Wood Combustion Nanoparticle Morphological/Elemental Characterization, and TEM Visualization of *in vitro* Nanoparticle *A*ttachment, Entry and Fate, within Human Lung Bronchial Epithelial Cells

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Cordwood-fired boilers used to collect combustion emission nanoparticles (Fuel: prepared Red Oak cordwood)

Indoor-- 2 advanced technology residential indoor cordwood-fired boilers,

Outdoor -- 1 conventional outdoor cordwood-fired boiler, and

-- 1 conventional advanced technology outdoor cordwood-fired boiler

Fuel: Red Oak cordwood

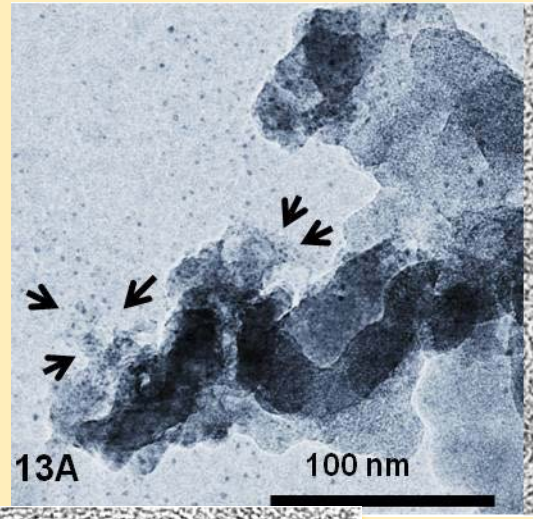
Combustion Emission Nanoparticles were collected from the dilution tunnel gas stream during steady state, late steady state, burnout, and shut-down

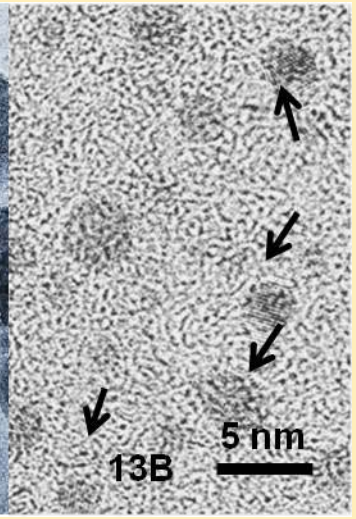
Characterization of Combustion Emission Nanoparticles from each boiler:

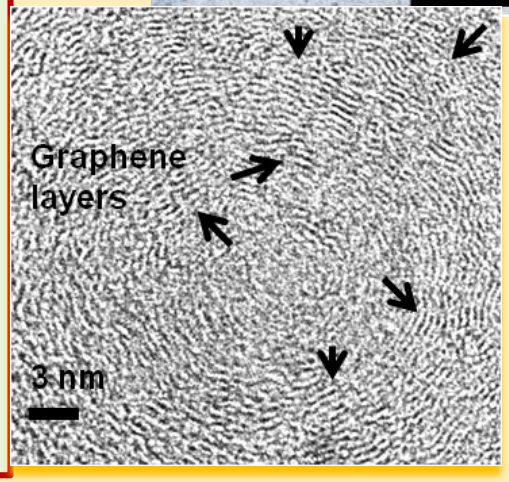
High Resolution Transmission Electron Microscopy (HRTEM) for determining combustion emission morphology.

X-ray microanalysis and Selected Area Electron Diffraction (SAED) to determine elemental composition and crystallinity, and verify graphene.

Toxicity Testing collected nanoparticles: carbon nanoparticles- and nano-salt crystalline nanosphere- interactions with human lung epithelial monolayers *in vitro (*related to dose/exposure time)



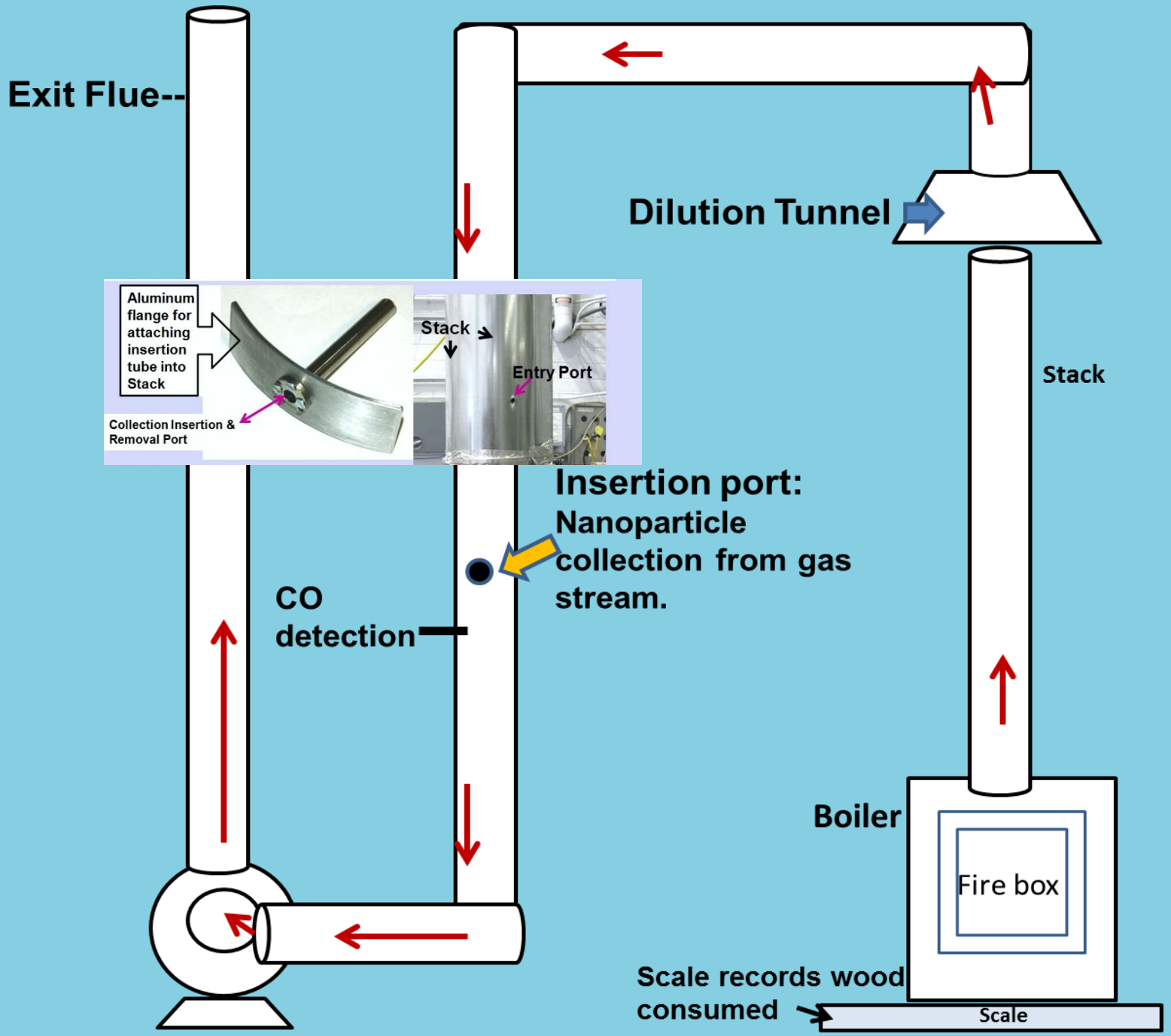




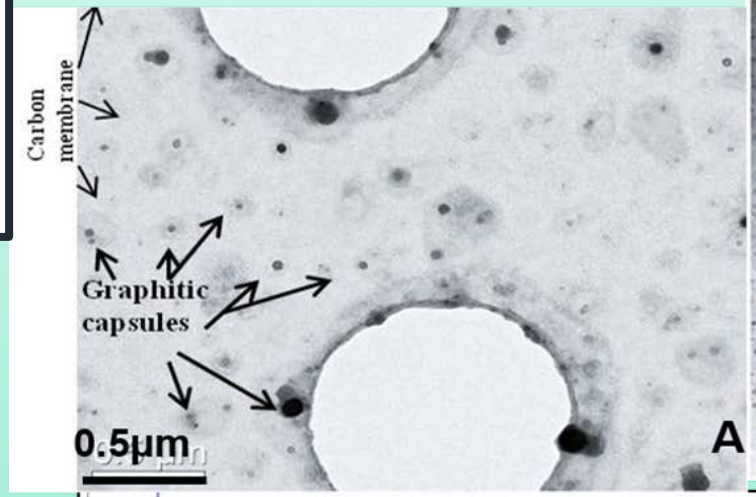
Brookhaven National Laboratory Test Facility-

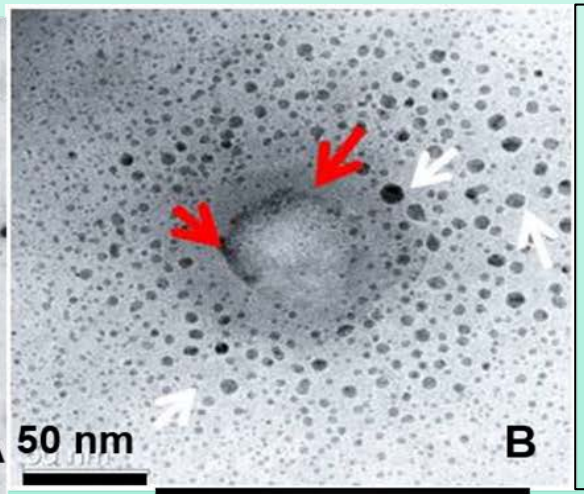
to measure and record combustion parameters of different boilers/wood stoves.

Here we collected wood combustion emission nanoparticles from the Dilution Tunnel gas stream (yellow arrow), during each stage in the burn cycle.



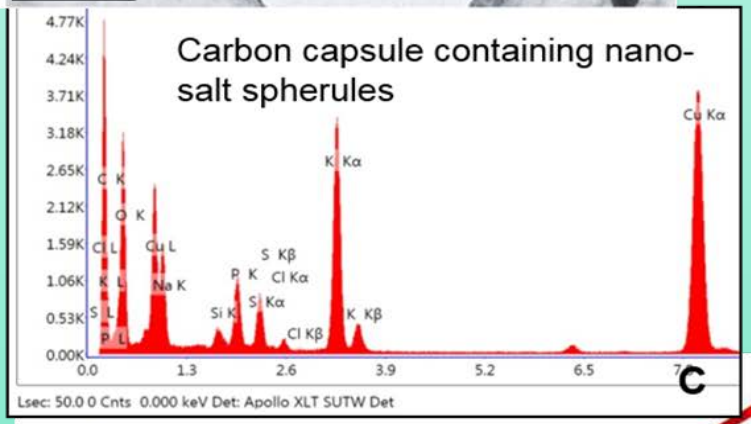
Advanced Technology indoor Cordwood Boiler, # 1, Red Oak fuel



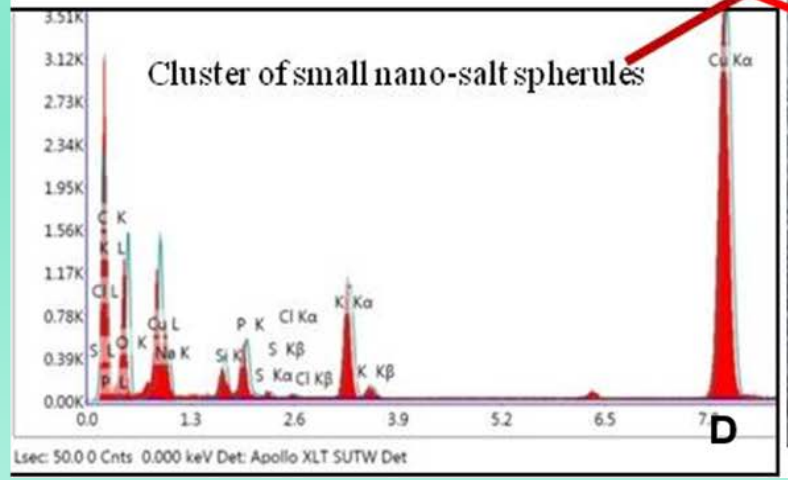


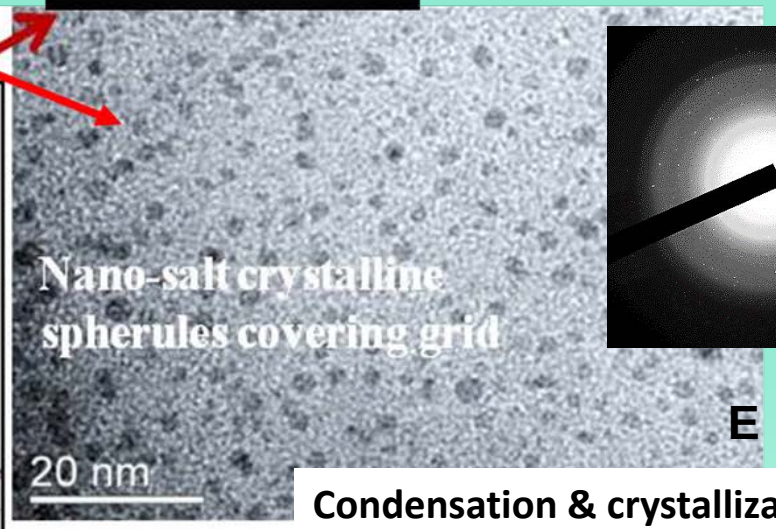
High magnification: carbon ghosts (red arrows) were present, surrounded by clusters of condensing nano-salt spherules (composed of C, K, O, Na, S,P and Cl).

Boiler: Two stage hydronic heater; best characteristics of traditional and downdraft boilers. Computer driven O_2 to combustion chamber.



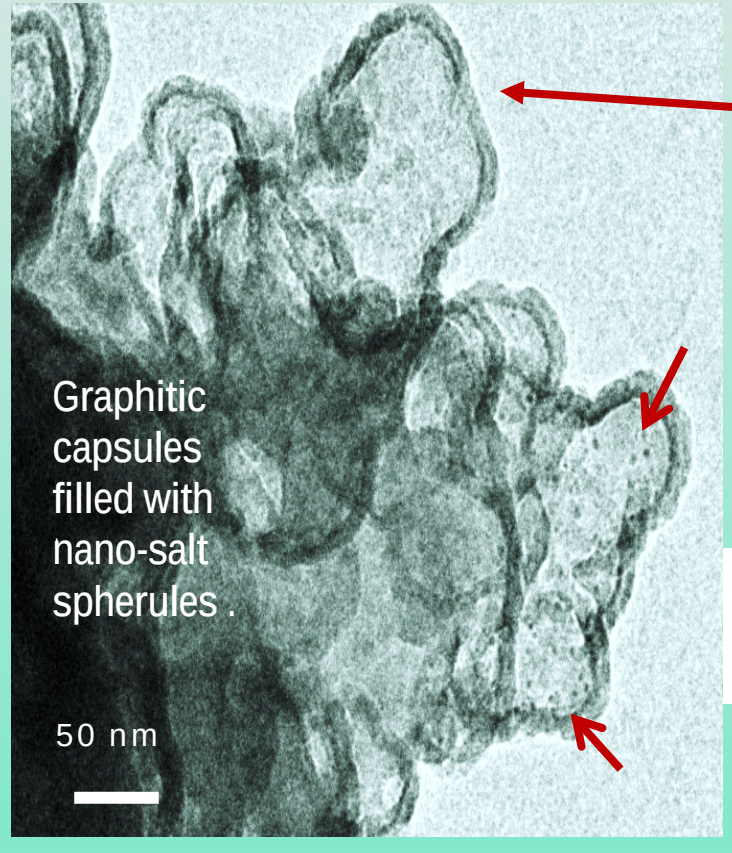
SAED of nano-salt crystalline spherules revealed soft carbon rings and salt crystals.

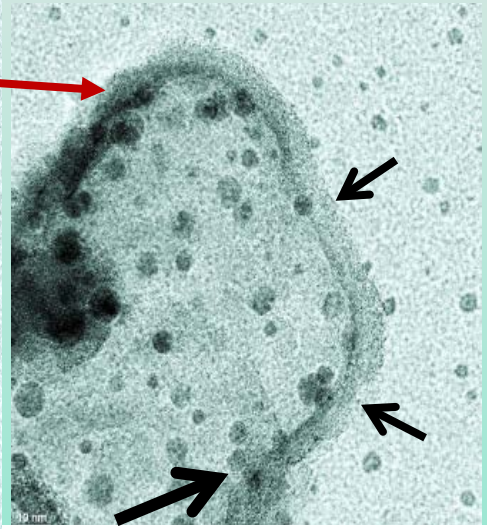




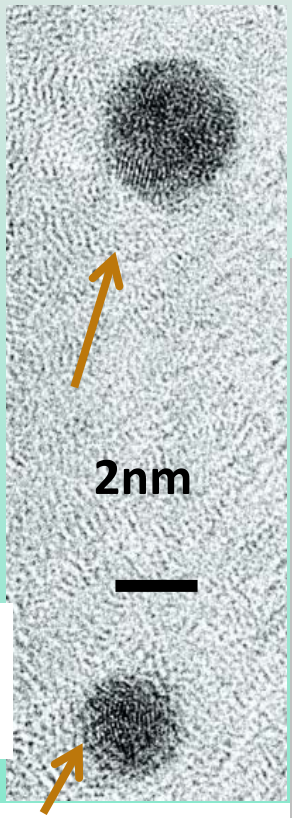
Condensation & crystallization

Wood Combustion Nanoparticles Collected in the gas stream of an Advanced Technology Indoor Wood Burning Boiler # 2 (fuel: red oak)



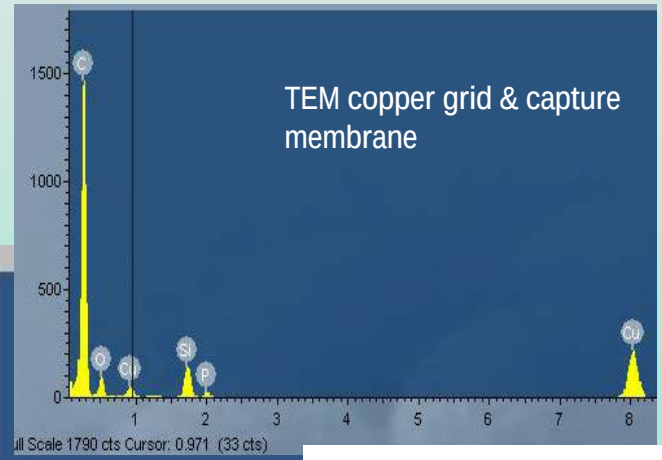


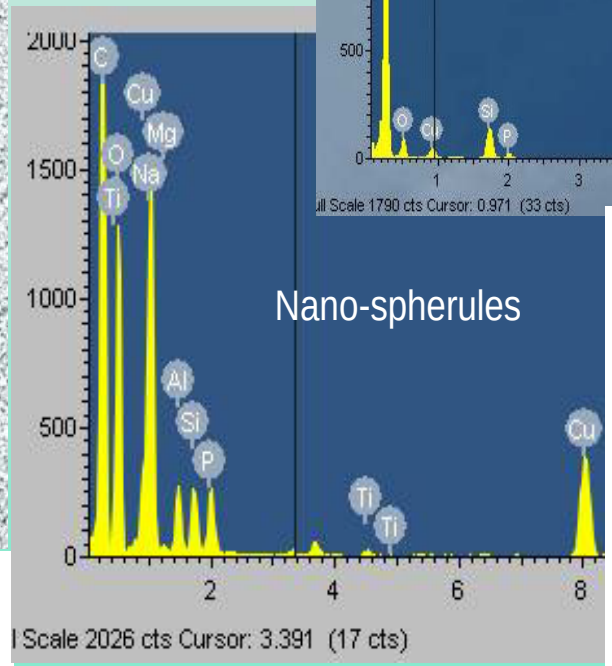
Release of nano-salt spherules (black arrows), from graphitic capsule.



Crystalline nano-salt-spherules (1.2-3.5 nm diameter).

Nano-Spherule Elemental Analysis

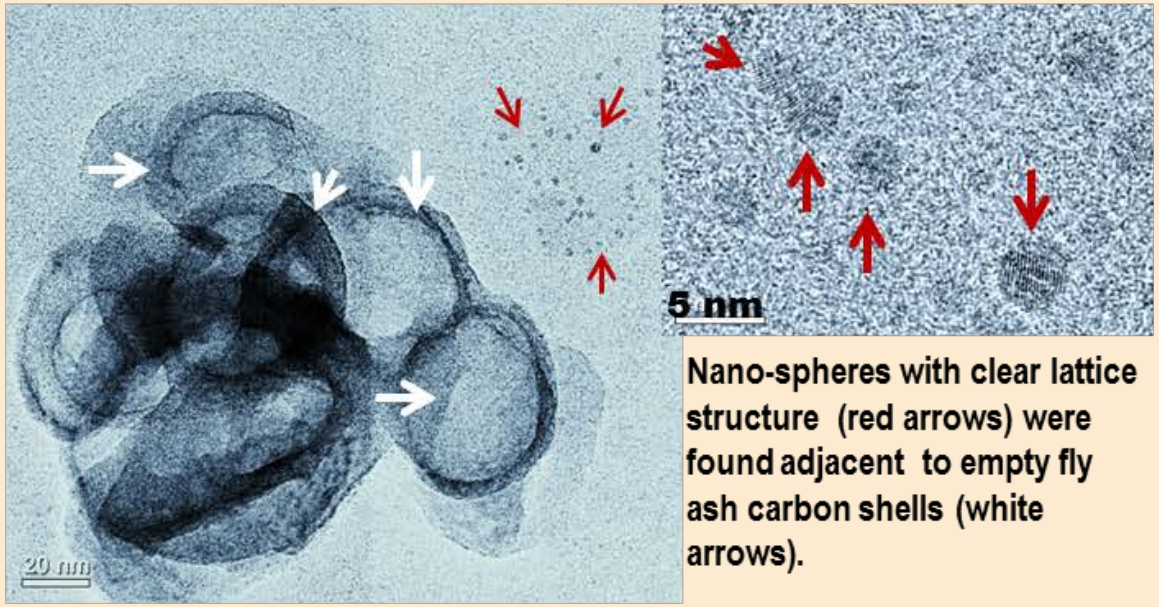




The combustion nano-spherules showed high amounts of C, O, Mg, Na, Al, P, and detectable Si and Ti. The control background analysis did not show any Al, Ti, Mg or Na, and minimal P and O.

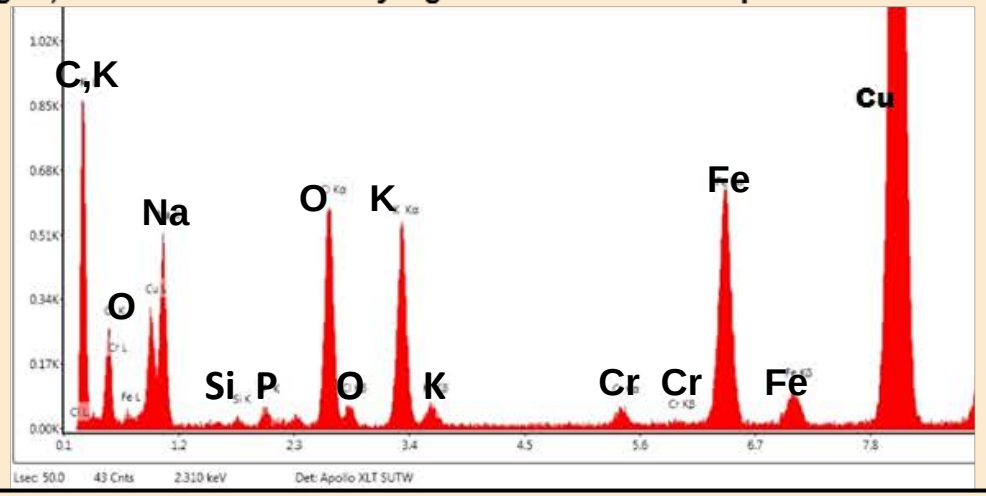
Boiler: Upper gasification section with lower secondary combustion zone; Hydronic heater; oxygen sensor and modern control system; air dampers to maintain target flu gas O_2 content.

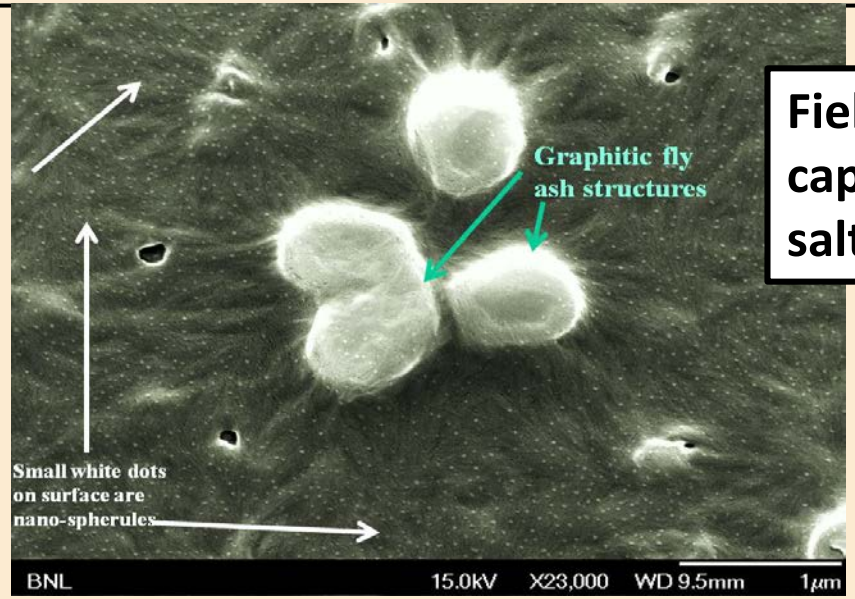
Combustion Emission Fly Ash Nanoparticulates Collected from Advanced Technology Outdoor Wood Burning Boiler (fuel: red oak)



Nano-spheres with clear lattice structure (red arrows) were found adjacent to empty fly ash carbon shells (white arrows).

Compositional analysis of fly ash nano-spheres revealed primarily K, Cl, Na, O and trace amounts of Si and P. The Cu x-ray events were from the copper grid, and the Fe and Cr x-ray signal was from the sample holder.



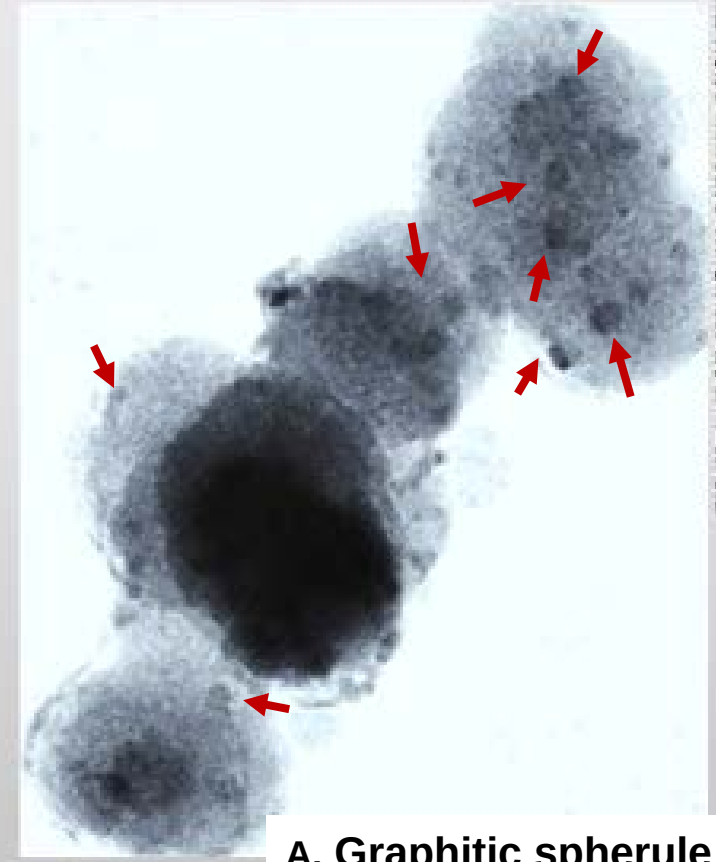


Field emission SEM image: graphitic capsules surrounded by released nano-salt spherules (small white dots).

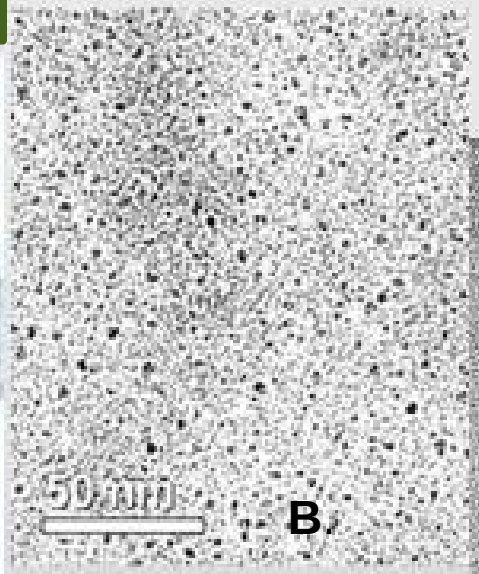
Hydronic heater, 2 stage combustion, with Clear Stak Catalyst

Conventional Outdoor Cordwood-fired Boiler (fuel: red oak)

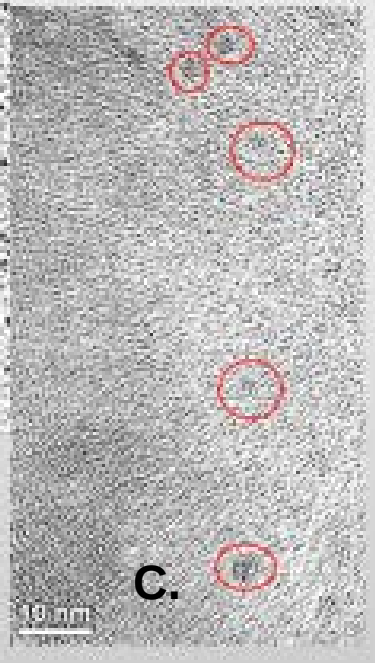
Hydronic heater; atmospheric draft; no mechanical draft fan; single stage combustion.



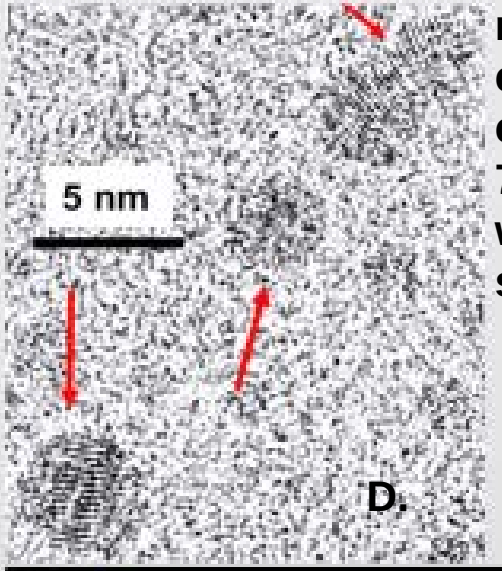
A. Graphitic spherule chain with nano-salt spherules visible (arrows) within the graphitic capsules.



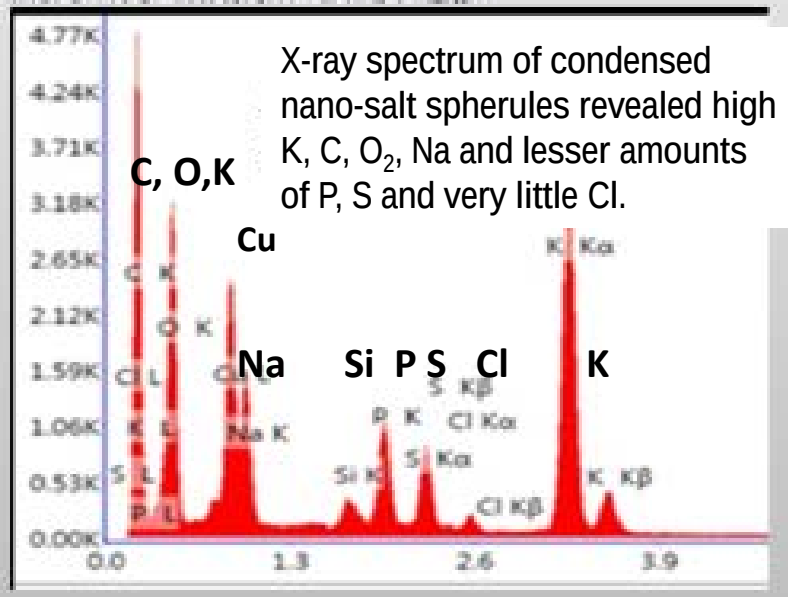
B. Accumulation of nanoparticles on collection membrane surface (mid to late steady state).



C. Some nano-salt nanoparticles in the last stages of condensation.

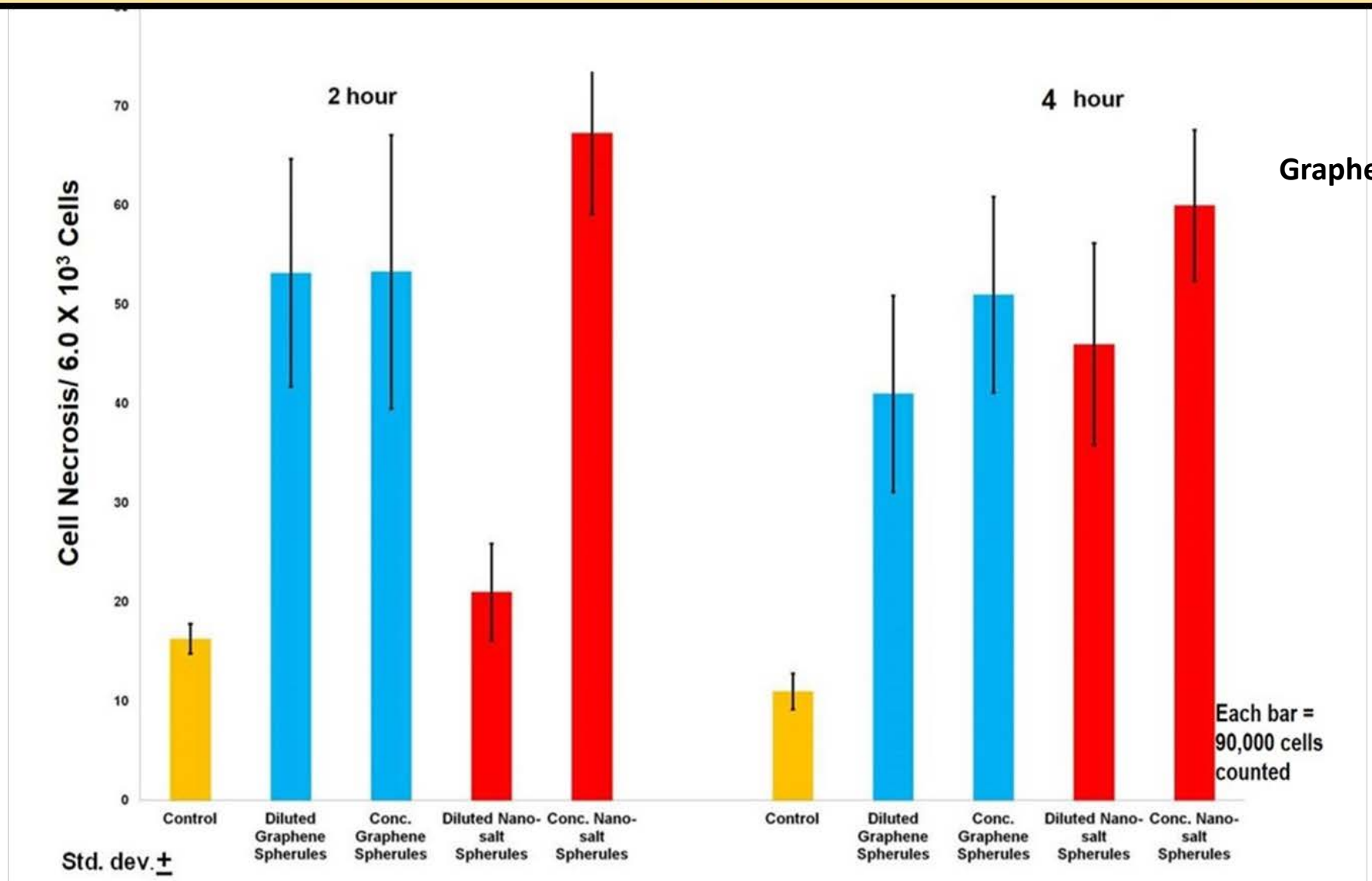


D. The smallest nano-salt spherules condensed and crystallized to 1.2 to 7.7 nm diameter, with surface lattice structure.



X-ray spectrum of condensed nano-salt spherules revealed high K, C, O_2, Na and lesser amounts of P, S and very little Cl.

Summary: Human Lung Bronchial Epithelial Cell *in vitro* Exposure to Wood Combustion Nanoparticles & Nano-Aggregates



Graphene/Graphitic chains

Nano-salt Spherules

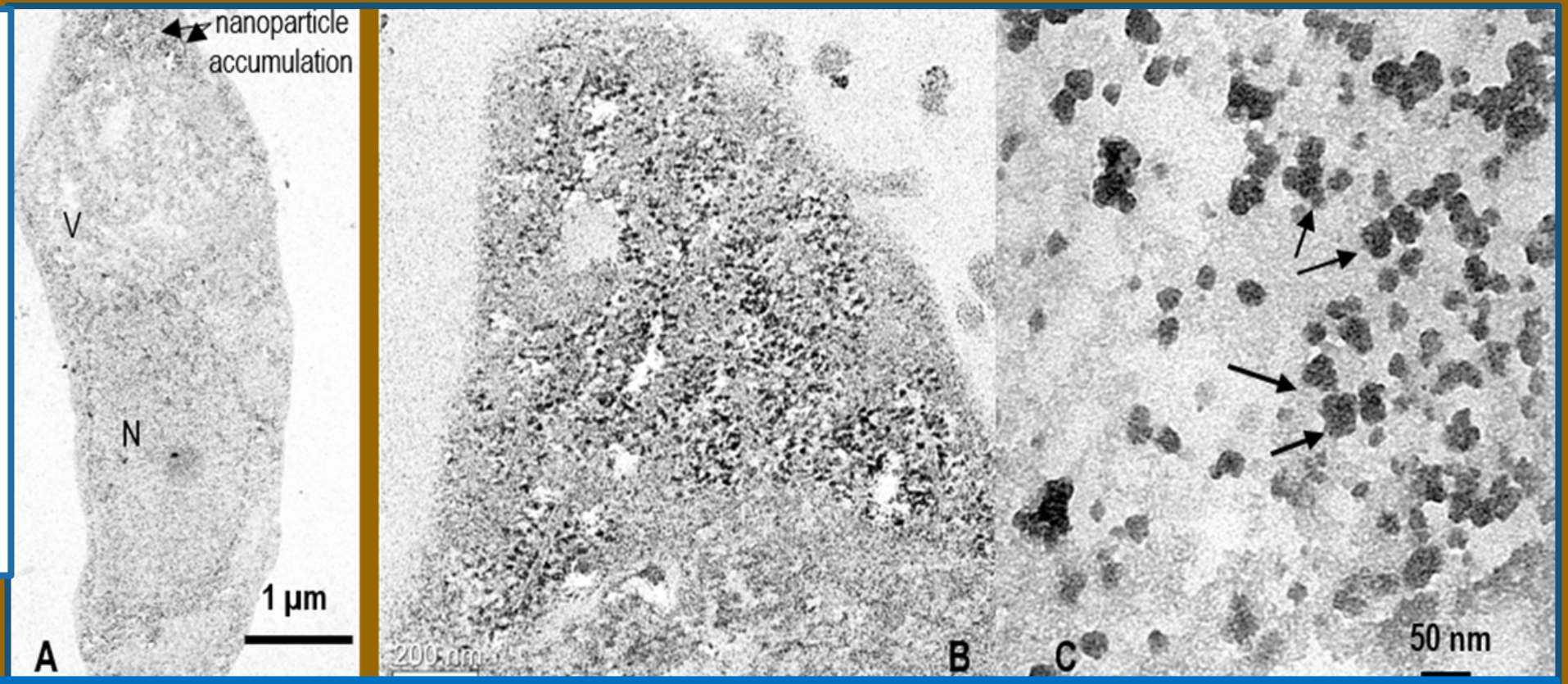
Control (PBS)

Concentrated nanoparticle preparation dispersed in PBS(2 µl added to 1.5 ml culture medium = 3.0 µg/ml high dose).
Diluted (1:5) nanoparticle preparation dispersed in PBS (2 ul added to 1.5 ml culture medium = 0.1 µg/ml low dose)
Control: Sterile phosphate buffered saline (PBS) (2 µl added to 1.5 ml culture medium) = Control
All preparations pH7.1.

NCI-292 cell monolayers grown to Confluency before testing.

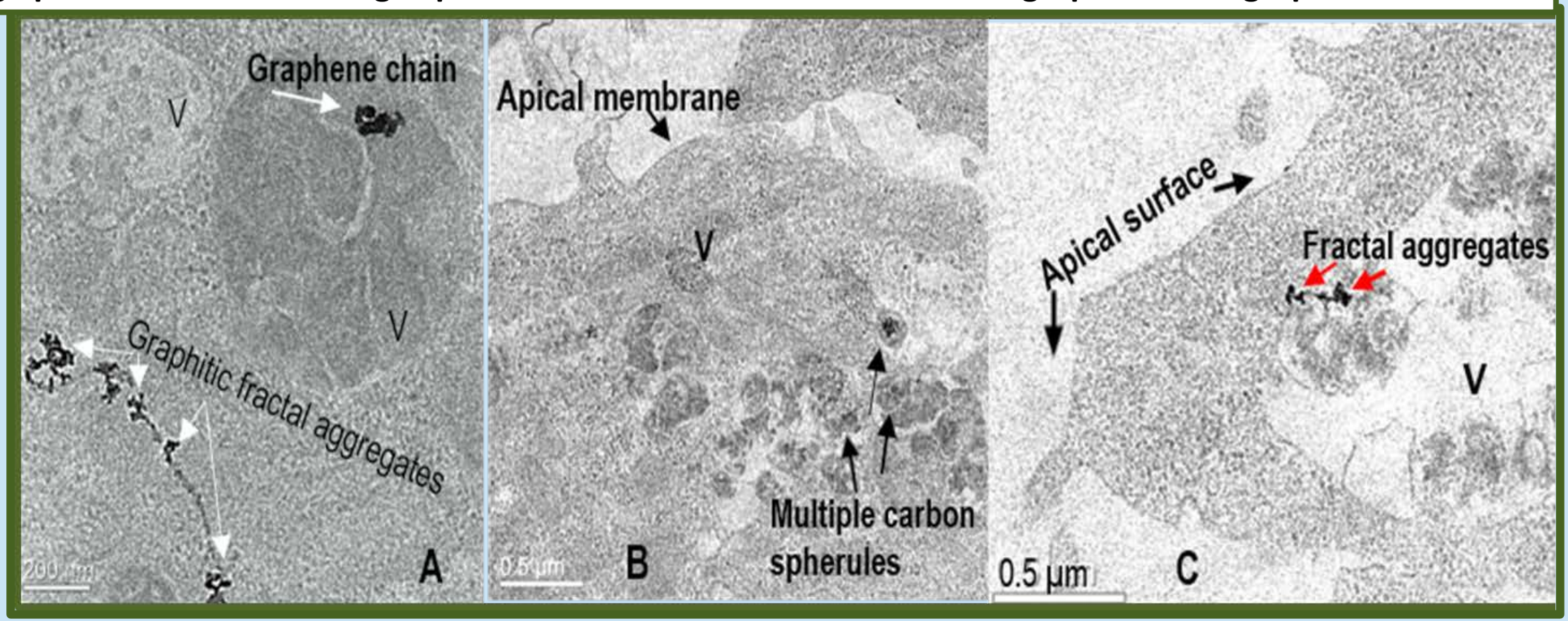
Lung Cell Exposure to nano-salt crystalline spherules 2 to 4 hours

Cells exposed to nano-salt crystalline nano-spherules showed uptake within 2 hours (Fig. A), with little cell death. By 3.5 to 4 hours incubation, nanoparticles clusters appeared in the cytoplasm, nuclei (N) and vacuoles (V).
The apical regions of the cells (Fig. B) filled with nano-spherules, and intracellular organelles were no longer present.



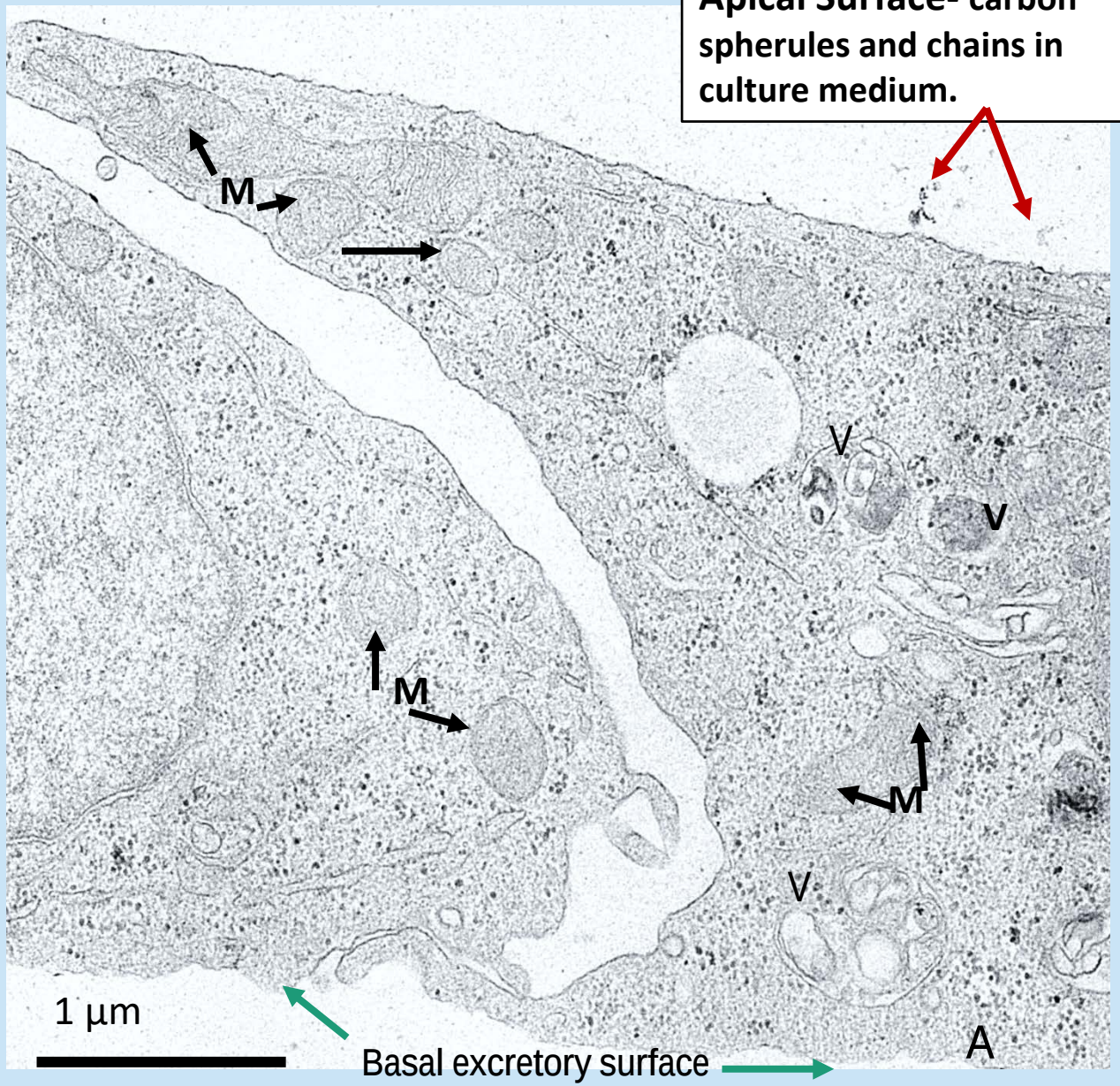
At 3.5 to 4 hours exposure (Fig.C) groups of nano-salt crystalline spheres had joined together into nanoparticle clusters of 5 to 60nm. These filled the cytoplasm, destroying the membranes of mitochondria, endoplasmic reticulum, the Golgi apparatus and digestive vacuoles. The digestive vacuoles' contents including acid and digestive enzymes was released into the cytoplasm, making the normal process of nanoparticle 'cell excretion' impossible.
By 4 hour exposure the numerous islands of joined crystalline nano-spherules filled the cell cytoplasm causing cell death due to Bio-accumulation.

Lung Epithelial Cells following exposure to wood combustion emission graphene and graphitic chains



<u>Cells exposed to wood combustion emission graphene /graphitic chains:</u> showed intact carbon graphene and graphitic chains and fractal aggregates within Cell Vacuoles (V), as well as embedded within organelles (Fig. A). Clusters of carbon spherules (Fig.B, arrows) were seen within vacuoles (V) that also contained digested amorphous carbon. Entire fractal aggregates and chains of graphitic or graphene spherules were also seen intact within cells (Fig. A and C). Sections unstained.

Apical Surface- carbon spherules and chains in culture medium.

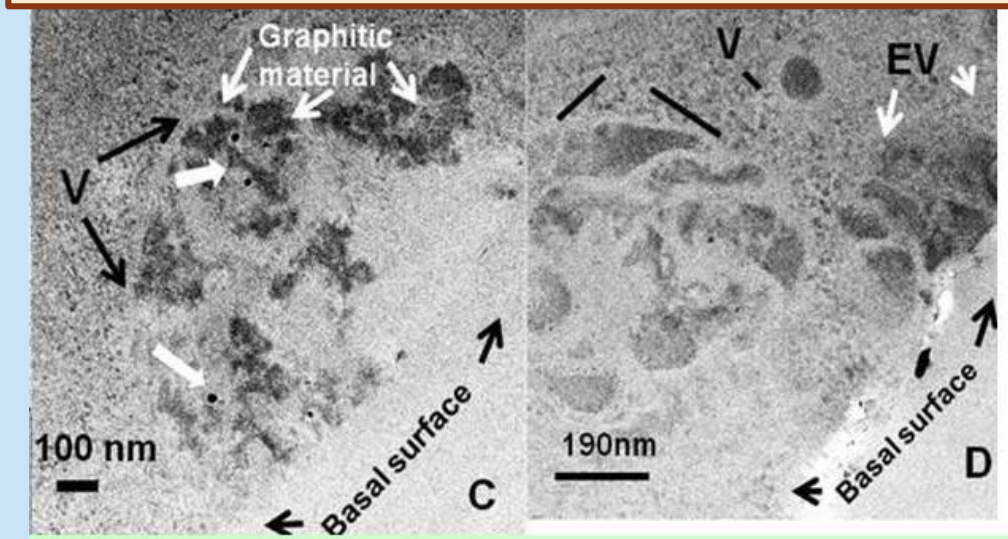


Findings

-At low doses, graphene and graphitic spherules entered the apical membrane, and passed into the cytoplasm (Fig. A)

-Loose nano-spherules and chains were seen throughout the cytoplasm and within digestive vacuoles (V), mitochondria (M), and at the basal cell surface (Fig. A).

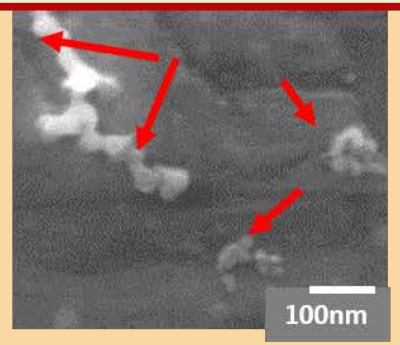
-Carbon spherules within digestive vacuoles (V) were transported to the basal cell surface, and excreted (Fig.C,D).

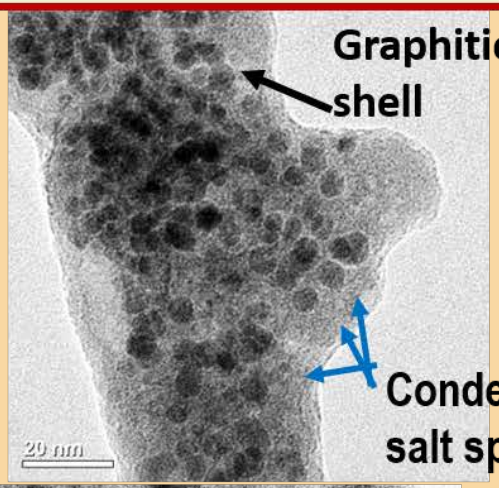


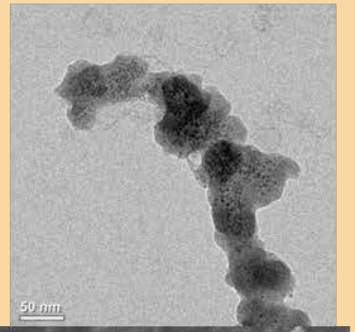
Graphene & Graphitic nanoparticles in cells

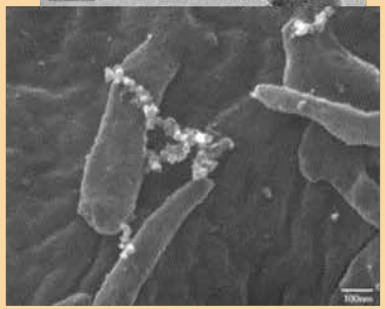
Lab-made Clean Graphene and Graphitic spherules & aggregates

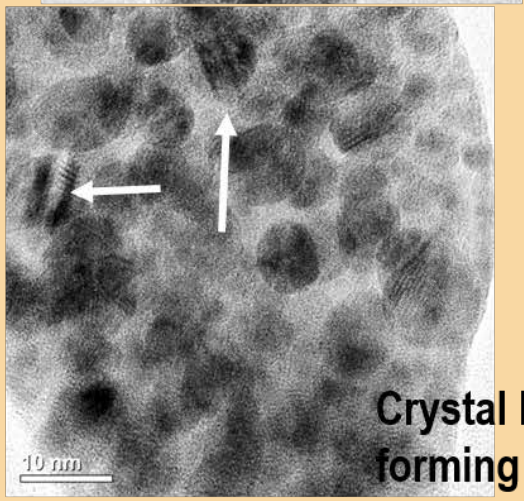
Pictures of Lab-made ultra-clean carbon spherule chains and nano-salt crystalline spherules



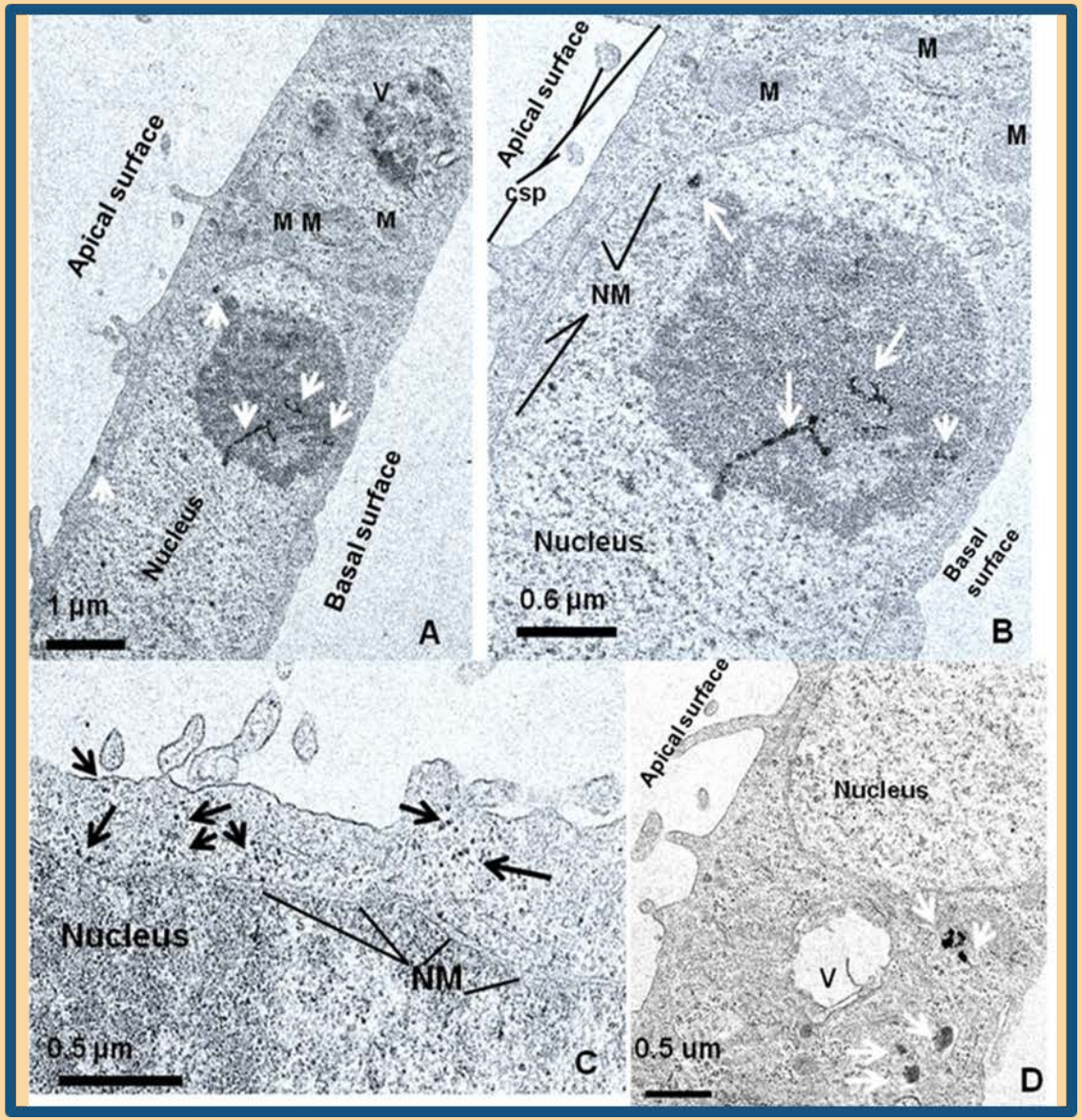




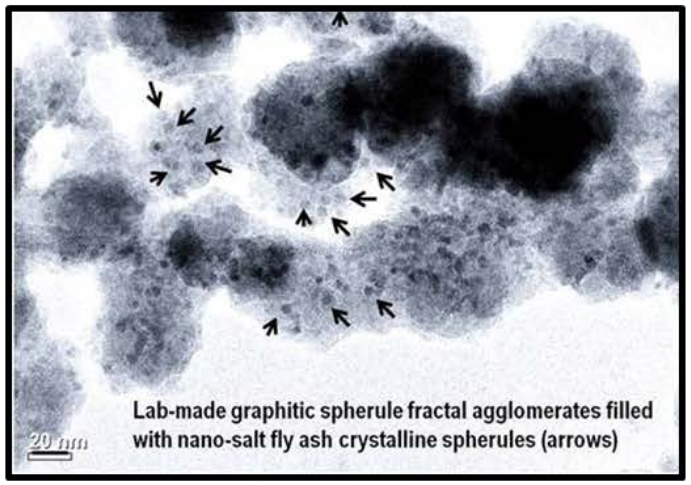


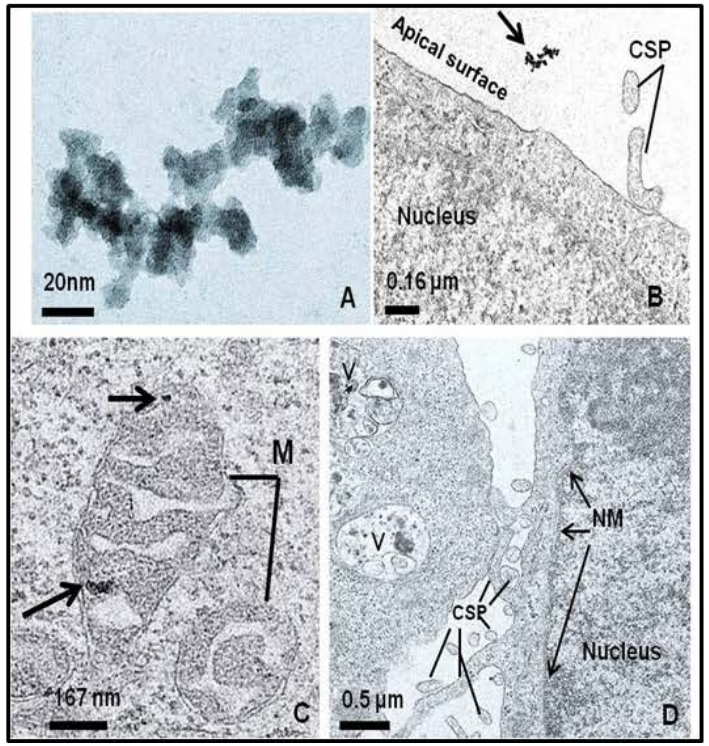


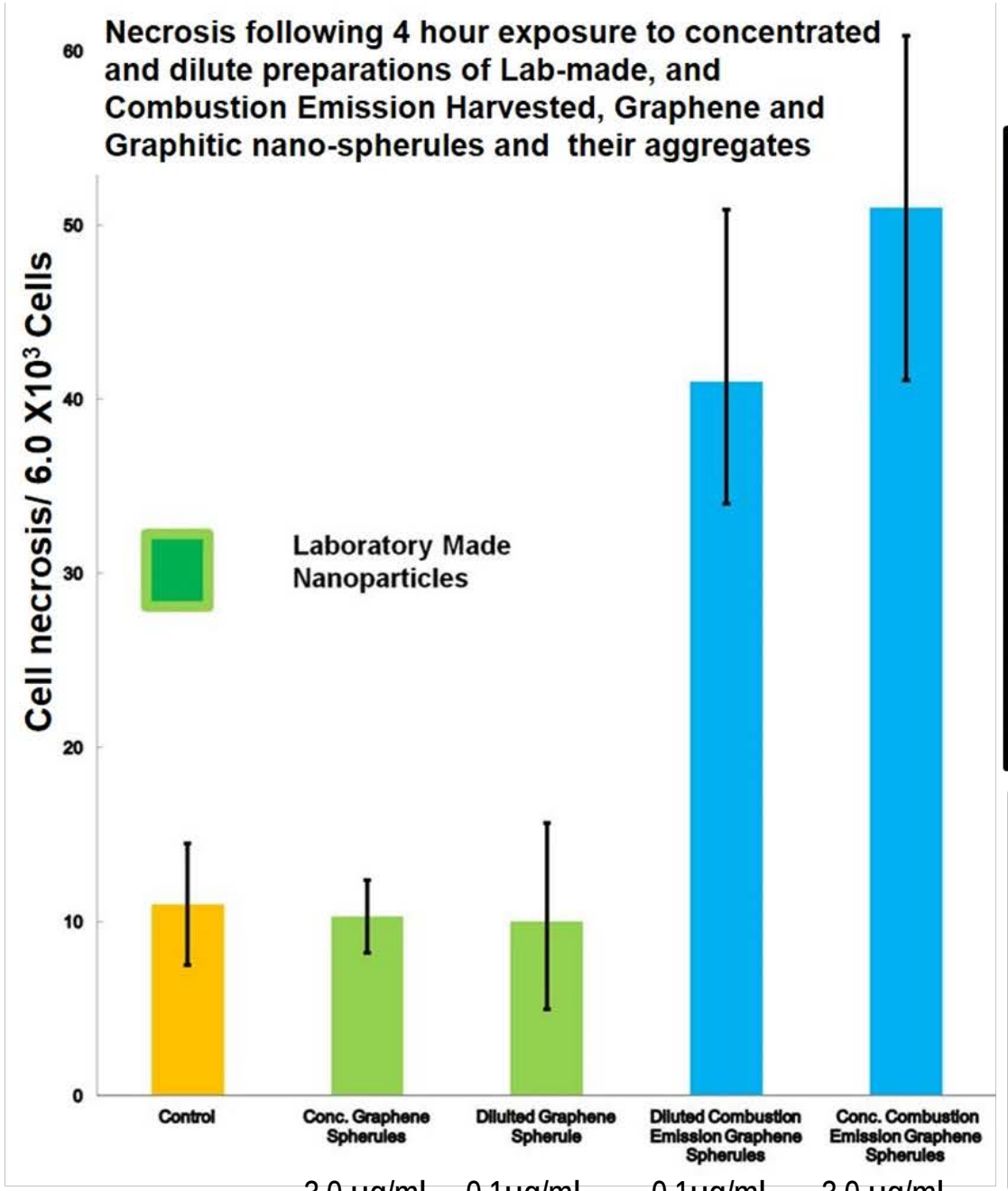
Lab-made Carbon Nano-spherule Cell Incubation



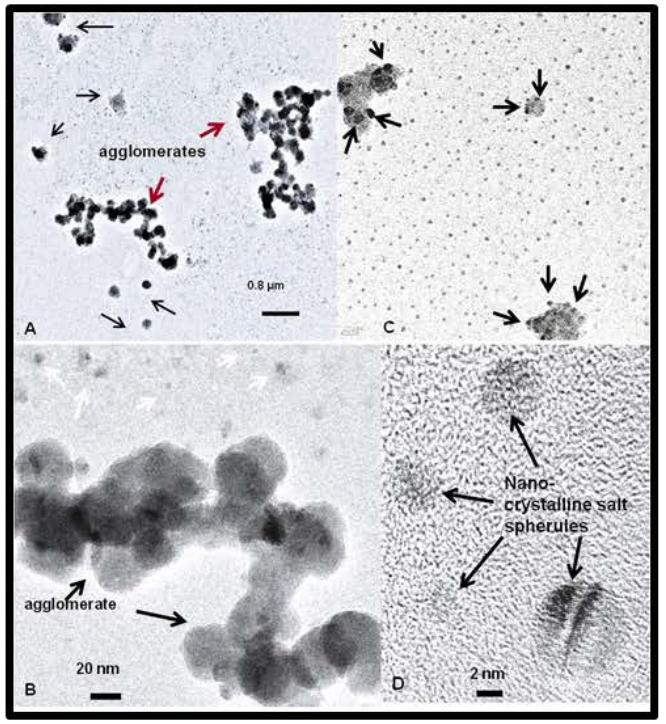
Lab-made Ultra-Clean Nanoparticles







Red Oak combustion nanoparticles



Wood Combustion nano-aggregates and isolated nanoparticles harvested from an advanced technology residential cordwood boiler. The nanoparticles were separated into (graphene agglomerates (Fig.A); some graphitic agglomerates(Fig.B); and isolated nano-salt crystalline spherules (Fig.D).

Lung Cell Viability studies following 4 Hour Exposure to wood combustion emission nanoparticles

Conclusions:

1. **Cell death was increased when healthy human lung bronchial epithelial monolayers were exposed to Boiler Wood Combustion Emission graphene and graphitic nanoparticles harvested directly from the gas stream within the dilution tunnel.**

2. No increase in cell death was seen following exposure of lung cell monolayers to the high and low doses of ultra-clean Lab-made carbon nanoparticles at high or low dose concentrations, when compared to Control cells (exposed to phosphate buffered saline (PBS).

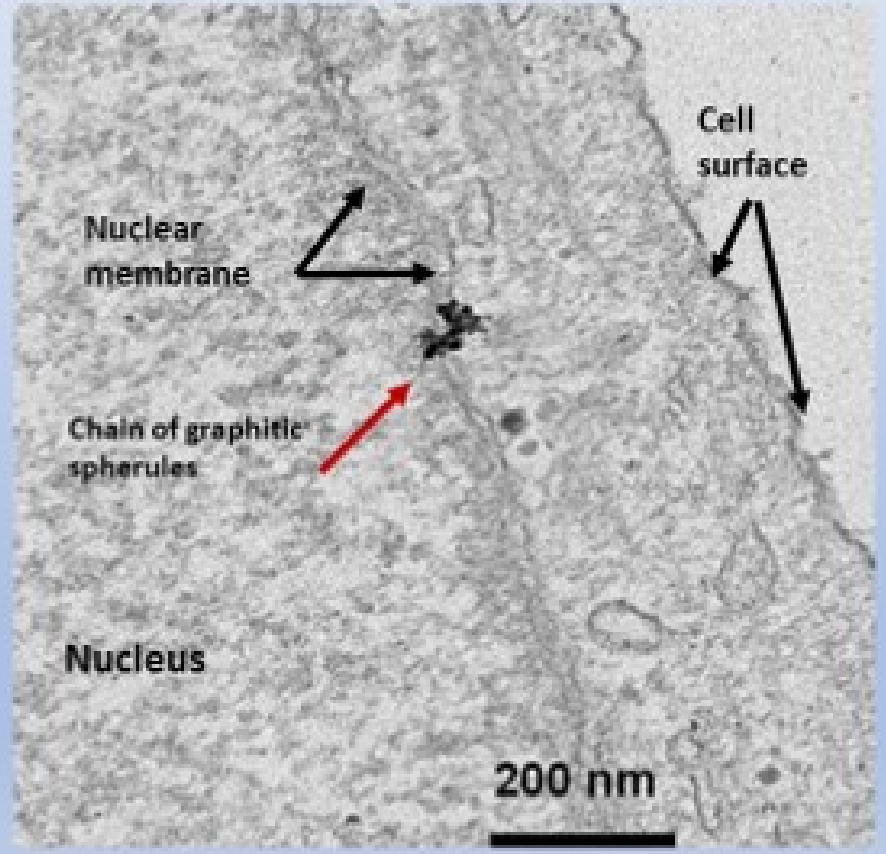
3. After 4 hr exposure, ultra-clean Lab-made carbon nanoparticles did not cause cell necrosis or cell death to the lung cell monolayers.

4. PAHs and resins produced during cordwood combustion /pyrolysis, are known to produce cytotoxic effects when they come in contact with living cells. These materials attach to the surfaces of carbon combustion emission nanoparticles, or can be embedded within the carbon layers of graphene and graphitic spherules (Murr, et al.). Conventional Cordwood fired-Boilers and stoves may not be able to remove all organic and PAH materials from exiting combustion nanoparticualtes.

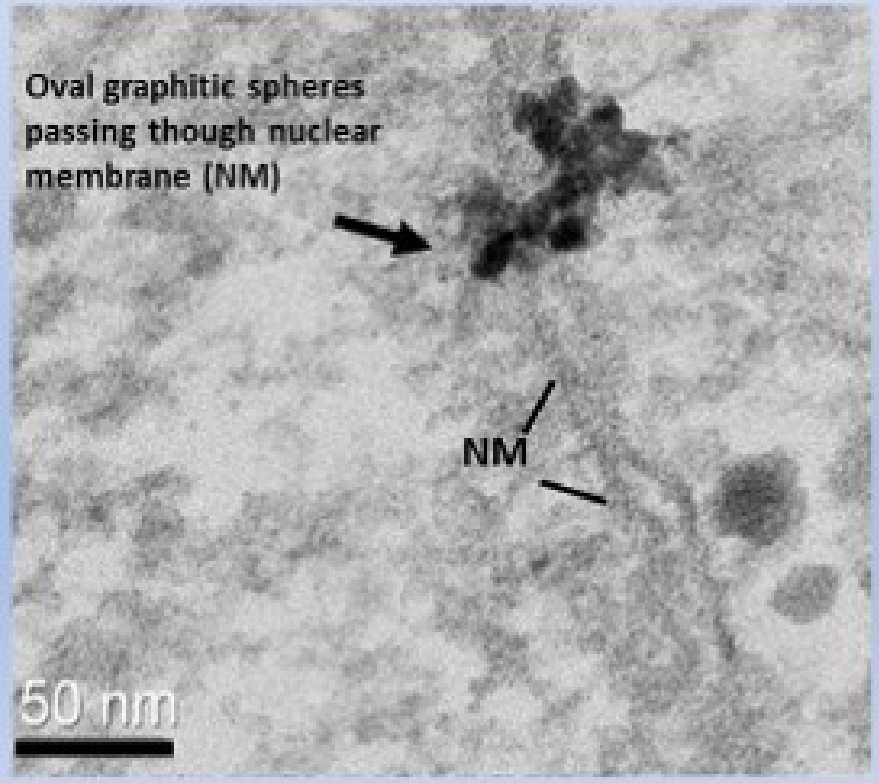
Like the Trojan Horse, the carbon nanoparticles and aggregate chains often carry on their surfaces, or within the carbon layers, these harmful organic materials, which if inhaled will cause cytotoxicity.

Passage of graphitic chains through the Nuclear Membrane in Human Lung Epithelial Cells

A fractal aggregate chain of individual graphitic spherules, (red arrow), entering the nuclear membrane of a human lung bronchial epithelial cell.



At higher magnification the individual spherules measured 11.6 X 11.6 nm; 9.6 X15.3 nm; 15.2 X 9.9 nm; 9.1nm X 11.6 nm; 11.6 X13.3 nm. The elongated spherule that successfully passed though the nuclear membrane, into the nucleus, measured 9.9 X 19.1 nm, suggesting that these graphitic spherules may be flexible and pass through the nuclear pore (which in humans is believed to be 9-12nm diameter). R. Wang and M .Brattain (2007) reported the maximal size of the nuclear pore complex in human nuclear membranes as 9-12nm in diameter. Therefore the graphitic spherules that have a central space filled with very small nano-salts, often can assume an elliptical shape within the appropriate size range to enter and pass through the nuclear pore.



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