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Electron microscopic and spectroscopic analysis of airborne ultrafine particles: its effects on the cell viability

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Abstract

Particulate matter (PM) is one of the most common air pollution sources causing various health-related conditions like cardiovascular diseases. However, among the three major PM types, UFPs have not yet been independently studied for their toxic effects on human health. In this study, we collected airborne dusts from Chuncheon-si, Republic of Korea, and analyzed it to understand the structural and chemical features of UFPs by using transmission electron microscope (TEM), energy-dispersive X-ray spectroscopy (EDX), and X-ray photoelectron spectroscopy (XPS). The TEM result showed UFP size to be within 100 nm, with some even appearing about 10 nm in size, while the X-ray spectroscopic studies implied the presence of sulfur to be a part of the UFPs chemical composition. We extended our study by carrying out in vitro cell analysis to understand the cellular response upon UFPs treatment. Our results serve as an analytical platform providing the preliminary information about the structural and compositional aspects of UFPs that can be attributed to further understanding of sulfur-induced human diseases.

Keywords: Particulate matter, Ultrafine particle, Cardiovascular diseases, TEM, EDX, XPS

Introduction

Particulate matter (PM) are categorized as very fine particles (less than 10 μm in diameter) which cannot be perceived by the naked eyes. Recently, it is recognized as one of the major health-related air pollution sources (Piao et al. 2018; Samet et al. 2000). PM is classified into three types according to its particle size, i.e., PM10 (less than 10 μm in diameter), PM2.5 (FP, less than 2.5 μm in diameter) and ultrafine particles (UFPs, less than 100 nm in diameter) (Brook et al. 2004). PM is found to circulate throughout the respiratory system and is therefore associated with increased respiratory, cardiovascular, and cardiopulmonary disease-related morbidity and mortality (Lee et al. 2018; Oberdorster et al. 2004; Pope et al. 2004). Short-term exposure to PM (for several minutes to several hours) can not only trigger myocardial infarction but also cardiac ischemia, arrhythmias, stroke, heart failure as well as lung cancer. More long-term exposure may also increase the possibility of acquiring chronic cardiovascular diseases (Brook 2008; Ren et al. 2017).

The toxicity of PM is correlated with the size of its particle where smaller the particle, more harmful is its impact on the body (Dockery et al. 1993). Therefore, UFPs which are the smallest in size are expected to have the largest adversity on human health. IARC (International Agency for Research on Cancer), which is an affiliated organization of WHO (World Health Organization), has designated PM to be a carcinogenic air pollutant. Thus, PM causes severe environmental issues for human health. In addition, the Asian Dust, which is known to contain air pollutants such as PM has been found beyond the heavily industrialized zone in China reaching the country of Republic of Korea. Consequently, some of the PM components found in Republic of

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Korea appear to be derived from the industrial emissions transported by the Asian dust (Park and Dam 2008).

PM is mainly generated by the combustion of fossil fuels such as natural gas and diesel fuel (i.e., automobile fuel, heating, industry) (Holmén and Ayala 2002; Murphy et al. 2009; Rogge et al. 1993; Xue et al. 2018). It is comprised of metallic elements (As, Be, Ca, Cd, Co, Cr, Fe, K, Mn, Ni, Pb, Sb, Se, Zn) (Park and Dam 2008), carbon sources (Adams et al. 2015; Funasaka et al. 2000), and inorganic compounds such as sulfate, nitrate, halide, and ammonium (Borgie et al. 2015; Brewer et al. 2015). Among them, carbon and sulfur sources are found to be highly correlated with cardiovascular diseases (Adams et al. 2015; Brook 2008; Lee et al. 2005).

Most of the sulfur-containing substances (sulfate, sulfoxides) are found under $PM_{2.5}$ level category, where the UFPs contain more amount of sulfur than the former (Adams et al. 2015; Funasaka et al. 2000; Wei et al. 2018). On a global level, a lot of research reported that the increase of SO_2 pollution is highly correlated with mortality and morbidity of cardiovascular diseases statistically (Sunyer et al. 2003; Tran et al. 2019; Yang and Liu 2019). In addition, systemic abnormalities caused by SO_2 such as rise in the blood pressure, inflammation, and thrombosis provide a plausibility of these being interrelated with sulfur (Delfino et al. 2005; Ren et al. 2017; Tran et al. 2019).

Here, we investigated UFPs which have not been studied much in comparison to other PM types partly because of its extremely small size. In general, the extent of the study of PM has been limited only to $PM_{2.5}$ level and very little is known about the much smaller, UFPs. Thus, we analyzed the structural and chemical composition of UFPs by using TEM, XPS, and EDX creating a platform to derive a better structural and conformational aspect of the UFPs.

Materials and methods

Preparation of the particulate matter

Since the probability of the Asian dust occurrence is maximum during the early summer period in Republic of Korea, particulate matter analyzed in this study was collected during this period. From that, days having the most conducive weather conditions and relatively higher occasion of PM2.5 incidences were taken into consideration. This particulate matter was collected twice on a petri dish for 24 h from the natural atmosphere at the height of 20 m from the ground level to avoid any obvious or deliberate artificial effect caused by drastic variables to maintain a constant condition during the sampling (Fig. 1). The obtained particulate matter was dissolved in 100% ethanol and stored in 1.5 ml Eppendorf tube. This was then centrifuged at 5000 rpm for 5 min to collect the "large particles" as a pellet. Note that the supernatant contained the "ultrafine particles" while the pellet contained PM10 and PM2.5 respectively.

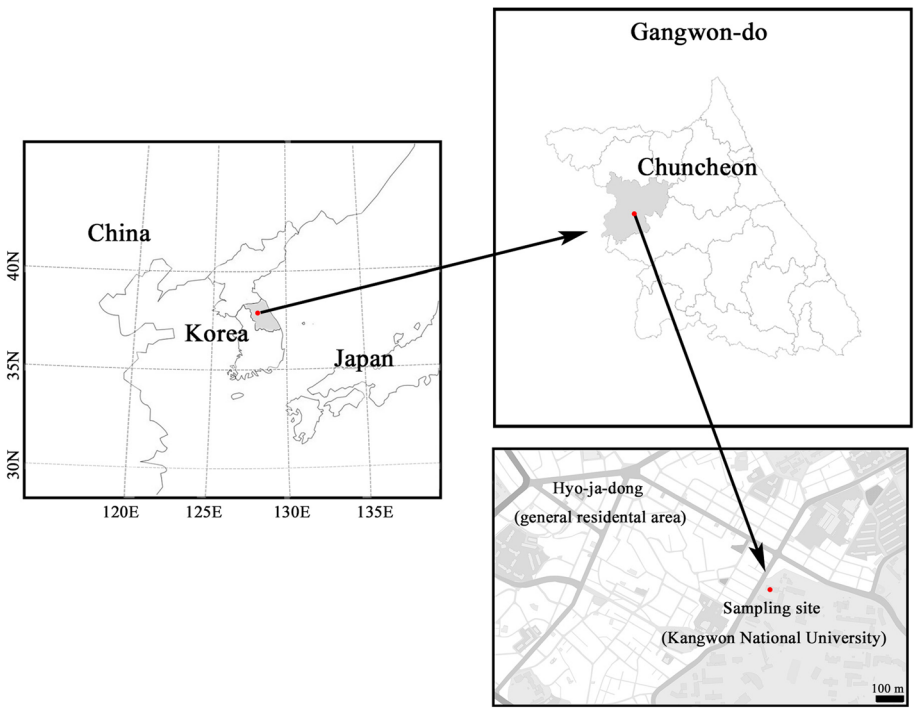

Fig. 1 Location of particulate matter collected in Republic of Korea. Particulate matter (PM) of this study was collected from Hyo-ja-dong (Kangwon National University, Chuncheon-si, Gangwon-do, Republic of Korea) with the location coordinate as 37°52′20.9″ N 127°44′31.3″ E. Here, Hyo-ja-dong area is shown as a general residential area in the university premises. Scale bar: 100 m

Transmission electron microscopy

We collected the PM from ambient circumstances and segregated the "whole particles" respectively to "large particles" (PM10 and PM2.5) and "UFPs" by centrifugation. The "large particles" were deposited as pellet while the UFPs remained in the supernatant itself. The supernatant containing UFPs was then visualized by transmission electron microscopy (TEM). 5 μl of the sample was directly applied to a carbon-coated grid without any staining process, and the grid was examined using Technai 10 (FEI, U.S) TEM operated at 100 kV. Images were then recorded on an UltraScan 1000 CCD camera (Gatan, USA) at a magnification of × 17,000 (0.62 nm/pixel). Instrumentation was used in Kangwon Center for Systems Imaging.

X-ray photoelectron spectroscopy and energy-dispersive X-ray spectroscopy

"Whole particles" include ultrafine particles whereas the "large particles" contained all other particles except the UFPs. Each set of the particle was dried to a powdered form and loaded onto an X-ray photoelectron spectroscopy (XPS) and energy-dispersive X-ray spectroscopy (EDX) which was performed at Korea Basic Science Institute (KBSI), Daejeon, Republic of Korea. X-ray photoelectron spectroscopy (XPS) (SUPRA, Kratos. Inc.) with monochromatic energy (Al-Kα, 1486.6 eV) as a photon source with a hemispherical analyzer was employed to investigate the surface elements in the sample. The resolution of electron analyzer was 0.47 eV as measured from the full width at half maximum (FWHM) of the Ag 3d5/2 peak. The binding energy of XPS spectra were calibrated by the position of C 1s (285.0 eV) of adventitious carbons in the air. High-resolution XPS spectra were obtained using an analysis area of 400 μm of the 40 eV pass energy. The surface morphological and microstructural analysis was studied using field emission scanning electron microscopy (FE-SEM, Hitachi S4800) equipped with an energy-dispersive X-ray (EDX)

Cell culture

Human non-small cell lung cancer cell line PC-9 was provided by L-Base Company, Seoul, Republic of Korea, and cultured in Dulbecco's modified minimal

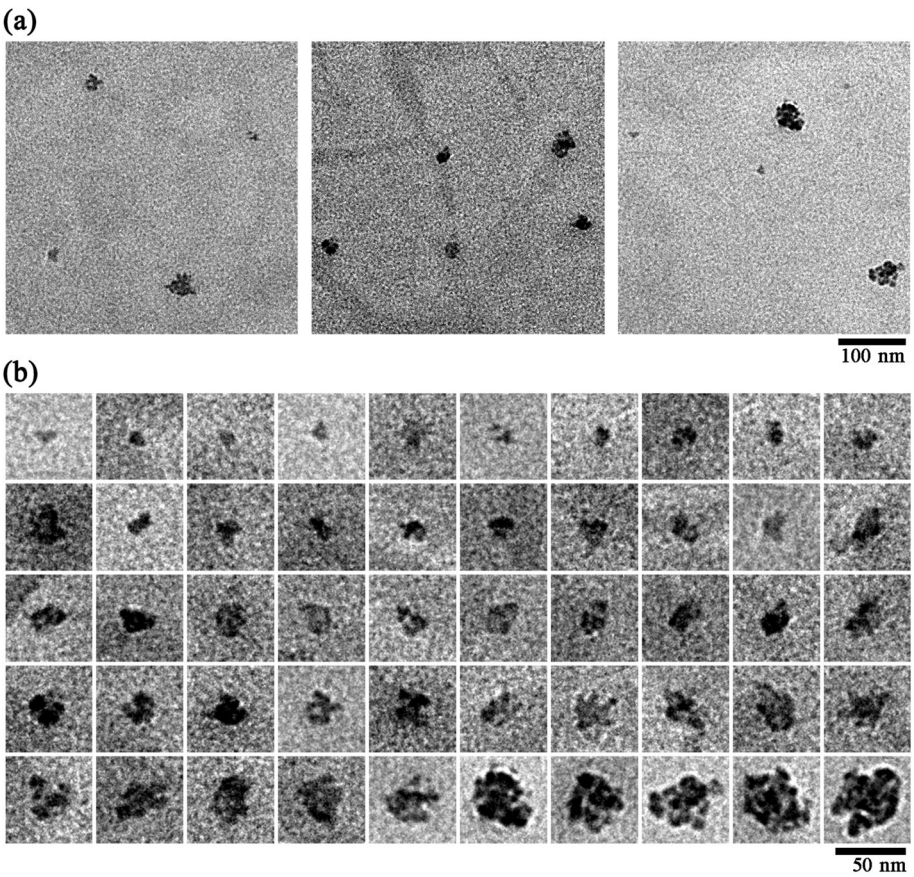

Fig. 2 Transmission electron microscopic analysis of ultrafine particles (UFPs). **a**, **b** Non-stained transmission electron microscopy (TEM) images of ultrafine particles and its particle set showed general appearances of ultrafine particle; images showed the presence of various size and shaped particles. Collected ultrafine particle set showed the smallest particle was 9 × 13 nm in length and width (left top) while the largest sized particle was 52 × 53 nm in length and width (right bottom). Majority of the particles appeared to have diameter smaller than 50 nm. Scale bars: **a** 100 nm, **b** 50 nm

essential medium (Corning) supplemented with heat-inactivated 10% fetal bovine serum (Serana) and antibiotics at 37°C in a humidified incubator with a mixture of 95% air and 5% CO2. For cell viability assay, cells were directly counted in a hemocytometer after 0.2% trypan blue (Lonza) staining.

Live cell imaging and cell viability assay

Cells were seeded at 5 × 10^4 cells per well in 96-well plate (SPL) and allowed to adhere for 24 h. Two-fold serial dilutions (25–100 μg/ml) of the UFP samples were dissolved in DMEM medium containing 10% fetal bovine serum and 1% penicillin. Subsequently, UFP solutions were treated to the cells (100 μl/well) for 48 h. During experiment, the plate was inserted into the IncuCyteTM (Sartorius) for real-time imaging of live cells and imaged per well under × 10 magnification every 2 h for a total of 48 h. Data were analyzed using the IncuCyte Confluence version 1.5 software (Sartorius), which quantified cell surface area coverage as confluence values. After 48 h of treatment, the cells were used in the MTT assay to determine cell viability. The cells were maintained in the culture medium containing 500 μg/ml MTT (Duchefa) for 2 h. The formazan dye was dissolved with dimethyl sulfoxide, and its concentration was determined by measuring the absorbance at 570 nm.

Results and discussion

Visualizing the structural appearance of UFPs

As mentioned earlier, PM has a diversity in its particle size distribution where the smallest-sized particle UFPs being critically harmful have not been explored in the structural aspect. Thus, we designed an experimental scheme to characterize them by TEM. From TEM imaging, we detected clusters of nano-sized particles. Their diameters being smaller than 100 nm are regarded to be UFPs (Fig. 2a). Thus, we grouped each UFP particle and constructed a collective set for standardization and analyzed its structural features (Fig. 2b). UFPs mostly showed irregular angular or aggregated characteristics in their overall shape. The collected UFPs appear to be extremely small in size ranging from 53 nm to 9 nm.

Analysis of the chemical properties of UFPs

After deriving the structural appearances of the UFPs by TEM, we then moved on to analyze the chemical composition of UFP to investigate if they contained any element known to be harmful to human health by using XPS and EDX. UFPs mainly share the same chemical elements as that with PM10 and PM2.5 sources and further analysis of XPS for 10 elements

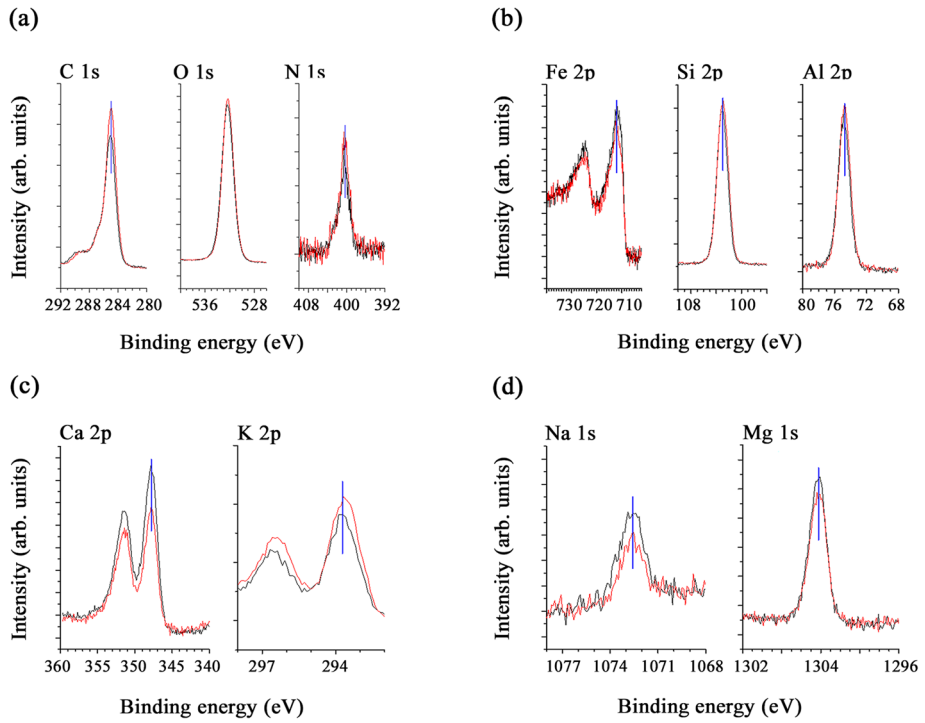

Fig. 3 X-ray photoelectron spectroscopy analysis comparing the "whole particles" with "large particles". **a**–**d** Chemical composition analyzed by X-ray photoelectron spectroscopy (XPS): black profile, "whole particles"; red profile, "large particles." Analysis was done to detect: carbon, oxygen, and nitrogen (**a**); iron, silicon, and aluminum (**b**); calcium and potassium (**c**); sodium and magnesium (**d**). Note that these representative results were taken from the data of five independent XPS analysis with three independent preparations of UFPs

(C, O, N, Fe, Si, Al, Ca, K, Na, and Mg) did not show any significant difference between the whole particles and large particles (Fig. 3). However, EDX assay conducted to analyze the detailed differences in the chemical composition showed the whole particles to specifically contain sulfur (Fig. 4). It can thus be said that UFPs and not large particles are the ones that contain sulfur solely. This inference can be supported by the replicated results for both particles.

In vitro cytotoxicity analysis of UFPs

Since the lungs are directly exposed from UFPs through respiration, we assessed in vitro cytotoxicity analysis using live cell imaging with PC-9 which is well known as a human lung adenocarcinoma cell line. During incubation, untreated control cells grew consistently while the UFP treated groups of different concentrations showed decreased cell confluency as UFPs dosage increased (Fig. 3a, b). Furthermore, we assessed MTT assay to determine cell viability at the end point of incubation where 25 and 50 μg/ml UFPs dosed classes declined up to 26 and 28% in their cell viabilities respectively. Especially, 100 μg/ml of UFPs treated test which is the highest exposure group displayed a remarkable decrease in cell viability as much as 96% (Fig. 3c). Cell confluency and viability showed a consistent outcome, thus indicating that UFPs from natural atmosphere have cytotoxic effect on the human lungs.

UFPs have been known to possess different composition based on different factors such as location and habitat environmental conditions (Dockery et al. 1993; Park and Dam 2008; Samet et al. 2000). We have collected UFPs from a general residential area in Chuncheon, Republic of Korea, to symbolize a typical city ambience and analyzed their structural and chemical properties by using TEM, XPS, and EDX. Here, we successfully demonstrated UFPs by TEM which showed many erratic forms and were uneven, angular and highly variable in shape. Although an accurate mechanism of UFPs affecting humans is unclear, in virtue of these structural characteristics such as hypervariable surface and a size small enough to permeate cells can provide a reliable evidence that UFPs are especially pernicious to living organisms. From such distinctive property of UFPs, it is able to penetrate via the respiratory-cardiovascular system facilitating invasion to any part of the organism and thereby affecting them (Dockery et al. 1993; Venners et al. 2003). Chemical composition of UFPs through XPS offered identical results for whole

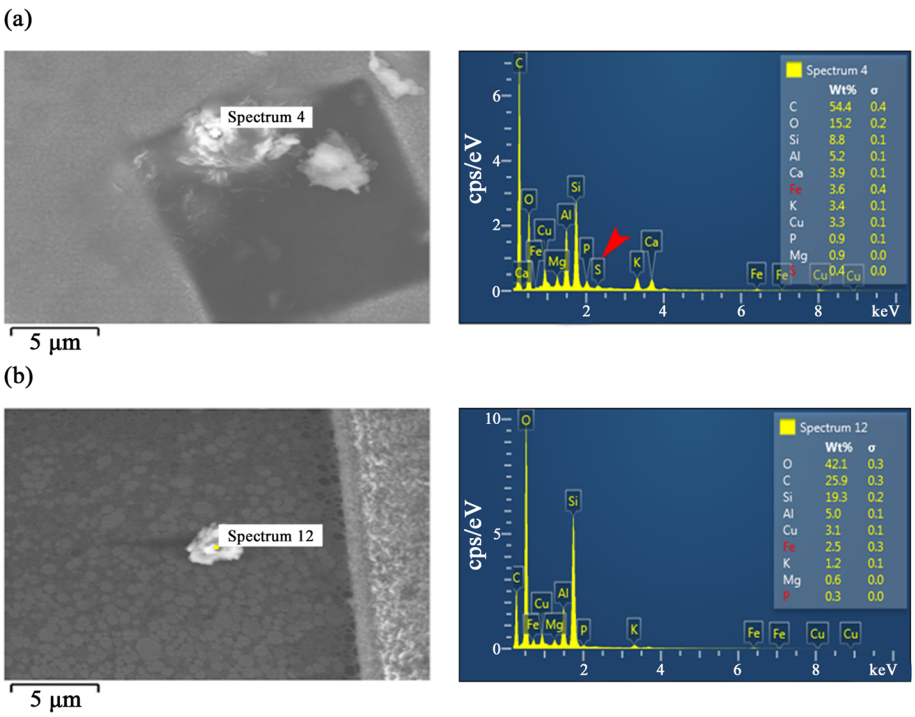

Fig. 4 Energy-dispersive X-ray spectroscopy profiles between twhole particlesofiles large particleso. **a**, **b** Scanning electron microscopic images (left panels) showing the "whole particle" and "large particle," indicated by the spectrum 4 and 12 (spectrum numbers refer to the characteristic location of the analyzed point), respectively, and their respective atomic compositions analyzed by energy-dispersive X-ray spectroscopy (right panels). For the energy-dispersive X-ray spectroscopy (EDX) profile of "whole particles" and "large particles" (right panels), each peak represents a different chemical element and specially sulfur element (red arrow) was detected in the "whole particles." Note that these representative results were taken from the data of five independent EDX analysis with three independent preparations of UFPs. Scale bars: **a** 5 μm, **b** 5 μm

particles and large particles but EDX in particular, depicted only UFPs to possess the sulfur element. In totality, sulfur accounted for only a small amount of the elements detected by EDX. Although the amount of sulfur detected is small, they have a significant relevance since they form a part of the UFPs which themselves occupy a very small part of the whole particles. Our technical demonstration by EDX and XPS provides a reliable evidence of UFPs to be linked with sulfur justifying the objective mentioned in the previous studies (Borgie et al. 2015; Brook et al. 2004; Zhu et al. 2019).

It can thus be said that the structural and chemical properties of UFPs having unique biochemical effect compared to other PM types are importantly associated with cardiovascular diseases (Gong et al. 2014). Thus, in this study, we strived to derive the basic characteristics of UFPs by using tools like TEM and EDX. In addition, most PM and sulfur related researches are restricted only up to PM2.5 level and not smaller than that like the UFPs (Sunyer et al. 2003; Tran et al. 2019; Venners et al. 2003; Yang and Liu 2019; Zhu et al. 2019). Nevertheless, we have reported the structural and chemical analysis of PM particles beyond PM2.5, particularly the UFPs. Consequently, we demonstrated the UFPs to possess sulfur, implying their possible potential in the toxicity of UFPs.

As an auxiliary study, in vitro assay by using live cell imaging and MTT assay determining the cell viability displayed significant role of the UFPs in understanding the lung cellular response. To data, we demonstrated that UFPs have a considerable cytotoxic effect on human lung adenocarcinoma cell line, PC9 (Fig. 5). This can serve to become a strong evidence to support our proposition about the critical toxicity of UFPs similar with other investigation (Steenhof et al. 2011). Surprisingly, all the groups of cells treated with UFPs demonstrated a noticeable decline in the cell viability, especially 100 μg/ml dosage showing the most outstanding severe effect on the PC 9 cells. Thus, there was a significant correlation between the concentration of UFPs and MTT cell viability of PC 9 cells.

In summary, the smallest PM, UFPs known to cause the strongest effect on human beings contains sulfur

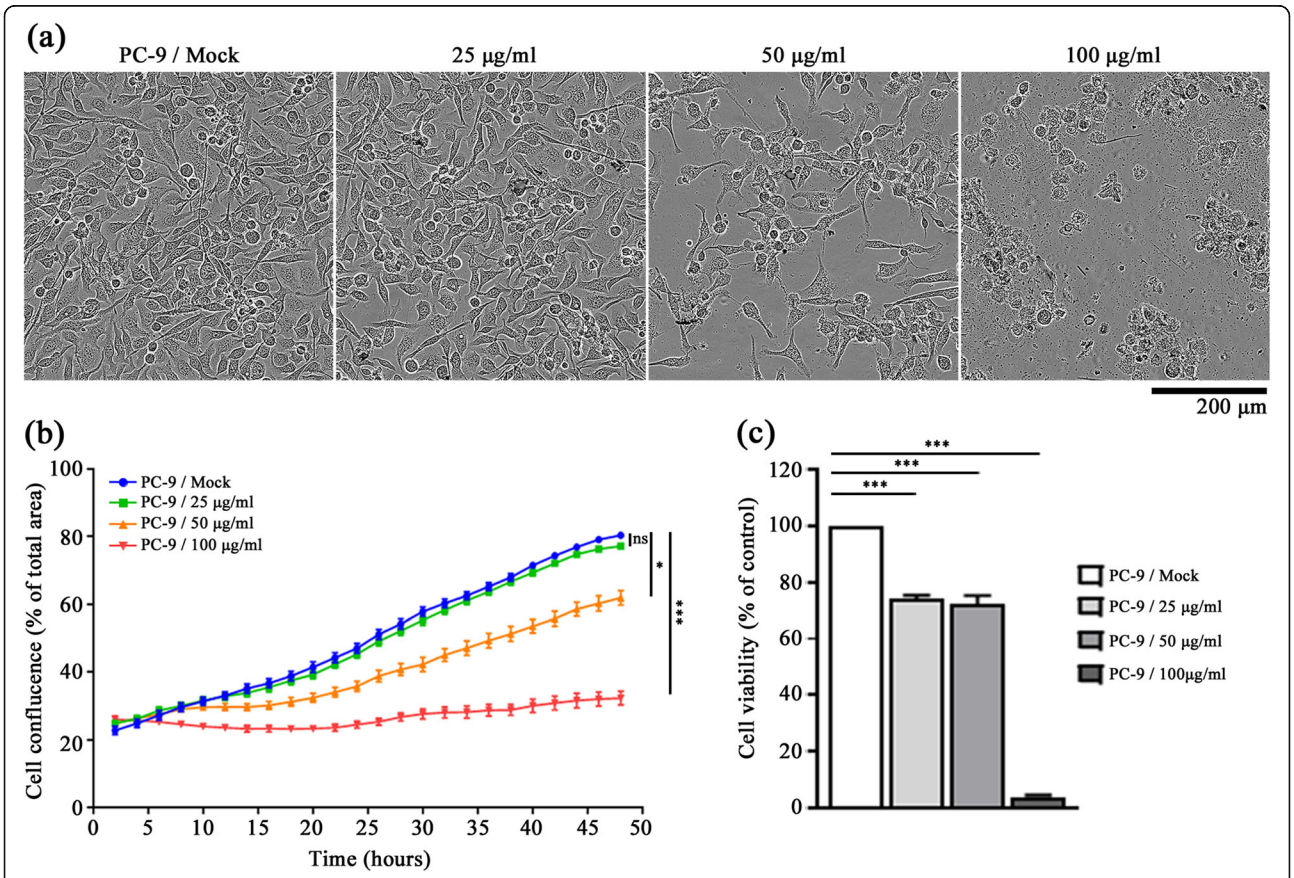

Fig. 5 Cytotoxic effect of UFPs on PC-9 cells. **a** Phase-contrast images of PC-9 cells at 48 h after untreated, 25 μg/ml, 50 μg/ml, and 100 μg/ml dose of UFPs (× 10 magnification). **b** Cell confluence of PC-9 cells after each treatment was determined by the IncuCyte Confluence version 1.5 software every 2 h for 48 h (means ± SEs, $n = 3$). **c** Cell viability of PC-9 cells after 48 h treatment of UFPs measured using 500 μg/ml as the final concentration of MTT solution (absorbance at 570 nm, means ± SEs, $n = 3$). p values were calculated using Student's t test; ns > 0.05; *$p < 0.05$; ***$p < 0.001$. Scale bar 200 μm

specifically. Thus, we presume that their harmful mechanisms are correlated with not only their nano-sized vascular permeability but also chemical composition containing sulfur. Until now, most of the research work has focused on understanding the sulfur induced mortality of cardiovascular diseases; however, they are limited to PM2.5 level. This work can serve as a structural base with straightforward manner to approach UFPs for carrying out further studies exploring the detrimental aspects of UFPs which can help to develop better emission regulations pertaining to the presence of UFPs in the near future.

Abbreviations

TEM: Transmission electron microscope; EDX: Energy-dispersive X-ray spectroscopy; XPS: X-ray photoelectron spectroscopy; FP: Fine particulate matter; UFP: Ultrafine particle; PM: Particulate matter; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

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Not applicable in this section.

Authors' contributions

J.B.Y. performed the preparation of PM, EM experiments, interpreted all obtained results, and was a major contributor in writing the paper. M.J.Y. performed live cell imaging, MTT assay and wrote the MTT assay part of the paper. H.J.Y. performed EDX and XPS experiments, and wrote method part of EDX and XPS. K.N.J. performed the editing of paper to add geological logic. D.I.J. supervised the live cell imaging and MTT assay part. H.S.J. supervised the whole project and wrote the paper. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this article and no datasets were generated or analyzed during the current study

Competing interests

No potential competing interest relevant to this article was reported.

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