MICROSTRUCTURES OF POTENTIALLY HARMFUL FIBROUS MINERALS

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HISTORICAL NOTES

Transmission electron microscopy and serpentine crossed destiny

In 1925, Louis de Broglie, was the first to theorize that electrons could be described as waves, but with a wavelength shorter than that of light (Ruska, 1980). Davisson & Germer (1927) pointed out that "slow" electrons were strongly diffracted by an ordered arrangement of atoms on the surface of a crystal. In the same year, G.P. Thomson, showed that with the increase of the accelerating potential from 50 V to 50 kV, the electrons were able to penetrate into the target material and were diffracted by the planes of atom within the material, thereby generating an electron diffraction pattern. These two research groups, independently, demonstrated the wave nature of electrons. Moving to Berlin, in 1931 Ernst Ruska completed the development of the first electronic lenses, coming to get the image of a metal grid. In short time, in an article written by Ruska & Knoll (1932), the term "electron microscope" was used for the first time. Just a year later, adding a third electronic lens, Ruska obtained the image of a cotton fiber and of an aluminium sheet; the resolution limit of the light microscope was overtaken. The observation of nuclei within biological cells was realized in Brussels in 1934 by Marton and his co-workers (Marton, 1934) who built a similar microscope. After two years the first commercial transmission electron microscope (TEM) was built and it was named "Metropolitan-Vickers EM1", but it seemed to not work properly (Marton, 1968). Only in the 1938, the real commercial production begun by Siemens and Halske, with instrument of a declared spatial resolution of 10 nm and an accelerating voltage of 80 kV. Long, curved and singly well resolved chrysotile fibers were photographed by Kühn in 1941. In the same year he published a picture of asbestos fibers extracted from human lungs. These fibers showed a cover made by a material rich in iron, produced by macrophages that had engulfed the foreign bodies (Kühn, 1941).

The newly invented Siemens Company's electron microscope was used to understand the asbestosis origins: this microscope with a magnifying power varying from 4000 to 40000 times was used to identify extremely small fibers in lung and tumor tissues (Proctor, 2000). Ruska itself published a paper, in the 1943 (Ruska, 1943), with images of mixed chrysotile and amphibole powders. Turkevich & Hillier (1949), and Bates *et al.* (1950) demonstrated that the chrysotile was hollow, highlighting also the possible different shapes: "cone in cone", concentric tubes and "Y" shaped intergrowths. After putting in evidence the diffraction contrast in 1955, the first study on the electron diffraction from serpentine minerals (chrysotile and lizardite) date back to 1957 by Zussman, Brindley and Comer (Zussman *et al.*, 1957). The silky from splintery chrysotile were then distinguished (the first more disordered than the second), as well as, "ortho" from "clino"-chrysotile and sharply crystalline lizardite. In 1958, Brindley overcame the magnification limitations of TEM and produced electron optical fringes which agreed in spacing and direction with the super-lattice parameters determined by electron diffraction (Mellini, 2013).The high resolution microscopy was reached by Yada (1967) with the first high-resolution image of a chrysotile fiber seen perpendicularly to the fiber axis, followed by chrysotile sections and stepped conical growth images.

The field emission gun (FEG) was invented by Albert Crew for his scanning transmission electron microscope (STEM) in the 1970 and the same year the model 100 B has been distributed by Jeol with an accelerating voltage of 100 kV and a resolution of 0.3 nm. With the model 2010, in 1990, the resolution reached a 0.2 nm with an accelerating voltage of 200 kV. Today TEM represents the most efficient and versatile instrument for the characterization of materials from the atomic- to the nano-scale.

Highlights in the study of asbestos dangerousness

In this chapter I'll briefly present some aspects of particular interest both from the mineralogical and biological point of view, in the light of this project approach.

In the '30 began the first experimental studies on animals (*in vivo*), when asbestosis was considered only an asbestos related industrial danger. A study carried out for years by Gardner on exposed rats and published *post-mortem* by Vorwald *et al.* (1951), seemed to evidence the higher carcinogenicity of chrysotile longer fibers compared to the shorter ones. Anyhow, Gardner's powders generation method didn't allow to obtain really specific sizing, as backward compared to the modern standards.

In 1960, Wagner observations on asbestos miners in South Africa, highlighted that mesothelioma seemed to have a major connection with amphiboles exposition, in particular with crocidolite, even if the accepted idea was that all the asbestos minerals could cause asbestosis and lung carcinoma at high exposition levels. Different system was implemented to study (*in vitro*) the toxicity of mineral dust and fibers; the first, by Harley & Margolis (1961), used erythrocytes in order to study interactions among particles and cells membrane. After two years from his last work, Wagner demonstrated that finely divided asbestos can generate mesothelioma if directly injected in the pleura. Allison *et al.* (1966) devoted themselves to the macrophages study. Gross was the first researcher which evidenced the development of lung cancer in experimental studies on rats (Gross *et al.*, 1967). Returning to the advancement of Wagner's work (Wagner *et al.*, 1974) he dispensed by inhalation to rats different kind of asbestos fibers at high doses for different periods of time. His experiment evidenced that chrysotile, amosite, crocidolite and anthophyllite asbestos generate fibrosis and cancers. In the '70s different authors, Stanton & Layard (1978), suggested the possibility of a relationship among mesothelioma and fibers morphologies. Pooley (1976), highlighted that tremolite presence in chrysotile samples has questioned the possibility that chrysotile itself can cause mesothelioma. In 1981 the Stanton's hypothesis was published in a paper (Smith *et al.*, 1981), when a lot of contemporary scientific work did not confirm this hypothesis and subsequent work deny his applicability. In 1983 and 1987 respectively Woodworth and Shatos (Woodworth *et al.*, 1983; Shatos *et al.*, 1987) dedicated their attention to the study of lung epithelial cells, fibroblasts and mesothelial cells as critical component of fibrogenic and carcinogenic mechanism.

Despite the size parameters are still used as reference, the idea that the dangerous effect of mineral fibers only derives from dimension and morphology is overcome. In fact, today is recognized that the contribution of different factors brings to the pathogenic effect of mineral fibers. Summarily the contribution to carcinogenicity of mineral fibers comes from mechanical and dimensional properties, metal cations and structural defects in the fibers (Hochella, 1993), and from surface properties with key factors that determines the cell-mineral interface behaviour (Schoonen *et al.*, 2006).

THE RESEARCH PROJECT AND THE RESEARCH PROJECT OF NATIONAL INTEREST (PRIN)

The commercial term of asbestos applies to a family of six silicate minerals having a specific fibrous morphology (defined by WHO, 1985) with a diameter below 3 μm, length above 5 μm and length/diameter ratio at least of 3. Five of them are asbestiform amphibole species: riebeckite (crocidolite), grunerite (amosite), tremolite, anthophyllite, and actinolite; the other one, the chrysotile, isa serpentine phyllosilicate. These minerals are notable for their fibrous habit, and bear useful attributes like considerable mechanical resistance, high flexibility, resistance to chemical agent, binder, sound-absorbing and heat insulating properties (Alleman & Mossman, 1997).

The modern asbestos industrial age began in the second half of the 19th century with the opening of large scale asbestos industries in Scotland, Germany, and England for the manufacture of asbestos containing materials (ACM). Since then, asbestos rapidly became an invaluable resource and every-day life commodities all over the world. Since the early 1960's, asbestos progressively began to lose its fortune in some countries due to the increasing knowledge on the danger to human healt (Alleman & Mossman, 1997); in fact it was found that high-dose exposure to asbestos, via inhalation and breathing, might provoke lethal lung diseases.

Today, all asbestos minerals are classified as carcinogenic substances and their use is consequently restricted or banned in 55 out of the 195 countries (28%) of the world (see www.ibasecretariat.org). Officially the amphibole asbestos are banned all over the world, whereas chrysotile is banned only in the countries strictly following the indication of the International Agency for Research and Cancer (IARC), which includes chrysotile in Group 1 "substance carcinogenic to humans". No ban was proposed for the asbestiform erionite zeolite. In the other countries worldwide, like in China and Brazil, only amphibole species are considered culprit for inducing mesothelioma, the major asbestos related lung disease, whereas use of chrysotile asbestos is allowed.

With this premise, since the 80's many countries worldwide have progressively reduced the use of asbestos. Notwithstanding, asbestos exposure is still a matter of concern because there are many ACM left "in opera", often deteriorated (and consequently dispersing fibers in air), for the execution of incomplete or superficial reclamation and because asbestos is naturally contained in rocks outcropped in many areas of the world. There are actually inconsistent data on the effects of cyto- and geno-toxicity of mineral fibers, with a growing concern for health risks in countries with a massive presence of asbestos products, or even where it is still produced and extracted or where reclamation plans are conducted.

The Ph.D. thesis project is part of a major long term research project of national interest (PRIN, Italy) aimed at the definition of a standard protocol for the characterization and testing to allow a full comparison of all the physic-chemical and mineralogical/structural characteristics of asbestos and asbestiform mineral fibers; moreover, their transformation during the interaction with the biological environment have been investigated, in order to explain the toxic [*]mechanism and the transformation processes of fibers. In particular, the target of my Ph.D. project, is the thorough investigation, in different conditions (raw to "cell cultures exposed fibers" and Ex vivo evidence), of the chemical, morphological, and structural characterization of different mineral fibers (*e.g.*, chrysotile, crocidolite, and asbestiform erionite), using transmission electron microscopy.

This Ph.D. project includes the following key issues from the PRIN research project: *i*) complete characterization of mineral fibers relevant for health issues and the industrial application; *ii*) chemical and mineralogical characterization of the fibers found in cytological and histological sections obtained from neoplastic tissues from patients affected by fatal lung disease or animals exposed to asbestos fibers.

The complete characterization of mineral fibers is the first step to provide a protocol of multidisciplinary analysis. The chemical, morphological, and structural characterization have been at first conducted on the raw fibers and later on the same fibers dispersed in different kind of cell cultures to understand the possible morpho-chemical changes of the fibers in contact with the biological medium. In the end a short confrontation on some *in vivo* samples have been performed. Two kinds of cell cultures were used: bronchoalveolar cells and mesothelial cells, considered the first and most important target of inhaled fibers.

The main three steps of the research project have been focused on: *i*)investigation on standard fibers (see note for the definition*); *ii*) investigation on fibers with drawed after 48 hours exposure to cell culture; *iii*) investigation on fibers after 96 hours exposure to cell culture.

For each step, the chemical composition and subsequently the variability of composition as a function of exposure time to the organic material have been determined, as well as morphological and crystallinity degree evidence. Moreover, identification of the most reactive materials in contact with the cells has been evaluated. In particular, attention have been paid to the morphological, boundaries, and surface variations observed in the fibers, searching for possible correlations with the composition and, using high resolution technique, in order to evaluate the eventual propagation of defects, the degree of amorphization or modification of the crystal lattice features in comparison with the starting material. This observation, always distributed on three levels (morphology, EDS chemical analysis, and crystallinity degree combining electron diffraction patterns and high resolution images when possible), is extremely interesting from a crystallographic point of view as it should shed

[*] The term standard fibers have been used for all the natural occurring and UICC prepared minerals that didn't came in contact with cell cultures, so, it's intended as starting materials before any interaction)

light on a possible transition from a crystalline lattice of the fibrous material towards amorphous phase or new crystalline phases. Moreover, particular attention has been paid to the variation of the density of structural defects, dissolution, boundaries amorphization and chemical leaching or re-deposition, compared to the time of exposure to cell cultures.

All the analytical results from the starting material have been processed through the use of the principal component analyses and the trend of the fiber transformation during the exposition evaluated through charts and variability considerations. Noteworthy, this study can be applied to potentially harmful other minerals (*e.g.*, asbestiform erionite) and mineral particulates, regardless of whether they are classified, or not classified, as asbestos.

CONCLUDING REMARKS

Starting fibers were characterized successfully by TEM and, in particular, mathematical analyses on dimensions and crystal chemistry data evidenced interesting aspects.

Applying theWHO's criteria (on 100 analyzed fibers per sample) a large number of fibers is not included in the defined dimensional category. The analyzed samples with the more dangerous fibers (according to the WHO's dimensional categories) are: anthophyllite (59 dangerous fibers), amosite (26 dangerous fibers), crocidolite (3 dangerous fiber), erionite (2 dangerous fibers), chrysotile UICC (no dangerous fibers), and chrysotile from Val Malenco (no dangerous fibers).

Conversely, ordering the dangerousness of the samples (100 fibers), according to the criteria set by the Stanton's Hypothesis, the list is the following: amosite (5 particularly reactive fibers), anthophyllite (3 particularly reactive fibers), crocidolite (1 particularly reactive fibers), chrysotile UICC, chrysotile Val Malenco, and erionite.

Obviously all of these valuable criteria do not take into account many important chemical, physical, and mineralogical characteristics of the fibers, in particular the characteristics that can have a strong influence in the interaction between fibers and the biological environment. All analyzed starting fibers proved to be highly crystalline, with a variable chemical composition, but coherent with those expected from literature and preliminary investigations. PCA application on the population of each sample was useful to get information and for the interpretation of data (composed by a lot of variables), proving to be a great tool applicable to the study of fibers (hence applicable to mineral dust or other particles populations). PCA also highlighted information that matches with aspects usually studied with other research techniques or present in literature (*e.g.*, cations influence on fibers dimensions, crystallographic site substitutions, etc.). The transformations of the three selected minerals (chrysotile, crocidolite, and asbestiform erionite) during the interaction with cell culture have been studied on three aspects: dimensional/morphological, degree of crystallinity, and crystal-chemistry. Transformations of variable intensity have been experienced by the three samples. The *in vitro* study allowed to shed light on the difference among studied minerals; moreover, it has been possible to compare the effects of bronchoalveolar cells and mesothelial cells on mineral fibers. Finally, it has been possible to appreciate the difference between the fibers exposed for 48 hours and 96 hours, in the same cell culture.

More sensitive samples to the biological environment resulted chrysotile and erionite; in particular the chemical transformation are more intense in the mesothelial cells for this two materials. Crocidolite fibers are really resistant on all investigated aspects. Greater morphological transformations occurs in chrysotile fibers, particularly after 96 hours of interaction with both the cell culture, followed by erionite, which, however, has a similar morphology after 48 hours and after 96 hours. At last, crocidolite preserves his characteristics (in particular the high degree of crystallinity) and experience fiber angles dissolution in some cases. Looking at the three aspects (dimensional/morphological, crystallinity degree, and chemistry) of the totality of the studied interactions, chrysotile was the one that suffer major transformations, followed by erionite and crocidolite. It must be emphasized, however, that most substantial chemical transformation took place in the fibers of asbestiform erionite. It is known that iron and in general metal cations, have a fundamental role in the generation

of reactive oxygen species inside mammalian cells and tissue. From this study, it is clear, that all the minerals that have interacted with the cells have released iron in different amounts (probably only Fe^{2+} for erionite and chrysotile and Fe^{2+} and Fe^{3+} for crocidolite). Dissolution is an important aspect in the interaction among studied mineral fibers and the biological environment, showing different path for each mineral. Biological environment shows a low concentration of Mg and Si and at these conditions chrysotile dissolves because of the undersaturated environment. Surface chemistry governs the dissolution, that starts from surface species that are hydrolysed and brought in solution.

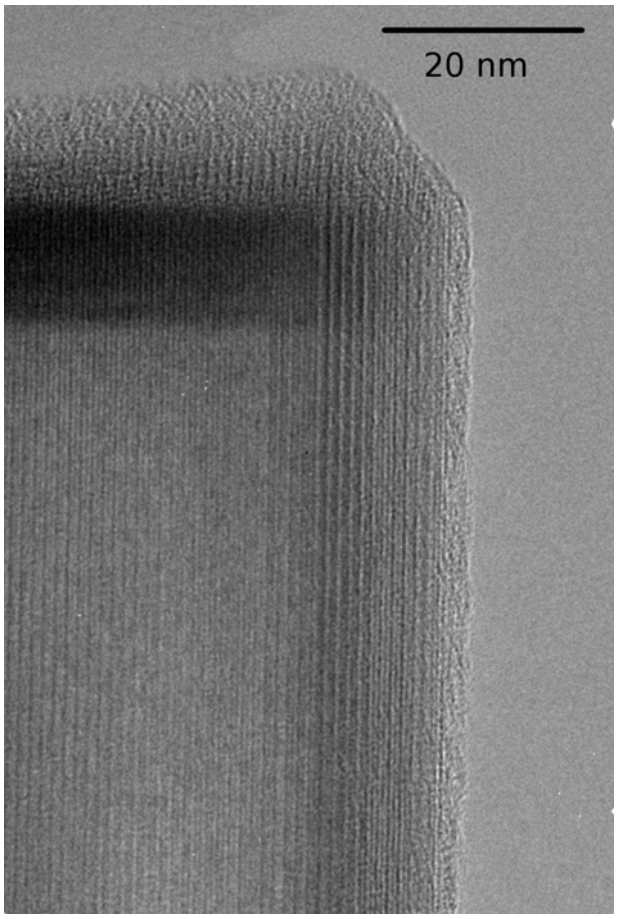

Fig. 1 - TEM image of a crocidolite fiber with probable Wadsley's defect on a side (right) and an amorphous layer of a few nanometer that rounds the whole fiber.

The octahedral external layer was the first to dissolve, whilst the tetrahedral sheet was the most resistant component of chrysotile. Crocidolite dissolution started with a loss of non-tetrahedral coordinated cations (Na, Mg, and Fe) due to the near-surface layers leaching, with the exception of calcium that increase (probably due to biological calcium deposition or absorption). If the iron has trivalent charge during the leaching, this can precipitate as ferric oxyhydroxide on the fibers surface. Fibers showed a typical amorphous shell (as visible in Fig. 1) that in some cases can reach the 30 nm of thickness. Finally, the cation exchange in erionite did not happen only on the surface or near-surface, but involves the interior of the mineral structure. The entire fiber act as buffer, preserving in several cases an high crystallinity degree (as visible in Fig. 2). To reach the aim of the improvement in the comprehension of the mechanisms provoking the onset of pathologies correlated with asbestos and asbestiform fibers, several further investigations and in-depth analyses of the present work should be performed, as follow: *i*) sample preparation for the fibers after the interaction with the cells cultures gives a good specimen for chemical and high resolution observation, but the cut doesn't allow us to make realistic dimensional evaluation. The combination of this sample preparation with the digestion of the cells and dispersion on the TEM gridas well as the possibility to work on two grids will allow to have better high resolution, dimensional, and chemical informations; *ii*) the experimental discrimination between Fe^{2+}/Fe^{3+} (*e.g.*, by EELS) is very important because of the role of this element in the biological processes; *iii*) increase the number of withdraws of fibers from the cell culture to have more exposition time (72 hours exposition cultures are already available); *iv*) increase the exposition time, which can allow to perform the PCA on the trend of the mean values of the concentration of elements (from the beginning of the experiment until the time *n)* and not only on a single sample population; *v*) reduction of variables (*e.g.*, using solutions at particular pH, particle with the same composition of fibers and so on) could help to better understand and modelling the transformation experienced by the fibers in a low temperature/low pressure environment (*i.e.*, the lung).

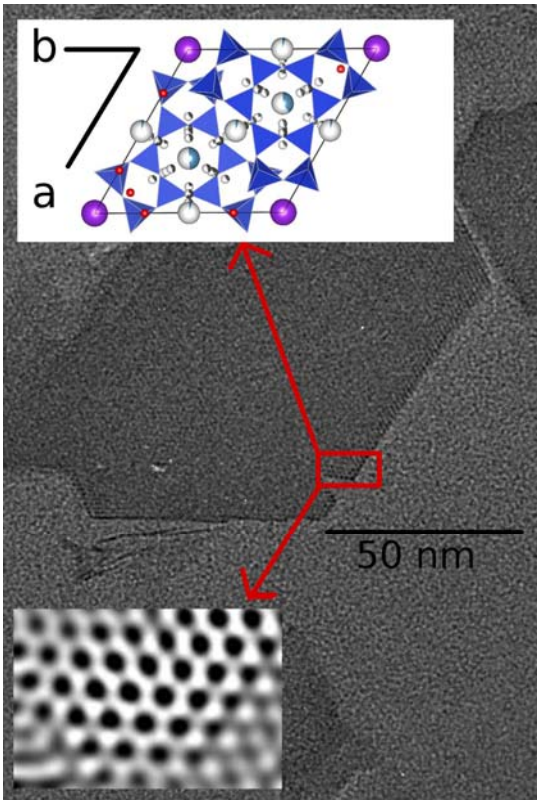

Fig. 2 - Asbestiform erionite fiber section, the high crystallinity degree is visible in the HRTEM-IFFT image. Structure and orientation are showed at the top of the image.

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