Preliminary observations on the interactions between fine atmospheric particulate matter (PM2.5) and human alveolar epithelial cells (A549)

G. Zerbi[1], A. Ferrugiari[1], G. Fustella[1], M. Tommasini[1], P. Mantecca[2], M. Gualtieri[2], F. Cetta[3], M. Camatini[2]
[1] Dipartimento di Chimica, Materiali ed Ingegneria Chimica "Giulio Natta", Politecnico di Milano, Piazza L. Da Vinci 32 - 20133 Milano
[2] Centro di Ricerca POLARIS, Dipartimento di Scienze dell'Ambiente e del Territorio, Università degli Studi di Milano Bicocca, Piazza della Scienza 1 - 20126 Milano
[3] Dipartimento di Chirurgia, Facoltà di Medicina e Chirurgia dell'Università di Siena

Fine atmospheric particulate matter (PM2.5) was collected in Milan during winter at Torre Sarca site, representative of background urban atmospheric pollution concentrations. Human alveolar epithelial A549 cells were subsequently exposed to different particle doses for increasing time. PM2.5 morphology, as well as cell-particle interaction, were characterized by light microscopy, transmission electron microscopy and Raman spectroscopy. Based on previous theoretical studies of molecular dynamics and vibrational spectroscopy (Zerbi et al. 2006, Zerbi 2007) we developed a method to extract from the Raman spectra information on the molecular properties of different carbonaceous materials. It has been shown that PM2.5 is progressively internalized in cells (Gualtieri et al., 2007). Of particular interest is the preliminary observation that the Raman spectra of a carbon particulate (PM2.5) isolated from its surrounding or encapsulated in a cellular matrix, like that of alveolar epithelial cells, showed differences. It indicates that some kind of interaction at the molecular level has occurred. Further work is in progress.

1. Introduction

Particulate matter and carbonaceous materials in general have been shown to appear in a variety of shapes and chemical compositions which have been sometimes related to the chemical and physical processes which have contributed to their formation (Van Setten et al., 2001).

Necessarily we neglect to discuss in this paper the use of carbonaceous materials in industry (tires, pharmaceuticals, filters, absorbers, etc.) and focus mainly on the particulate matter which at present is the centre of the interest of environmental scientists. The impact of particulate matter on humans is the object of many studies which coordinatively involves chemists, biologists, biochemists and medical doctors (Gualtieri et al., 2007).

Chemistry has contributed to the qualitative and quantitative determination of the chemical composition of various classes of particulate matter and has informed us that the particle may contain a variety of metals, inorganic ions, Organic Carbon (OC) and Elemental Carbon (EC). Among organic materials Polycyclic Aromatic Hydrocarbons (PAH) play the main role in the studies of carcinogenesis; the fraction of EC is generally associated with the graphitic component which is generated in all combustion processes from oil burning to industrial fumes, car exhaust, etc.

Our attention is focussed on the carbonaceous material generally labelled as “soot” which can be collected by suitably built filters which select the particles according to their size (Fermo et al. 2003, Owoade et al. 2006), by removing black crusts or black deposits from surfaces (e.g. works of art (Ferrugiari, 2008), chimneys, etc.)

Our work finds its justification by the observation that the characterization of all kinds of carbonaceous materials are mostly carried out by the observation of the particles with microscopic techniques which, starting from the optical microscope extends to, and is limited by, the spatial resolution available by electron microscopy. It follows that all the studies generally stop with the description of the carbonaceous matter at the micron level and relatively few information are available on the nature (structure and properties) of the molecules which make up the EC fraction.

Our aim is to explore the possibility to apply a “molecular approach” to the study of the “graphitic” portion of the EC fraction. Any additional information on the molecular aspects of the carbonaceous materials may provide new insight (if any) on the intermolecular interactions of such materials with the biological tissues when the particulate matter has been put in contact with living cells.

There should be no reason to deny the well known and well established evidence that intermolecular (atom-atom) interactions occur whenever “chemical” objects are brought together no matter whether the materials is in the gas, solid, liquid, amorphous, crystalline or solution phase (Machida, 1999). The only problem is to find a way to collect a “signal” specific of a certain kind of interaction (electrostatic, Van der Waals, covalent, magnetic, dispersive etc).

2. The molecular approach to graphite and to graphitic materials.

It is known that graphite, first considered as single crystal, is made up by planar sheets of polycondensed benzene, each sheet is stacked one on top of the others and the sheets are held together by weak dispersion forces. The origin of these weak forces lies in the fact that each carbon atom forming the hexagonal repeat motif in the graphitic sheet is in the sp^2 hybridization state with three electronic orbitals in a trigonal geometry (at 120°) linked by covalent co-planar σ bonds with the neighbouring atoms. The remaining pz orbitals are orthogonal to the plane and provide the dispersive atom-atom interactions which glue sheets together and allow graphite to exist in the perfect tri-dimensional crystalline state.

The additional very relevant structural feature is that p_z orbitals form CC bonds (π bonds) and are “delocalized” across the graphitic carbon network (Kelly, 1981). The chemistry and physics associated with the intramolecular delocalization of π bonds dominates materials science because the application of polyconjugated molecules (in 1-d e.g. polyacetylene, 2-d e.g. graphite and 3-d e.g. fullerene) have invaded science, technology and the market (Skotheim 1986, 1999).

Since a long time polyconjugated 1-d and 2-d organic materials have been the center of interest by the group at the Politecnico of Milano (Gussoni et al. 1991; Del Zoppo et al. 1999; Zerbi et al. 2006; Zerbi 2007). The tool of the studies were, and still are, electronic and mainly vibrational spectroscopy which are based on the recording and understanding of the vibrational spectra as seen from Infrared and Raman spectroscopy. The interpretation of the data collected are supported by quantum chemical and dynamical calculations which can also describe the oscillations of the atoms in the system (vibrational normal modes) (Castiglioni et al. 2004; Zerbi 2007).

In the case of graphite its Raman spectrum provides many information which we cannot obtain from Infrared. The Raman spectrum of single crystal graphite, supposedly infinite, is given in fig. 1a and lattice dynamical calculations on a perfect single sheet of graphite describe the normal mode near 1600 cm^{-1} as the in phase translation of all carbon atoms. Such a mode is universally labelled as G mode (fig. 1).

Facing real Nature we have to acknowledge the fact that the graphitic sheets cannot be "infinite" and that they must be terminated i.e. some kind of border must exist which defines a portion of the graphitic sheet (graphenes). Logically in graphenes the delocalization of π electrons is confined and the borders must take up a structure compatible with the electronic distribution of such a large, but finite systems. A collaboration with prof. K. Müllen of the Max Planck Institute on Polymerforschung made it possible to study the Raman spectra of a series of graphenes (i.e. large PAH) with different shapes and sizes. These large PAH are not a mixture of different molecules but are chemically pure (monodisperse) with a defined structure (fig. 2). For instance, in fig. 3 we report the Raman spectrum of C114 (a PAH belonging to D_{2h} point group symmetry) with the corresponding calculated atomic vibrational displacements for a mode near 1300 cm^{-1}.

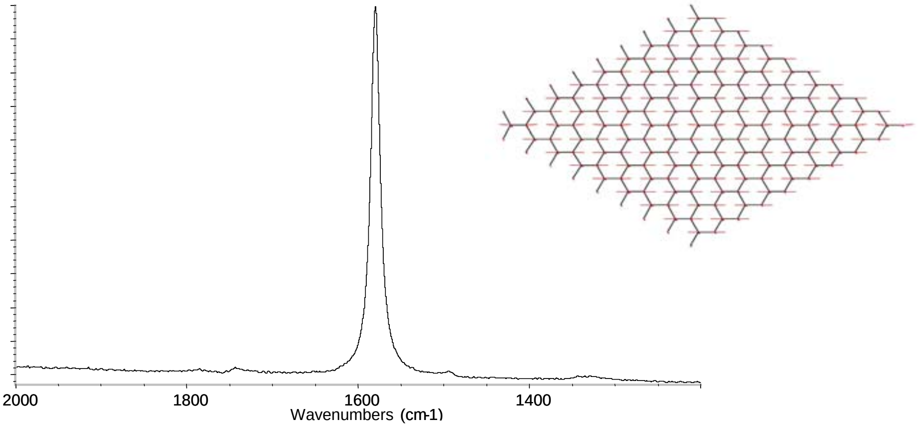

Fig. 1. *Left: Raman spectrum of single crystal graphite. Right: nuclear displacements associated to the G peak (one of the two degenerate E_{2g} normal modes is shown).*

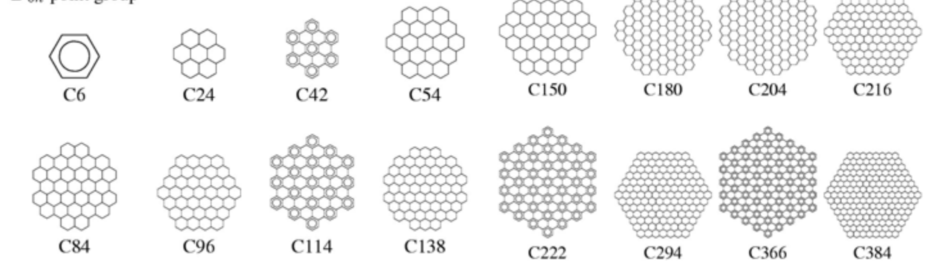

Fig. 2. *Sketch of the molecular structures of representative PAHs belonging to the D_{6h} point group. Samples C6, C24, C42, C114, C222 have been synthesized up to date.*

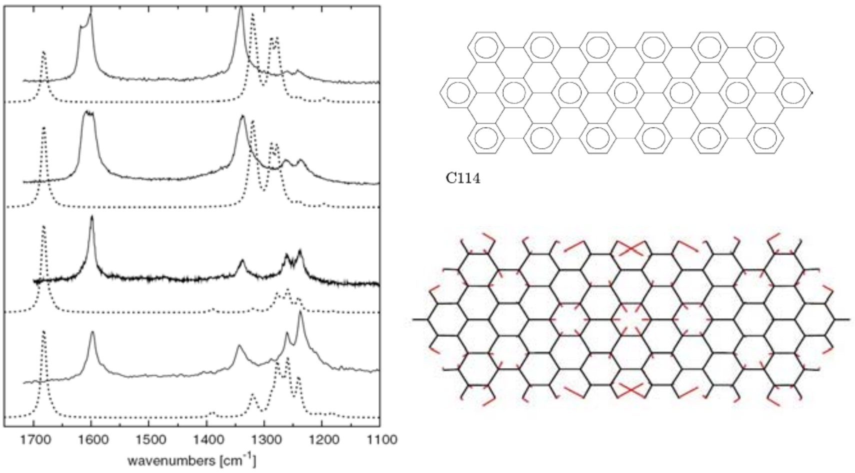

Fig. 3. *Comparison between the experimental (solid line) and the simulated (dashed line) Raman spectra of* D_{2h} *C114. The spectra were obtained by using different laser wavelengths; from bottom to top: 1064, 632.8, 514.5 and 457.9 nm. Nuclear displacements associated to the vibrational mode computed at 1277* cm^{-1} *of* D_{2h} *C114. Data taken from Di Donato et al., 2004.*

It then turns out that, whenever the graphitic sheet becomes smaller and boundaries are formed, a Raman peak near 1300 cm^{-1} has to develop (D line).

The observation in the Raman spectra of both G and D lines becomes a Raman “fingerprint” of the existence of carbonaceous graphitic materials. The Raman spectra of the many samples of carbonaceous materials and atmospheric particulate matter we have studied all show the characteristic G and D lines, thus indicating (a) the existence of a distribution of graphitic cores, (b) the existence of a distribution of confined sheets with some sort of boundaries and (c) the fact that the relative population of cores and boundaries do change in samples of different origin.

The next obvious questions we wish to answer are (i) what is the structure of the boundary regions of the graphenes? and (ii) what is the role of the boundary regions of the large graphenes in determining the interactions with the “chemistry” which forms the surrounding medium?

3. Materials and Methods

3.1 PM collection and preparation

PM2.5 samples from a urban site in Milan have been obtained by a low volume gravimetric samplers (EU system 38,33 l min-1, FAI Instruments, Rome, Italy) equipped with Teflon filters (47 mm Ø, 2 µm, Pall Gelman, USA). The filters were then preserved in the darkness at – 4°C (avoiding photo degradation and evaporation losses) until particle extraction.

Particles’ extraction process has been performed by exposing the filters immersed in two ml of sterile water to four ultrasounds cycle (20 min each) in a Sonica® ultrasound bath. Detached particles were then dried into a desiccators, weighed in a precision balance and re-suspended in sterile water to obtain aliquots at a final concentration of 4µg/µl which were stored at -20°C until use.

3.2 Cell culture and treatments

Human alveolar epithelial cells (A549) were routinely maintained in cultured medium in an incubator at 37°C, supplied with 5% CO_2. After 24h from seeding on, when confluent stage was reached, the cells were treated with PM2.5 suspensions obtained by multiple dilutions of the thawed particle aliquots to reach the final doses for treatments. PM2.5 exposures were extended for 3 and 24 h, with controls running parallel.

3.3 Cell and particle microscopical examinations

Prior to cell treatments, about 10 µl of sonicated suspensions of extracted PM2.5 were pipetted onto Formvar® coated 200 mesh copper grids. Water was then gently blot, and once dried, grids were directly inserted into a Jeol JEM-1220 transmission electron microscope (TEM) for the morphological characterization of particles.

For light microscopy and Raman spectroscopy, A549 cell monolayers have grown and been treated on coverslips placed on the bottom of 35 mm sterile Petri dishes. At the end of the treatments, the culture medium was removed, cells severely rinsed in PBS and finally fixed in 10% neutral buffered formalin for 1h. After being fixed, cells were routinely stained with Haematoxilin – Eosin (HE) for light microscopy (Mantecca et al., 2007).

For TEM analyses of cell-particle interaction, confluent cell monolayers grown on the bottom of plastic Petri dishes, were directly fixed with 2.5% glutaraldehyde in 0.2M sodium cacodilate buffer and routinely processed for embedding in EPON® 812 resin. Frontal 60 nm ultrathin sections of the cell layers were cut on a ultramicrotome and observed under the electron microscope after being stained with uranyl acetate and lead citrate.

3.4 Raman spectroscopy

Raman spectra of particles internalised in the cells were recorded using a Horiba Labram 800 spectrometer equipped with an Olympus BX 41 microscope. The 785 nm line of a solid state laser was used for the excitation. The laser beam was filtered in order to prevent damage of the sample. The slide with A549 cells was directly put on the stage of the microscope under a 50X objective.

4. Results

4.1 Particles' morphology

Transmission electron micrographs of particles extracted from the sampling filters and suspended in distilled water by sonication just before the cell treatments, are reported in fig. 4. Most particles resulted as aggregates of smaller round shaped nanoparticles, with different levels of bridging among particles' clusters. The smallest particles observed were about 40 nm in length, while only few particles overpass 1 µm in length.

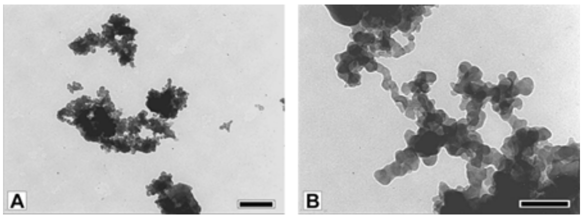

Fig. 4. *TEM images of particles pertaining to PM2.5. A, bar=500nm; B, bar=200nm*

4.2 Light and electron microscopy of cell-particle interaction

Under the light microscope, A549 monolayers showed the peculiar morphology of ephitelial like cells and different cell cycle stages could be observed. The quantity of particulate matter interacting with the cells appeared to increase with increasing exposure time (fig. 5). After 24h of exposure, particles appeared engulfed in the cytoplasms, where membrane lined vacuoles could be seen encircling degenerating organelles and particulate masses (fig. 6).

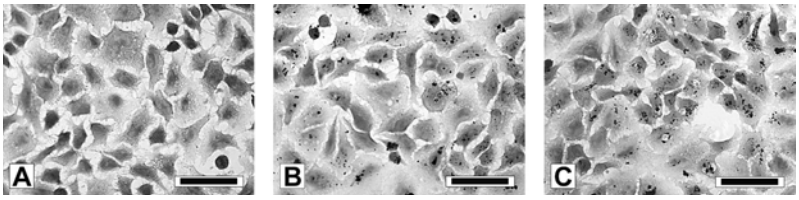

Fig. 5. *Light microphotographs of A549 monolayers. A, control cells; B,C, cells exposed to PM2.5 for 6 and 24h respectively. Bars=50µm*

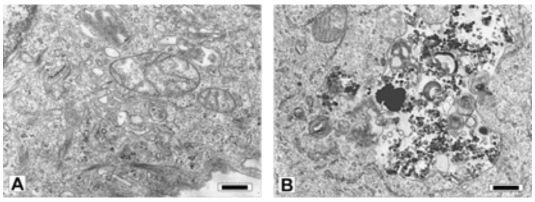

Fig. 6. *TEM images of A549 ultrastructure. A, control cell; B, cells exposed to PM2.5 for 24h. Bars=500nm*

4.3 Raman experiments

We delay to another paper a full discussion of our results; we report here on some recent results which have provided the way to define a new parameter which allows to characterize graphitic objects at the molecular level. The idea was the following: if by suitable mechanical treatment we can exfoliate graphite or break large graphitic sheets into smaller objects with a larger fraction perturbed by the borders, the intensity of the D line should increase relative to the intensity of the G line.

fig. 7 is a plot of the values of I_D/I_G (measured with three different exciting laser lines) vs. milling time. The intensity of the D lines unquestionably increases relative to the G line, thus proving that the boundary areas have increased with the time of milling. The mechanical attack to the graphenes by ball milling has been provisionally carried out in air; the breaking or the exfoliation of the sheets must be accompanied by some rearrangement of the electronic structure at the borders which is being studied at present in more detail.

This study is aimed at finding possible signals of the interactions of the boundaries or of the inner graphitic core with the chemical surrounding.

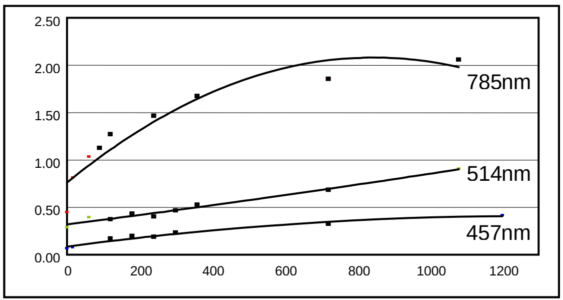

Fig. 7. *I_D/I_G ratios (measured with three different excitations) vs. ball milling time (minutes) for samples of graphite powder (Aldrich, CAS 7782-42-5).*

4.3.1 Interactions of PM2.5 with human alveolar epithelial cells (A549)

Our molecular approach to graphite and carbonaceous materials is actively being extended to the study of the structures of the graphenes in the atmospheric particulate matter and of their interactions with the human alveolar epithelial cells. We aim at disentangling the possible contribution to cell toxicity in lung cultured cells by the core or by the boundaries of the graphenes we have shown to exist in PM.

The chemical characterization of PM2.5 particles shows the existence of elemental and organic carbon, and PAHs. Moreover water soluble inorganic salts were detected. Following the methods described above in this paper the Raman spectrum shows that PM2.5 particles contain a non-negligible amount of a distribution of large graphenes whose role in the interaction with epithelial cells is our present interest. Raman spectra both of the original PM2.5 particles as well as of the particles embedded in epithelial cells were recorded from samples deposited as thin film on a glass support. Fig. 8 shows the noteworthy result that the G line is strongly affected by the interaction of the carbonaceous fraction and the epithelial cells. A more extended analysis on several samples is under way.

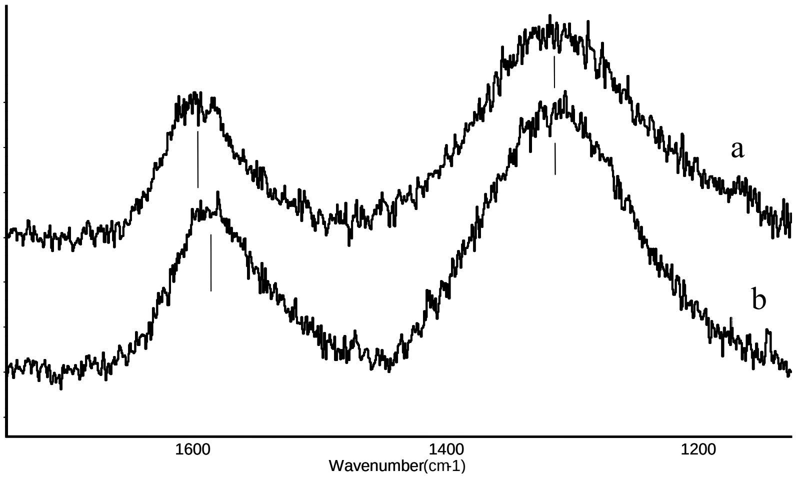

Fig. 8. *Raman spectra of pristine fine particles, PM2.5 (spectrum a) and of the same particles internalised in alveolar epithelial cells (spectrum b).*

Acknowledgments

This research has been partly supported by the strategic project PROLIFE 2007-2008 of the Comune of Milan.

References

Castiglioni, C., Tommasini, M., Zerbi, G., 2004, Raman spectroscopy of polyconjugated molecules and materials: confinement effect in one and two dimensions, Philosophical Transactions Of The Royal Society Of London Series A-Mathematical Physical And Engineering Sciences Vol 362, pp 2425-2459.

Di Donato, E., Tommasini, M., Fustella, G., Brambilla, L., Castiglioni, C., Zerbi, G., Simpson, C.D., Mullen, K., Negri, F., 2004, Chemical Physics Vol. 301, pp 81–93.

Del Zoppo, M., Castiglioni, C., Zuliani, P., Zerbi, G., 1999, “Molecular, electronic structure, nonlinear optics of polyconjugated materials from their vibrational spectra”, Handbook of conducting polymers, 2nd edition, pp 765, Eds. T.A. Skotheim, R.L. Elsembaumer, J.R. Reynolds, Dekker, New York.

Fermo, P., Gilardoni, S., Simarro, T.J., Bolzacchini E., Lasagni M., Gianelle V., Pozzoli L., Perrone G., Librando V., 2003, Annali di Chimica, Vol. 93, pp 389-396.

Ferrugiari, A., 2008, PhD thesis in Materials Engineering, Politecnico di Milano.

Gualtieri, M., Perrone, G., Corvaja, V., Ferrero, L., Mantecca, P., Bolzacchini, E., Camatini, M., 2007, Nanotoxicology, pp 77-78, Venezia.

Gussoni, M., Castiglioni, C., Zerbi, G., 1991, Spectroscopy of advanced materials, Eds. R.J.J. Clark & R.E. Hester, chapter 5, pp. 251–353, Wiley.

Kelly, B.T., 1981, “Physics of graphite”, Applied Science Publishers, Essex, ISBN: 0853349606

Machida, K., 1999, “Principles of Molecular Mechanics”, Wiley, ISBN: 0471357278.

Mantecca, P., Gualtieri, M., Andrioletti, M., Bacchetta, R., Vismara, C., Vailati, G., Camatini, M., 2007, Environ. Int. Vol. 33, pp 642-648.

Owoade, O.K., Olise F.S., Obioh, I.B., Olaniyi H.B., Bolzacchini E., Ferrero L., Perrone G., 2006, Nuclear instruments and methods in physics research, section A, Vol. 564, pp 315-318.

Van Setten, B.A.A.L., Makkee, M., Moulijn, A., 2001, Catalysis Reviews Vol. 43, pp 489-564 .

Skotheim, T. A. (editor) 1986, “Handbook of conducting polymers”, vol. 1–2, Dekker, New York; Skotheim, T.A., Elsembaumer, R.L., Reynolds, J.R. (editors) 1999, “Handbook of conducting polymers”, 2nd edition, Dekker, New York.

Zerbi, G., Tommasini, M., Centrone, A., Brambilla, L., Castiglioni, C., 2006, “A spectroscopic approach to carbon materials for energy storage”, in Carbon: The Future Material For Advanced Technology Applications, Eds. G. Messina, S. Santangelo, Topics in Applied Physics (Springer), Vol. 100, pp 23-53.

Zerbi, G., 2007, “Vibrational Spectroscopy of Conducting Polymers: theory and perspective”, in Vibrational spectroscopy of polymers: principles and practice, pp 487-536, Eds. N.J. Everall, J.M. Chalmers, P.R. Griffiths, Wiley.