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Surface reactivity and cell responses to chrysotile asbestos nano-fibers

Francesco Turci^{†,‡,#}, Massimiliano Colonna^{†,‡,#}, Maura Tomatis^{†,‡}, Stefano Mantegna[§], Giancarlo Cravotto^{†§}, Giulia Gulino^{†||}, Elisabetta Aldieri^{†,||}, Dario Ghigo^{†,||}, and Bice Fubini^{†,‡,}*

[†] “G. Scansetti” Interdepartmental Center for Studies on Asbestos and other Toxic Particulates, Università di Torino, Torino, Italy

[‡] Dipartimento di Chimica, Università di Torino, Torino, Italy

[§] Dipartimento di Scienza e Tecnologia del Farmaco, Università di Torino, Torino, Italy

^{||} Dipartimento di Genetica, Biologia e Biochimica, Università di Torino, Torino, Italy

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Prof. Bice Fubini

Dip. Chimica

University of Torino

Via P. Giuria, 7

I-10125, Torino, Italy

Tel +39 011 670.75.66

Fax +39 011 670.75.77

e-mail: bice.fubini@unito.it

FT and MC equally contributed to this work.

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Running Title: Nanometric asbestos on epithelial cells: lower or higher toxicity?

Abstract

High aspect-ratio nanomaterials (HARNs) have recently attracted great attention from nanotoxicologists because of their similarity to asbestos. However, the actual risk associated with the exposure to nanosized asbestos - which escape most regulations worldwide - is still unknown. Nanometric fibers of chrysotile asbestos have been prepared from two natural sources to investigate whether nanosize may modulate asbestos toxicity and gain insights on the hazard posed by naturally occurring asbestos which may be defined as HARNs because of their dimensions. Power ultrasound was used to obtain nano-fibers from two different chrysotile specimens, one from the dismissed asbestos mine in Balangero (Italian Western Alps), the other from a serpentine outcrop in the Italian Central Alps. Electron microscopy, X-ray diffraction and fluorescence spectroscopy revealed that the procedure does not affect mineralogical and chemical composition. Surface reactions relating to oxidative stress - free radical generation, bio-availability of iron and antioxidant depletion - revealed a consistent reduction in reactivity upon reduction in size. When tested on A549 human epithelial cells, the pristine but not the nano-sized fibers, proved cytotoxic (LDH release), induced NO production and caused lipid peroxidation. However, nano-fibers still induced some toxicity relevant oxidative-stress activity (ROS production) in a dose-dependent fashion. The reduction in length and a lack of poorly-coordinated bio-available iron in nano-chrysotile may explain this behavior. The present study provides a one-step procedure for the preparation of a homogeneous batch of natural asbestos nano-fibers and shows how a well-known toxic material might not necessarily become more toxic than its micrometric counterpart when reduced to the nanoscale.

Keywords: Chrysotile asbestos; nano-fiber; HARNs; iron; cellular toxicity; ultrasound

¹HARNs: High Aspect Ratio Nanomaterials; LDH: Lactate Dehydrogenase; ROS: Reactive Oxygen

Species; CNTs: Carbon Nanotubes; NOA: Natural Occurring Asbestos; SFA: Short Amosite Fiber; US: Ultrasound; SEM: Scanning Electron Microscopy; XRD: X-ray diffraction; XRF: X-ray Florescence; FBS: Fetal Bovine Serum; BCA: bicinchoninic acid; AA: Ascorbic Acid; DCFH-DA: 2',7'-dichlorodihydrofluorescein diacetate; TBARS: thiobarbituric acid-reactive substances; RNS: Reactive Nitrogen Species; DMPO: 5,5-Dimethyl-1-pyrroline *N*-oxide

1 **Introduction**

2 *Why investigate the potential toxicity of asbestos nano-fibers?* The general alarm on the possible
3 hazard associated to the exposure to nano-materials is often related to the change of toxicity
4 occurring when nano-dimension is achieved. For instance, substances generally considered safe for
5 humans in the micrometric range (e.g., TiO₂, Fe_xO_y, amorphous SiO₂ and carbon) might induce
6 detrimental effects on human health when at the nanometric scale.¹⁻³ The scientific community has
7 recently devoted some attention to the so-called high aspect ratio nanomaterials (HARNs¹),
8 indicating that their nanoscaled dimension is the source of toxic potency.^{4,5} Noticeably, several
9 studies⁶⁻⁸ and reviews⁹⁻¹² have been devoted to the comparison between the behavior of asbestos
10 and the most studied HARN so far, carbon nanotubes (CNTs). CNTs appear to show similarities
11 with asbestos in the induction of an inflammatory response and malignant mesotheliomas following
12 intraperitoneal injection in both rats and mice. In these studies CNTs were mostly compared to
13 amphibole asbestos, even if they more closely resemble chrysotile, the asbestos mineral form of
14 serpentine group, which is more flexible and curled than amphiboles (e.g., crocidolite, amosite, and
15 tremolite). In this respect a fibrous nano-chrysotile would be a more appropriate material for a
16 comparison with CNTs and in general with HARNs. Furthermore, asbestos nano-fibers may be
17 present both in urban areas and in natural environment. Most of the nano-fibers found in the past in
18 urban areas were released from several asbestos-containing products (e.g., automotive brake pads)
19 following mechanical stress.¹³ This latter source of airborne fibers is progressively losing importance
20 following the worldwide accepted asbestos ban for friction products. In the natural environment, the
21 vast majority of the nano-sized fibers are found in the turbulent waters that flow trough serpentinite

22 outcrops^{14,15} and in the surroundings of both active and inactive asbestos mines.^{16,17} These nano-
23 fibers may become airborne following agricultural irrigation or seasonal floods and may
24 subsequently become a source of hazard for rural workers and the general population.¹⁸ It is worth
25 noting that asbestos fibers, both amphibole and chrysotile, found in natural waters generally have
26 thinner diameters than fibers commonly investigated in toxicological studies (e.g. UICC samples).
27 Moreover the length of waterborne fibers - though highly source-dependent - is usually very short,
28 ranging from 0.1 to 3.0 μm .^{15,19} The chemical composition of waterborne asbestos – both
29 amphiboles and chrysotile – is not significantly modified by the leaching of the fibers in water,²⁰
30 whereas the surface area is usually increased, at least for chrysotile nano-fibers found in rivers,
31 likely due to the natural fiber-splitting effects due to turbulence.¹⁷

32 *Size dependent asbestos toxicity.* The toxicity and carcinogenicity of various asbestos forms is well
33 established¹. Inhalation of asbestos fibers causes asbestosis, lung cancer and pleural mesothelioma.
34 Three main factors contribute altogether to the development of the above pathologies: fiber length,
35 biopersistence and surface reactivity. It is generally agreed that thin and long fibers of both
36 amphiboles and serpentine cause more mesotheliomas in rodents than the shorter ones mainly
37 because of different mechanisms of clearance.^{21,22} Long fibers trapped in the lung would induce a
38 continuous release of fiber-derived free radicals, cell-generated ROS (Reactive Oxygen Species) and
39 cytokines, all contributing to chronic inflammation and eventually DNA damage.^{23,24}

40 The World Health Organization²⁵ rated consequently respirable asbestos fibers (diameter < 3 μm)
41 as a health hazard under worldwide regulations only if longer than 5 μm . Shorter fibers, however
42 should not be fully disregarded when considering the potential toxicity of a given fibrous mineral. In
43 a critical review on asbestos fiber length and pathogenicity, Dodson claimed that also shorter fibers
44 may contribute to the pathological response.²⁶ Nano-fibers (diameter of < 0.2 μm , length of few
45 micrometers) have been recovered both in exposed workers and in subjects exposed to
46 environmental pollution only.²⁷ It is still unclear if these nano-fibers are the result of the splitting up
47 of long fibers into sub-micrometric fibrils - which may take place in the lung²⁸ - or if they are
48 inhaled already in nanometric size. Therefore, studies on the toxicity of natural asbestos nano-fibers

49 which are often present in the environment,^{15,17,19} but are disregarded by regulations, are stringently
50 required.

51 *Previous attempts to prepare nano-fibers.* Data on the toxicity of real asbestos nano-fibers are in
52 fact extremely scarce, if any. Short crocidolite and chrysotile fibers were reported as cytotoxic to
53 macrophages in vitro.^{29,30} The large part of the studies on small fibers however refers to short but
54 relatively thick fibers, having a low aspect ratio. For instance 70% of the largely studied short
55 amosite fiber (SFA) had an aspect ratio below 3.³¹ A pure iron-free synthetic nano-chrysotile was
56 prepared and studied by some of us^{32,33} in the context of the role of iron in reactivity and toxicity of
57 asbestos, but the dimensional factor could not be considered at that time.

58 The lack of data on asbestos nano-fiber toxicity is due to the difficulty in obtaining homogenous
59 batches of short and thin fibers retaining all the mineral fiber characteristics. Mechanical milling
60 widely used in the past to prepare short amosite fibers mainly induces truncations perpendicularly to
61 the fiber axis^{31,34}. Consequently the diameter of short fibers obtained by milling is close to the one of
62 pristine sample with a marked decrease in aspect ratio. Furthermore mechanical fracturing may
63 increase the percentage of isometric particles,^{35,36} may modify crystallinity with partial
64 amorphization of surface layers^{35,37,38} and may also induce profound modifications in surface
65 reactivity.^{34,35} Alteration upon length reduction varies with different fiber types and it is related to
66 the time of milling³⁷. Milling as a means to prepare reference samples of asbestos fibers reduced in
67 size was therefore soon discarded.^{36,39}

68 Repeated centrifugations²⁹ or subsequent aqueous sedimentations⁴⁰ allow separation of fibers with
69 a shorter length and narrower diameter. However, both procedures require a large amount of the
70 pristine sample in order to obtain sufficient amount of short fibers. Moreover surfactants employed
71 to promote fiber bundles separation may likely remain adsorbed at the fiber surface, eventually
72 altering fiber behavior.

73 *Aim of the present study.* The present study was undertaken with the specific aims of:

74 a) Developing a size-selective procedure to prepare short (< 5 μm) asbestos nano-fibers similar to
75 natural waterborne nano-fibers and suitable for biological studies;

76 b) Testing the potential toxicity of these nano-fibers by comparing their behavior to pristine fibers
77 in toxicity related cell-free and cellular tests.

78 Previous studies have shown that application of ultrasound (US) can efficiently break down
79 chrysotile fibers.⁴¹ Low ultrasonic energy (< 20 kHz, power density 0.5 W/ml) or short exposure (<
80 10 minutes) time do not produce significant effects on both serpentine and amphibole asbestos
81 length and have practically no influence on the crystal structure.⁴² A few hours treatment at 50 kHz
82 (power density 0.1 W/ml) promotes the separation of fiber bundles in thinner fibrils.³⁶ while longer
83 exposure periods - several hours at 19.2 kHz, power density 3 W/ml - deeply reduce fiber length of
84 chrysotile asbestos.⁴³ Finally, when sonication is carried out in water suspension containing metal
85 chelators, chrysotile disappears following disruption of the crystal structure and full loss of the
86 original fibrous habit.^{41,43,44}

87 On the basis of the above findings we have here investigated the effect of ultrasonic treatments on
88 size, shape and structure in mild conditions to achieve separation without structural modification of
89 nano-fibers from natural chrysotile fiber bundles. A well characterized specimen⁴⁵ from the Italian
90 Central Alps (Val Malenco) was employed to identify the best conditions to produce homogenous
91 batches of short chrysotile asbestos fibers.

92 Ultrasonic treatment was carried out in water for different time periods (from 3 to 24 hrs). The
93 final products were checked for morphology (SEM), crystallinity (XRD) and elemental analysis
94 (XRF) to report any change occurred during sonication.

95 The best preparation protocol for nano-fibers was then applied also on a chrysotile specimen from
96 the Balangero mine, Italy. The potential toxicity of the two samples was compared with the well-
97 assessed toxicity of the original asbestos by evaluating:

- 98 a) Surface properties considered relevant in asbestos health effects;
- 99 b) Cellular responses in human lung epithelial cells.

100 Among the most relevant surface properties involved in asbestos toxicity²³ we have examined the
101 potential to cause oxidative injury within the lung through free radical generation, a simultaneous
102 depletion of antioxidant defenses and the amount of bio-available iron at the fiber surface following

103 previously set up procedures.^{34,46,47} Cytotoxic and oxidative effects of pristine and nano-sized
104 chrysotile fibers were measured on A549 cell line as leakage of lactate dehydrogenase (LDH) into
105 the extracellular medium, measurement of ROS production, lipid peroxidation⁴⁸ and nitric oxide
106 (NO) production.⁴⁹ A549, employed in milestone studies on asbestos toxicity⁵⁰ were chosen because
107 of their key role in inflammation, fibrogenesis, and carcinogenesis elicited by asbestos fibers⁵¹ and
108 have been described as the targets of asbestos-associated lung carcinomas.⁵²
109

Materials and methods

Asbestos

Two pure chrysotile specimens (see Table S1 in supporting materials), employed and thoroughly characterized in previous investigations^{44,45} have been considered for the present study: a mineral sample from Val Malenco (Italian Central Internal Alps) hereafter indicated as CTL-VM and a commercial sample (CTL-BM) from the Balangero dismissed asbestos mine (Italy), kindly provided by R.S.A. the society managing the mine. These two natural chrysotiles are made up of bundles of fibers with a diameter of about few microns where several fibers exhibit a length longer than tens of microns. To promote bundle separation and obtain specimens suitable for biological tests, the two natural chrysotiles have been suspended in water and sonicated for $t < 1$ min. at 10 W/ml and 20 kHz with a probe sonicator (Sonoplus, Bandelin, Berlin, Germany). These samples are hereafter referred as “pristine” with micrometric dimensions. The surface area of the pristine natural chrysotile specimens from Val Malenco and Balangero is 78 and 15 m²/g respectively.

Reagents

Fetal bovine serum (FBS) and RPMI 1640 medium were supplied by BioWhittaker (Verviers, Belgium); plasticware for cell culture was from Falcon (Becton Dickinson, Franklin Lakes, NJ). The protein content of cell monolayers and cell lysates was assessed with the BCA kit from Sigma Chemical Co (St. Louis, MO). When not otherwise specified, other reagents were purchased from Sigma Chemical Co.. For all experiments ultrapure Milli-Q (Millipore, USA) water was used.

Ultrasonic treatment of chrysotile

The ultrasonic apparatus was composed by a titanium air-cooled horn screwed on two piezoelectric rings (PZT403 type piezoceramic Rings O/D 50, 80 mm Morgan Ceramics). Pristine chrysotile specimens were suspended in Milli-Q ultrapure water (10 mg/ml) and placed into the US reactor. The horn frequency was set to 21 kHz and stabilized with an automated adjustment device (frequency hook). Three different sonication times (3, 6 or 24 hours) at an input power of ca. 100 W (2 W/ml) were tested. The suspension was cooled below 50 °C and evaporation of the liquid

136 minimized by sealing the US reactor. Following the US treatment, the nano-fibers were centrifuged,
137 rinsed with distilled water and dried.

138 On the basis of the results obtained a three hour-long optimized protocol was adopted to prepare
139 few grams of nano-fibers of both specimens.

140 **Crystallinity**

141 XRD analyses were performed on the solid residues with a Phillips PW 1830, with θ - 2θ geometry
142 and Cu K α radiation. The data were obtained by scanning the 2θ range 3–80° at a speed of 0.5°/min.
143 The patterns obtained were compared with those contained in the J.C.P.D.S. (Joint Committee of
144 Powder Diffraction Standard) archives.

145 **Chemical composition**

146 All samples were analyzed using an EDAX Eagle III energy-dispersive X-ray Florescence
147 spectrometer (micro-XRF) equipped with a Rh X-ray tube and a polycapillary exciting a circular
148 area of nominally 30 μ m diameter. Data collection was performed with 45 s detector live time, X-ray
149 tube settings adjusted for 30% dead time. About $1 \cdot 10^6$ Cps were counted per scan. At least 6 points
150 were collected for each sample.

151 **Size and morphology**

152 Fiber size analysis was performed by Scanning Electron Microscopy (SEM). SEM observations
153 were performed with a Stereoscan 410 Leica equipped with Oxford Link EDAX, using a secondary
154 electron detector. The images were obtained on gold-coated samples (coating time 60 s, current 19
155 mA), operating at very low current ($I = 5$ pA), with accelerating voltage of 15 kV and working
156 distance of 5 mm. SEM images were captured in the range 2000-10,000 \times , in order to visualize and
157 evaluate size of both long and thin fibers. To dimensionally characterize the fibers, SEM images
158 were analyzed using “ImageJ” software suite, developed at the National Institutes of Health –US
159 Federal Government, not subject to copyright protection and available to the public domain on the
160 Internet (<http://rsb.info.nih.gov/ij/>). For each sample a statistically significant number of fibers from
161 several microscopic fields were examined (see Table S2).

162 **Surface area**

163 Surface area of the pristine and treated chrysotile fibers was measured by means of the BET
164 method based on N₂ adsorption at 77 K (ASAP 2020 Micrometrics, Norcross, GA)

165 **ζ-potential**

166 The ζ-potential of the nano-fibers was evaluated by means of electrophoretic light scattering (ELS)
167 (Zetasizer Nano-ZS, Malvern Instruments, Worcestershire, U.K.). The chrysotile specimens were
168 suspended (6 mg / 10 ml) in ultrapure water (MilliQ) and rapidly sonicated (t < 1 min, 10 W/ml, 20
169 kHz, Sonoplus, Bandelin, Berlin, Germany). The ζ-potential was measured after adjusting the pH
170 step by step by addition of 0.1 M NaOH or 0.1 M HCl.

171 **Bio-available iron**

172 The total amount of removable iron was determined upon incubation in an aqueous solution of
173 ferrozine (a strong chelator specific for Fe²⁺) in the presence of ascorbic acid, which fully reduces
174 Fe³⁺ ions to Fe²⁺ 47. Pristine and nano-fibers (1 mg/ml) were suspended (up to a final volume of 20
175 ml) in a solution of ferrozine (1 mM) containing ascorbic acid (1 mM) and stirred for 16 days at 37
176 °C. At regular time intervals, aliquots of the suspension were taken and centrifuged at 10,000 rpm
177 for 20 minutes to remove the asbestos. The total amount of iron present in the supernatant was
178 determined spectrophotometrically on a Uvikon 930 dual beam spectrophotometer (Kontron
179 Instrument) by measuring the absorption of the iron-ferrozine complex at 562 nm (E_{mM} = 27.9 mM⁻¹
180 cm⁻¹). The experiments were performed in duplicate. The results are expressed per unit surface area
181 and reported as average values ± standard deviation.

182 **Cysteine and Ascorbic acid depletion**

183 *Cysteine*: Suspensions of micro- and nano-fibers were prepared by adding 20 mg of each sample
184 to 2 mL of a 0.1 M solution of cysteine in phosphate buffer (0.01 M, pH 7.4). The suspensions were
185 stirred for 1 hour at 37°C and after the fibers were separated from the solution by filtration. The
186 amount of cysteine in solution was measured spectrophotometrically (Uvikon 930) at 412 nm by the
187 Ellman's reagent.

188 *Ascorbic Acid (AA)*: Suspensions of micro- and nano-fibers (0.5 mg/mL) were prepared in a 0.09
189 mM solution of AA in phosphate buffer (0.01 M, pH = 7.4). The suspensions were stirred at 37°C

190 for 6 hrs. At regular time intervals, the suspensions were centrifuged (RCF = 8500 g, 10 min) and
191 the amount of AA in solution was measured spectrophotometrically (Uvikon 930) at 265 nm.

192 All experiments were performed in duplicate. The results are expressed per unit surface area and
193 reported as average values \pm standard deviation.

194 **Free radical generation**

195 The radical release upon incubation of chrysotile samples, with either H₂O₂ (yielding hydroxyl
196 radicals) or sodium formate (yielding carbon centered radicals following homolytic cleavage of C-H
197 bonds) was detected using the spin trapping technique with 5,5'-dimethyl-1-pyrroline-*N*-oxide
198 (DMPO) as trapping agent.⁴⁶ The radical adducts formed were monitored by Electron Paramagnetic
199 Resonance (EPR) spectroscopy. All spectra were recorded on a Miniscope MS 100 (Magnetech,
200 Berlin, Germany) EPR spectrometer. The instrument settings were as follows: microwave power 10
201 mW; modulation 1000 mG; scan range 120 G; centre of field approximately 3345 G. The number of
202 radicals released is proportional to the intensity of the EPR signal. The signals were double
203 integrated and numeric values were reported as arbitrary units, in order to compare the production of
204 free radicals by the mineral fibers. Blanks were performed in parallel in the absence of any fiber. All
205 the experiments were repeated at least twice.

206 - The generation of \bullet OH radicals was measured by suspending 25 mg of fibers in 500 μ L of 1 M
207 phosphate buffered solution (pH 7.4), then adding 250 μ L of 0.17 M DMPO (the spin trap
208 agent) and 250 μ L of 0.20 M H₂O₂. The radical formation was evaluated by recording the EPR
209 spectrum of the [DMPO-OH] \bullet adduct at 10, 30 and 60 min.

210 - The generation of \bullet COO⁻ radicals was measured by suspending 25 mg of fibers in 250 μ L of
211 0.17 M DMPO, then adding 250 μ L of 60 mM ascorbic acid (in phosphate buffer 1 M) and
212 500 μ L of 2 M of sodium formate (in phosphate buffer 1 M). The radical formation was
213 evaluated by recording the EPR spectrum of the [DMPO-COO⁻] \bullet adduct at 10, 30 and 60 min.

214 **Cellular tests**

215 Human pulmonary epithelial cells (A549) were provided by Istituto Zooprofilattico Sperimentale
216 “Bruno Ubertini” (Brescia, Italy). The cells were cultured in 35 or 100 mm-diameter Petri dishes in
217 RPMI-1640 + 10% fetal bovine serum (FBS) up to confluence, and then incubated for 24 hrs in the
218 absence or presence of natural or treated chrysotile fibers before the assays. The protein content of
219 the monolayers and cell lysates was assessed with the BCA kit.

220 **Measurement of cellular parameters**

221 *Cytotoxicity.* After a 24 hrs incubation in the absence or presence of 3, 6, 15 and 25 $\mu\text{g}/\text{cm}^2$ of
222 micro- and nano- chrysotile, the cytotoxic effect was measured as leakage of lactate dehydrogenase
223 (LDH) into the extracellular medium.⁵³ Briefly, the extracellular medium was collected and
224 centrifuged at 13,000 x g for 30 min. The cells were washed with fresh medium, detached with
225 trypsin/ethylenediaminetetraacetic acid (EDTA, a chelating agent; 0.05/0.02 % v/v), washed with a
226 phosphate-buffer solution (PBS), re-suspended in 1 ml of TRAP (triethanolamine 82.3 mM, pH 7.6),
227 and sonicated on ice with two 10 s bursts. LDH activity was measured in the extracellular medium
228 and in the cell lysate (solution produced during cell lysis), using a Synergy HT microplate reader
229 (Biotek Instruments, Winooski, VT). Both intracellular and extracellular enzyme activity was
230 expressed as μmol of NADH oxidized/min/dish, then extracellular LDH activity (LDH out) was
231 calculated as percentage of the total (intracellular + extracellular) LDH activity (LDH tot) in the
232 dish.

233 *ROS generation.* After 24 hr incubation in the absence or presence 3, 6, 15 and 25 $\mu\text{g}/\text{cm}^2$ of
234 micro- and nano- chrysotile, A549 cells were loaded for 15 min with 10 μM 2',7'-
235 dichlorodihydrofluorescein diacetate (DCFH-DA) to detect ROS generation.⁵⁴ DCFH-DA is a cell-
236 permeable probe that is cleaved intracellularly by (nonspecific) esterases to form DCFH, which is
237 further oxidized by ROS to form the fluorescent compound dichlorofluorescein (DCF). The cells
238 were washed twice with PBS and the DCF fluorescence was determined at an excitation wavelength
239 of 504 nm and emission wavelength of 529 nm, using a Perkin-Elmer LS-5 fluorimeter (Perkin
240 Elmer, Shelton, CT). The fluorescence value was normalized by protein concentration and expressed
241 as $\mu\text{mol}/\text{mg}$ cellular protein.

242 *Measurement of thiobarbituric acid-reactive substances (TBARS).* TBARS assay, used to detect
243 lipid peroxidation, was performed as previously described.⁵⁵ After 24 h incubation in the absence or
244 presence of 6 $\mu\text{g}/\text{cm}^2$ of the samples, the cells were washed with fresh medium, detached with
245 trypsin/EDTA, and resuspended in 1 ml of PBS. 500 μl of cell suspension, each containing the same
246 protein amount (0.1 mg), were added to 5 μl of Triton X-100 and 500 μl of TBA solution (0.375%
247 thiobarbituric acid and 30% trichloroacetic acid in 0.5 N HCl). Samples were boiled for 20 min at
248 100°C, rapidly cooled by immersion in an ice bath and centrifuged for 30 s at 12,000 rpm. The
249 absorbance of 300 μl of the reaction mixture at 532 nm was read with a Packard EL340 microplate
250 reader (Bio-Tek Instruments). TBARS values were expressed as pmol/mg cellular protein.

251 *Nitric oxide (NO) synthesis.* After a 24 hr incubation with control and 6 $\mu\text{g}/\text{cm}^2$ of the chrysotile
252 samples, the extracellular medium was removed and the concentration of nitrite (the stable product
253 of NO synthesis) in the culture medium was measured with the Griess method.⁴⁹ Nitrite was
254 measured at 540 nm with a Synergy HT microplate reader. A blank was prepared in the absence of
255 cells and its absorbance was subtracted from the one measured in the samples; absorbance values
256 were also corrected for the monolayer proteins and results were expressed as nmol/mg cellular
257 protein.

258 **Statistical analysis**

259 Data in text and figures are provided as means \pm SE. The results were analyzed by a one-way
260 Analysis of Variance (ANOVA) and Tukey's test. $p < 0.05$ was considered significant.

261

263 **Set up of a protocol for the preparation of chrysotile nano-fibers**264 *Mineralogical and elemental analysis*

265 The effect of ultrasound on fiber structure was investigated by X-ray powder diffraction to assess
266 changes in the crystallographic features of chrysotile. The X-ray diffraction patterns of CTL-BM and
267 CTL-VM before and after the sonication for 3 hours are compared in Figure 1A and 1B respectively.
268 Both pristine and US-treated fibers displayed the two strong basal reflections (interplanar spacing
269 7.36 Å and 3.66 Å corresponding to the 002 and 004 plane respectively) of chrysotile. The same
270 reflections were observed in the X-ray diffraction patterns of CTL-VM treated for 6 and 24 h (see
271 Figure S1 in supporting materials) confirming that under current experimental conditions ultrasound
272 does not affect the crystallographic features of chrysotile. Since ultrasound may promote
273 incongruent dissolution of the fibers⁴¹, the relative amounts of Mg and Fe with respect to Si were
274 measured by means of X-ray fluorescence spectroscopy. Figure 2 shows Mg/Si and Fe/Si ratio for
275 pristine and 3 hours-sonicated chrysotile samples, both from Balangero and Val Malenco. The
276 treatment did not modify Mg/Si or Fe/Si (Figure 2). Elemental composition of the fiber was
277 modified for longer sonication times. Following the 6 hrs treatment the Fe/Si ratio slightly
278 decreased, whereas, after 24 hours, both Mg/Si and Fe/Si ratios significantly increased with respect
279 to pristine fibers (see Figure S2 in supporting materials).

280 *Morphological analysis*

281 Figure 3 shows SEM images of CTL-BM and CTL-VM prior and after 3 hrs of sonication. Both
282 pristine chrysotiles are composed of thin fibrils, some of these longer than 10 µm. (Figure 3A and
283 B). After 3 hrs of sonication the fibers were dramatically shortened (Figure 3C and D). The samples
284 appeared very homogeneous in size and well dispersed. On the contrary, a sonication time of 6 and
285 24 hrs promoted fiber aggregation (Figure S3 in supporting materials). The surface area of both
286 chrysotiles increased after 3 hrs treatment likely because of the formation of nano-fibers, from 15
287 m²/g to 30 m²/g and from 78 m²/g to 93 m²/g for CTL-BM and CTL-VM respectively. Longer

288 treatments (6 and 24 hrs) reduced the surface area of CTL-VM to 88 and 78 m²/g respectively,
289 because of the aggregation of nano-fibers.

290 On the basis of all the above data, the 3 hrs sonication is proposed as the best standard treatment to
291 obtain chrysotile nano-fibers without inducing relevant alteration in the chemical and
292 crystallographic features of the mineral. Therefore further characterization, including dimensional
293 characterization, cell free and cellular tests, have been performed only on the nano-fibers obtained
294 with this protocol.

295 The size distribution of the pristine and nano-sized samples is reported in Figure 4 and
296 summarized in Table 1. Figure 4 shows the fiber diameter plotted against the length for CTL-BM
297 and CTL-VM micro (Figure 4A and B) and nano (C and D) respectively. Each point on the plot
298 (scatterplot) represents a single fiber analyzed. In each scatterplot three areas are highlighted: i) the
299 size parameters for respirable fibers according to the WHO definition are marked in red field; ii) the
300 fibers with nanometric diameter (< 100 nm) are highlighted out in the green field and iii) the straight
301 line on the left side of scatterplot graphically separates the fibers (length/diameter ≥ 3) from non-
302 fibrous particles (length/diameter < 3). CTL-BM micro is characterized by a very heterogeneous
303 length distribution, with a prevalence of long fibers (50% longer than 5 μm , see Table 1). All fibers
304 measured exhibited diameter lower than 350 nm, with 50% lower than 145 nm. CTL-VM micro is
305 also heterogeneous in length, but it is shorter and thinner than CTL-BM with only about 25% longer
306 than 5 μm (Table 1).

307 After sonication for 3 hours, a consistent reduction in length is observed for both CTL-BM and
308 VM, where the vast majority of the fibers exhibited length lower than 3 μm . Only 2% of CTL-BM
309 nano-fibers is longer than 5 μm (see Figure S4, supporting materials), with an overall maximum
310 length of 7 μm . No fibers longer than 5 μm are found in CTL-VM nano. Both nano samples are
311 totally fibrous in shape, according to the WHO definition, with CTL-BM nano being particularly
312 elongated with almost 90% of the samples showing an aspect ratio > 10 (see Table 1). CTL-BM
313 nano-fibers are generally thinner than CTL-VM nano, showing a 90% of the diameters lower than
314 200 nm or 300 nm respectively (Table 1).

315 **Chemical reactivity and toxicological studies**

316 *Surface reactivity relatable to oxidative stress*

317 The oxidative stress caused by asbestos^{24,50,56} is the consequence of the release of reactive oxygen
318 and nitrogen species (ROS, RNS) by cells attempting to phagocyte the fiber and to various surface
319 reactions also releasing ROS in the medium. These latter are mainly due to free radical generation at
320 poorly coordinated - surface bound - iron ions³³ as well as to free iron ions released in the solution⁵⁷.
321 Moreover the oxidative stress may be exacerbated by reaction with the fibers of the natural
322 antioxidant defenses in the lung lining layer. Free radical release and interaction with biomolecules
323 are thus among the surface properties most relevant to fiber toxicity. The potential of chrysotile
324 nano-fibers to generate radicals in solution at physiological pH and to induce a depletion of some
325 endogenous molecules involved in the antioxidant defenses (ascorbic acid and cysteine) have thus
326 been compared to their longer counterparts in the pristine chrysotile minerals. The surface charge (ζ
327 potential) has been evaluated only for the two nanometric samples, the pristine ones showing
328 dimensions exceeding the maximum allowed by the available instrumental specifics. The surface of
329 chrysotile asbestos is known to be positively charged at physiological pH, as indicated by positive
330 values of ζ potential⁵⁸. After 3 hrs sonication, the ζ potential remained positive at quasi neutral pH
331 for both CTL-BM nano and CTL-VM nano, over the whole pH range examined (see Figure S5 in
332 supporting materials).

333

334 *Potential to generate free radical decrease at the nano-level*

335 Asbestos minerals are highly reactive in releasing free radicals.^{34,46} Two radical-generating
336 mechanisms have been investigated:

337 a) $\cdot\text{OH}$ radical generation in the presence of hydrogen peroxide (Fenton activity). This test mimics
338 the contact with lysosomal fluids where H_2O_2 is released following phagocytosis by alveolar
339 macrophages;

340 b) $\cdot\text{COO}^-$ from the formate ion, used as a model target molecule for homolytic cleavage of a
341 carbon-hydrogen bond in several organic molecules and biomolecules.

342 The EPR signal of $\cdot\text{OH}$ and $\cdot\text{COO}^-$ radical adducts with the spin-trapping molecule (DMPO)
343 obtained incubating the two asbestos samples before and after 3 hrs of sonication with the respective
344 target molecule is reported in Figure 5A and B respectively.

345 All samples were able to produce $\cdot\text{OH}$ radicals, but to a different extent. In the pristine materials
346 chrysotile fibers from the Balangero mine were highly reactive, the signal intensity of the [DMPO-
347 OH] \cdot adduct being about three times greater than the intensity of the signal produced by CTL-VM
348 micro. The different Fenton activity may be related to the higher iron abundance on CTL-BM micro
349 (about 2.7 wt. % as oxide) if compared with CTL-VM micro (1.6 wt. %) or to a different exposure of
350 isolated and coordinatively unsaturated iron ions.^{33,59} Nano-fibers of CTL-BM chrysotile were less
351 reactive than the micrometric ones, whereas nano-fibers of CTL-VM had a similar reactivity when
352 compared *per* mass to the micrometric ones. Note that if compared *per* unit surface the radical
353 amount produced by the nano-fibers was about three times lower than what released by the
354 micrometric ones. Moreover, the generation of $\cdot\text{OH}$ radicals from the micrometric samples was
355 sustained up to one hour or even increased with time (CTL-VM micro). Conversely, the amount of
356 $\cdot\text{OH}$ generated by nano-fibers slightly decreased with time (data not show for brevity). This behavior
357 suggests a limited outburst of radicals from the nano-fibers opposite to a prolonged catalyzed release
358 from the pristine ones.

359 All the chrysotile specimens investigated were also able to cleave a C-H bond in the presence of
360 ascorbic acid with consequent production of the $\cdot\text{COO}^-$ radical. Also in this case micrometric fibers
361 produced more radicals than the nano ones. As with Fenton activity also the rupture of a C-H bond
362 was more pronounced in samples from Balangero than in the Val Malenco ones.

363 *Poorly coordinated removable iron is less abundant on chrysotile nano-fibers than on the*
364 *micrometric ones*

365 The presence of easily removable iron ions at the particle surface may play a role in the
366 biochemical reactions involved in the pathogenic processes (e.g. DNA damage).^{57,60,61} Bioavailable
367 iron was evaluated by mobilization of ferric and ferrous ions using ferrozine, a specific iron chelator,
368 following a protocol previously described.⁴⁷

369 Figure 6 shows the total amount of iron released per unit surface by micro- and nano-fibers during
370 16 days of incubation in a ferrozine solution containing ascorbic acid as a reducing agent.

371 All samples were able to release iron in solution. Both micro- and nano-chrysotile from Balangero
372 released more iron than micro- and nano-fibers from Val Malenco, accordingly with its highest iron
373 content. Interestingly at the end of the incubation iron mobilized from the surface of nano-fibers was
374 lower than what released from the pristine fibers for both CTL-VM and CTL-BM. In all cases iron
375 release was sustained during the first day of incubation, then the extraction kinetics progressively
376 decreased with time. After one week of incubation the amount of iron mobilized reached a plateau.

377 *The potential to deplete antioxidant defenses decreases at the nano-level*

378 The ability of the two sets of asbestos fibers to oxidize/adsorb molecules involved in the cellular
379 antioxidant defenses, namely ascorbic acid (AA) and cysteine (L-Cys), from aqueous solution at
380 physiological pH has been investigated. The kinetics of AA depletion and the consumption at
381 thermodynamic equilibrium conditions of AA and Cys contacted with the fiber suspension are
382 reported in Figure S6 (supplementary materials) and Figure 7, respectively. As this process takes
383 place at the fiber surface, the data are compared per unit surface. Micro- and nano-fibers from
384 Balangero reacted with AA and L-Cys more than the Val Malenco ones. The CTL-BM fibers in the
385 nano-form were less reactive than the pristine forms, while no significant differences were observed
386 between nano and pristine fibers of CTL-VM.

387 The lower potential to generate free radical and to deplete antioxidant defenses of nano-fibers by
388 respect to the pristine ones may be assigned to the decrease in accessible surface iron ions. Note that
389 such difference was more pronounced for CTL-BM, the more iron-contaminated chrysotile.

390

391 ***Cellular tests***

392 The results of cellular responses in human lung epithelial cells A549 of micro- and nano-fibers of
393 chrysotile are summarized in Figure 8 and below described in details.

394 *Chrysotile micro-fibers, but not chrysotile nano-fibers, induced cytotoxicity*

395 After a 24 hrs incubation with 3-6-15 $\mu\text{g}/\text{cm}^2$ CTL-BM micro or 6-15-25 $\mu\text{g}/\text{cm}^2$ CTL-VM micro,
396 A549 cells showed a significantly and dose-dependent increased release of LDH, used as sensitive
397 index of cytotoxicity. This toxic effect exerted by CTL-BM and CTL-VM micro exposure was
398 significantly decreased when A549 cells were incubated with equal amounts in mass of CTL-BM or
399 CTL-VM nano (Figure 8A). The cytotoxicity of different samples of CTL-BM and CTL-VM,
400 investigated by trypan blue cell staining, showed a pattern superimposable to that of LDH leakage,
401 thus confirming the lesser cytotoxic effect of chrysotile nanofibres previously observed and the
402 consistency of the two methods (see Figure S7 in supporting materials).

403 *Chrysotile micro-fibers, but not chrysotile nano-fibers, evoked cellular reactive oxygen species*
404 *(ROS) production*

405 After a 24 hrs incubation with 3-6-15 $\mu\text{g}/\text{cm}^2$ CTL-BM micro or 6-15-25 $\mu\text{g}/\text{cm}^2$ CTL-VM micro,
406 A549 cells exhibited a significantly and dose-dependent augmented ROS production (Figure 8B)
407 used as index of induction of oxidative stress. Similarly to what observed for LDH release, the CTL-
408 BM and CTL-VM nano-fibers evoked a significantly lower oxidative stress in A549 cells incubated
409 with the same concentrations of CTL-BM or CTL-VM micro. However, the ROS- generating
410 activity did not fully disappear. At the highest dose (15 $\mu\text{g}/\text{cm}^2$) CTL-BM, also in the nanometric
411 form, induced a significantly higher ROS production compared to the control.

412 The dose of 6 $\mu\text{g}/\text{cm}^2$ was used for subsequent experiments, as representative of a similar cellular
413 response towards each CTL sample.

414 *Chrysotile micro-fibers, but not chrysotile nano-fibers increased cellular lipid peroxidation*

415 The data on cellular lipid peroxidation are displayed in Figure 8C. After 24 hrs incubation with 6
416 $\mu\text{g}/\text{cm}^2$ of CTL-BM or CTL-VM micro, A549 cells showed a significantly increased cellular
417 membrane lipid peroxidation, a sensitive marker of induction of oxidative stress. Also in this
418 experiment, the incubation of A549 cells with CTL-BM or CTL-VM micro induced significant
419 lipoperoxidation, while CTL-BM or CTL-VM nano exposure did not evidence any increased
420 oxidative stress.

421 *Chrysotile micro-fibers, but not chrysotile nano-fibers evoked cellular nitric oxide production*

422 The level of nitric oxide (NO), another sensitive index related to a cellular oxidative stress status,
423 was measured as nitrite concentration in the medium of A549 cells incubated for 24 hrs with 6
424 $\mu\text{g}/\text{cm}^2$ CTL-BM or CTL-VM micro and CTL-BM or CTL-VM nano as reported in Figure 8D. Only
425 A549 cells exposed to CTL-BM or CTL-VM micro showed a significantly increased nitrite
426 production which was not observed when A549 cells were incubated with CTL-BM or CTL-VM
427 nano.

428

429 A decrease in both size and surface reactivity may be responsible for the lower toxicity to A549
430 cells. Only a relatively weak dose-dependent ROS-generating activity was observed for CTL-BM
431 nano. This activity may be either ascribed to the presence of few fibers longer than 5 μm or to a
432 residual surface reactivity in CTL-BM nano. CTL-BM nano was in fact more active in carboxyl
433 radical release and in the depletion of antioxidants than CTL-VM nano. Note that all cellular data are
434 collected and compared by equal mass, thus when compared per unit surface the differences in
435 responses elicited by micro and nano fibers would be even larger.

436

437

Conclusions

438 The treatment of chrysotile with ultrasound appears an appropriate method for the production of
439 nano-fibers from a natural micrometric-long asbestos source. The crystal structure is preserved and
440 the nano-fibers obtained are substantially homogeneous in size, show a high aspect-ratio and no
441 contamination or compositional alteration of the fibers occurred during the ultrasound procedure.
442 The reported procedure was mainly set up to reproduce the effect of rainwater erosion on serpentine
443 minerals as well as the splitting that takes place following the mechanical effect of turbulent water in
444 streams and rivers.

445 When reduced in smaller fibers the surface reactivity of both chrysotile sources decreased and
446 their potential to elicit several adverse responses in a human lung epithelial cell line was attenuated.
447 The reduction of adverse cellular responses may be due to both smaller size and reduced free radical
448 generation, likely dependent from the reduction of bio-available iron. More studies on other cell

449 lines and *in vivo* validation are required to properly evaluate the risk associated to the presence of
450 chrysotile nano-fibers in the environment, however the present study clearly shows that not always
451 the reduction of a fiber or particle⁶² to the nano-size implies an increment in toxicity.

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460 **Supporting Information Available:** chemical composition of CTL-BM and CTL-VM; method
461 used for the dimensional characterization of the micro and nano-fibers; characterization of CTL-
462 VM fibers obtained applying the 6 and 24 hrs sonication treatment; length and diameter distribution
463 of micro and nano CTL-BM and CTL-VM; ascorbic acid depletion kinetics; cytotoxicity of micro
464 and nano-fibers measured using Trypan blue assay.

Table 1. Length and diameter distributions and aspect ratio of micro and nano- CTL-BM and CTL-VM calculated from their SEM image. First, second (median) and third quartile for length and diameter distributions are reported. Aspect ratio ($AR=length/diameter$) is reported dividing the samples in: non-fibrous ($AR < 3$), fibrous (AR in the range 3-10) and highly fibrous ($AR > 10$). The high percentage of fibers in all the four samples with high aspect ratio ($AR > 10$) indicates that these fibers have to be considered HARNs.

	Length (μm)			Diameter (nm)			Aspect ratio		
	25%	50%	75%	25%	50%	75%	<3	3-10	>10
CTL-BM micro	2.6	5	9.8	115	145	187	0%	4.8%	95.2%
CTL-BM nano	1.3	1.8	2.4	90	117	146	0%	13.0%	87.0%
CTL-VM micro	1.9	3.1	5.4	56	79	103	0%	3.7%	96.3%
CTL-VM nano	0.9	1.2	1.5	84	121	165	0%	50.4%	49.6%

Figure Captions

Figure 1 – Effect of US treatment on the crystal structure of chrysotile. X-ray patterns of CTL-BM (A) and CTL-VM (B): (a) pristine and (b) treated with ultrasound for 3 hours (b). Both pristine and US-treated fibers display the two strong basal reflections of chrysotile marked with asterisk (interplanar spacing 7.36 Å and 3.66 Å corresponding to the 002 and 004 plane respectively). The reflections confirm that under the experimental conditions adopted ultrasound does not affect the crystallographic features of chrysotile.

Figure 2 – Effect of US treatment on the chemical composition of chrysotile. Atomic ratio (Mg/Si black, Fe/Si gray) calculated from the X-ray fluorescence (XRF) peaks of CTL-BM (A) and CTL-VM (B) pristine and treated for 3h with ultrasound. No significant differences were observed after the US treatment on the chemical ratio of structural element of the chrysotile fibers.

Figure 3 – Effect of US treatment on the fiber morphology of chrysotile. Secondary electron SEM images of CTL-BM (left column) and CTL-VM (right column): pristine (A, B) and treated with ultrasound for 3 hours (C, D). The long and thin fibers of the pristine chrysotile samples were dramatically shortened by US treatment.

Figure 4 – Analysis of fiber dimension. Diameter vs. length plots (scatterplot) of CTL-BM (left column) and CTL-VM (right column) of pristine (A, B) and treated with ultrasound for 3 hours (C, D) samples. Each point on the scatterplot represents a single fiber analyzed. In each scatterplot three areas are highlighted: i) the size parameters for respirable fibers according to the WHO definition are marked in red field (diagonal pattern; ii) the fibers with nanometric diameter (< 100 nm) are stressed out in the green field (crossed pattern) and iii) the straight line on the left side of scatterplot graphically indicates the aspect ratio (length/diameter, AR) = 3, virtually separating fibers (AR ≥ 3) from non-fibrous particles (AR < 3). Micrometric chrysotile is characterized by a very heterogeneous length distribution, with a prevalence of long fibers (A and B), while the sonicated samples are shorter and rather homogeneous in length and diameter.

Figure 5 – Free radical release. EPR spectra of DMPO- \cdot OH (A) and DMPO- \cdot COO $^-$ (B) adduct after 60 minutes of incubation of micro- and nano-CTL-BM (a and b) and micro- and nano-CTL-VM (c and d) with H₂O₂ (A) or sodium formate (B). Ascorbic acid was added as iron-reducing agent in the formate test (B) and small doublet at the centre of the spectra, due to the transient formation of the ascorbyl radical, was hence recorded. Shot fibers are slightly less effective than micrometric counterpart in releasing free radicals in solution, when incubated with H₂O₂ or CO₂ $^-$ as target molecules.

Figure 6 – Bio-available iron. Iron release in solution by micro- and nano-CTL-BM (■,□) and micro- and nano-CTL-VM (●,○) measured by means of ferrozine-Ascorbic Acid method and spectrophotometrically evaluated at 562 nm. The iron concentration measured in the supernatant is normalized per unit surface. Nano-fibers show minor amounts of bio-available iron are less effective in releasing.

Figure 7 – Depletion of antioxidant defenses. Cysteine consumption (Cys, black columns) was calculated spectrophotometrically by evaluating the difference of the intensity of the signal at 412 nm with Ellman's reagent before and after the incubation with the fibers. For ascorbic acid (AA, gray columns) the absorbance at 265 nm was measured. Cys and AA depletion is reported as relative % consumption per unit surface area of the incubated fibers, calculated as the relative variation of the antioxidant absorbance at the time t_i , according to the equation: $(Abs\% = [(Abs(t_0) - Abs(t_i)) / Abs(t_0)] \times 100)$, where t_0 is the absorbance of the freshly prepared solution and t_i the absorbance of the supernatant solution after 6 hours of incubation for both AA and Cys. All experiments were performed in duplicate. The results are expressed per unit surface area and reported as average values \pm standard deviation.

Figure 8 – Cell responses. Effect of micro- and nano-chrysotile fibers on the response evoked in A549 human lung epithelial cells. A549 cells were incubated for 24 h in the absence (CTRL) or presence of 3-6-15 μ g/cm² of chrysotile micro-fibers from Balangero (CTL-BM micro) or nano-fibers (CTL-BM nano) and 6-15-25 μ g/cm² of chrysotile micro-fibers from Val Malenco (CTL-VM

micro) or nano-fibers (CTL-VM nano).

- A. LDH leakage. Data are presented as means \pm SE (n = 8). Vs CTRL: ** p < 0.0001; * p < 0.05. n CTL-BM vs. its μ CTL-BM: \blacklozenge p < 0.001. n CTL-VM vs. its μ CTL-VM: $\bullet\bullet$ p < 0.0001; \bullet p < 0.01.
- B. ROS (reactive oxygen species) production. Data are presented as means \pm SE (n = 8). Vs CTRL: * p < 0.0001. n CTL-BM vs. its μ CTL-BM: \blacklozenge p < 0.001. n CTL-VM vs. its μ CTL-VM: \bullet p < 0.0001.
- C. TBARS (thiobarbituric acid-reactive substances). Data are presented as means \pm SE (n = 6). Vs CTRL: ** p < 0.0001; * p < 0.001. n CTL-BM vs. its μ CTL-BM: \blacklozenge p < 0.001. n CTL-VM vs. its μ CTL-VM: \bullet p < 0.001.
- D. NO (nitric oxide) production. Data are presented as means \pm SE (n = 6). Vs CTRL: ** p < 0.01; * p < 0.02. n CTL-BM vs. its μ CTL-BM: \blacklozenge p < 0.05. n CTL-VM vs. its μ CTL-VM: \bullet p < 0.001.

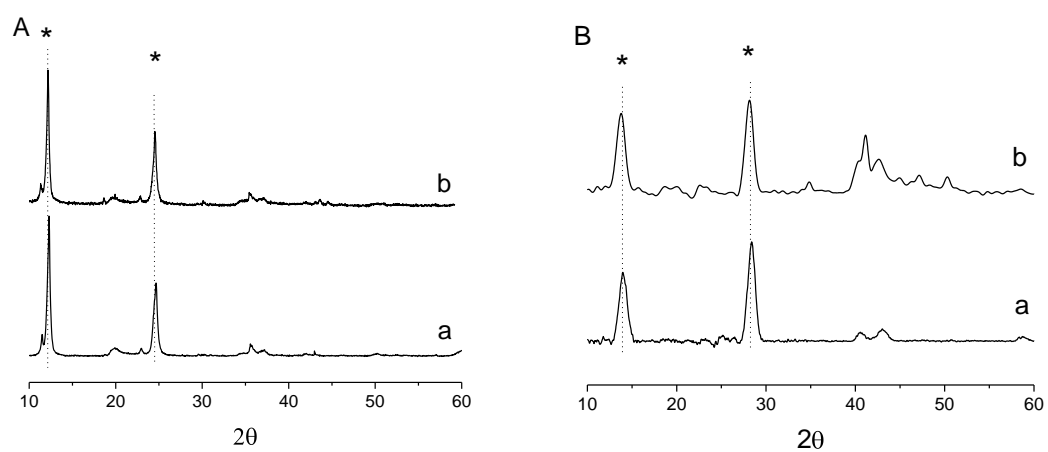


Figure 1.

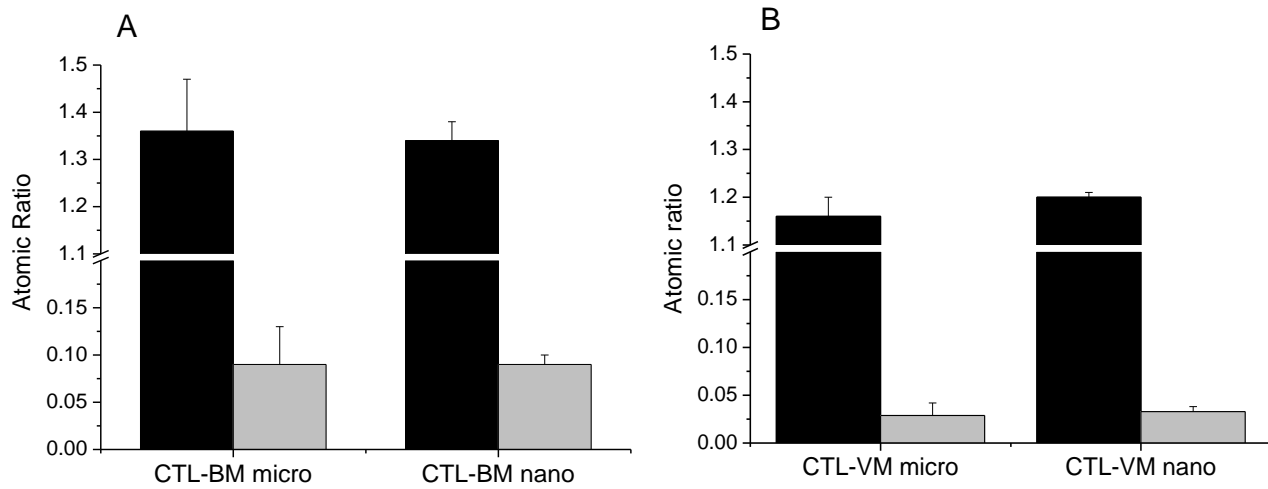


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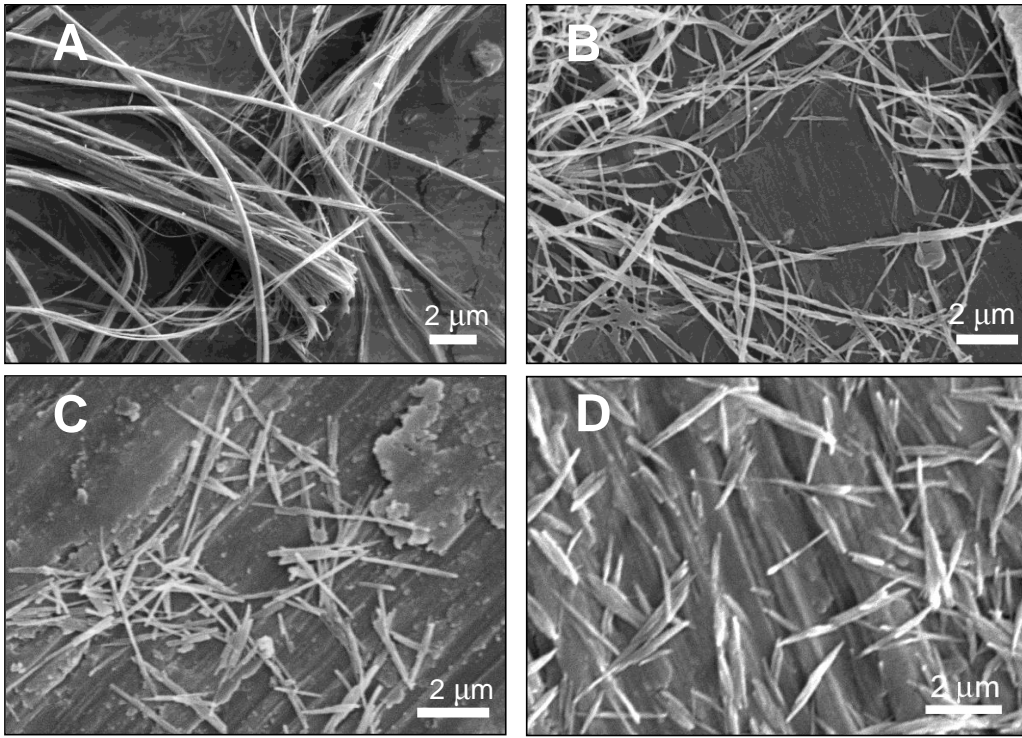


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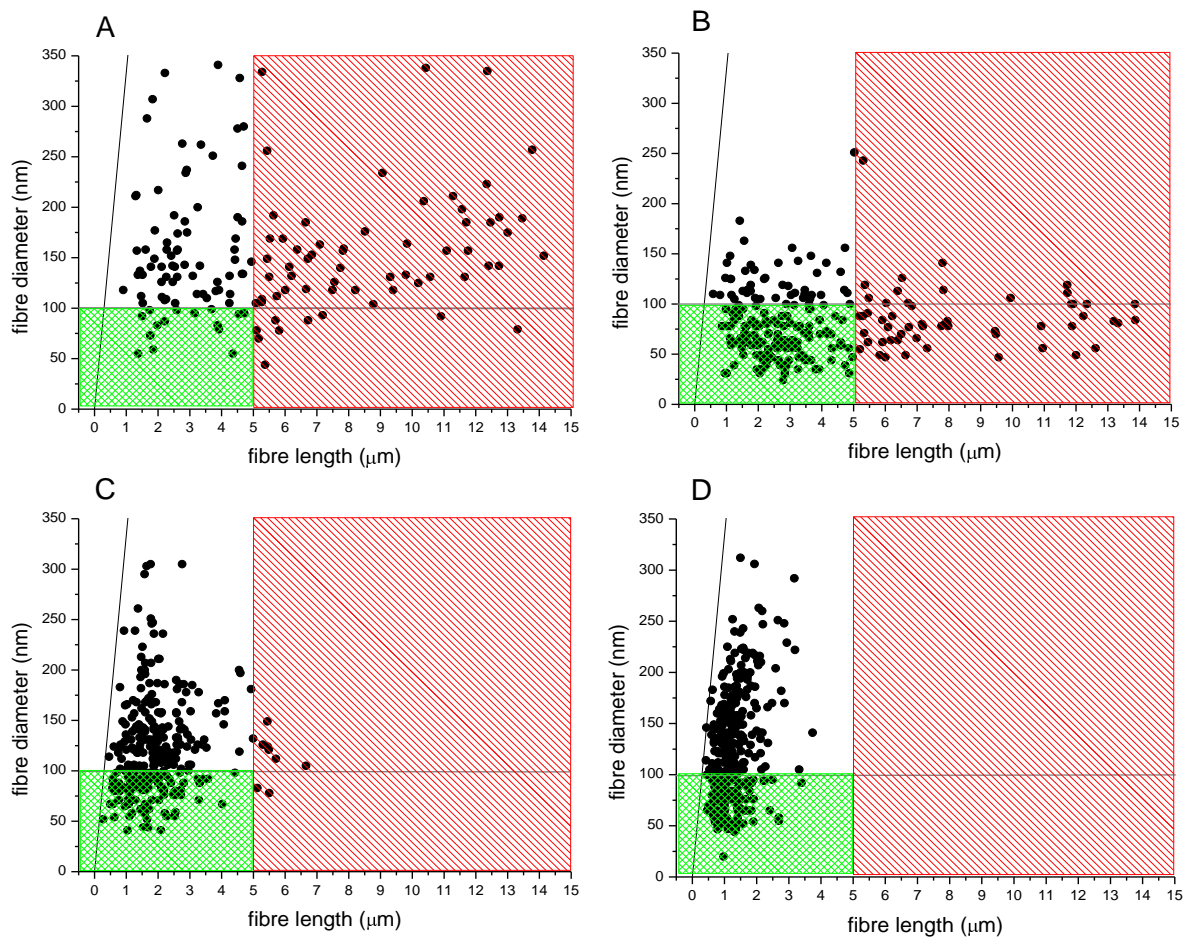


Figure 4.

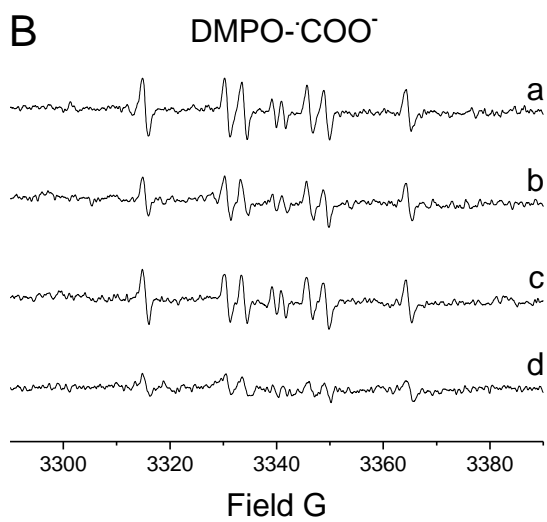
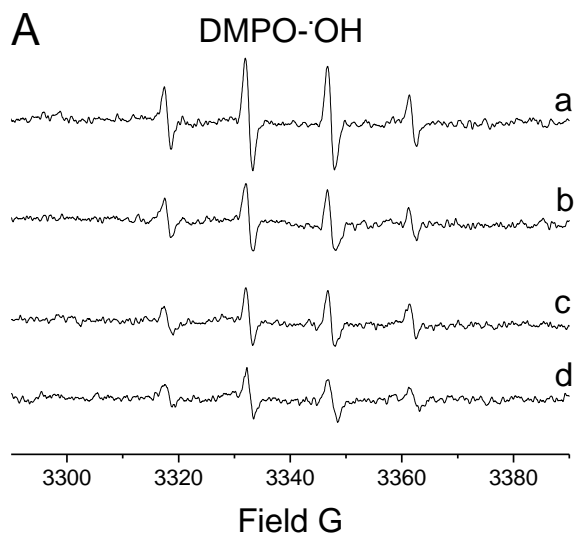


Figure 5.

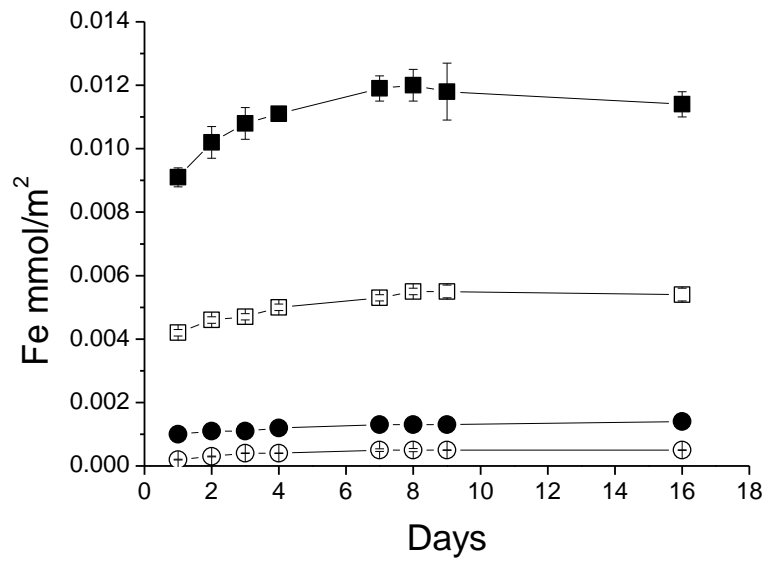


Figure 6.

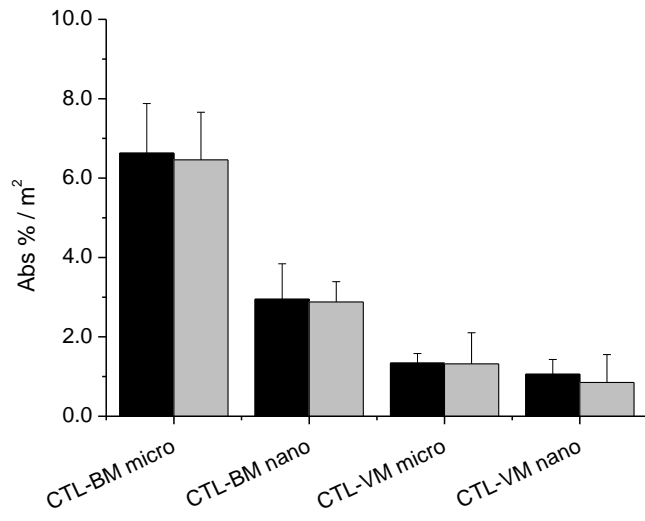


Figure 7.

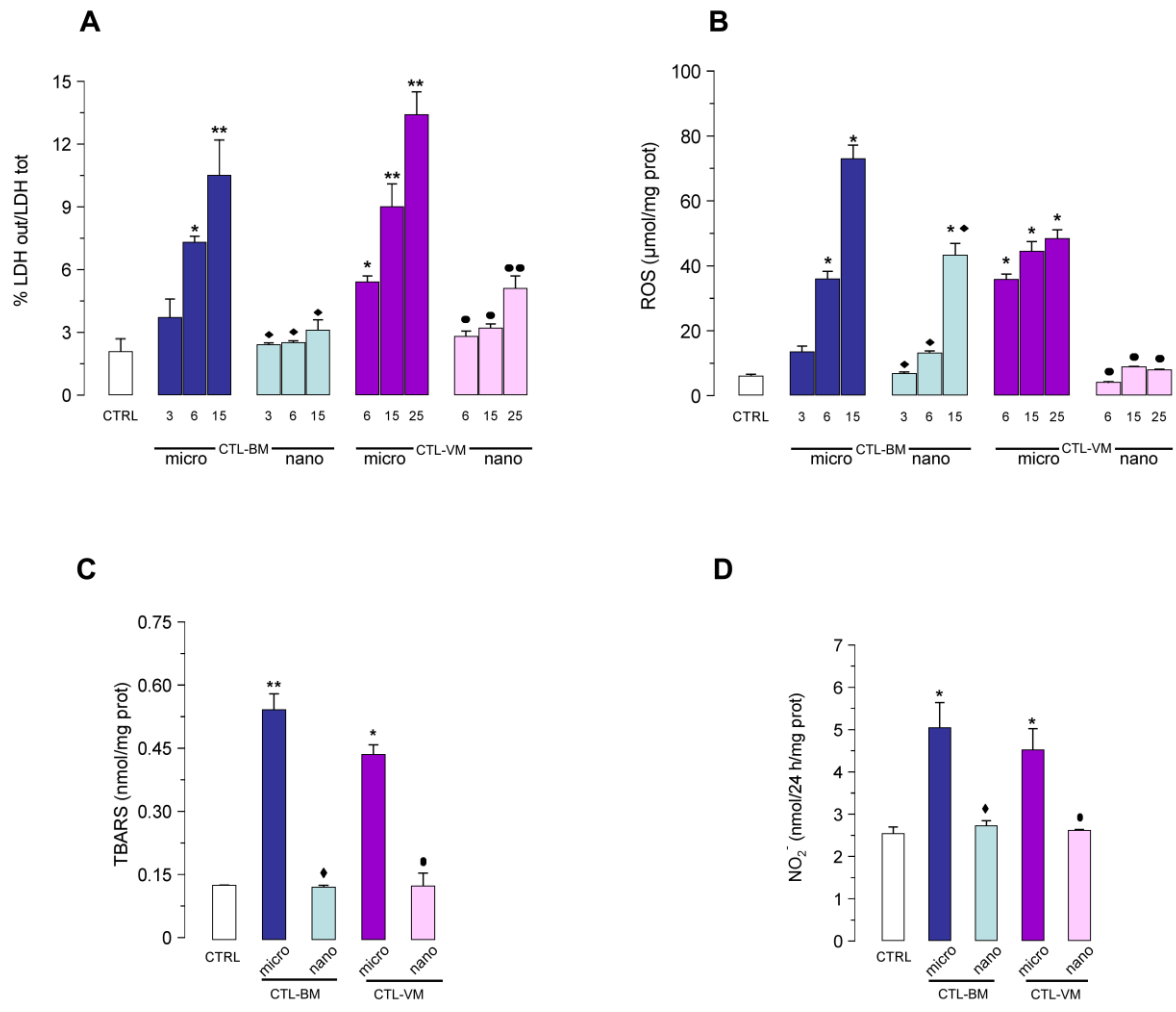


Figure 8.

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