We appreciate the careful consideration of our manuscript by the reviewers. We have carefully responded to all of the point-by-point comments and issues raised by the reviewers and have revised the manuscript accordingly. These revisions are described in detail below.

Referee #1

General Comments: This work deals with the very complex nature of how small particles affect human health. There is substantial evidence that shows that small particles do have adverse health effects, but it is still not clear what causes issues. The authors recognize this, and do a good job of presenting the problem and previous research (with one notable exception, which will be discussed below). They focus on different carbon-based nanoparticles, including engineered ones. Their results are generally in agreement with previous work, but their major new contribution is the identification of epoxide groups on graphene oxide surfaces having a significantly larger effect on DTT decay rates.

Response: Thank you for your positive comments.

I have no major issues with how the experiments were performed (including the analytical methods used), and why the different nanomaterials were used. However, I have some issues with the interpretation and implications stated. The title states "implication for toxicological evolution during atmospheric relevant aging of soot" but most of the results are for engineered nanomaterials, not atmospheric soot, which is a very different type of particle. While it is important to learn about the toxicity of engineered nanomaterials, those results should not be applied to atmospheric soot particles (which have very few engineered nanoparticles). The results themselves are interesting and important enough without the atmospheric extrapolation, and I suggest revising the title and the Conclusions section.

Response: Thank you for your instructive suggestions. We revised the title as "Influence of functional groups on toxicity of carbon nanomaterials".

In the Conclusion section, we removed the sentences related to soot aging. For example, the following sentences have been deleted in the revised manuscript. "It is also a primary process in the atmosphere relating to chemical aging of particles including soot and CB particles", "This means that oxidation potential enhancement of CB particles is also possibly resulted from the formation of epoxide during chemical aging in the atmosphere", "On the other hand, it has been found that aging rate of BC particles under highly polluted urban environment is faster than that under clean conditions (Peng et al., 2016). In the future, much work should be performed on the toxicity evolution of CB or BC particles under real atmospheric conditions".

In the abstract section (lines 32-33), the final sentence has been revised as "These results imply that epoxidation might enhance the oxidation potential of carbon nanomaterials". In the introduction section, the sentence "In the current study, both the cell-free toxicity and the cell cytotoxicity of carbon nanomaterials with different functionalities were evaluated to focus on the role of functionalization in their toxicities to understand the possible influence of different source or oxidation processes on the toxicity evolution of soot particles" has been revised as "In the current study, both the cell-free toxicity and the cell cytotoxicity of carbon nanomaterials with different functionalities were evaluated to focus on the role of functionalization in their toxicities" (lines 119-121).

A new sentence has been added into the conclusion to emphasize the toxicity of epoxide-containing carbon materials as "This means that exposure to epoxide-containing carbon materials should lead to high health risk regarding to oxidation potential" (lines 560-561 in the revised manuscript).

In the discussion section, reasons for the observed effects are given with very little evidence (though there are some references stated). For example, line 248 states: This means the cell membrane might be intact when exposed to SB4A. Another is line 293: For example, adhesions and/or covering on cells could be the main MOA for graphene/graphene oxide (2-D structure), while for carbon nanotubes (1-D structure),

piercing and/or internalization by cells could be the main MOA. I suggest moving these types of sentences to the Discussion section and providing more references or information about these assumptions.

Response: Thank you for your suggestion. In the original manuscript, we did not separate the discussion from the results section. In the revised manuscript, we divided them into two parts. These sentences you mentioned have been moved to discussion section (lines 348-364) as "As shown in Fig. 2, all the carbon nanomaterials showed decreased ATP activities as a function of the dose. This means the carbon nanomaterials investigated in this work are toxic to murine J774 cell line. This is consistent with the previous results that CNT and Printex U are toxic to J774 cells (Kumarathasan et al., 2012) and graphene oxide can induce dose-dependent cell death in normal lung fibroblasts (HLF), macrophages (THP-1 and J744A), epithelial (BEAS-2B) cells, lung cancer cells (A549) etc. (Zhang et al., 2016;Li et al., 2018). At the same time, the BrdU activities decreased as a function of the dose of carbon nanomaterials, which means they are inhibitor for cell proliferation of murine J744 (Cappella et al., 2015). In addition, except for SB4A, other carbon nanomaterials showed significant increases in LDH. This means that the integrity of cell membrane decreased when J774 cells were exposed to these engineered carbon nanomaterials, while the cell membrane might be intact when exposed to SB4A (Cho et al., 2008;Kumarathasan et al., 2015). This might be related to lipid peroxidation induced by these engineered particles (Li et al., 2018) and the non-sphere feature of these engineered particles as observed in Fig.S1. These results also consistent with the previous study that observed CNT cytotoxicity ranking was assay-dependent (Kumarathasan et al., 2015)". The corresponding references have

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been added in the revised manuscript as you suggested.

Specific Comments: As the authors correctly state, the particles studied have very different chemistry and morphology, making it almost impossible to discern the mechanisms or chemicals responsible for the observed results. While most of the particles have similar DTT decay rates, this could be a coincidence or the result of a similar mode of action. I do not think the authors have clearly identified which it is. The authors should reference the many works from the Prof. Barry Dellinger group at LSU (he is deceased, but the work continues). They have identified a new type of radical, called Environmentally Persistent Free Radicals, that produce cell damage in a catalytic cycle involving metals, nanoparticles, and quinones (they have many papers in ES&T). The catalytic cycle upsets the notion that it is simply the concentration of an active species that is important.

Response: Thank you for your instructive suggestion. We agree with you that the observed DTT activity could be a coincidence of the chemical composition, functional groups and morphology of these particles. This make it is difficult to clearly identify the crucial factor determining the toxicity. This is the reason why we mainly compared the toxicity among these particles with the similar morphology, in particular, between graphene and graphene oxide. On the other hand, we think the role of epoxide in the highest DTT activity of graphene oxide can be well supported by the different DTT activity between thermal treated graphene oxide and the pristine graphene oxide. In the revised manuscript (lines 488-493), we emphasized this point as "Although the observed toxicity including DTT activity and cytotoxicity could be a coincidence of the chemical composition, functional groups and morphology of these particles, the above results at least imply that these physiochemical properties such as morphology, metal and OC content should not be crucial factors as for the toxicity of these carbon nanomaterials because it is difficult to observe an obvious dependence of the toxicity on these factors".

As for the Environmentally Persistent Free Radicals, we added a paragraph in the

revised manuscript (lines 530-536) as "Recently, environmentally persistent free radicals (EPFRs) (a kind of surface stabilized metal-radical complexes characterized by an oxygen-centered radical) (Dugas et al., 2016) have been identified in different source of particles including biomass/coal combustion, diesel and gasoline exhaust, ambient $PM_{2.5}$ and polymer (Balakrishna et al., 2009;Truong et al., 2010;Dugas et al., 2016). However, it is unclear that whether epoxide in graphene oxide observed in this study contributes to the EPFRs formation. This is needed to be investigated in the future".

Technical Corrections: The paper, in general, is well written. However, there are several awkward sentences, missing or unnecessary words that should be corrected (as a native English speaker, I cannot imagine writing a paper in a different language). These are all minor and did not affect my review.

Some examples are: Line 21 were investigated for understanding - change to "were investigated to understand"

Line 63 Change NO3 to NOx

Line 94 Functionalized does not to be capitalized

Line 348 Do you mean bonded?

Line 388 awkward sentence

Line 447 add "the"

Line 487 don't need this sentence

Line 505 explain the difference between BC and CB. They are not the same type of particle.

Response: Thank you so much for your comments. We carefully corrected these errors.

Line 21 (line 19 in the revised manuscript): "were investigated for understanding" has been changed to "were investigated to understand".

Line 63 (line 81 in the revised manuscript): "NO₃" has been changed to "NOx".

Line 94 (line 108 in the revised manuscript): "fMWCNTs" has been changed to "FMWCNTs".

Line 348 (line 301 in the revised manuscript): This sentence has been revised as "This

can be ascribed to desorption of surface adsorbents including bonded organics and trace water".

Line 388 (lines 329-334 in the revised manuscript): This sentence has been revised as "Several oxygen-containing species were observed as shown in Fig. 4A-F. Adsorbed oxygen was observed at 535.2 eV in the O1s spectra. Carbon-oxygen single bond in hydroxyl group (C-OH) and epoxide (C-O-C) were at 533.5 and 532.6 eV, respectively. Carbon-oxygen double bound (C=O) was observed at 531.8 eV, while highly conjugated form of carbonyl oxygen such as quinone groups was identified at 530.5 eV (Schuster et al., 2011)".

Line 447 (line 499 in the revised manuscript): "the" has been added before C-O-C (epoxide).

Line 487: The sentence "The DTT decay rates of special black 4A (SB4A), graphene, graphene oxide, single wall carbon nanotubes (SWCNT), SWCNT-OH and SWCNT-COOH were 45.9±3.0, 58.5±6.6, 160.7±21.7, 38.9±8.9, 57.0±7.2 and 36.7±0.2 pmol min^{-1}µg^{-1}, respectively. Epoxide has been for the first time identified as a highly active functional group in the carbon nanomaterials as far as the oxidation potential is considered." has been deleted in the revised manuscript.

Line 505: The definition of CB and BC was added in the revised manuscript (lines 36-56) as "Carbon nanomaterials are predominantly composed of carbon atoms, only one kind of element, but they have largely diverse structures characterized by different degrees of crystallinity and different macro- and micromorphology (Somiya, 2013). Their basic structure is that of graphite with planes of honeycomb-arranged carbon atoms. Carbon black (CB), which is produced from incomplete combustion of heavy petroleum materials under controlled conditions (Apicella et al., 2003), has been widely used in industrial products, such as inkjet printer ink, rubber and plastic products (Lee

et al., 2016), electrically conductive plastics (Parant et al., 2017), paints, coatings and cosmetics (Sanders and Peeten, 2011) and so on. CB is a quasi-graphitic form of nearly pure element carbon (EC, consist of graphene layers). It is distinguished by its very low quantities of extractable organic compounds and total inorganics (Long et al., 2013) compared with soot or black carbon (BC) (Andreae and Gelencser, 2006). Soot, which originates from incomplete combustion of biomasses, biofuels, fossil fuels and natural fires in reduced or anoxic environments, is a mixture of elemental carbon and organic carbon (OC) compounds (Muckenhuber and Grothe, 2006). In addition, as a class of engineering nanoparticles, carbon nanotubes (CNTs) and graphene materials are also a large group of carbon nanomaterials although their graphene sheets are arranged more regularly (Hu et al., 2010) than that in CB (Nienow and Roberts, 2006). During production and use of these consumer products, they are prone to enter into the environment and ultimately the human body (Helland et al., 2007;Tiwari and Marr, 2010), subsequently, to pose risk of adverse health effect".

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Referee #2

In this work, the authors study the DTT and cytotoxicity response of several carbon nanomaterials and correlate them to their morphology and chemical composition. The main finding is that the epoxide content of graphene oxide is particularly high and also results in high apparent oxidative potential. This specificity is confirmed with thermal treatment of this substance to reduce the epoxide abundance (though also accompanied by morphological changes in the process). The manuscript is generally well written and addresses a current topic to interest of many researchers. The measurements appear technically sound, though further comments below could be addressed

Response: Thank you for your positive comments.

General comments.

First question is regarding the XPS measurements: 1) How do the authors go from counts per second to oxygen content in (%) in Figure 5? If no calibration is performed, then is it possible to state absolute differences among functional groups or only C-O-C content among different materials? 2) How are epoxides distinguished from ethers? 3) It's not clear that these functional group characterizations are representative of the overall OC that is separately measured given the small probing depth of XPS. Can the authors comment on this?

Response: Thank you for your instructive suggestions. 1) About the XPS measurements, the instrument directly outputs the signal of O1s or C1s in cps, which means the number of electrons that escape from surface of the material being analyzed. When calculating the surface atom contents (%), we scaled the peak areas of each element according to the relative sensitivity factors. However, the relative sensitivity factors for each oxygen-containing species in the envelope of O1s are unavailable at the present time. We simply assumed all these oxygen-containing species in O1s having the same sensitivity factors. We agree with you that this might lead to additional uncertainty, while this method are usually used to calculate the relative content of

oxygen-containing species (Chen et al., 2017) and absolute oxygen content of each species (Schuster et al., 2011) when comparing among different samples. Therefore, we calculated the relative fraction of each oxygen-containing species, then converted them into oxygen content.

On the other hand, Wepasnick et al. (2011) measured the surface oxygen-containing species in MWCNTs based on chemical derivation techniques in conjunction with XPS. The oxygen content of COOH, C=O and C-OH in oxidized MWCNTs were (~3.0%, ~1.3% and ~1.0%), respectively. Using the method based on peak fitting in this work, we calculated the oxygen contents of COOH, C=O and C-OH that had been identified in the MWCNTs after oxidized by 70% HNO_3. These values were ~3.9%, ~2.0% and ~1.1% and comparable with those measured with chemical derivation (Wepasnick et al., 2011). Therefore, we think the estimated absolute oxygen content in Fig. 5 should be reliable to semi-quantitatively discuss the influence of oxygen-containing species on the DTT decay rates although we agree with you that this might introduce additional uncertainty. In the revised manuscript (lines 447-453), we added the discussion about the possible uncertainty as "At the present time, the relative sensitivity factors for each oxygen-containing species are unavailable. Similar to the method used in the literatures (Chen et al., 2017;Schuster et al., 2011), we simply assumed all these oxygen-containing species in the envelope of O1s having the same relative sensitivity factors. It should be reliable when semi-quantitatively comparing the contents of oxygen-containing species among different samples although additional uncertainties might be introduced for the calculated oxygen content".

2) If other ethers present in the carbon nanomaterials, it should also contribute to the O1s band which might be closed to that of epoxide. However, at the present time, it has been recognized that oxygen species including epoxide, hydroxyl, carbonyl and carboxylic groups present in graphene layer (Inagaki and Kang, 2014;Hunt et al., 2012). Epoxide should dominate the band at 532.6 eV compared with ethers (Hunt et al., 2012). In particular, the TGA results also supported the high content of epoxide in graphene

oxide. For other samples, other ethers might overestimate their contents of epoxide. However, this should not have influence on our conclusion that epoxides are related to the high oxidation potential of graphene oxide. This discussion has been added in the revised manuscript (lines 520-529) as "It should be noted that if other ethers present in the carbon nanomaterials, they should also contribute to the O1s band which might be closed to that of epoxide. However, at the present time, it has been recognized that oxygen-containing species including epoxide, hydroxyl, carbonyl and carboxylic groups present in graphene layer (Inagaki and Kang, 2014;Hunt et al., 2012). Epoxide should dominate the band at 532.6 eV compared with ethers (Hunt et al., 2012). In particular, the TGA results also supported the high content of epoxide in graphene oxide. For other samples in this work, other ethers might overestimate their contents of epoxide. However, this should not have influence on our conclusion that epoxides are related to the high oxidation potential of graphene oxide".

3) These oxygen-containing species measured using XPS are not representative of the overall OC because the probe depth of XPS is around 10 nm. On the other hand, OC includes not only the oxygen-containing species but also the hydrocarbons without oxygen atoms. Thus, XPS results only reflect the relative element ratio on the surface. However, the surface property should be very important to understand the toxicity of nanoparticles from the point view of particle-cell interaction (Cedervall et al., 2007). In the revised manuscript (lines 343-346), we added sentence to clarify this point as "It should be noted that XPS results only represent the surface atom ratios, which are different from the OC content representing the bulk composition. However, the surface property of particle should be very important to understand the toxicity of nanoparticles from the point view of particle-cell interaction (Cedervall et al., 2007) ".

The oxidation of SO_2 by epoxides 2016 is cited as support for ROS generation observed mechanism that is different from the mechanism by which oxidative potential of ROS is meant to be measured by DTT. The authors may wish to clarify this point as this may also be related to the discrepancy with the lack of difference in apparent cytotoxicity.

Response: Thank you for your instructive suggestions. We agree with you that the mechanism of SO_2 oxidation by epoxide might be different from that of DTT oxidation. Here we cited the oxidation of SO_2 by epoxides to support the oxidative property of graphene oxide. In fact, DTT is a stronger reducer than SO_2. Both direct oxidation by epoxides and indirect oxidation by ROS generated on the particle surface contribute to the consumption of DTT. Therefore, DTT decay rate should include a part of oxidation reactivity which can be explained by SO_2 oxidation. It has also been found that ozone oxidized carbon nanomaterials showed decreased DTT decay rates after treated by SO_2 compared with the pristine particles (Xu et al., 2015). In the revised manuscript (lines 517-519), we clarified this point as "This result is also well consistent with the previous founding that epoxides in graphene oxide can oxidize SO_2 to sulfate (He and He, 2016) although their oxidation mechanism might be different."

The discrepancy of the observed strong oxidation potential of graphene oxide with the lack of difference in apparent cytotoxicity among these particles may also related to the different interaction mechanism between DTT assay and in vitro assays. In the revised manuscript (lines 400-405), it has been pointed out as "The interaction between target cells and particles should be much complicated than that between DTT and particles. As discussed above, the cytotoxicity of nanoparticles relied on not only the mode of action but also the chemical nature of particles. Therefore, the different responses of the oxidation potential and the cytotoxicity to the epoxide content in these carbon materials might be accounted for by different mechanisms of toxicity among these assays".

As with the other reviewer I agree that the connection to atmospheric soot particles is quite tenuous; due to my delay in response I already see that the authors have proposed

changes in this regard (which makes the work less relevant for ACP?).

Response: Thank you for your instructive suggestions. According to your suggestions, we removed the connection to atmospheric soot particles. We think this work is still atmospheric relevant because these carbon nanomaterials can be emitted into the atmosphere from different sources. The results of this work should be still interesting and important enough without the atmospheric extrapolation as commented by reviewer 1. In the revised manuscript (lines 53-56), we added a sentence to emphasize the importance of this work as "During production and use of these consumer products, they are prone to enter into the environment and ultimately the human body (Helland et al., 2007;Tiwari and Marr, 2010), subsequently, to pose risk of adverse health effect".

One additional point on this is that the authors refer to "BC" but perhaps "soot" is more suitable, and the "surface functionalization" of soot have been characterized previously (including ethers) - e.g., Cain et al. 2010, Vander Wal et al. 2011, Popovicheva et al. 2014. However, atmospheric aging not only includes surface functionalization but also condensation of co-emitted species and photochemical oxidation products which are particularly rapid under conditions of soot emissions (Johnson et al. 2005 and Adachi et al. 2010); it is unclear how much of the oxidation potential attributable to functional groups would be dependent on the carbon nanomaterial itself in the environmental context.

Response: Thank you for your instructive suggestions. In the literatures, soot and black carbon are usually exchangeable. In the revised manuscript (lines 47-50), we added a sentence "Soot, which originates from incomplete combustion of biomasses, biofuels, fossil fuels and natural fires in reduced or anoxic environments, is a mixture of elemental carbon and organic carbon (OC) compounds (Muckenhuber and Grothe, 2006)". We replaced the "BC" with "soot" in the revised manuscript according to your suggestion, such as in lines 86, 216, 438 and 549.

The references related to surface functionalization including ethers (Cain et al., 2010;Wal et al., 2011;Popovicheva et al., 2015) have been added in the revised

manuscript (lines 97-98).

We agree with you that atmospheric aging not only includes oxidation but also condensation or coating of co-emitted species and secondary products from photochemical oxidation. The relative contributions of these two processes in toxicity changes of soot or CB particles to the oxidation potential are unclear at present time and might be dependent on the carbon nanomaterial. It has been found that the DTT decay rates of SWCNTs (Liu et al., 2015) and engineered nanoparticles (SiO_2) (Liu et al., 2019) decreased significantly as a function of exposure time of these pollutants. In the revised manuscript (lines 582-591), we discussed the uncertainty of this work as "Finally, condensation of co-emitted species and photo oxidation products is particularly rapid under conditions of soot emissions (Johnson et al., 2005;Adachi et al., 2010;Peng et al., 2016). In previous our work, it has been found that condensation process significantly decreased the oxidation potential of the SWCNTs (Liu et al., 2015). A recent work has also found that condensation of organic aerosol leads to decrease in oxidation potential on engineered nanoparticles (Liu et al., 2019). Therefore, the contribution of functional groups to the oxidation potential should be greatly influenced by condensation of co-emitted species and photo oxidation products in the atmosphere. This might be dependent on the carbon nanomaterial itself and needs to be investigated in the future".

Minor comments:

The methods section is very sparse in citations except a few of the authors own work, but citations to primary sources would be relevant here.

Response: Thank you for your instructive suggestions. Several relevant references have been cited in the revised manuscript (lines: 143, 153-154, 168).

There are typographical and grammatical errors which can be corrected during the editorial process of Copernicus.

Response: Thank you for your suggestions. We carefully checked and corrected some

typographical and grammatical errors.

Reference:

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Influence of functional groups on toxicity of carbon nanomaterials

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Abstract:

It has been well recognized that carbon nanomaterials and soot particles are toxic for

human health, while it is still controversial about the influence of functionalization on

their toxicity as well as the evolution of the toxicity of carbon nanomaterials due to

chemical aging in the atmosphere. In the current study, the oxidation potential measured

by dithiothreitol (DTT) decay rate and the cytotoxicity to murine macrophage cells of

different functionalized carbon nanomaterials were investigated to understand the role

of functionalization in their toxicities. The DTT decay rates of special black 4A (SB4A),

graphene, graphene oxide, single wall carbon nanotubes (SWCNT), SWCNT-OH and

SWCNT-COOH were 45.9±3.0, 58.5±6.6, 160.7±21.7, 38.9±8.9, 57.0±7.2 and

23 36.7 ± 0.2 pmol min^{-1}µg^{-1}, respectively. Epoxide was found to be mainly responsible for

24 the largest DTT decay rate of graphene oxide compared with other carbon

25 nanomaterials based on comprehensive characterizations. Both carboxylation and

26 hydroxylation showed little influence on the oxidation potential of carbon

27 nanomaterials, while epoxidation contributes to the enhancement of oxidation potential.

28 All these carbon nanomaterials were toxic to murine J774 cell line. However, oxidized

29 carbon nanomaterials (graphene oxide, SWCNT-OH and SWCNT-COOH) showed

30 weaker cytotoxicity to J774 cell line compared with the corresponding control sample

31 as far as the metabolic activity was considered and stronger cytotoxicity to J774 cell

32 line regarding to the membrane integrity and DNA incorporation. These results imply

33 that epoxidation might enhance the oxidation potential of carbon nanomaterials.

34

1. Introduction

Carbon nanomaterials are predominantly composed of carbon atoms, only one kind of element, but they have largely diverse structures characterized by different degrees of crystallinity and different macro- and micromorphology (Somiya, 2013). Their basic structure is that of graphite with planes of honeycomb-arranged carbon atoms. Carbon black (CB), which is produced from incomplete combustion of heavy petroleum materials under controlled conditions (Apicella et al., 2003), has been widely used in industrial products, such as inkjet printer ink, rubber and plastic products (Lee et al., 2016), electrically conductive plastics (Parant et al., 2017), paints, coatings and cosmetics (Sanders and Peeten, 2011) and so on. CB is a quasi-graphitic form of nearly pure element carbon (EC, consist of graphene layers). It is distinguished by its very low quantities of extractable organic compounds and total inorganics (Long et al., 2013) compared with soot or black carbon (BC) (Andreae and Gelencser, 2006). Soot, which originates from incomplete combustion of biomasses, biofuels, fossil fuels and natural fires in reduced or anoxic environments, is a mixture of elemental carbon and organic carbon (OC) compounds (Muckenhuber and Grothe, 2006). In addition, as a class of engineering nanoparticles, carbon nanotubes (CNTs) and graphene materials are also a large group of carbon nanomaterials although their graphene sheets are arranged more regularly (Hu et al., 2010) than that in CB (Nienow and Roberts, 2006). During production and use of these consumer products, they are prone to enter into the environment and ultimately the human body (Helland et al., 2007;Tiwari and Marr, 2010), subsequently, to pose risk of adverse health effect.

57 The adverse effect of CB and soot particles on human health has attracted much

58 attention in the atmospheric chemistry community (Baumgartner et al., 2014). Overall,

59 exposure to CB is associated with high risk of cancer, respiratory and cardiovascular

60 diseases (WHO, 2013;Niranjan and Thakur, 2017). Mitochondrial damage in alveolar

61 macrophages and bronchial epithelial cells resulted from exposure of diesel exhaust

62 particles (DEPs) has been observed (Li et al., 2002a;Li et al., 2002b). Oxidation stress

63 or reactive oxygen generation (ROS) is one of mechanisms related to the toxicity of

64 particles including soot particles (Nel et al., 2006), and has been even used as a

65 paradigm to assess particle toxicity (Xia et al., 2006).

66   Dithiothreitol (DTT) decay rate is commonly used as a cell-free measure of the

67 oxidative potential of different particles (Cho et al., 2005;Charrier and Anastasio,

68 2012;Kumagai et al., 2002), such as ambient particles (Li et al., 2003;Fang et al.,

69 2016;Cho et al., 2005;Charrier and Anastasio, 2012;Wang et al., 2013), secondary

70 organic aerosol (SOA) (McWhinney et al., 2013b), DEP (Li et al., 2009;McWhinney et

71 al., 2013a), carbon nanotubes (CNT)(Liu et al., 2015), flame soot (Antinolo et al.,

72 2015;Holder et al., 2012;Li et al., 2013) and commercial carbon black (CB) particles

73 (Koike and Kobayashi, 2006;Li et al., 2009;Li et al., 2015;Li et al., 2013). However,

74 the reported DTT decay rate of soot and CB particles varied substantially, from 0.9 to

75 ~50 pmol min^{-1} μg^{-1}. The variation of DTT decay rate among different samples implies

76 the importance of the composition or structure of particles in their toxicities.

77   Although transition metals, element carbon, humic-like substances and quinones

78 are responsible for ROS generation on particle surface (McWhinney et al., 2013b;Li et

79 al., 2003), more work is still required to deeply understand the toxicity of soot and the

80 reason why the toxicity varies greatly among different soot samples. On the other hand,

81 soot particles are prone to undergo oxidation by O_3, OH and NOx etc. during transport

82 in the atmosphere. Subsequently, functionalization including formation of OH, C=O,

83 epoxide (C-O-C) and COOH occurs (Mawhinney et al., 2000;Liu et al., 2015;Holder et

84 al., 2012;Corbin et al., 2015). This make it more complicate to understand the toxicity

85 of soot particles. For example, several studies have found that atmospheric relevant

86 oxidation of CB or soot by O_3 leads to enhancement of their oxidative potential (Li et

87 al., 2009;Li et al., 2013;Li et al., 2015;Antinolo et al., 2015;Holder et al., 2012). In

88 particular, the DTT decay rate of soot particles has been found increasing as a function

89 of the content of quinone formed via ozone oxidation of organic carbons in soot

90 (Antinolo et al., 2015). However, some other studies have found that oxidation of CB

91 or soot by O_3 or OH under atmospheric related conditions has little influence on their

92 oxidative potential or cytotoxicity although surface functionalization is observable (Liu

93 et al., 2015;Peebles et al., 2011). Therefore, it is necessary to understand the role of

94 functional groups in the toxicity of soot and CB particles.

95   During combustion process, however, multiple functional groups including OH,

96 C=O, COOH, esters, ethers and so on are usually formed at the same time and present

97 in both OC and EC (Han et al., 2012a;Cain et al., 2010;Wal et al., 2011;Popovicheva et

98 al., 2015). Thus, it is difficult to differentiate the role of one kind of functional group

99 from others in the toxicity of soot particles. However, it is possible to investigate the

100 role of functional groups in the toxicity of carbon nanomaterials when using CB or

101 engineered carbon particles with different functional groups as model sample of soot

102 particles. Actually, it has been recognized that the surface properties of carbon

103 nanomaterials will influence their biological effects or toxicity (Lara-Martinez et al.,

104 2017;Liu et al., 2014b;Koromilas et al., 2014). For example, a recent study has found

105 that hydrated graphene oxide exhibited a higher cytotoxicity to THP-1 and BEAS-2B

106 cells as a consequence of lipid peroxidation of the surface membrane and membrane

107 lysis compared to pristine and reduced graphene oxide (Li et al., 2018). Functionalized

108 multiwalled carbon nanotubes (FMWCNTs) is highly cardioembryotoxic in

109 comparison with functionalized oxygen-doped multiwalled carbon nanotubes (Lara-

110 Martinez et al., 2017). As pointed out by Lara-Martinez et al. (2017), however,

111 cytotoxic effects of carbon nanomaterials at the cellular level generate considerable

112 controversy and more research is clearly needed to gain insight into the mechanism of

113 these adverse effects. In addition, passive diffusion and energy-dependent endocytosis

114 are the two methods suggested for particles entry into living cells (Foroozandeh and

115 Aziz, 2018). They can also be distributed to various parts of the body, from where they

116 can either remain, translocate, or be excreted. Therefore, it is meaningful to investigate

117 the influence of functionalization on other endpoints alone even for these carbon

118 nanomaterials.

119 In the current study, both the cell-free toxicity and the cell cytotoxicity of carbon

120 nanomaterials with different functionalities were evaluated to focus on the role of

121 functionalization in their toxicities. DTT decay rate representing the oxidative potential

122 and the cytotoxicity of murine macrophage cell were investigated. The carbon

nanomaterials were characterized with inductively coupled plasma-mass spectrometry (ICP-MS), thermal gravity analysis (TGA), X-ray photoelectron spectroscopy (XPS), transmission electron microscopy (TEM) and zeta potential analyzer. The role of oxygen containing species in the toxicity of carbon nanomaterials was discussed. This work will be helpful for understanding the toxicity of carbon nanomaterials with different functional groups.

2. Experimental Section

2.1 Chemicals and characterization of particle samples. Commercial carbon nanomaterials including Special Black 4A (SB4A), graphene, graphene oxide, SWCNT, SWCNT-OH and SWCNT-COOH were used in this study. All these functional groups have been identified in soot particles and chemical aged soot or CB particles. SB4A was supplied by Degussa. The other carbon nanomaterials with purity >98% were supplied by Timesnano. To obtain graphene oxide with low epoxide content, graphene oxide was thermally treated at 200 °C for 30 min in high purity (99.999%) nitrogen flow. Dithiothreitol (DTT) was supplied by Sigma-Aldrich. 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) was obtained from Alfa Aesar. Standard solutions of metal ions including Cr, Mn, Fe, Co, Ni, Cu, Zn, Cd, As, Sn and Pb were supplied by National Institute of Metrology, China. 30 % H_2O_2 solution was supplied by Sinopharm Chemical Reagent Co., Ltd.

A transmission electron microscope (H-7500, Hitachi) was used to investigate the morphologies of carbon nanomaterials (Golberg et al., 2012). Particles were ultrasonically dispersed in ultrapure water (18 MΩ) and a droplet of suspending liquid

7

145 was deposited onto a Cu microgrid. An acceleration voltage of 80 kV was used for

146 measurements. The morphologies were shown in Fig. S1. The diameter of primary

147 particles were analyzed by ImageJ 1.41 software (Liu et al., 2010). The diameter of the

148 primary carbon sphere for SB4A was 66±17 nm. The out diameter (OD) of SWCNT,

149 SWCNT-OH and SWCNT-COOH was <2 nm with fiber length of 1-3 μm according to

150 the product report and also confirmed by TEM (Fig. S1). Graphene and graphene oxide

151 were 2-dimensional materials with monolayer and the diameter of 0.5-3 μm.

152 XPS were measured using an AXIS Supra/Ultra (Kratos, Kratos Analytical Ltd.) to

153 identify the oxygen containing species on the surface of carbon nanomaterials (Wal et

154 al., 2011;Schuster et al., 2011). The samples were excited by Al Kα X-ray ($h\nu$=1486.7

155 eV) with 15 kV of working voltage and 40 mA of emission current. The spectra were

156 analyzed with XPS Peak software. The content of organic carbon (OC) in carbon

157 nanomaterials was measured by thermal desorption using a commercial TG instrument

158 (TGA/DSC1/HT1600, Mettler-Toledo Co., Ltd.). The amount of OC lost from the

159 particles was recorded when the temperature was ramped from 30 to 300 °C at 10 °C

160 min^{-1} in nitrogen flow according to the protocol reported in previous work (Han et al.,

161 2012a). Metals in the particles were measured with an inductively coupled plasma mass

162 spectrometer (ICP-MS 7500a, Agilent Technologies) after digested with concentrated

163 1:3 HNO_3/HCl. Transition metals were quantified with the standard solution. Zeta

164 potentials of the carbon nanomaterials were measured after sonicating for 30 min in

165 ultrapure water (18.2 MΩ) by using a Nanoparticle Size & Zeta Potential Analyzer

166 (Zetasizer Nano, ZS90).

2.2 DTT assay test. The DTT assay is an indirect chemical assay used for measuring the redox cycling capacity of PM (Xia et al., 2006). The added DTT is oxidized to its disulfide form by the ROS in particulate matter (Kumagai et al., 2002). Thus, the rate of DTT consumption is proportional to the concentration of the ROS in the sample (Cho et al., 2005). In this study, ~150 μg carbon nanomaterials were suspended in 10.0 ml phosphate buffer (0.1 M, pH 7.4) and sonicated for 15 min. 2.0 ml of 0.5 mM DTT solution was added to 3.0 ml aliquots of the sonicated suspensions. A redox reaction took place in a thermostat shaking chamber at 37 ºC. The remained DTT concentration was measured every 15 minutes by adding 0.25 ml of the reaction mixture filtration to 1.0 ml of 0.25 mM DTNB solution. DTNB reacted with the thiol groups in DTT to form a yellow compound (2-nitro-5-thiobenzoate, NTB), which could be detected by UV-vis absorption spectrometer (723N, Shanghai Ruiting Technology Co., Ltd) at 412 nm. Then, the amount of DTT consumed by PM was calculated according to the standard curves of DTT. The loss rate of DTT via a redox reaction in the presence of PM was monitored as the decrease of DTT concentration and normalized to the particle mass. Blank experiments were carried out without carbon nanomaterial particles in the buffer solution. For some samples, the response to the DTT assay was also measured for the water-soluble components of SWCNT by filtering aliquots of the samples with a 0.22 μm syringe PTFE filter, and measuring the activity of the solution without particles.

2.3 In vitro assays. Carbon nanomaterial particles were dispersed with 0.025% Tween-80 in 0.19% NaCl solution using a Dounce glass homogenizer, followed by sonication. A homogeneous and stable suspension of SWCNTs was obtained after the sonication

process. Cytotoxicity assessment of carbon nanomaterials was carried out using the

murine J774 cells. Three different assays targeting distinct mechanisms of cellular

metabolic perturbations were assessed simultaneously, including ATP (energy

metabolism), LDH (membrane integrity) and BrdU (incorporation into DNA) assays.

The experiments were carried out according to the corresponding protocol. Briefly, $4 \times$

10^5 J774 cells ml^{-1} were exposed to carbon particles in 96-well plates for 24 hours for

ATP and LDH assays, while the initial J774 cell concentration was 2×10^5 cells ml^{-1}

for BrdU assay. Carbon nanomaterials were dosed at 0, 10, 30 and 100 μg cm^{-2} in a

final volume of 200 μl $well^{-1}$ as similar to that reported in literatures (Kumarathasan et

al., 2014;Kumarathasan et al., 2012). The luminescence spectroscopy of the supernatant

after centrifugal separation at 1000 rpm for 5 min was measured after 24 h of cell

exposure using a Multimode Microplate Reader (Varioskan®Flash, Thermo Fisher

Scientific). The zero dose of carbon nanomaterials referred to the blank experiment and

also means the toxicity of 0.025% Tween-80 alone in 0.19% NaCl solution. Similar to

the literature results (Hadrup et al., 2017), they did not incur any obvious deleterious

effect on cells growth. In addition, it has been well recognized that carbon nanoparticles

tended to aggregate in water even after ultrasonic dispersion. Tween-80 has been

verified to be a biocompatible dispersant for carbon black (Kim et al., 2012). Negative

control experiments were performed in wells containing medium without cells to obtain

a value for background luminescence. Positive control experiments were carried out

with H_2O_2 solution for LDH assays (Fig. S2).

3. Results

3.1 Oxidative potential of carbon nanomaterials. Figure 1 shows the DTT decay

rates of SB4A, graphene, graphene oxide, SWCNT, SWCNT-OH and SWCNT-COOH.

They were 45.9±3.0, 58.5±6.6, 160.7.0±21.7, 38.9±8.9, 57.0±7.2 and 36.7±0.2 pmol

$min^{-1}\mu g^{-1}$, respectively. Except for graphene oxide, the measured DTT decay rates for

these carbon nanomaterials (with mean value of 47.4±10.1 pmol $min^{-1}\mu g^{-1}$) were

comparable with the DTT loss rates of soot reported in the literatures. For example, it

was 36.2±4.9 pmol min^{-1} μg^{-1} for Printex U (Li et al., 2015) and 59.3±7.4 pmol min^{-1}

for typical soot particles, such as 33.6 pmol min^{-1} μg^{-1} for methane flame soot (Holder

et al., 2012), 49±7 pmol min^{-1} μg^{-1} for propane flame soot (Antinolo et al., 2015), 27.0

pmol min^{-1} μg^{-1} for hexane flame soot (Li et al., 2013), as well as the typical ambient

$PM_{2.5}$ particles (34.7±19.1 pmol min^{-1} μg^{-1}) (Charrier and Anastasio, 2012;Liu et al.,

2014a). However, the measured DTT decay rates for these carbon nanomaterials were

significantly higher than that of diesel soot (6.1 pmol min^{-1} μg^{-1}) and graphite (0.9 pmol

min^{-1} μg^{-1}) (Li et al., 2013) reported in previous work. It should be noted that the DTT

decay rate of graphene oxide measured in this study was 160.7±21.7 pmol min^{-1} μg^{-1}.

Based on T-test, the DTT decay rate of graphene oxide was significantly higher than

that of other tested carbon nanomaterials at the 0.05 level (t=8.498, which is greater

than the critical value of 2.447). This means that graphene oxide definitely has a

stronger oxidative potential than other CB or carbon nanomaterials in this work.

3.2 Cytotoxicity of carbon nanomaterials to murine J774 cell line. At the present

time, the A549 (a human adenocarcinomia alveolar epithelial cell) and THP-1 (a human

leukemia monocytic cell line) cell lines were usually chosen as target cell lines

233   (Kumarathasan et al., 2012;Kumarathasan et al., 2014;Liu et al., 2015) to evaluate the

234   alveolar and pulmonary toxicity of CB particles. As the first barrier of the immune

235   system, macrophage cell lines will fight against the invaded particles in the lungs.

236   Macrophage cell lines like J774 cells are ideal model systems for establishing the

237   biophysical foundations of autonomous deformation and motility of immune cells (Lam

238   et al., 2009). It has been found that CB nanoparticles are able to stimulate the release

239   of macrophage chemo-attractants when exposed to type II epithelial cell lines (L-2 cells)

240   at sub-toxic doses (Barlow et al., 2005). CNTs exposure can also lead to biological

241   changes in J774 cells (Kumarathasan et al., 2012). Therefore, it is meaningful to

242   investigate the cytotoxicity of different carbon nanomaterials as well as the influence

243   of surface functional group on the macrophage cell lines.

244   Figure 2 shows the in vitro toxicities of SB4A, graphene, graphene oxide, SWCNT,

245   SWCNT-COOH and SWCNT-OH. The stars mean the indicator of the toxicity at a

246   certain dose of carbon nanomaterials is significantly different from the corresponding

247   blank experiments at 0.05 level. As shown in Fig. 2, the metabolic activity of J774 cell

248   line decreased monotonously as a function of the dose of all these carbon nanomaterials.

249   The relative ATP level (1.01 ± 0.02) at the SB4A dose of 10 μg cm^{-2} was almost the same

250   as that of the blank sample, while it significantly decreased to 0.89 ± 0.05 and 0.61 ± 0.07

251   when the dose of SB4A increased to 30 μg cm^{-2} and 100 μg cm^{-2}, respectively. Similarly,

252   the relative ratio of BrdU incorporation decreased from 0.74 ± 0.03 to 0.60 ± 0.04 when

253   the dose of SB4A increased from 30 to 100 μg cm^{-2}. However, the released LDH levels

254   were constant within experiment uncertainty at different SB4A doses.

255   As shown in Fig. 2B-F, the metabolic activity of murine J774 cell decreased more

256   significantly when exposed to engineered carbon nanomaterials than SB4A. For

257   example, the relative ratio of ATP level was 0.67±0.06, 0.84±0.03, 0.59±0.10,

258   0.93±0.01 and 0.88±0.02 even when the J774 cells were exposed to 10 μg cm^{-2}

259   graphene, graphene oxide, SWCNT, SWCNT-OH and SWCNT-COOH, respectively.

260   When exposed to high doses of engineered carbon nanomaterials, the reduction of

261   relative ATP level became more significant. These results mean the cytotoxicity of the

262   engineered carbon nanomaterials studied in this work are stronger than that of SB4A

263   regarding to metabolic activity. Graphene, graphene oxide and SWCNT-COOH

264   significantly enhanced release of LDH at different exposure levels, while SWCNT and

265   SWCNT-OH only led to significant increases of released LDH at high exposure level

266   (100 μg cm^{-2}).

267   It should be noted that the reduction of ATP ratio of J774 cells exposed to graphene

268   oxide was weaker than that of graphene. The reduction of ATP ratio of J774 cells

269   exposed to SWCNT-OH or SWCNT-COOH was also weaker than that of SWCNT.

270   However, compared with graphene, graphene oxide showed much stronger toxicity to

271   J774 cell as far as the membrane integrity was considered. The released LDH at

272   exposure level of 30 μg cm^{-2} graphene oxide was comparable with that when exposed

273   to 150 ppm H_2O_2 (Fig. S2). In addition, graphene oxide, SWCNT-OH and SWCNT-

274   COOH significantly inhibited DNA synthesis of J774 cells when the carbon

275   nanomaterials doses were above 10 μg cm^{-2}, while graphene and SWCNT did not show

276   significant inhibition of DNA synthesis for J774 cells. For instance, the relative ratio of

277 BrdU when J774 cells exposed to 100 μg cm^{-2} of graphene oxide was 0.61±0.10, while

278 it was 0.77±0.10 for graphene exposed cells at the same exposure level. They were

279 0.62±0.10 for SWCNT-OH and 0.56±0.09 for SWCNT-COOH treated cell at a dose of

280 10 μg cm^{-2} compared with 0.83±0.09 for 10 μg cm^{-2} of SWCNT treated J774 cell.

281 **3.3 Characterization of carbon nanomaterials.** As shown in Fig. S1, the

282 morphologies of these carbon nanomaterials varied greatly. SB4A was a zero-

283 dimensional material. SWCNT, SWCNT-OH and SWCNT-COOH were one

284 dimensional material. Graphene and graphene oxide were two dimensional materials.

285 The content of transition metals including Cr, Fe, Mn, Co, Ni, Cu, Zn, As, Cd, Sn

286 and Pb were measured by using an ICP-MS after the carbon nanomaterials were

287 digested with 1:3 HNO$_3$/HCl. As shown in Fig. S4A, Fe was the most abundant

288 transition metal in these carbon nanomaterials. Its concentration varied from 122 μg g^{-1}

289 ^{1} to 6596 μg g^{-1} among different carbon nanomaterials. The concentration of other

290 metals varied from zero to several hundred μg g^{-1} depending on both carbon

291 nanomaterials and the type of metals. Compared with SB4A, these engineered carbon

292 nanomaterials showed higher metal content. For example, the total metal content in

293 graphene was 6 times as high as that in SB4A, while it was 33 times in SWCNT as high

294 as that in SB4A. This can be explained by the fact that graphene and SWCNT materials

295 were catalytically synthetized using metal catalysts containing Fe, Co or Ni (Maruyama,

296 2018).

297 Figure 3 shows the thermo gravity and differential thermal analysis curves for these

298 CB materials when the temperature was ramped from 30 to 300 °C at 10 °C min^{-1} in

14

299 nitrogen flow. Weight loss (Fig. 3A) accompanied with an endothermic process (Fig.

300 3B) were observed below 60°C for all of these samples. This can be ascribed to

301 desorption of surface adsorbents including bonded organics and trace water. As shown

302 in Fig. 3B, the saddle points of these differential thermal analysis curves were observed

303 at 35, 35, 41, 42, 56 and 58 °C for graphene, SWCNT, SB4A, SWCNT-OH, SWCNT-

304 COOH and graphene oxide, respectively. It should be noted that the oxidized carbon

305 nanomaterials such as SWCNT-OH, SWCNT-COOH and graphene oxide showed

306 higher saddle points of the heat curves than graphene, SWCNT and SB4A. This implies

307 stronger interaction between the adsorbents and these three oxidized carbon

308 nanomaterials compared with the counterpart. Therefore, it is reasonable to deduce that

309 the adsorbed water mainly contributes to the weight loss in this stage. The sample

310 weight slightly decreased as the temperature further increased for all of these carbon

311 nanomaterials except for graphene oxide and accompanied with a gradual increase of

312 the heat flow. This can be ascribed to desorption of adsorbed organics from the surface

313 of the carbon nanomaterials. The relatively small increase rate of the heat in this stage

314 was consistent with the small heat capacity of organics when compared with the first

315 one which was ascribed to desorption of water. For graphene oxide, however, weight

316 loss (from 32% to 60%) was significantly observed accompanied with an acute

317 exothermic process when the temperature increased from 150 to 200 °C as shown in

318 Fig. 3B. This implies that release of pyrolysis products and structure collapse of

319 graphene oxide occur. It also means a high reactivity of graphene oxide and highlights

320 the distinctive property of graphene oxide among these investigated carbon

321 nanomaterials. The adsorbed organics were estimated based on the thermogravimetric

322 curves when the possible contribution of water was ruled out. For graphene oxide,

323 150 °C was taken as the endpoint, while 300 °C was chosen for other samples. The

324 content of adsorbed organics on SB4A, graphene, graphene oxide, SWCNT, SWCNT-

325 OH and SWCNT-COOH was 6 %, 13 %, 15 %, 9 %, 5 % and 9 %, respectively.

326   To further investigate the role of surface oxygen in the toxicity of carbon

327 nanomaterials, the oxygen-containing species of these carbon nanomaterials were

328 identified with X-ray photoelectron spectroscopy. Fig. 4 shows the typical O1s and C1s

329 spectra of these carbon nanomaterials. Several oxygen-containing species were

330 observed as shown in Fig. 4A-F. Adsorbed oxygen was observed at 535.2 eV in the O1s

331 spectra. Carbon-oxygen single bond in hydroxyl group (C-OH) and epoxide (C-O-C)

332 were at 533.5 and 532.6 eV, respectively. Carbon-oxygen double bound (C=O) was

333 observed at 531.8 eV, while highly conjugated form of carbonyl oxygen such as quinone

334 groups was identified at 530.5 eV (Schuster et al., 2011). In the C1s spectra (Fig. 4G-

335 L), the band at 291 eV was attributed to the shakeup peak associated with π-π^*

336 transition (Simmons et al., 2006). The band at 289 eV corresponded to carbonyls and

337 epoxides was observed at 287 eV (Kuznetsova et al., 2001). The band at 285 eV and

338 284.6 eV was assigned to graphite and sp^3 carbon, respectively. In particular, the

339 intensity of C-O-C at 532.6 eV in graphene oxide was very strong compared with other

340 carbon nanomaterials. At the same time, the band of C-O-C at 287 eV was also much

341 stronger than that of other carbon nanomaterials in the C1s spectrum. These results

342 mean that epoxides (C-O-C) is the predominating species (Fig. 5C and I) in graphene

343   oxide. It should be noted that XPS results only represent the surface atom ratios, which

344   are different from the OC content representing the bulk composition. However, the

345   surface property of particle should be very important to understand the toxicity of

346   nanoparticles from the point view of particle-cell interaction (Cedervall et al., 2007).

347   **4. Discussion**

348   As shown in Fig. 2, all the carbon nanomaterials showed decreased ATP activities

349   as a function of the dose. This means the carbon nanomaterials investigated in this work

350   are toxic to murine J774 cell line. This is consistent with the previous results that CNT

351   and Printex U are toxic to J774 cells (Kumarathasan et al., 2012) and graphene oxide

352   can induce dose-dependent cell death in normal lung fibroblasts (HLF), macrophages

353   (THP-1 and J744A), epithelial (BEAS-2B) cells, lung cancer cells (A549) etc. (Zhang

354   et al., 2016;Li et al., 2018). At the same time, the BrdU activities decreased as a function

355   of the dose of carbon nanomaterials, which means they are inhibitor for cell

356   proliferation of murine J744 (Cappella et al., 2015). In addition, except for SB4A, other

357   carbon nanomaterials showed significant increases in LDH. This means that the

358   integrity of cell membrane decreased when J774 cells were exposed to these engineered

359   carbon nanomaterials, while the cell membrane might be intact when exposed to SB4A

360   (Cho et al., 2008;Kumarathasan et al., 2015). This might be related to lipid peroxidation

361   induced by these engineered particles (Li et al., 2018) and the non-sphere feature of

362   these engineered particles as observed in Fig.S1. These results also consistent with the

363   previous study that observed CNT cytotoxicity ranking was assay-dependent

364   (Kumarathasan et al., 2015).

365   As shown in Fig. S3, all these carbon nanomaterials revealed negative zeta potential

366   from -42 mV to -20 mV. SB4A, graphene oxide and SWCNT-COOH almost borne the

367   same zeta potential (-42 mV), while SWCNT, SWCNT-OH and graphene showed

368   comparable zeta potential. This observation suggested the stability of dispersed SB4A,

369   graphene oxide and SWCNT-COOH in water and the interaction between these

370   particles with cells was comparable. However, the cytotoxicity of SB4A, graphene and

371   SWCNT showed an increase trend regarding the metabolic activity of J774 cell (Fig.

372   2). This can be explained by the different mode of action (MOA) when the cells were

373   exposed to different types of nanomaterials. For example, adhesions and/or covering

374   on cells could be the main MOA for graphene/graphene oxide (2-D structure) (Gupta

375   et al., 2019;Keshavan et al., 2017), while for carbon nanotubes (1-D structure), piercing

376   and/or internalization by cells could be the main MOA (Lacerda et al., 2013). This

377   means morphology should plays a role in determining the cytotoxicity of the carbon

378   nanomaterials studied in this work. Therefore, in the following section we mainly

379   discuss the cytotoxicity among these materials having same dimension, such as

380   SWCNT-OH and SWCNT-COOH verse SWCNT and graphene oxide verse graphene.

381   It should be noted that oxidized carbon nanomaterials including graphene oxide,

382   SWCNT-OH and SWCNT-COOH showed weaker reduction of ATP ratio of J774 cells

383   than the counterparts (Fig. 2). These results suggested that functionalized carbon

384   nanomaterials caused a low cytotoxicity of murine J774 cell line regarding to the cell

385   apoptosis, while a stronger toxicity was demonstrated for cell proliferation and the

386   membrane integrity. This finding was true, in particular, for graphene oxide. However,

we did not observe a clear dependence of cytotoxicity to murine J774 cell line on the morphology, the transition metal content, the OC content and the content of oxygen-containing species on the surface of carbon nanomaterials although oxidized CB materials showed reduced toxicity to J774 cell lines as far as metabolic activity was considered. In particular, the difference in surface oxygen content between graphene oxide and graphene was much higher than that between SWCNT-OH/SWCNT-COOH and SWCNT (Fig. 5A), while the differences in metabolic activity to J774 cell line between graphene oxide and graphene was similar to that between SWCNT-OH/SWCNT-COOH and SWCNT. The pathways of cellular toxicity induced by particles reside in both oxidative stress (ROS) and non-oxidative stress dependent (Shvedova et al., 2012). Oxidative stress leads to selective oxidation of mitochondrial CL, NADPH oxidase activation and MPO activation in neutrophils, while non-oxidative stress results from interference with mitotic spindle and actin cytoskeleton, and steric hindrance of ion channels. The interaction between target cells and particles should be much complicated than that between DTT and particles. As discussed above, the cytotoxicity of nanoparticles relied on not only the mode of action but also the chemical nature of particles. Therefore, the different responses of the oxidation potential and the cytotoxicity to the epoxide content in these carbon materials might be accounted for by different mechanisms of toxicity among these assays.

The DTT decay rate (Fig. 1) did not show obvious dependence on their morphologies in this work. For example, except for graphene oxide, the DTT decay rates were comparable among all the other materials regardless of the morphology.

409   Graphene and graphene oxide showed similar particle size, graphene layer and

410   morphologies (Fig. S1), while they showed totally different toxicity as shown in Fig. 1.

411   Transition metals in the particles have been identified to be the important contributor

412   to ROS generation (McWhinney et al., 2013b;Li et al., 2003). It should be noted that

413   although the metal content of SB4A was very low compared with other materials (Fig.

414   S4), the DTT decay rate of SB4A was still comparable with these engineered carbon

415   nanomaterials except for graphene oxide as shown in Fig. 1. On the other hand,

416   SWCNT had the highest metal content, while graphene oxide rather than SWCNT

417   showed the strongest DTT decay rate. In addition, the soluble metal contents were in

418   the following order: SWCNT-COOH > SWCNT > SB4A > graphene oxide > graphene >

419   SWCNT-OH (Fig. S4B), after being sonicated for 30 min in water. Graphene oxide

420   (103.7 $\mu g\ g^{-1}$) did not show a significant difference compared with SB4A (106.3 $\mu g\ g^{-1}$)

421   and graphene (93.7$\mu g\ g^{-1}$). These results indicated that the high oxidative potential of

422   graphene oxide relative to other materials cannot be attributed to their difference in

423   bounded or soluble transition metals. This can be explained by the following reasons.

424   First, metal content was measured after digested with 1:3 HNO_3/HCl. The speciation of

425   metals should be quite different from that presenting in the pristine carbon

426   nanomaterials. For example, the contents of soluble metal ions after sonicated for 30

427   min (Fig. S4B) varied from zero to 356 $\mu g\ g^{-1}$. These values were much lower than the

428   corresponding metal contents of digested samples as shown in Fig. S4A. Second, metal

429   might be in the inner pores of carbon nanomaterials. This will decrease the efficiency

430   of metals to generate ROS. Finally, the concentration of carbon nanomaterials was 10-

431   40 μg ml^{-1} in DTT assay tests. This meant the concentration of transition metals was at

432   ng ml^{-1} level even if all of the transition metals were available. The low concentration

433   of metals released might lead to negligible contribution to ROS formation. This was

434   further confirmed by the very small DTT decay rate of the SWCNT filtered solution

435   (1.66±0.15 pmol min^{-1} μg^{-1}) compared with that of SWCNT suspension (38.9±8.9 pmol

436   min^{-1} μg^{-1}) even though SWCNT had the highest metal concentration (Fig. S4A). This

437   was consistent with the previous conclusions that redox activity originates from the

438   surface of CB or soot particles but not from water-soluble substances (Liu et al.,

439   2015;McWhinney et al., 2013a).

440   As shown in the insert graph of Fig. 3A, the content of organics cannot explain the

441   sequence of the DTT loss rate (Fig. 1) of these carbon nanomaterials. For example, the

442   content of organics on graphene and graphene oxide were almost the same, while the

443   DTT decay rate of graphene oxide was as about 2.5 times as that of graphene (Fig. 1)

444   This means the different DTT loss rate observed in this study cannot be explained by

445   the adsorbed organics among these materials. Fig. 5A summarizes the distribution of

446   the oxygen species mentioned above normalized to O atoms in these carbon

447   nanomaterials. At the present time, the relative sensitivity factors for each oxygen-

448   containing species are unavailable. Similar to the method used in the literatures (Chen

449   et al., 2017;Schuster et al., 2011), we simply assumed all these oxygen-containing

450   species in the envelope of O1s having the same relative sensitivity factors. It should be

451   reliable when semi-quantitatively comparing the contents of oxygen-containing species

452   among different samples although additional uncertainties might be introduced for the

453   calculated oxygen content. Highly conjugated form of carbonyl oxygen (quinone) and

454   adsorbed oxygen contributed little to the total oxygen on the surface (<1 %), while C=O,

455   C-O-C and C-OH were predominating oxygen-containing species. Our results agree

456   well with the previous work that C=O, C-O-C and C-OH dominated oxygen-containing

457   species on natural chars, diesel soot, hexane soot and activated charcoal (Langley et al.,

458   2006). Although quinone has been well recognized to contribute to ROS generation on

459   the surface of fine particles (Kumagai et al., 2002;Li et al., 2002b), the content of

460   quinone was lower than 0.35% and showed little difference among all of these tested

461   carbon nanomaterials (Fig. 5A and B). It did so for adsorbed oxygen content. Therefore,

462   we can conclude that the very large DTT decay rates of graphene oxide compared with

463   other carbon nanomaterials as shown in Fig. 5C cannot be explained by the content of

464   quinone or adsorbed oxygen.

465   As shown in Fig. 5A, the total oxygen content of SB4A, graphene, SWCNT,

466   SWCNT-OH and SWCNT-COOH was 6.68%, 2.41 %, 2.88%, 3.60% and 9.21%,

467   respectively. They were comparable with that of diesel soot (2.1%-12.2%) (Schuster et

468   al., 2011). However, the oxygen content of graphene oxide (29.0%) was significantly

469   higher than the other carbon nanomaterials (Fig. 5A). At the same time, the distribution

470   pattern of the surface species on graphene oxide was quite different from the other

471   carbon nanomaterials. Fig. 5B compared the content of the oxygen-containing species

472   of graphene oxide with other carbon nanomaterials. The red stars indicate the content

473   of oxygen-containing species in graphene oxide, while the blue boxes show that of other

474   carbon nanomaterials. It can be seen that the content of quinone and adsorbed oxygen

475 showed no difference between graphene oxide and other carbon nanomaterials. The

476 concentration of C=O and C-OH in graphene oxide was slightly higher than that in the

477 other carbon nanomaterials. However, the content of epoxide in graphene oxide was

478 significantly higher than the other carbon nanomaterials. The content of epoxide in

479 graphene oxide normalized to O atoms was 20.8 %, which was 71.7 % of its total

480 oxygen content (Fig. 5B), while it was less than 2.7 % in other carbon nanomaterials.

481 This well corresponded to the large DTT decay rates of graphene oxide (160.7 pmol

482 min^{-1} μg^{-1}) compared to other carbon nanomaterials (less than 60 pmol min^{-1} μg^{-1}) as

483 shown in Fig. 5C. It should be noted that the content of epoxide was not linearly

484 correlated to the DTT activity. This can be explained by the typical nonlinear

485 relationship between the dose of toxicant and toxicity (Antinolo et al., 2015). It should

486 be pointed out that multiple parameters of particle may have influence on its toxicity,

487 in particular, on the cytotoxicity. For example, particle size and morphology may have

488 influence on the material mobility and uptake by cells. Although the observed toxicity

489 including DTT activity and cytotoxicity could be a coincidence of the chemical

490 composition, functional groups and morphology of these particles, the above results at

491 least imply that these physiochemical properties such as morphology, metal and OC

492 content should not be crucial factors as for the toxicity of these carbon nanomaterials

493 because it is difficult to observe an obvious dependence of the toxicity on these factors.

494 In the meantime, we can propose that epoxides in graphene oxide are mainly

495 responsible for the high ROS activity of graphene oxide. The high ROS formation

496 potential of graphene oxide might also explain its strong cytotoxicity to J774 cell line

497   regarding to the cell membrane.

498   To further confirm this assumption, we measured the ROS activity of the thermally

499   treated graphene oxide at 200 °C in nitrogen flow because the C-O-C (epoxide)

500   structure can be broken under this condition as shown in Fig. 3 and discussed above.

501   XPS spectra confirmed the broken of epoxide by the fact that the content of epoxide in

502   thermally treated graphene oxide decreased to 4.3% from 20.9% in graphene oxide as

503   shown in Figs. S5 and S6. In addition, TEM results also showed that graphene oxide

504   broke into small sheets, whose morphology and particle size were close to that of SB4A

505   (Fig. S1). At the same time, the DTT decay rate of the thermally treated graphene oxide

506   decreased to 54.9 ± 9.8 pmol min^{-1} µg^{-1} (Fig. 6). This value was comparable to the DTT

507   decay rates of other carbon nanomaterials, in particular, graphene (58.5 ± 6.6 pmol min^{-1}

508   µg^{-1}) (Fig. 1), while it was significantly lower than the graphene oxide ($160.7.0\pm21.7$

509   pmol min^{-1} µg^{-1}) as shown in Fig. 6. It should be noted the total oxygen contents of

510   thermally treated graphene oxide was 19.3 %, which was lower than that of graphene

511   oxide (29.0 %) but significantly higher than that of other carbon nanomaterials.

512   However, the DTT decay rate of thermally treated graphene oxide was still comparable

513   with other carbon nanomaterials. This further highlights the importance of functional

514   group in the DTT decay rate. Therefore, it means that epoxides in graphene oxide are

515   the highly reactive site for ROS formation on the surface of graphene oxide. This is for

516   the first time to observe that epoxide is a highly reactive site for ROS formation besides

517   quinone on carbon nanomaterials. This result is also well consistent with the previous

518   founding that epoxides in graphene oxide can oxidize SO_2 to sulfate (He and He, 2016)

24

although their oxidation mechanism might be different.

It should be noted that if other ethers present in the carbon nanomaterials, they should also contribute to the O1s band which might be closed to that of epoxide. However, at the present time, it has been recognized that oxygen-containing species including epoxide, hydroxyl, carbonyl and carboxylic groups present in graphene layer (Inagaki and Kang, 2014;Hunt et al., 2012). Epoxide should dominate the band at 532.6 eV compared with ethers (Hunt et al., 2012). In particular, the TGA results also supported the high content of epoxide in graphene oxide. For other samples in this work, other ethers might overestimate their contents of epoxide. However, this should not have influence on our conclusion that epoxides are related to the high oxidation potential of graphene oxide.

Recently, environmentally persistent free radicals (EPFRs) (a kind of surface stabilized metal-radical complexes characterized by an oxygen-centered radical) (Dugas et al., 2016) have been identified in different source of particles including biomass/coal combustion, diesel and gasoline exhaust, ambient $PM_{2.5}$ and polymer (Balakrishna et al., 2009;Truong et al., 2010;Dugas et al., 2016). However, it is unclear that whether epoxide in graphene oxide observed in this study contributes to the EPFRs formation. This is needed to be investigated in the future.

5. Conclusion and atmospheric implications

Oxidation is a useful method to obtain functionalized CB materials with distinctive performance in industry. This process unusually leads to formation of carbonyls, hydroxyls, carboxylic acids, esters, ethers and epoxides on the surface of CB or soot

541   particles. Previous work have found that oxidation of carbon nanomaterials (SWCNT)

542   by O_3 or OH under atmospheric related conditions has little influence on their oxidative

543   potential or cytotoxicity although carbonyls, carboxylic acids and esters were formed

544   (Liu et al., 2015). Similarly, surface functionalization was observed for commercial CB

545   materials by ozone oxidation, while increase in the cytotoxicity of murine macrophages

546   and release of inflammation markers upon exposure to the oxidized CB were not

547   observed (Peebles et al., 2011). However, some other studies observed that oxidation

548   process enhanced the oxidation potential (Li et al., 2015;Li et al., 2013;Antinolo et al.,

549   2015) as well as the cytotoxicity (Holder et al., 2012) of CB and soot particles. Using

550   the model carbon nanomaterials with different dominate surface functionalities in this

551   work, we have found that hydroxyl and carboxyl functionalized CB particles had little

552   influence on their oxidation potential, while epoxide functionalized CB (graphene

553   oxide) led to a very strong oxidation potential. Epoxide has been identified as a surface

554   product on SWCNT when treated with high concentration of ozone (Mawhinney et al.,

555   2000;Yim and Johnson, 2009). Besides carboxylic acids, esters (Liu et al., 2015),

556   ketone, lactone and anhydride species (Liu et al., 2010;Han et al., 2012b), epoxides has

557   also been identified as the surface product during oxidation of SWCNT in atmosphere

558   relevant conditions (Liu et al., 2015). On the other hand, graphene oxide was an

559   important commercial product, while showed strong oxidation potential as observed in

560   this work. This means that exposure to epoxide-containing carbon materials should lead

561   to high health risk regarding to oxidation potential. Therefore, Mussel-inspired

562   chemistry is necessary for fabrication of functional materials and decreasing their

563   toxicity and for biomedical applications (Liu et al., 2014b;Zhang et al., 2012).

564   It has been found that CB particles (Printex 90) can induce opening of plasma

565   membrane calcium channels leading to a calcium influx and cause significant release

566   of proinflammatory cytokine TNF-α by the murine J774 cells (Brown et al., 2004),

567   subsequently potentially induce migration of macrophages (Barlow et al., 2005). This

568   could initiate the recruitment of inflammatory cells to sites of particle deposition and

569   the subsequent removal of the particles by macrophages. The metabolic activity of these

570   hydroxyl, carboxylic acid and epoxide functionalized carbon nanomaterials increased

571   when compared with the corresponding sample as observed in this work. This implies

572   functionalization of carbon nanomaterials might not pose an enhanced cytotoxicity risk

573   to macrophages compared with the corresponding control materials although the

574   oxidized carbon nanomaterials were still toxic as far as metabolic activity was

575   considered. However, the oxidized carbon nanomaterials might pose enhanced

576   cytotoxicity to macrophages regarding to membrane integrity and DNA synthesis. It

577   should be pointed out that exposure experiments were performed under high particle

578   concentration with short exposure time in this work. More work needs to be done at

579   low particle concentration with long exposure time in the future. On the other hand, the

580   interaction between particles and biological entities such as proteins or cells has not

581   been considered in this work. Therefore, the in vivo toxicological effect of these

582   functionalized particles needs to be further evaluated in the future. Finally,

583   condensation of co-emitted species and photo oxidation products is particularly rapid

584   under conditions of soot emissions (Johnson et al., 2005;Adachi et al., 2010;Peng et al.,

2016). In previous our work, it has been found that condensation process significantly decreased the oxidation potential of the SWCNTs (Liu et al., 2015). A recent work has also found that condensation of organic aerosol leads to decrease in oxidation potential of engineered nanoparticles (Liu et al., 2019). Therefore, the contribution of functional groups to the oxidation potential should be greatly influenced by condensation of co-emitted species and photo oxidation products in the atmosphere. This might be dependent on the carbon nanomaterial itself and needs to be investigated in the future.

Data availability. The experimental data are available upon request to the corresponding authors.

Supplement. The supplement related to this article is available online at:

AUTHOR INFORMATION

Author contributions. YL, HH and SL designed the experiments. YL wrote the paper. YL, HJ and YG did the DTT assay tests. CL and LW did the cytotoxicity assessments. HJ and BZ performed the characterization of samples.

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Figure captions

Figure 1. DTT decay rates of several black carbon materials compared with literature results (Li et al., 2013;Charrier and Anastasio, 2012;Liu et al., 2014a;Li et al., 2015;Liu et al., 2015;Holder et al., 2012;Antinolo et al., 2015).

Figure 2. Cytotoxicity of (A) SB4A, (B) graphene, (C) graphene oxide, (D) SWCNT, (E) SWCNT-OH and (F) SWCNT-COOH toward murine J774 cell line. The stars mean the difference is significant at 0.05 level for a certain dose of carbon nanomaterials compared with the corresponding blank experiments.

Figure 3. (A) Thermo gravity curves of carbon nanomaterials in nitrogen gas flow; (B) the corresponding differential thermal analysis curves. The insert graph shows the weight loss due to desorption of organics.

Figure 4. XPS spectra of carbon nanomaterials. (A)-(F) are O1s spectra and (G)-(L) are C1s spectra for SB4A, graphene, graphene oxide, SWCNT, SWCNT-OH and SWCNT-COOH, respectively.

Figure 5. (A) Distribution of oxygen containing species on the tested carbon nanomaterials; (B) comparison of oxygen-containing species and (C) DTT decay rate between graphene oxide and other carbon nanomaterials.

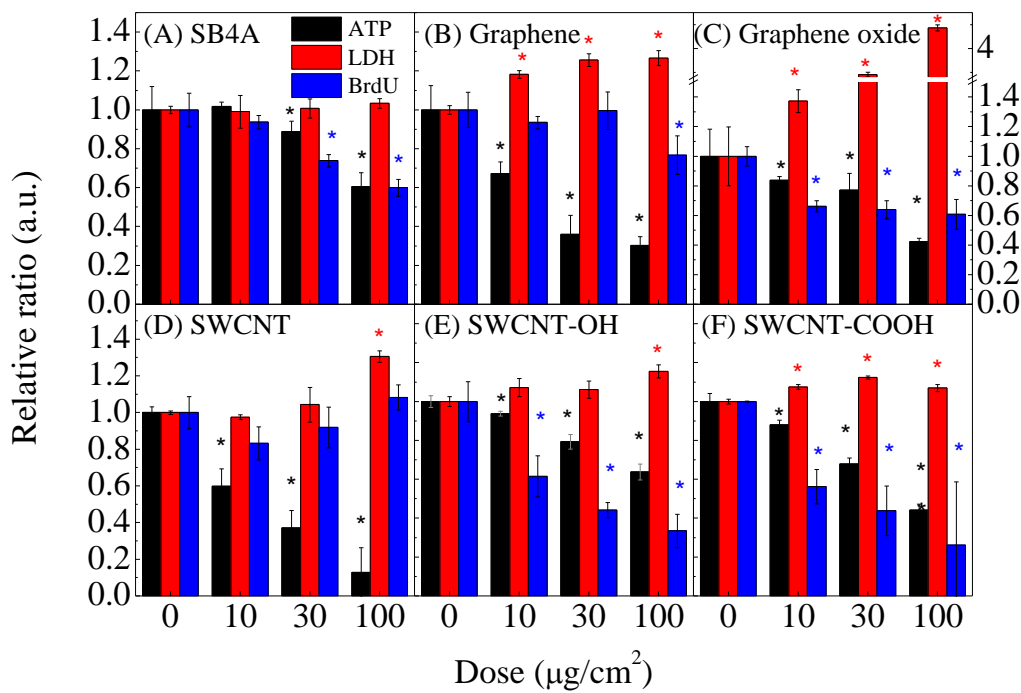**Figure 6.** DTT decay rate for graphene oxide and thermally treated graphene oxide in N_2 flow at 200 °C.

Figures

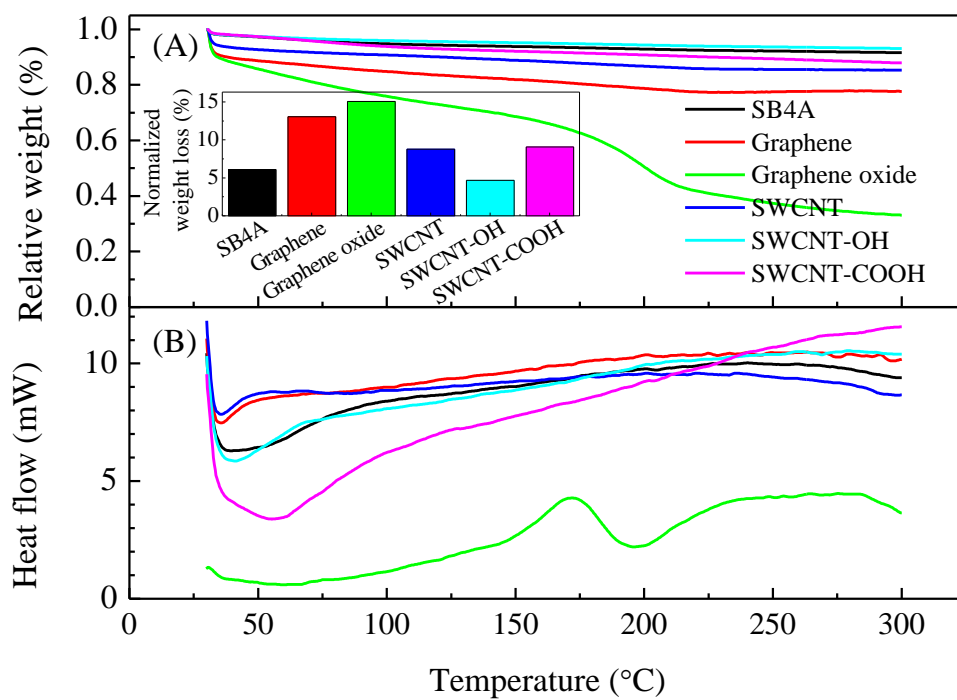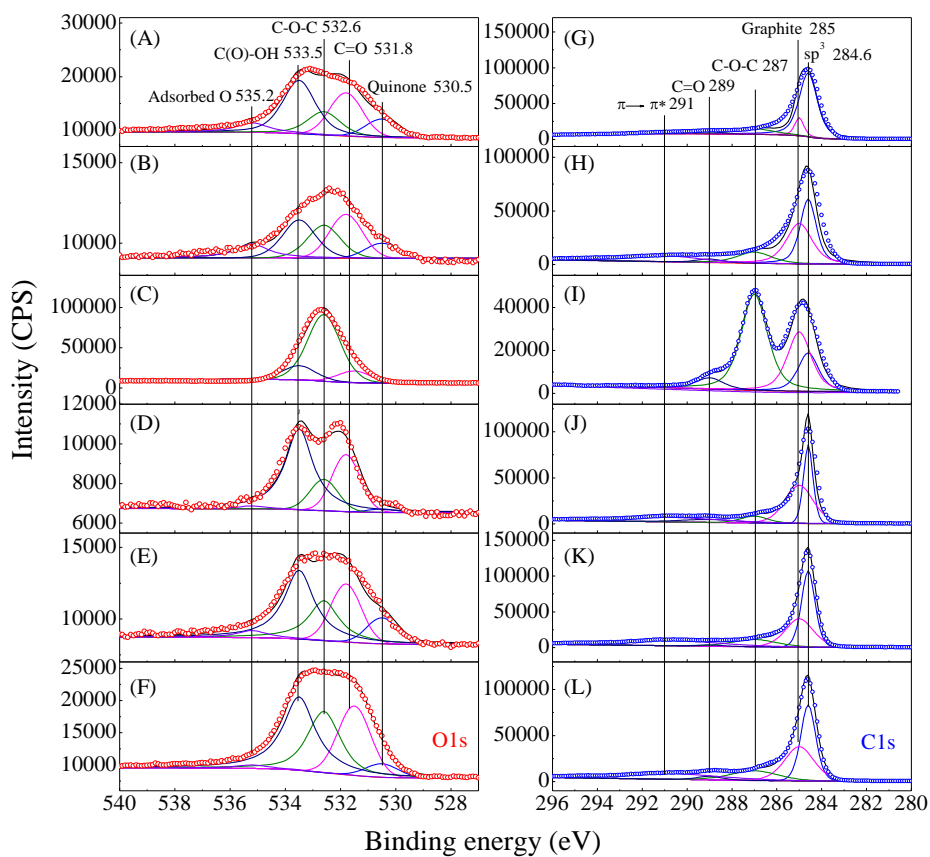Fig. 1

Fig. 2

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Figure 6.