ESRF	**Experiment title:** Respiratory Effects Of Carbon Nanotubes: Localization And Induced-Modifications	**Experiment number**: MD-280
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Report:

Carbon nanotubes (CNT) are cylinders with diameters in the nanoscale range, possessing unique properties that make them candidates to promising applications. The forecast increase of CNT use in manufacture will lead to an increase of human exposure to these nano-objects. Therefore, a better understanding of the potential human health impact associated with such nanomaterials is urgently needed. Since the respiratory tract could be the main route of exposure to these volatile materials, the pulmonary toxicity of CNT and effects on cells involved in the immune respiratory response (such as macrophage cells) are target issues.

One key-point in understanding CNT biological effects is to characterize their interactions with cells. This is a difficult issue since CNT, and single-walled CNT in particular, are difficult to visualize in biological environments. Therefore, analysis of the fluorescence signal of iron catalyst nanoparticles, remaining attached to or within the CNT, using synchrotron-based X-ray fluorescence microscopy (microXRF) is an interesting mean to study CNT-cell interactions and in particular CNT cellular localization. Investigation of intracellular chemical element distribution also allows detecting concomitant chemical modifications in cell due to CNT exposure. The first phase of our investigations thus consisted in evaluating whether different carbon nanotubes (single-walled carbon nanotubes (SWNT) and multiwalled carbon nanotubes (MWNT)) enter macrophages when they are brought into contact, and if they are internalized the same way. A second step aimed at evaluating biologically relevant chemical modifications.

Materials & Methods

The incident wavelength was 7.2 keV in order to excite the K-lines XRF of elements of atomic number Z between 8 (O) and 26 (Fe). The X-ray spot had dimensions of 1.5*0.5 µm², allowing a micron resolution. Three types of CNT were used: MWNT, Raw-SWNT and purified-SWNT, all synthesized by Chemical Vapor Deposition (CVD) method, with iron as catalyst. Iron content was 4.4wt% for MWCNT, 20wt% for

raw-SWNT and 1.8wt% for purified-SWNT as determined by thermogravimetric analysis. SEM and TEM analysis showed that MWCNT contained iron-based particles at their basis (catalyst particles) and inside their hollow core. TEM analysis of SWCNT (raw and purified) showed that iron is present in the form of nanoparticles at the end or on the side-walls of nanotubes. Murin macrophages (RAW 264.7 cell line) were exposed to different concentrations of CNT, namely 10 µg/mL and 100 µg/mL, the choice of macrophage cells being dictated by their crucial role in inflammatory and respiratory responses to exogenous agents. The exposure times were of 6 or 24 hours. The sample fixation was achieved by two different methods in order to test which one was the most appropriate for our special application: i) by chimio-fixation in a para-formaldehyde solution and then air-drying and ii) by cryo-fixation consisting in quickly plunging the sample into isopentane cooled with liquid nitrogen, the sample being further freeze-dried at -60°C under vaccum. Non-exposed cells were used in order to get a XRF "reference" spectrum of macrophages, which was used as a comparison with the data obtained on cells exposed to CNT. XRF spectra of control CNT samples (CNT not incubated with cells) were also collected.

Results & discussion

Conventional Transmission Electronic Microscopy (TEM) of macrophage cells showed the presence of MWCNT inside the cells, but SWCNT could not be clearly/ unambiguously visualized. Examination of iron mapping by microXRF allowed us to follow iron particles attached to or within both SW and MWCNT. It is thus possible to detect the incorporation of non-labelized CNT inside cells and to observe intracellular localization of both MW and SWCNT (figure 1). A dose-response effect is measured for the cellular iron signal in CNT-exposed cells (100 vs 10 µg/mL). The high sensitivity of microXRF also allowed us to detect CNT (i.e. purified SWCNT) with less than 2wt% of remaining iron catalyst particles.

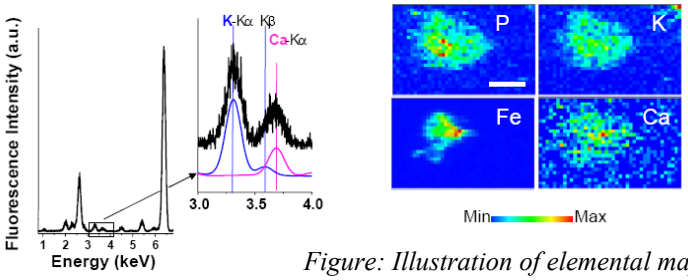

Figure: Illustration of elemental mapping by microXRF of a macrophage exposed to R-SWCNT

Furthermore, elemental mapping of calcium showed increased concentrations of calcium in some of the cells exposed to CNT (figure 1), in particular MWCNT and raw-SWNT (see figure) as compared to non-exposed macrophages. Calcium is an element involved in inflammatory mechanisms and also plays a key role in many cellular signalling pathways. The modification of its cellular concentration is thus of special interest. We moreover noticed that chimiofixation -as opposed to cryofixation- may induce cell membrane porosity and could interfere with interpretation of the results, especially concerning calcium concentration. In consequence we derived our results only from measurements performed on cryofixed samples. The influence of Ca on the biological effects of CNT was further demonstrated by complimentary pharmacological assays.

Conclusion

Our results demonstrate for the first time the validity of the use of metal catalyst attached to or within CNT to visualize CNT distribution at a single cell level by analyzing the fluorescence signal of the metal particles by microXRF using synchrotron X-ray source. We also demonstrate the modification of intracellular calcium content in cells exposed to CNT, especially those with the higher iron content (MWCNT and Raw-SWCNT). This finding could be of strong importance in the perspective of understanding the toxicological effects of CNT, since excess of calcium could be linked to functional disorder like cytotoxicity, oxidative stress and inflammation.

The detailed experimental results have been reported through different communications and published in a Nano Letters, **8(9): 2659-2663, 2008**. doi : 10.1021/nl800914m

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Carbon Nanotubes in Macrophages: Imaging and Chemical Analysis by X-ray Fluorescence Microscopy. Bussy C [1,2], Cambedouzou J [2], Lanone S [1],, Leccia E[2], Heresanu V [2], Pinault M [3], Mayne-L'hermite M [3], Brun N [2], Mory C [2], Cotte M [4], Doucet J [2], Boczkowski J [1,5,#], Launois P[2,#]; ***Nano Lettesr*, 8(9): 2659-2663, 2008.**

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X-ray fluorescence microscopy (μXRF) is applied for the first time to study macrophages exposed to unpurified and purified single-walled (SW) and multiwalled (MW) carbon nanotubes (CNT). Investigating chemical elemental distributions allows one to (i) image nanotube localization within a cell and (ii) detect chemical modification of the cell after CNT internalization. An excess of calcium is detected for cells exposed to unpurified SWCNT and MWCNT and related toxicological assays are discussed.