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Application of Atomic Force Microscopy in Dental Investigations

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Abstract: ***Atomic force microscopy provides a detailed characterization of various dental tissues and processes in the nanometric scale. It is currently applied in dental investigations focused on demineralization of dental hard tissues, observation of dentinal surface and collagen network, bacterial cells and biofilms, dental materials and appliances, etc. Results from various studies in the field of dental caries, tooth hypersensitivity, endodontology, prosthetics, restorative and preventive dentistry can be of clinical benefit in the development of future treatment methods and products. An attempt is made to summarize and analyse the existing data concerning the basic characteristics, advantages and limitations of the application of atomic force microscopy in dental investigations.***

Keywords: atomic force microscopy, dental investigations, nanocharacterization

1. Introduction

There is a broad spectrum of two-dimensional imaging approaches used in different research fields. Nevertheless, they may provide insufficient description of various sample features such as its topography and mechanical properties [1]. Recently, dental investigations adopt newly-developed nanoscale technologies in order to enable a better understanding and manipulation of various biological materials and processes. Through the utilization of nanocharacterization methods oral diseases can be examined at the molecular and cellular levels, thus allowing precise diagnostics and proper therapeutic strategies [2].

A new level of data acquisition is possible through the application of the so-called Scanning probe microscopes (SPM) [1]. SPM comprises of several experimental methods designed to investigate the physics and chemistry of surface structures at subatomic resolution [3]. Along with the scanning tunneling microscopy (STM) and near-field scanning optical microscopy (NSOM), atomic force microscopy (AFM) is a branch of SPM [4, 5]. Since its invention by *Binning et al.* in 1986 [6], AFM has evolved into one of the most widely used imaging tool in the fields of physics, biology and material science [5]. AFM is based on mapping of an atomic-force field on a surface with non-destructive probes [3, 5]. This technique overcomes the constrains of the optical microscope resolution as well as the requirements for sample conductivity [1]. It provides a detailed topographical three-dimensional image of an object surface with vertical and lateral resolution from 0.1 nm, depending on the type of the sample [3].

One of the main advantages of the AFM method is the flexibility of the conditions in which it can operate. AFM imaging can be conducted in an ambient or liquid environment with minimal compromise to its resolution [3, 4]. A specifically designed cell can ensure the scanning in fluids, thus enabling *in situ* monitoring of a real-time biological processes and materials that cannot survive *ex situ*. Additionally, the artefacts caused by dehydration of the sample could be eliminated [3, 4, 5]. Another important benefit of this method is that measurements can be made with minimal or no specimen preparation. Neither physical or chemical fixations, nor coating of the surface by metal sputtering are required for better contrast and conductivity [3]. Consequently, the tested materials and their biomechanical properties are kept close to their original state [5]. Due to the non-destructive nature of the methodology, objects can be visualized repeatedly, acting as their own control [3, 7]. However, AFM imaging demands a smooth surface and a limited size of the sample [4, 5].

2. Basic components and operation modes of AFM

A simplified schematic view of AFM hardware is shown in figure 1. The main parts of the AFM are [1, 8, 9]:

- A cantilever with a sharp tip at the end;
- A detection system of the cantilever's bending;
- A movement system that scans the sample surface in the three dimensions with respect to the cantilever;
- A feedback control of the applied force and the distance;
- A graphical visualization system of the acquired data.

The main component of the AFM is the cantilever/probe assembly that interacts or probes the surface of the underlying sample to provide the information of interest. Generally, the cantilevers are made by micromachining silicon (Si) or silicon nitride (Si_3N_4) and have either rectangular or triangular geometry [1, 4, 8]. The probes are located at the end of the lever and vary in shape, size and sharpness. The dimensions of the tip (shape and diameter) affect the resolution of the topography image [1, 4]. The cantilever/probe body is mounted into a specific cantilever holder with piezoceramic element that can vibrate the lever at a given frequency [4].

Measurement of the bending of the cantilever as it scans a surface is achieved through a laser beam focused on the rear

side of the lever [1]. In order to provide better reflectivity for magnetic or electrical imaging, cantilevers can be treated with thin metal coatings [4]. The reflected light is directed to a quadrant photodiode that registers its positional alterations. Any change of the interaction force between the tip and the sample surface results in bending of the cantilever that leads to a change in the position of the laser spot on the photodiode. The measured signals are transferred to the feedback panel, processed and converted into a voltage which is used to retract or extend the piezo. The movement of the piezo is used to change the position of either the experimental object or the cantilever [1]. A dedicated software is used to transform and analyze the data acquired from the AFM scanning. The area of interest is displayed both two- and three-dimensionally.

The scanning mechanism of AFM is based on the tip-sample interactions. Approaching the surface, the tip of the probe experiences attractive forces until it jumps into contact with the surface. According Newton's third law the specimen also exerts the same force on the tip causing it to withdraw. Considering this process various operation modes are available [1]. The most widely used AFM modes in dental studies are the contact and tapping mode. In the contact mode the imaging is based on the contact between the tip and the surface throughout the entire scanning period. It is a fast and straightforward imaging mode suitable for biological samples. In the oscillating modes (tapping modes) the tip touches the surface periodically and the interaction forces are minimized [1, 9].

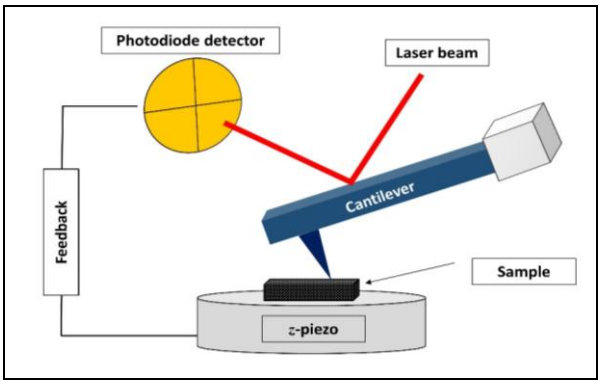

Figure 1: Schematic set-up of the AFM technique

3. Application in dental investigations

AFM is used as a nanocharacterization tool to quantitatively probe topographic, mechanical and biomechanical properties of different organic and artificial dental surfaces and structures [2]. Dental investigations focus on the structure characterization, chemical and mechanical properties of dentin substrate [5], biomechanical properties of teeth, implants, oral bacteria and biofilms as well as diagnostic and clinical tools for oral healthcare [2].

3.1 Demineralization of dental hard tissues.

Reports in literature concerning the utilization of AFM as a research tool in dentistry date back to the mid-90s**.** The focus of the first work was the inspection of native and acid-etched dentin [10]. Recent investigations utilize the AFM method to study the enamel erosion and dentin demineralization as well as the protective effect of different newly developed products against the action of soft drinks' acids [11-14]. Demineralized enamel demonstrates a high degree of surface porosity. This morphological aspect is reflected in the increase of its surface roughness [14]. Application of casein phosphopeptides-containing pastes [12] as well as of different recent formulation toothpastes [15] is reported to prevent dentin/enamel erosion produced by bevarages. In 2016, *Ceci et al.* examined the remineralizing potential of the recently introduced biomimetic self-assembling peptide P11-4 (*Curodont™ Protect/Credentis*). The analysis of the AFM images indicated significant differences when comparing softened enamel with softened and further remineralized enamel, and enamel treated with the protective paste between two acid attacks. The lowered surface roughness after Curodont application verified the protective ability of the tested material [16]. In another experiment *Medeiros et al.* concluded that the thickness and nanomechanical properties of the protective layer formed by TiF_4 varnish were superior to those of the NaF one [17].

3.2 Observation of dentinal surface and collagen network

Atomic force microscopy is a frequently used method for imaging of dentin [3]. It is largely applied in studies of the collagen network of dentin and its changes caused by different chemical agents [3, 18, 19]. Investigation of changes in intertubular and peritubular dentin resulting from treatment with phosphoric acid, self-etching primers, conditioners and other agents used in bonding of restorative materials can be conducted as well [3, 20-23]. Furthermore, AFM enables the understanding of the interaction between dentin adhesives with tooth hard tissues [3, 24, 25]. The AFM scanning of dentin substrate is able to evaluate its roughness, chemistry and mechanical properties by measuring roughness and elasticity of hydrated peritubular and intertubular dentin [26, 27].

Information about surface topography in dental investigations is commonly obtained by scanning in contact mode where the probe is in permanent contact with the surface. However, inherent problems of this methodology are the damage of both sample and probe as well as creation of artefacts in the recorded image [5]. In an attempt to overcome the aforementioned obstacles caused by the dragging motion of the probe tip and the adhesive forces between the tip and the tested surface in this modality, some authors preferred scanning with tapping AFM mode [5, 28]. Despite its increased accuracy and higher resolution, the latter is more time-consuming than the contact mode.

The data acquired from the images after AFM scanning provide quantitative information on surface changes such as dentin tubule diameter and depth. The roughness analysis software of the AFM can be used to obtain different roughness parameters of the altered dental materials being examined [5]. The three commonly used parameters for accurate characterization of surface architecture in vertical dimensions are: average surface roughness (Ra), root mean squared surface roughness (Rq), and maximum surface roughness (Rmax). Both Ra and Rq measure the spread of

the height values around the mean, whereas Rmax resembles the difference between the highest and lowest points on a surface and can be significantly affected by surface damage or contamination. Additional description of the horizontal dimensions is achieved by skewness (Rskw), kurtosis (Rkur), surface area increase (Rsa), and peak counts (Rpc) parameters [29].

Atomic force microscopy enables to observe micro-morphology of non-carious cervical lesions as well as functional width of the dentino-enamel junction [3, 30]. This method is also suitable for monitoring of any changes in nanomechanical properties of dentin during its storage [27]. A difference in local mechanical properties surrounding the tubules has also been observed by AFM. The findings of *Kinney et al.* (1996) showed fourfold hardness (~2.3 GPa) for peritubular vs. intertubular dentin [31]. Inspecting the dentin surface structure, dentinal tubules and collagen network at the nanoscope scale can facilitate improvements in the field of restorative and regenerative dentistry [2].

The AFM methodology is used to measure the surface roughness and wettability of root canal dentin subjected to irrigation with common endodontic solutions such as NaOCl and EDTA [7, 32]. The chelating action of EDTA softens the root canal wall by dissolution of its inorganic components. Thus, dentin tubules become patent and the surface roughness increases as a result of the smear layer removal [33]. The organic dissolving properties of NaOCl on the collagen components of dentin matrix might explain its increased roughening effect over dentin surface. Uneven surfaces could be a clinical benefit in cases of micromechanical bonding of the adhesive materials that need irregularities on the surface [7]. However, rough surfaces with free energy are favorable sites for colonization and biofilm formation [34, 35]. Several investigations show that irrigants used in different concentrations and application time influence the physico-chemical properties of dentin surface [7]. Markedly higher roughness is evaluated after the removal of smear layer by using 17% EDTA in comparison with 2.5% to 5.25% NaOCl and other solutions such as 3% H_2O_2 and chlorhexidine [7, 35-40].

Stiffness and adhesion force of dentin are other biomechanical characteristics of dentin surface after treatment with endodontic irrigants that can be assessed via AFM technology [39]. Dentin stiffness provides information on the capacity of the dentin to withstand the force applied by the nanoindentor and is measured by the mark left by the indenter tip (coupled to the microscope) upon its contact with the dentin (entry stiffness). The resistance offered by the tip as it comes off the dentin is the value of the adhesion force [41]. In the study of *Barón et al.* EDTA caused reduction in dentin stiffness and adhesion force due to the demineralization effect. This morphological change was more pronounced in the peritubular dentin due to its greater mineralization compared with the intertubular dentin [39].

One of the main advantages of AFM is the ability to monitor the real-time *in situ* observation and quantification of demineralization process in radicular dentin. The possibility to follow the very same area of a sample during the process is important for the understanding of the phenomenon and for establishing an optimal duration of chelating substances effects in clinical conditions [42]. The major shortcoming of this method is the impossibility of reliable evaluation of surfaces presenting great height variations due to the saturation in the image formation system of the AFM. Thus, it is necessary to prepare the sample through grinding and polishing, to render a relatively flat surface, before the experiment [3, 5, 42]. Moreover, the size of the examined object cannot be very large in order to fit into the scanner. The x-y scan area can be a maximum of 240µm x 240µm [5]. Another limitation of AFM is that the information about the sample surface is obtained through scanning of a small area just below the tip. Any information about the rest of the its surface is missing which complicates statistical analysis of the results [3].

AFM technology was used to gain comprehensive knowledge about the mechanism of action of substances used for treating sensitive teeth, as well [2]. One of the main approaches to alleviate dentin hypersensitivity is the sealing of dentine tubules in order to isolate them from the external stimuli [43]. The desensitizing prophylaxis paste containing 8% arginine, calcium carbonate and prophylaxis-grade silica has been clinically proven to effectively plug and seal dentinal tubules [2]. In an attempt to gain insight of the mechanism of action of the paste, *Petru et al.* imaged the surface of dentin samples with open tubules and those that were occluded by the desensitizing agent by means of AFM. The untreated dentin surfaces exhibited open tubules with helical fine structure. In the AFM images of the experimental group a protective layer was formed, thus confirming the occluding effect of the arginine-calcium carbonate technology [44].

3.3 Bacterial cells and biofilms

In vivo methodologies such as AFM provide a novel, nondestructive way for *in situ* studying of the critical properties of bacterial cells and their surface proteins. AFM has evolved into a powerful research tool not only for detailed observation of the microorganism ultrastructure surfaces but also for assessing their associated mechanical properties and intermolecular forces [2]. *Cross et al.* (2006) demonstrated that the surface roughness of *S. mutans* strains harbouring genetic mutations of specific surface proteins correlated with their different cariogenic potential [45]. Previous studies explored the surface the surface properties of different *Lactobacillus* strains using AFM to quantify tip–cell-surface adhesion forces. Authors claimed that *Lactobacilli* had the ability to adhere to surfaces, clustering, auto- and co-aggregation [46, 47]. *Van der Mei et al.* (2000) applied the same technology for determination of the cellular stiffness of fibrillated and non-fibrillated strains of *Streptococcus salivarius* [48].

Streptococcus mutans is one of the main cariogenic pathogens. By metabolizing carbohydrates, it emits extracellular polymeric substance (EPS) – glucan, which is an essential virulence factor for the biofilm constitution process [2]. In an attempt to determine the role of glucans in cell adhesion and aggregation of *S. mutans*, *Cross et al.*

(2007) examined cells treated with 1% sucrose compared with non-treated control ones by using the force-measuring aspect of AFM [49]. The inter- and intra-molecular forces are evaluated through recording of the force–displacement curves [50]. An AFM probe is brought close to the cell surface, indented into the cell surface, and then retracted from it. Macromolecules, such as glucan-polymers, attach to the tip during extension of the probe towards the cell surface. The piezoelectric transducer retracts the probe from the cell surface, causing an extension of the attached macromolecule(s) as the tip–cell distance is increased. The force required to separate the probe from the cell-surface molecules is determined by the bending of the AFM cantilever [51]. Upon separation of the tip and cell-surface macromolecules, rupture events occur. This process often results in the formation of sawtooth-like patterns representative of the sequential stretching, unfolding and breakage of the cell surface macromolecules, thus revealing the adhesive nature of the biomolecules native to the cell surface [49]. The authors report direct measurement of the mechanical properties associated with glucan molecules demonstrating that the local adhesion strength increases in a time-dependent process, with a decrease in the average number of rupture events between the AFM tip and the surface proteins of the cell. This finding suggests that *S. mutans* attaches mainly through glucans to surfaces in the presence of sucrose [2].

Together with the mechanical analysis of the cell-surface protein interactions, these studies clearly demonstrate the potential use of AFM for characterization of bacterial surface properties under physiological conditions using intact bacterial cells. As these studies were conducted with uncompromised cells under *in situ* conditions, the results add substantial new information regarding the cell-adhesion and cariogenic properties of *S. mutans* and the individual role of the glucosyltransferases in its adhesion properties [2].

3.4 Dental materials and appliances

AFM can be used for the characterization and fabrication of dental restorative composites in terms of their surface morphology and elastic properties [52]. The increased surface roughness and porosity of dental materials predispose bacterial and fungal colonization that can impair the esthetics and jeopardize their long-term survival [53]. Polymerized resin composites and non-polymerized monomers are reported to accelerate the growth of cariogenic bacteria [54-57]. Moreover, plaque accumulation in clinical conditions is higher compared to other types of restorative materials [54]. This undesirable phenomenon is due to the incomplete polymerization of the restorative material. By using atomic force microscopy for surface roughness measurement of polymerized resin composites that had been exposed to bacterial biofilm, *Beyth et al.* concluded that *S. mutans* outgrowth was accelerated following direct contact with the surface of aged composites. Furthermore, the nanometric surface changes caused by bacteria, increase surface roughness, which in turn, benefits the adhesion and colonization of more microbes. In time, the roughness continues to increase, which may be the reason for restoration failure [54].

A significant increase of the roughness of either restorative materials or intact dental surfaces is also observed after clinical procedures for dental plaque removal by hand instruments or oscillating scalers [58, 59]. Thus, a faster re-growth of biofilm might be expected [59]. Air polishing (AP) with simultaneously ejected water and pressurized air containing abrasive powders has been advocated in dental practice. Although the technique is the least invasive towards dental surfaces, it can cause their damage when the working parameters, type of abrasive powder, spray time and distance are not accurately adjusted [58-60]. In an *in vitro* investigation of surface roughness of dental restoration after air-polishing *Salerno et al.* compared the effect of two abrasive powders (glycine and bicarbonate), at different treatment times (5, 10 and 30s) and two distances (2 and 7 mm). The authors claimed that the least surface change was found when glycine powder was used for 5 s treatment at jet distance of 2 mm. Glycine performed better than bicarbonate at 7 mm. Roughening resulting after 10 and 30 s was comparable for bicarbonate and glycine [59]. This was assessed by measuring the change in the most common parameter of surface roughness, namely the root mean square (RMS) of heights [52, 59]. Other authors measured the same parameter to analyse the effect of polishing for dental restoration with different polishing protocols [61, 62]. Dental clinicains aim to achieve smooth surfaces for both medical (decrease of bacterial adhesion) and esthetical reasons (improved reflectiveness and translucency). *Salerno et al.* investigated the effect of different polishing procedures on the surface morphology of a commercial dental restorative resin composite. The two experimental systems used in the study were Venus Supra (VS) (*Heraeus Kulzer*) and Enhance (EN) (*Dentsply/Caulk, DE, USA*). VS is a one-step polishing system based on silicone-impregnated polishing points. EN is a multi-step polishing system, combining pointed shape polishing points with a polishing cup with abrasive paste, with overall alumina abrading particles in the range of 0.3–1μm diameter. The findings of this research suggested that both tested polishing systems performed equally in terms of surface roughness change. In fact, using either of the two protocols decreased the surface roughness with respect to the positive control (milled but non-polished specimens). However, both polished samples resulted in greater surface roughness when compared to the negative control group (non-polished, non-milled samples) [62].

During the past decade, AFM has become the nanotechnology standard for elastic measurements at high accuracy and spatial resolution, by means of force-spectroscopy of force-volume modes [52, 62]. Atomic force microscope has been used by *Thorat et al.* (2012) to investigate the elastic modulus of experimental restorative composites based on fillers of titania, nanosilica, and milled glass. These composites were studied in a simplified model version, without the use of a coupling agent bonding the fillers and the matrix [63]. In an attempt to develop coupling-agent-free novel dental restorative materials, *Thorat et al.* (2013a,b) changed the vitreous filler materials with hard metal oxides such as titania and alumina. After the AFM characterization of the material, authors concluded that

the nano-size fillers provide some advantage with respect to microscale fillers of similar materials, probably due to the higher interfacial area connecting with the matrix, when properly dispersed [52, 64, 65]. In another study focused on the surface morphology and mechanical elastic properties of flowable composites *Salerno et al.* correlated the material properties to peculiar morphology of the matrix-filler distribution [52, 66].

In orthodontics, the contact mode of AFM was used to map the surface of wires, obtained after a 3-week exposure to the oral cavity [5, 67]. Similar investigation was conducted for another orthodontic appliance used for retraction of teeth – the elastic orthodontic chains. The alteration of the surface roughness of the chains lowered the effectiveness of chain-guided tooth movement [5].

Several investigations in the field of prosthetic dentistry postulate that the improper choice of disinfection mode can increase the roughness of the denture surfaces, thus favoring plaque accumulation [68-70]. In a recent study, *Yankova et al.* (2018) evaluated the roughness change of two types resilent relining materials for complete dentures subjected to five different cleaning methods. The AFM analysis demonstrated that greatest reduction in roughness in all test samples was established when using Protefix cleaning tablets (*Queisser Pharma, Germany*) and ultrasonic cleaner. The surface of the lining materials was more uneven after mechanical brush cleaning alone and chemical treatment by oxidizing agents with enzymes such as Corega tablets (*GlaxoSmithKline, UK*) [68].

AFM can be used in the field of dental implantology for advanced imaging of the implant surfaces and interfaces. The interfaces of interest in dental implants occur between the tooth ceramic crown on the top, the cement joining the crown to the metallic (Ti alloy) parts of the abutment, and the implant itself [52]. In a recent work AFM was used to identify possible morphological damage occurring on the surface of implant screws. The results from the images revealed that most implants exhibited no major changes in their surface topography after in vitro implantation tests in a model bone similar to the typical clinical bone [52, 71]. AFM is also used to characterize the hydroxyapatite modifications of the implants' surface and their effects on the biocompatibility and osseointegration [2]. Although the design and use of such materials is well-known, their initial application resulted in high failure rates that were solely associated with their mechanical properties [72]. Further studies applied the nanoscale characterization approach in an attempt to clarify the physiochemical mechanisms that occur. The results from the investigation of *Lin et al.* (2009) associated the higher success rates (*in vitro* and *in vivo*) and favorable biocompatibility with the nanotopography of the modifiers rather than with the substrate chemistry [73].

4. Conlusions

The use of atomic force microscopy for nanocharacterization in dental investigations is an effective and versatile analytical tool. The ability of thorough characterization of topology and quantitative biomechanical and biophysical analysis of dental surfaces and materials enables the application of this method in various dental fields. The results obtained from a broad spectrum of *in vitro* studies of dental caries, tooth hypersensitivity, endodontology, prosthetics, restorative and preventive dentistry can be translated from research to *in vivo* application by stimulating the development of contemporary methods and products of possibly great benefit to the treatment results and prognosis. However, AFM scanning is an expensive, labour and time-consuming process that requires a dedicated software and trained operator for acquisition and interpretation of the results. Other drawbacks of the methodology are the limited size of the tested sample as well as the requirement for surface flatness prior the scanning.

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