# Study of the asbestos bodies and chemical-physical modification of mineral fibres in rat histological tissues using scanning electron microscopy, and high-resolution transmission electron microscopy

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## SUMMARY

This work presents a systematic FEG-SEM and HR-TEM characterization of the morpho-chemical characteristics of both asbestos bodies and fibres found in the tissues of Sprague Dawley rats after an intrapleural injection of UICC chrysotile, UICC crocidolite and erionite from Jersey, Nevada (USA). The characterization of the fibrous materials and asbestos bodies within the organic tissues, were performed using a FEI Nova NanoSEM 450 FEG-SEM located at the CIGS (Centro Interdipartimentale Grandi Strumenti) of University of Modena. Parts of histological tissue were dissolved, and the mineral fibres recovered were subsequently analysed with a FEI TECNAI G2 200kv TEM located at The Friedrich Schiller University of Jena, and with a JEOL ARM 200F TEM located at National Institute of Chemistry of Ljubljana. Notwithstanding, the results of this study may help to better understand the mechanism of formation of asbestos bodies and why do they not form on the fibrous erionite specimen used for this study. No major change in term of crystal habit, crystallinity and chemistry of all fibre species were observed during the contact time with the organs of rats, except for chrysotile which shows signs of leaching of Mg. The combination of both scanning and high-resolution transmission electron microscopy, allowed to study in detail the structural and morpho-chemical changes that occurred in the three mineral fibres that remained in contact with the organic medium for different residence time.

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## Introduction

"Mineral fibre" is a general term that refers to a group of minerals distributed worldwide with a fibrous crystal habit. Among them, the most relevant are asbestos minerals and the fibrous zeolites erionite (Mossman *et al*., 1990; Baumann *et al*., 2013). These minerals possess a dreadful reputation because they may provoke fatal lung diseases (mainly lung carcinoma and pleural/peritoneal mesothelioma) if inhaled. The term "asbestos", is a generic industrial-commercial term used to identify a group of hydrate silicate minerals that occur as bundles of flexible fibres, that can be separated into thin, durable threads. From a mineralogical point of view, the term asbestos has no significance, but it is applied to several fibrous minerals, which under certain circumstances crystallize with an asbestiform habit (Case *et al*., 2011). The term "fibrous" refers to a mineral phase whose crystal habit consists of thin separable fibres, the term "asbestiform" refers to a mineral with a flexible structure made up from single fibres called "fibrils" (Skinner *et al*., 1988; Veblen and Wylie, 1993; Hawthorne *et al*., 2012). This peculiar crystal habit is called fibrous-asbestiform and describes minerals that grow in a fibrous aggregate of long, flexible, and thin crystals that readily separate (Institute of Medicine, 2006). Some of these minerals have been used extensively thanks to their unique physical-chemical and technological properties such as flexibility, heat resistance and ability to be woven (Williams *et al*., 2013). These include serpentine asbestos and amphibole asbestos; the fibrous-asbestiform variety of serpentine is called chrysotile (white asbestos) (Figure 1a) and represents the most commonly used form of asbestos. The amphiboles family includes five minerals: fibrous actinolite, amosite, fibrous anthophyllite, crocidolite (Figure 1b) and fibrous tremolite. This subdivision is based on the mineralogical properties which are common only to this kind of minerals, namely, long, thin, flexible fibres with high tensile strength and resistance to heat and chemicals (Case *et al*., 2011). The regulatory definitions, in addition to mineral species identification based on its mineralogy, specify physical parameters, such as length and width, which apply to and define particles that meet specific counting rules, with the purposes of identifying them and limiting the human exposure (*i.e*., ISO 10312 "ISO, 1995", NIOSH 7400 "NIOSH, 2003"). The mechanisms by which mineral fibres induce cyto- and genotoxic damage is still a matter of debate because the cause-effect relationship between exposure to the fibres and the onset of mesothelioma and other lung diseases remains unclear. Although it was proven that mineral fibres, if inhaled, may induce lethal lung diseases (Doll, 1955; Mossman *et al*., 1996; Stayner *et al*., 1996; Becklake *et al*., 2007; Kamp, 2009), the toxicity degree of chrysotile asbestos has become a controversial issue in recent years. Some researchers suggest that chrysotile has little potential in inducing mesothelioma (McDonald *et al*., 1980; Mcdonald *et al*., 1982; Sebastien *et al*., 1989; McDonald and

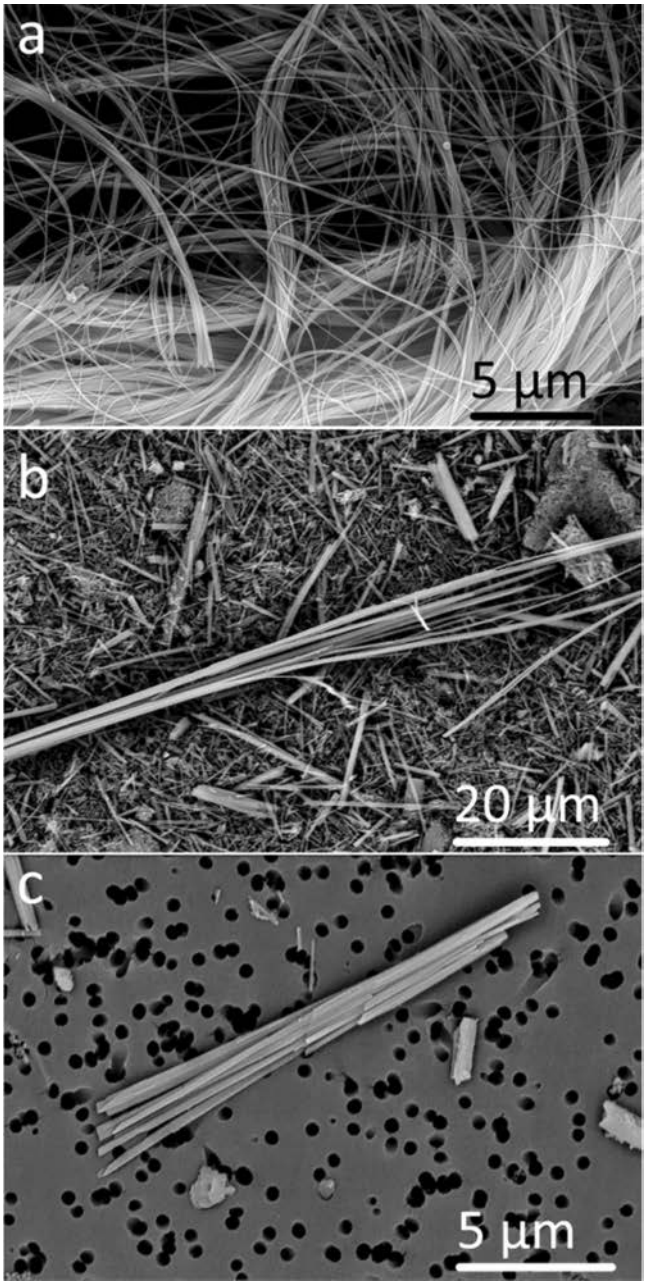


**Figure 1. SEM pictures of: a) bundle of UICC chrysotile fibres; b) UICC crocidolite fibres; c) erionite fibres.**

McDonald, 1997) promoting the safe use of chrysotile, assuming that the potential toxicity of this fibre is much lower with respect to that of fibrous amphiboles and erionite. This is the so called "*Amphibole hypothesis*", and it is supported by the evidence that chrysotile dissolves reasonably quickly (low biodurability) in the intracellular macrophage environment during phagocytosis, whereas amphiboles are much more durable (high biodurability) and remain in the lungs for a very long time (Bernstein *et al*., 2008). Others researchers claim the opposite, assuming that all above mineral fibres are indistinctly classified as potentially toxic substances (Dement and Brown, 1994; Stayner *et al*., 1996), suggesting that chrysotile produces a risk of lung cancer similar to other asbestos fibres types (Acheson *et al*., 1982; Hughes *et al*., 1987; Dement and Brown, 1994; Giaroli *et al*., 1994; Tarchi *et al*., 1994; Stayner *et al*., 1996). On the other hand, the "asbestos industry" continues to generate endless debate on the relative hazards of different forms of asbestos, without taking into account the fact that in the real world exposure is almost always to mixtures of asbestos fibres of different types and sizes (Ramazzini, 1999, 2005, 2010). Epidemiologic, experimental, and molecular evidence suggests that the arguments for the role of fibre size relative to dose, dose-response effect, and genetic susceptibility are fraught with enormous uncertainties (Tomatis *et al*., 2007), rejecting the assertion that chrysotile is safe (Smith and Wright, 1996; Stayner, Danhovic and Lemen, 1996; Landrigan *et al*., 1999; Bang *et al*., 2001; Lemen, 2004; Tomatis *et al*., 2007; Ramazzini, 2010) and demonstrating that the so-called "controlled use" of asbestos is a mistake (Lemen, 2004). At the moment all amphibole asbestos minerals are banned worldwide whereas chrysotile is banned only in the countries where the line of the International Agency for Research on Cancer (IARC) of the World Health Organization and the National Toxicology Program has been fostered (Mossman and Churg, 1998; Holland and Smith, 2001; Yano *et al*., 2001; Roggli *et al*., 2002; Pfau *et al*., 2005; Yarborough, 2007). Asbestiform erionite (Figure 1c) is a potent human carcinogen listed by the IARC as a Group 1 Carcinogen which has not been banned to date simply because it has never been used for industrial applications like asbestos minerals. This work is part of a long-term Italian Research Project of National Interest (PRIN) in progress since 2011, aimed to better understand the nature of the biological interaction mechanisms of mineral fibres and developing a general model of fibres' toxicity/pathogenicity. The outcome of this multidisciplinary long-term study allowed to define the macro- and micro-parameters responsible for the bio-chemical activity of the fibres and assess their specific role. As a first step all the physical-chemical and crystallographic parameters related to toxicity (mineralogical characteristics such as the presence of impurities, morphometric parameters, iron and toxic elements, biodurability, surface charge) have been identified and systematically investigated using a set of standard fibres (Pollastri *et al*., 2014; Bursi Gandolfi *et al*., 2016). This project involved also a collaboration with the Ramazzini Cancer Research Institute of Bentivoglio (Bologna) that used the same set of mineral fibres studied within the PRIN2011 for a long term *in vivo* experiments with rats, for the determination of their cancerogenicity. The rats were let to live until spontaneous death, so it was possible to collect histological tissue at different contact time (ct) with the purpose to understand the structural and physical-chemical variation of the inoculated mineral fibres, and the morphological and chemical characteristics of the asbestos bodies found in the tissues (Bursi Gandolfi *et al*., 2016).

## Asbestos bodies formation

When asbestos fibres are found in the lung parenchima usually look like surrounded by a thick and dense coating called asbestos bodies (ABs). ABs formation is a complex mechanism involving different factors, such as the nature of the fibre, its morphometry and its surface activity. They were first described by Marchand in 1906 as peculiar pigmented crystals that contain asbestos fibres (Marchand, 1906; Churg and Warnock, 1981), and their formation is essentially due to a biological coating process that begins with deposition of a ferritin layer (organic Fe-storage complex) (Richter, 1958; Davis, 1965) around the fibre. In general, fibres coated by ABs always coexist with uncoated fibres (Pooley, 1972). Fibre size and diameter also play a key role. It was observed that ABs generally occur on fibres 10-60 µm long and with a mean diameter greater than d > 0.5 µm (Gloyne, 1929). They rarely occur on asbestos fibres with L <10 µm, because phagocytosis acted by macrophages occurs successfully. Regarding the chemistry of ABs, Harrison *et al*. (1967) showed that the ferritin core is a ferric oxyhydroxide, presumably (FeOOH) or ($FeOOPO_3H_3$), if phosphate is present. Meyer (1970) confirmed the presence of Ca and P in ABs. According to those authors, ABs formation is an extracellular mechanism and the various configurations found might reflect repeated contact of the same AB with different macrophages. This work reports the results of the combined systematic field emission gun-scanning electron microscopy (FEG-SEM) study aimed to understand the structural and physical chemical variation of the mineral fibres used for the *in vivo* experiments, and the morpho-chemical characteristics of the ABs found in the tissues of rats (Bursi Gandolfi *et al*., 2016). The structural modifications, with special attention to the fibre crystallinity, of the same fibrous specimen has been investigated by

high resolution electron transmission microscopy (HR-TEM) (Gualtieri *et al*., 2017).

## Materials and Methods

The choice of chrysotile, crocidolite and erionite fibres was prompted by the relevance of these mineral fibres. Chrysotile is a serpentine layer silicate with ideal formula $Mg_3(OH)_4Si_2O_5$; crocidolite is the fibrous variety of riebeckite, an amphibole double-chain silicate with ideal composition $Na_2Fe_3^{2+}Fe_2^{3+}Si_8O_{22}(OH)_2$; erionite is a fibrous zeolite with mean chemical formula $K_2(Na, Ca_{0.5})_8[Al_{10}Si_{26}O_{72}]\cdot 30H_2O$. The complete characterization of the raw mineral fibres has been fully described elsewhere (Pollastri *et al*., 2014; Pollastri, 2015; Bloise *et al*., 2016). These mineral fibres were used in the past during *in vivo* tests conducted at the Ramazzini Cancer Research Institute of Bentivoglio Bologna, Italy (Maltoni and Minardi, 1989).

The tests were carried out using groups of 40 (20 males and 20 females) 6/8-week-old Sprague-Dawley rats, treated by a single 25 mg injection of each mineral fibre in 1 ml of $H_2O$. The tested animals were let to live until spontaneous death (no euthanasia). The results of the *in vivo* study, involving 3 years long experimental bioassays, proved that all the tested fibres are mesotheliomatogenic and prompted the ban of such mineral fibres worldwide (Ramazzini, 1999). All the morphological and chemical information were obtained using a FEG-SEM FEI Nova Nano-SEM 450 available at CIGS (Centro Interdipartimentale Grandi Strumenti) at the University of Modena and Reggio Emilia. The fibres were first studied *in situ* in the histological slides through the *low vacuum* operation mode of the instrument, in order to avoid the coating of the specimen ready to be analysed with other different techniques. To perform accurate dimensional and structural analyses of both mineral fibres and ABs, it was necessary to separate the fibres from the organic medium. To do this, a standardized tissue digestion protocol was applied (Vigliaturo *et al*., 2016). The HR-TEM data for chrysotile were collected using a FEI TECNAI G2 200kv TEM located at The Friedrich Schiller University of Jena. For the TEM observations, fibres were deposited over a Cu grid with lacey carbon support films. A liquid nitrogen cold trap was used to prevent the formation of vapour in the electron beam column. Through the use of HR imaging, it was possible to detect the presence of a halo of amorphous material around the fibres. The selected area electron diffraction patterns (SAED) allowed to confirm this observation and to identify the mineral phases. Semi-quantitative analysis was performed through the EDSX detector of the instrument with the aim to show possible variation of the chemistry of the mineral fibres at different ct with the organic medium. Crocidolite and erionite-Na fibres were also studied through HRTEM, using an 80 kV Cs-probe-corrected cold-field-emission transmission electron microscope JEOL ARM 200F TEM, located at National Institute of Chemistry of Ljubljana.

## Results

### SEM-FEG

A detailed description of the obtained results is present in Bursi Gandolfi *et al*., (2016). Figure 2 is a gallery of FEG-SEM images of the observed fibres *in situ*, and after the digestion process of the surrounding histological tissue. ABs occur in a variety of forms, and different morphologies were observed. They may vary from cylindrical, elliptical, curved and may produce globular clusters at one or both ends of the fibre. The length of ABs formed on chrysotile fibres (Figure 2a, *in situ* sample) ranges from 1.5 to 20 µm and diameters range from 0.6 to 15 µm. Although very large structures as long as 40-80 µm were also observed in correspondence to the termination of the chrysotile bundles (Figure 2b, sample from digestion process). Concerning crocidolite (Figure 2c, *in situ* sample), the size of ABs varies in length from 4 µm to 25 µm, and diameter from 4 µm to 8 µm. Figure 2d (sample from digestion process) shows globular structures that tend to develop on a fibre with a globular and elliptical shape. The systematic observation of the histological samples highlighted that ABs do not coat the erionite fibres (Figure 2d, *in situ* sample) used for this study. Figure 2e shows erionite fibres after the digestion process of the tissues, they display the typical fibrous habit with stocky short fibres. The subsequent morphometric analysis allowed to calculate the fibre length that ranges from 2.7 to 3.7 µm and the diameters that ranges from 0.75 to 0.98 µm. All the examined samples present uncoated fibres. The ratio of coated fibres was 3.3% for chrysotile and 6% for crocidolite. ABs appear in chrysotile and crocidolite in after 40 weeks from the fibres injection. This times of formation are in concert with literature data (Roggli, 2014). All examined Abs are mainly composed of Ca and P and small contents of Mg, Si and Fe. Micro-Raman analysis performed on ABs of both chrysotile and crocidolite displayed the typical pattern of apatite. Concerning only chrysotile, the EDS spot semi-quantitative analyses of fibres with different ct highlighted an increase of the Si:Mg ratio with respect to the pristine standard chrysotile UICC sample. This result suggests that a Mg leaching process could occur during the contact of the fibre with the biological environment (Bursi Gandolfi *et al*., 2016).

## TEM

A detailed description of the obtained results is present in Gualtieri *et al*., (2017). To verify if the leaching of Mg observed during the FEG-SEM study of the chrysotile fibres leads to changes in the crystal structure, a HR-TEM analysis campaign was performed. Figure 3 shows TEM pictures of chrysotile, crocidolite and erionite fibre from the tissues of the rats at different ct. Pristine UICC chrysotile (Figure 3a)

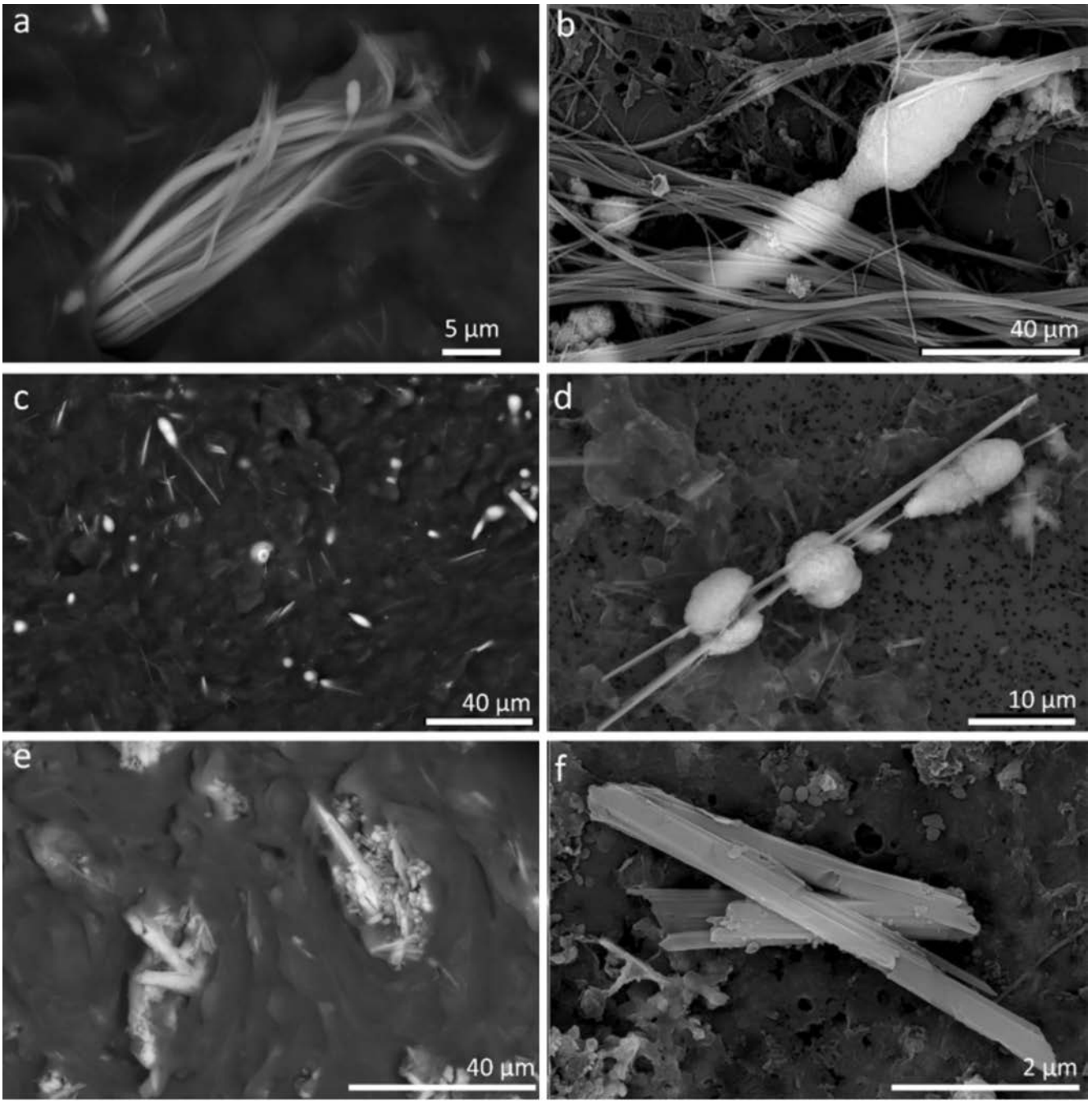


**Figure 2. SEM pictures of: a) *in situ* chrysotile fibres and asbestos bodies after 83 weeks of contact time; b) chrysotile fibres from the tissues digestion process; c) *in situ* crocidolite fibres and asbestos bodies after 80 weeks of contact time; d) crocidolite fibres from the tissues digestion process; e) *in situ* erionite fibres after 95 weeks of contact time; f) erionite fibres from the tissues digestion process.**

was analysed as reference standard. After 71 weeks of ct (Figure 3b) it is evident the presence of an outer layer of amorphous material formed due to the contact of the fibre with the organic tissue. This prolonged contact with the biological environment prompts also changes in the morphology of the mineral, where the surface of the fibre looks like eroded (Figure 3c). These changes highlight a decrease of the whole crystallinity of the fibres confirmed by the respective electron diffraction patterns After 80 weeks of ct the fibres have lost their original crystal habit (Figure 3d) and

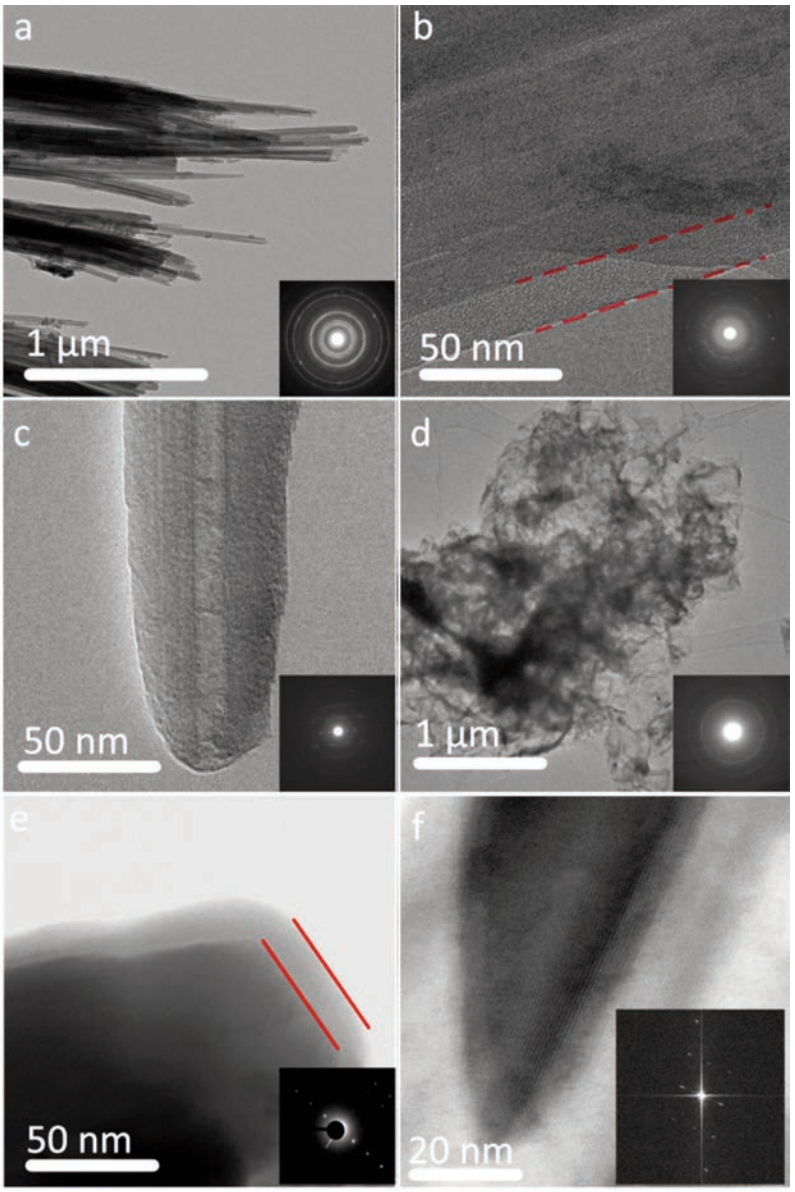


**Figure 3. a) TEM picture of a pristine bundle of UICC chrysotile fibres used as a standard. b) HR-TEM picture of a chrysotile fibre after 71 weeks of contact time, the dashed red lines show the presence of an outer layer of amorphous material, confirmed by the respective diffraction pattern. c) HR-TEM picture of a chrysotile fibre after 71 weeks of contact time, the surface of the fibre is eroded due to the contact with the organic tissue. d) TEM picture of a chrysotile after 80 weeks of contact time, the fibres have lost their original crystal habit and are fully dissolved, the respective electron diffraction patterns confirm the amorphous nature of this material. e) HR-TEM picture of a erionite fibre after 104 weeks of contact time, the red lines show the presence of an amorphous layer of few nm that surround the fibres, but the mineral is still crystalline. f) HR-TEM picture of a crocidolite fibre after 80 weeks of contact time, the mineral is still crystalline as confirmed by the diffraction pattern.**

are fully dissolved. The result of the prolonged contact with the organic environment is the dissolution of the chrysotile fibres. This residue is formed of silica rich nanoparticles of amorphous nature, confirmed by the weak rings and halos of the diffraction pattern. The HR-TEM study conducted over the erionite (Figure 3e) and crocidolite (Figure 3f) fibres evidenced the presence of an amorphous layer of few nm that surround the fibres, but the crystallinity of the mineral phase is still preserved after 104 (erionite) and 80 (crocidolite) weeks of ct.

## Discussion

To better understand the mechanism of formation of asbestos bodies and the subsequent morphological and chemical modification of the mineral fibres surrounded by the coating, a systematic FEG–SEM investigation was performed to study the characteristics of both fibres and ABs found in tissues of rats from *in vivo* experiments. The characterization of UICC chrysotile, UICC crocidolite and fibrous erionite samples after prolonged ct with organs of rats, evidenced the occurrence of chemical and morphological changes in respect to pristine specimen. The most interesting results is that chrysotile undergoes partial decomposition with leaching of Mg due to the prolonged contact with the biological environment, and that ABs do not nucleate over erionite fibres. The ABs formation model (presented in Bursi Gandolfi *et al*., 2016) reports that ABs only form over long fibres that cannot be engulfed by macrophages. Erionite displays very short fibres (mean L = 3.2 µm) and phagocytosis occurs successfully (no frustrated phagocytosis as for long chrysotile and crocidolite fibres). The first defence mechanism of the body against airborne particles, is the filtration of the breathed air in the nasopharyngeal region (nose, nasal cavity and throat). If a fibre reaches the lung parenchyma, the second defence mechanism of the body is the phagocytosis process acted by alveolar macrophages. Fibres shorter than macrophage mean diameter (< 10 µm) can be engulfed and embedded within phagolysosomes (pH = 4-4.5), fibres longer than macrophage mean diameter cannot be engulfed (Stanton *et al.*, 1981; Donaldson *et al.*, 2010) and frustrated phagocytosis accompanied by inflammatory burst occurs with release extracellularly of highly reactive cyto- and genotoxic substances. The engulfment mechanism permits to dissolve only chrysotile but not crocidolite and erionite fibres that are very stable at acid pH. The last available defence mechanism is then the formation of the ABs with the aim to isolate the fibre from the cellular environment. Following the SEM-FEG study, the HR-TEM characterization allowed to achieve a thorough and comprehensive picture of the changes in the crystal structure, chemistry and morphology of the fibres after the interaction with the biological medium. The decrease of the overall crystallinity with partial decomposition of the chrysotile fibres during ct with the organs of rats, is a consequence of the leaching of Mg. The result of this prolonged chemical interaction is the formation of an amorphous silica-rich relict. The earlier step of this process of dissolution is called *pseudomorphosis* (Giacobbe *et al*., 2010; Gualtieri *et al*., 2013), and may evolve later with the physical destruction of the fibres as described by Bernstein *et al*., (2013). On the other hand, crocidolite and erionite fibres demonstrate their stability even at very long ct within the tissues of the rats. Only a thin amorphous layer (in a range of 1-2 nm) that surrounds the fibres was observed (Gualtieri *et al*., 2017).

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