AFM ANALYSIS OF ENAMEL DAMAGE DUE TO ETCHING WITH ORTHOPHOSPHORIC ACID

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Summary: Bearing in mind that in the field of jaw orthopedics and related scientific fields, there are no scientific studies that use the most modern technology based on the Atomic Force Microscopy (AFM) to investigate the nanostructure of tooth enamel after etching with 37% orthophosphoric acid, in this paper we will use this method, which is currently the most reliable, to determine the degree of tooth damage after its etching in the process of fixing orthodontic brackets. Considering the fact that the degree of enamel damage after etching cannot be realistically comprehended by the method called 'network', because in this way the damage cannot be seen three dimensionally (the depth of the damage), but only the damage in a single plane, a more precise analysis can be obtained with the application of the most contemporary method that we can apply in this case, and that is the application of AFM technology.

Keywords: Atomic Force Microscopy of enamel damage, etching, orthophosphoric acid.

1. INTRODUCTION

The development of the properties of adhesion of composite materials to hard tooth tissues, primarily enamel and dentin is one of the most important achievements in contemporary dentistry. The property of adhesion to enamel was found by Buonocore in 1955 [1], where he used the phosphoric acid to etch the surface layer of enamel due to the increase of retention area and the free energy of the surface. Etching enamel creates pores in which later penetrates resin or adhesive system. Different morphological forms of etched enamel were found by Silverstone. The surface of enamel after laying the phosphoric acid was demineralized in a 5−10 μm layer, which is the area of etched enamel. The pores about 20 μm thick are created beneath the surface, and these are the areas of qualitative pores, while beneath that area there is the area of quantitative pores, about 20 μm thick.

Apart from these, there are self-etching adhesives too, which can also achieve good adhesion. Low-viscosity monomers penetrate into the enamel surface and form, both within and around enamel prisms, a hybrid layer forming micromechanical retention. Having in mind that these structures are visible only under very high magnification (electronic microscope), we should speak here about nanoretenion on enamel surface. And finally, we should note here that the action of self-etching adhesives on enamel surface is less aggressive than phosphoric acid, where the demineralization of surface is 1−2 μm.

Enamel prisms are formed through complex interaction of ectodermal and ectomesenchymal tissues and through the coordination of cells responsible for their ameloblasts synthesis. Ameloblasts are created from the enamel body under the inductive influence of ectomesenchymal cells that migrate into the area of stomatodeum. Ectomesenchymal cells lead to ribbon-like multiplication of ectodermal cells and the creation of a horseshoe designated as dental lamina. At ten places in lines subsequent multiplication of epithelial cells occurs in the form of the ball-like cell piles designated as dental bud. Epithelial cells exert inductive influence on ectomesenchymal cells, they multiply and deepen the ball which now assumes the shape of a hat. At this stage all the formative structures are differentiated with the bud, from which future dental tissues will arise [2−6].

Enamel surface is not flat. It has a wavy structure because at places where Retzius’ striae end, these striae overlap in the form of steps, with the appearance of shallow grooves referred to as perikymata (Figure 1). In certain places, especially with primary (baby) teeth, there a few microns of enamel without prismatic organization on the surfaces – aprismatic enamel (Figure 2) [7,8].
Although enamel has pronounced hardness, it is also fragile at the same time and similar to glass, so that for these reasons it could appear to be susceptible to breaking. Despite that, enamel can take loads higher than 1000 N several times during the day. The overall enamel microstructure is formed in such a way to adjust to such loads. This is also owing to the support of elastic dentin and the structures such as enamel tufts at the dentin-enamel junction [4,6,9].

Enamel is in constant dynamic communication with the ecosystem of oral cavity. The processes of demineralization and remineralization are always present and their balance ensures the enamel integrity. If the external aggressive factors direct the balance toward the demineralization activities, the integrity of the crystal lattice weakens, reducing the hardness and resistance of enamel, which, after crossing a certain limit of mechanical resistance of enamel leads to rupture and creation of cavities, as the beginning of an irreversible damage [2,10].

Etching enamel with acid causes selective demineralization which increases the free surface energy, but at the same time porosity and the contact surface too. Bonding to enamel (adhesion) [11] depends on the capacity of the resin to penetrate into the area between the crystal prisms [12] which leads to macromechanical retention. The infiltrated resin surrounds the individual crystals of hydroxyapatite forming microthorns [13] thus creating a hybrid layer which accomplishes the retention mechanism at the nanolevel between the tooth and resin [14]. These micro thorns probably contribute to adhesion more than macro thorns which entering the area between the enamel prisms [15].

The retention capacities of etched enamel depend on the chemical structure of enamel mineral phase, the type of acid and time of etching [16]. Research has proved that the variations in time of etching from 15 to 90 seconds with 35–37% phosphoric acid do not influence much the shear bond strength or orthodontic brackets [17].

With etching time damage is greater and this is primarily manifested by capturing complete enamel prisms which happens in the first 15 seconds. In the further course, between 15 and 30 seconds destruction spreads mainly in the depth covering the central prism regions [18].

Something that has not been investigated in detail so far is the surface roughness at the microscopic level [19] where the nano characterization of surface roughness could provide biophysical mechanisms on enamel surface [20]. AFM with high lateral and vertical resolution allows investigating roughness at micro and nano levels without major interference of macroscopic components such as the wavy structure [21]. AFM microprobe does not require preparation of the sample and consequently endangering of the original surface. Thus it represents a direct way to experimentally detect and quantify the surface roughness.

2. MATERIAL AND METHODS

When analyzing the teeth after etching with 37% phosphoric acid, the material of work comprises 50 teeth of permanent dentition, which are fully intact (without caries, non repaired through conservative methods and without similar intervention therapeutic dentistry procedures); the total of 256 lines were done on each of these 50 teeth (*the total 12,800 lines*).

After extraction, the teeth were immediately placed in the solution of artificial saliva, in order to preserve the physiological mineralization of enamel. After this procedure, further preparation for the
study included the following: the etching material (in the concentration of 37% of orthophosphoric acid), was applied on enamel surfaces on which, in clinical orthodontic practice, brackets are bonded to teeth. This material was left to act for 30 seconds, i.e. in the way that it is done in clinical practice. After this, the surface on which the etching agent had been laid was rinsed with demineralized water and after thorough drying tooth enamel was cut to the dimensions 3mm x 2mm x 2mm, and then the surfaces to be scanned were polished. Tooth enamel samples were fixed to the AFM microscope holder.

Nanotechnological device JSPM-5200 which is located in NanoLab module for biomedical engineering at the Faculty of Mechanical Engineering of Belgrade University was used to test the nanostructure of tooth enamel. This is an integrated nanosystem with multiple operating modes that enables the following functions: STM, AFM, MFM, ECSPM etc.

JSPM-5200 consists of AFM base, anti-vibration table, AFM amplifier, SPM controller, computer and optional components such as a microscopic system with CCD camera, vacuum system etc. [22].

The schematic presentation of the system with interconnected components is given in Figure 3. The following parts of the system were presented:
- SPM base: the measuring part of the microscope that consists of AFM head, table and scanner,
- anti-vibration table: its function is to prevent transfer of vibrations to the sample,
- AFM amplifier: interface between SPM base and SPM controller that contains the signal-monitor AFM and on-off switch for laser diode,
- It also includes: console (cantilever) holder, holder of the type and holder of the sample.

The image was analyzed by the program WinSPM (Processing). This program packet allows the user to perform different processing functions in order to improve the quality of the image obtained by the scanning program. These functions include the following: leveling the image, adjusting brightness and contrast, applying different filters, etc. The analysis of the profile on the image of the scanned surface may be done in a number of ways: by Single, Multi, Extra and Multiple Images. With Single Analysis one production line may be placed in any direction within the image, while the distance between two points and the height difference between up to three pairs of markers are measured. With Multi Analysis up to five arbitrary lines in any direction within the image may be placed. With Extra Analysis the roughness of the scanned surface is measured within the set rectangular area, while with Multiple Images Analysis, up to three images may be placed.
and the profile is analyzed on the same line. We have used the Multi Analysis of Profile.

This program, WinSPM (Processing), also allows generation of three-dimensional images of scanned surface (bird’s-eye view). The adjustable parameters are Position (direction of display), Zoom (height along the Z-axis) and Centering (centering the surface in relation to the screen).

We finally use the report making feature, which is used to display images, measurements, profiles and 3D images in the form of the report for printing that are presented in the results of research. It is understood that the format of the A4 page is in vertical layout. The measurement data for the selected 2D image may be displayed.

3. RESULTS

The results of research of enamel surface morphology will be presented by the regression analysis of analyzed etched-treated and non-treated teeth with roughness parameters Ra, Rq, Rzijs and Rz.

In the analysis of the results of obtained roughnesses through AFM the most used are: Roughness Average (Ra), Root Mean Square roughness (Rq), Ten Point Mean Roughness (Rzijs) and Mean Roughness Depth which is determined as the biggest height difference (Rz). Roughnesses are expressed in nanometers (nm) [23,24].

The Figures below, due to space limitation, present one AFM image (each) with appropriate displays of measurements places. Figure 4 shows the general data of AFM image of non-treated sample, while Figure 5 presents the general data of AFM image of etched-treated sample. Figure 6 gives a presentation of measurement places and arithmetic means of average roughnesses of non-treated sample, and Figure 7 presents the measurement places and arithmetic means of average roughnesses of etched-treated sample.

![Figure 4. General data of AFM image – (Sample: 1; Non-treated, scan 1)](image)

![Figure 5. Presentation of measurement places and arithmetic means of average tooth roughnesses – (Sample: 1; Non-treated, image 1).](image)
Due to the size of the table, we will here present only the mean values for roughness parameters. In Table 1 the mean values of parameters $R_a$, $R_q$, $R_{zijs}$ and $R_z$ for non-treated teeth are presented.

In Table 2 the mean values of the parameters $R_a$, $R_q$, $R_{zijs}$ and $R_z$ for etched-treated teeth are presented.
### Table 1. Mean values of parameters Ra, Rq, Rzisj and Rz for non-treated teeth

<table>
<thead>
<tr>
<th>Samples: 1-50 Non-treated</th>
<th>Mean values of non-treated samples</th>
<th>Ra [nm]</th>
<th>Rz [nm]</th>
<th>Rq [nm]</th>
<th>Rzisj [nm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xsr-U1-NET</td>
<td>66.260</td>
<td>300.320</td>
<td>80.220</td>
<td>792.800</td>
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<tr>
<td>Xsr-U2-NET</td>
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<tr>
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<td>265.000</td>
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<tr>
<td>Xsr-U4-NET</td>
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<tr>
<td>Xsr-U5-NET</td>
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<tr>
<td>Xsr-U6-NET</td>
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<td>1000.000</td>
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<tr>
<td>Xsr-U7-NET</td>
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<td>414.440</td>
<td>104.020</td>
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<tr>
<td>Xsr-U8-NET</td>
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<td>590.640</td>
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<tr>
<td>Xsr-U9-NET</td>
<td>86.960</td>
<td>418.100</td>
<td>105.120</td>
<td>841.700</td>
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</table>

<table>
<thead>
<tr>
<th>Samples: 1-50 Non-treated</th>
<th>Mean values of non-treated samples</th>
<th>Ra [nm]</th>
<th>Rz [nm]</th>
<th>Rq [nm]</th>
<th>Rzisj [nm]</th>
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</thead>
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<td>Xsr-U14-NET</td>
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<tr>
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<tr>
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<td>39.720</td>
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<tr>
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<td>36.320</td>
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<td>333.540</td>
<td>84.680</td>
<td>467.200</td>
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<tr>
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<td>27.940</td>
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<tr>
<td>Xsr-U26-NET</td>
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<td>299.380</td>
<td>85.320</td>
<td>864.900</td>
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</tr>
<tr>
<td>Xsr-U27-NET</td>
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<td>76.840</td>
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<td>Xsr-U28-NET</td>
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<td>25.020</td>
<td>293.200</td>
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<td>Xsr-U29-NET</td>
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<tr>
<td>Xsr-U31-NET</td>
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<td>492.520</td>
<td>131.020</td>
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<tr>
<td>Xsr-U32-NET</td>
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<td>454.540</td>
<td>100.760</td>
<td>836.600</td>
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</tr>
<tr>
<td>Xsr-U33-NET</td>
<td>145.000</td>
<td>519.700</td>
<td>177.820</td>
<td>900.600</td>
<td></td>
</tr>
<tr>
<td>Xsr-U34-NET</td>
<td>89.140</td>
<td>451.180</td>
<td>98.220</td>
<td>733.700</td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Mean values of parameters $Ra$, $Rq$, $Rzisj$ and $Rz$ for etched-treated teeth

<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td>$Ra$ [nm]</td>
<td>150.600</td>
<td>183.800</td>
<td>107.020</td>
<td>69.460</td>
<td>72.240</td>
<td>43.512</td>
<td>40.520</td>
<td>35.280</td>
<td>34.660</td>
<td>38.000</td>
<td>147.100</td>
<td>191.720</td>
<td>103.780</td>
<td>68.060</td>
<td>70.800</td>
<td>5.492</td>
<td>40.900</td>
<td>33.360</td>
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<tr>
<td>$Rq$ [nm]</td>
<td>175.600</td>
<td>210.120</td>
<td>123.820</td>
<td>74.940</td>
<td>82.580</td>
<td>67.230</td>
<td>50.220</td>
<td>43.240</td>
<td>41.360</td>
<td>45.000</td>
<td>169.460</td>
<td>206.400</td>
<td>123.780</td>
<td>74.460</td>
<td>83.880</td>
<td>7.330</td>
<td>51.360</td>
<td>42.240</td>
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<tr>
<td>$Rzisj$ [nm]</td>
<td>1750.000</td>
<td>1040.000</td>
<td>568.300</td>
<td>507.400</td>
<td>762.000</td>
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<td>366.700</td>
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<td>277.800</td>
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<td>102.700</td>
<td>382.600</td>
<td>294.900</td>
</tr>
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</table>
The regression parameters of samples present the functional dependence of roughness in relation to the place of measurement of each specified roughness of etched-treated as opposed to non-treated samples.

Table 3 presents the regression parameters of non-treated teeth by roughnesses (Ra, Rq Rzijs, Rz) of the adhesive.

Table 4 presents the regression parameters of etched-treated teeth by roughnesses (Ra, Rq, Rzijs, Rz) of adhesives.
Table 3. Regression parameters of sample 1, 1-50, non-treated – Total data (with arithmetic means of measurement images)

<table>
<thead>
<tr>
<th>Parameter designation</th>
<th>Regression equation ((y=ax+b))</th>
<th>a</th>
<th>b</th>
<th>Determination coefficient ((R^2))</th>
<th>Correlation coefficient ((r))</th>
</tr>
</thead>
<tbody>
<tr>
<td>NET-Ra</td>
<td>(y = -0.0093x + 61.988)</td>
<td>-0.0093</td>
<td>61.988</td>
<td>0.0002</td>
<td>0.0141</td>
</tr>
<tr>
<td>NET-Rz</td>
<td>(y = 0.0121x + 273.57)</td>
<td>0.0121</td>
<td>273.57</td>
<td>0.00002</td>
<td>0.0045</td>
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<tr>
<td>NET-Rzis</td>
<td>(y = -0.2249x + 581.08)</td>
<td>-0.2249</td>
<td>581.08</td>
<td>0.0025</td>
<td>0.0500</td>
</tr>
<tr>
<td>NET-Rq</td>
<td>(y = 0.0027x + 73.699)</td>
<td>0.0027</td>
<td>73.699</td>
<td>0.00001</td>
<td>0.0032</td>
</tr>
</tbody>
</table>

Table 4. Regression parameter of samples 1-50, etched-treated – Total data (with arithmetic means of measuring images)

<table>
<thead>
<tr>
<th>Parameter designation</th>
<th>Regression equation ((y=ax+b))</th>
<th>a</th>
<th>b</th>
<th>Determination coefficient ((R^2))</th>
<th>Correlation coefficient ((r))</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRE-Ra</td>
<td>(y = -0.0105x + 86.439)</td>
<td>0.0105</td>
<td>86.439</td>
<td>0.0001</td>
<td>0.0100</td>
</tr>
<tr>
<td>TRE-Rz</td>
<td>(y = -0.1813x + 359.07)</td>
<td>-0.1813</td>
<td>359.07</td>
<td>0.0031</td>
<td>0.0557</td>
</tr>
<tr>
<td>TRE-Rzis</td>
<td>(y = -0.0367x + 689.32)</td>
<td>-0.0367</td>
<td>689.32</td>
<td>0.00003</td>
<td>0.0055</td>
</tr>
<tr>
<td>TRE-Rq</td>
<td>(y = -0.0398x + 102.08)</td>
<td>-0.0398</td>
<td>102.08</td>
<td>0.0016</td>
<td>0.0400</td>
</tr>
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</table>

4. DISCUSSION

The reason we chose the investigation by AFM is in the fact that AFM is currently the most reliable possible method for determining the degree of damage of enamel caused by its etching. There are numerous studies that showed the convenience of using AFM analyses to monitor both qualitative and quantitative changes on enamel surface [25–30]. Only with the development of AFM technology, a possibility was opened to monitor more subtle changes in enamel surface [31]. The AMF studies start to be used more and more in the researches in dentistry too, which monitor surface changes such as dental plaque and mineralized and coloured layers, surface properties of different materials and morphological and mechanical changes in mineralized tissues.

AFM technology is suitable for monitoring the enamel structure ranging from individual crystals to prisms (from nano to micro level) [32–34]. At lower magnifications it presents the enamel prisms as deep recesses. Individual crystals are arranged in parallel and present the elongated hydroxyapatite plane. The crystal surfaces are unevenly compacted in this way forming the enamel surface roughness. At higher magnifications the crystals, show characteristic hexagonal appearance, each set at an angle of 60 degrees.

AFM has a number of significant advantages when it comes to investigation of dental tissues compared to the other techniques and especially SEM (Scanning Electron Microscopy); the basic possibility is in obtaining a three-dimensional profile of the test surface.

There are numerous papers [29,30,35–37] that underline and confirm that the AFM technique is especially suitable for investigation of the erosions on enamel surface, even those at the nano level. AFM can be used to clearly monitor the enamel structures from individual crystals to prisms (from nano to micro level) [33,35].

A big number of researches have been undertaken to date in order to determine the degree of enamel damage caused by the etching procedure, with the results that were compared between certain researchers, and which were confusing, to say the least [38–43]. The explanation of these and similar findings is sought in partly different methodology, the size of the sample, insufficient precision at work, different methods of preparation-treating the preparations intended for research, etc. However, based on a thorough and very extensive research i.e. analysis of these results (over 90% of papers that dealt with this problematic in the past 15 years) we believe that the differences in the results between individual researches and researchers, even when the identical methodological procedures were applied, are probably due to different mineralization of enamel of tested teeth. This factor – the degree of tooth mineralization is hard or almost impossible to objectivize in this type of research, and which would give a fuller picture of the state of tooth enamel. This factor is influenced by the race (it is different with the white and the black race), and is also different within the same race with different nations. Having in mind that mineralization is a dynamic process, i.e. that enamel is in constant interaction with the oral cavity ecosystem [44,45], the demineralization and remineralization processes are always present i.e.
they are carried out permanently, while their balance ensures the enamel integrity. The current state depends on the patient’s age, diet, type of food and drinks (special influence is exerted by fizzy drinks [46–51]), the method and regularity of keeping oral hygiene, individual tendency to plaque creation, etc.

Based on tables 1 and 2 with the values of the parameters Ra, Rq, Rzijs and Rz, and their mean values for non-treated and etched-treated teeth we conclude that the mean values of roughnesses Ra, Rz, Rq, Rzijs of etched-treated samples are higher compared to the mean values of parameters of roughnesses of non-treated counterparts. Expressed in the percentages, the mean value of parameters Ra of etched-treated teeth is by 39.93% higher, that of Rz roughness is by 22.26% higher, while Rq and Rzijs roughnesses are by 31,14% and 23,85% higher, respectively, compared to the same roughness parameters of non-treated samples.

The obtained results of the analysis of etched enamel as a preparation for bonding brackets suggest significant distortions in the structure compared to non-treated. All the parameters for measuring roughness are significantly higher with the surfaces treated with acid. Investigating etched enamel areas show on AFM images, in addition to micro concavities, the devastation areas of prismatic structures in wider intervals. Nevertheless, it is not possible to completely compare the findings due to the different degree of enamel mineralization with different persons and teeth. Similar results were recorded in other studies too. Endo et al. reported that enamel treated with 37% phosphoric acid becomes very porous with exposure to numerous crystals and the honeycomb structure [52]. The main advantage of AFM compared to other technologies is in the fact that it provides roughness quantification [53]. The changes of enamel surface compared to the type of surface treatment were reported [54–57]. Changes were observed in the morphology, surface roughness and the profile depth of treated enamel. The erosion effects are different compared to the acidity of treating agents (acid, adhesives, etc.) and are related with clinical effects [58]. Surface hardness of enamel may be determined on AFM images based on the curve of cantilever force [59,60]. The easiest way to achieve this is to record the curves while the cantilever tip performs raster scanning through the sample [61,62]. Bayer et al. [63] investigated the creation of erosions under the influence of different acids on human enamel and discovered the existence of its loss, and also that pH is not the most significant in determining the erosive potential. Surface hardness may be determined by AFM on the basis of the curves after scanning [64, 65]. Thus, it was determined that different acids, and especially phosphoric acid decreases the surface hardness, whereby pH is not a decisive factor. A number of possible factors are mentioned, whereby the self-bonding adhesives have higher hardness without the influence of thermal changes unlike the classical etching with acid. In etching with phosphoric acid, there is penetration of water in the contact zone that makes the enamel softer due to the loss of calcium [66]. Hanning et al. [67] reported that individual hydroxyapatite crystals on enamel surface are in a way encapsulated in the self-bonding adhesive system at the nano-level.

5. CONCLUSIONS

Based on the obtained results of research, their statistical processing and detailed analyses, the following conclusions may be made:

– Based on the obtained values for the parameters Ra, Rq, Rzijs and Rz and their mean values for non-treated and etched-treated teeth, we come to a conclusion that the mean values of roughnesses of Ra, Rq, Rzijs and Rz of etched-treated samples is higher than mean values of parameters of roughnesses of their non-treated counterparts. Expressed in percentages, the mean value of parameters of roughness of Ra of etched-treated samples is higher by 39.93%, of Rz roughness by 22.26%, and of Rq and Rzijs roughnesses are by 31,14% and 23,85% higher, respectively, compared to the same roughness parameters of non-treated samples.

– The obtained results of the analysis of etched enamel as preparation for bonding brackets show significant distortions in the structure compared to the treated counterparts. All the parameters for measuring roughness are significantly higher with the surfaces treated with acid. Investigating the etched enamel areas show on AFM images, in addition to micro concavities the areas of devastation of prismatic structures at bigger intervals.

– Preparing enamel for bonding orthodontic bracket by etching with acid causes extensive changes in the nanostructure of enamel crystals that can influence its biomechanical properties and resistance toward the action of acids from oral biofilm (caries) or microenvironment (erosion).

– Enamel treated with acids may be considered risky and requires enhanced preventive care because brushing the teeth with toothpaste containing with fluoride is not sufficient.

– After debonding of orthodontic brackets bonded with composite material by enamel etching a
long and complex treatment of enamel remineralization is necessary.
- Manufacturer’s instruction on the time of enamel etching should be strictly adhered to, because the longer it is, the bigger is the damage of the central prism areas.

6. REFERENCES


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АФМ АНАЛИЗА ОШТЕЋЕЊА ГЛЕЋИ УСЉЕД ЈЕТКАЊА ОРТОФОСФОРНОМ КИСЕЛИНОМ

Сажетак: Имајући у виду да у области ортопедије вилица и сродних научних областима не постоје научна истраживања која користе данас најсавременију технологију залиноване на атомској микроскопији (АФМ) за испитивање наноструктуре глеђи зуба након јеткања 37% ортофосфорном киселином, у раду ће се утврдити, на данас најпouзданији могући начин, АФМ (Atomic Force Microscopy) методом степен оштећења глеђи зуба након њеног нагризања (јеткања) у циљу фиксирања ортофосфорних брацица. Степен оштећења глеђи након јеткања не може се реално сагледати методом тзв. „мреже”, јер се оштећење, на овaj начин, не може сагледати просторно (у дубини оштећења), већ се може видjetи само оштећење у једној равни. Прецизнија анализа може се добити примјеном АФМ технологије, као најсавременије методе коју имамо у овом случају.

Кључне ријечи: атомска микроскопија, оштећење глеђи, јеткање, ортофосфорна киселина.