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Atomic force microscopy studies of the effects of chemicals on the dentin tissue

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Atomic force microscopy (AFM) was used to monitor the effect of several chemicals on the dentin tissue. The action of three different etching agents, namely aqueous solutions of phosphoric acid, polyacrylic acid and EDTA (at clinically relevant concentrations) on dentin was followed in real time. The etched dentin was then treated with an aqueous solutions of glutaraldehyde and hydroxyethylmethacrylate (part of a commercial bonding agent), and the effect of the latter on the dentin morphology was studied as a function of the etching agent used. Results show different mode of actions of the chemicals tested, and that hydroxyethylmethacrylate undergoes a polymerization reaction on EDTA-treated dentin. More generally, it is shown that the use of AFM for real-time imaging of wet samples under normal pressure conditions can give important information on the effects of chemicals on the dentin tissue.

1. Introduction

In the field of dental biomaterials, a very important aspect of liquid–solid interaction involves dentin adhesives or dentin bonding agents: in order to promote effective and durable bonding between composite restorative materials and dentin tissue, the latter is subjected to treatment by several chemicals [1]. Typically, dentin must be treated by an etching agent, whose purpose is to remove the so-called smear layer, which is a tenacious layer of tissue debris produced by cutting instruments or by polishing routines. The smear layer cannot be easily removed by being rinsed or scrubbed with water, but can be readily attacked by several chemicals (note, however, that the matter of smear layer removal is rather debated, and several authors claim that it is better to leave the smear layer in place [2]). Then, "conditioning liquids" are put on the treated dentin surface. This stage, which depending on the particular adhesive, involves one or more steps, is often the core of the dentin adhesive system. After that, a lightly filled or unfilled resin is put on the primed dentin surface, where it is chemically or (more often) light-polymerized. The composite restorative resin is finally copolymerized with the underlying structure.

A huge literature exists on the interaction between dentin bonding agents and dentin tissue [1, 2]. Several mechanisms have been suggested and several strategies have been adopted to produce effective dentin primers: among the suggested mechanisms of bonding are surface precipitation of inorganic layers, chemical bonding to the inorganic dentin phase, chemical bonding to the organic dentin phase, and mechanical interlocking.

Despite this great research effort, the subject matter of dentin adhesives is still much debated. A recent report by the Accredited Standards Committee MD 156 Task Group on Test Methods for the Adhesion of Restorative Materials underlined that correlation between *in vitro* and *in vivo* performances of dentin adhesive is often poor [2]. All too often, dentin bonding agents which show promising *in vitro* performances produce disappointing clinical results.

Undoubtedly, part of the problem resides in the still not complete understanding of the events going on at the dentin surface. The unravelling of the mechanism which controls the effectiveness of a given bonding agent requires the understanding of adhesive interaction at the basic level. Unfortunately, the characterization of the effect of dentin primers and dentin adhesives is a rather difficult matter from an analytical point of view. As previously discussed, it occurs through interfacial interactions at a liquid phase/solid phase boundary. Most surface sensitive techniques requires high vacuum for analysis [3]. In this way, real-time observations of the evolution of the dentin/liquid phase system is impossible and the threat of vacuum-induced analytical artefacts is always present.

From this brief introduction, it can be easily understood that the coming of age of the atomic force microscope (AFM) [4] could represent an important step forward in the understanding and development of dentin adhesives. In fact, AFM can work with non-conducting samples and it does not require a vacuum chamber for analysis, so that biological samples can be imaged at normal pressure conditions and even underwater. Thus, in principle, AFM analysis allows one to follow in real time, with very high lateral and vertical resolution, the effect of the chemicals used in dentin bonding agent on the dentin topography.

A few reports showing the usefulness of AFM analysis in the study of the effect of chemicals on dentin have already appeared. Marshall and co-workers discussed the effect of etching by HNO_3 on dentin morphology [5]. We presented a preliminary report on the

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evaluation of the mode of action of a commercial bonding agent [6].

In this paper, three different etching agents are used, and their effect on the surface morphology of dentin is evaluated by AFM. Then, the etched samples are treated with an aqueous mixture of glutharaldehyde and HEMA (Gluma), which are the components of a dentin bonding agent. Since it has been reported that the chemical nature of the etching agent has a marked effect on the bond strength of Gluma [7], it is of interest to determine if AFM can give some clues on the reason of the reported behaviour.

2. Materials and methods

2.1. Teeth samples.

Freshly extracted human, non-carious, erupted third molars, which were stored in distilled water at 4 °C until prepared were used. Samples for analysis were obtained by removing the occlusal one-third of the crown using a low speed saw, and phosphate-buffered saline (pH 7.4, 0.9% NaCl) as a coolant. The surface of each specimen was then polished with abrasive discs using a rotating disc machine (LSI, Remet, Italy). A 0.5 μm alumina slurry was used for the final polishing step. For each step, the specimen was oriented so that the grinder rotated from the coronal to the apical aspect.

From the polished tooth, samples for AFM analysis were obtained by making a section, using the low speed saw, about 2 mm thick and parallel to the polished surface. The polished surface was used for AFM analysis.

2.2. Chemicals

Doubly distilled water was used for aqueous solutions. H_3PO_4 (PhA), 99% pure and polyacrylic acid (PA), both from Fluka, were used as aqueous solution at 35% and 10%, respectively. A 0.5 M solution was prepared from the disodium salt of EDTA (> 99% pure, Fluka). The pH was adjusted to 7.4 by adding NaOH (Fluka).

HEMA (95% pure, Fluka) and glutaraldehyde (50% solution in water, Fluka) were used as received, without further purification

2.3. Atomic force microscopy.

A Nanoscope III AFM (Digital Instruments, USA) was used. Samples were imaged using a Si_3N_4 cantilever, with a spring constant of about 0.12 N/m and a 125 × 125 μm scanner (J scanner). The "Height" data type mode was used, that is data corresponding to the change in the piezo height needed to keep the cantilever deflection constant.

The nominal contact force was calculated from the cantilever spring constant and the force calibration graph (accessible through the instrument control software). A 15 nN contact force between the cantilever tip and the samples surface was measured.

2.4. Teeth treatment.

Samples were imaged as follows: a polished tooth section was mounted on the AFM and fixed to the sample holder by double-sided adhesive. First of all, imaging of the as-polished dentin surface was performed. Scanning was suspended and, without moving the sample, a 10 μl drop of the selected solution was put on the dentin surface by a microsyringe, about 1 mm away from the cantilever tip. After a given time, discussed later the tooth surface was dried by gently blotting with soft paper and rinsed with water by a microsyringe. Then, the tip was engaged again and imaging was resumed. This procedure was repeated several times, in order to evaluate the effect of etching time on the dentin morphology.

After that, a 10 μl drop of the HEMA/Glutaraldehyde solution was put on the etched samples, about 1 mm away from the cantilever tip. After a specified time, the tooth surface was gently blotted with soft paper and imaging was resumed.

3. Results

3.1. Effect of the etching agents on dentin morphology

The polishing routine used for the preparation of the samples produced a dentin surface covered by the smear layer, characterized by an uneven surface topography resulting from the debris covering the underlying dentin tissue.

The polished dentin was etched with three different etching agents: a 35% PhA solution, a 10% PA solution and a 0.5 M solution of the disodium salt of EDTA, whose pH was adjusted to 7.4 by adding NaOH.

Figs. 1 and 2 show the evolution of the dentin surface treated by the PhA solution, as detected by AFM. Fig. 1 (after 30 s etching), shows that the acid attack begins in the peritubular region: a few tubules readily open, exhibiting a funnel shape, while the intertubular region seems more resistant. In Fig. 2, after 45 s etching, the latter shows clear signs of attack and much oblong debris can be seen.

After another 15 s of etching, the dentin surface was completely free from the smear layer and the dentin tubules were completely open (Fig. 3).

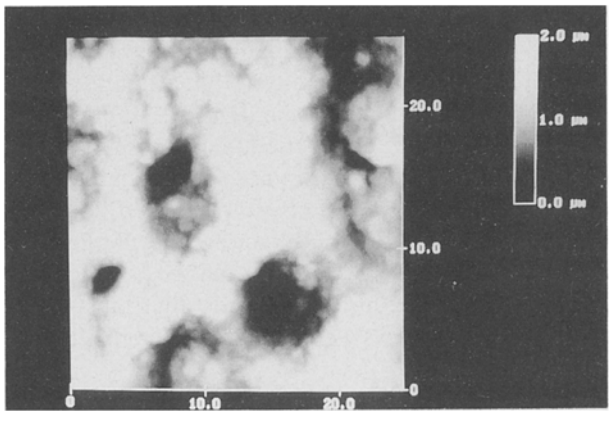

Figure 1 AFM image of dentin after 30 s etching with phosphoric acid.

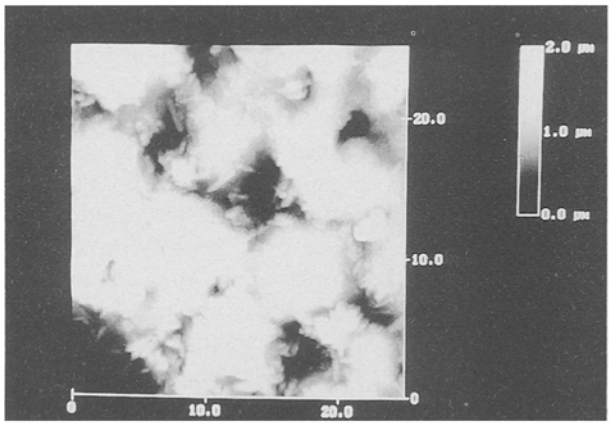

Figure 2 AFM image of dentin after 45 s etching with phosphoric acid.

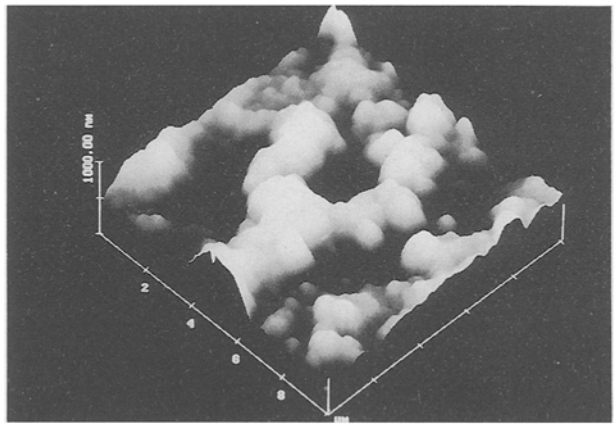

Figure 4 AFM image of dentin after 30 s etching with EDTA.

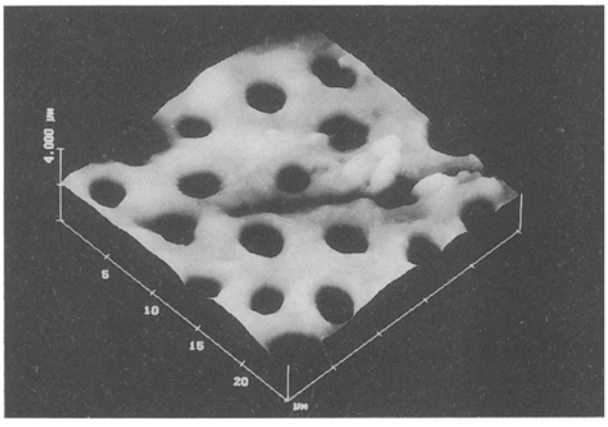

Figure 3 AFM image of dentin after 60 s etching with phosphoric acid.

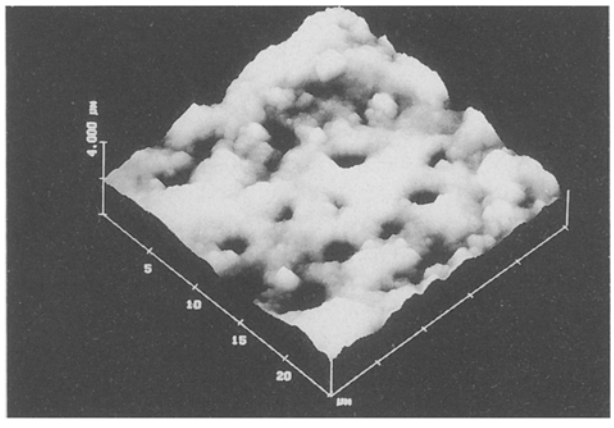

Figure 5 AFM image of dentin after 60 s etching with EDTA.

The effect of PA was similar to that of PhA. In general, the kinetics of the attack appeared a little slower.

For EDTA, Fig. 4 shows details of a dentin tubule at an early phase of the attack (30 s). The picture is different from that shown in Fig. 1 or 2. In fact, EDTA etching seems to leave, at least in this early phase, a relieved ring around the tubule. After 60 s etching many tubules are open, but the surface topography is rougher than in the case of acid etching (Fig. 5).

3.2. Effect of Gluma as a function of the etching agent

Figs. 6 and 7 show the morphology of 60 s PhA and 60 s EDTA etched dentin after Gluma treatment (the dentin before Gluma treatment is shown in Figs. 3 and 5, respectively). The effect of Gluma was followed as a function of time. The first image was taken after 60 s, which is the suggested time of application in clinical practice. The evolution of the surface morphology was then followed for up to 24 h, and 24 h images are shown. It must be noted, however, that most of the morphological effects were already observed after 60 s treatment.

The figures show that the effect of Gluma on the surface morphology of dentin depends on the etching agent used. In particular, in the case of PhA etching,

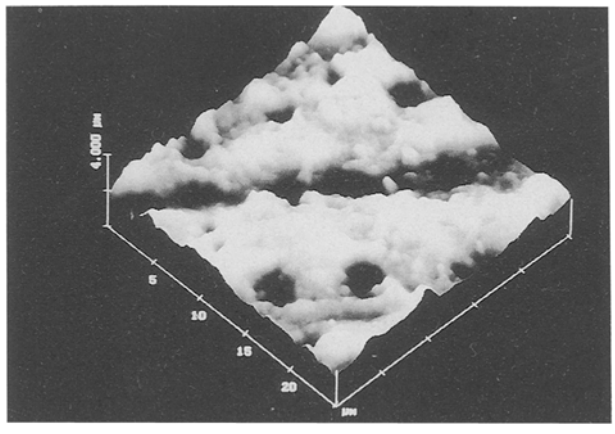

Figure 6 AFM image of dentin etched for 60 s with phosphoric acid and treated with Gluma.

only minor effects are observed: in general the intertubular regions seem more uneven after Gluma treatment, but most tubules are open and clearly visible (the same results were observed on PA etched dentin). On the other hand, when Gluma is applied to EDTA-treated dentin, a marked effect is detected. As shown in Fig. 7, tubules are no longer observed and a new surface morphology develops. Tubules are apparently covered by a "precipitate", which forms almost instantaneously upon application of Gluma. A closer

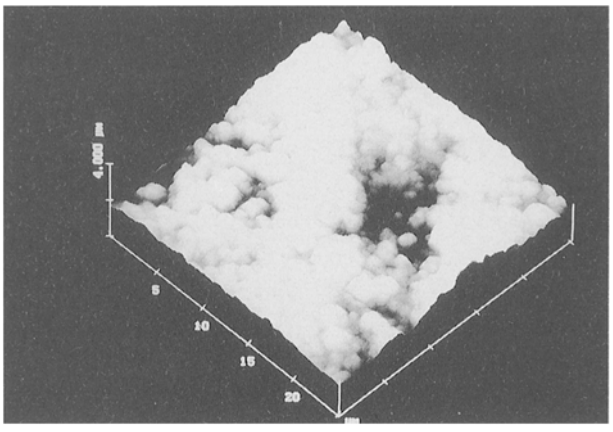

Figure 7 AFM image of dentin etched for 60 s with EDTA and treated with Gluma.

look a the Gluma-induced surface structure on EDTA-etched dentin reveals a closely packed array of 500–600 nm wide bumps.

4. Discussion

The effect of chemicals on dentin tissue is a topic of great relevance in the development of new and improved dentin bonding agents. The understanding of the events going on at the liquid phase/biological tissue (dentin) interface is key to this endeavour.

The effect of etching agent on the dentin surface morphology, as detected by scanning electron microscope (SEM) analysis, has been discussed in many papers. Here, our purpose was to determine if the peculiar characteristics of AFM can provide new information on this topic. In this respect, Figs. 1–3 show that AFM analysis can add a dynamic dimension to the study of the effect of the etching agents. By real-time monitoring of the evolution of dentin morphology, and removing possible artefacts induced by the need for putting the samples in a vacuum chamber for SEM analysis, AFM allows one to closely control the mode of action of the different etching agents.

The results of the effect of Gluma application to the treated dentin surface require consideration. Concerning the mode of action of Gluma, a mechanism was proposed by Munksgaard after the recognition that aqueous mixtures of HEMA with glutaraldehyde polymerize by the addition of catalytic amounts of amines or amino acids [8]. According to this mechanism, on application of Gluma, amino-group-containing substances in dentin react with glutaraldehyde and start the formation of poly(HEMA) (PHEMA). In this way, the system glutaraldehyde–amino groups behaves as a polymerization initiator for HEMA. Since, as discussed by Nakabayashi and Takarada [9], HEMA readily penetrates the dentin tissue, probably driven by a Lewis acid–base interaction [10], the amine-induced polymerization of HEMA produces a strictly interlocked dentin–PHEMA material, possibly with some covalent bonding with dentin by glutaraldehyde fixation to dentin proteins. The composite restorative applied to the Gluma-primed dentin will then bond by co-polymerization to the PHEMA layer.

The present results show that, when Gluma is applied to dentin etched by EDTA (which is the etching agent used, in clinical practice, before Gluma application), a marked modification of dentin morphology occurs (Fig. 7). In particular, a "precipitate" is observed, which covers and occludes the dentin tubules. Based on the behaviour of Gluma–amines or amino acids solution, and on the observation that this "precipitate" does not appear if either glutharaldeyde or HEMA are removed from the solution, it is possible to suggest that the solid phase which appears on Gluma-EDTA-treated dentin is indeed the amino-polymerized HEMA suggested by Munksgaard. The bumped morphology of the precipitate probably arises from polymerization starting at many different sites, as Gluma spreads and penetrates to the amino-groups-rich dentin surface.

Fig. 6 shows that PhA-etched dentin failed to produce, after Gluma treatment, the marked modification observed on EDTA-etched dentin (and the same was observed on PA-etched dentin). The reason for this behaviour is probably linked to the mode of action of the etching agents. Possibly, acidic etching agents reduce the number of amino groups available for the amino-induced polymerization of HEMA. While the complete explanation of the observed behaviour requires more than a morphological analysis, it is interesting to observe that the bond strength of a composite restorative to dentin mediated by Gluma was 13.4 MPa in the case of EDTA etching and 1.9 MPa in the case of phosphoric acid etching [7]. The finding that the amino-induced polymerization of HEMA occurs extensively only on EDTA-etched dentin, offers an explanation for the bond strength behaviour and implicitly, underlines the importance of the mechanism suggested by Munksgaard in the Gluma-mediated bonding of restorative resins to dentin [8].

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