United States
Consumer Product Safety Commission

June 28, 2023

CPSC Staff Statement on: Physicochemical, Morphological and Toxicological Studies of Engineered Nanoparticles Released from Printing Equipment During Consumer Use

The report titled, “Physicochemical, morphological and toxicological studies of engineered nanoparticles released from printing equipment during consumer use,” provides an overview of studies performed by Harvard University in collaboration with the Centers for Disease Control and Prevention (CDC)/National Institute for Occupational Safety and Health (NIOSH). The studies were supported by interagency agreements CPSC-I-15-0018 CDC and CPSC-I-15-0019 between the Consumer Product Safety Commission (CPSC) and NIOSH.

This statement was prepared by the CPSC staff. NIOSH prepared the following report for CPSC staff. This statement and the report have not been reviewed or approved by, and do not necessarily represent the views of, the Commission.

The use of engineered nanomaterials (ENMs) in toners of laser printers has improved both printing quality and performance. With the exponential growth of computing and business machine technology, workers and the public heavily use laser printers and photocopiers in many homes and commercial environments. Limited studies on copier operators reported respiratory symptoms, such as cough, nasal blockage, excessive sputum production, and breathing difficulties related to copier operation. These adverse effects induced by printer emitted particles (PEPs) can be exacerbated via chronic exposures and in individuals susceptible to inhaled particles. The release of ENMs from laser printers during consumer use similarly has raised concerns for human health.

To characterize PEPs and their potential health effects, a Printer Exposure Generation System (PEGS) was developed to generate and sample airborne PEPs for subsequent physiological, morphological, and toxicological analyses.

For physicochemical and morphological characterization, the sampled PEPs were qualitatively compared to toner powders directly from toner cartridges by comparative chemical analyses for metals, volatile organic compounds, as well as organic and elemental carbon. The physicochemical and morphological analyses identified that laser printers release engineered nanoparticles up to 1.26 million particles/cm^3. The analyses presented a unimodal size distribution of emitted particulate matters with aerodynamic diameters ranged from 39 to 122 nm; most particle sizes were less than 100 nm (*i.e.*, nanoparticles). The PEPs contained similar chemical composition compared to that of toner powder. The analysis indicated that toner powder is the main source of the released airborne ENMs.

A toxicological assessment of PEPs was conducted using both *in vitro* and *in vivo* experimental models.

For the *in vitro* studies, mono-cultures of selected cells (*i.e.*, human small airway epithelial cells (SAEC), macrophages (THP-1), and lymphoblasts (TK6)) were exposed to PEPs at a range of concentrations (0.5 to 100 µg/ml) that correspond to human inhalation exposure levels during printer use for 8 hours or more. PEPs caused cytotoxicity, oxidative stress, and pro-inflammatory cytokine release in the cell lines. An alveolar-capillary co-culture system using SAEC and human microvascular endothelial cells (HMVEC) was used to evaluate the effect of PEPs on paracrine signaling between the two cell lines; cytological changes and increased reactive oxygen species production as well as angiogenesis were observed.

For the *in vivo* studies, effects of PEPs on various pulmonary endpoints were investigated in a mouse model. With mice exposed to PEPs by intratracheal instillation, a pulmonary immune response, but no changes in the lung membrane integrity, was detected by elevated levels of neutrophils and macrophages. In addition, PEPs caused upregulated expression of genes and changes in DNA methyltransferase levels leading to changes in DNA methylation that are associated with repair processes from oxidative damage and the initiation of immune responses.

The scientific findings from the *in vitro* and the *in vivo* studies suggest that PEPs may cause immune responses and modifications of gene expression in the lung at doses that are comparable to human exposure during laser printer use. This observation raises concerns for human health during heavy use of laser printers in areas with poor ventilation.

Appendix

Pirela SV, Lu X, Miousse I, Sisler JD, Qian Y, Guo N, Koturbash I, Castranova V, Thomas T, Godleski J, Demokritou P. Effects of intratracheally instilled laser printer-emitted engineered nanoparticles in a mouse model: A case study of toxicological implications from nanomaterials released during consumer use. *NanoImpact*. 2016, 1:1–8. Available at https://doi.org/10.1016/j.impact.2015.12.001.

Pirela SV, Miousse IR, Lu X, Castranova V, Thomas T, Qian Y, Bello D, Kobzik L, Koturbash I, Demokritou P. Effects of laser printer-emitted engineered nanoparticles on cytotoxicity, chemokine expression, reactive oxygen species, DNA methylation, and DNA damage: A comprehensive *in vitro* analysis in human small airway epithelial cells, macrophages, and lymphoblasts. *Environ Health Perspect.* 2016, 124(2):210–9. Available at https://doi.org/10.1289/ehp.1409582.

Lu X, Miousse IR, Pirela SV, Moore JK, Melnyk S, Koturbash I, Demokritou P. *In vivo* epigenetic effects induced by engineered nanomaterials: A case study of copper oxide and laser printer-emitted engineered nanoparticles. *Nanotoxicology*. 2016,10(5):629-39. Available at https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4958020/.

Lu X, Miousse IR, Pirela SV, Melnyk S, Koturbash I, Demokritou P. Short-term exposure to engineered nanomaterials affects cellular epigenome. *Nanotoxicology*. 2016, 10(2):140–50. Available at https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4633390/.

Physicochemical, morphological and toxicological studies of engineered nanoparticles released from printing equipment during consumer use

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Centers for Disease Control and Prevention
National Institute for Occupational Safety and Health

Ethics Approval

All procedures performed on animals were approved by the Harvard University Institutional Animal Care and Use Committee (IACUC).

Disclaimer

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention.

A report for Interagency Agreements:

1) CPSC-I-15-0018 CDC IAA #15-NS15-07
2) CPSC-I-15-0019 CDC IAA #15-NS15-06

The main focus of the study project is the physicochemical, morphological and toxicological characterization of engineered nanoparticles released from printing equipment during consumer use. A printer exposure generation system (PEGS, Figure 1) suitable for the physicochemical, morphological, and toxicological characterization of printer-emitted particles (PEPs) was developed and used to assess the properties of PEPs generated during the use of commercially available laser printers(Pirela, Pyrgiotakis et al. 2014). The system consists of a glovebox type environmental chamber for uninterrupted printer operation, real-time and time-integrated particle sampling instrumentation for the size fractionation and sampling of PEPs and an exposure chamber for inhalation toxicological studies.

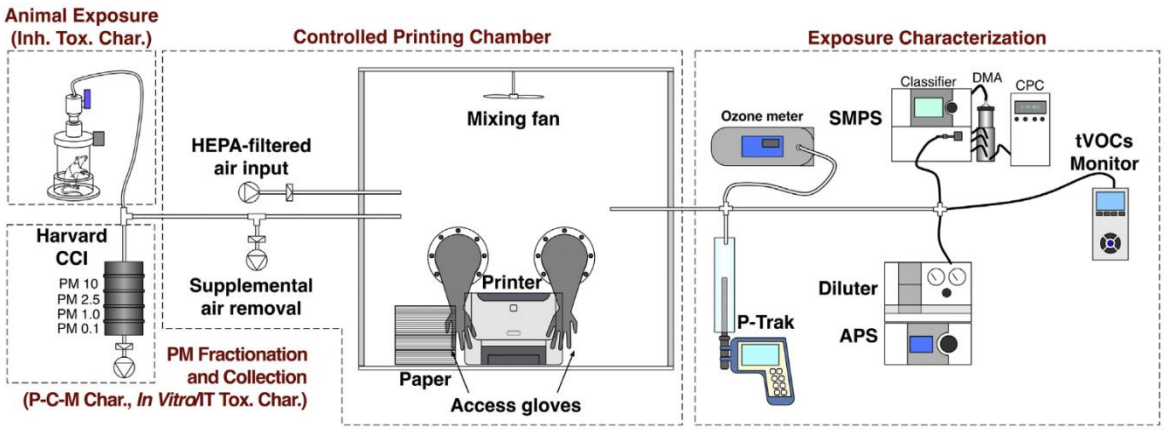

Figure 1 | Experimental design overview showing the exposure platform for *in vitro and in vivo* characterization of PEPs from a high PEP emitting laser printer. The experimental setup includes the Harvard compact cascade impactor that allows for size-fractionated sampling of the particulate matter (PM) emitted by the laser printer to be used for cellular and intratracheal instillation experiments, as well as an animal exposure system that allows for whole-body inhalation exposures to both PM and gaseous pollutants emitted by the laser printer. (Pirela et al. 2016)

Physico-chemical and morphological characterization of both toner and PEPs

Eleven monochrome commonly used laser printers were evaluated and ranked based on their PM emission profiles. The six highest emitting laser printers were selected to have their PEPs evaluated during a continuous print job utilizing both real-time and time-integrated sampling instrumentation. The sampled PEPs and toner powders (collected directly from the toner cartridge) from these laser printers underwent a detailed chemical characterization that included analysis for total and water-soluble metals, total volatile organic compounds, as well as organic and elemental carbon. Transmission and scanning electron microscopy (TEM/SEM) and energy dispersive spectroscopy (EDX) were also used to assess the morphology as well as the chemistry of both the PEPs and the toner powder.

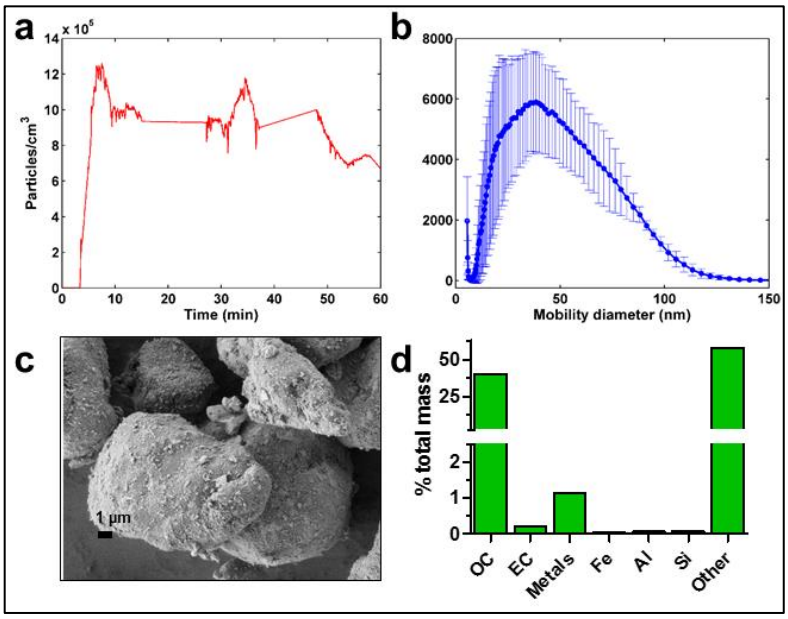

Figure 2 | PEPs generation and properties. a, Air concentration of PEPs during 60-min print job. **b**, Size distribution of PEPs sampled at 10-20 min (±SD). **c**, SEM of PEPs, **d**, Chemical composition of PEPs from IC-PMS. (Pirela et al., 2016)

Thorough physicochemical and morphological assessment (Figure 2) showed that laser printers were found to release engineered nanoparticles up to 1.26 million particles/cm^3. The emitted PM had a unimodal size distribution and aerodynamic diameters that ranged from 39 to 122 nm, but the majority of the particles were less than 100 nm (Pirela, Pyrgiotakis et al. 2014, Pirela, Sotiriou et al. 2015). Scanning transmission electron microscopy (STEM) analysis of both the toner powder and PEPs showed presence of engineered nano materials (ENMs) in the toner that become airborne during printing; thus, identifying the toner powder as the main source of the released aerosol. Additionally, electron microscopy analysis showed that the size of the PEPs and chemical composition is in agreement with the results of real time aerosol monitoring instruments and chemical analysis. The chemical composition of both the toner and PEPs revealed a similar chemical fingerprint. The PEPs had a complex chemical makeup that included 42% elemental carbon (EC)/ organic carbon (OC), 1.5% metal/metal oxides (aluminum, titanium, cerium, zinc and copper) and 56% other (phosphorus, sulfur, chlorine). In particular, one of the highest emitting laser printer, Printer B1, had PM peak emissions of 1.26x10^6 particles/cm^3, modal diameters ranging from 37 to 43 nm, with the majority of PEPs in the nanoscale (<100 nm) size, and mass concentrations of approximately 50 µg/m^3 for $PM_{2.5}$ and 15 µg/m^3 for $PM_{2.5\text{-}10}$ in a print job lasting 60 minutes. Moreover, levels of gaseous pollutants were 13.8 ppbv and 681 ppm for ozone and CO_2, respectively.

Based on the data obtained, further physicochemical characterization of both the emitted particles and the toner powder was done in the interest of understanding the source of these nano-emissions. It was our hypothesis that the toner formulations had been modified to

incorporate ENMs to improve the charge of the toner particles and enhance quality of adhesion and fusion of the toner to the paper at the time of printing. It was confirmed that a number of ENMs were incorporated into toner formulations (*e.g.*, silica, alumina, titania, ceria, iron oxide, zinc oxide, copper oxide and carbon black among others) and released into the air during printing. For instance, toner from Printer B1 contained large amounts of organic carbon (OC, 62.03%), metals/metal oxides (<3%), and some elemental carbon (EC, 9.71%). The PEPs possess a composition similar to that of toner and contained 96.51% OC, 0.48% EC and <4% metals. The chemical analysis report of the airborne PM and toner revealed the similarity in the metal composition of both samples and TEM/EDX results provided further confirmation that the toner particles have engineered nanoparticles (*i.e.*, Al, Si, Te, Cu, S) on their surface and thus, that the emitted nanoparticles released during printing can actually be sourced back to the toner powder that is not being completely fused to the page.

Toxicological assessment of PEPs

Initial toxicological analysis was performed to test the biological reactivity of PEPs from Printer B1 using both *in vitro* and *in vivo* experimental models (Figure 3). In order to do this, the sampled size-fractionated PEPs were extracted from the sampling substrates and a particle suspension was prepared to be used for both mono- and co-culture systems (Pal, Watson et al. 2015). Further, physical characterization of the PEPs suspension was performed using dynamic light scattering technology to measure the hydrodynamic diameter, zeta potential, polydispersity index, and specific conductance while taking into account dosimetry considerations for the *in vitro* toxicological characterization studies.

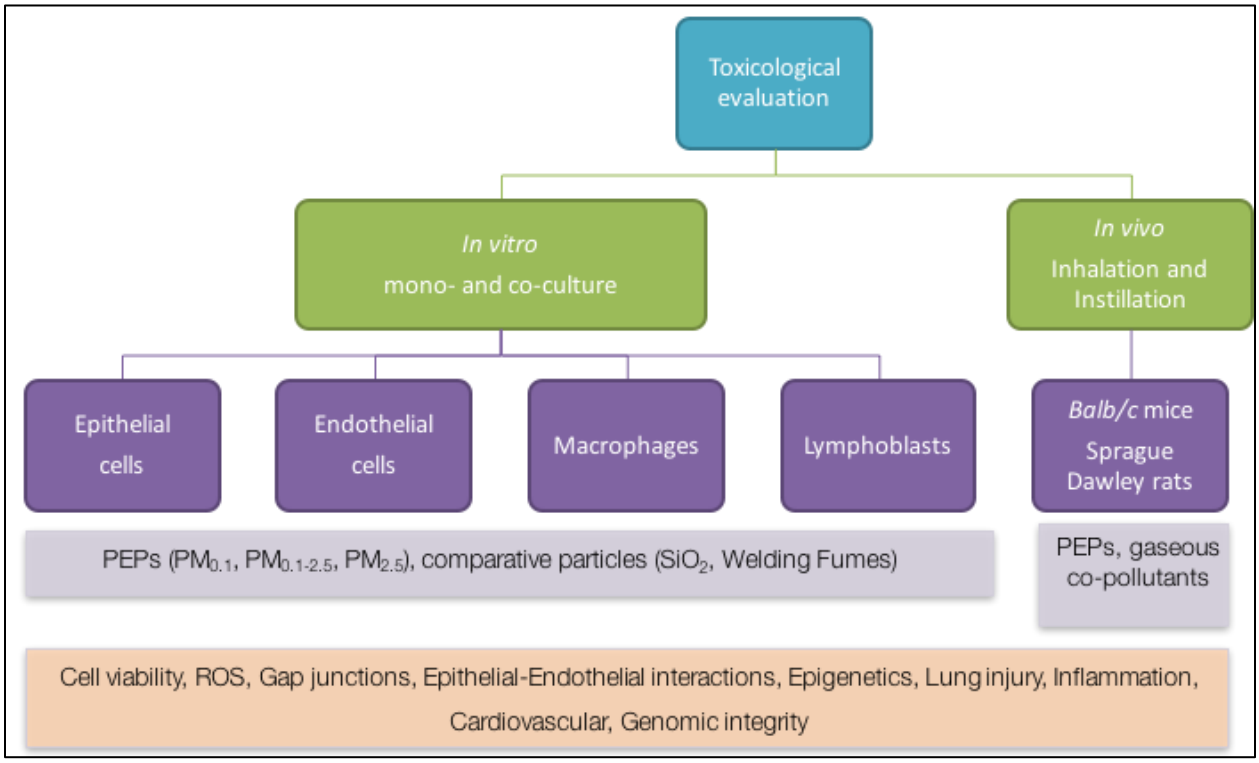

Figure 3| Experimental design of the toxicological assessment of PEPs. Understanding the adverse health effects exposures to PEPs may have on the body was studied using both cellular and animal experimental models. (Balb/C mice were used in this phase)

First, for the *in vitro* toxicological assessment, mono-cultures of small airway epithelial cells (SAEC), macrophages (THP-1) and lymphoblasts (TK6) were exposed to PEPs at a wide range of doses (0.5-100 μg/ml) that correspond to human inhalation exposure durations at the consumer level of ~ 8 hours and higher. PEPs caused significant membrane integrity damage, an increase in reactive oxygen species (ROS) production as well as a rise in pro-inflammatory cytokine release in different cell lines at doses relevant to exposure durations from 7.8 to 1,500 hours (Lu, Miousse et al. 2016, Pirela, Miousse et al. 2016).

Secondly, an alveolar-capillary co-culture system using SAEC and Human Microvascular Endothelial Cells (HMVEC) was used to test the effect of PEPs on paracrine signaling between these two cell lines. It was found that direct exposure of SAEC to low concentrations of PEPs (0.5 and 1.0 μg/ml) caused morphological changes of actin remodeling and gap formations within the endothelial monolayer. Furthermore, increased production of reactive oxygen species (ROS) and angiogenesis were observed in the HMVEC. Analysis of cytokine and chemokine levels demonstrates that interleukin (IL)-6 and MCP-1 may play a major role in the cellular communication observed between SAEC and HMVEC and the resultant responses in HMVEC (Sisler, Pirela et al. 2015).

Regarding the *in vivo* toxicological characterization of PEPs from Printer B1, an initial investigation on the effects of PEPs on various pulmonary endpoints was completed. In summary, mice were exposed by intratracheal instillation of the same size-fractionated sampled particles extracted as previously described (Lu, Miousse et al. 2016, Pirela, Lu et al. 2016). Additionally, nine-week old male *Balb/c* mice (by inhalation) were exposed to freshly generated, size-fractionated airborne particulate matter as well as gaseous co-pollutants relevant to the instillation component. Bronchoalveolar lavage (BAL) was performed post-exposure at various time points and the recovered lung cells analyzed for viability, necrosis, ROS generation, mitochondrial membrane toxicity, epigenetic changes and pro-inflammatory responses, using similar assays as in the *in vitro* component of the study. Our results show that while intratracheal instillation of PEPs caused no changes in the lung membrane integrity, there was a pulmonary immune response, indicated by an elevation in neutrophil and macrophage percentage over the vehicle control and low dose PEPs groups. Additionally, exposure to PEPs led to upregulated expression of the *Ccl5* (Rantes), *Nos1* and *Ucp2* genes (Figure 4) in the murine lung tissue and modified components of the DNA methylation machinery (Dnmt3a) and expression of transposable element (TE) LINE-1 compared to the control group. Such genes are involved in both the repair process from oxidative damage and the initiation of immune responses to foreign pathogens (Pirela et al, Env. Health Perspectives, 2017, Liu et al, Nanotoxicology 2016, Pirela et al. NanoIMPACT, 2016)

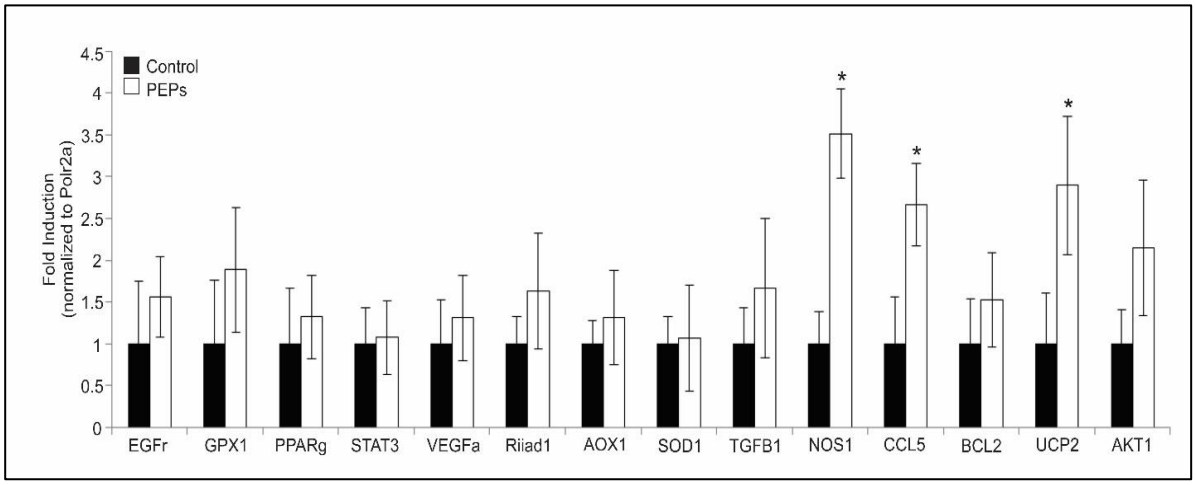

Figure 4 | Expression analysis. qRT-PCR analysis of gene expression in mouse lungs treated with PEPs. RNA was isolated from control and 2.5 mg PEPs treated mouse lungs. cDNA was amplified using a High Capacity cDNA Reverse Transcription kit. The cDNA was used to analyze the genes indicated using TaqMan Universal PCR Master Mix and TaqMan primers. Relative gene expression was analyzed using the $2^{-\Delta\Delta CT}$ method with POLR2a as the internal control. $n = 3$ male *Balb/c* mice and * indicates $p < 0.05$. (Pirela et al., EHP 2016)

The results are in agreement with findings from our previous *in vitro* studies and suggest that PEPs may cause immune responses in addition to gene expression modifications in the murine lung

at doses that can be comparable to real world exposure scenarios; thereby, raising concerns of deleterious health effects.

All details for the two interagency agreements (IAAs) are outlined in the following publications.

- Pirela SV, Lu X, Miousse I, Sisler JD, Qian Y, Guo N, Koturbash I, Castranova V, Thomas T, Godleski J, Demokritou P. Effects of intratracheally instilled laser printer-emitted engineered nanoparticles in a mouse model: A case study of toxicological implications from nanomaterials released during consumer use. *NanoImpact*. 2016, 1:1–8. Available at https://doi.org/10.1016/j.impact.2015.12.001.
- Pirela SV, Miousse IR, Lu X, Castranova V, Thomas T, Qian Y, Bello D, Kobzik L, Koturbash I, Demokritou P. Effects of laser printer-emitted engineered nanoparticles on cytotoxicity, chemokine expression, reactive oxygen species, DNA methylation, and DNA damage: A comprehensive *in vitro* analysis in human small airway epithelial cells, macrophages, and lymphoblasts. *Environ Health Perspect.* 2016, 124(2):210–9. Available at https://doi.org/10.1289/ehp.1409582.
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