Characteristic Fe and Cu compounds in particulate matter from subway premises in Japan and their potential biological effects

Takuma Okamoto¹, Ayumi Iwata¹.²*, Hiroko Yamanaka¹, Kako Ogane¹, Tatsuhiro Mori¹, Akiko Honda³,⁴, Hirohisa Takano³,⁴, Tomoaki Okuda¹*

¹Department of Applied Chemistry, Faculty of Science and Technology, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Yokohama, Kanagawa 223-8522, Japan
²Meteorological Research Institute, Japan Meteorological Agency, 1-1 Nagamine, Tsukuba, Ibaraki 305-0052, Japan
³Department of Environmental Engineering, Graduate School of Engineering, Kyoto University, Kyoto University Kastura, Sakyou-ku, Kyoto 615-8540, Japan
⁴Graduate School of Global Environmental Studies, Kyoto University, Kyoto University Kastura, Sakyou-ku, Kyoto 615-8540, Japan

* Corresponding author.

Ayumi Iwata
Tel: +81-29-853-8702
E-mail address: iwata@mri-jma.go.jp

Tomoaki Okuda
Tel: +81-45-566-1578
E-mail address: okuda@apple.keio.ac.jp

Abstract

Suspended particulate matter (PM) in subway premises contain high concentrations of metal components and distinctive compounds owing to their unique emission processes. However, little is known regarding the detailed chemical states of airborne PM and their biological effects. Therefore, to demonstrate the unique chemical states of the PM collected from subway premises and outdoors, this study compared the chemical speciation of iron (Fe) and copper (Cu) components using X-ray absorption fine structure analysis. The potential biological effects of these chemical states on humans were also investigated in vitro by assessing cell damage and its pathways in cells after exposure to several compounds. Compared with a reference outdoor PM sample, Fe was enhanced by at least 10 times in subway PM and the concentrations of several metal components, including Cu, contained in railway bodies, rail, overhead wires, and tunnel walls, also increased. In these chemical speciations, the compounds derived from wear processes with relatively high-temperature oxidation (Fe₃O₄, γ-Fe₂O₃, and monovalent Cu compounds)
were detected among the Fe and Cu components in subway PM. Our cell-based bioassay suggested that the contribution of the Fe component to cell damage can be enhanced by the predominance of Fe$_3$O$_4$ in subway PM. In contrast to typical bivalent Cu compounds in the atmosphere, monovalent Cu compounds, which are characteristically identified in subway PM, exacerbate cell damage via different cell death pathways. Our results indicate that the chemical states of the distinctive compounds in the PM of subway premises differ from those in the typical atmosphere, thus exerting different biological effects. These findings suggest that the detailed chemical speciation is an important factor in accurately understanding their PM toxicities.

**Keywords:** Particulate matter, Subway premises, Chemical speciation, Cytotoxicity, X-ray absorption fine structure

**Abbreviations:**

3-MA: 3-methyladenine; CRMs: certified reference materials; EDXRF: energy-dispersive X-ray fluorescence; IL-6: interleukin-6; LCF: linear combination fitting; Nec-1: necrostatin-1; PM: Particulate matter; PMA: phorbol 12-myristate 13-acetate; WST-1: water-soluble tetrazolium salt; XAFS: X-ray absorption fine structure; zVAD: pan-caspase inhibitor benzoyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone
1 INTRODUCTION

Particulate matter (PM) adversely affects human health in various ways (Gauderman et al., 2007; Hoek et al., 2002; Pope et al., 2004), such as increasing in the risk of respiratory, cardiovascular, and immune system diseases, including bronchial asthma, and cancer (Kappos et al., 2004; Tecer et al., 2008). The duration spent by individuals in semi-closed environments such as indoor and subway premises is long (Klepeis et al., 2001). PM suspended in some semi-closed environments, including unique sources, exhibits properties that significantly differ from those of general atmospheric PM; therefore, the effects of PM on human health in these semi-closed environments warrant in-depth assessment. Subways are extremely important in urban areas for transportation. Several studies have reported higher PM concentrations in subway stations, which is one of the most typical semi-enclosed environments, compared with outdoors, due to the inefficiency of air circulation (Steenhof et al., 2011; Wen et al., 2020; Seaton et al., 2005). In particular, the concentrations of transition metal components such as iron (Fe) and copper (Cu) has significantly increased due to PM emissions by the wear and friction processes of rail–wheel–brake and catenary–pantograph interfaces (Querol et al., 2012; Okuda et al., 2019; Lee et al., 2018).

The metal components in atmospheric PM are major contributors to its toxicity (e.g. Kurihara et al., 2022; Wu et al., 2016). Therefore, the contributions of transition metals of PM in subway premises remain a major concern since they may cause significant adverse health effects.
The PM in subway premises causes more damage DNA than PM in road traffic in cell-based assay (Karlsson et al., 2005). The indices of adverse cardiovascular effects and inflammatory response have increased in the blood of subway workers after remaining in subway premises for several hours (Klepczyńska Nyström et al., 2010), while patients with asthma have a higher inflammatory response to subway PM (Klepczyska-Nyström et al., 2012).

Conversely, the lack of data on the detailed chemical speciation of metal compounds in the environment has limited our understanding of their consequent health effects. Metallic components in PM are not only simple substances, but also have a variety of chemical states depending on the chemical reactions with constituents at the emission source and during their transport (Lorenzo et al., 2006). Previous studies have indicated that different oxidation states of the same metal element have vastly different biological effects (Ghio and Devlin, 2001; Heal et al., 2005; Sugimoto et al., 2021; Honda et al., 2015). For example, hexavalent Cr is a carcinogenic substance that has more harmful effects on the respiratory and gastrointestinal tract systems than those of Cr in other oxidation states (Costa and Klein, 2006; Abd-Elfatah Mohammed Hassan et al., 2019; Husain and Mahmood, 2017; Wise et al., 2018). Subway PM can have different chemical properties and toxicities from those of general atmospheric PM due to definite sources of emission. The chemical speciation in subway particles has been previously demonstrated through previous investigations of Fe components, which showed the
predominance of Fe$_3$O$_4$, γ-Fe$_2$O$_3$, and α-Fe$_2$O$_3$, although regional variations due to differences in
train bodies and brake systems have also been reported (Eom et al., 2013; Jung et al., 2012;
Moreno et al., 2015; Querol et al., 2012). However, the findings of those studies cannot be
applied investigate the particles size related to human inhalation or provide quantitative
representation due to methodological limitations. Some of these results, for example, are based on
X-ray diffraction analysis of floor dust and coarse particles (Jung et al., 2012; Querol et al.,
2012), and individual particle analysis by using micro-Raman spectroscopy and electron
diffraction with electron microscope (Eom et al., 2013; Moreno et al., 2015; Salma et al., 2009). Therefore, accurate assessments and prediction of the risks associated with inhalation of PM in
subway premises require characterization of the chemical state of each metal component of
airborne fine particles. Furthermore, there is insufficient even fundamental knowledge about the
biological effects of these chemical state characteristics.

Therefore, this study investigated the chemical states of Fe and Cu components in PM
collected from subway premises using X-ray absorption fine structure (XAFS) spectroscopy. We
focused on Fe and Cu as they are the primary factors of toxicity in atmospheric PM (Font et al.,
2019; Lee et al., 2018; Wen et al., 2020). The detection of high concentrations of Fe and Cu in
subway PM in this study confirms the need to focus on these components. We described the
potential differences in the induced biological responses by the characteristic chemical states of
Fe and Cu based on the viability of cells following exposure to each of the standard chemical species identified from the speciation results. Furthermore, we determined the induction of cell death mechanisms by these species based on their chemical properties to characterize their biological effects. Previous studies have recognized that the adverse health effects caused by PM are linked to their ability to initiate signaling pathways that regulate various cell fates, including apoptosis, necrosis, and autophagy, along with DNA damage and the production of reactive oxygen species (Peixoto et al., 2017; Mohammadinejad et al., 2019). Thus, cell death induced by exposure to PM provides insights into mechanisms underlying PM cytotoxicity. Furthermore, dysregulation of cell death pathways is a typical feature of cancer and neurological diseases (Peixoto et al., 2017). The contributions of the chemical states of PM to its biological effects presented in this study expand our understanding of the toxicological mechanisms and provide a risk assessment of the health adverse effects caused by PM not only on subway premises but also in other environments.

2 MATERIALS AND METHODS

2.1. Chemical speciation of Fe and Cu components in PM of subway premises

2.1.1. PM Sampling

Sampling of subway PM was performed in June 2019 at three locations (spots A, B, and C) in one underground station in Tokyo, Japan (Fig. 1a,b). This station is one of the major stations in
the Tokyo Metropolitan Area that is daily used by hundreds of thousands of passengers. The three sampling points in the station differ in their distance from the ground and from the train’s platform. Spot A was located at the deepest point near many train platforms, spot B was located on the floor above another small platform, whereas spot C was closest to the ground (Fig. 1c).

Ambient particles at each point were collected using the filter collection method, the most common particle collection method. The ambient air was drawn at 5.0 L min\(^{-1}\) using a vacuum pump (VP0125, Nitto Kohki Co., Ltd., Tokyo, Japan), controlled by a needle valve (4411-F4-R, Flowell corp., Kanagawa, Japan). The contained ambient particles were collected on a polytetrafluoroethylene membrane filter (PM2547050, Merck, Darmstadt, Germany) in a filter holder (NL-I, Innovation nilu AS, Kjeller, Norway). The air inlet was set at a height of approximately 1 m from the floor. At the same time and by the same method, furthermore, atmospheric aerosol particles were collected as a reference sample from the fifth-floor balcony of a building at the Yagami campus of Keio University located within approximately 20 km from the station (Fig. 1a, b).

2.1.2. Determination of metal components and their chemical species

The concentrations of metal components in the collected samples such as Fe, Cu, Mg, Al, Si, P, S, Cl, K, Ca, Ti, V, Cr, Mn, Fe, Ni, Cu, Zn, and Pb were measured using energy-dispersive X-
ray fluorescence (EDXRF) spectrometer (EDXL 300, Rigaku Corp., Tokyo, Japan) according to our previous method (Okuda et al., 2014).

The chemical speciation of Fe and Cu in the samples was characterized by analyzing XAFS spectra, which are absorption spectra of synchrotron radiation obtained at Beam Line 11 in the Kyushu Synchrotron Radiation Research Center (the electron beam energy in the electron storage ring was approximately 1.4 GeV). In the XAFS spectroscopy, the change in the X-ray absorption coefficient near the characteristic absorption edge of the element of interest was recorded as a function of energy. Analysis of the XAFS spectra, including X-ray absorption near edge structure and extended XAFS spectral regions, provides structural information regarding the oxidation state, symmetry, and identity of the coordinating ligand environment as well as information on more distant neighboring atoms (Gaur and Shrivastava, 2015; Tannazi and Bunker, 2005). Due to its sensitivity and direct and non-destructive analysis, XAFS spectroscopy has been previously applied to identify and quantify the chemical state of several metals, including Fe and Cu, in PM and environmental samples (D’Amore et al., 2005; Yinsong et al., 2007; Datta et al., 2013; Gaur and Shrivastava, 2015; Jing et al., 2022).

XAFS spectra of the reference standard materials (Fe: Fe, FeO, Fe₃O₄, Fe₂O₃ (α, γ), FeO(OH), Cu: Cu, Cu₂O, CuO, CuCl₂, CuSO₄, and CuS) have been measured in our previous study (Jing et al., 2022). The reference samples were measured using the transmission method.
with foil or a mixture pellet with boron nitride; however, it was difficult to apply this method to measure the filter samples since the sampling filter itself absorbs transmitted X-rays owing to its thickness. Furthermore, creating a pellet from the collected particles requires the sampling of tens of milligrams of particles, which is not practical for field sampling. Therefore, the XAFS spectra of filter samples were measured using the fluorescence yield method with a seven-element silicon drift detector. The atoms core-excited by X-ray absorption emit fluorescent X-ray during the relaxation process, which is proportional to the intensity of X-ray absorption. In the fluorescence yield method, the XAFS spectra can be obtained by measuring the intensity of fluorescent X-rays, and are applied to samples with high noise and blanks such as dilute and thin film samples (Newville, 2014). The difference in the measurement methods used for the reference and filter samples results in strict spectral differences attributed to the self-absorption of incident X-ray in the fluorescence yield method. However, in practice, we have previously shown that it is difficult to identify significantly different results (Saito et al., 2020).

Data treatment of the XAFS spectra involved energy calibration, background subtraction, and normalization. Energy calibration of XAFS measurement was conducted using a common method as follows. A foil of the target element was set the downstream of sample between two transmitted intensity monitors in the path of X-ray, and the absorption spectrum was obtained. The energy position of the known element absorption edge in the obtained spectrum was referred
to adjust the energy scale of the samples. Background subtraction and normalization were achieved by drawing pre- and post-edge lines to unify the absorbance height. The absorption edge was defined as the midpoint of the rising of the absorption peak, while the pre- and post-edge range were set as energy range from -150 to -20 eV and from +150 to +500 eV with respect to the absorption edge, respectively. Measurement spectra were normalized using these polynomial-fitted lines for each edge range as absorbance from 0 to 1. The semi-quantitative fraction of each chemical species in the filter sample was calculated using linear combination fitting (LCF) based on the least-squares method. These treatments and analyses were calculated using the analysis software Athena (Ver. 0.9.26, Ravel and Newville, 2005). The LCF reconstructs the sample spectrum using a combination of reference sample spectra. Here, we report the percentage of each reference compound that contributed to the fit.

2.2. Exposure of cells to standard PM samples from subway premises

2.2.1. Samples to which cells are exposed

During experiments on exposure to cells, we focused on the potential difference in the induced biological responses by signature compounds in the subway premise. Thus, we exposed five Fe standard samples (FeO, Fe$_3$O$_4$, α-Fe$_2$O$_3$, FeO(OH), and FeCl$_3$) and seven Cu samples (Cu, Cu$_2$O, Cu$_2$S, CuO, CuS CuCl$_2$, and CuSO$_4$), including compounds with different oxidation states.
based on the chemical speciation of subway premises and atmospheric filter samples. In addition to these Fe and Cu compounds, the following five samples were exposed to cells as standard samples of atmospheric aerosols: environmental certified reference materials (CRMs) #28 and #30 produced by the National Institute for Environmental Studies, Japan, which consist of atmospheric urban PM and mineral dust in the Gobi Desert, respectively; graphite as a substitute for elemental carbon particles; \((\text{NH}_4)_2\text{SO}_4\) and oxalic acid as representative inorganic and organic compounds in the atmosphere. Particle size distribution (Fig. S1) and volume median diameters (Table 1) of insoluble samples among the samples were measured using a laser scattering particle size distribution analyzer (Partica, LA-960V2, HORIBA, Kyoto, Japan).

These standard samples were suspended in culture media (Supplementary material (SM)) at four concentrations, namely, 20, 50, 100, and 200 \(\mu\text{g mL}^{-1}\), 24 h before exposure. After sample preparation and immediately before exposure, the suspensions were thoroughly dispersed in the medium for 20 s using a vortex mixer.

\subsection*{2.2.2 Measurement protocols for cell viability induced by exposure to PM}

The contributions of each sample to biological responses were assessed by exposing them to two types of cells: A549, a human adenocarcinoma alveolar basal epithelial cell line, and macrophage-like cells, representative antigen-presenting cells. Macrophage-like cells were
obtained by differentiating the human monocytic leukemia cell line THP-1 with phorbol 12-
myristate 13-acetate (PMA; FUJIFILM Wako Chemicals Corporation, Osaka, Japan) according
to the methods of Daigneault et al. (2010). The detailed culture methods for these two cell types
are provided in SM.

The A549 cells seeded at 2,000 cells well\(^{-1}\) in 96-well plates were exposed to the sample and
cultured for 24 h. THP-1 cells were seeded at 4,000 cells well\(^{-1}\) in 96-well plates along with a
PMA (200 nM) to differentiate macrophage-like cells. After incubation for 72 h, the medium was
replaced with PMA-free medium, and cells were cultured for an additional 72 h. We confirmed
differentiation by cell attachment to the plate and an increase in cytoplasmic volume (Sokol et al.,
1987; Daigneault et al., 2010). The viability of each cell type exposed to sample suspensions for
24 h was measured with a colorimetric assay using the Premix WST-1 cell proliferation assay
system (MK-400, TAKARA BIO Inc., Shiga, Japan). The reaction time after adding the water-
soluble tetrazolium salt (WST-1) solution (10 µL well\(^{-1}\)) was 4 h for both cell types. After the
reaction, absorbance was measured at wavelengths of 450 and 630 nm using a microplate reader
(MPR-A100, AS ONE Corp., Osaka, Japan). The viability of the exposed cells was expressed as
the ratio of the measured result of the unexposed cells (0 µg mL\(^{-1}\)).

To assess the ability of the samples to induce cell death, each sample was distinguished by
changes in cell viability upon exposure to A549, which inhibited each of the three major cell
death pathways (apoptosis, autophagy, and necroptosis). In this study, we used the pan-caspase inhibitor benzylloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (zVAD, AdipoGen Life Sciences, Inc., San Diego, CA, USA), 3-methyladenine (autophagy inhibitor, 3-MA, Santa Cruz Biotechnology, Inc., Dallas, TX, USA), and necrostatin-1 (Nec-1, AdipoGen Life Sciences, Inc.) as inhibitors to cell death type of apoptosis, autophagy, and necroptosis, respectively (Sun et al., 2012; Farasat et al., 2020). A549 cells were seeded at 2,000 cells well⁻¹ in 96-well plates and cultured for 24 h. The inhibitors zVAD-fmk, 3-MA, and Nec-1 were added to the wells at 20, 10, and 20 μM, respectively, 2 h before exposure to samples. For this measurement, cells were the sample suspensions at a concentration of 20 μg mL⁻¹ were exposed to the cells for 6 h, at which point the effect of each inhibitor was clear. The viability of the cells was measured using the WST-1 assay as described above.

In the measurements of viability and viability with the inhibitors, differences between the samples exposed to cells and unexposed cells or those without inhibitor treatment, respectively, were analyzed using one-sided Dunnett’s test with statistical computing software, R. Differences in viability among groups were analyzed using the Tukey multiple comparison test. A p-value < 0.05 was considered statistically significant.

3 RESULTS AND DISCUSSION
3.1. Chemical elements and speciations of Fe and Cu components in subway PM

As shown in Fig. 2, the total element concentrations measured using EDXRF were significantly higher in the TSP samples at spots A and B near the platform compared with those of the referenced outdoor PM. However, no obvious differences were found in the total metal concentrations of the TSP in spot C and PM$_{2.5}$ in all subway spots from those in outdoor PM. The concentrations of some metal components in the subway spots were higher compared with those in the outdoor sample, even in PM$_{2.5}$. In particular, for Fe components, spot A was 30.2 and 25.7 times higher for TSP and PM$_{2.5}$ than outdoors, respectively (Table S1). Cu component was also significantly increased compared with that in the outdoor; therefore TSP in Spot A was 10.8 times higher than that in the outdoor. Elements such as Ti, Mn, Cr, and Ca also indicated more than twice the concentrations in TSP and PM$_{2.5}$ in the subway samples compared with those in the outdoor samples. These enhanced elements, including Fe, are contained in the train body and various parts of the railroad line. The train slides and overhead wires contain Cu, while the wheels, rails, and brake systems contain Ca, Cr, Mn, and Fe (Abbasi et al., 2011; Eom et al., 2013; Furuya et al., 2001). The Ca component, which showed a high concentration in the subway premise compared with the outdoor, is present as calcite in friction materials (Moreno et al., 2015). Although the enhancement of Si from the outdoor was not clear, relatively high concentrations of Ca and Si might have contributed to the emissions from building materials of
ballast and tunnel walls in addition to the inflow from the outside. Previous studies have reported that the PM in the subway is dominated by those emitted from these components related to subway operations (Font et al., 2019; Ngoc et al., 2020; Loxham et al., 2013; Minguillón et al., 2018). Comparisons of the concentrations of the enhanced metals in TSP and PM$_{2.5}$ from spots A to C in the subway premises indicated that these are mainly emitted in coarse regions and spatially transported toward the ground level. The fine S component in the subway premises had lower concentrations than that in the outdoor PM. This may be not only due to the slightly distant sampling location of the outdoor PM, but also because the influx of outdoor sulfate particles may be suppressed by the air-conditioning and ventilation system inside the subway premises.

Next, we determined the chemical states of Fe and Cu based on the enrichment of the outdoor PM. The speciation of Fe (Fig. 3a and Table S2a) showed that most of it was oxidized in all samples, including the outdoor samples. Compared with the outdoor samples, the subway samples indicated high proportions of Fe$_3$O$_4$ in both TSP and PM$_{2.5}$. Furthermore, $\alpha$-Fe$_2$O$_3$ in TSP and $\gamma$-Fe$_2$O$_3$ in PM$_{2.5}$ were present in relatively higher proportions in the subway sample compared with the outdoor. In contrast, FeO(OH) is a characteristic fraction of the Fe component in the outdoor environment. In particular, this compound was predominant in the outdoor PM$_{2.5}$ sample (48%) and could not be detected in the TSP samples in the subway. Our results for Fe speciation are supported by previous studies showing that $\alpha$-Fe$_2$O$_3$, $\gamma$-Fe$_2$O$_3$, and Fe$_3$O$_4$ dominate
the Fe component in the subway premise (Ngoc et al., 2020; Salma et al., 2009; Jung et al., 2010). The increase in $\alpha$-Fe$_2$O$_3$ in the subway premises, especially in coarse particles, is explained by the natural oxidation on the surface of the iron materials in humid environment and mechanical emissions such as abrasions. The Fe$_3$O$_4$ contained in the coarse particles can be attributed to the friction process, which involves mechanical emission following oxidation at high temperatures. This fraction of Fe was also contained in the fine particles and could be reformed via the vaporization of Fe at high temperatures. In addition, these fine Fe$_3$O$_4$ particles can be oxidized to $\gamma$-Fe$_2$O$_3$ even at low temperatures. Our frequent detection of FeO(OH) in outdoor samples is supported by previous studies that have indicated the predominance of iron oxyhydroxide in the typical atmosphere (Petroselli et al., 2018; Hoffmann et al., 1996). However, our identification of FeO(OH) remains a possibility that was confused with ferrihydrite, which was not referenced in our measurements. This is because this ferrihydrite, which was not referenced in our measurements, has a similar XAFS spectrum to FeO(OH) (Natori et al., 2022) is also reported to be dominant in the typical atmosphere (Petroselli et al., 2018). Although further investigations are needed to fully understand the emission processes for each Fe fraction, the chemical speciation of Fe components in the PM of subway premises is clearly different from that of the outdoor site.

Furthermore, the analysis of Cu components (Fig. 3b and Table S2b) showed that Cu was mostly oxidized in both samples from the subway and outdoor sites. Most of Cu fractions in
PM$_{2.5}$ from the subway premises were Cu$_2$O or CuSO$_4$. In addition to these Cu components, the measurements of TSP also classified them as CuO. Most of the Cu fractions in the outdoor sample were CuCl$_2$; however, caution must be exercised regarding this result. Although the XAFS spectrum of Cu$_2$O, which predominantly detected in the subway sample, has a clear pre-edge peak, the spectrum of CuCl$_2$ is very similar to that of other divalent Cu compounds because these spectra do not have a clear pre-edge peak (Fig. S2). Our previous results of Cu speciation analysis using the same method indicated that most Cu in atmospheric PM powders collected at the same outdoor site was CuO (Jing et al., 2022). In addition, previous studies have reported that divalent Cu species, including CuO, CuCO$_3$, and CuSO$_4$·xH$_2$O, were abundant in urban PM and standard samples of urban PM (Osán et al., 2010; Huggins et al., 2000; Wang et al., 2007). Therefore, we concluded that our identification of CuCl$_2$ in the outdoor sample is compatible among divalent Cu due to the similarity of these XAFS spectra and those of previous studies. However, our results clearly showed that subway PM contained monovalent Cu components; in contrast, the outdoor sample was dominated by divalent Cu compounds. These speciations, including Fe, are consistent with previous studies on the Shanghai subway (Lu et al., 2015), although our comparison with the outdoor sample provides more information. We propose that monovalent Cu in the PM of subway premises is emitted from the friction between the pantographs and overhead trolley wires, which is similar to those of previous studies (Lee et al.,
The wear of the Cu wires under electric current causes oxidation to Cu$_2$O, and its formation is further promoted with sparks and high temperatures (Yamashita and Sugahara, 2014).

### 3.2. Biological effects of standard Fe and Cu samples on cells

Fig. 4 shows that the trends for cell damage by Fe compounds are similar in both cell types except for $\alpha$-Fe$_2$O$_3$ and FeO(OH). Exposure of FeO(OH) to A549 cells resulted in a concentration-dependent decrease in cell viability; however, no clear concentration dependence was observed in macrophage-like cells following exposure. Meanwhile, $\alpha$-Fe$_2$O$_3$ showed opposite trends. FeCl$_3$ caused a significant decrease in cell viability even at low exposure concentrations (20 µg mL$^{-1}$) compared to the other Fe compounds ($p < 0.05$; one-sided Dunnett’s test), but the viability was independent of the exposure concentration. Fe$_3$O$_4$ induced concentration-dependent cell damage.

Exposure of the two cell types to the Cu samples showed similar trends in their cytotoxicity, as did exposure to Fe compounds, although the Cu compounds caused more damaged to cells. In particular, some Cu compounds, except for Cu$_2$S, CuS, and CuO, induced the highest rate of cell deaths following exposure to low concentrations (20 µg mL$^{-1}$).
Cu\(_2\)O (monovalent) had lower cell viability than CuO (divalent), and this trend was particularly significant at the exposure to 20 µg mL\(^{-1}\) concentrations (\(p < 0.01\), Table S3). Meanwhile, the quantitative difference in survival between CuS (divalent) and Cu\(_2\)S (monovalent) was only small although a low significant difference was observed (\(p < 0.05\), Table S3). The comparison results differed between the cell types, which suggests that it is difficult to characterize the cell damage caused by compounds with the same element using their oxidation states. Of course, other characteristics must be considered such as ion solubility, PM diameter, and active surface area. However, the higher cell damage caused by Cu\(_2\)O with a smaller specific surface area (calculated from the PM diameter, Table 1) suggests that even compounds of the same element cause various types of cell damage, based on their detailed chemical states rather than their physical properties.

Graphite, environmental standard PM (CRM#28 and CEM#30), and oxalic acid decreased the viability of A549 and macrophage-like cells based on their concentrations. In contrast, \((\text{NH}_4)_2\text{SO}_4\) did not affect cell viability.

To further characterize the biological effects of the samples, especially damage to A549 cells, we evaluated the pathway of cell death in each sample (Fig. 5). Among the Fe compounds investigated in this study, exposure to FeO and \(\alpha\)-Fe\(_2\)O\(_3\) significantly increased the viability of A549 cells treated with the apoptosis inhibitor (zVAD). This suggests that these compounds...
mainly induce cell death via apoptosis. In the results of FeO(OH), a major Fe compounds in the typical atmosphere, inhibitors of autophagy, necroptosis, and apoptosis were increased the viability compared with non-inhibitor. This implies that all three processes are involved in cell death induced by this compound. Among the Cu compounds, we could not identify the compounds that caused cell damage via apoptosis cell death alone. Rather, the results for CuO suggest that the autophagic process is a relatively large contributor to induced cell death. Sun et al. (2012) have reported that CuO nanoparticles induce autophagic cell death by altering cell viability using A549 cells and inhibitors. In contrast, autophagy may have a low contribution to cell death by Cu$_2$O. Samples imitating typical atmospheric aerosols, which contribute to the inflammatory response (i.e., graphite, CRM#28, and CRM#30), tended to induce the predominance of apoptotic cell death. The validity of our results is supported by previous reports that suggest black carbon particles and mineral dust led A549 to apoptotic death (An et al., 2019; Ardon-Dryer et al., 2020).

In summary, these responses by the cell-based bioassay show that compounds of the same element have various and inconsistent contributions to cell death and its pathway based on their detailed chemical state. For example, cell damage by $\alpha$-Fe$_2$O$_3$ is induced only via apoptotic cell death, despite the similar particle diameter of Fe$_3$O$_4$. Our observations, which only demonstrated a fraction of the biological effects of the samples, can lead to different results for different cell
types and particle sizes. However, we emphasize that various biological effects and processes are selected by also the chemical complexity among compounds with the same metal element, not only the complexity of the receiver, such as the types of affected cells and mechanisms in humans.

3.3. Cytotoxic effects caused by PM from subway premises

The elements in PM from subway premises have different chemical states from typical atmospheric PM. Moreover, these standard chemical compounds can lead to different biological effect. Especially, Fe$_3$O$_4$, which is the most characteristic Fe compound in subways, consistently damaged the two cell types. The difference in damage to some cells per unit mass caused by subway and outdoor Fe components may be insignificant due to the similarity in the damage caused to A549 cells by Fe$_3$O$_4$ and FeO(OH) (Table S3). However, the concentration-dependency of the effects of FeO(OH) exposure to the macrophage-like cells is small. Therefore, the contribution of the increase in Fe components with high level of Fe$_3$O$_4$ in subway premise, which damage macrophage cells in a concentration-dependent manner, differs from that of FeO(OH) in the outdoor. In other words, the larger concentrations and enhancement in the Fe components in the subway premises and the cell damage caused by Fe$_3$O$_4$ based on the concentration mean that the risk assessment of air pollution in subway premises should be more concentrated the toxic effects of Fe particles.
The monovalent Cu compounds detected only in the subway PM contributed to further cell damage compared with divalent Cu compounds that dominated the outdoor PM. Furthermore, the damage caused by atmospheric Cu compounds may have a relatively high proportion of the autophagic process compared with subway Cu compounds. Conversely, the increase in subway Cu compounds with significant biological damage through the apoptotic pathway can also promote bias in the cell death pathways.

In this study, our toxicity of subway PM based on the cell viability in vitro obtained here just characterized the final cellular response and its pathway induced by the chemical species in the particles at the subway premise and atmosphere. Thus, concluding in their toxic effects further require to comprehensively evaluate these cytotoxicity effects by multiple toxicity tests based on biological systems. Also, to understand the effects of subway PM on health, it is necessary to link exposure levels, complex reactions, and interactions of various chemical species.

However, our findings suggest that subway PM can not only simply increase in toxicity associated with richness of metallic components, but also have different contributions by their unique chemical state compared with the atmospheric PM.

The implication of these findings is not limited to particle exposure in the subway premise. Fe and Cu are widely used in various industries, such as the electrical sector, due to their low cost, hence the PM containing their metals is emitted into the atmosphere from smelters, refineries,
power plants, and municipal waste incinerators. Fe$_3$O$_4$ is also emitted from road traffic (Liati et al., 2015), and this anthropogenic magnetite can be widely distributed both locally and globally (Koop and Zobrist, 2009). Therefore, the difference in the biological response among the chemical states of the Fe and Cu components also provides useful information for assessing the exact toxicity of the complicated atmospheric PM.

4 CONCLUSIONS

This study compared the chemical speciation of each Fe and Cu component, which significantly enhanced the concentration of the ambient PM$_{2.5}$ and TSP collected from the subway premises. We further explained the potential biological effects of these characteristic chemical species using standard compounds based on results of speciations.

The chemical analysis of three filter samples collected from an underground station in Tokyo, Japan, revealed that the emissions with oxidation at relatively high temperatures caused by the wear process of rail–wheel–brake and catenary–pantograph interfaces enhanced the concentration of Fe and Cu components, including Fe$_3$O$_4$, γ-Fe$_2$O$_3$, and monovalent Cu compounds. Exposure of human alveolar cells and macrophage-like cells as representatives of antigen-presenting cells to these standard samples showed that different biological effects attributed to these characteristic compounds in the subway, such as cell damage and pathway to cell death compared with compounds that are dominant in the outdoor and atmospheric standard
PM. As findings from previous research, the high concentrations of PM in subway premises and metals contained in it may increase its toxicity. However, our findings suggest that unique chemical states in subway PM may have different effects on human health compared with a simple prediction based on the typical chemical states of certain metal components in atmosphere. Our conclusions are supported by sample size distributions, which cannot consistently explain the differences in the biological responsiveness of some compounds with the same element. These findings emphasize that the modulation of detailed states in chemical components of PM is an important factor in determining its toxicities for an accurate understanding of its adverse health effects in various environments.

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Figure and Table Captions

Table 1. Volume median diameters of standard particles used for cells exposure by the Mie scattering theory.

Fig. 1. Location map of the sampling in subway premises and outdoor (a, b) and sampling spots in the subway premise (c).

Fig. 2. Mass concentrations of metallic components in TSP (a) and PM$_{2.5}$ (b) collected from the subway premises and outdoor. The total concentration of all metal components obtained was indicated as the total metal component concentration (Tot).

Fig. 3. Chemical speciations results in the Fe and Cu components (b and c) in the particulate matter (PM) collected from subway premises and outdoors. Chemical speciations of PM in the underground station are shown in spots A to C based on the diameter classification (TSP and PM$_{2.5}$ samples) of particles.

Fig. 4. Cell viability of the A549 and macrophage-like cells (a and b) by exposing standard samples. Each pillar and error bar shows the average value and standard error for three independent experiments. The results of each experiment were averaged from measurements of four wells. The dashed red line indicates the cell viability of cells without exposure to particulate matter (i.e.,
The asterisk indicates a significant difference ($p < 0.05$) compared with cells without sample exposure, which was calculated using one-sided Dunnett’s test.

**Fig. 5.** A549 cell viability following treatment with inhibitors by exposing standard samples. The columns of each color show the ratio of the quantity of activity of cells exposed with the standard sample to that of cells without the sample containing only each inhibitor solutions (zVAD, 3-MA, or Nec-1). The white column indicates the viability of cells exposing only the standard samples. Each column and error bar shows the mean and standard deviation of measurements in six wells. The asterisk indicates a significant difference ($p < 0.05$; one-sided Dunnett’s test) compared with the viability of cells with only the standard samples (white column).
Table 1. Volume median diameters of standard particles used for cells exposure by the Mie scattering theory.

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<tr>
<th>VMD [μm]</th>
<th>FeO</th>
<th>Fe₃O₄</th>
<th>α-Fe₂O₃</th>
<th>FeO(OH)</th>
<th>FeCl₃</th>
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<th>Cu₂S</th>
<th>CuO</th>
<th>CuS</th>
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<th>CRM #30</th>
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<td>13.8</td>
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(a) TSP

(b) PM$_{2.5}$