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Effects of ultrasonic treatment on dithiothreitol (DTT) assay measurements for carbon materials

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ABSTRACT

The dithiothreitol (DTT) assay is the most commonly used method to quantify the oxidative potential of fine particles. However, the reported DTT decay rates of carbon black (CB) materials vary greatly among different researchers. This might have resulted from either the intrinsic toxicity of CB or the unsuitability of the DTT assay protocol for CB particles. In the current study, the protocol of the DTT assay for CB materials has been carefully evaluated. It was found that the dispersion degree of CB particles in water has a great influence on the DTT decay rate of CB materials. For CB particles (special black 4A (SB4A) and Printex U) and single-walled carbon nanotube tube (SWCNT), the DTT decay rate after sonication for 10 min became 4.2, 4.6 and 1.7 times higher than that without sonication. The rate continued to grow as a function of ultrasound time up to 30 min of sonication. Although the concentration of soluble transition metals and surface oxygen-containing species such as carbonyls increased slightly with sonication, they had no significant effects on the measured DTT activity, while the increase in the dispersion degree of aggregates was found to play a vital role in the observed enhancement of the DTT decay rates for different CB materials. Based on our results, 30 min of sonication is recommended for sample dispersion when measuring the DTT decay rate of CB materials.

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Introduction

Reactive oxygen species (ROS) are accompanied by the generation of oxidative stress, which is considered a possible

cause of respiratory disease and is one of the indicators used to evaluate oxidative damage to the body (Xia et al., 2006). A number of methods such as the dithiothreitol (DTT) assay (Cho et al., 2005), electron paramagnetic resonance (EPR) assay

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(Borm et al., 2007), ascorbic acid (AA) assay (Janssen et al., 2014) and dichlorofluorescein (DCFH) assay (Venkatachari et al., 2005) have been developed to quantify the oxidative potential of atmospheric particles with different sensitivities to ROS-generating compounds, whereas the DTT assay is the most commonly used method (Cho et al., 2005). DTT has a strong reducing ability and can be oxidized by the ROS in particles (Kumagai et al., 2002). Thus, the DTT assay is considered to be a chemical substitute for cell reduction reactions (Charrier and Anastasio, 2012; Li et al., 2003, 2009b; Verma et al., 2009).

Carbon black (CB), generated from the incomplete combustion of heavy petroleum materials (Apicella et al., 2003), has been mainly used for the manufacture of master batch, printer ink, paint, rubber, electrically conductive plastics (Lee et al., 2016; Parant et al., 2017), cosmetics and so on. CB is mainly composed of elemental carbon (EC) and contains low amounts of extractable organic and inorganic substances (Long et al., 2013) which are different from black carbon or soot. Carbon nanotubes (CNTs) can also be considered as CB-like materials, although their graphene sheets are arranged more regularly (Hu et al., 2010) than those in CB. During the lifetime of the relevant products, CB can enter the environment and eventually enters the human body (Tiwari and Marr, 2010). CB is also a component of atmospheric particles (Lai et al., 2017), and can contribute around 20% to particulate matters with a diameter $\leq 2.5 \mu\text{m}$ ($\text{PM}_{2.5}$) by mass concentration (Zhang et al., 2016). It has been well recognized that CB is associated with ROS formation (Chaudhuri et al., 2017; Zhen et al., 2017).

At the present time, the reported DTT decay rate for CB varies substantially from 0.9 to ~ 50 pmol/(min $\cdot\mu\text{g}$). For example, the decay rate was 0.9 pmol/(min $\cdot\mu\text{g}$) for graphite (Li et al., 2013), while it was 36.2 ± 4.9 pmol/(min $\cdot\mu\text{g}$) for CB particle (Printex U) (Li et al., 2015) and 59.3 ± 7.4 pmol/(min $\cdot\mu\text{g}$) for single-walled carbon nanotube (SWCNT) (Liu et al., 2015). Similar variations have also been observed among different soot particles. For example, the decay rate was measured at 6.1 pmol/(min $\cdot\mu\text{g}$) for diesel soot (Li et al., 2013), 33.6 pmol/(min $\cdot\mu\text{g}$) for methane flame soot (Holder et al., 2012), 49 ± 7 pmol/(min $\cdot\mu\text{g}$) for propane flame soot (Antinolo et al., 2015), and 27.0 pmol/(min $\cdot\mu\text{g}$) for hexane flame soot (Li et al., 2013). In addition, the DTT activity of CB particles has also been found to increase after ozonization, nitrogen oxides (NO_x) oxidation and anthraquinone coating (Li et al., 2009b), while it decreased after particles were coated with sulfate when compared with pristine CB samples (Li et al., 2015). These results mean that the DTT activity of CB particles should depend on the chemical properties of CB particles. However, it should be pointed out that CB are hydrophobic materials and prone to aggregate into fractal particles (Gray and Muranko, 2006). ROS formation mainly originates from the water-insoluble components or particle surfaces of CB particles rather than soluble components, because the filtrate from CB suspensions showed much less DTT activity than the suspensions themselves (Li et al., 2015, 2013). This means that experimental conditions might also have a great influence on the measured DTT activity of CB.

In general, the concentration of ROS in particles is measured by the DTT decay rate under conditions where an

excess of DTT reacts with particles suspended in buffer solutions (Li et al., 2009a). However, the protocols of DTT assays were inconsistent among different researchers in previous works when measuring the oxidative potential of CB materials. In one example, CB samples (without sonication) were directly incubated at 37°C in 1 mL of 0.1 mol/L potassium phosphate buffer (pH = 7.4) with 100 $\mu\text{mol/L}$ DTT from 15 to 90 min. Then, the reaction was quenched by 10% trichloroacetic acid (1 mL), and the remaining DTT was measured by the absorbance at 412 nm of 5-mercapto-2-nitrobenzoic acid, which was formed by mixing the incubation mixture (0.5 mL aliquot) with 0.4 mol/L Tris-HCl (1 mL, pH 7.4) containing 20 mmol/L of ethylene diamine tetraacetic acid (EDTA) and 10 mmol/L 25 μL 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) (Cho et al., 2005). In contrast, CB particles in other studies were sonicated for different amounts of time to obtain suspensions before reacting with DTT, followed by the same or similar measurement procedure mentioned above. The sonication time employed usually varies from 1 (Xu et al., 2015) to 15 min (Antinolo et al., 2015; Liu et al., 2015). Therefore, it is necessary to evaluate the suitability of the DTT assay protocol for CB samples to obtain credible results.

In the present study, we investigated the influence of sonication time on the DTT activity of three CB samples. Heavy metals, functional groups and oxygen-containing species for CB samples with different ultrasound treatment times were analyzed by inductively coupled plasma mass spectrometry (ICP-MS), attenuated total reflectance infrared spectroscopy (ATR-IR) and X-ray photo-electron spectroscopy (XPS), respectively, to determine the influence of sonication time on the measured DTT decay rates. We also analyzed the differences in morphology and particle size of three carbon black samples including CB particles (special black 4A (SB4A) and Printex U) and SWCNT at different sonication times using transmission electron microscopy (TEM) and a Zetasizer Nano instrument. The present work is important to establish a reliable method suitable for DTT assay of CB particles and is also meaningful for understanding the toxicity of CB in the atmosphere.

1. Materials and methods

1.1. Chemicals

Three kinds of commercial CB materials, including SB4A, SWCNT and Printex U, were used in the present study. SB4A and Printex U were obtained from Degussa, while SWCNT was supplied by Timesnano. DTT was provided by Sigma-Aldrich. DTNB was purchased from Alfa Aesar. A heavy metal mixed standard solution was purchased from Aladdin. Sodium dihydrogen phosphate dehydrate and disodium hydrogen phosphate dihydrate were supplied by Sinopharm.

1.2. DTT assay

CB samples of about 100 μg were suspended in 10 mL phosphate buffer (pH 7.4, 0.1 mol/L) and sonicated for 0, 5, 10, 30 and 60 min, respectively. Then, DTT (3.0 mL, 0.5 mmol/

L) was mixed with 3.0 mL of the post-sonicated suspensions or filtrates without further sonication. The mixture was placed in a thermostatic shaking chamber at 37°C. The reaction mixture (0.25 mL) was withdrawn every 5 min and filtered by a 2 μm syringe poly(tetrafluoroethylene) (PTFE) filter, then added to a cuvette containing 1 mL DTNB (0.25 mmol/L). The concentration of 2-nitro-5-thiobenzoate was detected at 412 nm by ultraviolet–visible (UV–Vis) absorption spectroscopy (7230G-N2, Jingke, China).

The rate of DTT decay rate was expressed as the ratio of the concentration decrease of DTT and the quantity of CB particles. The concentration of DTT was measured using a standard curve. The reaction between buffer solution and DTT was used as the blank control for the experiment.

1.3. Coating of carbon materials

Oxygen-containing species on the surface of CB samples with different sonication times were measured by XPS (AXIS Supra, Kratos, UK). According to the XPS results, we used benzoquinone to compensate the increase of oxygen content during sonication. Coating of quinones on fresh black carbon has been performed in previous work to illustrate the contribution of quinones to the DTT activity of black carbon (Chu et al., 2017). The carbon materials and benzoquinone were dissolved in dichloromethane with a ratio of 7:1. The mixture was rotary evaporated and dried to obtain a carbon material with 9.17% of oxygen content, which was the same as the highest oxygen content among different carbon materials after sonicated for 30 min.

1.4. Particle characterization

Total metals in the particles were measured after the CB samples were digested with concentrated 1:3 HNO_3/HCl , using an ICP-MS (7500a, Agilent Technologies, USA). The soluble metal concentration was also measured after samples were sonicated for 10 and 30 min. Transition metals were quantified with the standard solution. The infrared (IR) spectra were measured with a Fourier transform infrared (FT-IR) spectrometer (Nicolet 6700, Thermo Nicolet Instrument Corp, USA) equipped with a high sensitivity mercury cadmium telluride (MCT) detector to analyze changes in functional groups of samples at different sonication times. Samples with different sonication times were sprayed evenly on the ZnSe crystal of the ATR cell. After drying, the spectra of functional groups were recorded using the clean ZnSe crystal as a reference spectrum. All spectra reported here were recorded at a resolution of 4 cm^{-1} for 100 scans. Oxygen-containing species on the surface of CB samples with different sonication times were measured by XPS (AXIS Supra, Kratos, UK). The samples were excited by Al $K\alpha$ X-rays (high voltage ($h\nu$) = 1486.7 eV) with 15 kV working voltage and 40 mA emission current. The morphologies of the CB samples were characterized by TEM (H-7500, Hitachi, Japan). The CB samples were ultrasonically dispersed in ultrapure water for different times and the suspensions were evenly distributed on copper microgrids. The test accelerating voltage was 80 kV. A Zetasizer Nano instrument (ZS90, Malvern Panalytical, UK) was used to analyze the particle sizes at different ultrasound times.

2. Results and discussion

2.1. Oxidative potential of CB materials at different sonication times

Fig. 1 shows the DTT decay rates of SB4A, Printex U and SWCNT, measured at different sonication time. They were 46.1 ± 2.3 , 32.4 ± 4.6 and 52.5 ± 1.1 $\text{pmol}/(\text{min}\cdot\mu\text{g})$ without sonication. The measured DTT decay rates for these three CB samples were comparable with those reported in the literature with different sonication times. For example, the rate was 31 $\text{pmol}/(\text{min}\cdot\mu\text{g})$ for Printex U without sonication (Li et al., 2013) and 36 $\text{pmol}/(\text{min}\cdot\mu\text{g})$ with 1 min of sonication (Xu et al., 2015) and 59.3 ± 7.4 $\text{pmol}/(\text{min}\cdot\mu\text{g})$ for SWCNT with 15 min of sonication time (Liu et al., 2015). These values were also similar to reported values for soot particles, such as 33.3 $\text{pmol}/(\text{min}\cdot\mu\text{g})$ from methane flame soot (Holder et al., 2012), as well as diesel emission particles (39 ± 5 $\text{pmol}/(\text{min}\cdot\mu\text{g})$) and gasoline emission particles (25 ± 3 $\text{pmol}/(\text{min}\cdot\mu\text{g})$) (Geller et al., 2006).

The DTT decay rate of the three samples increased monotonically as a function of sonication time. The DTT decay rates of SB4A, Printex U and SWCNT sonicated for 10 min became about 4.2, 4.6 and 1.7 times higher than the corresponding values without sonication. This indicates that sonication has a great impact on DTT activities. After 30 min of sonication, the growth rate of DTT activity slowed down. Therefore, we think that 30 min of sonication should be required for the DTT assay test with CB materials. It should be noted that the DTT decay rate of SB4A and Printex U grew faster than that for SWCNT under the same conditions. This means that further verification of the sonication time is required when comparing DTT activities among different CB materials.

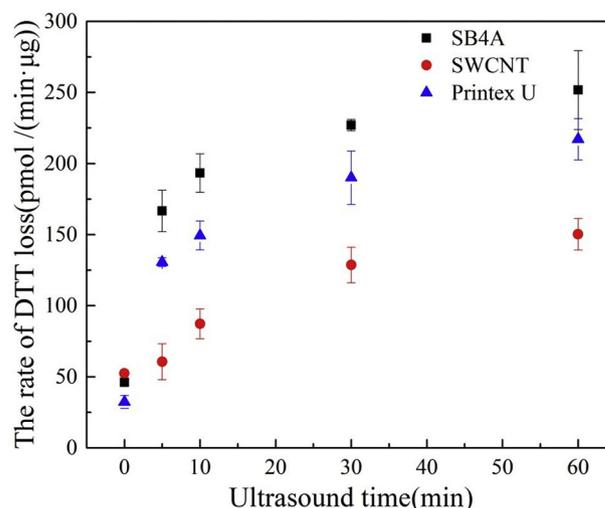


Fig. 1 – Rate of dithiothreitol (DTT) loss of carbon black particles (special black 4A (SB4A) and Printex U) and single-walled carbon nanotube tube (SWCNT) under different ultrasound times.

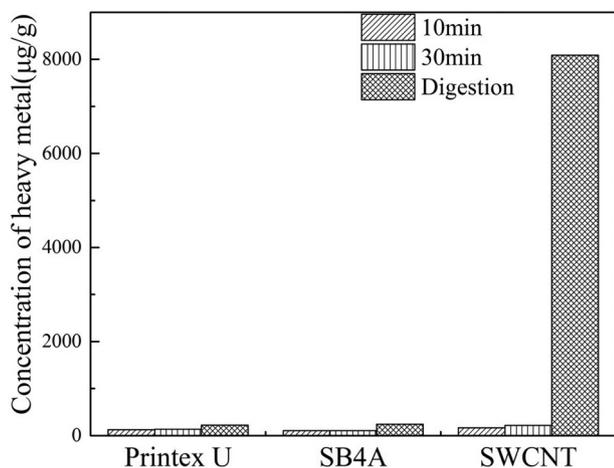


Fig. 2 – Content of heavy metals dissolved in sample suspensions under different conditions.

2.2. Influence of sonication on chemical composition of CB

To identify the possible reasons for the variation of DTT activities, the physicochemical properties of the sample suspensions after sonication were analyzed. Transition metals were considered to be the main factor responsible for the production of DTT activity in black carbon (BC) particles in previous studies (Li et al., 2003; McWhinney et al., 2013). The contents of metals including V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Cd, Sn, Pb and Ba were measured using an ICP-MS. Samples after 10 and 30 min of sonication were analyzed and compared with the corresponding samples digested by using concentrated 1:3 HNO₃/HCl.

As shown in Fig. 2, the total metal contents in SB4A, Printex U and SWCNT samples were 223, 242 and 8087 µg/g, respectively, while the corresponding soluble metals after 10 min of sonication were 106, 128 and 161 µg/g. When further increasing the sonication time, the concentration of dissolved

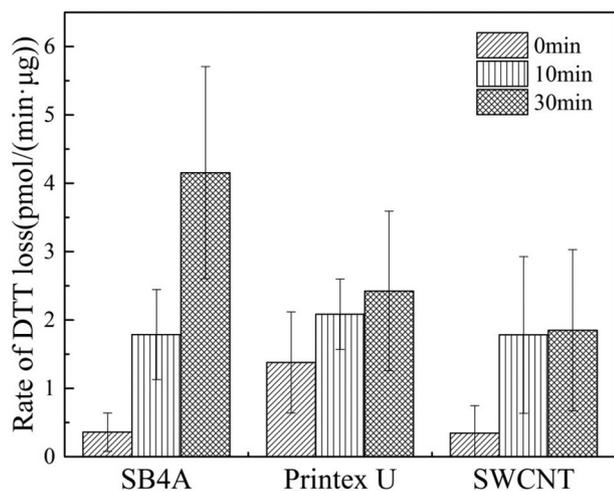


Fig. 3 – DTT activities of filtrates of SB4A, Printex U and SWCNT under different ultrasonic times.

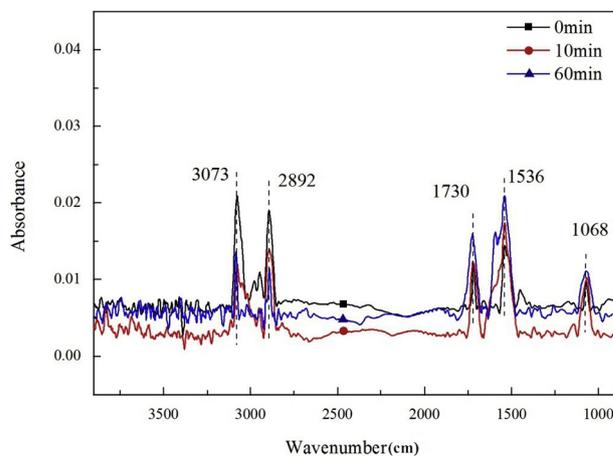


Fig. 4 – Comparison of infrared (IR) spectra of SB4A samples with different ultrasound time.

metals slightly increased. For example, the dissolved metal concentrations after 30 min of sonication were 107, 136 and 217 µg/g, respectively. This means that the soluble metals contributed 56.9%, 42.9% and 2.0% to the total metals for SB4A, Printex U and SWCNT, respectively. However, the DTT activity of these CB particles significantly increased from 10 to 30 min of sonication. This means the increase of DTT activity of different CB particles as a function of sonication time is not related to the dissolution of metal ions. In addition, it should be noted that SWCNT had the lowest DTT activity at all the sonication times, although it contained the highest content of total metals or soluble metals. In addition, The DTT activities of the filtrates after sonication at different times were measured to further confirm the role of soluble metals in the measured DTT decay rate of different CB materials. As shown in Fig. 3, although the DTT activities of the filtrates slightly increased as a function of sonication, the DTT decay rate of the filtrates contributed less than 1.8% to the corresponding

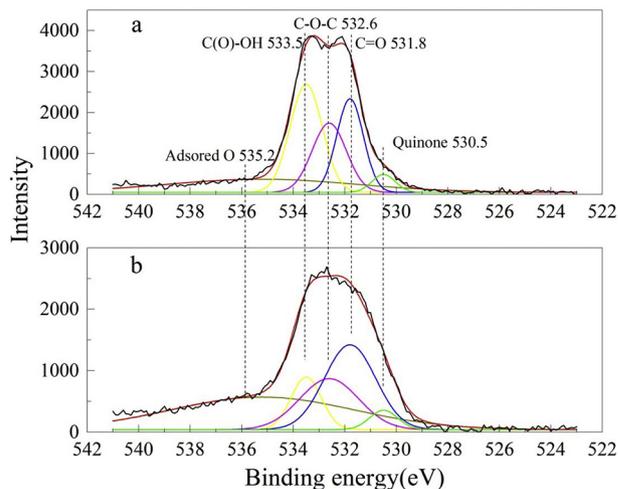


Fig. 5 – Comparison of oxygen content of SB4A with (a) 30 min and (b) 0 min of ultrasound time.

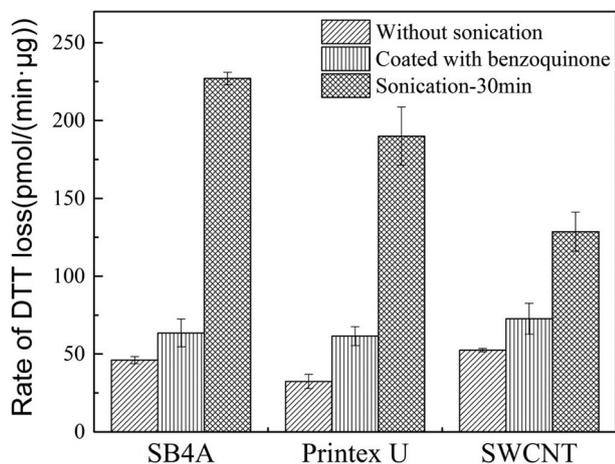


Fig. 6 – Comparison of the DTT decay rates for benzoquinone coated carbon materials with the corresponding material without and with sonication for 30 min. The oxygen contents after coating were 9.17%.

DTT activities of CB suspension (Fig. 1). These results mean that dissolution of metals might have a little influence on the increase of DTT activity during sonication.

It has been recognized that sonication might lead to modification of surface functional groups on CB particles (Xue et al., 2011). Fig. 4 shows the IR spectra of SB4A with sonication time varying from 0 to 60 min. The peaks at 3073 and 2892 cm^{-1} were attributed to olefin and alkane C–H (Han et al., 2016). The peaks at 1730 and 1536 cm^{-1} were ascribed to carbonyl and ether ketone C=O (Cain et al., 2010), while the peak at 1068 cm^{-1} was assigned as ether C–O–C (Al Lafi, 2014). As shown in Fig. 4, the intensities of olefin and alkane C–H decreased, while the intensities of carbonyl and ether C=O peaks increased slightly as the sonication time increased. These results suggest that sonication might lead to weak oxidation of CB materials. In previous work, several studies found that oxidation of CB or BC by O_3 leads to enhancement of their oxidative potential (Antinolo et al., 2015; Holder et al., 2012; Li et al., 2015, 2009b). In particular, the DTT decay rate of soot particles has been found to increase as a function of the content of quinones formed from ozone oxidation of organic carbons in soot (Antinolo et al., 2015). However, other studies found that oxidation of CB or BC by O_3 or OH has little influence on their oxidative potential, although surface carbonyls, carboxylic acids and esters can be formed (Liu et al., 2015; Peebles et al., 2011). Therefore, we think that the weak oxidation occurring during sonication of CB observed in the present study should not lead to an obvious enhancement of DTT decay rate.

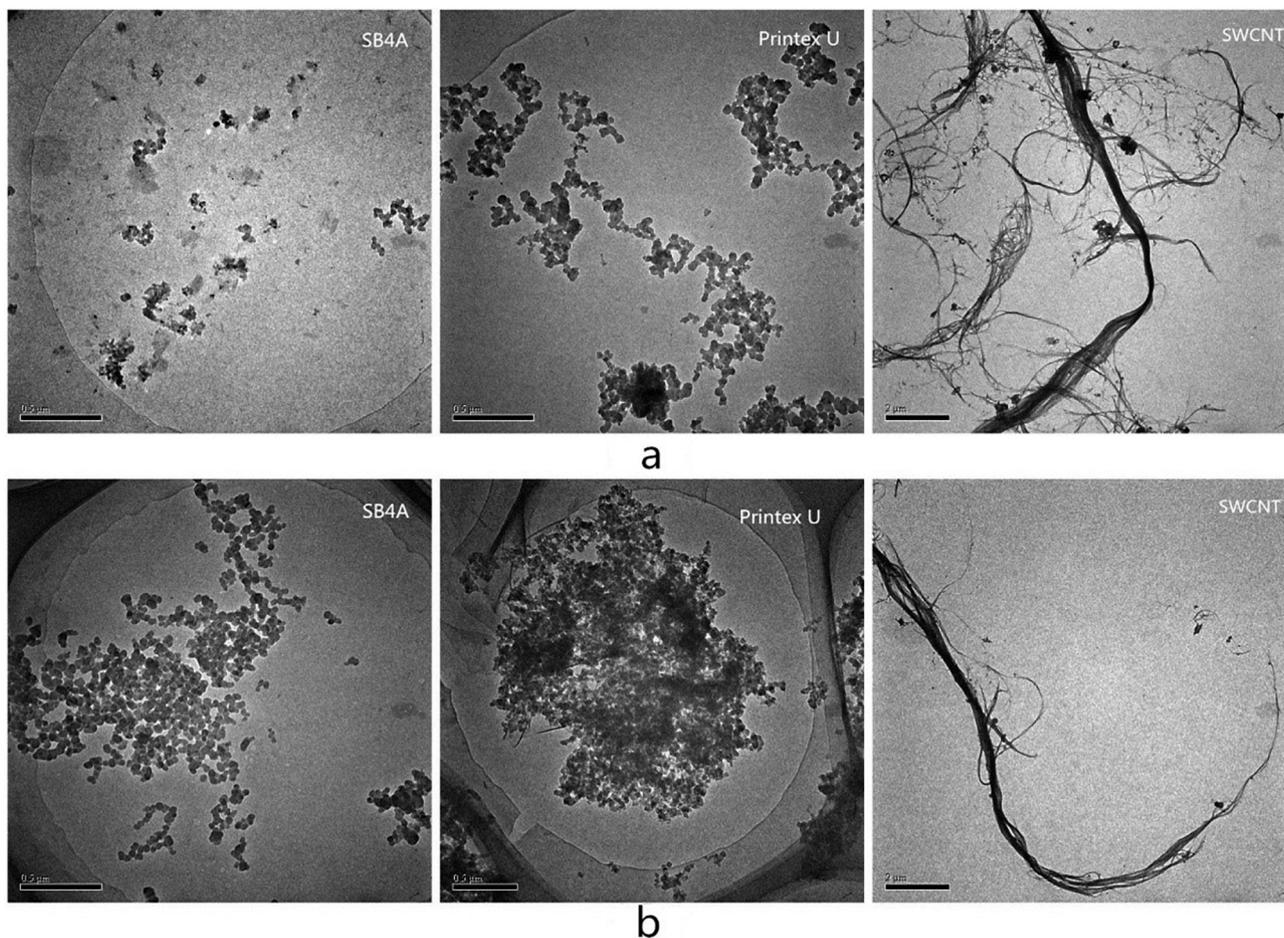


Fig. 7 – Transmission electron microscopy (TEM) images of SB4A, Printex U and SWCNT with (a) 5 min and (b) 60 min of ultrasound time.

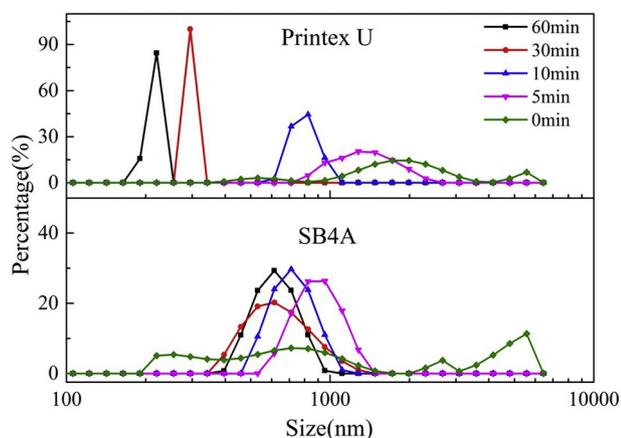


Fig. 8 – Particle size distribution in Printex U and SB4A suspensions at different ultrasound times.

To further analyze the relationship between changes in oxygen-containing groups and changes in DTT activity, the surface oxygen of SB4A without ultrasonic or with 30 min of sonication were analyzed by XPS and the results are shown in Fig. 5. Highly conjugated forms of carbonyl oxygen, such as quinone groups at 530.5 eV, carbon–oxygen double bond (C=O) at 531.8 eV, carbon–oxygen single bond in epoxide (C–O–C) at 532.6 eV, carbon–oxygen single bond in hydroxyl group (C(O)–OH) at 533.5 eV and adsorbed oxygen at 535.2 eV (Schuster et al., 2011) were present in these CB samples. In particular, the intensity of C(O)–OH, C–O–C and C=O in samples with 30 min of ultrasound were higher than in samples without sonication. We compared the change of oxygen content between two samples by calculating the total peak area. The mass percentage of surface oxygen of the sample after 30 min of sonication was 9.17% while that for the sample without ultrasound was 6.42%, indicating that weak oxidation occurs during sonication of samples. The results of XPS and ATR-IR together show that sonication makes the surface oxygen content of the samples increase slightly, however, this is not directly related to the significant increase observed in the DTT activities of samples after sonication.

Carbon black was coated with benzoquinone to investigate the influence of reactive oxygen species which might be produced during sonication. According to the XPS results (Fig. 5), the surface oxygen species content of SB4A was increased from 6.42% to 9.17% after ultrasonic. Therefore, carbon materials with 9.17% of oxygen content was obtained by coating benzoquinone. The DTT decay rates of benzoquinone coated carbon materials are shown in Fig. 6. The DTT activity of SB4A, Printex U and SWCNT after coating were 63.5 ± 8.9 , 61.5 ± 6.1 and 72.6 ± 9.8 pmol/(min· μ g). The DTT decay rates of benzoquinone coated carbon materials increased slightly compared with the corresponding control experiments without sonication (46.1 ± 2.3 , 32.4 ± 4.6 and 52.5 ± 1.1 pmol/(min· μ g)), while they were significantly lower than the corresponding DTT decay rate of the sample sonicated for 30 min (221.3 ± 6.7 , 199.7 ± 4.7 and 128.6 ± 12.6 pmol/(min· μ g)) as show in Fig. 6. It should be noted that besides quinone, other oxygen containing species including carbonyl, hydroxyl and epoxides are the newly generated products during sonication

as shown in Fig. 5. On the other hand, quinone is a compound with high activity to generate ROS. Therefore, the DTT decay rates of the quinone-coated samples should represent the upper limit for the increase of DTT activity result from oxidation by sonication. This means that the increase effect resulted from oxidation during sonication should be insignificant compared to the increase in DTT resulted from other factors due to sonication.

It should be noted that we cannot rule out trace ROS generated in the solution during sonication. If the ROS formed by sonication in the solution is still alive after stopping sonication, the direct contribution of sonication on the measured DTT decay rate has been subtracted by the blank experiments because the blank experiments are always carried out at the same time in the DTT assay test. On the other hand, we measured the DTT decay rate of the filtration after sonication of SWCNT. It was 1.66 ± 0.15 pmol/(min· μ g). Even if this value is resulted from the ROS formed during sonication, it can be ignored when compared with the DTT decay rate of the SWCNT suspension (128.6 ± 12.6 pmol/(min· μ g)).

2.3. Influence of sonication on the morphology of CB aggregates

It has been found that the size distribution and morphology of particles are important factor of DTT activities (Samara, 2017). Fig. 7 shows the particle morphology images of CB materials at different sonication times. SB4A and Printex U are aggregates of spherical particles with the primary diameter of ~30 nm. After 5 min of sonication, a large amount of carbon black particles were still in an aggregated state with a relative large particle size (Fig. 7a), while the size of aggregated particles decreased obviously after these samples were ultrasonically dispersed for 60 min (Fig. 7b). As for SWCNT, it is tubular and not easy to disperse. With the increase of ultrasound time, more thin tubes appeared near the tubular structure, indicating that ultrasound separated the tubular aggregates. As the ultrasound time further increasing, the

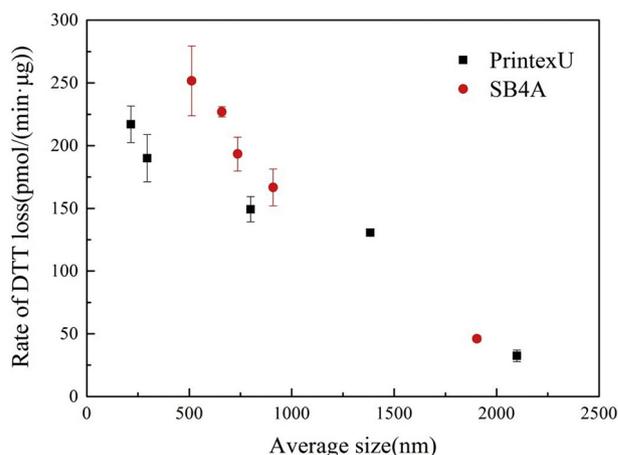


Fig. 9 – Relationship between the average particle size in SB4A and Printex U suspensions and DTT activity.

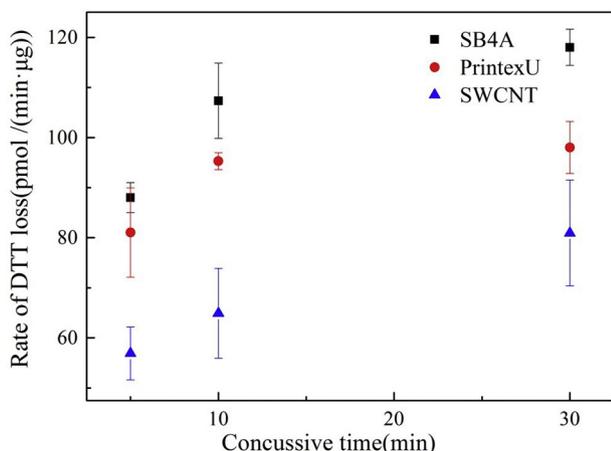


Fig. 10 – Rate of DTT loss of SB4A, Printex U and SWCNT under different concussive times.

aggregation state of the particles continuously changed, which is consistent with the increase of DTT decay rate.

To further analyze the effects of size and morphology, the distribution of the particle size after different sonication time in water was measured by a Zetasizer Nano. As shown in Fig. 8, the CB samples without sonication showed two peaks. The peaks at 5 µm were account of aggregation of large particles. The particle size distribution showed a clear left shift trend with the increase of ultrasonic time. For the ultrasonic times of 5, 10, 30 and 60 min, the median particle sizes of the suspension of SB4A particles were 955, 712, 615 and 531 nm, respectively. The median particle sizes of Printex U were 1280, 825, 295, 220 nm when the ultrasonic times were 5, 10, 30 and 60 min, respectively. As shown in Fig. 9, the average particle size of the particles is negative related to the DTT activity as a function of sonication time. This further confirmed that the increase in dispersion degree of the aggregates determined the DTT decay rate of CB materials.

2.4. Oxidative potential of CB materials at other dispersion methods

In order to verify the effect of particle size dispersion, we also tried to use the oscillating method to disperse CB, and measured the change of DTT activities under different concussive time. The results are shown in Fig. 10. Obviously, The DTT activities also showed an upward trend due to the turbulent treatment. However, the measured DTT decay rates of these samples dispersed by sonication were as around two times as that dispersed by turbulent manner with the same dispersion time. Even if quinone is the only oxidation product during sonication, it cannot explain the increment of DTT decay rates as shown in Fig. 6. As discussed above, the particle surface rather than the filtrates of suspensions of CB particles contributes to the consumption of DTT (Cao et al., 2014). The increase of the dispersion degree of the particles in the suspension means that more active sites will be available for the reaction with DTT, which may explains the increase of DTT activities as observed in Figs. 1 and 10. Although the DTT decay rates of CB materials and soot particles have been

measured in previous works, the dispersion degree of the samples was not well characterized. This means that the oxidation potentials reported by different researchers might be greatly influenced by the experimental conditions. If the CB samples are not well dispersed, the measured oxidation potential would be heavily underestimated. When comparing SB4A and Printex U, however, Printex U was dispersed much better with smaller size than SB4A, but Printex U showed a smaller DTT decay rate than SB4A. This means that the chemical nature of the particles is also important in determining oxidative stress.

3. Conclusions

In the present study, the protocol of the DTT assay for CB materials has been evaluated. It was found that the dispersion degree of aggregates played a vital role in the measured DTT decay rates of different CB materials. As sonication time increased, the size of the particles in the suspension became smaller, leading to an increase in the DTT activity. Although soluble transition metals and surface oxygen-containing species such as carbonyls slightly increased during sonication, they had no significant effects on the measured DTT activities. The morphological structure of the samples themselves showed a different response to the sonication. For SB4A, Printex U and SWCNT samples, at least 30 min of sonication is required to obtain a well-dispersed suspension.

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