1. Introduction

1.1. Properties of titanium

Titanium (Ti) the ninth most abundant element in the Earth's crust, occurs in a number of minerals and clays. It is always bonded to other elements in nature. It was discovered in Cornwall, United Kingdom, in 1791 by an amateur geologist and pastor, William Gregor (Boase, 2008). Ti readily reacts with oxygen, when exposed to air. Its alloys are very strong and lightweight, and are widely used in industrial processes, e.g. desalination plants, the aerospace and automotive industries and agriculture, and the element is utilized in medical prostheses, orthopedic implants, dental implants and dental and endodontic instruments (Wang and Fenton, 1996), and also in sporting goods, jewelry and mobile phones. The most important properties of this metal are its corrosion resistance and high strength-to-weight ratio. In an unalloyed condition, Ti is as strong as some steels, but 45% lighter. A passive and protective oxide coating that forms on its surface protects it from further oxidation and other reactions. Nevertheless, it does corrode slowly (Schutz and Thomas, 1987). A surface oxide layer is formed within nanoseconds when Ti is exposed to air (Hanawa, 1998).

Passivation treatment provides a controlled and uniformly oxidized surface state. Passivation decreases ionrelease, and leads to a dense and stable oxide film which improves the resistance to corrosion. The treatment with nitric acid eliminates metallic contamination from the surface, but has practically no influence on the overall topography of the Ti surface. The result is a passive layer of TiO_2 (titanium dioxide) with an initial thickness of 2-6 nm (Puippe, 2003), but it slowly grows until it attains a thickness of 25 nm. Oxidative agents are well known to exert a corrosive effect on the alloys used in dentistry, with the exceptions of Ti and some other bioinert materials. Indeed, oxidative processes can thicken and condense the TiO_2 layer on the surface, improving the corrosion stability of the underlying Ti. On the other hand, reducting agents, such as fluoride (F^-), may have the opposite effect and attack this layer (Strietzel et al., 1998). Ti ion release is enhanced in the presence of F^-, and this effect is accelerated at low pH. High F^- concentrations and an acidic pH are known to impair the corrosion resistance of Ti (Toumelin-Chemla et al., 1996) and consequently crevice formation and pitting corrosion occur (Reclaru and Meyer, 1998; Schiff et al., 2002).

Ti is a relatively cheap metal, and commonly used to make surgical and dental implants. However Ti crowns, bridges and the framework of partial removable dentures

(Huget, 2002; Wang and Fenton, 1996) are not common in prosthetic dentistry because the technology of the operation process is very expensive. The material used to produce dental implants is generally “commercially pure” Ti (CP Ti) or the most common Ti alloy, Ti-6Al-4V (Mändl et al., 2005; Park and Kim, 2000). For the production of Ti brackets for orthodontic dentistry (Harzer et al., 2001), another Ti alloy with special features is used. Dental arch wires and orthodontic braces are usually made from the TiNi shape memory alloy.

Large numbers of patients with dental implants also have natural teeth in the oral cavity. Furthermore, orthodontic patients are at highrisk of the development caries and in need of F^- protection. To maintain good oral hygiene and prevent caries, patients use toothpaste, gels or mouthwash, almost all of which contain F^-. The hydroxyapatite [$Ca_5(PO_4)_3OH$] of the enamel is attacked by F^- to form fluorapatite, $Ca_5(PO_4)_3F$. Fluorapatite makes the enamel more resistant to caries (Triller, 1998). However, as F^--containing mouthwashes have been found to cause galvanic corrosion between orthodontic wires and brackets, it has been suggested that a new type of mouthwash for use during orthodontic therapy could be an interesting development in this field (Schiff et al., 2006).

The effects of the use of ordinary F^--containing toothpastes to clean the teeth of patients with brackets have also been reported (Harzer et al., 2001). The surface roughness and biocompatibility of Ti facilitate plaque adherence. The surface of the Ti brackets became very rough due to the use of toothpaste for 5.5-17 months, and in a few cases corrosion occurred in the brackets. It was concluded that such toothpaste enhanced plaque accumulation and discoloration of the bracket surface, but the level of corrosion was not significant.

Nakagawa et al. (2001) investigated the Ti alloys Ti-6Al-4V, Ti-6Al-7Nb and Ti-0.2 Pd form the aspect of their corrosion behavior and established that even a low F^- concentration caused corrosion in an acidic environment. However if the Ti alloy contained at least 0.2% Pd, corrosion did not take place. The high corrosion resistance of this alloy is due to the enrichment of Pd in the surface, which promotes the repassivation of Ti.

1.2. Types of dental implants

During the history of dental implantology since 1952, when the first dental implants were introduced many different types have been employed, but endosteal implants are currently most common. On the next figures some forms of implants (subperiosteal, transosteal, endodontic, endosteal) are shown.

a. Intramucosal implants (Evasic, 1983).

b. Subperiosteal implants are illustrated in Fig. 1 (Zwerger et al., 2007).

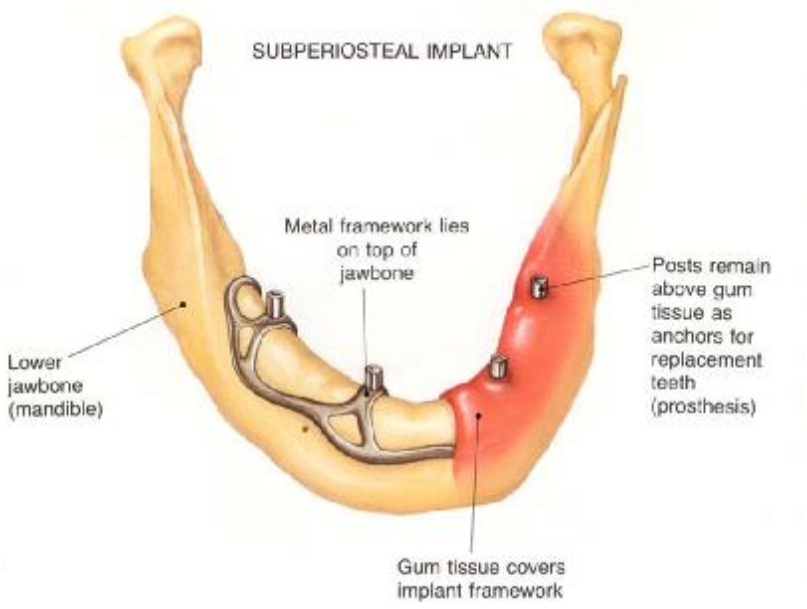


Figure 1. Subperiosteal implants

c. Typical transosteal implants are shown in Fig. 2 (Knapp and Small, 1990).

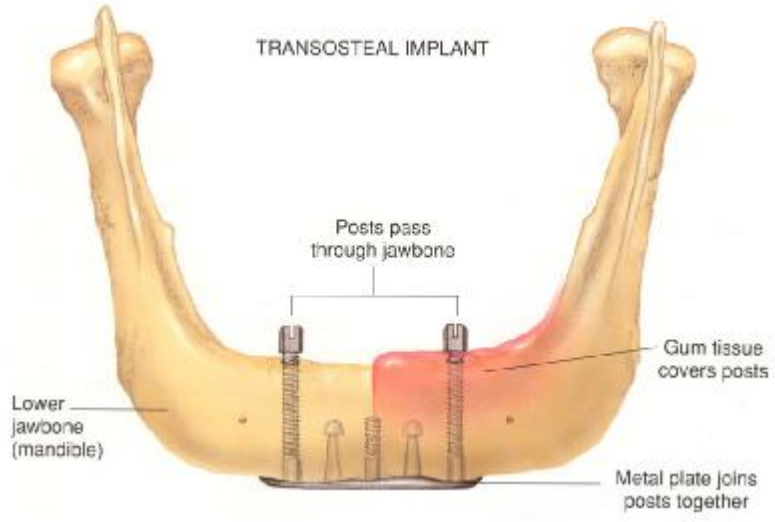


Figure 2. Transosteal implants

d. An endodontic implant is to be seen in Fig. 3 (Frank, 1967).

Figure 3. Endodontic implant

e. Endosteal implants

Endosteal implants (Fig. 4) are the most widely used type of implants in bone. The various types include screws, cylinders or blades placed surgically into the jawbone. A typical implant consists of a Ti screw (resembling a tooth root) with a roughened or smooth surface. Roughened surfaces may be modified by plasma spraying, anodizing, etching or sandblasting to increase the surface area and the integration potential of the implant. The endosteal implants used almost exclusively nowadays comprise a root portion, which is placed in the jawbone, a cervix (transmucosal part), located at the level of the gums, and a head, which supports the crowns, bridges and in general any structure. The transmucosal part between the root portion and the head is assorted with the gingival margin (Fig. 4).

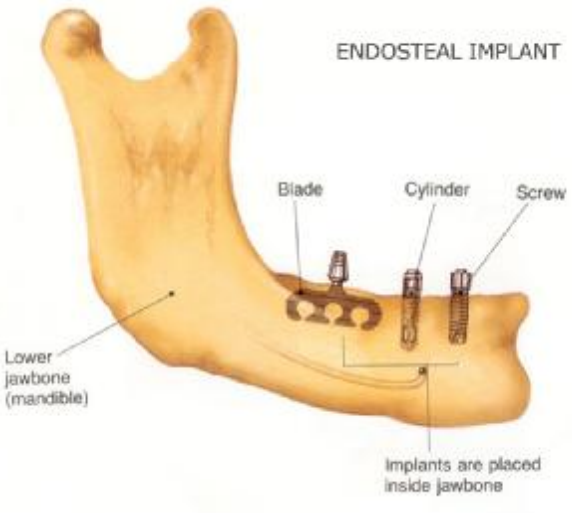


Figure 4. Endosteal implants

The majority of dental implants are made of CP Ti, which is available in 4 grades depending on the carbon and iron contents (Park and Kim, 2000, Table 1). For reasons of economy and mechanical quality, some implant systems are fabricated from Ti-6Al-4V alloy (Parr et al., 1985).

Table 1. Chemical compositions of various grades of Ti and one of its alloys [Park and Kim, 2000 (ASTM, 1992)]

Element	Grade 1	Grade 2	Grade 3	Grade 4	Ti-6Al-4V[a]
Nitrogen	0.03	0.03	0.05	0.05	0.05
Carbon	0.10	0.10	0.10	0.10	0.08
Hydrogen	0.015	0.015	0.015	0.015	0.0125
Iron	0.20	0.30	0.30	0.50	0.25
Oxygen	0.18	0.25	0.35	0.40	0.13
Titanium			Balance		

[a]Nominally: aluminum 6.00% (5.50 ~ 6.50), vanadium 4.00% (3.50 ~ 4.50), and other elements: 0.1% maximum individually or 0.4% total. All data are maximum allowable weight percentages.

1.3. Indications of dental implants

Dental implants are used to replace one or more or all missing teeth, and can serve as an abutment for various prosthetic appliances. Their use allows dentists to avoid the preparation of teeth for fixed partial dentures, or a more stable and more esthetic treatment option can be provided for the patients. A well-functioning dental implant can prevent further tooth loss and preserve the alveolar bone.

The main indications for prosthetic implant treatment are:

(a) One or more missing teeth. Single tooth implants for cases with a bounded saddle: without preparation of the adjacent teeth for a bridge, the crown is retained only on the implant.

(b) A posterior edentulous ridge: without implants, only removable partial dentures can be made; with implants, the patient may wear crowns or bridges with great comfort.

(c) A completely edentulous jaw: an implant-retained overdenture, a removable or fixed bridge, or crowns can be provided for the patient, who will achieve a higher level of quality of life.

In the first two groups, Ti implants and natural teeth are present together in the oral cavity. For caries prevention, dentists often suggest various alternative prevention solutions, mainly mouthwashes.

1.4. Macroscopic features of the gingiva

The success and long-term prognosis of endosseous implants depend predominantly mainly on three factors: osseointegration (anchorage in the host bone), gingival attachment and the appropriate transmission of masticatory force or load transfer capacity (Fig. 5). This section focuses on the macroscopic features of the gingiva, which is important for a clear understanding of the purpose of my studies.

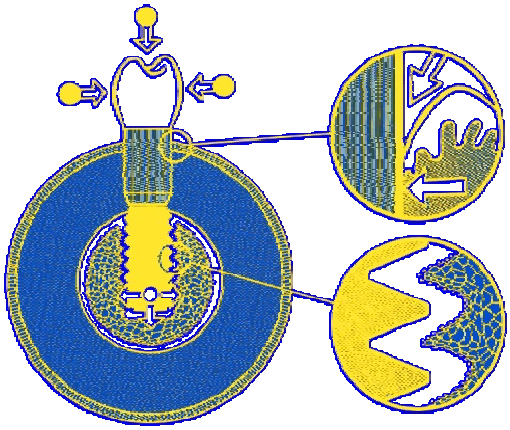


Figure 5. The success and long-term prognosis of endosseous implants depend mainly on three factors: osseointegration (anchorage in the host bone), gingival attachment and the appropriate transmission of masticatory force (load transfer capacity).

The gingiva is divided anatomically into free gingiva, and attached and interdental papillae. The marginal gingiva is the terminal edge of the gingiva surrounding the teeth in a collar. In around half of the population, it is demarcated from the adjacent, attached gingiva by a shallow linear depression, the free gingival groove. It forms the soft tissue wall of the gingival sulcus, which is usually about 1 mm deep. The marginal gingiva is supported and stabilized by the gingival fibers. The attached gingiva is continuous with the marginal gingiva. It is firm, resilient, and tightly bound to the underlying periosteum of the alveolar bone. The facial aspect of the attached gingiva extends to the relatively loose and movable alveolar mucosa, from which it is demarcated by the mucogingival junction. It is resistant to masticatory forces and always keratinized. The peri-implant mucosa is the soft tissue that surrounds dental implants. The interface between the implant and the mucosa consists of an epithelial and a connective tissue component. The presence of the attached mucosa around dental implants is important because it is bound very tightly to the underlying alveolar bone and provides protection for the mucosa during functional use of the structures of the oral

cavity, such as chewing. The gingival and the peri-implant mucosa are covered by a keratinized oral epithelium. The connective tissue barrier and the junctional epithelium around the tooth or the implant are about 2 mm wide (Fig. 6). The width of the supraalveolar connective tissue around the tooth is 1 mm, but in the case of a Ti implant it is 1.5 mm (Berglundh et al., 1991); it is separated from the alveolar bone by collagen-rich connective tissue. This biological barrier which has a thickness of 3 to 4 mm, has been called the "biological width"; it protects the zone of osseointegration from factors caused by plaque and bacteria in the oral cavity (Berglundh and Lindhe, 1996).

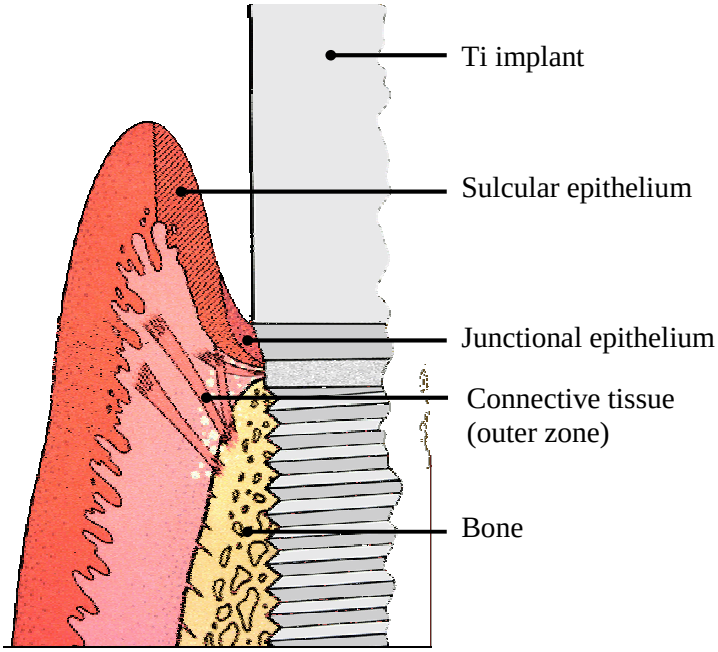


Figure 6. Details of the epithelial attachment on a Ti implant surface

Both epithelium types develop a hemidesmosomal connection between the tooth and the implant surface (Gould et al., 1984; Swope and James, 1981). Pending around the natural tooth, the main filaments which constitute the connective tissue radiate from the cementum, in the event of a Ti implant, the main filaments start from the periosteum of the bone and run parallel to the bone surface. Without attached gingiva, the freely movable alveolar mucosa (which is more fragile) would suffer injury during eating and cleaning activities, such as brushing of the teeth. The interdental gingiva occupies the gingival embrasure, which is the interproximal space beneath the approximal contact area of the teeth. It can be pyramidal or have a col shape, like a pass in a mountain range (Schroeder and Listgarten, 2000).

It has been reported that a basal lamina and hemidesmosomes develop 2 weeks after implant placement. *In vitro* and *in vivo* studies have demonstrated a chemical attachment between the Ti implant surface oxide layer and the epithelium. There is a similar attachment between the epithelium and natural tooth surfaces, mediated by a glycoprotein. Connective tissue fibers adjacent to Ti implant surfaces may bring the tissue into tight apposition to the implant without the formation of an absolute biological attachment between the implant and the connective tissue. Modification of the Ti surface morphology may selectively intensify the attachment of either epithelial cells or fibroblasts. The mechanisms of attachment and of the factors which enhance the integrity of the biological seal between the implant and the soft tissues are very important for the prognosis of Ti implants. (Donley and Gilette, 1991).

A very important factor in the implant survival is the state of health of the peri-implant soft tissue. Any damage to the junctional epithelium results in it being irregular in texture, and in the formation of "pocket" epithelium, which is a primary symptom of gum disease. The long-term success of a dental implant depends to a large extent on the gingival attachment to the neck of the implant. This mucosal seal ensures protection against bacterial attack and other injurious effects exerted by the oral environment. The epithelial attachment may be anchored onto a rough or a smooth surface by hemidesmosomes through a preformed glycoprotein layer. A rough surface is more favorable for plaque accumulation, which is an undesired effect in this very sensitive region of the implant. Accordingly, in order to avoid pathogenic plaque accumulation, the neck of an implant abutment must be polished (Fig. 5) (Bollen et al., 1996).

1.5. Causes of dental implants failure

The most common causes of implant failures are:

1. overloading
2. bacterial infection
3. secondary complications in the incomplete bone tissue

This section will deal only with bacterial infections as these are related to the studies and aims of the thesis.

The plaque formed on the surface of a dental implant may be indicative of a reaction in the tissue which causes inflammation primarily in the soft tissue, and the bone too may soon be is involved. Peri-implantitis is an inflammatory reaction which, without treatment, sooner or later results in boneloss around the implant (Albrektsson and Isidor, 1994). Peri-

implant diseases are caused by inflammatory conditions that affect the soft and hard tissues around the implant fixtures. The extent and location of peri-implant inflammation differ greatly from inflammation around the teeth in similar environments (Schou et al., 1993). Peri-implantitis progresses deep into the bone, which is in contrast with periodontitis, when the inflammation initially developes only in the connective tissue. Gotfredsen et al. (2002) reported that the resistivity of peri-implant tissues was less than that of the tissues around the teeth. Peri-implantitis may involve bone resorption, and the treatment outcome following nonsurgical management is less predictable. Adjunctive decontaminating treatment includes the use of antimicrobials, and resistant cases may sometimes be managed with a surgical approach (Heasman, 2010). The proportion of failed dental implants is higher amount patients who display poor oral hygiene. Histories of periodontitis and cigarette smoking have been found to be risk indicators for peri-implant disease (Heitz-Mayfield, 2008).

Fibroblasts play an important role in establishing and maintaining the mucosal seal, this fibroblast-rich barrier exhibiting a high cell turnover next to the Ti surface (Berglundh et al., 1994). The microbial biofilm adheres to the surface of the transmucosal abutment of osseointegrated dental implants. The peri-implant mucosa which covers the alveolar bone is closely adapted to the implant, in a similar way as the gingival crevice around the natural tooth (Figure 7.).

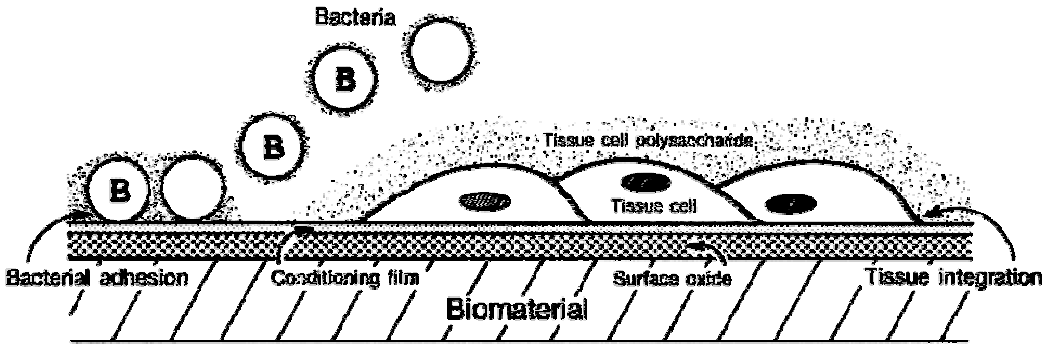


Figure 7. On a biomaterial such as a dental implant there is always a competition for the surface between the tissue cells and the bacteria (Anderson et al., 1996)

1.6. Periodontal pathology diseases and peri-implant infections

Periodontal pathology diseases have been divided into two main types: gingival diseases and periodontal diseases. The gingival diseases can be subdivided two groups, depending on the origin: dental plaque-induced or non-plaque-induced (Table 2).

Table 2. Classification of the gingival diseases (Armitage, 1999)

A. Dental plaque-induced gingival diseases	**B. Non-plaque-induced gingival lesions**
Gingivitis associated only with dental plaque	Gingival diseases of specific bacterial origin
Gingival diseases modified by systemic factors	Gingival diseases of viral origin
Gingival diseases modified by medication	Gingival diseases of fungal origin
Gingival diseases modified by malnutrition	Gingival lesions of genetic origin
	Gingival manifestations of a systemic condition
	Traumatic lesions
	Foreign body reactions
	Not otherwise specified

Periodontal diseases can be grouped as shown in Table 3 (Armitage, 1999).

Table 3. Types of periodontal diseases

Chronic periodontitis
Aggressive periodontitis
Periodontitis as a manifestation of a systemic disease
Necrotizing periodontal diseases
Abscesses of the periodontium
Periodontitis associated with endodontic lesions
Developmental or acquired deformities and conditions

In peri-implant tissues, microbial colonization and consequent inflammatory reactions may occur in a similar manner to the pathogenesis of periodontitis. In partially edentulous patients, the microbiota developing around an implant closely resembles the microflora of the crevicular sulcus around natural teeth. A history of periodontitis and the presence of putative periodontal pathogens are factors that can influence the condition of peri-implant tissues in partially edentulous subjects (Leonhardt et al., 1993; Mombelli et al., 1995). The subgingival

microflora around implants and the gingival sulcus around the teeth in the same jaw have a similar bacterial morphotype, because the teeth may serve as a reservoir for bacterial colonization (Quirynen and Listgarten, 1990).

The pockets around the teeth can serve as a reservoir for putative periodontal pathogens, which cause the early colonization of the peri-implant sulcus in partially edentulous subjects, when the oral hygiene is not meticulous. In several studies on partially edentulous patients, the microbiologic flora was found to be analogous in the sulcus around teeth and implants (van Winkelhoff et al., 2000). The complex peri-implant microbiota resembling that in adult periodontitis can cause infection and implant failures. *Aggregati-bacter actinomycetemcomitans* (previously: *Actinobacillus actinomycetemcomitans)* and *Porphyromonas gingivalis* are not as frequently associated with peri-implant infection in edentulous individuals as in dentate subjects (Mombelli et al., 1987).

The oral cavity is a favorable environment for a great variety of bacteria. Oral bacteria include streptococci, lactobacilli, staphylococci and corynebacteria, with a large number of anaerobes, especially Gram-positive rods. Bacterial cells account for 60-70% of the volume of the dental plaque. The other components are: salivary polymers and bacterial extracellular products. The plaque is regarded as a naturally-constructed biofilm, in which the consortia of bacteria may reach a thickness of 300-500 cells on the surfaces of the teeth or dental implants. High concentrations of bacterial metabolites accumulated on the teeth and gingival tissues can cause dental diseases. The dominant bacterial factors in the dental plaque are *Streptococcus* species (*Streptococcus sanguis* and *Streptococcus mutans*), both of which are considered to be major factors responsible for the formation of plaque.

The development of a dental biofilm (dental plaque) on a surface is determined by the physical properties of the surface, the environmental humidity and the free energy of the bacterial surface. Salivary glycoproteins form an acquired pellicle on a hard surface, which contains a small amount of immunoglobulins too (Almsthäl et al., 2001). This pellicle can interconnect with the hard surfaces via van der Waals and electrostatic forces (de Jong et al., 1984; Quirinen et al., 1989) and its width does not exceed 1 µm. At first, the pellicle is transparent. The formation of plaque is initiated by a loose attachment of Gram-positive cells; this connection is reversible. Later, a strong attachment develops, when the extracellular glycocalyx, a sticky polymer produced by bacterial cells, promotes the connections on the surface. More and more bacterial cells appear among these polysaccharides and can modify their phenotype, which results in rapid growth of the bacteria and produces large amounts of extracellular polymeric substances. The bacterial cells synthesize the glycoprotein from

dietary sugars (principally sucrose). The extracellular polymeric substances help the emerging biofilm community to develop a complex, three-dimensional structure. Biofilm communities can be formed within some hours, the coccoid bacteria develop several layers, and the form is usually cubic. After a few days, the Gram-positive rods and filaments (mainly *Actinomyces* sp.) outnumber the cocci. A reticulation of water channels serve as the pathways for nutrients and help with the efficient removal of the waste products for use by the bacteria on the surface. The oxygen concentration within the biofilm changes. It is increasingly difficult for oxygen to reach the deeper layers and mainly anaerobes can proliferate near the hard surface until virtually only aerobes or facultative anaerobes can be found on the surface of the biofilm. The many interactions between the different bacterial species include a number of synergistic and antagonistic biochemical interactions. For example, when obligate anaerobes and aerobes are involved in co-adhesion, they interact to ensure the survival of the anaerobic bacteria in the oxygen-rich oral cavity (Marsh 2004, 2006). As the biofilm thickens and becomes more mature, it can consist of 100-300 cell layers. In time, the growth of the plaque slows down and dead bacterial cells can be seen deep inside the plaque. Around the pellicle Ca-containing crystals appear in the interbacterial matrix; this is the first sign of calculus. On the surface, the bacterial colonies demonstrate an active life, reflected in a typical corn-cob formation. The center is this occupied by long filament- shaped bacteria, connected to a number of cocci and short rods on the surface (Listgarten, 1976).

The initial dental biofilm bacteria are: *Streptococcus* species, *Veillonella* sp., Gram-negative anaerobic cocci, Gram-positive rods (including *Actinomyces* sp.) and Gram-negative rods (*Capnocytophaga* sp.) Specific anaerobic species subsequently appear: *Fusobacteria* and *Prevotella intermedia*. The late colony bacteria are *P. gingivalis,* mobile Gram-negative rods and the spirochetes. Figure 8 illustrates of the biofilm formation and maturation, on the surface (Rickard et al., 2003).

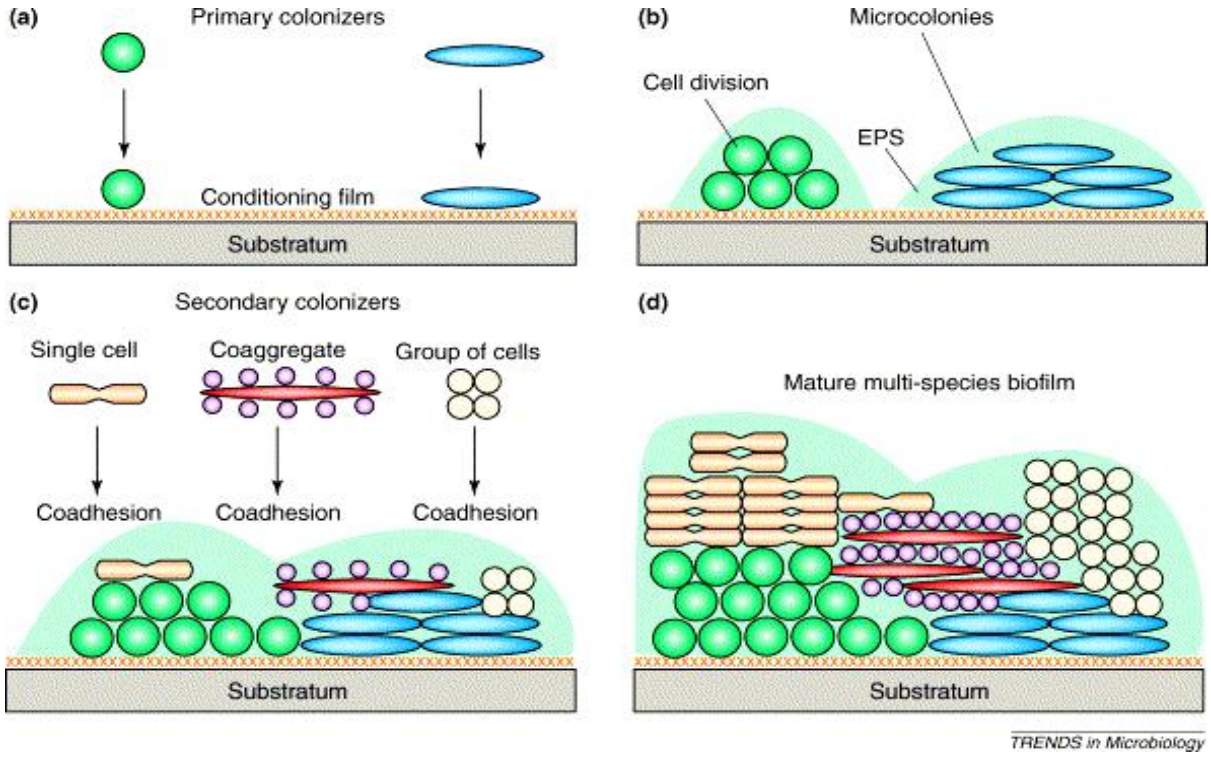


Figure 8. (a) Free-floating or planktonic bacteria come into contact with a coated surface and within minutes can become attached. (b) The bacteria begin to produce slimy extra-cellular polymeric substances (EPS). (c) The biofilm can spread through detachment of small or large clumps of cells, or by "seeding dispersal" that releases individual cells. (d) Either type of detachment allows bacteria to attach to the surface or the down-stream biofilm of the original community. Maturation and the formation of clonal mosaics within the multi-species biofilms can be seen (Marsh 2004, 2006).

2. Aims of the study

The physical and chemical changes that may take place on a Ti surfaces as a result of the use of prophylactic agents such as those which are widespread in everyday oral hygiene regimens may be important factors as concerns the accumulation of plaque on that surface. In view of the evidence of the causative effect of plaque accumulation in the development of peri-implantitis, it is very important to identify the effects of F^- on Ti surfaces and the consequences relating to bacterial colonization.

The aims of my study were as follows:

- To apply atomic force microscopy (AFM) to investigate whether a commonly used F^--containing mouthwash, NaF solution and a prophylactic gel can modify the surface roughness of CP Ti.
- To identify the chemical changes caused on a Ti surface caused by F^--containing materials (mouthwash, NaF solution and prophylactic gel) through the use X-ray photoelectron spectroscopy (XPS).
- To evaluate whether the surface changes mentioned above could influence the accumulation and colonization of different microorganisms.
- To assess the changes in the number and maturation of the bacteria after modification of a Ti surface by F^--containing materials.

3. Materials and methods

3.1. Cleaning of Ti samples

A total of 72 polished CP Ti discs (9 mm in diameter, 2 mm in thickness, CP grade 4, Camlog, Biotechnologies AG, Switzerland and Protetim, Hungary) were used. This type of Ti contains only very small amounts of other elements: the oxygen content is less than 0.40%, the nitrogen not more than 0.05%, and the carbon less than 0.10%. The discs were mechanically polished until the surface was analogous to the polished surface of the neck of the dental implant abutment, i.e. the roughness did not exceed 0.2 μm. They were first cleaned in an ultrasonic bath with acetone for 15 min then in absolute ethanol for 15 min, and they were finally rinsed with distilled (ultrapure) water 3 times for 10 min. The discs were always held only with Ti forceps, so as not to contaminate the surfaces (Stájer et al., 2008). Each of the cleaned discs was used in only one subsequent investigation.

3.2. Fluoride treatment and sterilization

Patients are recommended to use a prophylactic rinse once a day for 30 s and the gel once a week for 2 min. In our investigations, the duration of trials of cleaned Ti discs with F^- was 1 h. This corresponds to the accumulated effect of the regular usage of the rinse for 4 months or of the gel for 7.5 months. In all cases relating to the determination of numerical data reported in this thesis, groups of 4 discs were treated for 1 h with one or other of the following materials:

– a mouthwash (Elmex, GABA International AG, Switzerland) containing 250 ppm F^- (pH 4.4),
– an aqueous 1% solution of NaF, containing 3 800 ppm F^-, with the pH set to 4.5 with lactic acid,
– an aqueous 1% solution of NaF, containing 4 159 ppm F^-, with the pH set to 6.5 with lactic acid (prepared according to the instructions of Nakagawa et al., 1999),
– a gel (Elmex, GABA GmbH, Germany, containing 12 500 ppm F^-, 2 500 ppm (0.25%) in the form of amine fluorides: Olaflur or Dectaflur (hexadecylamine hydrofluoride), with pH 4.8.

The solutions were always prepared fresh and filtered through a Millipore filter with a 0.22 μm filter cartridge.

After the 1 h treatment, the discs were removed from the solution or gel, washed with ultrapure water and dried in air. They were then subjected sterilization with steam at 160 ºC for 20 min in order to eliminate bacteria from the surfaces. Subsequent investigations were always performed within the sterilization validity time, which was 14 days. (Stájer et al., 2012).

3.3. Bacterial incubation

3.3.1. Microbiological sampling, isolation and identification of the oral flora from the mouth of a patient with chronic periodontitis

The area to be sampled was isolated with cotton rolls, and the tooth surface was cleaned with 70% ethanol and dried with sterile cotton swabs. Samples were obtained from the 3 deepest pockets or most diseased sites with individual sterile paper points, which were placed in the gingival crevice for 15 s and moved around the tooth, and then sent to the microbiology laboratory in Portagerm multitransport medium (bioMérieux, S.A., Marcy l'Etoile, France). Culturing was always commenced within 1 h of sampling (Jousimies-Somer et al., 2002).

Sample culturing: All three paper points were placed together into 1.0 ml of reduced BHI (brain heart infusion, pH 7.2) solution and mixed on a Vortex shaker for 30 s. After gentle dispersion, the suspensions were diluted (10^{-1}–10^{-6}) in reduced BHI broth and 100-µl samples of each dilution and 100-µl samples of the corresponding undiluted suspension were immediately plated on selective and nonselective media. Columbia agar base (Oxoid, Basingstoke, United Kingdom) supplemented with 5% (v/v) cattle blood, hemin and vitamin K_1 was used to quantify the total cultivable facultative and anaerobic bacterial flora. *Veillonella* spp. were isolated from Veillonella agar (Oxoid, Basingstoke, United Kingdom), while Rogosa agar (Oxoid, Basingstoke, United Kingdom) was used for the selective isolation of lactobacilli, CFAT (cadmium, fluoride, acriflavine, tellurite) (Oxoid, Basingstoke, United Kingdom) agar was used for *Actinomyces* spp., and chocolate agar was used for determination of the total aerobic bacterial flora. For the selective growth of streptococci and *Entero-bacteriaceae*, Mitis Salivarius (Oxoid, Basingstoke, United Kingdom) and Endo (bioMérieux, S.A., Marcy l'Etoile, France) agar, respectively, were used. For aerobic bacteria, the plates were cultured at 37 °C in a 5% CO_2-containing environment for 48 h. For the isolation of anaerobic organisms, cultures were set up and incubated in an atmosphere of 90% N_2, 5% H_2 and 5% CO_2 in an anaerobic chamber (Concept 400) for 5 days at 37 °C. The selective agar

media for the isolation of *Enterobacteriaceae* were incubated at 37 °C for 24 h. Each different colony type from positive cultures was subcultured for purity and identification. The results of Gram staining and the atmospheric growth requirements of the different colony types were utilized to determine the additional biochemical tests required for identification of the isolates. API 20A and ATB ID 32 ANA (bioMérieux, S.A., Marcy l'Etoile, France) tests were applied to identify anaerobic bacteria, facultative anaerobic Gram-positive cocci and bacilli (Jousimies-Somer et al., 2002).

Other conventional tests for different bacteria were used where appropriate. A very complex aerobic-anaerobic bacterial flora was isolated from the given patient. The total CFU/sample was $7.5x10^8$. Besides Gram-positive anaerobes (*Eubacterium* sp., *Actinomyces naeslundii, Bifidobacterium* sp, *Finegoldia magna, Parviromonas micra* and *Propionibacterium* sp.), Gram-negative anaerobes were dominant (*Prevotella disiens, P. gingivalis, Tannerrella forsythia, Bacteroides ureolyticus* and *Fusobacterium nucleatum*). Fresh colonies of these bacteria, which were incubated in an atmosphere of 90% N_2, 5% H_2 and 5% CO_2 in an anaerobic environment (Bactron from Sheldon Manufacturing Inc., Cornelius, Oregon, USA) for 2 days at 37 ºC were suspended in reduced BHI broth (Oxoid, Basingstoke, United Kingdom) and used after gentle dispersion (McFarland 1.0 dilution). 2-ml aliquots of this mixture were immediately plated onto 24-well sterile microtitre plates (two were used simultaneously), containing different Ti discs. The Ti discs were removed from the microbiological environment after 2, 4 or 7 days.

3.3.2. *Streptococcus mutans* preparation

Fresh colonies of the *S. mutans* ATCC 25175 control strain, incubated in a 5% CO_2 atmosphere for 24 h at 37 ºC, were suspended in reduced BHI broth (Oxoid, Basingstoke, United Kingdom) and used after gentle dispersion (McFarland 0.5 dilution). 20-ml aliquots of this mixture were immediately plated onto sterile Petri dishes containing the different Ti discs. After 5 days of incubation under 5% CO_2, the samples were removed from the incubation (Stájer et al., 2009).

3.3.3. *Porphyromonas gingivalis* preparation

Columbia agar base (Oxoid, Basingstoke, United Kingdom) supplemented with 5 v/v% cattle blood, hemin and vitamin K_1 was used for the culturing of *P. gingivalis*. Fresh colonies of *P. gingivalis* strain ATCC 33277, incubated in an atmosphere of 90% N_2, 5% H_2 and 5% CO_2 in an anaerobic environment (Bactron from Sheldon Manufacturing Inc.,

Cornelius, Oregon, USA, for 2 days at 37 ºC), were suspended in reduced BHI broth (Oxoid, Basingstoke, United Kingdom) and used after gentle dispersion (McFarland 1.0 dilution). 2-ml aliquots of this mixture were immediately plated onto 24-well sterile microtiter plates containing different Ti discs. After 5 days of anaerobic incubation (under 90% N_2, 5% H_2 and 5% CO_2), the samples were removed from the bacterial culturing.

3.4. Characterization of the Ti surface

3.4.1. AFM Study of the physical properties of Ti surfaces

The surface structure of the discs was analyzed by AFM. A PSIA XE-100 instrument (PSIA Inc South Korea) was used to acquire information on the roughness of the sample surface on the micron to nanometer scale, through a technique that measures the forces on the tip of the instrument probe as it approaches and retracts from the investigated surface. The tips were P/N 910M-NSC36 contact silicon cantilevers purchased from MikroMasch Eesti OU (Estonia). Cantilevers with spring constants of 0.95 and 1.75 N/m were used. The measurements were performed in contact mode, and the height, deflection and 3D images with areas of 10x10 μm and 5x5 μm were captured. The surface roughness (R_a) was determined via the AFM software program (at least 10 independent measurements) as the arithmetic average of the surface height relative to the mean height. R_{pv} was also determined, as the difference between the highest (peak) and deepest (valley) values of the surface. R_a was depicted graphically following section analysis.

3.4.2. XPS study of the chemical properties of Ti surfaces

The chemical composition of the Ti surfaces was studied by XPS, with photoelectrons generated by Mg Kα primary radiation (hν = 1253.6 eV) and analysis with a hemispherical electron energy analyzer (Kratos XSAM 800). The X-ray gun was operated at 210 W (14 kV, 15 mA). The binding energies were normalized with respect to the position of the C (1s) peak of adventitious carbon, which was taken as 285.1 eV. Changes in the XPS spectra were measured after 10 min of bombardment (repeated several times) with Ar^+ generated with an ion gun energy of 3 kV, at an incident ion beam current density of 4 μA/cm^2. This bombardment led to the removal of ~10 nm from the surface in 10 min. Wide-range scans and higher-resolution narrow scans of the characteristic Ti 2p peaks were recorded.

3.5. Examination of bacterial proliferation and colonization

3.5.1. Scanning electron microscopy (SEM) studies

After treatment with F^- and bacterial incubation, the Ti discs were treated with the following method for fixation: dehydration of the surface bacteria and bacterial biofilm, first by rinsing with ethanol solutions with increasing etanol content (30–50–70–100%) then by a mixture of ethanol–acetone (90–10, 70–30, 50–50, 30–70, 10–90, 100% acetone). Critical point drying (an SPI 1320 apparatus) was applied, after which the discs were gold-coated by means of an Edwards sputter coater and subjected to SEM with a Hitachi S 2400 instrument.

3.5.2. Protein assay studies

The quantity of *P. gingivalis* bacterial protein was determined with a micro Coomassie (Bradford) protein assay kit (Pierce, Rockford, IL USA) in order to check the survival and the proliferation of the bacteria on the Ti discs surfaces treated with the materials containing different amounts of F^-. For removal of the adhered bacteria, the samples were washed with a lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4 and 1 μg/ml leupeptin).

4. Results

4.1. AFM measurements

AFM testing of the polished and cleaned Ti samples before F^- treatment (Fig. 9) revealed almost parallel grooves on the surface; these grooves originated from the mechanical machining of the samples (the grooves appear lighter on proceeding from the depths toward the surface in the picture). The AFM measurements yielded a value of R_a = 37.0 ± 2 nm for the control samples.

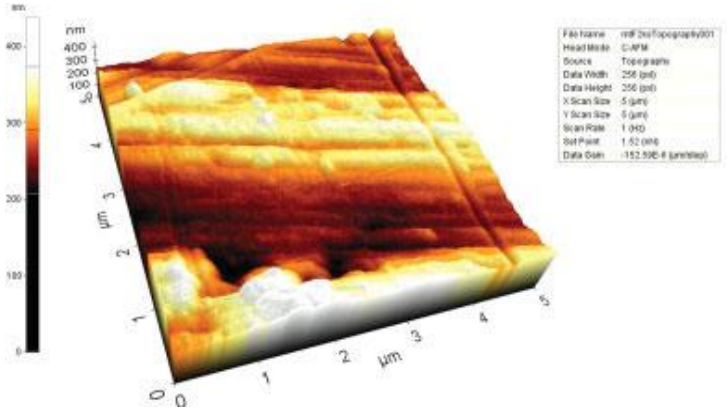


Figure 9. 3D AFM image of a control Ti surface, revealing the almost parallel grooves due to the mechanical machining. Image dimensions: 5x5 µm.

On the Ti discs treated with mouthwash (250 ppm F^- content, pH 4.4), the average surface roughness was R_a = 51.3 ± 4 nm (Fig. 10). Although major differences could not be observed relative to the control samples, the R_a value was significantly higher (p = 0.007).

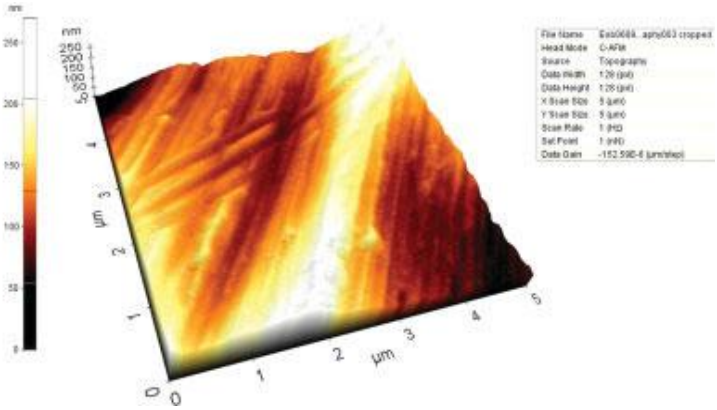


Figure 10. 3D AFM picture of a mouthwash-treated (250 ppm F^-, pH 4.4) surface. The R_a is significantly higher, than the control value was. Image dimension: 5x5 µm

After treatment with 1% NaF solution (3800 ppm F^-, pH = 4.5), the Ti discs displayed the greatest increase in R_a (Fig. 11): the depth of the grooves was almost 7 times that of the control : R_a = 254.8 nm ± 20 nm ($p < 0.001$).

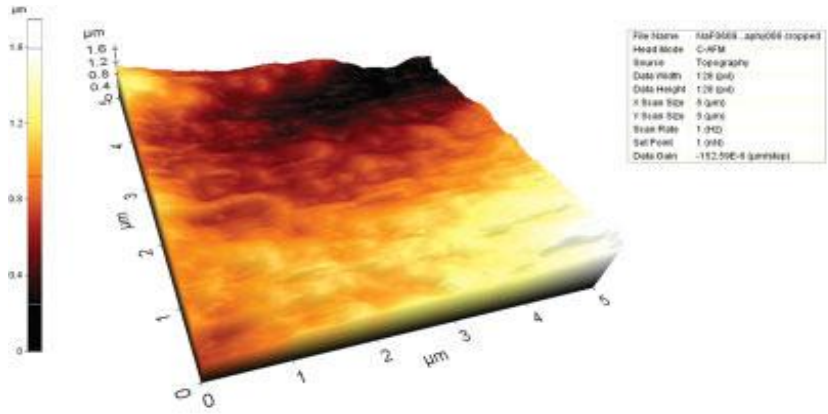


Figure 11. 3D AFM image of a surface treated with 1% Na.
The surface roughness became much higher. Image dimensions: 5x5 µm

For demonstration of the disc surface modification caused by the Elmex gel (12 500 ppm F^-), we smeared it with this preventive gel and then rinsed it with distilled water. After drying, it could be clearly seen that the surface was not as shiny and smooth as previously (Fig. 12). The AFM picture (Fig. 13) showed deep corrosive regions and granular forms. The average roughness of the gel-treated surface was significantly increased as compared with the control: R_a = 48.6 ± 3 nm (p = 0.005).

Figure 12. Macroscopic image of a disc smeared with the gel (12 500 ppm F^- and pH 4.8) revealing the macroscopic surface change caused by the high concentration of F^-.

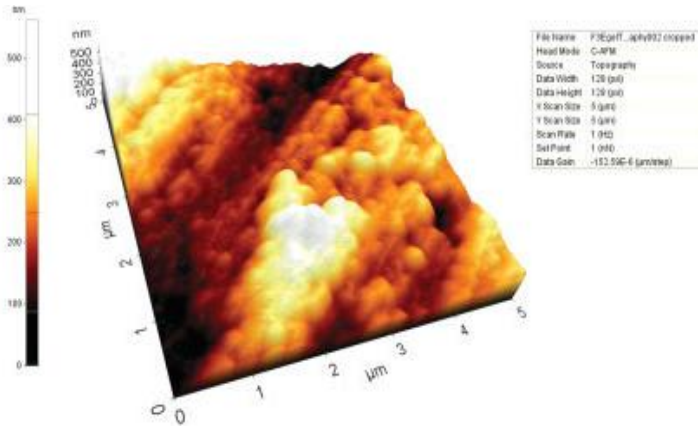


Figure 13. 3D AFM picture of a gel-treated Ti discs surface. High differences on the surface between the higher and deeper parts can be observed. Deep corrosive regions and granular forms were detected on the surface. Image dimensions: 5x5 µm.

Thus the AFM measurements demonstrated that R_a was increased significantly on all the treated Ti discs as depicted in Fig. 14: control surface R_a = 37.0 ± 2 nm; mouthwash-treated discs, R_a = 51.3 ± 4 nm (p = 0.007); 1% NaF solution-treated discs R_a = 254.8 ± 19 nm (p < 0.001) and gel-treated discs R_a = 48.6 ± 3 nm (p = 0.005). R_a = 254.8 ± 19 nm (p < 0.001)

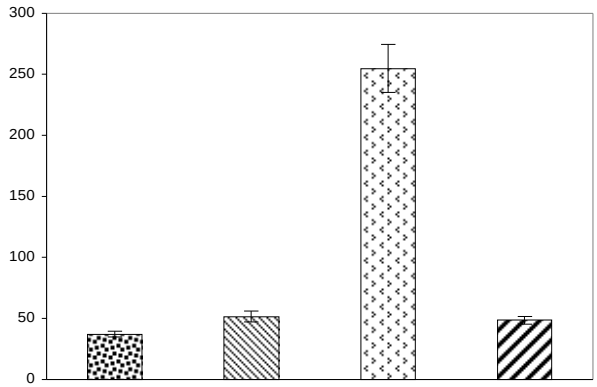


Figure 14. Bar-graph comparison of the surface roughness (R_a) of the Ti discs after the various treatments.

4.2. XPS measurements

The XPS spectra of control and mouthwash-treated (250 ppm F^-, at pH 4.4) Ti discs are to be seen in Fig. 15A and 15B. In both recordings, the presence of O, C and Ti was confirmed. The C 1s signal indicates the presence of carbonaceous contamination, resulting

from C-containing molecules remaining after chemical cleaning or adsorbed later on the air-exposed surfaces. These are typical elements that can be found on Ti implant surfaces. The double peaks of Ti (Ti 2p at binding energies of 458 and 464 eV) and the O 1s signal (530 eV) demonstrated the presence of the TiO_2 layer. Trace amounts of Ag, Cu, Zn and Na could also be detected on the surface, originated from external contamination. The chemical composition of the surface of the mouthwash-treated disc did not differ from that of the control.

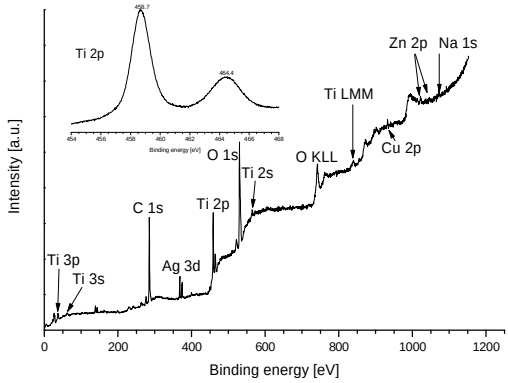


Figure 15A. XPS spectrum of a control Ti disc. The XPS survey spectra confirmed the presence of O, C, and Ti. These elements are typically observed on Ti implant surfaces.

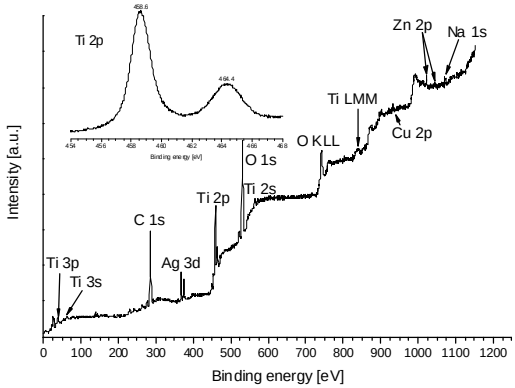


Figure 15B. XPS spectrum of a mouthwash-treated Ti disc. The chemical composition of the surface did not change.

After treatment with 1% NaF (3 800 ppm F^-, pH 4.5) for 1 h (Fig 16), 3 new peaks were observed in the spectrum. One of them, at a binding energy of 1080 eV, was the characteristic line of Na 1s. Two other peaks appeared in the binding energy range 600-700 eV: that at 600 eV originated from F KLL, and the F 1s peak at 685.3 eV from Na_2TiF_6, which modifies the TiO_2 layer of the surface.

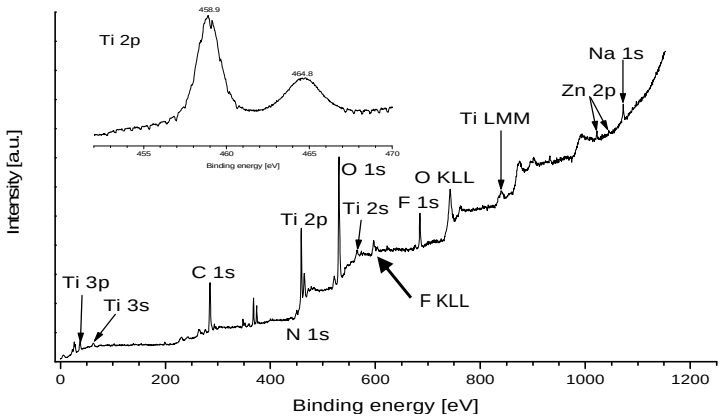


Figure 16. XPS spectrum of the surface treated with NaF. The usual peaks (O, C and Ti on the spectra were found and new F KLL peak and F1s peak appeared, which proves the modification of the surface composition.

The XPS spectrum of the surface treated with Elmex gel (12 500 ppm F^- and pH 4.8) was similar to that of the NaF-treated surface. Three new peaks were observed: the characteristic line of Na 1s at a binding energy of 1080 eV resulted from the NaF content, the line at 600 eV originated from F KLL, and the F 1s peak at 685.3 eV pointed to the presence of Na_2TiF_6, modifying the TiO_2 layer on the surface (Fig. 17).

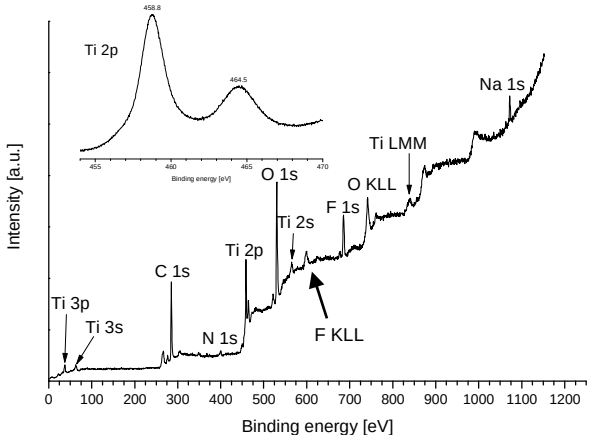


Figure 17. XPS spectrum of a Ti discs treated with Elmex gel. The large arrow indicates the two new peaks: F KLL and F 1s, proving the formation of Na_2TiF_6.

After Ar^+ bombardment for 10, 20, and 30 min, repeated XPS investigation revealed that ~10 nm was removed from the surface of the material, but the F 1s peak at 684.7 eV persisted (Fig. 18), proving that the binding between the Ti and F^- was very strong.

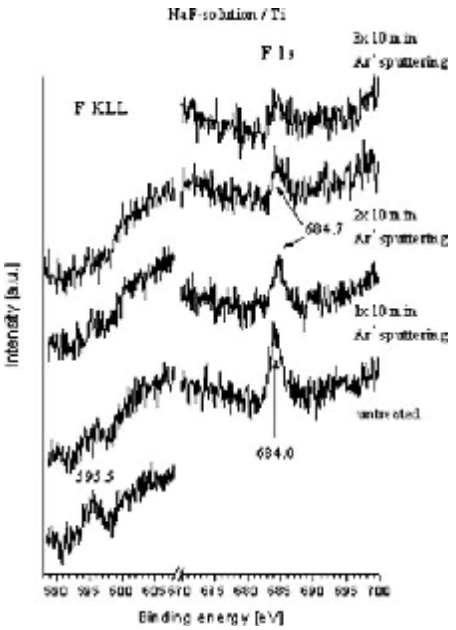


Figure 18. High-resolution XPS spectrum of the NaF-treated (3 800 ppm F^-, pH 4.5) Ti disc after 10, 20, and 30 min of Ar^+ bombardment. After 10 min of Ar^+ bombardment, repeated XPS investigation revealed that about 10 nm was removed from the surface of the material, but the F 1s peak at 684.7 eV persisted, proving that the binding between the Ti and fluoride was strong.

4.3. SEM results on Ti samples incubated with periodontal pathogenic bacteria

Figures 19A, B and C show typical SEM images of **control** Ti discs incubated for 2, 4 or 7 days in a suspension of oral bacteria from a patient with chronic periodontitis. The beginning of biofilm development can readily be observed, and the steps of multiplication and maturation of the oral bacteria can be clearly followed. The microcolony spreads first in the surface plane and then upwards, creating palisades of cells. The pioneer species acted as the substrate for further colonization through a process of coaggregation between different species.

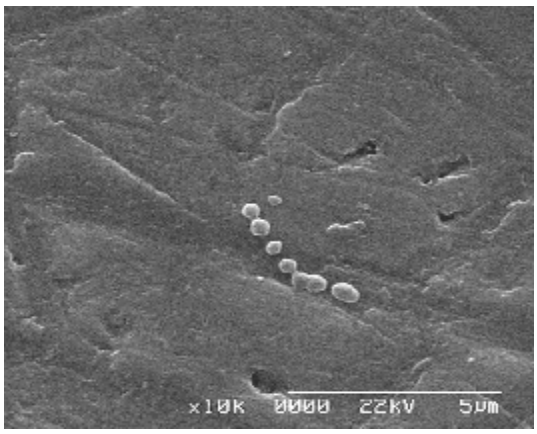


Figure 19A. SEM image of a control Ti disc incubated for 2 days with pathogenic bacteria from a patient with chronic periodontitis. Only a few Streptococcus sp. can be seen. (Magnification: 10 000x)

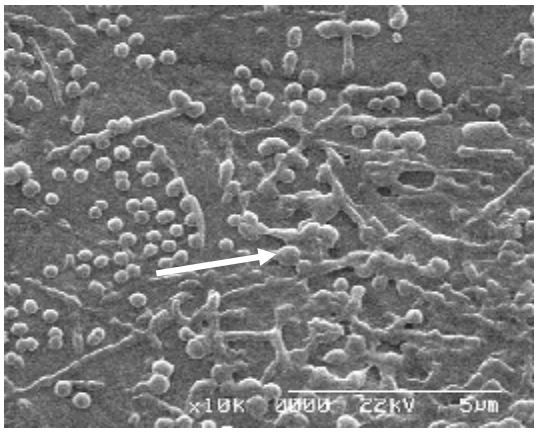


Figure 19B. SEM image of a control Ti disc incubated for 4 days with pathogenic bacteria from a patient with chronic periodontitis. In several places bacteria proliferation can be observed (arrow). Both Gram-positive organisms and the filamentous predominantly Gram-negative anaerobic bacteria can be seen. (Magnification: 10 000x).

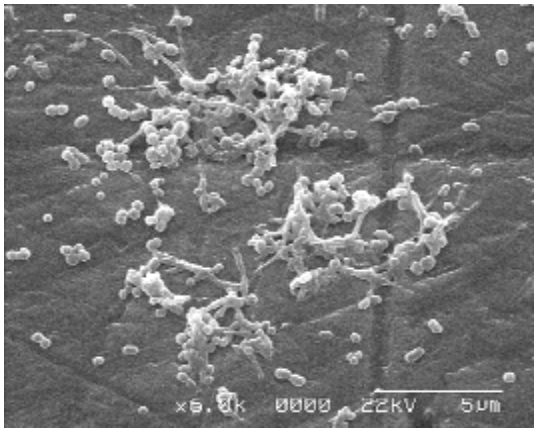


Figure 19C. SEM image of a control Ti disc incubated for 7 days with oral bacteria harvested from a patient with chronic periodontitis. After the filamentous bacteria appeared, connection with other specimens developed, and maturation began. (Magnification: 6 000x)

Figures 20A, B, C, D and E depict Ti disc surfaces treated **1% NaF**, at **pH 4.5**, and then incubated for 2, 4 or 7 days with bacteria from a patient with chronic periodontitis. The maturation of the biofilm began earlier than in the case of the control disc: the proliferated bacteria were interconnected already on day 2, and the glycocalyx surrounded the cells. The number of bacteria on this surface was not higher than that on the control, and each coaggregation partner cell type displayed an independent metabolic capability.

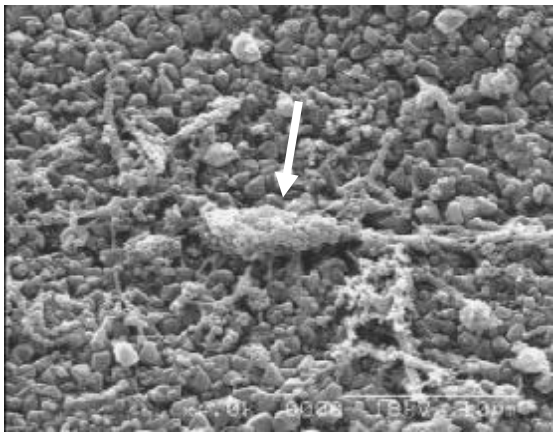
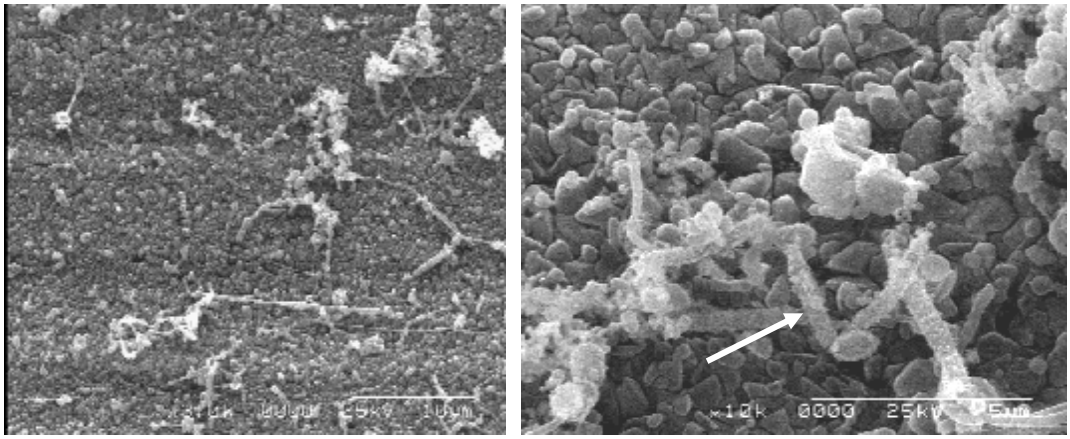
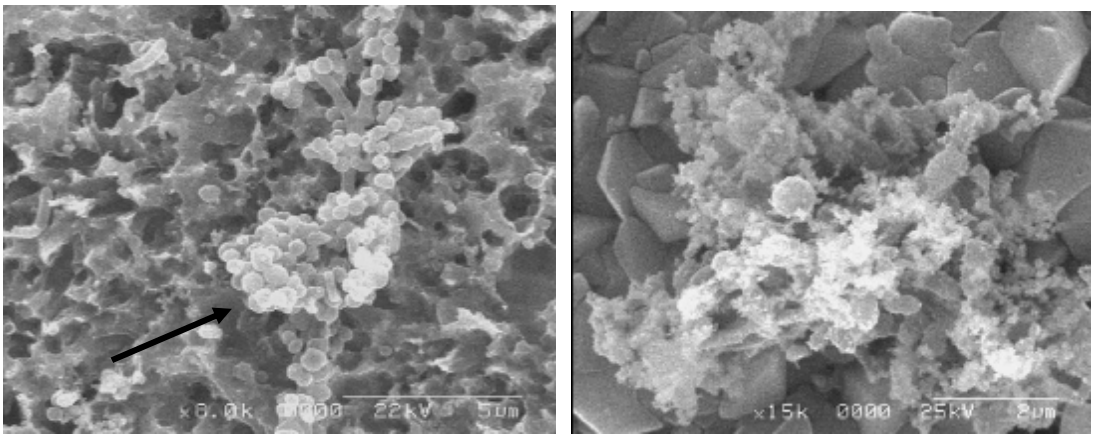


Figure 20A. SEM image of a Ti disc treated with 1% NaF solution (pH 4.5) and then incubated for 2 days, with oral bacteria from a patient with chronic periodontitis. The rough, etched surface beneath the bacteria can be observed, with the interactions between specific bacterial surface molecule. The ordered arrangement of the bacteria within the biofilm indicate a highly organized process (Magnification: 4 000x).



Figures 20B and C. SEM images of the surface of Ti discs treated with 1% NaF at pH 4.5 and then incubated for 4 days with the bacteria from a patient with chronic periodontitis. A more mature biofilm was visible in a few places. The corn-cob morphological structure was present in the plaque biofilms. The arrow indicates attached oral cocci and growing on the surface of filamentous microorganisms. This formation provided a possibility for cell-cell communication. (Magnifications: 3 000x and 10 000x).



Figures 20D and E. SEM images of the surface of Ti discs after treatment with 1% NaF solution at pH 4.5, and then incubated for 7 days with mixed bacterial flora. (Magnifications: 8 000x and 15 000x).

Figures 21A, B, C and D show the surfaces of Ti discs treated with **1 % NaF** at **pH 6.5**, illustrating the steps of biofilm development. Initially Gram-positive cocci were situated on the Ti disc surface; secondary colonizers associated with other Gram-positive rods and filamentous bacteria allowed other bacteria to colonize the Ti implant. Maturation of the biofilm occurred through the attachment of filamentous, predominantly Gram-negative aerobic bacteria to the basal biofilm. After 4 days on the surface the corn-cob form appeared too with longer filament bacteria at the center and a great number of cocci and short rods were connected with it. After incubation for 7 days there were several layers of bacteria on the surface. The biofilm was very thick, with all types of cells attached to the surface and interconnected to each other.

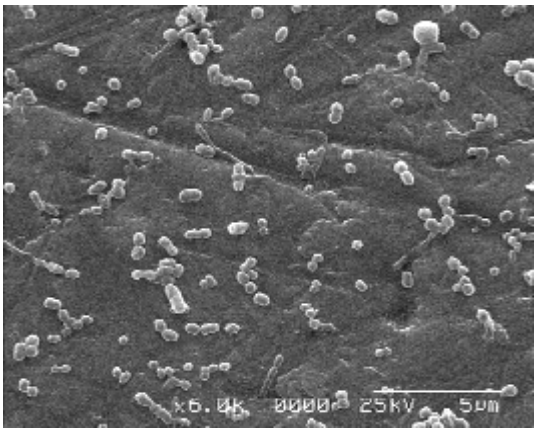


Figure 21A. SEM image of the surface of a Ti disc after treatment with 1% NaF solution at pH 6.5, and incubation for 2 days with bacteria from a patient with chronic periodontitis. The bacterial proliferation began earlier than on the control sample as reproduction was clearly visible and the filaments appeared on the surface. The arrow indicates one filamentous bacterial cell. (Magnification: 6 000x).

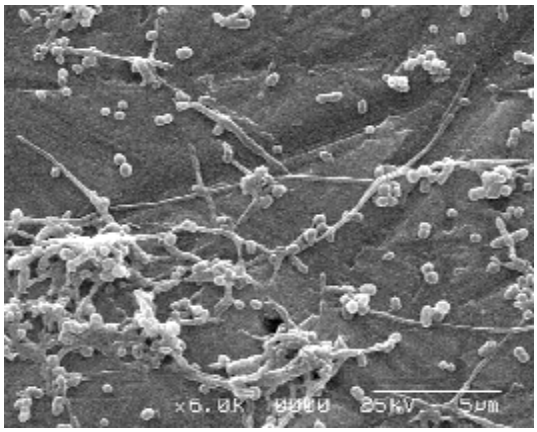
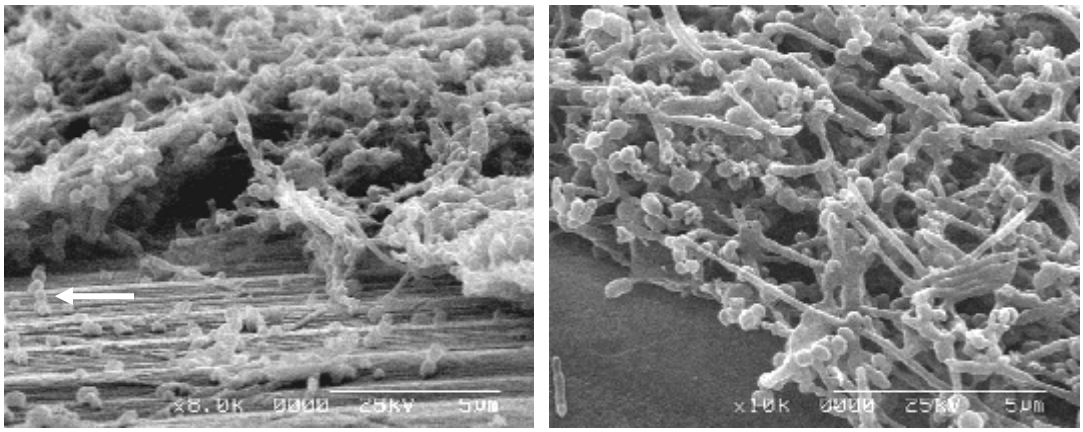


Figure 21B. SEM image of the surface of a Ti disc treated with 1% NaF solution at pH 6.5 and then incubated for 4 days with bacteria from a patient with chronic periodontitis. The pioneer cocci cells have begun to interconnect with the rods and filamentous. (Magnification: 6 000x).



Figures 21C and D. SEM images of Ti disc surfaces treated with 1% NaF solution at pH 6.5. and then incubated for 7 days with bacteria from a patient with chronic periodontitis. Mature biofilm is seen on the surface, with several layers of bacteria. Cocci, rod-shaped and filamentous bacteria are also present. Numerous Streptococcus sp. are situated adjacent to the surface and multiply (see arrow) to cover an ever greater surface area. (Magnifications: 8 000x and 10 000x).

4.4. SEM results onf Ti samples incubated with *Streptococcus mutans*

Figure 22 illustrates the adhesion of *S. mutans* on a **control** Ti disc surface, demonstrating the connection between the cells, and the bacterial chain on the surface. A continuous monolayer is not seen, only separated bacteria cells, in some cases interconnected (Fig. 22).

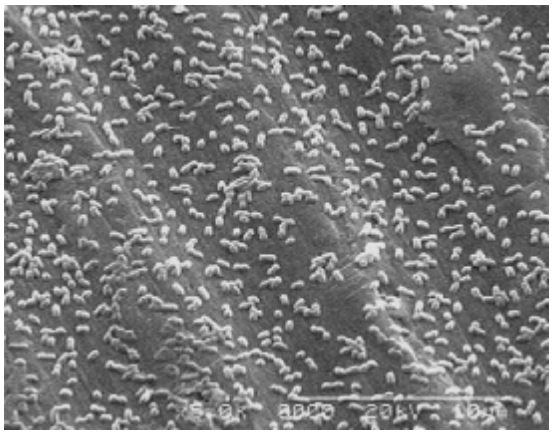


Figure 22. SEM image of the surface of an untreated (control) Ti disc incubated for 5 days with S. mutans. There is only one, not continuous bacterium layer on the Ti surface. (Magnification: 5 000x).

Figure 23 depicts a Ti disc after treatment with **mouthwash** containing 250 ppm F^- at pH 4.4 and incubated with *S. mutans* for 5 days. The SEM picture reveals cocci cells covering the surface in only one layer, and readily observed adhesion in several places. There are a large number of interconnected multiplied bacteria on the treated Ti surface.

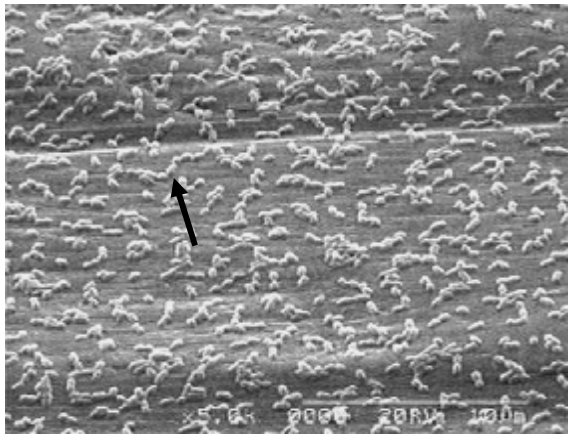


Figure 23. SEM image of a Ti disc treated with mouthwash containing (250 ppm F^-) at pH 4.4 and then incubated with S. mutans for 5 days. The coccus bacteria are connected to the surface and the arrow indicates the formation of coccus chains (Magnification: 5 000x).

Figures 24A, B and C relate to the Ti disc treated with **1% NaF solution** containing 3 800 ppm F^- at pH 4.5 and incubated with *S. mutans* for 5 days. Figure 24A shows the macroscopic features of a gold-coated Ti disc prepared for SEM examination.

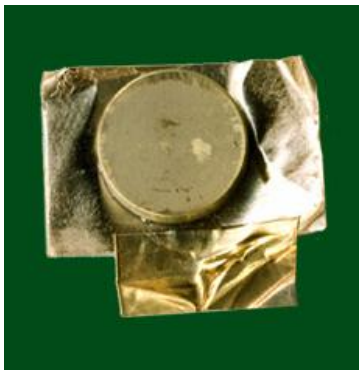


Figure 24A. Macroscopic picture of a Ti disc treated with 1% NaF at pH 4.5 and incubated with S. mutans for 5 days. The bacterial cells cover almost the whole surface.

Figures 24B and C present SEM images of the surface with the microbial adhesion, revealing a mature biofilm in several layers, covering the whole surface area. The bacteria may proliferate inside the polysaccharide matrix produced by the bacteria or some other biological surface (e.g. the mucosa). Other microorganisms can infiltrate into this formation, and may cause the growth of a microbial consortium. The glycocalyx provides protection for the *S. mutans* and promotes the proliferation of bacterial cells.

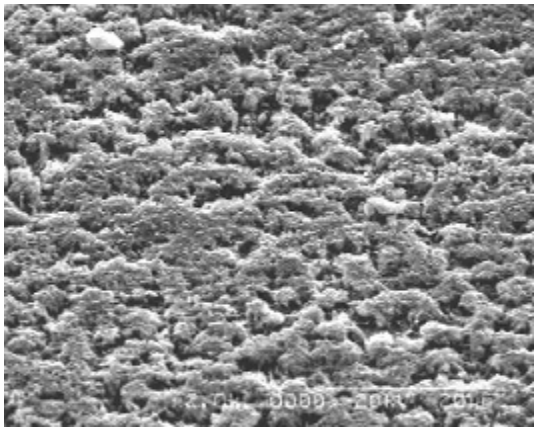


Figure 24B. SEM image of a Ti disc treated with the 1% NaF (3 800 ppm F^-) at pH 4.5 and incubated with S. mutans for 5 days. The biofilm can be seen, its form is maturated which developed in several layers. The bacteria covered the whole area of this Ti surface (Magnification: 2 000x).

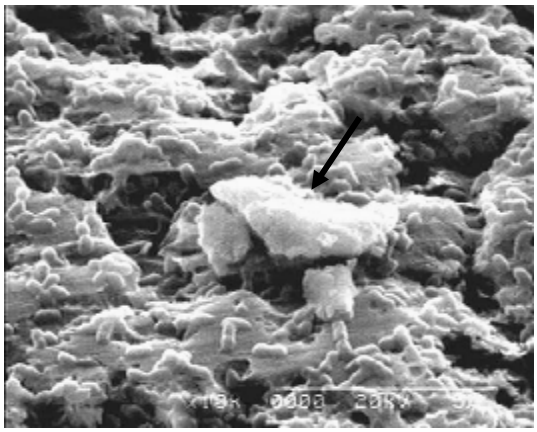


Figure 24C. Higher magnification of an SEM image of a Ti disc treated as in Fig. 24B. The arrow indicates the thicker protective glycocalyx around the bacteria. (Magnification: 10 000x).

Figures 25A, B and C show SEM images of Ti surfaces treated with **Elmex prevention gel**, containing 12 500 ppm F^- at pH 4.8, with huge numbers of bacteria on the surface. At some sites, the bacterial cells have joined together, and biofilm formation is in an advanced stage. On the surface of the bacteria, a polysaccharide layer (glycocalyx) is visible as a product of the cells. These Figures reveal holes on the surface of the Ti discs, due to the Elmex gel treatment causing pitting corrosion. Similar holes could also be observed in the AFM pictures (Fig. 13). This is an unfavorable effect, destroying the surface itself, and allowing, the bacteria to attach to the inner surfaces of the deep holes, where they may adhere and proliferate. Figure 25C demonstrates that the biofilm can develop inside these holes.

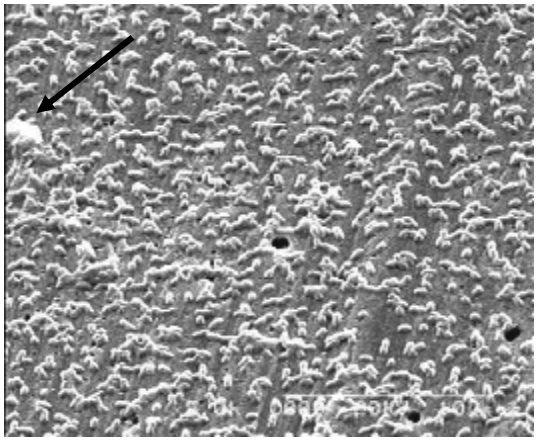


Figure 25A. SEM image of the surface of a Ti disc treated with Elmex gel (12 500 ppm F^-) at pH 4.8 and then incubated for 5 days with S. mutans. Bacterial cell proliferation has started and at some sites biofilm development has begun (arrow). The holes reflect pitting corrosion. (Magnification: 8 000x).

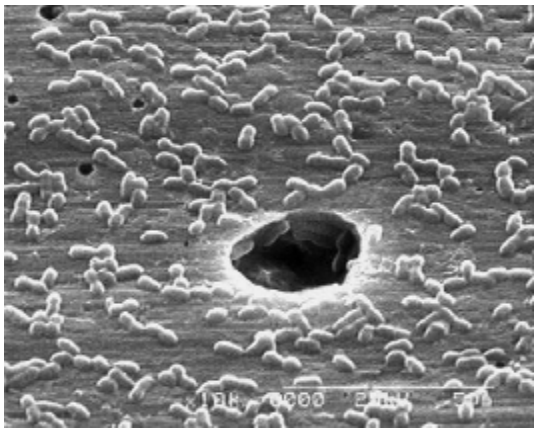


Figure 25B. Higher magnification of an SEM image of a Ti disc treated as in Fig. 25A. The holes in the Ti surface were caused by the high fluoride content of the gel. S. mutans bacteria can attach the inner surface of the deep hole, where they may adhere and proliferate. The biofilm can develop inside these holes (Magnification: 10 000x).

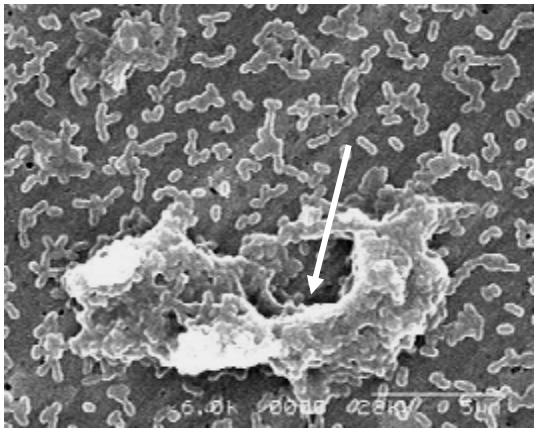


Figure 25C. Higher magnification of an SEM image of a Ti disc treated as in Fig. 25A, revealing a typical bacterium biofilm formed in several layers with glycocalyx. Adhesion to the protective mucous substance of the bacterium promots reproduction (Magnification: 6 000x).

4.5. Results of incubation with *Porphyromonas gingivalis*

4.5.1. SEM results

After incubation for 5 days active reproduction of the bacteria was observed on the **control** discs (Fig. 26). The bacterial cells did not cover the entire Ti surface. The formation of a monolayer had not yet started and transverse bacterium chains could be seen as the *Porphyromonas* multiplied.

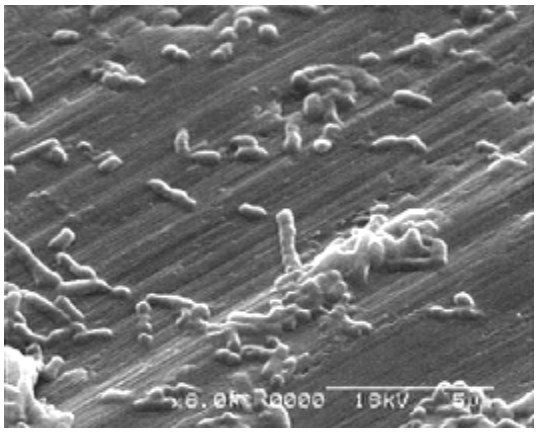


Figure 26. SEM image of a control Ti disc incubated with P. gingivalis for 5 days, showing active reproduction, with some interconnected cells (Magnification: 8 000x).

Figure 27 illustrates the bacteria on a Ti disc treated with **Elmex mouthwash** containing 250 ppm F^- at pH 4.4, and then incubated with *P. gingivalis* for 5 days. Only one layer of bacteria appeared on the surface, but it was not continuous. At some locations biofilm appeared.

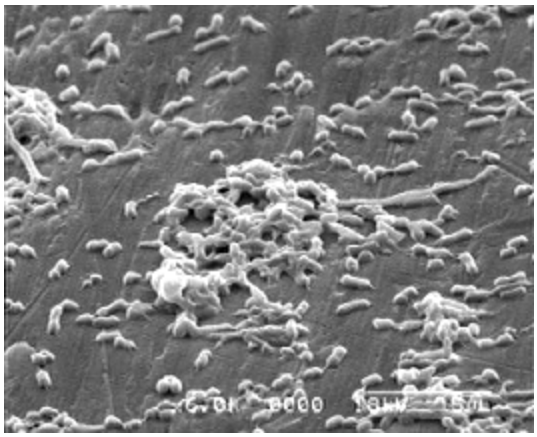


Figure 27. SEM image of a Ti disc treated with Elmex mouthwash (250 ppm F^-) at pH 4.4 and then incubated with P. gingivalis (Magnification: 6 000x).

Figures 28A and B reveal an extremely rough Ti surface following treatment with **1% NaF** (3 800 ppm F^-) at pH 4.5. After incubation with *P. gingivalis for* 5 days, a typical bacterium biofilm was formed in several layers and glycocalyx was produced on the surface. Adhesion to the protective mucous substance of the bacterium promoted reproduction.

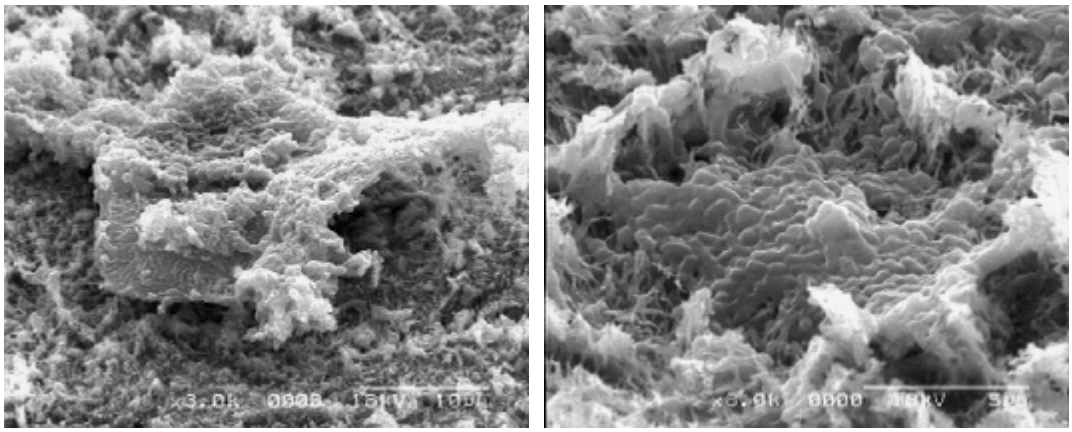


Figure 28A and B. SEM images of bacteria on Ti discs treated with 1% NaF (3 800 ppm F^-) at pH 4.5 and then incubated with P. gingivalis for 5 days. Biofilm developed on the surface. (Magnifications: 3 000x (A) and 8 000x (B)).

Figures 29A and B show SEM images of the surfaces of Ti discs after treatment with **Elmex gel** (12 500 ppm F^-) at pH 4.8 and incubation with *P. gingivalis* for 5 days. Deep corrosive regions and holes can be seen. Some of the bacteria are present in multiple layers, in an interconnected manner, and additionaly a biofilm has been formed. The polysaccharide coating (glycocalyx) forms a protective layer around the bacterial population.

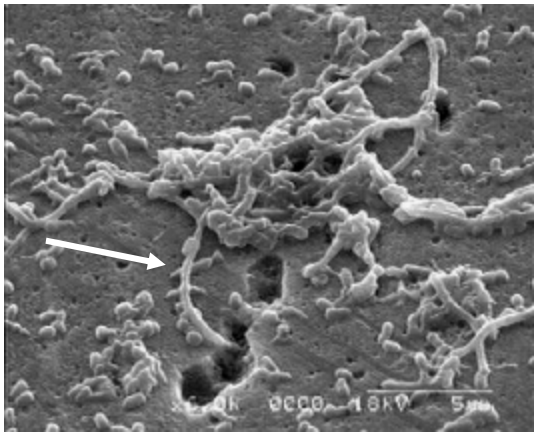


Figure 29A. SEM image revealing holes and bacteria on the surface of a Ti disc treated with Elmex gel (12 500 ppm F^-) at pH 4.8 and incubated with P.gingivalis for 5 days. The arrow shows the beginning of corn-cob formation on the surface (Magnification: 6 000x).

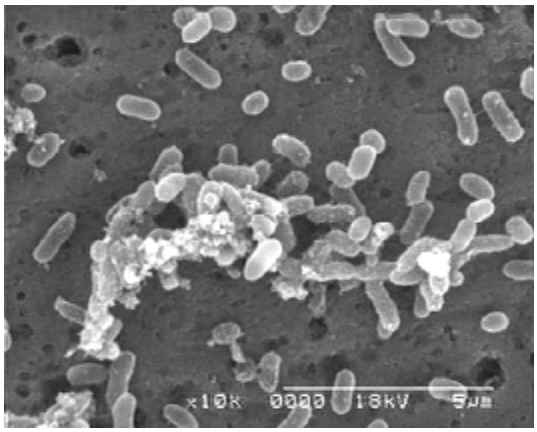


Figure 29B. Higher magnification of an SEM image of a Ti disctreated as in Fig. 29A, demonstrating thick glycocalyx growth (Magnification: 10 000x).

4.5.2. Protein content assays

The quantity of *P. gingivalis* bacterial protein was determined with a micro Coomassie protein assay kit in order to check the survival and proliferation of the bacteria on the Ti disc surfaces after different treatments.

After incubation with *P. gingivalis for* 5 days, the protein assays indicated that there were no significant differences in the number of bacteria on the surfaces of Ti discs treated with Elmex mouthwash (250 ppm F^- at pH 4.4), with 1% NaF solution (3 800 ppm F^- at pH 4.5) or with Elmex gel (12 500 ppm F^- at pH 4,8) as compared with the control (Fig. 30), with levels of 96.7 ± 25%, 106.7 ± 24% and 118.6 ± 26% respectively, vs. 100%).

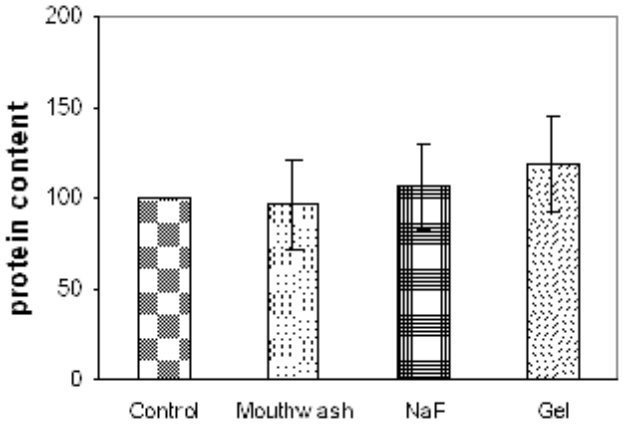


Figure 30. Protein contents on the surfaces of Ti discs incubation with P. gingivalis for 5 days following treatment with Elmex mouthwash, NaF solutions or Elmex gel, relative to the amount measured on the control

5. Discussion

5.1. AFM measurements

In the mouth, the transmucosal area of Ti implants and abutments must be smooth; these surfaces are therefore polished during production. It has been found (Rimondini et al., 1997) that a Ti surface with $R_a \leq 0.088$ µm strongly inhibits the accumulation and maturation of plaque in a 24-h period. At higher R_a plaque colonization accumulation occur (Bollen et al., 1996). A great number of studies concerning the bone-promoting activity of fluorides have proved that fluorides incorporated into the TiO_2 layer increase the retention of implants significantly, especially if they are rough-surfaced (Ellingsen, 1995; Ellingsen et al., 2004). Studies have been made of the effects of pH in the interval 3.5-7.0 and of F^- concentration up to 1 000 ppm. (Nakagawa et al., 1999) When the F^- concentration in toothpastes reaches or exceeds 0.1-0.15% (1 000-1 500 ppm) the rubbing, cleaning or foaming materials present form calcium compounds which are able to reduce the effectiveness of the F^- by 25-50% (Neubert and Eggert, 2001).

Our AFM examinations revealed that our polished Ti discs were similar in roughness to the mechanically polished surface of the neck of dental implants and abutments as the average surface roughness was $R_a = 37.0 \pm 2$ nm. When we applied various forms of F^- treatment (mouthwash, gel or a NaF solution produced in our laboratory) to the Ti disc surfaces, R_a changed as compared with the control surface: for the mouthwash-treated discs, $R_a = 51.3 \pm 4$ nm ($p = 0.007$); for the gel-treated discs, $R_a = 48.6 \pm 3$ nm ($p = 0.005$), and for the 1% NaF solution-treated Ti discs, $R_a = 254.8 \pm 19$ nm ($p < 0.001$) i.e. a 6.9-fold increase, due to the presence of hydrofluoric acid (HF) in aqueous solution at acidic pH (Stájer et al., 2006, 2008). Thus R_a was changed significantly in comparison with the control Ti sample by all three treatments. Although the F^- concentration in the gel was higher, the different additives present in the product Olaflur and Dectaflur chemically bind the F^-, impeding the formation of HF.

5.2. XPS measurements

Our XPS investigations of the chemical compositions of the control and treated Ti discs demonstrated the presence of the basic elements, O, C and Ti on every surface. The C 1s signal was due to carbonaceous contamination from molecules containing carbon that remained on the surface after chemical cleaning. On the other hand, such contamination can

be adsorbed later on air-exposed surfaces (NIST XPS Database). These elements are typically observed on Ti implant surfaces. Slight amounts of Ag, Cu, Zn and Na were also detected on these samples, which originated from external contamination (Handbook of X-Ray Photoelectron Spectroscopy. 1979). The presence of the TiO_2 layer was confirmed by the double peaks of Ti (Ti 2p at binding energies of 458 and 464 eV) and the O 1s signal (530 eV) (Ameen et al., 1993, Kilpadi et al., 1998). After NaF solution or gel treatments, 3 new peaks were seen in the spectra. One of them, at a binding energy of 1 080 eV, was the characteristic line of Na 1s, which resulted from NaF. Two other peaks appeared in the region 600-700 eV. That at 600 eV originated from F KLL (Huang, 2002) and the F 1s peak at 685.3 eV from Na_2TiF_6, which modifies the TiO_2 layer of the surface (Huang, 2003; NIST XPS Database). XPS revealed that the high F^- concentration and acidic pH of the gel and the 1% NaF solution resulted in strong corrosion and modification of the composition of the Ti surface.

This result is in accordance with literature findings (Huang, 2003). Our XPS investigations further demonstrated that, after 10 min of Ar^+ bombardment, ~10 nm was removed from the surface of the discs, but the F 1s peak at 684.7 eV persisted, proving that the binding between Ti and F^- was very strong. The complex Na_2TiF_6 modifies the TiO_2 layer on the surface (Stájer et al., 2006, 2008). The smoothness and the chemical stability of the Ti surface may deteriorate following the application of 1% NaF solution or Elmex gel, favoring the adhesion of bacterial flora.

5.3. Incubation with bacteria from a patient with chronic periodontitis

On the control discs, the process of biofilm development could be followed. Initially only a few *Streptococcus* sp. cells were observed to be adhered to the surface. After 2 days, they were associated with Gram-positive organisms containing surface proteins that bind to specific salivary glycoproteins in the pellicle. After 4 days, proliferation had started, and other types of bacteria also appeared on the Ti discs. After 7 days, the maturation of the biofilm was in advanced stage, but was not yet completed. Large numbers of different bacteria, fungi and protozoa can live in the oral cavity. When these organisms adhere to a surface, they form an organized mass called dental plaque or biofilm.

Pontoriero et al. (1994) allowed plaque to accumulate around implants and teeth for 3 weeks and found a correlation between the levels of plaque accumulation and peri-implant mucositis and the similar response of the soft tissues around the teeth and implants when

these were exposed to plaque. Our understanding of how these organisms arrive in the dental biofilm community and how they interact with each other and their hosts has been improved immeasurably by applying the principles of ecology to the study of this complex system. In addition to their ability to coaggregate into complex structures, a number of other interactions occur between the microbial species that comprise bacteria. These interactions can be either inhibitory or stimulatory, depending on the species involved. On the control Ti discs, the steps of biofilm (dental plaque) formation could be followed clearly. This plaque contains more than 600 different microorganisms, and is typically the precursor of tooth decay, contributing to the overall dynamic environment in the oral cavity that frequently undergoes rapid changes, depending on the pH, nutrient availability and oxygen tension (ten Cate, 2006).

Maturation of the biofilm (after 4 days in our study) occurs via the attachment of filamentous, predominantly Gram-negative aerobic bacteria to the basal biofilm. This process also reflects interactions between specific bacterial surface molecules. The ordered arrangement of bacteria within the biofilm indicates a highly organized process. In a recent study of microbial colonization on Ti implants, periodontal pathogens were demonstrated to be present in different proportions on all failed implant surfaces (Shibli et al., 2007). Specific bacteria within the biofilm community were able to interact with other species to either help or impair the host, besides to providing positive cooperation between the different species of the biofilm. After 7 days, clustered groups of bacteria appeared too. Each cluster is created on the basis of similarities and differences in nutritional and atmospheric environments. Polymer production results in the development of the extracellular matrix, which consists of soluble and insoluble glucans, fructans and heteropolymers. This matrix is one of the key structural aspects of the plaque biofilm, much like that of other biofilms.

We performed SEM analyses of Ti discs treated with 1% NaF solutions (pH 4.5) and incubated with oral bacteria from the sulcus of a patient with chronic periodontitis for 2, 4 or 7 days. The SEM images clearly showed that the proliferation of the cells had alredy begun after 2 days, the cells becoming interconnected and producing glycocalyx. The steps of biofilm formation continued without difficulty. Corn-cob formation and clustered groups of bacteria could be observed on the treated Ti discs. The bacteria did not cover the surface everywhere. Initiation was associated with Gram-positive organisms (mostly *Streptococcus* sp.) that contain surface proteins that bind to specific surfaces of Ti implants. Negatively charged adhesions on bacterial cells may bind to negatively charged glycoproteins through bivalent cations (usually Ca^{2+} or Mg^{2+}). Secondary colonizers associated with other Gram-positive rods and filaments (mostly *Lactobacillus* sp.) allow other bacteria to colonize Ti

implants. The maturation of biofilm occurs through the attachment of filamentous predominantly Gram-negative aerobic bacteria to the basal biofilm. The early composition of the biofilm reflects the ability of the early colonizing biomass to create a low redox potential suitable for anaerobes (e.g. *P. gingivalis*) (Marsh, 2004). Anaerobes survive in the high oxygen concentrations present in the oral cavity, without having much protection from other bacteria. Immediately after tooth brushing, such a thin biofilm is almost always present on the tooth surface (Sbordone and Bortolaia, 2003).

In our study, Ti discs were treated with 1% NaF solution at pH 6.5 and incubated for 2, 4 or 7 days with oral bacteria flora from the patient with periodontal disease, in order to investigate the changes in the bacterial cells on the Ti surface. The multiplication of the bacteria began after day 2, and after day 4 the maturity of the biofilm had reached the second stage. At the end of this investigation (after 7 days), several layers of bacteria cells were present on the surfaces. While the discs were removed from the incubation medium, many bacterial cells were lost because the huge amount could not remain on the surface. SEM revealed that after 7 days more and more *Streptococcus sp.* strived to adhere to the empty surface to grow the biofilm. Subramani et al. (2009) established that increases in surface roughness and surface free energy facilitate biofilm formation on dental implant and abutment surfaces. The surface chemistry and the design features of the implant-abutment configuration also play significant roles in biofilm formation. (Subramani et al., 2009). The maturation of the biofilm seemed to be advanced relative to the previously mentioned samples. The immersion of the Ti discs in the 1% NaF solution at pH 6.5 apparently led to the surface changes that generated a very favorable environment for the oral bacteria and the biofilm developed more easily. These results demonstrate that the changes caused on Ti surfaces by the above-mentioned treatment can promote favor the development of a biofilm.

5.4. Incubation with *Streptococcus mutans*

The behavior of *S. mutans* on the Ti surface was examined because it is considered to be the primary etiologic agent of dental caries a global health problem that affects 60-90% of the population. *S. mutans* has 4 different serotypes. The Gram-positive cocci bacterium is facultative aerobic; although it is often found in the normal, healthy human oral cavity, it is a significant contributor to tooth decay. The microbe was first described in 1924 (Clarke, 1924). Our investigations with *S. mutans* demonstrated a monolayer of separate bacterial cells, on the control Ti discs, which did not multiply after 5 days. The primary colonizing bacteria may

connect to the surface in two ways: 1. Hydrophobic interactions, when lipophilic adhesions on the bacterial cell surfaces come into contact with hydrophobic receptors on the epithelial cells. *S. mutans* is frequently exposed to toxic compounds from oral healthcare products, food additives and tobacco. Streptococci account for about 20% of the oral bacteria and determine the development of the biofilm. Although *S. mutans* can be antagonized by pioneer colonizers, when it becomes dominant in oral biofilms, dental caries can develop and thrive (Biswas and Biswas, 2011). 2. *S. mutans* prossesses an enzyme, glycosyl transferase, which is involved in the initial attachment of the bacterial cells to the tooth surface. Through the transformation of sucrose to dextran polymers (glucans) it facilitates the formation of plaque. It appears as a regular component of human normal oral flora in relatively large numbers. The salivary component mucin contains glycoproteins which can easily colonize on the tooth surfaces and form a thin film called enamel pellicle on the tooth. The adsorbed mucin probably serves as a molecular receptor for ligands on the bacterial cell surface. The lactic acid that originates from the utilization of dietary carbohydrate can demineralize the tooth enamel. The extracellular glucans formed by *S. mutans* and other organisms can also produce intracellular polysaccharides from sugars, which are stored in the cells and then metabolized to lactic acid. *S. mutans* produces more lactic acid and is more acid-tolerant than most other streptococci. *S. mutans* is important in the initiation of dental caries, because its presence leads to bacterial colonization on the tooth surfaces, plaque formation and the localized demineralization of tooth enamel. However, it is not the only bacterium which causes dental decay. After bacteria appear on the enamel, various oral bacteria begin to grow in the interior regions of the tooth. Lactobacilli, actinomyce, and various proteolytic bacteria are secondary invaders, which assist the progression of the lesions (Todar's online textbook of bacteriology).

Prevention is very important in oral health. During orthodontic treatment, dentists recommend different types of preventive solutions and gels, and prophylactic agents are used in dental surgery too. Unfortunately, the pH of the mouth rinses and gels used for caries prevention in dentistry range between pH 3.5 and pH 7.0, and their F^- concentration is between 1 000 and 10 000 ppm (Nakagawa et al., 1999). For dental practitioners, it is essential to know whether F^- attacks Ti surfaces, i.e. whether the prophylactic solutions are able to modify the corrosion resistance of the surfaces of Ti dental implants, dental prostheses or wires in the case of orthodontic braces.

When Elmex mouthwash was applied on Ti discs, we observed that the number and proliferation of bacterial cells did not change on the surface relative to the control ones. The treatment with Elmex gel resulted in an increase in the number of *S. mutans* cells which grew

on the samples. At some some sites a biofilm appeared, with a glycocalyx wrapping. In the presence of corrosion holes, the cells adhered inside the deep areas and proliferated there. If the surface of Ti exhibits crevices and pits, *S. mutans* can adhere there (Barbour et al., 2007).

SEM analysis of Ti discs treated with NaF solution before incubation showed that several layers of bacteria cells covered the surface, and at higher magnification the biofilm covered the whole of the Ti discs. We earlier reported (Stájer et al., 2009), in agreement with Rimondini et al. (1997), that the amount and rate of maturation of the biofilm correlated with the roughness of the Ti surface. Both Gram-positive colonies and Gram-negative cocci were observed to be present as in a previous study (Li et al., 2001). A change in free energy on the Ti surface influences the adherence of *S.* (Fujioka-Hirai et al., 1987).

5.5. Incubation with Porphyromonas gingivalis

5.5.1. SEM studies with *Porphyromonas gingivalis*

We carried out an SEM investigation of the proliferation of *P. gingivalis* on Ti discs treated with Elmex mouthwash, 1% NaF or Elmex gel. On the controls the formation of a monolayer had not yet started after 5 days of incubation; only transverse bacterial chains could be seen as the *Porphyromonas* multiplied. The colonization of *P. gingivalis* on polished and F^--modified Ti surfaces can play an important role in the development of peri implantitis inside the mouth. On intact Ti surfaces, *P. gingivalis* can proliferate in association with other bacterial strains and together they may develop the biofilm of the surface.

P. gingivalis is a non-motile Gram-negative, rod-shaped, anaerobic pathogenic bacterium, which produces dark-brown or black porphyrin pigments. *Porphyromonas* sp., commonly found in humans, and especially in the oral cavity, grow in black colonies on blood agar. They cause gingivitis or other oral inflammatory processes in approximately 70-90% of the pubescent population, which could be a possible precursor of adult periodontal diseases (Choi et al., 1990). As in *Bacteroides*, they form an outer membrane, the peptidoglycan layer, and a cytoplasmic membrane. The cell surface adhesion molecules on *Porphyromonas* interact with other bacteria, when epithelial cells and extracellular matrix proteins assist them to live in their human host, attaching equally well to both smooth and grooved coated Ti surfaces (Wu-Yuan et al., 1995). *P. gingivalis* is present in periodontal tissues: it receives energy from dissolved sugars and other simple carbohydrates which the mouth generally contains. The clinical evidence points to the presence of *P. gingivalis* in periodontal diseases (Haffajee and Socransky, 1994). It is found in elevated levels in

periodontal lesions, in contrast with the low levels observed at healthy sites (Choi et al., 1990; Moore et al., 1991). These bacteria may be eliminated by successful therapy (Ali et al., 1992), but infections are liable to recur.

The smoothness and the chemical stability of the Ti surface may deteriorate in response to treatment with Elmex mouthwash, 1% NaF or Elmex gel, the environment becoming suitable for the oral bacteria flora to adhere to the surface more easily. After incubation for 5 days with *P. gingivalis,* some of the bacteria were present in multiple layers, in an interconnected manner, and in addition a biofilm was formed. The bacterial population developes a protective layer by producing a polysaccharide coat (glycocalyx), and adhesion to the protective mucous substance of the bacterium promoted reproduction (Stájer et al., 2012).

The occurrence and multiplication of these bacteria is an important factor in the development of periodontal diseases. In a published study of microbial colonization on Ti implants, periodontal pathogens, e.g. *P. gingivalis*, were demonstrated to be present in different proportions on all failed implant surfaces (Shibli et al., 2007). Our study proved that only the smooth Ti surface inhibited the adherence of bacterial cells. Chemical changes to the TiO_2 may accelerate the adherence of bacterial cells and maturation of the biofilm. It has been established that rough surfaces promote plaque formation and maturation, but high-energy surfaces collect more plaque and it can bind more strongly. These two factors interact with each other, the influence of the surface roughness canceling out that of the surface free energy (Quirynen and Bollen, 1995). The initial bacterial adhesion to differently textured Ti surfaces is primarily influenced by R_a, whereas the influence of the surface free energy seems to be only of minor importance. The micro-structured parts of an implant that are exposed to the oral cavity should therefore be highly polished to inhibit plaque accumulation (Bürgers et al., 2010).

Periodontal pathogens, e.g. *P. gingivalis*, are very important factors, because they are present in different proportions on all failed implant surfaces, as demonstrated by Shibli et al. (2007). The influence of R_a and bacterial cell growth was studied earlier by Rimondini et al. (1997), who found that the number of bacterial cells (either cocci, or short and long rods) depended on R_a. They established a threshold limit of $R_a \leq 0.088$ µm, which depended both on the type of the Ti, and on the treatment method.

Amoroso et al. (2006) reported that the adhesion of *P. gingivalis* to Ti was inhibited by a R_a of ~ 35 nm, which is appreciably below the levels generally encountered for implant abutments ($R_a \approx 350$ nm). Our SEM results are in accordance with this finding. The F^- treatment significantly changed the R_a values of the Ti surfaces relative to that of the control

(R_a = 37.0 ± 2 nm), on which fewer bacteria were observed. R_a increased to ~ 51 nm (Elmex mouthwash), 49 nm (Elmex gel) and 255 nm (1% NaF solution), the SEM investigations revealing a more pronounced bacterial growth on these surfaces.

The XPS studies demonstrated modifications in the chemical composition of the surfaces of the Ti discs treated with 1% NaF or Elmex gel, due to the concomitant effect of the high F^- concentration (3 800–12 500 ppm) and the acidic pH (4.5-4.8).

The findings of this thesis clearly prove that the surface characteristics induced by F^- treatment affected the growth and maturation of *P. gingivalis*, with the roughness of the surface as the predominant factor.

5.5.2. Protein assays

The SEM investigations of the surfaces of Ti discs treated with different F^--containing substances indicated more extensive growth of the bacterial biofilm and earlier maturation. Although the protein content assays revealed slight increases relative to the control in the number of bacteria on the surfaces of Ti discs treated with NaF solution or Elmex gel, the changes were not significant. These apparently contradictory results can be explained with regard to the relatively short incubation time (5 days). Mabilleau et al. (2006) established that R_a was highly increased when Ti discs were immersed in F^-, H_2O_2 and lactic acid containing artificial saliva. They found that the number of bacteria was significantly increased on discs incubated with activated J774.2 cells or *Streptococcus mitis* for 21 days.

6. Conclusions

The main conclusions of my Ph.D. work are as follows:

1. AFM measurements demonstrated that R_a was increased significantly on all Ti discs treated with F^--containing materials. Elmex rinse was tested, because many patients use this for caries prevention worldwide. Treatment with 1% NaF resulted in a roughness enhancement of almost 7-fold. After Elmex gel treatment, the AFM picture revealed deep corrosive regions and granular forms, but the roughness change was not so high. The reason for this is that, although the F^- concentration in Elmex gel is higher than that in 1% NaF solution, the Olaflur and Dectaflur in the gel chemically bind the F^-, impeding the formation of HF.

For a dental practitioner, it is essential to know whether fluorides attack Ti surfaces, i.e. whether prophylactic solutions are able to modify the corrosion resistance of the surface of Ti dental implants, crowns and bridges, partial prostheses, orthodontic brackets and wires. We were the first to study in detail the changes in roughness of CP Ti surfaces due to these treatments.

The SEM and AFM measurements revealed that crevice and pitting corrosion can occur on the surface of Ti implants, and it may therefore be concluded that these destructive effects can also take place on fixed and removable dentures, orthodontic braces and wires made of Ti.

2. Dentists often offer preventive mouthwashes (e.g. Elmex rinse) for home use. Our XPS investigations indicated that these rinses did not change the chemical composition of the Ti surface. The XPS spectra of 1% NaF solution and Elmex gel-treated surfaces showed that a high F^- concentration and acidic pH can cause significant corrosion and result in the formation of the complex Na_2TiF_6. Our results are in accordance with the findings of other studies. We have shown that this complex binds strongly to the surface and modifies the TiO_2 layer. The prophylactic gels (e.g. Elmex gel) are mainly used in pediatric dentistry to prevent caries while orthodontic braces and wires are situated in the oral cavity.

We are not aware of previous investigations the effects of preventive mouthwashes and gels (e.g. Elmex gel) on the physical and chemical properties of CP Ti surfaces.

3. The above-mentioned surface treatments favored the accumulation of *S. mutans* and of *P. gingivalis* on CP Ti discs during incubation with bacteria from a patient with periodontal disease. The SEM pictures showed that after a few days the total number of bacterial cells was

higher and the development of maturation was faster than on the untreated Ti surfaces. The steps of bacterial plaque development were similar in every case, even when oral bacteria flora and individual bacteria were used. The acidic environment and the F^- concentration of these materials were the determining factors. Higher F^- content caused deeper corrosions, where the bacteria cells could adhere, and could not be removed by the patients or oral hygienists.

4. The protein content assay of *P. gingivalis* did not indicate any significant differences between the untreated and treated samples, although slight increases in the number of bacterial cells were displayed by the Elmex gel and NaF-treated discs. The incubation time was only 5 days, and this short time can probably account for this result, which is in contrast with the literature data.

7. Summary

Similarly to other reductive agents, F^- may affect the structure of the oxide layer on a Ti surface. Prophylactic mouthwashes or gels used for the prevention of caries usually contain F^- and are applied at low pH. Hence, the aim of the present work was to study whether various concentrations of F^- at acidic pH cause changes in the surface structure of polished dental Ti implants and abutments, and alter the adherence, colonization and proliferation of bacteria.

Polished CP grade 4 Ti discs were treated with a mouthwash containing 0.025% F^- (pH 4.4), a 1% aqueous solution of NaF (3800 ppm, pH 4.5) or a gel containing 1.25% F^- (pH 4.8). The surfaces were investigated by AFM and XPS, to assess the physical and chemical changes in the smooth Ti surface.

The AFM measurements revealed that the roughness (R_a) of the treated sample surfaces was increased significantly: 1.3-fold for the gel, 1.4-fold for the mouthwash, and 6.9-fold for the 1% NaF solution relative to the untreated control surface. The high F^- concentration and acidic pH characteristic of the 1% NaF solution and the gel caused significant corrosion and resulted in the formation of the complex Na_2TiF_6, revealed by XPS measurements. This binds strongly to the surface and modifies the native TiO_2 layer. XPS revealed that the high F^- concentration and acidic pH of the gel and 1% NaF solution resulted in strong corrosion and modification of the composition of the Ti surface.

In microbiological tests, the bacterial flora was used from the sulcus of a patient with periodontal disease. We observed the development of a biofilm on the control and later the treated surfaces; the incubation period was 2, 4 or 7 days. In this case the initial treatment was with 1% NaF solution at pH 4.5 or pH 6.5.

We next treated Ti disc surfaces with Elmex mouthwash, 1% NaF solution or prophylaxis gel before incubation for 5 days with *S. mutans* (the initial bacterium responsible for the formation of the biofilm) or *P. gingivalis* (which causes gingivitis or other oral inflammatory processes). Bacterial colonization was observed by SEM, and the quantity of the bacterial protein of *P. gingivalis* was determined by means of protein assay.

The microbiological investigations showed that the steps in the formation of a biofilm occurred on all of the Ti surfaces. The number of bacterial cells was increased on the treated surfaces and the proliferation began earlier than on the control discs. When the surface of the Ti was not sufficiently smooth and chemical changes took place on the surface. The biofilm

underwent maturation earlier. The quantity of *P. gingivalis* protein was slightly (but not significantly) increased on the surfaces treated with 1% NaF and gel in comparison with the control surface.

Agents with high F^- concentration at acidic pH increased the roughness of the Ti surface. The SEM images revealed a correlation between the roughness and the chemical composition of the surface and thickness and maturity of the bacterial biofilm. This study suggested that a high F^- concentration at acidic pH (as in the preventive products) may promote the adhesion of the different bacteria on the Ti surface. This may therefore affect the development and maintenance of a healthy transgingival epithelial junction on Ti implants. The predictability and success rate of oral Ti implants in patients depend mainly on a healthy periodontal environment.

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10. LIST OF ABBREVIATIONS

AFM: Atomic force microscopy

ATCC: American Type Culture Collection

BHI: brain heart infusion

CFAT: cadmium, fluoride, acriflavine, tellurite

CFU: colony-forming unit

NIST: National Institute of Standards and Technology

SEM: Scanning electron microscopy

XPS: X-ray photoelectron microscopy