Engineered nanoparticles emitted from laser printers: Quantifying the health implications from nano-enabled products during consumer use

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Session A | Quantifying Potential Acute and Chronic Exposure from 3D Printing/Additive Manufacturing.

QEEN II

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CENTER FOR NANOTECHNOLOGY AND NANOTOXICOLOGY
http://hsph.harvard.edu/nano

Background: Knowledge gap

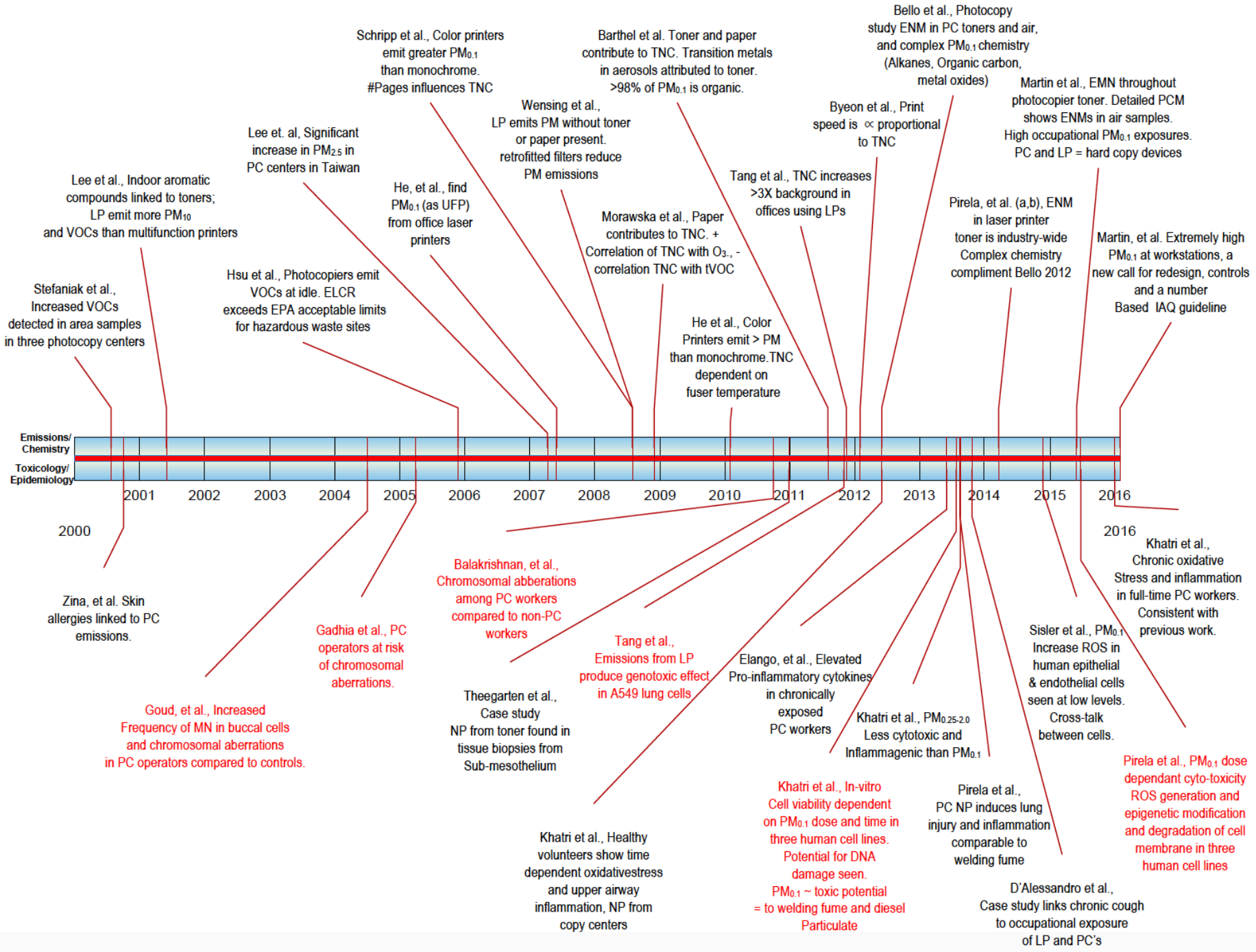
NANOTECHNOLOGY

- Superior physical, chemical and optical performance of nanoparticles in comparison to micron-sized components
- Thousands of nano-enabled products (NEPs) introduced to the market (textiles, paints, cosmetics, pharmaceutical, personal care products)

Exposure at the consumer level is inevitable

RESEARCH GAPS

- Risk assessment requires both exposure data as well as toxicological data
  - Exposure evidence is critical to understand adverse health effects from exposures across the life cycle of NEP
- No standardized methodology for the systematic investigation of real world exposures of particulate matter released across life cycle of NEPs (LCPM)
  - No link from LCPM exposure during consumer use or end-of-life to toxicology
  - Limited exposure data beyond manufacturing stage
  - Life cycle perspective toxicology


Emissions/ Chemistry
Toxicology/ Epidemiology
2000
2001
2002
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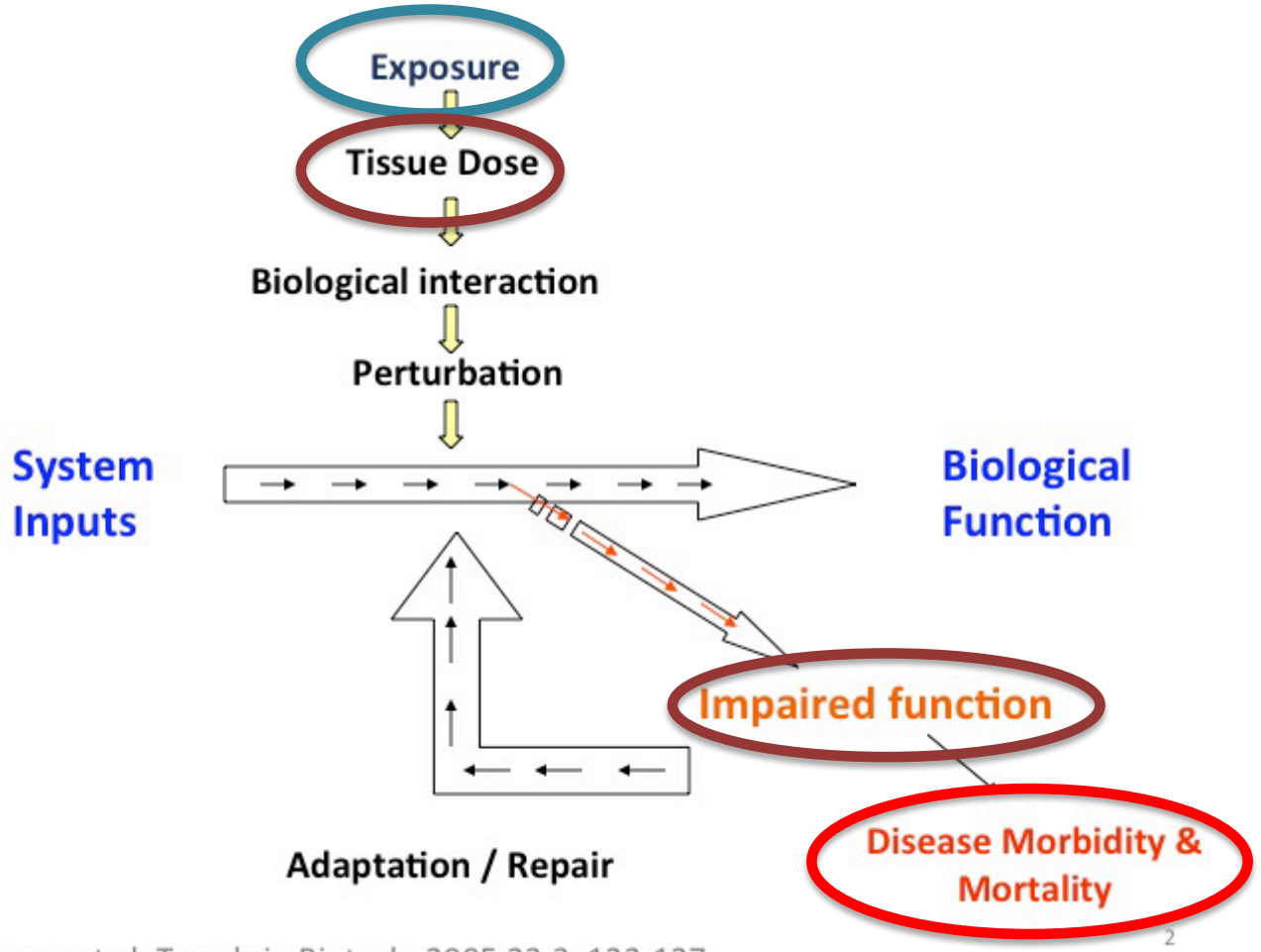
Zina, et al. Skin allergies linked to PC emissions.
Stefaniak et al., Increased VOCs detected in area samples in three photocopy centers
Lee et al., Indoor aromatic compounds linked to toners; LP emit more PM10 and VOCs than multifunction printers
Goud, et al., Increased Frequency of MN in buccal cells and chromosomal aberrations in PC operators compared to controls.
Hsu et al., Photocopiers emit VOCs at idle. ELCR exceeds EPA acceptable limits for hazardous waste sites
Gadhia et al., PC operators at risk of chromosomal aberrations.
Lee et. al, Significant increase in PM2.5 in PC centers in Taiwan
Schripp et al., Color printers emit greater PM0.1 than monochrome. #Pages influences TNC
He, et al., find PM0.1 (as UFP) from office laser printers
Wensing et al., LP emits PM without toner or paper present. retrofitted filters reduce PM emissions
Barthel et al. Toner and paper contribute to TNC. Transition metals in aerosols attributed to toner. >98% of PM0.1 is organic.
Morawska et al., Paper contributes to TNC. + Correlation of TNC with O3., - correlation TNC with tVOC
He et al., Color Printers emit > PM than monochrome.TNC dependent on fuser temperature
Balakrishnan, et al., Chromosomal abberations among PC workers compared to non-PC workers
Theegarten et al., Case study NP from toner found in tissue biopsies from Sub-mesothelium
Tang et al., Emissions from LP produce genotoxic effect in A549 lung cells
Khatri et al., Healthy volunteers show time dependent oxidativestress and upper airway inflammation, NP from copy centers
Tang et al., TNC increases >3X background in offices using LPs
Elango, et al., Elevated Pro-inflammatory cytokines in chronically exposed PC workers
Bello et al., Photocopy study ENM in PC toners and air, and complex PM0.1 chemistry (Alkanes, Organic carbon, metal oxides)
Byeon et al., Print speed is ∝ proportional to TNC
Khatri et al., PM0.25-2.0 Less cytotoxic and Inflammagenic than PM0.1
Khatri et al., In-vitro Cell viability dependent on PM0.1 dose and time in three human cell lines. Potential for DNA damage seen. PM0.1 ~ toxic potential = to welding fume and diesel Particulate
Pirela et al., PC NP induces lung injury and inflammation comparable to welding fume
D'Alessandro et al., Case study links chronic cough to occupational exposure of LP and PC's
Martin et al., EMN throughout photocopier toner. Detailed PCM shows ENMs in air samples. High occupational PM0.1 exposures. PC and LP = hard copy devices
Pirela, et al. (a,b), ENM in laser printer toner is industry-wide Complex chemistry compliment Bello 2012
Martin, et al. Extremely high PM0.1 at workstations, a new call for redesign, controls and a number Based IAQ guideline
Khatri et al., Chronic oxidative Stress and inflammation in full-time PC workers. Consistent with previous work.
Sisler et al., PM0.1 Increase ROS in human epithelial & endothelial cells seen at low levels. Cross-talk between cells.
Pirela et al., PM0.1 dose dependant cyto-toxicity ROS generation and epigenetic modification and degradation of cell membrane in three human cell lines

Conceptual Framework

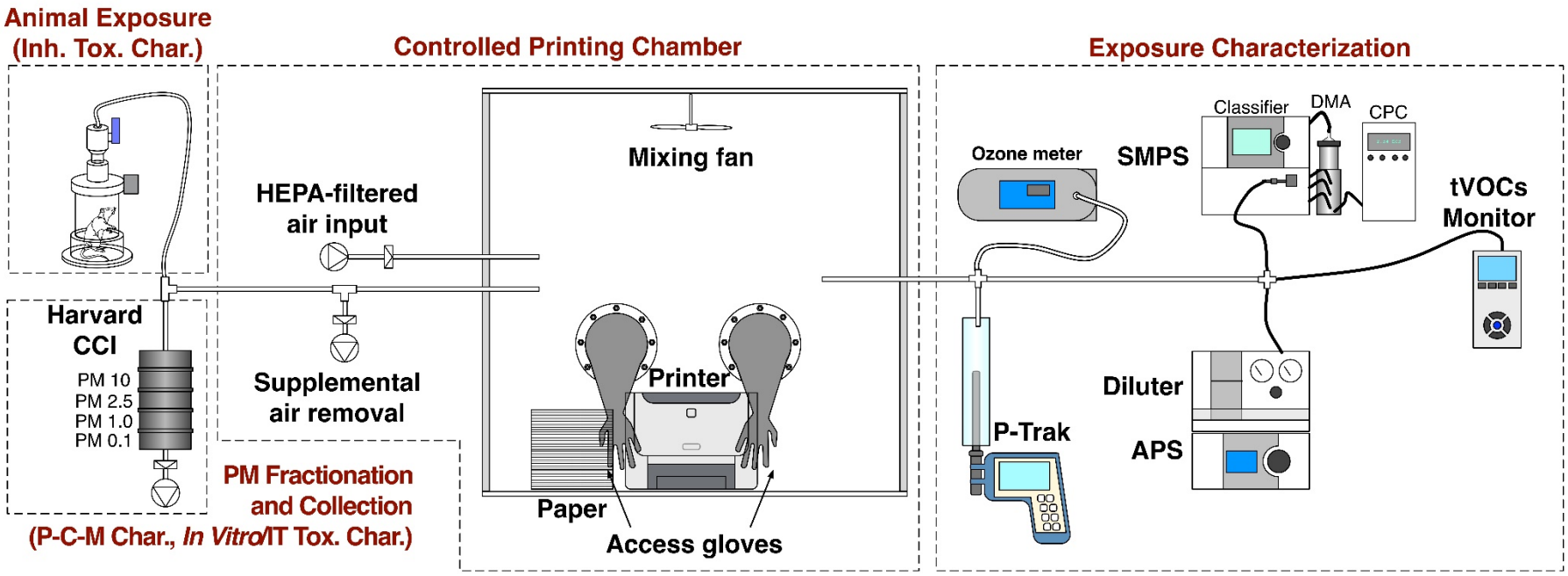


Andersen et al, Trends in Biotech, 2005,23,3, 122-127

Research Objectives

- Develop lab-based exposure platform to generate real-world PEPs
- Utilization of developed platform to evaluate PEPs and gaseous co-pollutants released by laser printers currently in the market
  - Is the toner a nano-enabled product (NEP)? Physico-chemical and morphological characterization of toner powders and PEPs
  - Are ENMs emitted during a print job? Assess emission profile of laser printers (*i.e.*, PM and gaseous co-pollutants)
  - Are there operational parameters that affect the emission profile of laser printers?
- Toxicological evaluation of PEPs
  - *In vitro*: mono- and co-culture systems
  - *In vivo*: whole-body inhalation and intratracheal instillation of PEPs

Development of Printer Exposure Generation System (PEGS)

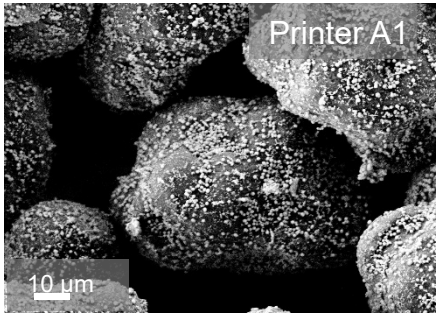


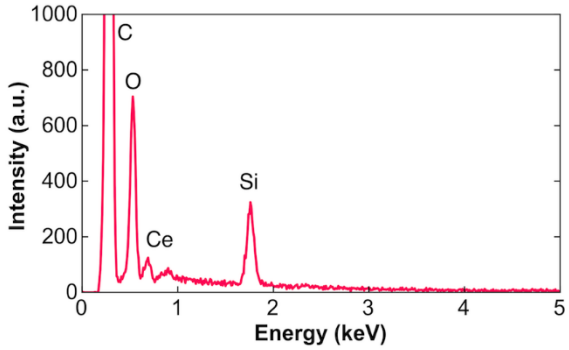
Features

- Uninterrupted operation
- Real time aerosol and gaseous emission monitoring
- Particle generation and collection
- Animal exposures
- Simulation of different exposure scenarios (ACH)
- Versatile: can be used for characterization of particle released from various NEPs

Physicochemical and morphological assessment of toner powder and PEPs

Toner powder

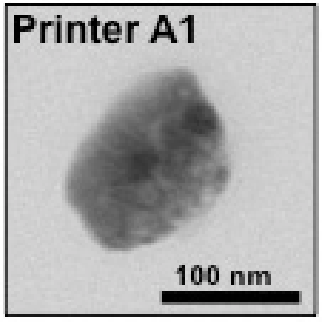


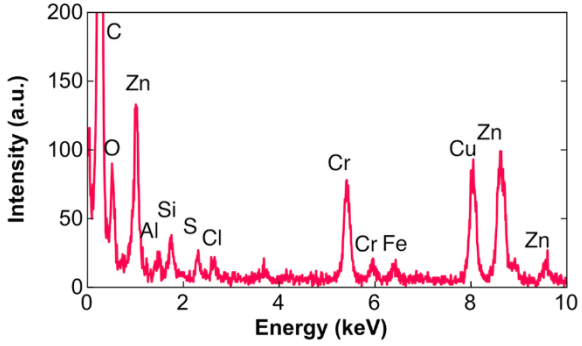


- Diameter 10-15 µm
- ENMs on the surface and embedded in the toner particle
- *EDX:* traces of carbon, oxygen, aluminum, silicon, cerium, iron, among others

Toner formulations are nano-enabled products

PEPs



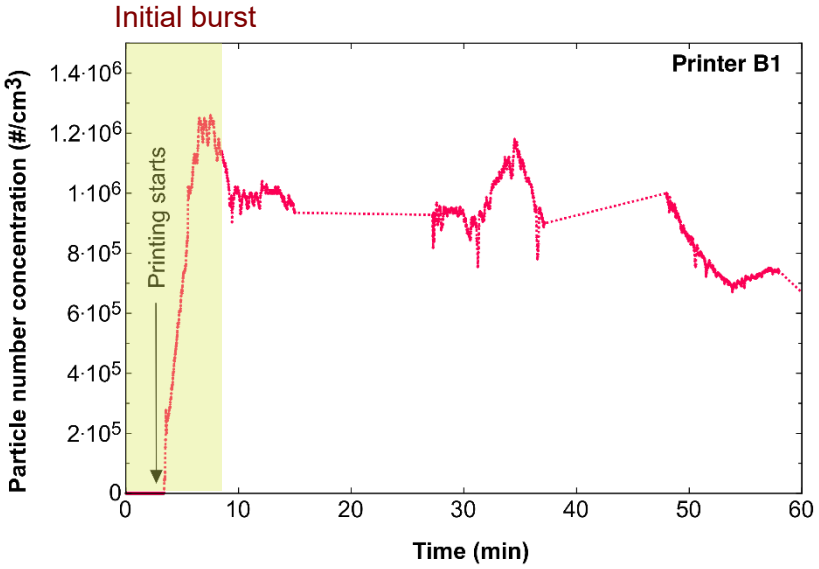


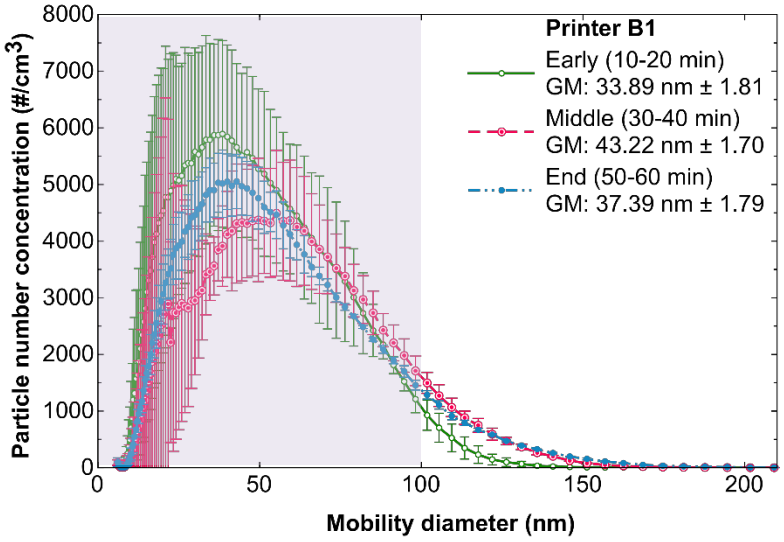
- Different aggregate shapes/sizes of ~ 20 – 200 nm
  - Consistent with RT monitoring data
- *EDX:* traces of carbon, oxygen, aluminum, silicon, zinc, iron, cerium, copper, tellerium, titanium, sulfur, among others

ENMs become airborne during consumer use of laser printer

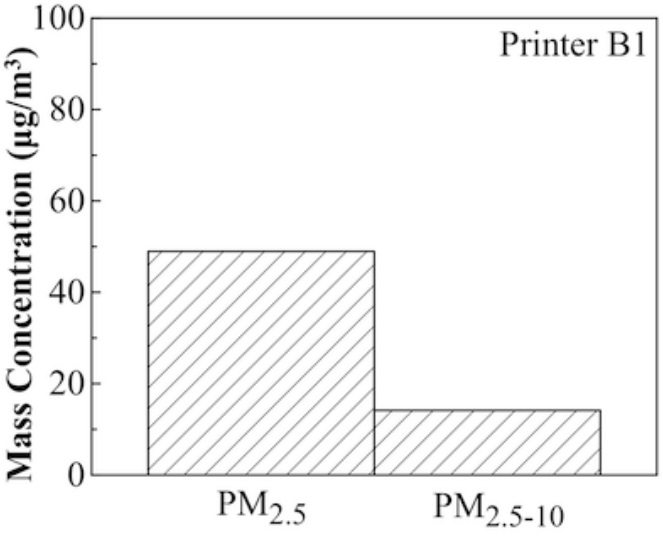
Pirela et al., Inhalation Toxicology, 2014
Pirela et al., Nanotoxicology, 2014

Assessment of laser printer emission profiles: Size distribution and number concentration of PEPs

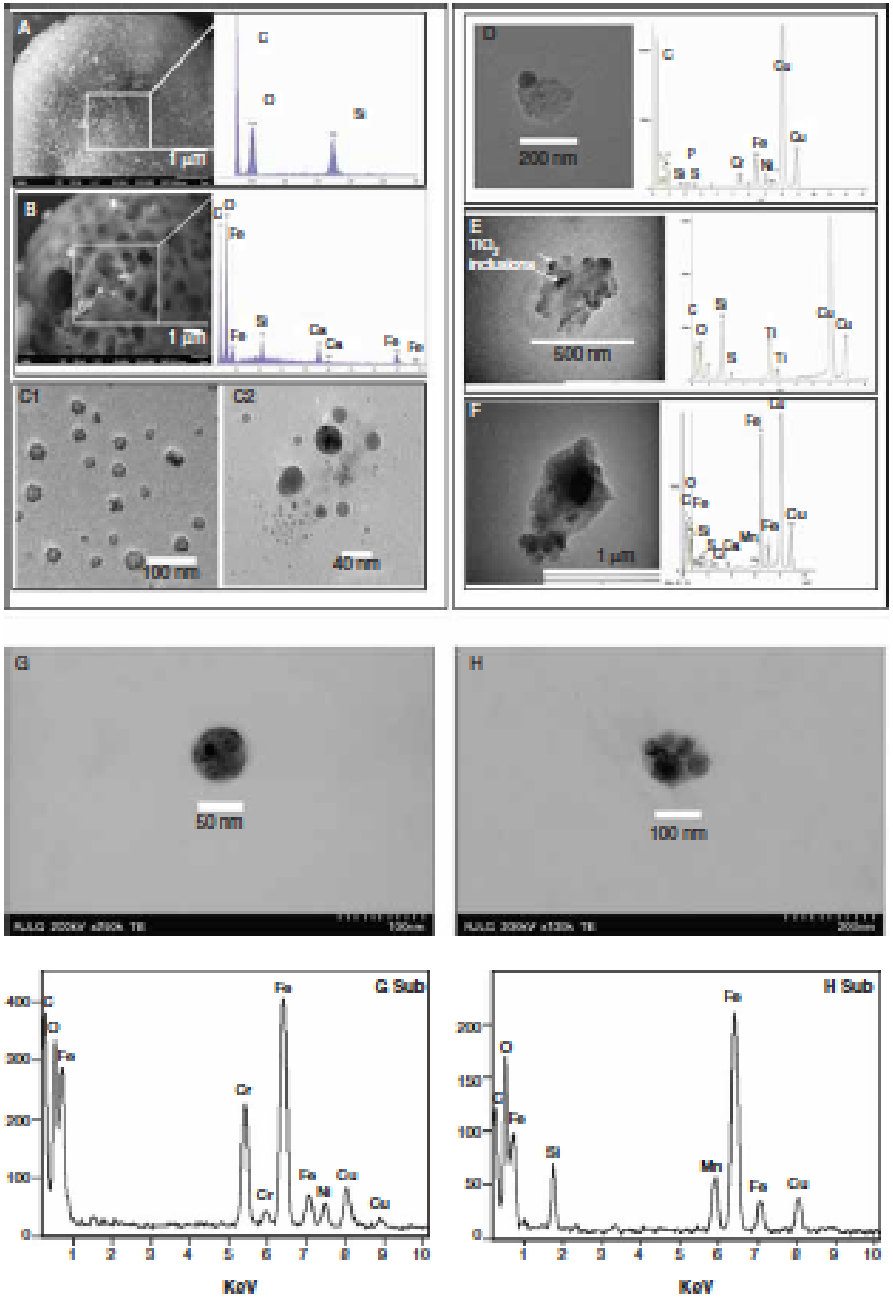


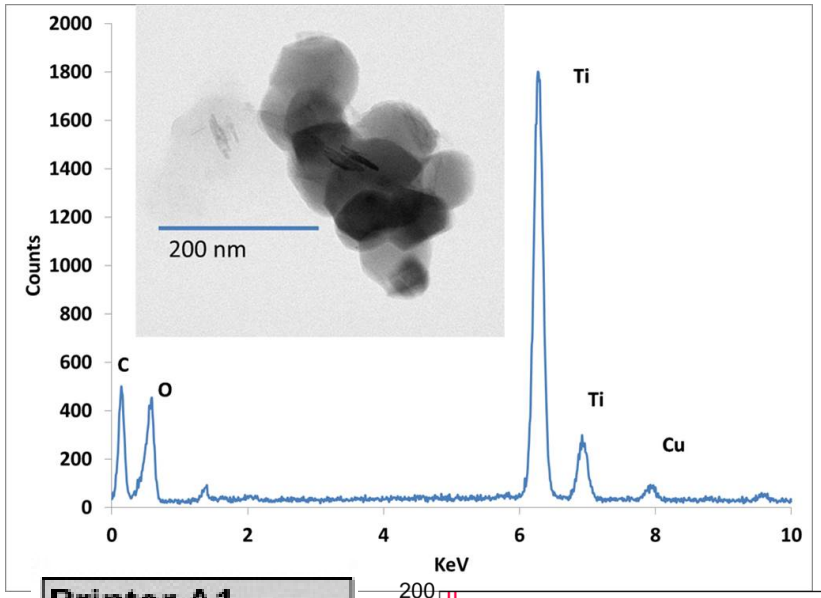


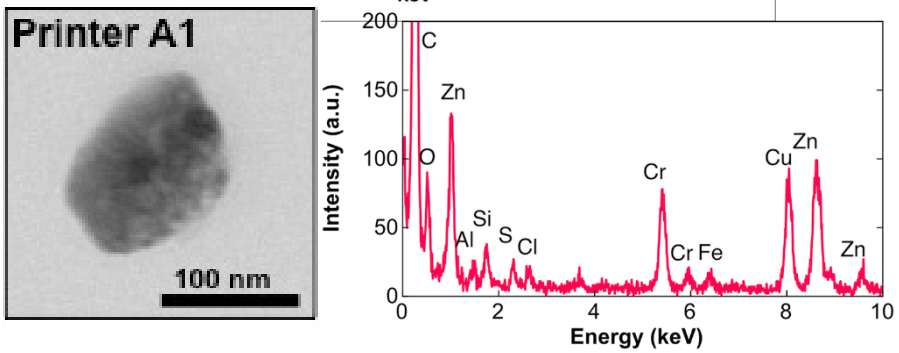
- Emission profiles of 11 laser printers (4 manufacturers)
  - No association between emission profile and brand/model
  - Peak emissions: 2,990 - 1.27 million particles/cm^3
  - Initial burst within 10-12 min
  - Mean diameters: 39 - 122 nm, majority < 100 nm
  - Mass concentrations of up to 100 µg/m^3
- Emission profiles identified for printers → rank them based on maximum particle released



ENM in the breathing zone



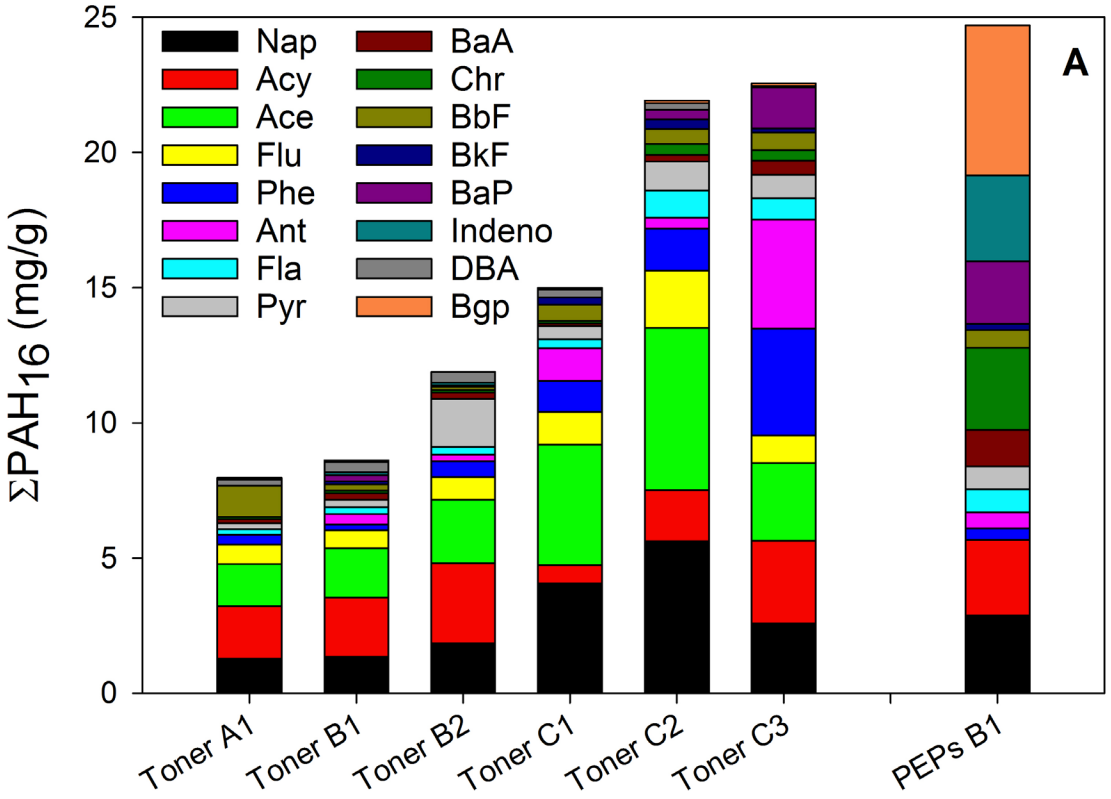




Fe, Mn, Cu, Si, Cr, Ti, Al, C, Zn, Fe, Ce, Te, S, Ni, and others

Pollen et al Nanotoxicology 2013; Martin et la 2015, J Hazardous Materials; Pirela et al 2014/2015

Chemical speciation of PAHs in toner powder and PEPs



PEPs (B1)

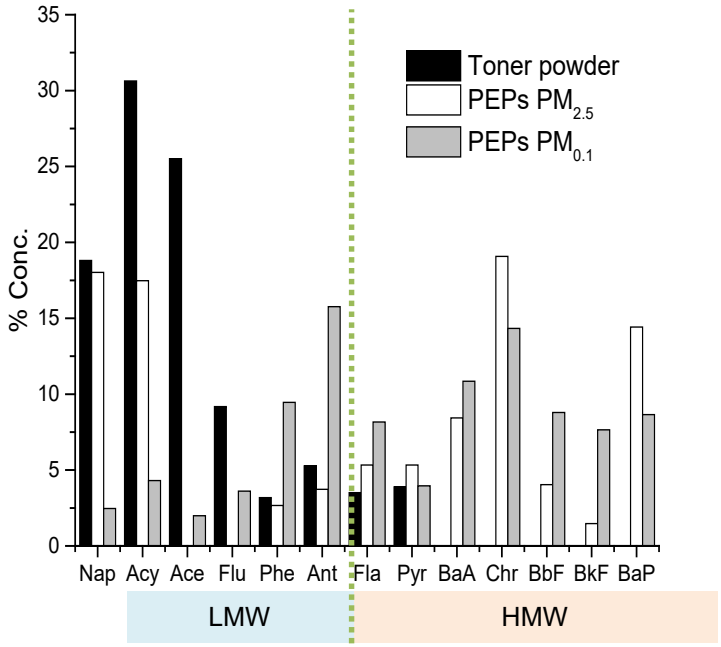
- Concentration of PAHs: 24.71 ng/mg
- Major contribution to the PAHS were high molecular weights

Toner powders (A1, B1, B2, C1, C2, C3)

- Concentration of PAHs: 22.5, 21.93, 14.99, 11.88, 8.62 and 7.97 ng/mg.
- Relatively high fraction of low molecular weight PAH compounds that made up 73-85% of the sample
- 1.86 fold increase of PAHs concentration in PEPs compared to toner (B1)

Chemical speciation of PAHs in toner powder and PEPs

Relative distribution of PAHs

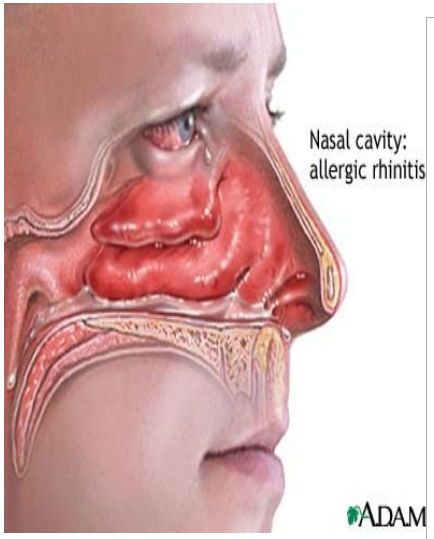


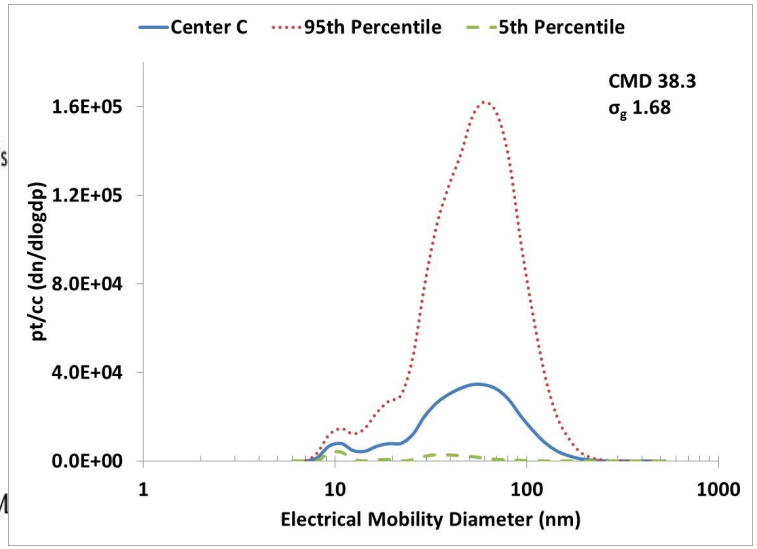
- Relative distribution of PAHs changes from low to high molecular weight PAHs from toner to high molecular weight in PEPs
- PEPs $PM_{0.1}$ appears to have a higher concentration of high molecular weight PAHs than PEPs $PM_{2.5}$
- Higher PEF associated with high molecular weight PAHs found mainly in the PEPs rather than the toner → toxicological implications?

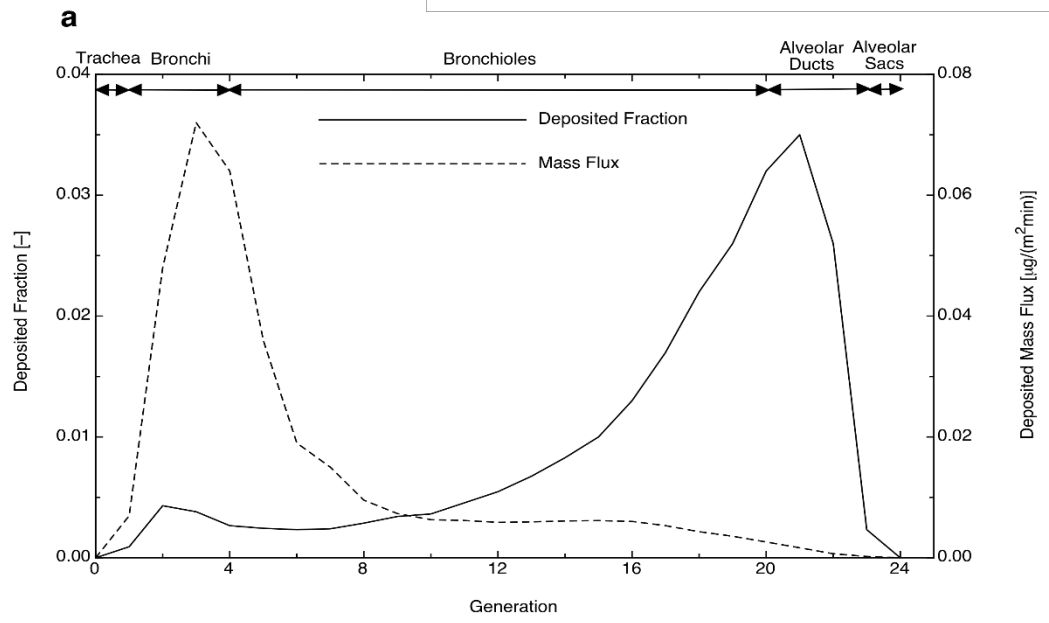
Mean PAHs and BaP-equivalent concentrations estimated using cancer potency-equivalent factor (PEF)

	Compound	PEF	Concentration (ng/g) Toner powder	PEPs $PM_{0.1}$	PEPs $PM_{2.5}$
LMW	Naphthalene	0.001	1.3	2.9	1.7
LMW	Acenaphthylene	0.001	2.2	2.8	2.9
LMW	Acenaphthene	0.001	1.8	0.0	1.3
LMW	Fluorene	0.001	0.7	0.0	2.4
LMW	Phenanthrene	0.001	0.2	0.4	6.3
LMW	Anthracene	0.001	0.4	0.6	10.6
LMW	Fluoranthene	0.001	0.3	0.9	5.5
LMW	Pyrene	0.001	0.3	0.9	2.6
HMW	Benzo[a]anthracene	0.1	0.0	1.3	7.3
HMW	Chrysene	0.01	0.0	3.0	9.6
HMW	Benzo[b/j]Fluoranthene	0.1	0.0	0.6	5.9
HMW	Benzo[k]Fluoranthene	0.1	0.0	0.2	5.1
HMW	Benzo[a]pyrene	1	0.0	2.3	5.8
	Total PAHs conc.		7.2	16.0	67.0
	Total PEF-equivalent conc.		0.0	2.6	7.8
	% PEF-equivalent/total conc.		0%	16%	12%

High Dose and Dose Rate in the Nasal Cavities







Mass Flux 0.072 μg/(m^{2}min)
Exposure time of 480 min (8 hr)

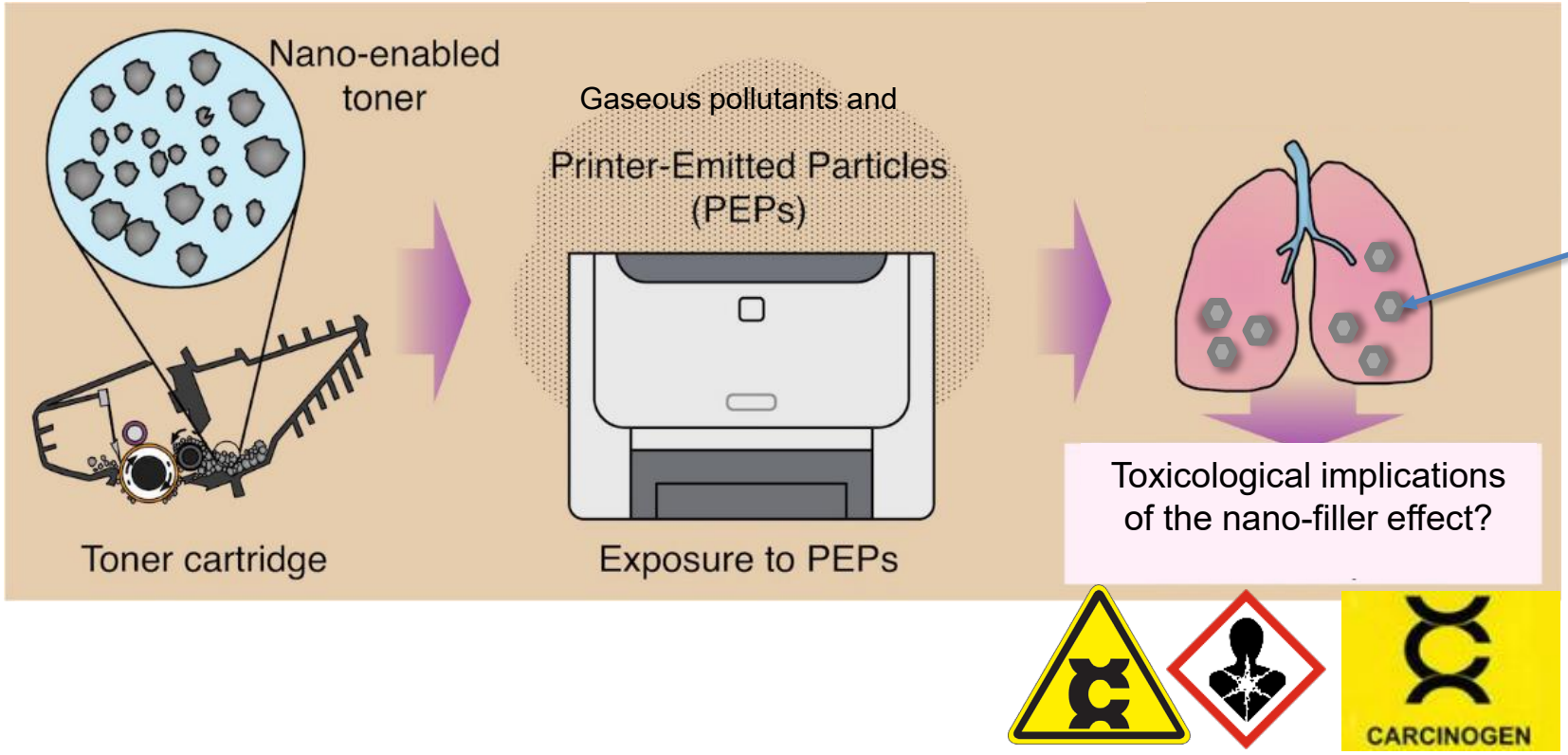
Estimated lung surface dose of 34.6 μg/m^2

Nasal Cavity: 150 cm^2
Deep Lungs: 120 m^2

Lungs/Nasal SA Ratio = ~8000
Deposited Fraction ~5x

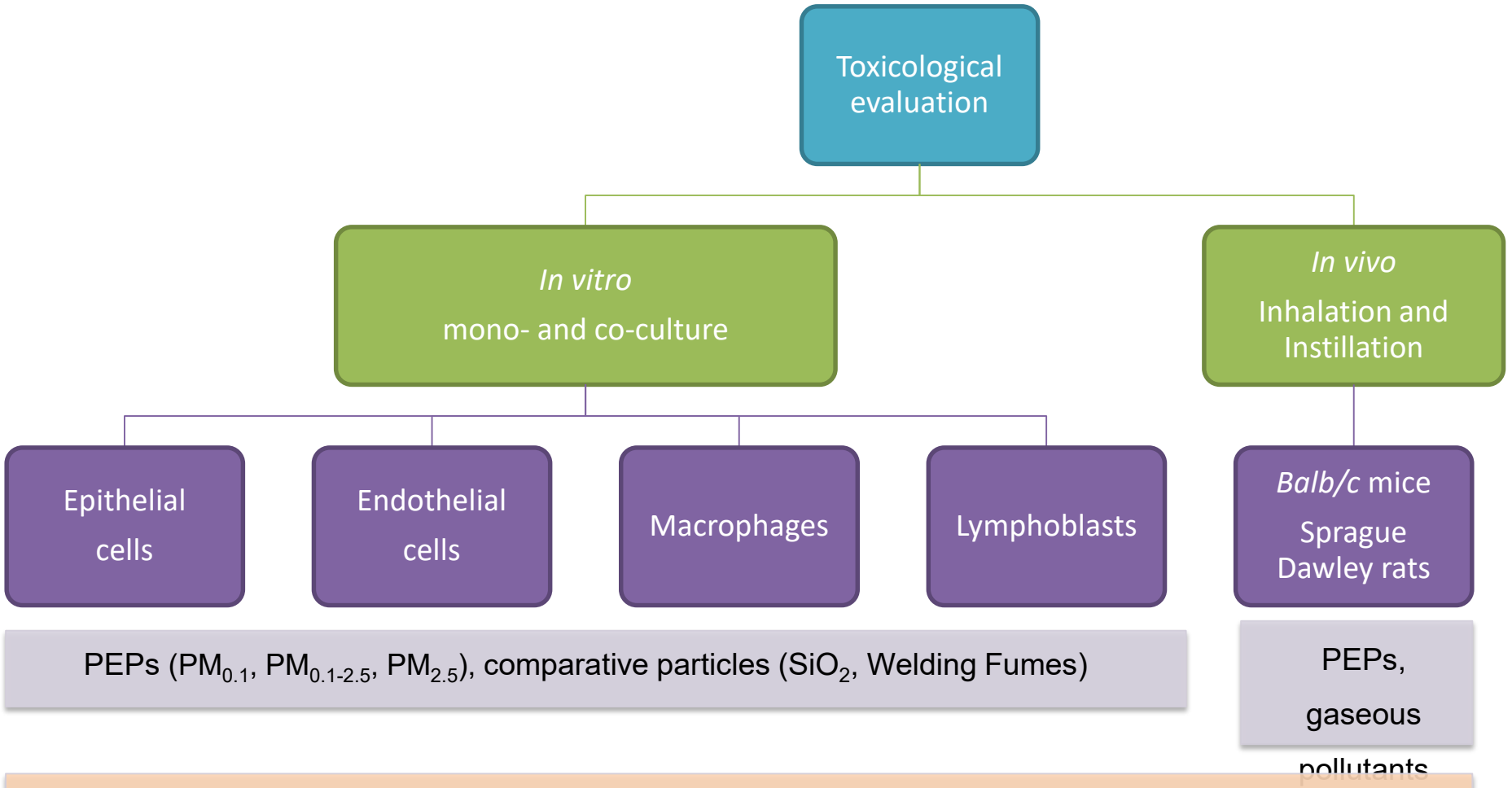
Nose/Alveolar Dose (cm^{-2})
~ 2,500x

Chemical speciation of tVOCs present in toners and PEPs



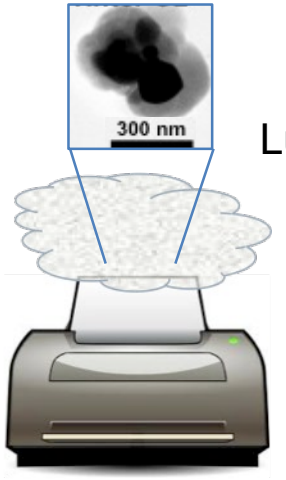
Chalbot et al., Environmental Science: Nano, 2017

Toxicological assessment of PEPs – Study design



[1] Pirela et al., EHP 2015 | [2] Lu et al., Nanotoxicology, 2015 | [3] Sisler et al., Nanotoxicology, 2014

Dosimetric considerations for toxicological assessment

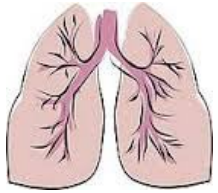


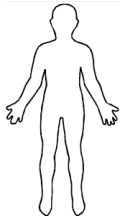
Source of emissions

Lung deposition model [1]

Estimating lung deposited mass of inhaled particles

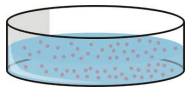
Deposited mass In the lung





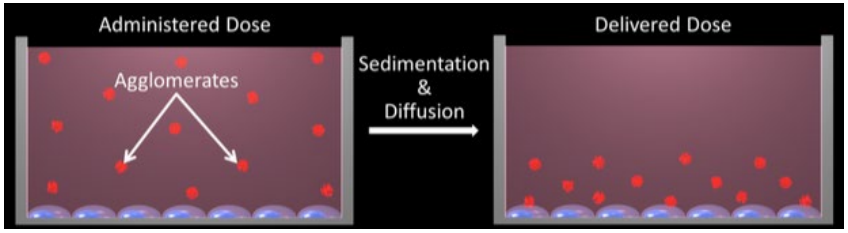
Breathing parameters + Airborne PEPs properties

Deposited mass *in vitro*



Media + Cell line + Particle

Estimating *In vitro* Administered dose using the Harvard In vitro dosimetric platform [2-4]



[1] Angilvel, 1995 | [2] Demokritou et al., 2013 | [3] Cohen et al., 2014 | [4] DeLoid et al., 2014

Summary of results from *in vitro* toxicological assessment

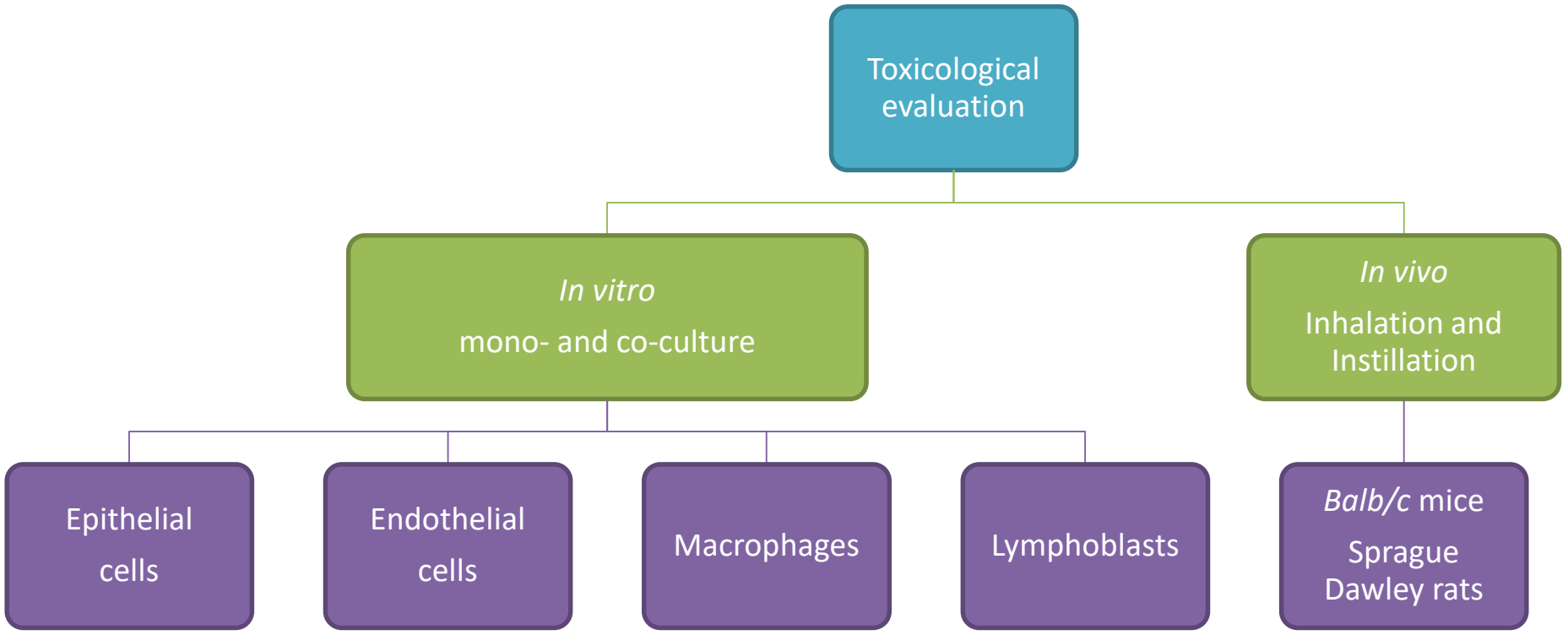
Mono-culture system

- Significant cell death in epithelial cells (at highest delivered mass) and in macrophages in a dose-dependent pattern
- Dose dependent increase in ROS production in epithelial cells and in macrophages
- PEPs affect cytokines associated with cell division and immune responses
  - Recruitment of leukocytes to injury site, immune response stimulation, neutrophil production
- Decreased expression levels of in DNA methyltransferases (DNMTs) and TET in a dose-response pattern
  - Possible change in methylation patterns affecting overall gene expression

Co-culture system

- Co-culture system allows for investigation of alveolar-capillary interaction
- Following epithelial cell treatment with PEPs, endothelial cells exhibited:
  - Increased reactive oxygen species
  - Actin filament remodeling (stress fibers, filopodia, lamellipodia)
  - Angiogenesis
  - Substantial gap formation
  - Elevated cytokines levels: IL-1β, IL-8, IP-10, FGF-basic, IL-1RA, IL-6, MCP-1, MIP-1b, RANTES

Toxicological assessment of PEPs – Study design



Cell viability, ROS, Gap junctions, Epithelial-Endothelial interactions, Epigenetics, Lung injury, Inflammation, Cardiovascular

[1] Pirela et al., EHP 2015 | [2] Lu et al., Nanotoxicology, 2015 | [3] Sisler et al., Nanotoxicology, 2014

In vivo toxicological assessment: Instillation exposure

Experimental Design

- Animals: male Balb/c mice
- Exposure by intratracheal instillation
  - $PM_{0.1}$ (sampled/extracted from CCI)
  - Control group: DI H_2O
- Doses: 0.5, 2.5 and 5.0 mg/kg bw
- Assessment done 24-hrs post exposure
- Samples collected: blood, heart, liver, spleen, lungs, bronchoalveolar lavage
- Parameters examined: lung injury and inflammation, epigenetics, oxidative damage

Summary of results from *in vivo* toxicological assessment

Intratracheal instillation

- No effect observed on pulmonary membrane integrity and neutrophil degranulation.
- Significant differences in white blood cell population (neutrophils, macrophages and lymphocytes) after PEPs exposure (5 mg/kg).
- Expression of a number of genes (*Nos1*, *Ccl5* and *Ucp2*) involved in inflammatory and oxidative damage responses was elevated after PEPs exposure.
- Leukemia inhibitory factor (LIF) was considerably upregulated by exposure to PEPs.
- Significant loss of DNA methyltransferase Dnmt3a and an elevated expression of TE LINE-1 observed in the whole lung tissue of mice instilled with PEPs.

In vivo toxicological assessment: Inhalation exposure (1/2)

PEPs exposure

Study repeated 2016 and 2017

9 weeks old Sprague-Dawley rats

HEPA exposure (Control)

Exposed for 5 hours a day

Exposure Days	1	5	9	13	17	21

Animals Sacrificed

Real-time exposure measurement

Multiple pathway particle dosimetry modelling

Multivariate statistical analysis

Nasal lavage fluid collection

- LDH release
- Peroxidase activity
- GSH levels
- Multiplex cytokine and chemokine analysis

Bronchoalveolar lavage fluid collection

- LDH release
- Peroxidase activity
- GSH levels
- Albumin levels
- Hemoglobin levels
- Total and differential WBC analysis
- Multiplex cytokine and chemokine analysis

Pulmonary and cardiac tissues analysis

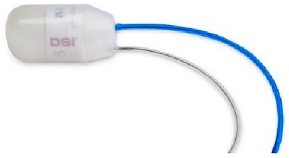
- Histopathological analysis
- In situ chemiluminescence based oxidative stress analysis

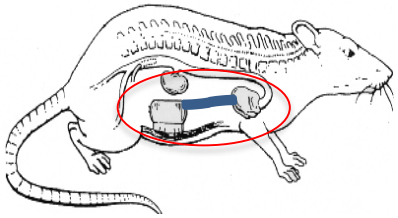
Blood serum collection

- Oxidative stress and inflammation markers.
- Metabolomics analysis.

Determination of dosimetry: NOAEL and LOAEL

In vivo toxicological assessment: Inhalation exposure (2/2)





Animal assignment:
HR and Contractility

PEPs (n= 4)
HEPA filtered air (n= 4)

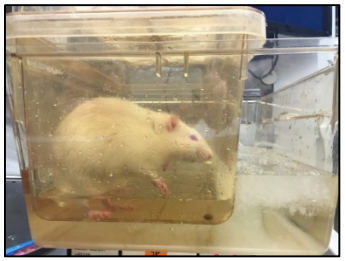
Baseline				Exposure (21d)										Post-Exposure Days (*: cold-water stress)					Sac
1	2	3	4	1	2 - 4	5	6 -8	9	10-12	13	14 - 16	17	18 - 20	21	23*	50 , 57	58*	65 , 86	91*	93

HEPA filtered air: All

5 hrs exposure to PEPs and HEPA filtered air (Control)

1h Monitoring

- 20 min pre
- 20 min Stress
- 20 min post

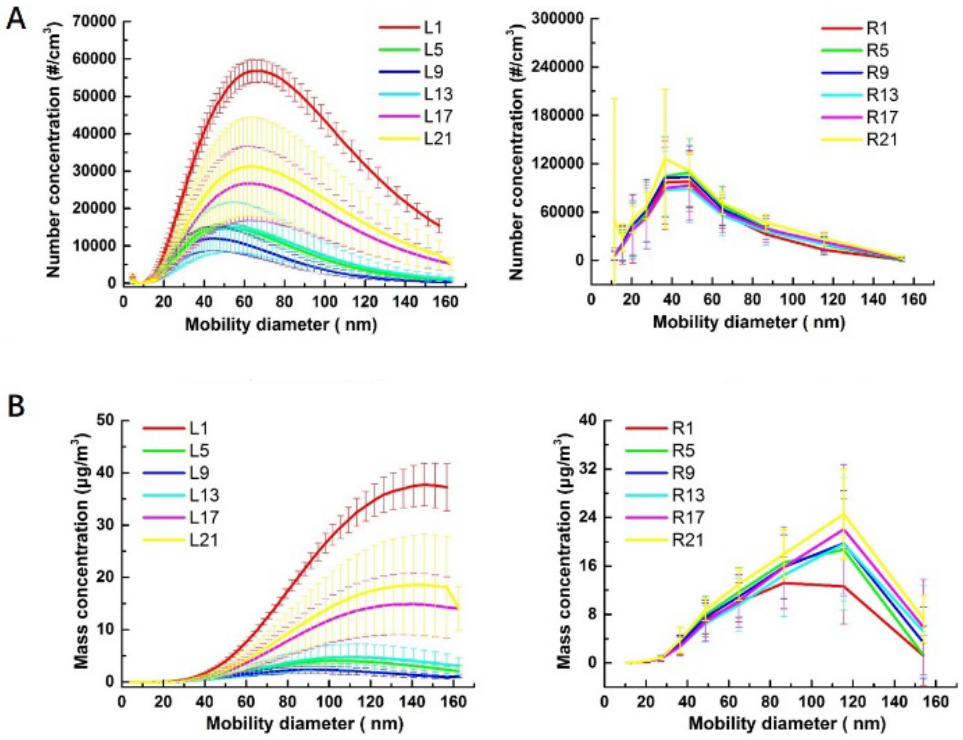


Effects of PEPs on Cardiac & Autonomic Responses to Stress

Detection of stress related metabolites in urine

Real-time exposure measurement

2016 2017



- Real-time mean particle diameter: ~45 nm
- Total particle number concentration: ~4-5 x10^5 #/cm^3
- Highest mean particle diameter: 67.62 nm
- Particle mass 737.90 μg/m^3
- VOCs 364 ppb
- Variation between exposure days was detected in the 2016 study
  - This was due to use of different printers, wear and tear.

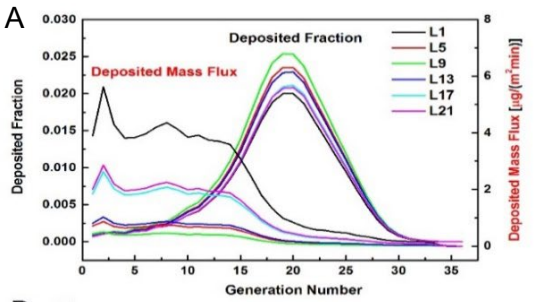
Multiple particle pathway analysis

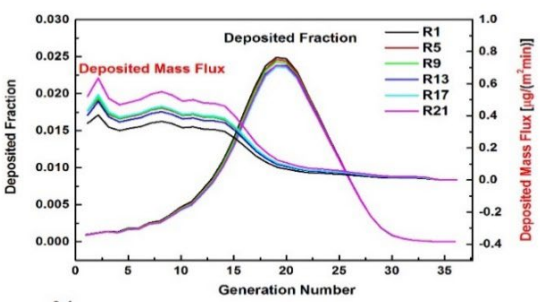
Rats

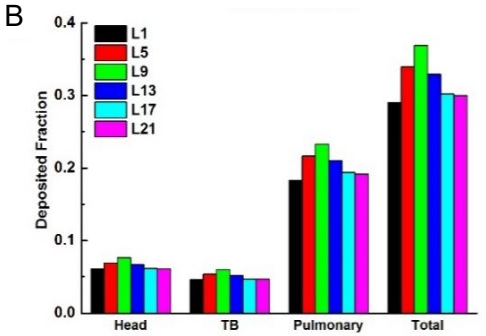
Human

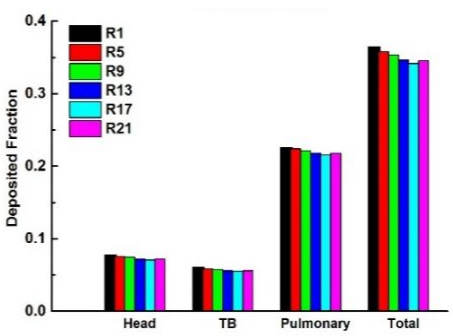
L = 2016 study

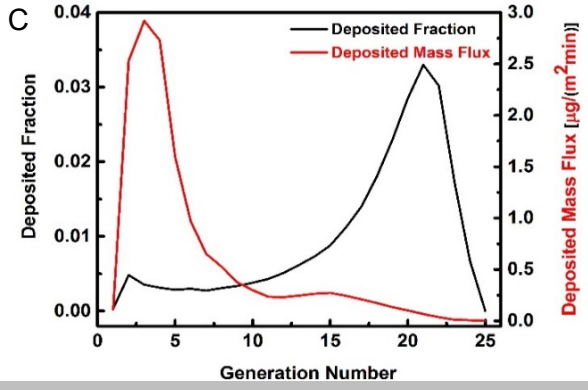
R = 2017 study

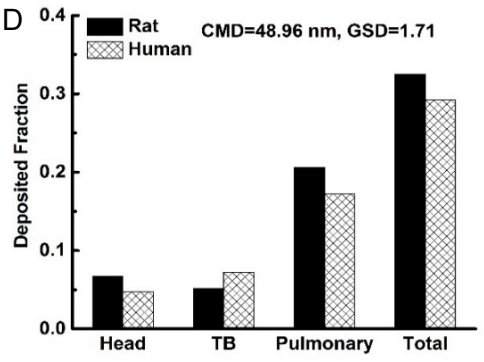












Deposition:

~ 7%: head

~6%: TB region

21%: alveolar region

Rats dose-response analysis relationship

Exposure day	Deposition rate (μg/hour)	Retained mass dose (μg/m²)	Biological outcomes for control versus PEPs exposed Sprague-Dawley rats (p value ≤0.05)
L1	2.34	1.06	----
R1	0.19	2.51	----
R5	0.24	15.27	----
R9	0.24	26.55	----
L5	0.43	28.2	BALF LDH ↑
L9	0.25	28.24	----
R13	0.23	35.86	----
R17	0.25	49.31	IL-18 ↓
R21	0.30	70	BALF Hemoglobin ↑; BALF IL-2 ↑
L13	0.50	79.31	BALF LDH ↑
L17	1.23	252.06	----
L21	1.31	322.75	----

NOAEL

LOAEL

NOAEL= No adverse effect levels LOAEL= Low adverse effects levels

0.29 m^2 alveolar surface area in rat

EPA, 1995

Summary of results from *in vivo* toxicological assessment (1/2)

Inhalation – *Work in progress*

- PEPs induced mild cytotoxicity, inflammation and oxidative stress in the respiratory region of the rats.
- Responses were in the form of modest release of pro-inflammatory cytokines and chemokines, influx of immune cells and modest increase in peroxidase activity and glutathione levels both in the NLF and BALF of the exposed animals.
- Histological and *in situ* ROS studies demonstrated no negative and pathological effects from PEPs exposure to both pulmonary and cardiac region of the exposed animals.
- Repeated PEPs exposure causes hypertension and sympathetic excitation.

Summary of results from *in vivo* toxicological assessment (2/2)

- Serum markers for oxidative stress and inflammation showed upregulation in response to PEPs exposure.
  - 8-Isoprostane and 4-HNE are well established markers of oxidative stress originating from free radical oxidation of arachidonic acid *in vivo*.
  - Leukotriene B4 (LTB4) is an important, well-established inflammatory mediator generated from activated innate immune cells such as neutrophils, macrophages, and mast cells.
- Extrapolating the obtained results to human exposure to PEPs for 8 hrs/day, 5 days/week, 3 weeks: NOAEL and LOAEL after pulmonary clearance were determined at 4.71 mg/m^2 and 7.53 mg/m^2.
- Based on the measured biological responses the PEPs concentration of 28.2 $\mu g/m^2$ was found to be the transition point from NOAEL to LOAEL.

Impact of the study

- Addressed the importance of evaluating life-cycle implications of NEPs.
- Assessing real world exposures and their associated toxicological properties rather than focusing on “raw” materials used in NEP synthesis.
- Multidisciplinary approach and methodology to investigate toxicological implications of consumer exposures to released PM from NEPs.

Major Knowledge Gaps

- Estimates of the disease burden in workers and consumers are lacking.
  - Respiratory, cardiovascular, immune system, genotoxicity
  - Carcinogenicity, neurological and reproductive toxicity
- Exposure-dose-effect relationships are needed for every endpoint.
- Exposure biomarkers for routine exposure monitoring purposes are currently lacking.
- Exact molecular mechanisms not fully elucidated.

Thank you for your attention!
Questions?

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In vitro doses of PEPs and corresponding consumer inhalation exposure duration

Table 2. *In vitro* doses of PEPs and the corresponding consumer inhalation exposure duration.

	SAEC		THP-1	
Administered dose (cells)[a] (µg/mL)	Delivered dose (cells)[a] (µg/mL)	Corresponding consumer inhalation exposure duration to PEPs (hr)[b]	Delivered dose (cells)[a] (µg/mL)	Corresponding consumer inhalation exposure duration to PEPs (hr)[b]
0.5	0.5	15.0	0.26	7.8
5	5	75.2	2.6	39.0
10	10	150.4	5.2	77.9
20	20	300.7	10.4	155.8
30	30	451.1	15.6	233.7
40	40	601.4	20.8	311.5
100	100	1503.6	52.0	778.9

[a]*In vitro*–administered and delivered doses were based on a 24-hr *in vitro* exposure. [b]Calculations of the corresponding consumer inhalation exposure duration (hours) were based on the added values of deposition mass flux (µg/m^2 • min) in the various human airways, excluding head airways: the conducting zone (generations 0 to 16) and the transitional and respiratory zones (generations 17 through 23).

In vivo doses of PEPs and corresponding consumer inhalation exposure duration

Table 2
Comparison of doses of murine PEP exposures used in the study by intratracheal instillation with comparable human inhalation exposures to PEPs.

PEP exposure by intratracheal instillation (mg/kg bw)	Duration of consumer inhalation exposure of PEPs (h)
0.5	13.7
2.5	70.9
5.0	141.9