 DOI: 10.1002/masy.200650613

Atomic Force Microscopy Applied to the Study of Whatman Paper Surface Deteriorated by a Cellulolytic Filamentous Fungus

G. Piantanida,[1,2] *F. Pinzari,*[*1] *M. Montanari,*[1] *M. Bicchieri,*[1] *C. Coluzza*[2]

Summary: In this study AFM technique has been used to observe the degradation of cellulose fibres in paper samples used as substrata for the growth of a filamentous fungus known to be responsible for damage to art works made from or supported on paper. Images obtained from the analysis of artificially deteriorated samples have been used for comparison with topographies obtained by AFM from naturally affected samples. As ''control'' images, AFM topographies of samples obtained from the same paper quality utilised as substrata for fungal inoculum were considered. The samples that were artificially deteriorated and those naturally affected by biological agents showed, at the molecular level, distinct surface differences when compared with control images. The biodegradation of cellulose fibres that appeared in AFM images can be attributed to the activity of both cellulolytic enzymes and acidic compounds produced by the fungal cells. Further studies using the AFM technique could reveal interesting aspects of paper biodeterioration caused by different microrganisms and might allow for a better description of the different stages of fungal attacks on cellulose fibres.

Keywords: AFM; biodegradation; cellulose; fungi; paper

1. Introduction

In paper-supported works of art (documents, books, prints, etc.) the colonisation of cellulose substrata by specific groups of micro-organisms often represents something more than a mere aesthetic problem. In fact, the alterations regard not only the visual appearance of the objects (stains, patinas, etc.) but often correspond to a deep modification of its chemical and physical structure, both at macro and microscopic levels.[1] Atomic Force Microscopy (AFM) can be used to study cellulose fibres degradation directly on a paper's surface, and it can provide both qualitative and semi-quantitative information on their deterioration and ageing.[2–7] The technique can produce topographical maps of paper consisting of three-dimensional images of the surface ultra structure at molecular level, in real time, under atmospheric conditions, and without the necessity to fix samples. Molecular interactions, surface hydrophobic properties, surface charges, and mechanical properties can be investigated with AFM, and therefore, can provide new insights into the relationships between spoiling organisms[5] and substrata surfaces.[2–4]

In this study AFM technique has been used to observe the degradation of cellulose fibres in paper samples used as substrata for the growth of a filamentous fungus known to be responsible for damage to art objects made from or supported on paper. Images obtained from the analysis of artificially deteriorated samples have been used for a comparison with topographies obtained by AFM from naturally affected samples.

[1] Istituto Centrale per la Patologia del Libro, Rome, Italy
Fax (+39) 06 4814968
E-mail: icplbio@tin.it
[2] Università degli Studi di Roma ''La Sapienza'', Dipartimento di Fisica. Rome, Italy

As ''control'' images, AFM topographies of samples obtained from the same paper quality utilised as substrata for fungal inoculum were considered.

2. Samples

2.1 Mycelial Cultures and Inoculum of Fungi on Paper

Mycelial cultures of *Aspergillus terreus* Thom were inoculated on MEA (Malt Extract Agar)[8] and incubated at 25 °C. The spores from *A. terreus* were obtained by gently scraping the surface of 7-day-old cultures with a swab. Spores were then suspended in 30 ml of sterile, distilled water containing 0.02% Tween 80 (Merck-Schuchardt- Germany). The spore density was defined for each strain by counting the elements in a Thoma Chamber to obtain a concentration of 4×10^2 spores μl^{-1}. A defined volume of each spore suspension was diluted with diluted nutritive broth (Sabouraud Broth by Difco, Becton Dickinson, USA) in order to inoculate the paper strips. All the inoculations were performed under a laminar flow hood to assure sterility in the procedures.

We used 100 μl of broth for each paper strip, distributing 4 droplets of this on to the same. As control-paper a Whatman 1CHR (for chromatography) Cat. N° 3001 917 made of pure cotton linter and not sized was used. Four replicates (a–d) for each treatment were considered. Each inoculated paper strip was placed in a 15 cm polystyrene Petri dish and incubated at 100% of relative humidity (RH) with T = 27 °C for 7 days.

2.2 AFM Topographies were Obtained from:

1) Whatman paper samples as control.
2) Artificially biodeteriorated samples obtained by inoculating *Aspergillus terreus* Thom on to Whatman as described in 2.1.
3) Artificially deteriorated samples aged for 49 days in climate chamber at 80 °C, 65% R.H.
4) Naturally biodeteriorated paper samples taken from ''Letter from Innocenzo Coreti'' (a.D. 1568).

Prior to observation with the AFM, artificially deteriorated samples were cleaned of spores and aerial mycelium using a soft brush used for paper restoration within a biological hazard cabin; this operation, although carried out using repeated delicate strokes, can lead to changes in the paper surface-fungus relationship.[3]

3. Characteristics of the Instrument

The Atomic Force Microscope used in this study was a SNOM/AFM instrument assembled at ISM-CNR (Figure 1), operating on air, and using a quartz fibre probe with conical tip (diameter ≅ 30nm). Such a setup allows needle-sensor AFM performance.[9] The fibre probe is held, in fact, to a piezoelectric bimorph parallel to its axis, and is forced into oscillation (close to its resonance frequency) normal to such direction. As it approaches the sample, the fibre tip becomes sensitive to the effect of surface interaction. This instrument allows scanning within a maximum x-y-z range of about 20 microns. The topographies can be visualised as ''False colour scale'' images (Figure 2d-2e; 4b) or as ''surface plots'' (Figure 3).

4. Results and Discussion

The roughness of some paper samples attacked by filamentous fungi can render them difficult to measure; in these circumstances the use of AFM is limited to imaging portions of fibre and fungus surfaces, and not the whole objects. The AFM imaging of paper samples covered by a widespread biofilm of fungal mycelium proved to be problematic in contact mode and even in tapping mode, since hyphae and aerial structures can trap the AFM probe, causing it to break. The whole

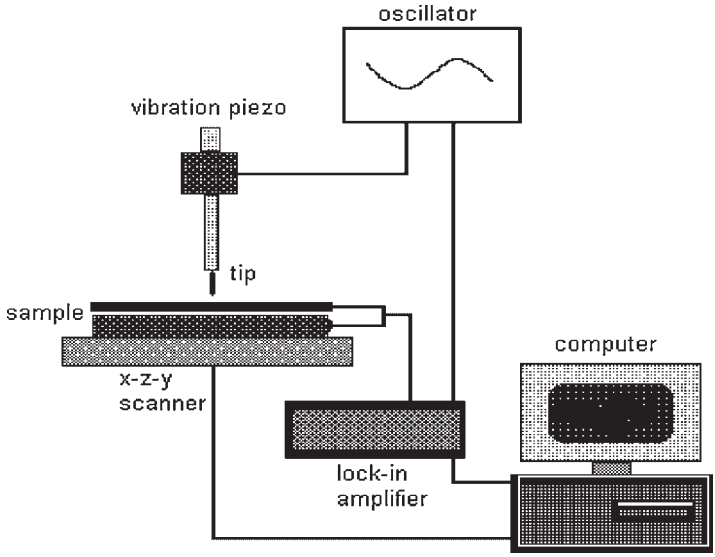

Figure 1.
Simplified scheme of the SNOM/AFM instrument assembled at ISM-CNR.

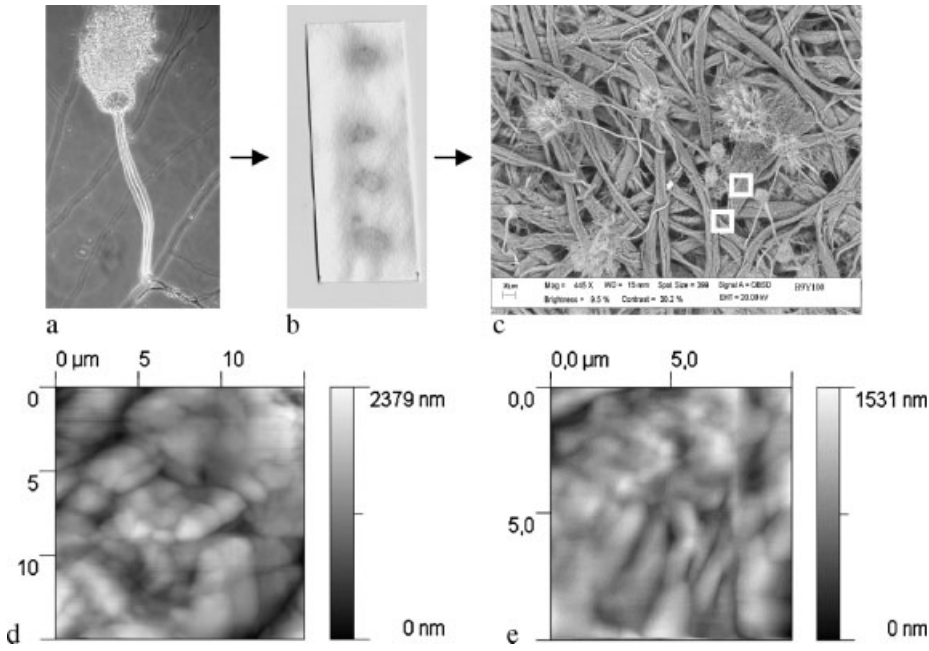

Figure 2.
a) Conidophores with conidia of *Aspergillus terreus* Thom; b) Whatman paper strip with artificially induced stains; c) Picture of *A. terreus* conidiophores and hyphae growing on Whatman paper at the Scanning Electronic Microscope, with reported, as dimensional reference, the $15 \times 15\ \mu m^2$ frames from which were obtained AFM topographies; d-e) AFM topographies of artificially biodeteriorated samples visualised in false colour scale.

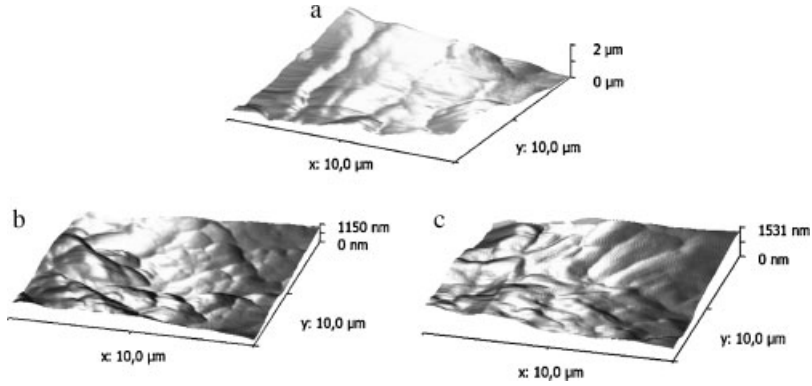

Figure 3.
a) Control sample consisting of 1CHR Whatman paper (for chromatography) Cat. N° 3001 917 made of pure cotton linter, not sized. b, c) Surface AFM plot. Induced biodeterioration. Samples obtained by inoculating *A. terreus* on to 1CHR Whatman paper made of pure cotton linter. Surface AFM plot.

experiment was therefore carried out in non-contact mode.

Both the artificially deteriorated samples (Figure 2, 3b, 3c) and those naturally affected by biological agents showed, at the molecular level, pronounced surface differences when compared with control images (Figure 3a). The biodegradation of cellulose fibres that appeared in AFM images can be attributed to the activity of both cellulolytic enzymes and acidic compounds produced by the fungal cells.[10,11]

For each sheet, samples measuring 1cm × 1cm were selected from different locations (ranging from the border to the centre of the sheet). AFM images acquisition was performed on several areas of each sample, using 10×10 μm^2 and 15×15 μm^2 frames (Figure 2). Typical target objects are expected to be only portions of the fibre surface: the fibre diameter in cotton cotton linter paper is known to be mainly of the order of magnitude of 10–20 µm. Quantification of degradation effects can be evaluated by means of the ''surface roughness'' parameter.

Local surface roughness is defined, on each 10×10 μm^2 frame, as the root mean square deviation of the surface height, from its average value. Sample surface roughness is intended here as the average, among 10 areas measured on the same sample, of the

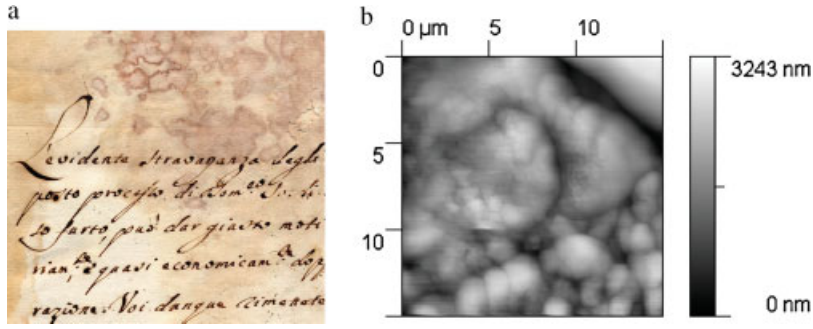

Figure 4.
a) Naturally occurred biodeterioration on ''Letter from Innocenzo Coreti'' (a.D. 1568). b) False colour scale AFM image.

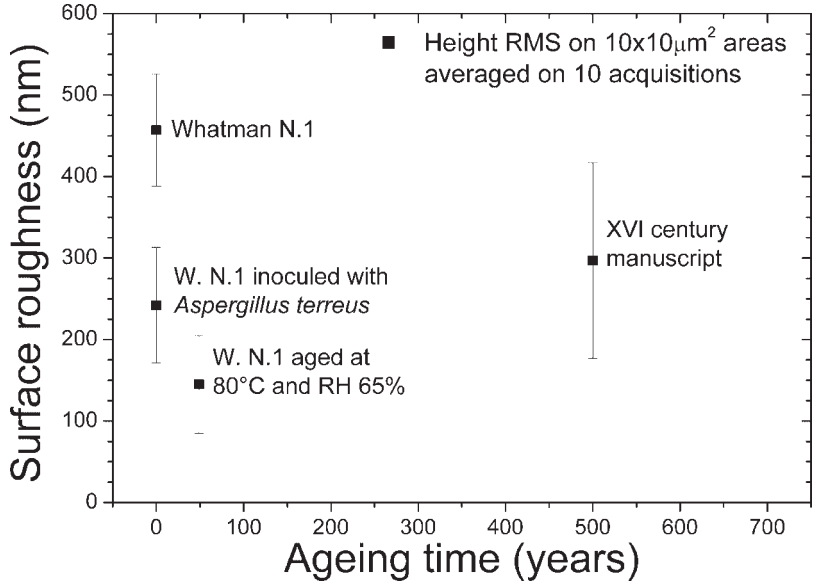

Figure 5.
Comparison between samples by means of the surface roughness parameter.

local roughness values. Therefore, fibres in good conservation state will result in higher height deviation (i.e. surface roughness) values than fibres degraded into fibril aggregates and other deterioration products. Surface roughness parameter analysis also provides a clear indication of the fact that original degraded library objects

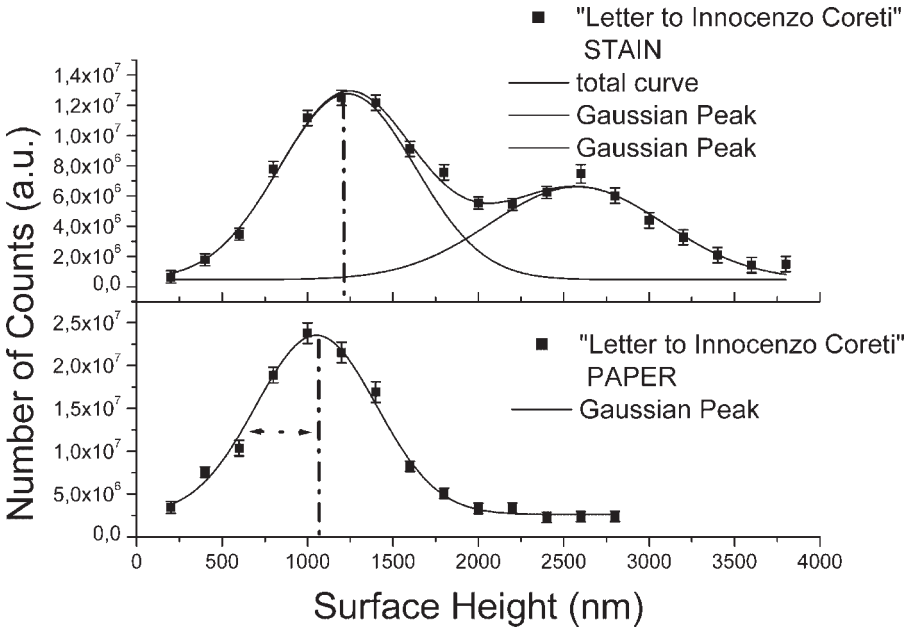

Figure 6.
Surface height distribution of ''Letter from Innocenzo Coreti'' sample. Two separate peaks are found on the stain.

cannot easily be modelled using a single process. Whereas single deterioration processes, applied in laboratory experiments on the same kind of paper induce a surface roughness variation without affecting experimental uncertainty, a major deviation was found in the roughness of stain samples taken from the naturally biodeteriorated manuscript ''Letter from Innocenzo Coreti (a.D. 1568)'' (Figure 4a–b, 5, 6).

A deeper insight on such a dramatic increment seemed necessary, since it was not found in artificially biodeteriorated samples nor in control samples: the entire surface height distribution on the samples was therefore analysed. As can be seen in Figure 6, it turned out that the increment error is not due to a higher distribution curve spread across the stain rather than in the paper, but, in fact, is determined by the presence of two distinguished peaks in the surface height distribution on the stain. Whereas a single peak characterises paper samples, in actual fact two peaks appear on the stain, one of them being compatible with the one found on paper, whilst the other, which is smoother, is definitely separate, and can be interpreted as the contribution of all those structures, in the stain, that do not belong to the paper.

Paper samples with artificially induced stains represented built-up model-systems fundamental to study the behaviour of the AFM with such heterogeneous surfaces, but naturally deteriorated samples offers a wider number of variables that call for a wider study. Further studies using AFM technique could reveal interesting aspects of paper biodeterioration caused by different micro-organisms, and could allow for a better description of the different stages involved in a fungal attack on cellulose fibres.

A comprehensive understanding of the degree of interaction between microbial metabolism and substrata is fundamental for deciding which kind of restoring intervention should be undertaken. The set-up of new techniques and methodologies for a better understanding of biodegradation of paper represent a step towards a more conservative policy of cultural heritage restoration.

[1] R. Kowalik, *Restaurator.* **1980**, *4*, 135.
[2] A.A. Baker, W. Helbert, J. Sugiyama and M.J. Miles, *Biophysical Journal.* **2000**, *79*, 1139.
[3] G. Piantanida, M. Bicchieri, F. Pinzari and C. Coluzza, in: R. Salimbeni and L. Pezzati, eds. *Proc. of SPIE V. 5857: Optical Methods for Art and Archaelogy*. **2005**, 217-227, Munich, Germany. SPIE.
[4] G. Piantanida, M. Bicchieri and C. Coluzza, *Polymer.* 2005, V.46, *26*, 12313.
[5] Y. F. Dufrêne, C. J. P. Boonaert, P. A. Gerin, M. Asther, and P. G. Rouxhet, *J. Bacteriol.* **1999**, *18*, 5350.
[6] J. Gustafsson, L. Civica, J. Peltonen, *Polymer.* **2003**, *44*, 661.
[7] C. Kugge, *J. Pulp Paper Sci.* **2004**, *30*(4), 105.
[8] J. Pitt, A.D Hocking, in: Pitt J., Hocking A.D., Fungi and Food Spoilage. CSIRO-Division of Food Research-Sydney. Academic Press Australia, 1985, p. 5.
[9] C. Barchesi, A. Cricenti, R. Generosi, C. Giammichele, M. Luce and M. Rinaldi, *Rev. Sci. Instrum.* **1997**, *68*(10), 3799.
[10] Lee, B. R. Evans, J. Woodward, *Ultramicroscopy*, **2000**, *82*, 213.
[11] J. K. Magnuson, and L.L. Lasure, *Advances in fungal biotechnology for industry, agriculture and medicine*, J. Tkacz, Kluwer Academic Publishers, Norwell, Mass, **2005**.

