistry behind the DTT assay, the challenge and limitations of using OP^{DTT} to predict health outcomes, and outlined research needs for utilizing the DTT assay to evaluate OP of atmospheric aerosols in future studies.

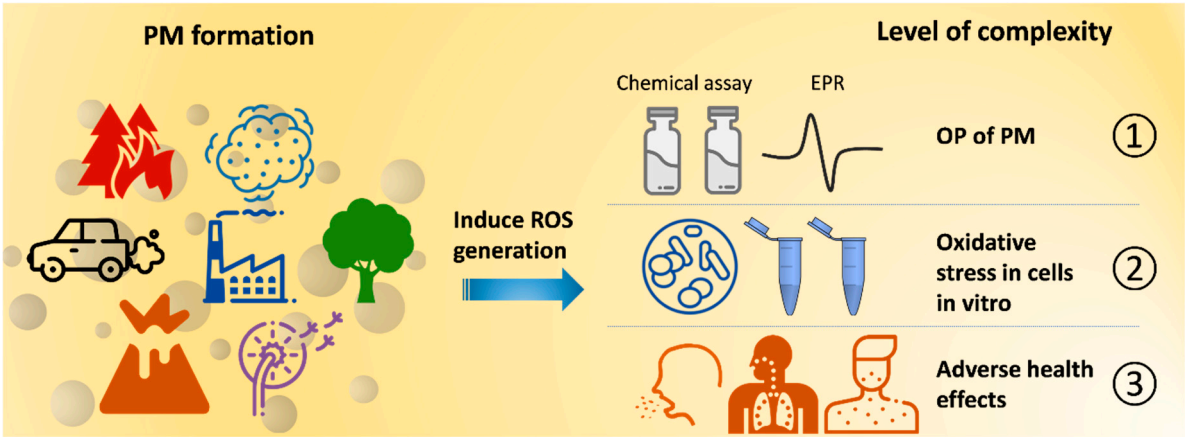

Figure 1. Sources, toxicity measurement, and adverse health effects of particulate matter (PM). Increasing levels of complexity involved in (1) the oxidative potential (OP) measurement of PM, (2) the quantification of oxidative stress in cells in vitro, and (3) PM exposure and epidemiological studies on the adverse health effects of PM.

2. Current Status of Research Using DTT Assay

2.1. Principle of Measuring Oxidative Potential Using DTT Assay

As a strong reducing agent [19], DTT is oxidized to form disulfides in the DTT assay when electrons are transferred from DTT to molecular oxygen through the redox reactions that can be accelerated in the presence of catalytic components (e.g., quinones and transition metals) in PM [18,24]. Over time, the remaining reduced DTT can be quantitatively determined with colorimetric analyses by adding 5,5′ dithiobis (2-nitrobenzoic acid) (DTNB, Ellman's reagent) to immediately form

the chromophoric 2-nitro-5-thiobenzoic acid (TNB), which has a large absorption coefficient (14,150 M^{-1} cm^{-1}) at 412 nm [27]. The DTT consumption rate depends on the concentration of DTT-reactive species in PM [13]. The oxidative potential is defined by the DTT consumption rate normalized by PM mass, as shown in Equation (1),

$$OP^{DTT} = \frac{\Delta DTT}{t \times m} \quad (1)$$

where ΔDTT (nmol) is the DTT consumption over the specified reaction time t (min), and m is the PM mass (μg or ng) applied to the DTT assay. Typically, to ensure the pseudo-first order reaction (excess DTT compared to PM components), m is restricted so that less than 50% of DTT is consumed within the reaction period. In order to compare DTT data obtained from various studies with different experimental settings, OP^{DTT} of aerosols is sometimes normalized to that of 1,4-naphthoquinone (1,4-NQN), and expressed as the normalized index of oxidant generation and toxicity (NIOG) [28-32], as shown in Equation (2):

$$NIOG = \frac{OP^{DTT}}{OP^{1,4-NQN}} \quad (2)$$

2.2. The OP^{DTT} of PM from Various Sources

The DTT assay has been extensively used to evaluate the OP^{DTT} of ambient PM and laboratory-generated aerosol samples in many studies. Summaries of reported OP^{DTT} values from various studies can be found in recent review articles by Shiraiwa et al., Bates et al., and references therein [1,16]. Overall, the OP^{DTT} values of ambient PM are highly variable (from less than 1 to 300 pmol min^{-1} μg^{-1}), depending on the sampling sites, sampling time and contributing sources [1,16]. For example, the OP^{DTT} of fuel emission particles largely depended on the engine types, driving cycles and fuel compositions. Exhaust particles from alternative fuels like biodiesel tend to have much lower OP^{DTT} than conventional diesel exhaust particles [33]. The addition of ethanol to gasoline blends can largely decrease the OP^{DTT} of exhausted particles [34].

The OP^{DTT} of laboratory-generated secondary organic aerosols (SOA) is determined by its precursor, oxidant and aging status. Among all SOA systems that have been studied so far, naphthalene SOA has shown the largest OP^{DTT} due to its high concentration of quinones [35].

NO_x conditions have been reported to have a significant impact on the formation pathways and chemical composition of resulting SOA, which will also directly influence OP^{DTT} [36]. In the study of Jiang et al. [36], isoprene (the most abundant non-methane hydrocarbon)-derived SOA has higher OP^{DTT} under low-NO_x conditions (favorable for hydroperoxide formation [37]), although no NO_x effect was shown on the OP^{DTT} of isoprene SOA in the study by Tuet et. al. [38].

The transformation processes of aerosol aging, such as the heterogeneous oxidation by atmospheric oxidants and dilution during transport dynamically alter the chemical composition of aerosols through the formation, degradation and oligomerization of oxidized molecules [39,40]. Thus, the aging process can impose a large influence on OP^{DTT} of SOA as well [29,35,41–43]. For example, the formation and oligomerization of oxidative compounds in the initial aging stage has been reported to increase the OP^{DTT} of SOA [35]. However, the degradation of DTT-reactive species in a later aging stage may lead to the decrease of OP^{DTT} of SOA [41]. In addition, other factors such as relative humidity, temperature and the existence of seed aerosols can also alter the chemical compositions [44–46] and the OP^{DTT} of SOA [38,47]. Even though the OP^{DTT} of various types of PM have been investigated, the reactive PM components that contribute to OP^{DTT} have not been fully elucidated.

2.3. The Oxidative Properties of Various Chemical Compositions

To characterize how chemical compositions of PM contribute to DTT consumption, the OP^{DTT} of model organic compounds with specified functional groups as well as transition metals are summarized in Table 1. The catalytic redox-active compounds like quinones and transition metals have been recognized as the main contributors to the OP^{DTT} of PM [48–52]. In the presence of reduced

molecules such as DTT, quinones can catalyze the transfer of electrons and undergo redox cycling, resulting in the formation of oxidized DTT, semiquinone radical anions (Q^{-}) and hydroquinones (QH_2). The semiquinone and hydroquinone will then react with dissolved molecular oxygen to regenerate the quinone and produce superoxide anions (O_2^{-}) [28,35], which can further form hydrogen peroxide (H_2O_2) [53–56]. Some quinone compounds can significantly modify DTT (Table 1) through catalytic reactions [35,47,49,57]. Among them, 5-hydroxy-1,4-naphthoquinone (5-H-1,4-NQN), 1,2-naphthoquinone (1,2-NQN) and 1,4-NQN have been reported to contribute to 30 ± 5% of OP^{DTT} in naphthalene SOA [35]. However, other quinones, such as 1,4-benzoquinone, 2-methyl-1,4-benzoquinone, 2-chlorobenzoquinone, 2,3,5,6-tetramethyl-1,4-benzoquinone, pyrroloquinoline quinone, 2-anilino-1,4-naphthoquinone, lapachol, 2-chloroanthraquinone, 5,12-naphthacenequinone, 9,10-anthraquinone, or mytomycin c, 5,12-naphthacenequione, acenaphthequionone, benzoanthraquione, phenanthrenequinone, methyl anthraquinone, dimethyl anthraquinone, benz[*a*]-9,10-anthraquinone, benz[*a*]-7,12-anthraquinone, chrysenequinone, benzo[*a*]pyrene-6,12-quinone, benzo[a]pyrene-3,6-quinone, and benzo[a]pyrene-1,6-quinone do not have significant DTT responses [21,47,58]. The reactivity of quinones with DTT is determined by the reduction potentials of the Q/Q^{-} and Q^{-}/QH_2 one-electron couples [59,60].

Transition metals, likely originating from fuel combustion, lube oil emissions, and, to a lesser extent, brake and tire wear [61,62], can be significant contributors to OP^{DTT}. For example, Fe, Cu, and Mn can be highly reactive with DTT through Fenton reactions to produce H_2O_2, as shown in reactions (3)–(7) [49,63]. In these reactions, M stands for a metal.

$$\mathrm{DTT(red)} + 2\mathrm{M}^{n+1} \rightarrow 2\mathrm{M}^{n} + \mathrm{DTT(ox)} + 2\mathrm{H}^{+} \quad (3)$$

$$\mathrm{M}^{n} + \mathrm{O}_2 \rightarrow \mathrm{M}^{n+1} + \mathrm{O}_2^{-\cdot} \quad (4)$$

$$\mathrm{M}^{n} + \mathrm{O}_2^{-\cdot} + 2\mathrm{H}^{+} \rightarrow \mathrm{H}_2\mathrm{O}_2 + \mathrm{M}^{n+1} \quad (5)$$

$$2\mathrm{H}^{+} + \mathrm{O}_2^{-\cdot} \rightarrow \mathrm{H}_2\mathrm{O}_2 + \mathrm{O}_2 \quad (6)$$

$$\mathrm{Sum:\ DTT(red)} + \mathrm{O}_2 \rightarrow \mathrm{H}_2\mathrm{O}_2 + \mathrm{DTT(ox)} \quad (7)$$

In addition, Fe^{2+} can convert H_2O_2 into highly reactive hydroxyl ·OH radicals, as shown in reaction (8) [64,65]. Charrier and Anastasio determined that transition metals (i.e., Cu (II) and Mn(III)) attributed ~80% of DTT consumption by ambient particles, while the remaining 20% was contributed by quinones and other redox-active species [49].

$$\mathrm{Fe(II)} + \mathrm{H}_2\mathrm{O}_2 \rightarrow \mathrm{Fe(III)} + \mathrm{OH}^{-} + \cdot\mathrm{OH} \quad (8)$$

SOA, which contribute substantially to ambient PM [66], are understudied for their capability to generate ROS [35,36,67–72]. Except for those generated from aromatic precursors (e.g., naphthalene SOA), SOA typically have negligible amounts of quinones but some SOA systems exhibit high OP^{DTT} [38,57]. Thus, non-catalytic reaction pathways may contribute to the noticeable OP^{DTT} of SOA. For example, Wang et al. reported that α-pinene, β-pinene and toluene SOA all generated H_2O_2 in aqueous conditions, and the formation of H_2O_2 could be attributed to the decomposition of hydroxy hydroperoxides and organic hydroperoxides at pH > 7 [73]. Both H_2O_2 and organic hydroperoxides (ROOH), such as isoprene hydroxyhydroperoxide (ISOPOOH) (Tables 1 and 2), have been found to significantly contribute to OP^{DTT} [30,36,71]. ROOH can oxidize DTT into disulfide, sulfenic acid, sulfinic acid or sulfonic acid [74]. Jiang et al. reported that the DTT consumption by isoprene derived SOA could be attributed exclusively to ROOH [36]. Furthermore, Michael acceptors (e.g., conjugated carbonyls) that constitute a large fraction of aromatic SOA can contribute to DTT consumption notably through nucleophilic or conjugate additions [36,41,75]. A recent study by Chen and Jiang et al. has demonstrated that atmospheric electrophiles such as carbonyls can react with DTT through nucleophilic additions and form DTT-carbonyl adducts [75]. In addition, quinones, though known as catalytic DTT-reactive species, can also react with DTT through non-redox cycling pathways such as sulphydryl arylation [76]. These findings highlight the significance of previously unrecognized non-catalytic PM components to OP^{DTT}, which requires further research.

Table 1. The summary of dithiothreitol (DTT) responses of oxidative compounds.

Compounds	$(DTT)_0$ [a] (μM)	Incubation & Shaking Method	$(Sample)_0$ [b] (μM)	DTT_r [c] (nmol/min/μg)	NIOG [d]	Reference
Formaldehyde	20	37 °C, Incubator	$0.54–2.69 \times 10^6$	8.50×10^{-6}	3.79×10^{-5}	Chen & Jiang et al.[75]
2-Furaldehyde	20	37 °C, Incubator	$1.91–9.60 \times 10^3$	1.05×10^{-4}	4.69×10^{-4}	Chen & Jiang et al.[75]
Benzaldehyde	20	37 °C, Incubator	$0.78–3.88 \times 10^5$	1.53×10^{-5}	6.83×10^{-5}	Chen & Jiang et al.[75]
4-Formylbenzoic acid	20	37 °C, Incubator	$0.26–1.30 \times 10^3$	1.67×10^{-4}	5.51×10^{-9}	Chen & Jiang et al.[75]
2-Nitrobenzaldehyde	20	37 °C, Incubator	$0.05–1.10 \times 10^3$	6.43×10^{-4}	2.87×10^{-3}	Chen & Jiang et al.[75]
3-Nitrobenzaldehyde	20	37 °C, Incubator	$0.05–1.04 \times 10^3$	2.06×10^{-4}	9.20×10^{-4}	Chen & Jiang et al.[75]
4-Nitrobenzaldehyde	20	37 °C, Incubator	$0.05–1.09 \times 10^3$	3.52×10^{-4}	1.57×10^{-3}	Chen & Jiang et al.[75]
Mesityl oxide	20	37 °C, Incubator	$0.88–4.46 \times 10^3$	1.02×10^{-4}	4.55×10^{-4}	Chen & Jiang et al.[75]
Citral	20	37 °C, Incubator	$0.30–1.51 \times 10^3$	1.53×10^{-4}	6.83×10^{-4}	Chen & Jiang et al.[75]
trans-Cinnamaldehyde	20	37 °C, Incubator	2.82×10^4	1.51×10^{-3}	6.74×10^{-3}	Chen & Jiang et al.[75]
1,4-NQN	20	37 °C, Incubator	6.46×10^{-1}	2.24×10^{-1}	1.00	Chen & Jiang et al.[75]
Isoprene epoxydiol	20	37 °C, Incubator	NA	$7.00 \pm 1.39 \times 10^{-5}$	$4.93 \pm 0.98 \times 10^{-5}$	Kramer et al.[30]
2-Methyltetrol	20	37 °C, Incubator	NA	$4.44 \pm 0.92 \times 10^{-5}$	$3.13 \pm 0.65 \times 10^{-5}$	Kramer et al.[30]
Methacrylic acid epoxide	20	37 °C, Incubator	NA	$9.84 \pm 0.97 \times 10^{-5}$	$6.93 \pm 0.68 \times 10^{-5}$	Kramer et al.[30]
2-Methylglyceric acid	20	37 °C, Incubator	NA	$2.51 \pm 0.37 \times 10^{-4}$	$1.77 \pm 0.26 \times 10^{-4}$	Kramer et al.[30]
ISOPOOH	20	37 °C, Incubator	NA	$4.90 \pm 2.20 \times 10^{-1}$	$3.45 \pm 1.55 \times 10^{-1}$	Kramer et al.[30]
1,4-NQN	20	37 °C, Incubator	NA	1.42	1.00	Kramer et al.[30]
Acrolein	100	37 °C, Sonicator	5.40×10^1	$8.60 \pm 0.36 \times 10^{-2}$	$2.95 \pm 0.12 \times 10^{-2}$	Jiang et al.[57]
Methacrolein	100	37 °C, Sonicator	1.77×10^2	$3.26 \pm 0.10 \times 10^{-2}$	$1.12 \pm 0.03 \times 10^{-2}$	Jiang et al.[57]
2,4-Hexadienal	100	37 °C, Sonicator	2.13×10^2	$6.32 \pm 2.39 \times 10^{-3}$	$2.16 \pm 0.82 \times 10^{-3}$	Jiang et al. [36]
9,10-PQN	100	37 °C, Sonicator	2.50×10^{-2}	$2.54 \pm 0.10 \times 10^1$	8.72 ± 0.34	Jiang et al.[57]
1,2-NQN	100	37 °C, Sonicator	3.00×10^{-1}	9.07 ± 0.29	3.11 ± 0.10	Jiang et al.[57]
1,4-NQN	100	37 °C, Sonicator	6.00×10^{-1}	2.92 ± 0.12	1.00	Jiang et al.[57]
tert-Butyl hydroperoxide	100	37 °C, Sonicator	280	$1.17 \pm 0.19 \times 10^{-2}$	$4.01 \pm 0.65 \times 10^{-3}$	Jiang et al. [36]
9,10-PQN	100	37 °C, Dry bath	$0.25–2 \times 10^{-1}$	6.77×10^1	2.01×10^1	Charrier and Anastasio [49]
1,2-NQN	100	37 °C, Dry bath	0.01–1	2.59×10^1	7.67	Charrier and Anastasio [49]
1,4-NQN	100	37 °C, Dry bath	0.5–1.5	3.37	1	Charrier and Anastasio [49]
BQN	100	37 °C, Dry bath	1–4	1.17	0.35	Charrier and Anastasio [49]
Co (II)	100	37 °C, Dry bath	1	4.58	1.36	Charrier and Anastasio [49]

Table 1. (Continued)

Compounds	$(DTT)_0$ [a] (μM)	Incubation & Shaking Method	$(Sample)_0$ [b] (μM)	DTT_r [c] (nmol/min/μg)	NIOG [d]	Reference
Co (II)	100	37 °C, Dry bath	1	4.58	1.36	Charrier and Anastasio [49]
Ni (II)	100	37 °C, Dry bath	0.1–5	1.81	0.54	Charrier and Anastasio [49]
V (V)	100	37 °C, Dry bath	1–5	1.98	0.59	Charrier and Anastasio [49]
Pb (II)	100	37 °C, Dry bath	1	0.31	0.09	Charrier and Anastasio [49]
Fe(II)	100	37 °C, Dry bath	0.5–5	0.93	0.28	Charrier and Anastasio [49]
Fe (III)	100	37 °C, Dry bath	0.5–10	0.30	0.09	Charrier and Anastasio [49]
5-H-1,4-NQN	100	Room temp	NA	7.8	3.7	McWhinney et al. [35]
1,2-NQN	100	Room temp	NA	5.7	2.7	McWhinney et al. [35]
1,4-NQN	100	Room temp	NA	2.1	1.0	McWhinney et al. [35]

[a] $[DTT]_0$ is the initial DTT concentration in μM. [b] $[Sample]_0$ is the initial sample concentration in μM. [c] DTT_r is the DTT consumption rate or OP^{DTT} in nmol/min/μg. [d] NIOG is the normalized index of oxidant generation and toxicity, calculated with equation (2).

2.4. Recent Advancements of DTT Assay to Increase Throughput

To more efficiently determine the OP^{DTT} of aerosol samples on either a bench scale or portable device, improved designs of DTT measurements have been pursued to increase the assay throughput. Various offline and online systems for analyzing DTT have been developed. One example of an off-line method is the semi-automated system, which is equipped with a programmable syringe pump to add reagents to the reaction vials and a spectrophotometer to measure the absorption of the reaction mixture after certain reaction time. This type of system has been employed by Fang et al. for the improved offline OP measurements of PM filter extracts [77]. This experimental setup could be operated in an unattended manner but still with high analytical precision compared to manual operation. Additionally, this semi-automated system can be used for other chemical assays, such as ascorbic acid (AA, also known as vitamin C) assay [51]. However, the lifetime of some reactive components in PM might be short and could be degraded before the DTT measurement, potentially leading to the underestimation of OP^{DTT}. Thus, an online sampling system is desired in many studies to minimize the degradation of short-lived reactive species.

Several online methods have been developed to facilitate the effort of automated aerosol collection and the subsequent DTT measurement with reduced assay time. For example, Puthussery et al. used a mist chamber design to wash off the particles from the filter samples [78]. In some other studies, a particle-into-liquid sampler or liquid spot sampler has been used to directly collect aerosols into liquid [78–81]. These online sampling devices could be coupled to an online DTT measurement system. Either a syringe pump or a peristatic pump can be used to continuously deliver the reagents to reaction mixture in sample cells for UV-Vis measurements. In addition to the commonly used spectroscopic measurement for the DTT detection and quantification, microfluidic electrochemical sensors [79,80] and the electrochemical system with commercialized electrodes [27] have been developed to directly measure DTT concentration.

2.5. The Correlation between Biological Responses and OP^{DTT}

Increasing evidence suggests that exposure to $PM_{2.5}$ (aerodynamic diameter less than 2.5 μm) is linked to adverse health outcomes, such as respiratory diseases (e.g., asthma, chronic obstructive pulmonary disease, and bronchitis), cardiovascular diseases (e.g., myocardial infarction, coronary heart disease, and stroke), and emergency hospital visits and admissions [2,82–84]. The induction of ROS generation, followed by oxidative stress and inflammation, is believed to be one of major toxicological pathways initiated by PM exposure. Based on this hypothesis, the OP^{DTT} has been widely used to predict adverse health outcomes from PM exposure. However, no conclusive associations have been found between OP^{DTT} and cellular ROS generation measured by the macrophage ROS assay and H2DCFDA assay [68,85,86]. Also, contradictory results have been reported between OP^{DTT} and gene expressions of oxidative stress and inflammation-associated biomarkers in cellular assays. A strong correlation was observed between OP^{DTT} and the expression of heme oxygenase-1 (*HMOX-1*) (a relevant biomarker for oxidative stress) in BEAS-2B cells exposed to PM collected from the Los Angeles basin in California [87]. However, OP^{DTT} was found not to be associated with the *HMOX-1,* but with the expression of tumor necrosis factor-alpha (*TNF-α*) (a pro-inflammatory cytokine) in a mouse macrophage cell line (Raw 264.7) after exposing to diesel and biodiesel emissions [86]. Additionally, a recent study by Tuet et al. reported no significant correlations between OP^{DTT} and cellular inflammatory biomarkers (i.e., *TNF-α* and interleukin-6*)* when exposed to various types of SOA in immortalized murine alveolar macrophages [68]. Therefore, OP^{DTT} alone does not appear to adequately predict the PM-induced cellular ROS generation nor oxidative stress and inflammatory gene expression.

When comparing OP^{DTT} with the outcomes of epidemiological studies, inconclusive findings remain [84,88–91]. Abrams et al., found that Lag 0–2 days of OP^{DTT} was strongly associated with emergency hospital visits for multiple cardiorespiratory health effects [84]. A strong association of OP^{DTT} with respiratory health, such as lung function in children, was also observed by Yang et al. [90]. However, the study by Atkinson et al. reported no significant correlations between daily average OP^{DTT} and numbers of deaths and hospital admissions for respiratory and cardiovascular diseases

[88]. Similarly, OP^{DTT} was found to be not consistently associated with lung function, vascular inflammatory and coagulation parameters in the blood samples of 31 volunteers after exposure to ambient air pollution for 5 h, as reported by Janssen et al. [91].

OP^{DTT} represents an isolated chemical reaction between PM and thiols, while cellular assays involve more physiological processes in response to PM exposure. It has been reported that various intracellular processes could produce endogenous ROS, suggesting that the cellular oxidative stress in response to PM exposure could be driven by different mechanisms at the same time [8,89,92], which will be further discussed in Section 4. Thus, extra caution should be taken when using OP^{DTT} as an indicator for PM toxicity. For a more complete scenario of PM-induced oxidative responses, biological mechanisms should also be considered to explain the overall PM-induced health effects. Nevertheless, as illustrated in Figure 1, different levels of complexity are involved in acellular assays, cellular assays and the human body. As a result, even cellular assays have limitations that may not faithfully represent the human physiological conditions, and thus results may not necessarily correlate with PM-induced health outcomes.

3. Additional Acellular Assays in Determination of OP

In addition to the DTT assay, the OP of atmospheric aerosols has also been assessed by several other acellular assays, such as GSH assay, AA assay, electron paramagnetic or spin resonance (EPR or ESR), and fluorescent probes for·OH, O_2^- and H_2O_2 to characterize the particle-bound ROS generation from PM. Direct comparisons among these assays have been conducted within the scope of OP measurements [17,51,54,93–96]. Some assays such as EPR/ESR and 2′,7′-dichlorofluorescin (DCFH) probes can be used in either a cellular or an acellular environment.

The basic principle of most acellular assays is to measure the depletion of antioxidants (or surrogates) that may exist in the biological system, while the cellular methods typically involve the use of fluorescent probes to indicate the ROS-initiated oxidation within the cells [97,98]. Compared to cellular assays that require cell culture and PM exposure, acellular assays only require incubation of PM samples and reagents before measuring OP, providing cost-effective screenings for the oxidizing capacity of PM from large sample sizes. However, since acellular assays are not performed within the biological system, OP values determined by acellular assays are not able to characterize the endogenous cellular defense mechanisms or immune responses, making them more difficult to connect to PM-induced health outcomes. Moreover, while ROS encompasses a wide range of reactive molecules and free radicals, the results of DTT assay have been found to correlate only with production of H_2O_2, but not ·OH or superoxide O_2^- that may be generated in the presence of PM and lead to a cascade of other ROS [53,99]. Thus, OP^{DTT} does not characterize the full spectrum of ROS.

Since there is no gold standard that can provide a comprehensive picture of the intrinsic OP of PM, a combination of different acellular assays is necessary to obtain complementary and reliable information. For example, an ·OH probe such as disodium terephthalate (TPT) [54,99] and a O_2^- probe such as luminophore coelenterazine [100,101] can be applied along with the DTT measurement to detect ·OH and O_2^- generated by metals. EPR or ESR can be applied to detect the short-lived free radicals that cannot be easily detected by the DTT assay.

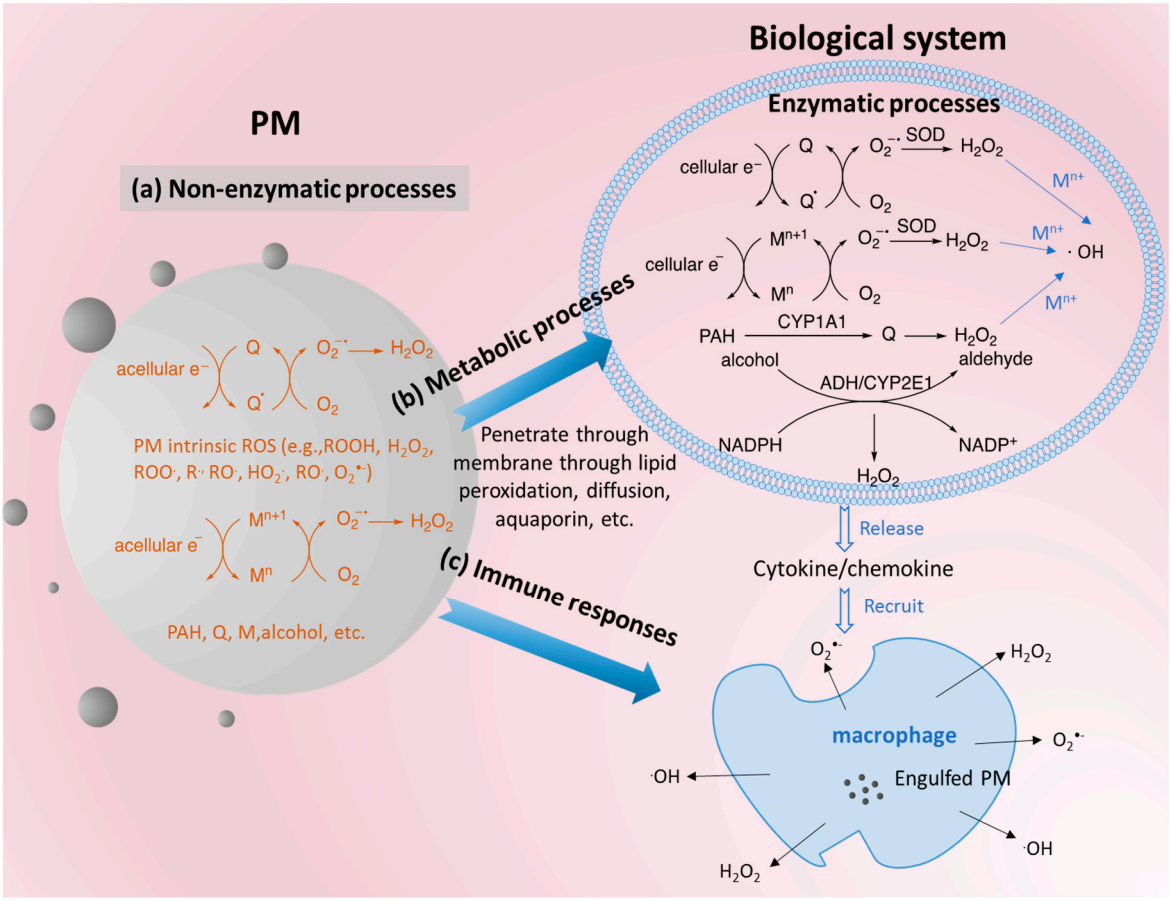

Figure 2. The production of PM-bound reactive oxygen species (ROS) through (**a**) non-enzymatic processes, and PM-induced ROS through (**b**) metabolic processes (i.e., enzymatic reactions) and (**c**) immune responses. Damaged/injured cells could produce pro-inflammatory cytokines or chemokines to recruit and activate immune cells (e.g., macrophages) to combat against foreign substances (i.e., PM). ADH/CYP2E1: alcohol dehydrogenase/ cytochrome P450 2E1, enzymes involved in metabolism of alcohols; M: metals (e.g., Fe, Cu, Zn, and Mn); $NADP^+$: nicotinamide adenine dinucleotide phosphate, a cofactor in metabolism reactions; NAPDH: the reduced form of $NADP^+$; CYP1A1: cytochrome P450 1A1, an enzyme involved during the metabolism of PAH; PAH: poly aromatic hydrocarbons; Q: quinones; SOD: superoxide dismutase.

4. PM-Associated ROS: PM-Bound ROS and PM-Induced ROS

As discussed above, ROS are molecules and free radicals with high reactivity and oxidative ability, including·OH, 0_2^-, organic radicals (e.g., alkoxyl radical RO·), nitroxyl radical NO·, singlet oxygen $^1O_2$, H_2O_2, and ROOH [102]. The unpaired electrons can lead to the high reactivity of free radicals that can damage most organic biomolecules [103]. For example, ·OH is extremely reactive in the aqueous solution, with a half-life estimated to be ~10^{-9} s [104–106]. Compared to ·OH, H_2O_2 and ROOH are less reactive (with longer half-lives), but higher stabilities allow them to diffuse and reach distant cellular compartments to produce ·OH and cause damage [107].

To differentiate the origins of PM-associated ROS and their implications for health effects, below we discuss the PM-bound ROS and PM-induced ROS separately.

Table 2. PM-bound reactive oxygen species (ROS) in various aerosol systems.

Aerosol System	Method	PM-Bound ROS [a]	Sample Concentration (nmol μg^{-1})	Reference
α-Pinene SOA	BPEAnit	Radicals	0.0200 ± 0.0050	Campbell et al. [108]
Limonene SOA	BPEAnit	Radicals	0.0059 ± 0.0010	Campbell et al. [108]
β-caryophyllene	BPEAnit	Radicals	0.0025 ± 0.00080	Campbell et al. [108]
Roadside $PM_{2.5}$	BPEAnit	Radicals	0.1–10	Crilley et al. [109]
Biodiesel combustion	BPEAnit	Radicals	0.001–1	Pourkhesalian et al. [110]
Diesel combustion	BPEAnit	Radicals	0.04	Stevanovic et al. [111]
SOY biodiesel	BPEAnit	Radicals	1.5	Stevanovic et al. [111]
Side stream cigarette smoke	BPEAnit	Radicals	0.02–0.05	Miljevic et al. [112]
Biodiesel combustion	BPEAnit	Radicals	0.05–0.4	Hedayat et al. [113]
$PM_{2.5}$ EPFR	EPR	Radicals	0.2–1.0 × 10^{-3}	Arangio et al. [114]
$PM_{2.5}$ water extract	EPR	Radicals	4.0 × 10^{-5}	Arangio et al. [114]
Naphthalene SOA EPFR	EPR	Radicals	0.02–0.05	Tong et al. [71]
$PM_{2.5}$ EPFR	EPR	Radicals	0.05–0.40	Gehling and Dellinger [115]
Wood smoke particles	NPBA	ROOH	1.60–2.56	Jiang et al. [41]
Gasoline LNOX SOA	NPBA	ROOH	2.18–2.28	Jiang et al. [41]
α-Pinene LNOX SOA	NPBA	ROOH	3.81–7.34	Jiang et al. [41]
Toluene LNOX SOA	NPBA	ROOH	3.53 ± 1.90	Jiang et al. [36]
Toluene HNOX SOA	NPBA	ROOH	5.41 ± 0.73	Jiang et al. [36]
Isoprene LNOX SOA	NPBA	ROOH	2.80 ± 0.37	Jiang et al. [36]
Isoprene HNOX SOA	NPBA	ROOH	1.13 ± 0.64	Jiang et al. [36]
α-Pinene + O_3 SOA	KI	ROOH, ROOR	0.79 ± 0.17	Epstein et al. [116]
α-Pinene + O_3 SOA	KI	ROOH, ROOR	0.95–2.03	Docherty et al. [117]
Δ-^{3} Carene + O_3 SOA	KI	ROOH, ROOR	0.82–1.45	Docherty et al. [117]
β-Pinene + O_3 SOA	KI	ROOH, ROOR	2.42–4.00	Docherty et al. [117]
Sabinene + O_3 SOA	KI	ROOH, ROOR	3.09–3.44	Docherty et al. [117]
Isoprene + O_3 SOA	KI	ROOH, ROOR	1.0 ± 0.1	Nguyen et al. [118]
Isoprene LNOX SOAA	KI	ROOH, ROOR	0.80–2.06	Surratt et al. [119]

[a] Type of PM-bound ROS measured in the studies cited, including particle-bound radicals, organic hydroperoxides (ROOH) and organic peroxides (ROOR).

PM-bound ROS, as illustrated in Figure 2, are formed non-enzymatically on particles during the particle formation processes or from the catalytic reactions of inhaled PM components (e.g., quinones and transition metals) in the presence of O_2, which has been described in Section 2.3. The photooxidation of hydrocarbon precursors by atmospheric oxidants (e.g.,·OH, O_3 and ·NO_3) can generate a large amount of ROS including ROOH and free radicals (e.g., RO·, R·, ROO· and HO_2·), as shown in reactions (9)–(13) [120]. These reactive species (with lifetimes ranging from minutes to days in the atmosphere) are key intermediates leading to formation of SOA [120]. As shown in Table 2, PM-bound ROS such as radicals and ROOH have been characterized in prior research directly by 9,10-bis (phenylethynyl) anthracene-nitroxide (BPEAnit), EPR/ESR techniques, and 4-nitrophenyl boronic acid (NPBA) assay. Part of Table 2 was adapted from Campbell et al. [108].

$$RH + \cdot OH \xrightarrow{UV} R\cdot + H_2O \quad (9)$$

$$R\cdot + O_2 \rightarrow ROO\cdot \quad (10)$$

$$ROO\cdot + NO \rightarrow RO\cdot \quad (11)$$

$$ROOH \xrightarrow{UV} RO\cdot + \cdot OH \quad (12)$$

$$HO_2\cdot + HO_2\cdot \xrightarrow{UV} H_2O_2 + \cdot OH \quad (13)$$

PM-induced ROS refers to ROS generated through the interactions among PM components within biological systems during the cellular metabolic processes (enzymatic bioactivation) or immune responses (Figure 2). Due to its small size, $PM_{2.5}$ can deposit deeply in the lungs, PM (as a whole) or its reactive constituents may enter the cells through active or passive transport (e.g., diffusion) [121,122]. Reactive constituents in PM (e.g., PM-bound ROS) can oxidize the membrane phospholipids, leading to the impairment of membrane function, inactivation of membrane-bound enzymes and receptors and the increase of membrane permeability [121,123] Reactive PM components like quinones can also form ROS enzymatically in the presence of NADPH-dependent P450 reductase in microsomes and NADPH oxidase on cell membranes or phagosomes in macrophages [124]. Although polyaromatic hydrocarbons (PAHs) do not produce ROS directly, they can be converted to quinones or quinone-like compounds through biotransformation of cytochrome P450 CYP1A1. Additionally, the metabolism of alcohols by alcohol dehydrogenases (ADH) and CYP2E1 has been shown to generate ROS and reactive aldehydes, releasing H_2O_2, reactive lipid hydroperoxides and O_2^- [125,126]. As a carrier of various toxic compounds, PM can serve as adjuvant or antigen and stimulate immune responses in immune cells, such as alveolar macrophages [127]. Recruitment and activation of neutrophils, eosinophils, monocytes, and lymphocytes by immune cells induce the generation of intracellular ROS [64,128]. For example, macrophages and neutrophils can generate O_2^-, which can then be rapidly converted to H_2O_2 by superoxide dismutase (SOD). These endogenous ROS species contribute to intracellular perturbation on the redox homeostasis [129]. Additionally, the inhaled particles can also bind with pathogen-associated molecular patterns or pattern recognition receptors (e.g., toll-like receptors), initiating intracellular signaling and stimulate the generation of ROS, which can further activate nuclear factor kappa B (NFκB) and induce the expression of pro-inflammatory cytokines [130,131]. Although elevated levels of ROS can be mitigated by biological antioxidants (e.g., GSH and AA) within their limited capacities, excessive production of ROS induced by PM exposure will eventually lead to the redox imbalance, so-called oxidative stress. Oxidative stress and damages to biomolecules (e.g., DNA, lipid, and protein) may in turn result in a wide range of adverse health effects, from cardiovascular diseases to cancer [83,89,102,129,132].

The cellular assays measure the combined effects of both PM-bound and PM-induced ROS, while OP^{DTT} measures the PM-bound ROS (most directly related to H_2O_2 production). Then to connect back to the inconclusive associations between biological responses and OP^{DTT}, the lack of correlation may not be surprising. In carefully controlled multi-assay studies, it is still possible that OP^{DTT} could be correlated with biological responses. To advance the current understanding of PM-induced health effects, it is important to take into account both PM-bound ROS and PM-induced ROS, as well as their interactions.

5. Challenges in Intercomparison and Interpretation of OP^{DTT}

5.1. The Non-Standardized Protocols

Since the DTT assay has been widely used to determine the OP of PM, major challenges remain to intercompare results from different studies because there are no existing standardized protocols for the DTT assay. One aspect that differs among studies is the incubation method during the reaction. While most studies are carried out at 37 °C during the reaction as a physiologically relevant temperature, incubation at room temperature has also been applied [72]. As the temperature of assay

affects the reaction rates, the results may not be directly comparable. Also, different sample mixing approaches (e.g., incubator with or without shaking, ultrasonic bath, etc.,) have been used in different studies [14,49,57,75], which may lead to different DTT consumption rates.

Another discrepancy could arise from the time allowed for reaction. Most often, extracted PM samples are incubated with buffer solutions (pH 7–7.4) and the DTT reagent for a specified amount of time before the reaction is terminated by adding DNTB. Some studies allow incubation for up to 30 or 60 min before absorption measurement (i.e., a fixed period of time) [28], while other studies measure the DTT consumption at different time intervals and estimate the fitted slope for DTT consumption over a period of reaction time [24,54,133,134]. The methods of calculation could lead to inconsistent DTT results. Specifically, using the DTT consumption rate calculated with equation (1) can lead to a non-negligible bias when estimating the OP^{DTT} of PM for prolonged reaction time. Equation (1) is derived based on the assumptions of a pseudo-first order reaction between DTT and PM and for a short-term reaction (i.e., with limited time) [18,49,57]. Under these two conditions, the relationship between DTT consumption versus time can be approximated using a form of linear regression that in turn represent a zero-order reaction. However, the DTT consumption with reaction time may not always follow the zero-order trend if the major contributors to DTT consumption are not catalytic species[36]. For example, the DTT consumption rate of isoprene SOA decreased sharply after 50 min in the study by Jiang et al. because the major DTT consumption pathway is the oxidation by organic hydroperoxides [36]. Measuring the DTT consumption within a short-term reaction may attenuate the bias, depending on the corresponding rate constants of DTT-active components in PM, but such effects have generally not been considered. Thus, systematic studies on kinetics between DTT and test compounds are required to better evaluate the measured OP^{DTT}.

Furthermore, the initial concentrations of DTT have also been reported to influence OP^{DTT} results, showing that the DTT consumption rates were proportional to the initial DTT concentrations for both humic-like substances (HULIS) and metal samples. Namely, higher initial DTT concentrations resulted in higher OP^{DTT} with the same tested samples [135]. As shown in Table 1, different initial DTT concentrations have been applied in published studies, ranging from 20 μM to 100 μM [13,49,54,133,136]. This observation highlights the need to consider the reaction kinetics in order to unify the assay results.

To improve the extraction efficiency of PM components, many studies use different solvents to ensure that most PM species can be fully dissolved and available to react with DTT [30,89,94,137,138]. Solvents used for extraction range from pure water to organic solvents (i.e., methanol, ethanol/deionized water, dichloromethane and dimethyl sulfoxide). Notably, the solvent used for PM extraction and DTT measurement affects the OP^{DTT} measurement because the solubility of PM components varies greatly in different type of solvents. Rattanavaraha et al. and Verma et al. both demonstrated that the OP^{DTT} for methanol extracted samples was significantly higher than that of water extracted samples [29,139]. Yang et al. also reported that the extraction solvent has a significant effect on OP^{DTT} for urban PM samples [94]. Thus, when intercomparing OP^{DTT} from different studies, extraction solvents should be considered.

Moreover, many studies applied a chelating agent such as EDTA directly to the reagents or reaction mixtures of DTT assay [13,28,140,141]. However, EDTA has been found to suppress the DTT activity of both metal and quinones [49]. As a result, OP^{DTT} could be underestimated if EDTA is added to the assay. As an alternative, Chelex resin has been chosen to replace EDTA to remove trace metals in reagents [50,135].

Lastly, potential bias of OP^{DTT} may result from sample extraction procedure using an ultrasonic treatment. Jiang et al. [142] reported that the OP^{DTT} of carbon materials increased when the samples were treated with sonication before incubation with DTT and attributed the increment to the dispersion of particles during sonication. While sonication can increase the extraction efficiency of PM in filter samples, it has been reported that sonication-derived ROS could be produced from PM components through thermal degradation or other mechanisms [143], which might alter the OP^{DTT} results.

Here, we provide a few recommendations for future studies that seek to standardize the DTT assay for a better inter-comparison of DTT assay results: (1) as most studies have been carried out, incubation at 37 °C and preferably without sonication during incubation would improve the consistency; (2) considering the reaction kinetics of DTT assay and the definition of DTT consumption rate, reaction times should be constrained within the linearly responsive range (to ensure a zero-order reaction), given the presence of the non-catalytic DTT-reactive species and the non-linearity of DTT consumption versus prolonged reaction time; (3) if the PM mass-normalized DTT consumption rate is used to represent OP^{DTT}, the initial DTT and sample concentration must be standardized; (4) EDTA is not recommended to be added during the DTT measurement.

5.2. The Understudied DTT Reaction Mechanisms

As discussed in Section 2, transition metals and quinones are commonly recognized as major contributors to DTT consumption by PM through catalytic redox reactions. However, other potential reactions that could also contribute to DTT consumption have not been widely studied. For example, as described in Section 2.3, organic hydroperoxides and Michael acceptors constitute prominent fractions of SOA, and they could contribute to DTT consumption through non-catalytic redox reactions and Michael additions [36,41,144,145], respectively. In addition, autooxidation of DTT in the presence of O_2 could lead to production of superoxide [146]. These DTT depletion pathways have not been fully understood, and how they will influence the interpretation of OP^{DTT} is largely unknown.

Other understudied mechanisms include the interaction among PM components. Verma et al. indicated that although water-insoluble metals are dominant components in PM, OP^{DTT} has been only mildly correlated with either water-soluble or insoluble metals [139]. The interaction between metals and other PM components might attenuate the correlation between metals and OP^{DTT} results. When determining DTT consumption of reactive PM constituents, individual chemical compounds are commonly assessed. Little is known about the effect of the interaction among PM components on DTT measurement. Below we summarized possible interactions among PM components.

(1) Organic hydroperoxides may interact with dissolved transition metal ions through Fenton-like reactions, leading to the formation of a variety of radical forms of reactive oxygen species including carbon and oxygen-centered organic radicals,·OH, and O^{-}_{2} [72].
(2) The formation of metal-organic ligand complexes may also complicate the elucidation of the DTT consumptions by PM. In the study by Yu et al., it was found that when interacting with quinones, Fe showed additive and synergistic effects in DTT consumption and ·OH, respectively, but Cu showed antagonistic effects in both measurements [54]. Meanwhile, Mn interacting with quinones showed synergistic effects in DTT consumption but antagonistic effects in ·OH generation [54]. As a comparison with the interactions with quinones, Fe, Mn, and Cu showed similar interaction pattern with HULIS, but their interactions with HULIS were weaker in DTT consumption than ·OH generation. [54]. In another study by Wei et al., Fe and Cu complex with Suwanee river fulvic acid (SRFA) showed a strong synergistic and additive effects in ROS generation, respectively [65]. DTT itself can also form specific and very stable polymeric and monomeric complexes with all of these metal ions, Zn(II), Cd(II), Pb(II), Ni(II) and Cu(I) [147].
(3) Interactions among organics have been shown to affect OP^{DTT}. For example, nitrogen-containing bases, such as pyridine, imidazole and their alkyl derivatives that are commonly found in HULIS were shown to significantly enhanced OP^{DTT} in the presence of quinones. This observation has been attributed to the presence of unprotonated N atom in nitrogen-containing bases that can act as H-bonding acceptors to facilitate hydrogen atom transfer in the ROS generation cycle of quinones, and thus, enhance the DTT consumption [148,149].
(4) High molecular weight organic compounds are commonly found in ambient PM samples. These compounds are often featured with multiple reactive functional groups within one molecule [43]. The presence of proximal reactive functional groups within high molecular weight organics could possibly influence DTT consumption, but the exact effect has not yet been fully investigated.

6. Conclusions

In this review, we presented a critical evaluation of DTT-based OP measurements. As a robust and cost-effective acellular method to measure the contribution of PM-bound ROS, the DTT assay has the advantage to screen for a large sample size of PM samples within a relatively short amount of time, providing initial insights into the oxidative capacities of PM to deplete thiol antioxidants. Recent advances in the automated DTT assay design have increased the assay throughput and further broadened its application. However, due to the non-standardized protocols and several understudied reaction mechanisms (e.g., non-catalytic pathways and interactions among PM components), intercomparisons between different studies and the interpretation of assay results remain major challenges. Furthermore, given that OP^{DTT} correlates well only with the generation of H_2O_2, but not with other types of ROS [54], a combination of various ROS measurements will be necessary to provide a more comprehensive picture of PM-bound ROS production. Nevertheless, since the PM-bound ROS and endogenous ROS generated by cells through metabolic processes and immune responses to inhaled particles (i.e., endogenous respiratory burst or PM-induced ROS) both occur in biological systems, to account for the overall ROS-associated health effects by PM exposure, integration of acellular and cellular ROS detection techniques will provide valuable information to identify the most influential factors that lead to adverse health outcomes. Overall, it is essential for future studies to continue elucidating the chemistry behind the DTT assay. Improved understanding of underlying mechanisms will allow for a more accurate interpretation of OP^{DTT} and possibly reconciliation of currently discrepant research findings.

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