MASARYK UNIVERSITY

Faculty of Science

DIPLOMA THESIS

Use of silver containing compounds as bacteriocides in
dimethacrylate based dental materials

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DECLARATION

I hereby declare that this diploma thesis was written independently using the listed references, and that all references are quoted correctly.

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SUMMARY

ABSTRAKT

Cílem práce bylo prověřit možnosti modifikace pryskyřic používaných v zubním lékařství pomocí sloučenin obsahujících stříbro. Kompozitní dentální materiály nabízí vynikající mechanické i estetické vlastnosti, nicméně k jejich organické fázi (pryskyřici) adherují ve zvýšené míře bakterie orální mikroflóry, což může vést k e zvýšené tvorbě organického plaku s následným vznikem sekundárního zubního kazu, vývoji nepříjemného pachu až k onemocnění dutiny ústní. Předložená diplomová práce se proto zabývá možnostmi modifikace existujících dentálních materiálů sloučeninami s bakteriocidními účinky.

Jako modelový mikro organismus byl zvolen *Streptococcus mutans*, který je prokazatelně nejvíce kariogenickým druhem bakterie přítomné v lidské ústní dutině. Jeho nárůsty byly vyhodnoceny Biuretovou metodou spektrofotometricky, a to jak na discích, tak paralelně v živném médiu, které obsahovalo disk. Jako referenční materiál byla zvolena zubní keramika.

Studovanými pryskyřičnými materiály byly komerční kompozit Adoro a neplněná uretan-dimetakrylátová pryskyřice. Tato pryskyřice byla testována jednak čistá a jednak modifikovaná pomocí činidel obsahujících stříbro, a to formou přídavku komerčně dostupného práškového biocidního aditiva 1 a jako polymeračně imobilizovatelné aditivum bylo použito biocidního aditiva 2. Disky byly sterilizovány teplem a ultrafialovým světlem.

Jejich povrch byl dále studován pomocí konfokálního mikroskopu za účelem stanovení drsnosti.

Disky obsahující antibakteriální modifikace byly statisticky významně méně kolonizovány bakteriemi oproti nemodifikovaným (rozdíl větší než 60%). Nárůsty na UV světlem sterilizovaných discích byly menší než na discích sterilizovaných teplem. Nárůsty volných bakterií v médiu byly stejné u všech typů materiálů stejné v rámci experimentální chyby, tedy nezávislé na typu materiálu i způsobu sterilizace , navíc prokázaly, že bakteriocidní látky nejsou z pryskyřice vymývány .

Modifikace dentálních materiálů sloučeninami stříbra by mohla představovat řešení problému akumulace plaku na výplňových materiálech a tvorbu sekundárních kazů.

ABSTRACT

The aim of this thesis is to study the possibilities of modifying resins used in dentistry by compounds containing silver. Composite dental materials offer excellent mechanical and esthetical properties, nevertheless, bacteria of the oral microflora adhere to their organic phase (resin) in a greater degree, which may lead to the increased formation of organic plaque with the consequential development of secondary caries, unpleasant smell, and even oral cavity diseases. The presented diploma thesis is therefore focused on exploring the possibilities of modifying existing dental materials by compounds with bacteriocide effects.

Streptococus mutans was selected as a model microorganism, it has been proven to be the most cariogenic strain of bacteria present in the human oral cavity. The amount of adhering bacteria was quantified spectrophotometrically by the Biuret method on the polymer discs, and also a parallel measurement was carried out to state the amount of bacteria in the liquid medium that contained the disc. Dental ceramics was selected as a referential material. The investigated materials include a commercial composite Adoro and an unfilled urethane-dimethacrylate resin. This resin was tested with no modification and with modification by agents containing silver, specifically by adding the commercially available powder biocide additive 1 and an additive capable of immobilization on polymerization - biocide additive 2. The discs were sterilized by heat and ultraviolet light. The structure of their surface was further studied under a confocal microscope in order to assess their roughness.

Discs containing antibacterial modification were colonized to a statistically significant smaller degree in comparison to the unmodified ones (the difference exceeding 60%). The UV light sterilized discs were colonized less than those sterilized by heat. The amount of free planktonic bacteria in the liquid medium was the same in all types of materials within experimental error, it did not depend on the type of material or sterilization, thus proving that the bacteriocide agents did not leach out from the resin.

The modification of dental materials by silver-containing agents could present a solution to the problem of plaque accumulation on dental filling materials and the formation of secondary dental caries.

1. INTRODUCTION

Composite materials are widely used in dentistry. Their main applications include various cavity filling composites, veneers, adhesives, crown build-up composites, etc. Main advantage of dental composites include biocompatibility, good mechanical properties and esthetics. Although the mechanical properties and wear resistance of these materials have been improved substantially over last 30 years, the problem of enhanced organic plaque deposition which may result in secondary caries and poor oral hygiene when compared to ceramics and metal alloys, has not, however, been addressed yet.

Despite the numerous advantges dental composites offer in comparison with metal alloys such as amalgam, they still accumulate more plaque than these alloys. Efforts to prevent bacterial colonisation of these composites have focused on the modification of the polymer surfaces to induce bactericidal properties and at the same time preserve the bulk mechanical properties of the device.

The reasons that composite antibacterial properties have not been completely resolved yet are the complexity of their structure, complicated immobilization of these agents, their cytotoxicity and absence of appropriate testing methods. In addition, the oral microflora consists of a large variety of bacteria, each of which has a different life cycle, adhesion mechanism and optimal growth conditions. Information in the area of antimicrobial restorative materials in general has been limited both in the number of materials evaluated and in the variety of oral microorganisms tested. Thus, research in the area of suitable biocide additives for these composites to reduce microbial colonization is highly desired, since these additives can enhance the performance of existing dental composites, primarily to reduce the frequency of secondary caries and to improve the overall oral hygiene.

2. THEORETICAL

2.1. THE ORAL ENVIRONMENT

It is essential to briefly lay out some terms and definitions in order to understand the complicated procedures that take place in the oral environment, thus also stating factors that will and will not be taken into account in this thesis.

2.1.1. The oral cavity

The oral cavity is the beginning of the gastric system, an environment including hard and soft tissue, saliva, oral microflora and synthetic materials in the form of fillings, orthodontic replacements or implants, etc. This complex system is constantly affected by changing conditions associated with food intake and its processing - mechanical and chemical - the beginning of its digestion. However, the temperature inside it remains a more or less constant 35 - 36°C, thus enabling optimal conditions necessary for the growth of a wide spectrum of microorganisms including Streptococci, Actinomyces, Rothia, Nocardia, Bacterionema, Leptotricha, Veillonella. [1]

2.1.1.1. Teeth

The tooth is a hard structure in the oral cavity of most vertebrates, they serve to grasp, separate and masticate food. Human teeth present approx. 20% of all the oral cavity surface and comprise three parts, the root embedded in the alveolar bone, neck (cervix), and crown (corona), the visible part protruding into the oral cavity carrying the mastication surface. The structure of the tooth is presented in Fig. 1.
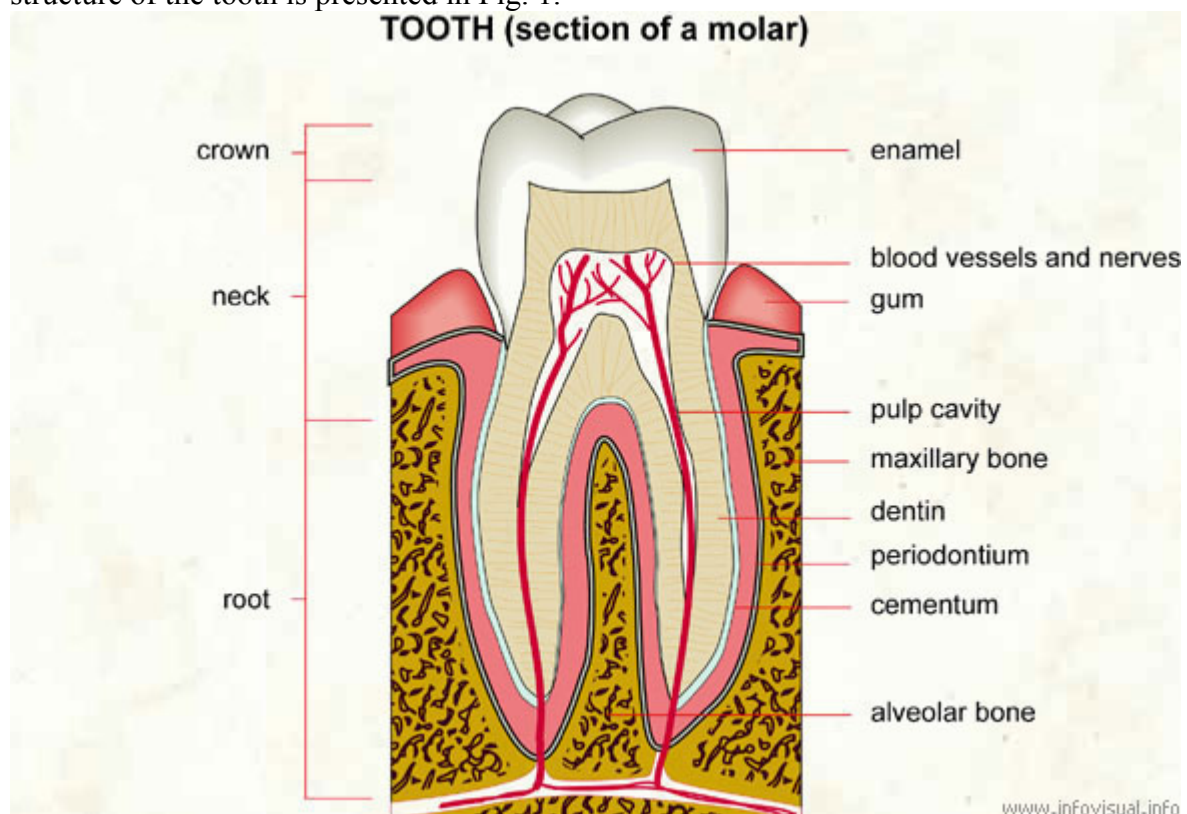

Fig. 1. Structure of the adult human tooth. Source: www.infovisual.info

The adult tooth consists of five components; pulp (the soft inner tooth material), surrounded by the main tooth material dentine (a highly calcified material that consists 70-80% of inorganic hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$ and 20-30% of organic material in the form of collagen fibers and glycosaminoglycans), the crown is covered by enamel (hardest part of the human body, 90% composed of hydroxyapatite, covered at the root by cement (similar to bone tissue, calcified, contains collagen), and the periodontal ligament (dense collagen and fibrocytes) anchors the tooth in the jaw. [2] Dentine contains a small amount of dentine producing cells, whereas enamel contains practically no such cells, therefore in adults, it is a "dead" tissue.

2.1.1.2. Saliva

Saliva, the liquid inside the oral cavity, contains mostly water, (glyco)proteins and electrolytes. It is secreted by the small and large salivary glands, in humans ranging between one to one and a half litre, and always contains gingival sulc fluid, remainders of food, microorganisms and the products of their metabolism, making it difficult to state its composition exactly. The pH of saliva is slightly acidic (pH 6,7), decreasing upon the consumption of food. Most microorganisms require pH in the neutral region for their growth, but the product of their metabolism is lactic acid. Saliva is expected to have a buffer effect enabling it to balance the pH decreased by the secreted acids. [3,4]

Saliva plays many roles in digestion, tooth maintenance and protection - calcium and phosphates from the saliva are used for the mineralization of new teeth and white spot repair on enamel. Although it does lower the amount of bacteria in the oral cavity by mechanically washing its surfaces, bacteriostatic and bactericidal effects of saliva have not been confirmed, its components may act as a first line of defense by slowing down the penetration and adhesion rate of bacteria to the mucosa.

The salivary proteins cover teeth with a protective film called the acquired pellicle, which plays an important role in bacterial adherence.[2]

2.1.1.3. Food

Food affects saliva secretion by means of voluntary and involuntary reflexes, other participating factors include smell and the time of mastication. During food processing, both the quality and quantity of the saliva changes - dry food evokes more watery saliva secretion, consuming meat produces saliva with a higher mucoid substance content. These parameters return back to normal approx. 20 minutes after the incidence of food.

Some components of food are considered to have a mechanical cleaning effect in removing film from the surface of teeth, e.g. some types of fruit and vegetables (apples, carrots). This mechanism, however, only functions in areas that are available - the cervix and interdental spaces. Some foods, such as carbonized soft drinks with a pH around 2-4, act as acids and accelerate dissolution of hydroxyapatite resulting in an enhanced occurence of carries.

The chemical effect of food is most important, especially due to the fact that for a great number of oral microorganisms, sacharides represent the main (and for some the only) nutritive substrate. Refined sugars (especially sucrose) are the most dangerous in increasing microbial film cariogenity, since microorganisms are capable of synthetizing intra- and extracellular polysacharides from them. The effect of protein and fat is not as unambiguous as the effect of sacharides.[3]

2.1.1.4. Restorative dental materials

Varying by region, 30-90% of adult population worldwide has at least one carrious cavity repaired with an artificial dental material. The extended life expectancy in significant portion of the world causes an increasing number of patients requiring prosthetic devices made of metal alloys, ceramics, polymers and polymer composites and, in many times, replacing their entire teeth arch. These materials are also exposed to the oral environment and, depending on their chemical composition and surface roughness, they are colonized by oral bacteria.

Unlike natural oral tissues, prosthetic devices are made of a wide variety of materials and are manufactured by dentists or dental technicians. Hence, the extent of adhesion of oral bacteria onto these devices varies greatly in respect to their chemical composition, placement in the arch and the actual hanufacturing technique used as well on the dentist or technician skills. Despite considerable volume of literature published on this topic, there are many contradictory results and no conclusions have been accepted by the dental community unambigiously, yet.

Among the dental material, polymers and polymer matrix composites are increasingly important due to their easy handling, excellent esthetics and biomechanics tunable according the the actual tooth tissue. Poorer wear perfomance compared to ceramics and greater biofilm adherence compared to amalgam are the main shortcomings of these materials. Hence, investigating the mechanisms of bacteria adhesion on polymer based dental materials is of great importance for their future applications.

2.1.1.5. Most common oral bacteria

The oral cavity is inhabited by microflora composed of a very wide spectrum of organisms, bacteria comprising the dominant part (approx. 70% Streptococci), there are also smaller numbers of virae, mycoplasmates, yeasts and protozoa. The composition of the oral microflora changes with age and is influenced by host factors, including the eruption and the loss of teeth, and the insertion of partial or complete dentures, the level of oral hygiene, saliva quality and quantity, perorally administered medicaments and the general state (age, immunity system, illness). [5,6]

Microorganisms present in the oral cavity can be both pathogenic as well as symbiotic. Pathogenic bacteria can cause various diseases, both of the oral cavity and the entire human organism. Acids that are emitted as a product of the metabolism of symbiotic bycteria into the oral environment dissolve the hydroxyapaptite (main component of enamel, as stated above). The acid attack affects both enamel and the area between tooth crown and gingiva causing periodontal disease resulting in a loss of alveolar bone tissue and eventually in a loss of affected teeth.

The most frequent problem, however, remains dental caries. About 65% dental procedures include filling the cavities left when the carious tooth material is removed. The most popular materials applied in prosthodontics include amalgam (historical), composite dental materials dominate the field since the 70´s.

2.2. *THE STREPTOCOCCUS SPECIES*

Streptococci comprise the majority of oral microorganisms. They are Gram-positive non-sporulating cocci of circular to ovoid shape, facultative anaerobes (some requiring CO_2), catalase negative, homofermentative chemoorganotrophes with complex nutritional requirements. They are typically assembled into chains and are widespread in nature. Forty of

the currently known species of Streptococci are found predominantly on the mucosa surfaces of humans and animals, the upper respiratory tract, on skin, the gastro-intestinal tract, they can also be found in soil, dairy products, other foods and plants.

It has been possible to isolate Streptococci from all locations in the oral cavity. On average they are represented by 28% in tooth plaque, 29% in the gingival crevice, 45% on the tongue, and 46% in saliva. [7,8]

Insoluble extracellular polymers play an important role in colonizing exposed tooth surfaces by some oral streptococci. Polymer production is also a key test in identifying schemes of these organisms. [8] Examples of Streptococci that participate on the formation of biofilms in the oral cavity:

2.2.1. Streptococcus mutans

This strain is considered to be the most cariogenic of all bacterial species, which is why it has been chosen for research in this thesis. *Streptococcus mutans* synthesizes soluble and insoluble extracellular polymers from sucrose (mutane, soluble glucane, fructane) that incorporate other components and lead to the formation of plaque. Plaque protects the streptococci, and they damage the enamel by their acidic metabolites which lead to caries. The insoluble polymers are important in the adhesion of this species to hard surfaces of the oral cavity. [7]

Fig. 2.a) Streptococcus mutans, Source: http://www.zahnarzt-stuttgart.com/prophy_streptococcus_mutans.png
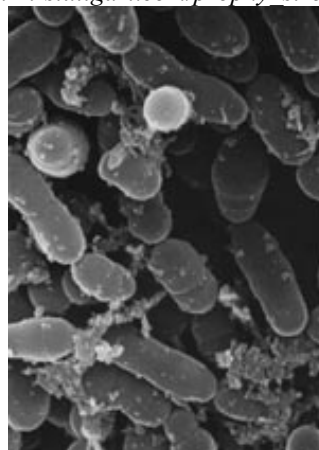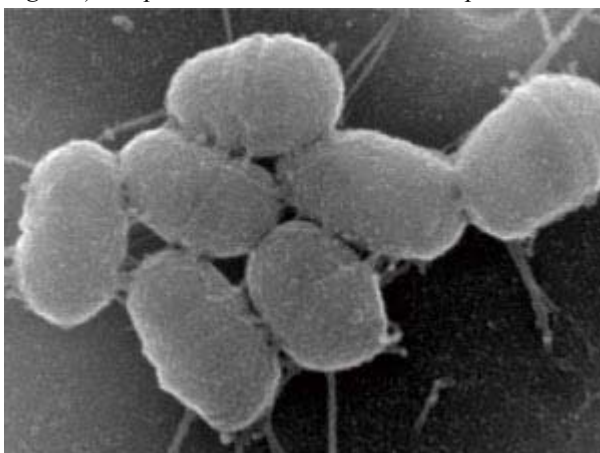

Fig. 2.b) S. mutans, Source: http://www.den.hokudai.ac.jp/rikou/akasaka/homemenu/DentSugar
Fig.2.c) S. mutans, Source: http://www.sciencenews.org/articles/20020202/a1353_412.jpg

2.2.2. Streptococcus sanguis

Studies of this strain have proven this to be one of the primary colonizers of the clean tooth surface. These strains produce extracellular insoluble and soluble glucanes from sucrose. They firmly adhere to the surface of epithelial cells, thus helping to shield the surface from other bacterial species, that can be inhibited by both occupying receptors and bacteriocine production. [7]

Fig. 3. Streptococcus sanquis. Source: biology.kenyon.edu

2.2.3. Streptococcus mitis (mitior)

This is probably the most frequently isolated species from tooth plaque. Some strains produce extracellular polymers from sucrose. [8] It colonizes hard dental tissue as well as mucous membranes, especially the cheeks and tongue. Although *S. mitis* may cause caries, in most cases it is considered a harmless member of the oral microflora. [9]

Fig.4.a) Streptococcus mitis on Mitis-Salivarius-Bacitracin agar. Source: smccd.net
Fig 4.b) S. mitis culture on Columbian agar. Source: www.szu.cz/cem/ehk/ehk172/ehk172.htm

2.2.4. Streptococcus salivarius

S. salivarius strains can be isolated from all locations in the mouth, though they prefer to colonize epithelial surfaces. S. salivarius produces an extracellular polymer levane from sucrose. This is a highly unstable polymer that can be metabolized by other microorganisms. [10]
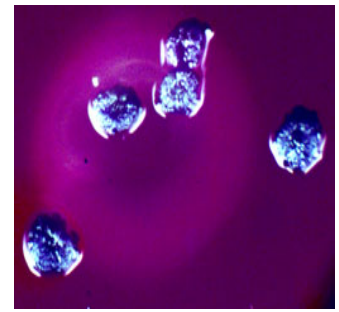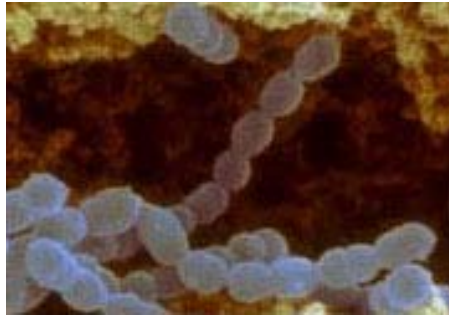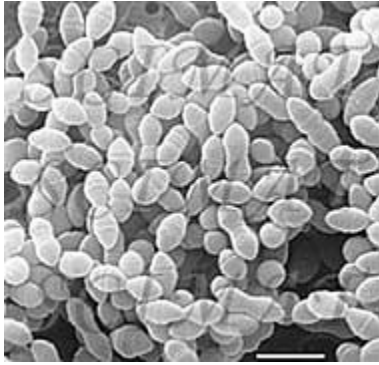
Fig.5.a) Streptococcus salivarius, microscopic view. Source: wwww.uiowa.edu;
Fig.5.b) S. salivarius in yoghurt. Source: www.zum.de/Faecher/Materialien/beck/bs11-3.htm
Fig. 5.c) S.salivarius on Mitis-Salivarius-Bacitracin agar.Source: www.smccd.net

Bacteria in the oral environment do not grow freely, but firmly attach themselves to hard and soft tissue and form a so-called biofilm.

2.3. CELL ADHERENCE, BIOFILMS AND THEIR FORMATION

2.3.1. Cell adhesion

The term adhesion has several definitions according to the area of its use. In general, adhesion can be defined as a state, where two surfaces are so close to one another, that their separation requires energy. In biological systems, adhesion is more complicated – it is a situation where a cell firmly adheres to a surface by means of complex physico-chemical interactions between the organism and substrate. The formation of an adhesive connection between a bacterium and a soft or solid surface requires energy. The term adherence is frequently used in biological systems, as it describes bacterial adhesion in general (the initial process of bacterial connection directly to a surface). [8]

The evolution of biofilms may have arisen from reasons such as protection from harmful conditions in the host (defense), sequestration to a nutrient rich area (colonization), utilization of the cooperative benefits (community), another possibility being that biofilms normally grow as biofilms and planktonic cultures are an in vitro artefact (biofilms as the default mode of growth). [11]

The formation of a biofilm encompasses the following four phases:

1) a reversible phase, the initial phase of adhesion includes primary interactions between microorganism and substrate, defined as deposition or adsorption onto a surface, followed by
2) an irreversible phase, where interconnection of polymers between organism and surface plays a key role in anchoring the bacterium, and
3) a repetition of phases 1) a 2), where bacteria adhere to the ones that are already adsorbed onto the surface in a process called coaggregation, and finally
4) the proliferation of organisms adhering to the surface, which consequently leads to the formation and growth of biofilm. [8]

Fig.6. Biofilm. Source: http://pathmicro.med.sc.edu/mycology/biofilm1.jpg
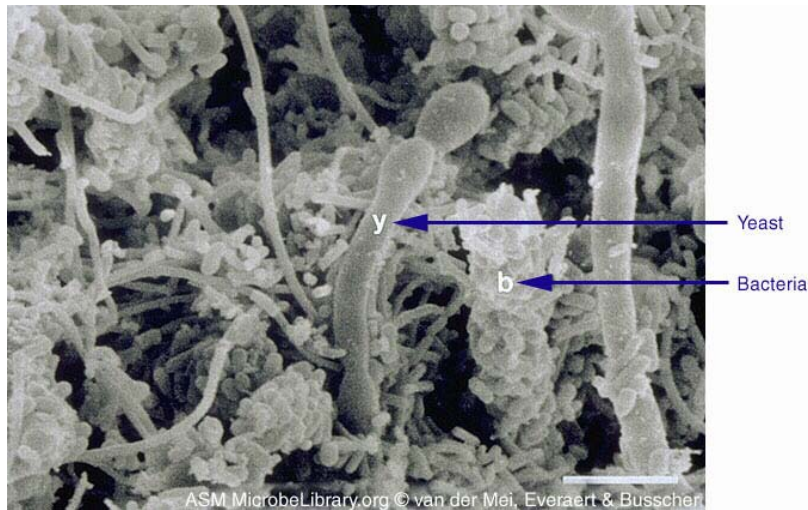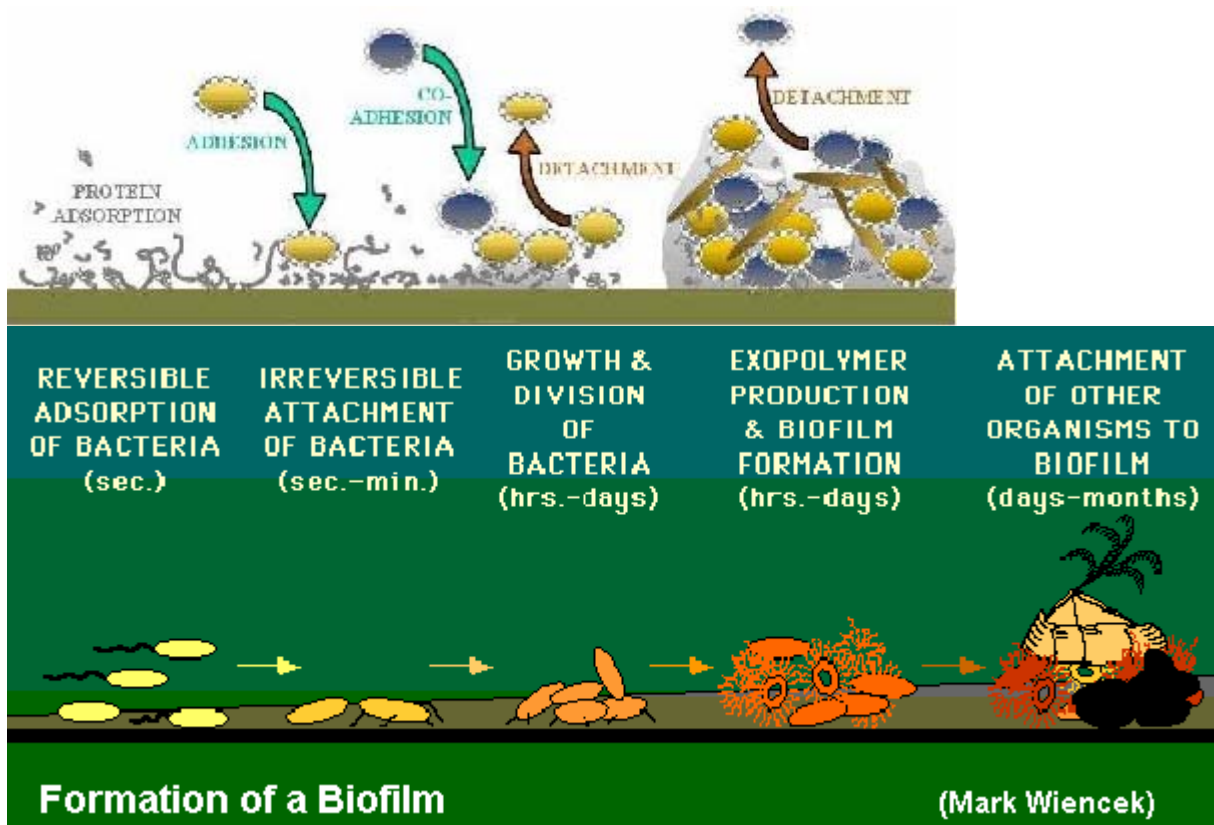
Cartoon representations of biofilm formation.
Fig.7.a) Biofilm formation. Source: http://www.mah.se/ImageVault/Images/scope_4
Fig.7.b) Biofilm formation. Source: http://images.google.cz/imgres?imgurl=http://www.rlc.dcccd.edu/mathsci/

2.3.2. Factors affecting adhesion

Bacterial adhesion is a complicated process affected by numerous factors including the characteristics of the bacteria themselves, the target surface and environmental factors. Cells do not interact with an exposed surface directly neither in vivo, nor in vitro. Contact is always mediated by some biological liquid component. Surface energy may influence the adsorption of proteins and their structural assembly on the surface of the material.

The first stage of adhesion involves primary interactions between microorganism and substrate including short-term physico-chemical interactions like electrostatic forces, van der Waals forces, etc. The second stage is long-term and involves a number of biological molecules like extracellular matrix proteins, cell membrane and cytoskeletal proteins that interact together with the aim of signal transduction, which sets off transcription factor action, and this leads to regulating gene expression. [12]

Factors affecting adhesion include:

2.3.2.1. Bacterial polymers

Streptococci are capable of producing extracellular polysacharides (as part of the bacterial capsule) which enables them to adhere to the hardest surfaces including glass, enamel, wire, etc.[8]

2.3.2.2. Sucrose

Sucrose can facilitate long glucose residue polymer production.

2.3.2.3. Lipoteichoic acid

Many oral G+ bacteria carry a negative charge as a consequence of LTA penetration of their cell wall, it is the chief factor responsible for the hydrophobic character of the cell surface.[13]

2.3.2.4. Surface hydrophobicity

A characteristic of bacteria (varies according to species), an important factor influencing adhesion (especially if the material surface is either hydrophobic or hydrophilic). In general bacteria with hydrophobic properties prefer hydrophobic material surfaces and those with hydrophilic characteristics prefer hydrophilic ones. [1]

2.3.2.5. Fuzzy coat

Some populations of oral bacteria have a trypsine-sensitive fuzzy coat, whose chemical and physical properties will affect their interaction with other organisms or surfaces.

2.3.2.6. Bacteria surface charge

The surface charge of bacteria is considered one of the important physical factors influencing bacterial adhesion. Bacteria in water solution are always negatively charged, their surface charge varies according to species, and is affected by growth medium, bacteria age and bacterial surface structure.

2.3.2.7. Long-distance electrostatic forces

Electrostatic forces may influence the initial phase of bacteria adhesion to solid surfaces. [1]

2.3.3. DLVO theory

When a particle such as a bacteria contacts a surface, their mutual interactions will decide, whether the particle adheres or not. The best small-particle interaction description is

considered to be the Deryagin, Landau, Verwey and Overbeek theory (DLVO) on colloid stability, combining van der Waals and electrostatic forces. It claims that the total energy of an interaction of two small particles is given by the sum of van der Waals attractive forces, electrostatic repulsive forces and hydrogen bridges.

The final, basically irreversible adhesion, is created due to specific groups on the cell as well as on the surface of the substrate that can reorientate themselves in a way that leads to strongly irreversible „primary adhesion". This bond is caused by the same physico-chemical mechanisms as mentioned above. In these specific interactions, stereochemic groups come to close proximity with a great mutual physico-chemical attraction, which causes the formation of a strong bond. To achieve such a close proximity of their positions, water must be removed from the area between the cell and substrate. This could explain the effect of cell and substrate surface hydrophobicity on bacterial adhesion.

As soon as a monolayer of microorganisms forms, further plaque growth proceeds by the proliferation of the adhering organisms, and also by coadhesion between bacterial species. During the period of reversible bondage, the microorganism may become detached if the external forces are sufficient. On unlevel surfaces of rough substrates, the microorganisms are well protected from such shear forces. Therefore, theoretically, irreversible bond formation should be easier and therefore more frequent. Furthermore, because cell and substrate hydrophobicity play an important role, free surface energy change should lead to a change in bacterial colonization.

Research has confirmed that bacterial cell adhesion depends on the level of roughness and hydrophobicity. Microbial adhesion and growth vary in individual species of adherent bacteria, each has varying characteristics and prefers different conditions. [12]

After the curing process the denture bases can be polished with rotary instruments in order to produce a smoother surface. This may however produce grooves or irregularities on the surfaces, thus promoting bacterial retention. Both surface free energy and roughness of the material are important factors in microbial adherence and colonisation. A negative change in interfacial free energy, which thermodynamically favors adhesion, has been shown for interactions of oral streptococci with solid substrata like polymethylmethacrylate and polyurethane containing materials. Another important factor for adhesion especially to inert materials is the hydrophobicity both of the microorganism and the material. [1, 6]

2.3.4. Biofilm on biomaterials

Scientific interest in the process of biofilm formation has erupted in the recent years and studies on the molecular genetics of biofilm formation have begun to shed light on the driving forces behind the transition to the biofilm mode of existence. Established biofilms can tolerate antimicrobial agents at concentrations of 10 - 1000 times that needed to kill genetically equivalent planktonic bacteria, and are also extraordinarily resistant to phagocytosis, making biofilms extremely difficult to eradicate from living hosts. [11]

The term „biomaterial" may refer to biological matter, biocompatible material, biologically derived or based material. Factors affecting bacterial adhesion to biomaterial surfaces include the chemical composition of the material, its surface charge, hydrophobicity, and also the roughness of the surface and physical configuration, [12]; for example as further research indicates, filler particles exposed by finishing and polishing after composite restoration, play an important role in plaque formation on composite resin surfaces. [14]

From a biological point of view, the oral cavity represents an open dynamic system of extreme complexity. Nutrients are constantly brought in and removed. In order to remain attached to their positions in the oral cavity, microorganisms must withhold forces such as the flow of saliva, gingival fluid, mastication forces, epithelial cell removal and the mechanical oral hygiene procedure. Therefore, only microorganisms firmly adhering to intraoral surfaces will survive. Deposition, growth, removal, and re-attachment of bacteria are continual processes and microbial film like tooth plaque undergoes constant reorganization. [12]

Streptococci are among the first bacteria to colonize oral surfaces and may make up 70% of the cultivable bacteria found in human dental plaque. Streptococci bind most human salivary proteins present in pellicles, including mucins, proline-rich proteins, glycoprotein agglutinin, albumin, fibronectin, lysozyme and α-amylase. [15, 16]

Different Streptococci species preferentially colonize different oral sites and coadhere to a different range of bacteria, but evidence available so far, however, suggests that at least some of the adhesins and receptors expressed by different species of oral Streptococci are structurally similar. Furthermore, cell-surface proteins with structural or metabolic functions may also act as adhesins. [15]

2.3.5. Dental plaque and its formation

Dental plaque is a complex biofilm that accumulates on the hard tissues (teeth) of the oral cavity. It is a general term describing the complex microbial community found on the surface of teeth embedded into a polymer matrix of bacterial and salivary origin. Although over 500 bacterial species (75% of the total plaque volume, the remaining 25% is an intermicrobial substance that serves e.g. to prevent the penetration of antimicrobiotics) comprise plaque, colonization follows a regimented pattern with adhesion of initial colonizers to the enamel salivary pellicle followed by secondary colonization through interbacterial adhesion. A variety of adhesins and molecular interactions underlie these adhesive interactions and contribute to plaque development and ultimately to diseases such as caries and periodontal disease.

Human dental plaque is a complex biofilm that is present on tooth tissues as well as on restorative materials. Oral biofilm may harbour many bacteria that are involved in the development of disease conditions such as secondary caries and demineralisation processes of marginal enamel and dentin. A number of studies have well documented that biofilm formation occurs on the surface of materials of different chemical nature shortly after placement in the oral cavity. The influence of material surface is not well defined, but different studies suggest that several restorative materials may have antibacterial activity or may induce the growth of several bacteria. [17]

Dental plaque is not a uniform structure but in fact varies from tooth to tooth and from location to location on each tooth. Plaque is formed by a progression of organisms with the Streptococci being the dominant pioneer species followed by increasing proportions of Actinomyces and eventually the conversion of the plaque to a mature community with high levels of Gram-negative anaerobic filamentous organisms. One of the prominent factors in plaque formation is the condensed layer of salivary pellicle that forms at the base of the plaque. This emphasizes the importance of this biologically active substrate to which pioneer organisms must attach. [18]

Dental biofilms harboring cariogenic bacteria (caries-associated microorganisms) are among the virulent factors associated with the progression of tooth decay and periodontal

diseases. Dental biofilms may be found on any hard surfaces in the oral cavity such as enamel, implants, orthodontic appliances or restorative materials. These dental biofilms are composed of host constituents, cell-free enzymes, polysaccharides and bacteria. The biofilm development process involves several progressive stages, the initial coat on the hard surface is a conditional film which is composed mostly of salivary proteins and cell-free enzymes. Bacteria-free enzymes such as glucosyltransferase which synthesize sticky polysaccharides are an important component of that stage of biofilm formation since they provide binding sites for the succession of bacteria onto the hard surface. The biofilm matures upon adhesion and colonization of numerous species of cariogenic bacteria that populate the dental plaque. [19]

Plaque accumulation on intraoral surfaces depends on complicated physio-chemical interactions between the solid adsorbens (i.e., tooth or restoration surface) and liquid adhesive (i.e., saliva or sulcus fluid). The surface free energy changed by the formation of the so-called"acquired pellicle", bacteriostatic or bactericidal effects of the actual adsorbent material, and the microstructure in particular have a fundamental effect on the initial plaque formation. In addition, biological interactions between plaque microorganisms and the human immune system should be considered in vivo. [20]
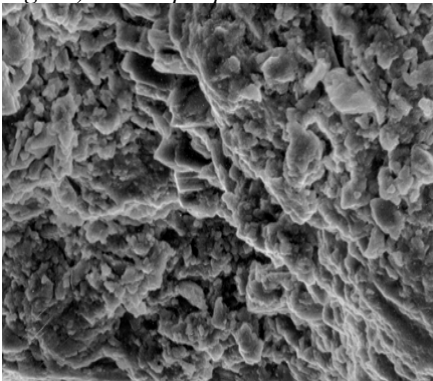

Fig.8.a) Dental plaque. Source: www.scharfphoto.com

Fig.8.b) Dental plaque. Source: www.uiowa.edu

2.3.5.1.Phases of plaque formation

There are three phases of plaque formation to be differentiated:

1. phase – formation of a dental (acquired) pellicle

The pellicle is composed of precipitated salivary glycoproteins or glycoproteins from the gingival fluid. Some of its properties enable microbial adhesion to its surface.
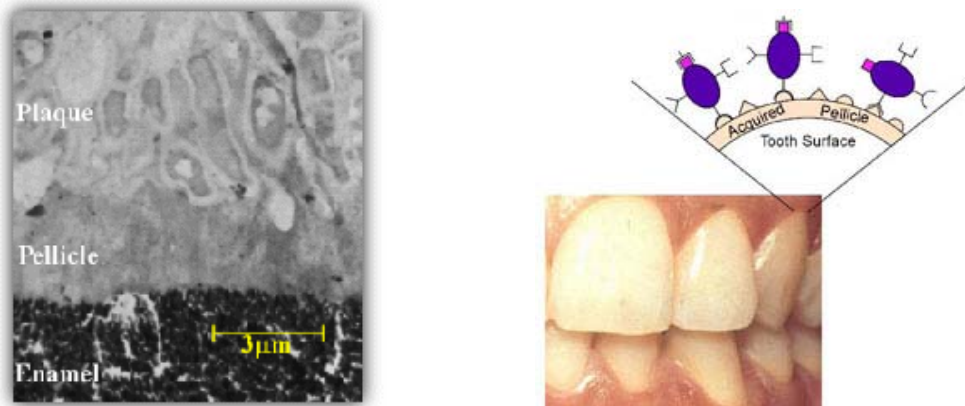

Fig.9.a) Acquired pellicle and plaque on enamel. Source: www.ncl.ac.uk
Fig.9.b) An acquired pellicle on the surface of a tooth. Source: www.uiowa.edu

2. phase - primary colonization

G+ cocci start to attach themselves to the surface of the acquired pellicle, they are equipped by fibrous fimbriae whose ends carry so-called adhesins enabling bondage between the structure of the pellicle and fimbriae covering the surface of the microbes. These organisms also have the capability of producing high-molecular substances of a polysaccharidic character serving as a binding agent (glue) for the microbes and simultaneously as an energy reservoir for periods with no food intake.

3. phase – secondary colonization and plaque maturation

Microorganisms that have inhabited the surface of the dental pellicle increase in number and produce an extracellular intermicrobial substance. Dental plaque increases in volume, oxygen penetration through this larger mass of plaque becomes increasingly difficult. Anaerobic conditions necessary for the colonization of G- anaerobic microorganisms that do not have the capability of firm bonding with other microorganisms are thus created.

These bonds enable the formation of the solid plaque structure. The mutual bond between microorganisms is called coaggregation. [21] The formation of microbial plaque is a quick process. As early as hours after tooth surface cleaning, microbes start to colonize the secondary enamel pellicle. The major species are always Streptococci and fibrous organisms, ecpecially on the surface of plaque. The composition of plaque varies with location on the tooth.[22]

2.3.5.2.The accumulation of plaque

Bacteria come into direct contact with enamel very rarely. Several seconds after brushing, salivary glycoproteins are adsorbed onto teeth and create an acquired pellicle. Coccal bacteria are adsorbed onto pellicle-coated enamel approximately within two hours of brushing. These include Neisseria and Streptococcus species (up to 95% of all cultivable flora after a 24hr cultivation). These populations multiply and form microcolonies that become embedded into the matter of extracellular polysacharides and more layers of adsorbed   salivary

glycoproteins. During this period of colonization, the growth rate of the organisms is higher than in comparison with the climax of the community.

After approx. 7 days, during which Streptococci remain the dominant type of organism, fibrous bacteria start to appear. After 14 days, under the microscope these organisms seem to be dominant, even though they contribute to the overall flora only by 10-13%. A decrease in the total number of Streptococci is associated with this onset of anaerobic fibrous bacteria. The accumulation of plaque on teeth is the result of a balance between deposition, growth, and organism removal. [23]
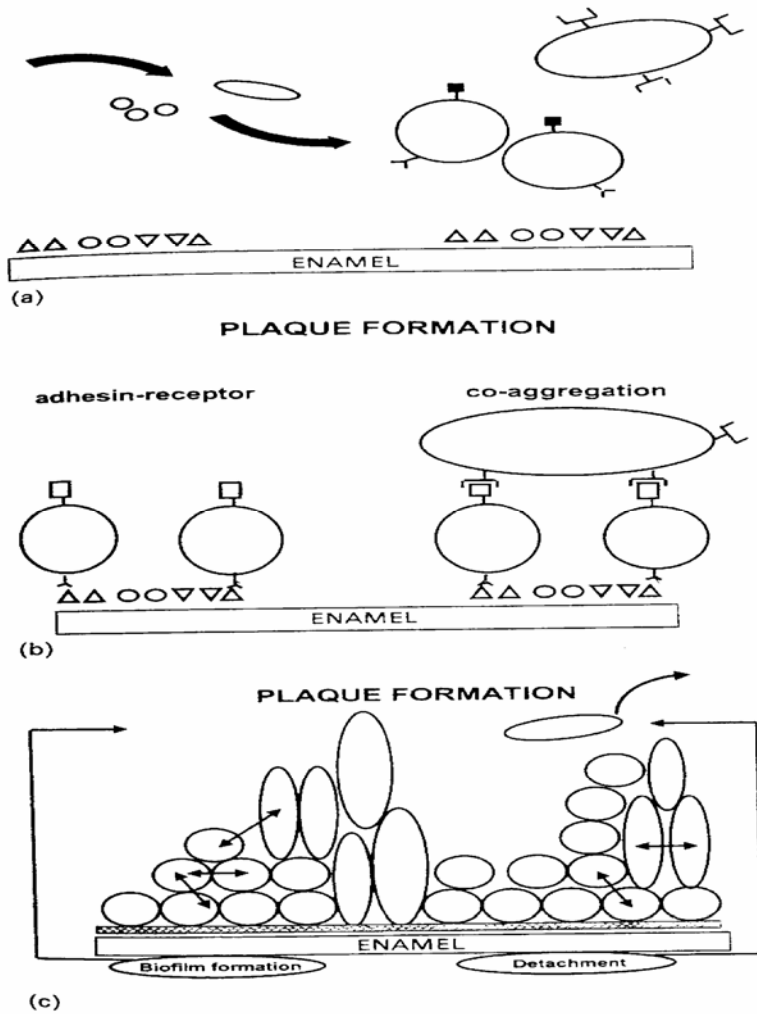

Fig.10. The formation of plaque [59]

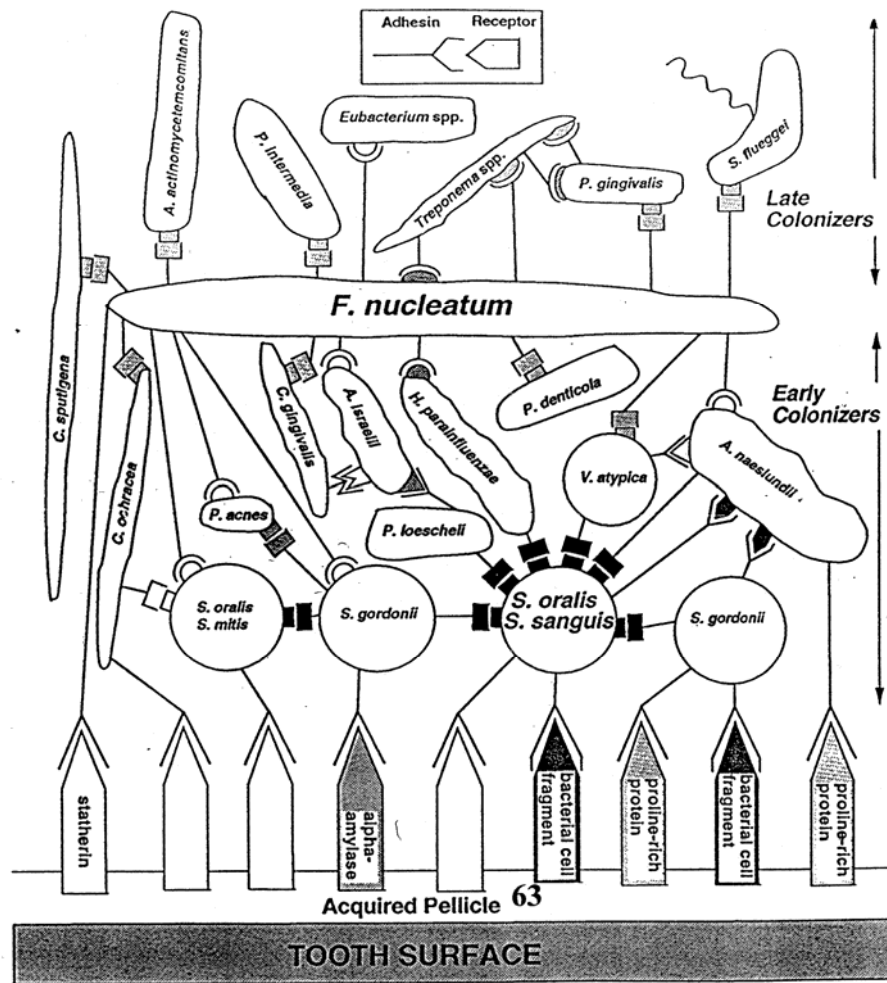Fig: Some intermolecular interactions involved in plaque formation.

Fig.11. Some intermolecular interactions involved in plaque formation [59]

2.4. ORAL BIOFILM-RELATED DISEASES

Cell adherence is central to microbial colonization of the human oral cavity, home to over 300 different species of bacteria, including 10 - 20 different species of Streptococci. Adherence to oral tissues allows bacteria to resist clearance, and so is the prelude to infection and invasion. The Streptococci have a vast repertoire of adherence mechanisms enabling them to bind to salivary components that are deposited on the various surfaces of the mouth. They also adhere to other oral bacteria in a process called coaggregation and which usually involves protein-carbohydrate interactions leading to the formation of plaque. [16]

Dental caries and the various periodontal diseases are among the most common bacterial infections in humans. Dental caries, a chronic endogenous infectious disease caused by commensal flora [24], is a consequence of a disproportionate increase (often site-specific) in the dental plaque content of strongly acidogenic and aciduric Gram positive facultative aerobes (e.g. *mutans Streptococci*, *Lactobacilli* and *Actinomyces*). In contrast, the more common forms of periodontal disease are aetiologically linked to subgingival plaque communitities containing prominent mixtures of generally anaerobic and proteolytically active Gram negative bacteria including *Porphyromonas, Prevotella, Treponema* and *Actinobacillus*. [25]

Today, tooth restoration is a widely accepted dental clinical procedure. Large surface areas of the tooth may be covered with restorative material. These materials are covered with biofilm once placed into the oral cavity which serves as a reservoir of bacteria. Accumulation of bacteria on restorative materials can lead to secondary dental caries. Recurrent human dental caries has been associated with the deterioration of dental restorative materials - the breakdown of marginal areas between enamel and restorative material can provide potential pathways for bacterial reinfection. Cariogenic microorganisms can then more easily penetrate into the underlying dentine through these defects. Reducing, or preferably preventing, such marginal breakdowns is an important element in reducing the incidence of recurrent caries. [26] In addition, accumulation of plaque on restorative materials adjacent to the gingivae should be minimized in orded to avoid tissue irritations that may lead to periodontal diseases. [19]

It is now widely recognized that environmental changes (like the placement of an orthodontic appliance) may shift the bacterial community from a healthy one to one that is able to cause disease. The placement of a fixed orthodontic appliance leads to an increase in the volume and number of bacteria within dental plaque and a disproportionate increase in the numbers of mutans streptococci within the bacterial community. *Streptococcus mutans* and *Streptococcus sobrinus* are closely associated with decalcification and several studies have reported an increase in the number of S. mutans following the placement of orthodontic appliances. [27]

Because these diseases are not life-threatening, risk to benefit considerations often encourage treatment which is less agressive and more preventative in nature than for other more serious infectious diseases. Nonetheless, the treatment of dental diseases remains extremely expensive and it is desirable to prevent and eliminate them if possible. [25]
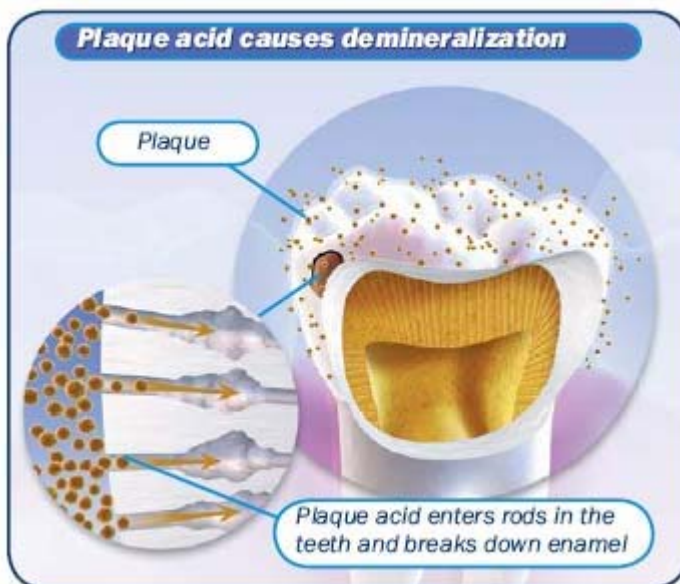

Fig. 12. Plaque acid causes demineralization. Source: www.crest.com/en_CA/prohealth/plaque.jsp

Fig. 13. Human dental caries. Source: www.mja.com.au/.../she10494_fm-1.jpg
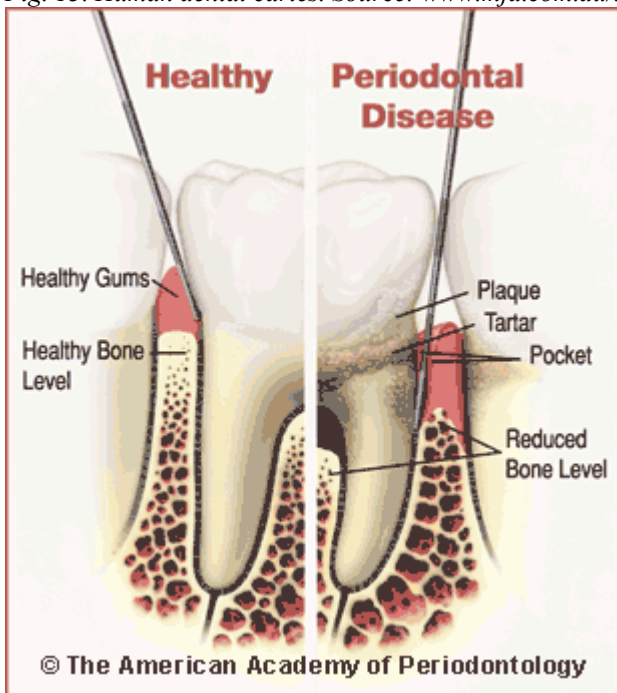

Fig. 14.a) Periodontal disease. Source: myhomepage.ferris.edu/.../periodontitis.html
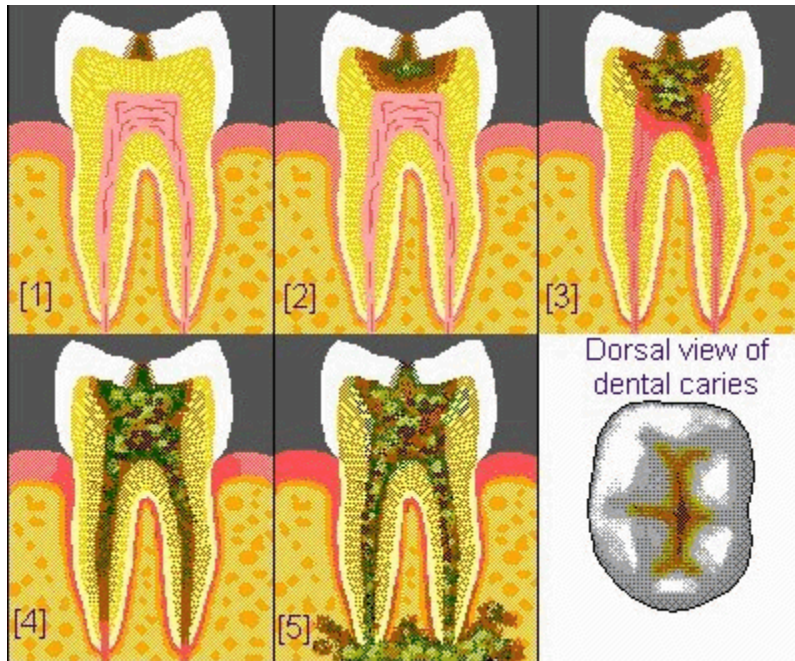
Fig.14.b) Periodontal disease. Source: http://scienceblogs.com/afarensis/2007/03/18/sunday_paleopathology
[1] Early stages : acids dissolve the enamel in the crown of the tooth
[2] Moderate tooth decay : here the dentin is attacked by acids and bacteria invade the cavity.
[3] Advanced tooth decay : inflammation of the pulp.
[4] Necrosis (death) of the pulp tissue.
[5] Periapical abcess forms at the apex of the root

2.5.   BRIEF OVERVIEW OF DENTAL MATERIALS

Four types of materials are used most commonly for direct aesthetic dental restorations. Silicates were first used in the late 19th century and frequently used until approx. 1970. They were highly soluble and not resistant to degradation in the oral environment. Silicates also changed color, became opaque when in contacts with dyes and as a consequence of dehydration, their aesthetic qualities decreased with time.

The primary use of dental polymers includes prosthetic applications such as denture bases, they are also used as dental replacements, cements, crown facets and bridges, splints, implants, temporary crowns, endodontic filling and athlete mouth protectors. [1] Acrylic polymers (unfilled) were used in the 60´s of the last century. They had improved resistance against dissolving and had no problems with dehydration, stains, however, remained a problem. Some of the less favorable properties of unfilled acrylates include significant changes in size upon curing and temperature change, low mechanical strength and toughness, low abrasion resistance, problems with recurrent dental caries.
Composites were introduced around 1960 and at present are dominant amongst materials used for direct aesthetic restorations. [1]

Polymers for dental applications must meet several strict requirements, such as:

1) Favorable physical properties - The material must have appropriate mechanical properties such as Young´s modulus, fracture toughness, etc. according to the specific application. Dental devices must withstand the constant chemical changes in the oral cavity, flow of saliva, mastication forces and the mechanical wear caused by abrasion from frequent brushing. In addition, optical properties (refractive index, electrical conductivity) are of importance for applications in dentistry.

2) Biocompatibility - The material must be able to perform its function with an appropriate reaction from the host´s body in a specific application. This is closely connected with the behavior of the cells in contact with the material, especially cells adhering to its surface.

3) Surface characteristics of the material (such as topography, chemical composition or free surface energy) play an important role in adhesion to biomaterials, must therefore be appropriate for the intended application.

4) No toxicity to humans

5) Esthetics and patient comfort.

2.5.1. Dental acrylates

Acrylates have been introduced to dentistry in the late 30s of the 20th century in the form of methylmethacrylate. Later, poor wear and fragility of PMMA has resulted in the development of dimethacrylates in the late 60´s. Eventually, filled dimethacrylates termed dental composites have been introduced in the early 70´s of the 20th century and quickly captured large portion of prosthetic applications. Even though lightly cross-linked PMMA still remains the most important material for removable prostheses and orthodontic devices, majority of cavity fillings and restorations are made of dental composites, based on cross-linked dimethacrylates filled with varying amount of ground barium glass and silica of different particle size and size distribution.

In the last 15 years, fiber reinforced dimethacrylates have been introduced to the dental market. These structural materials can replace metal alloys in load bearing substructures in many prosthodontic procedures, in orthodontics and maxilofacial surgery. Hence, research in this thesis was focused on the bacterial adhesion onto dimethacrylates, thus, they will be described in more detail in the following section (Fig.18). [28, 29]

Most dimethacrylate resins used in dentistry polymerize by chain radical polymerization initiated by light or heat. Use of low temperature activating agents allowed to produce dimethacrylates curing at room temperature (termed chemically or self-cured), however, these materials require two paste packing and their properties are more sensitive to the handling procedures. In the 20th century, the popularity of light cured dental materials increased significantly, as their use is very simple and comfortable. The initiation system is a two-component system usually containing diethylaminomethacrylate as a redox system and camphorquinone as a light-sensitive substance capable of excitation when exposed to blue light with a wavelength of 460 nm. The active free radicals react with the double bond in methyl-methacrylate monomer molecules; as soon as monomer molecules are activated, they can react with those added, thus leading to polymer chain growth. [30, 31, 32, 33]
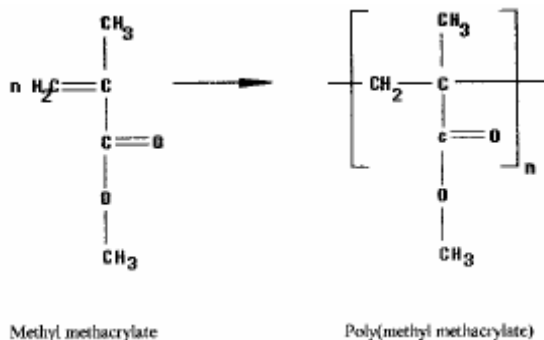

Methyl methacrylate   Poly(methyl methacrylate)

Fig.15. Methacrylate polymerization. [1]

2.5.1.1.Applications

Methacrylates have become increasingly popular as materials for bases of complete or partial dentures. They meet a number of criteria, among them high biocompatibility and good aesthetic quality, they are easy to process, and are fairly inexpensive. [1, 6] They are widely used in dental clinical practice for the replacement of hard tissues due to the compatibility of their mechanical properties with human tissues. Although the mechanical properties and wear resistance of these materials have been improved substantially, their antibacterial properties are still limited. These materials accumulate more dental plaque than other restorative materials both in vitro, and in vivo, which may result in secondary caries. [34]

Acrylate plastics may be soft and elastic or hard and brittle, they can therefore be used for a wide variety of applications, their main use remaining denture bases that serve as a support device for artificial teeth (an artificial gingiva). The properties vary according to the degree of conversion and monomer matrix.

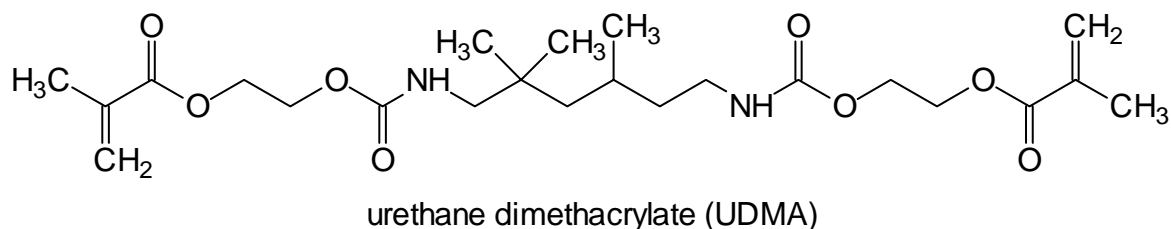*Fig.16. Acrylic Denture Base. Source: www.pro-artdentallab.com/.../eclipse3_p.jpg*

urethane dimethacrylate (UDMA)

Fig.17. UDMA. [1]
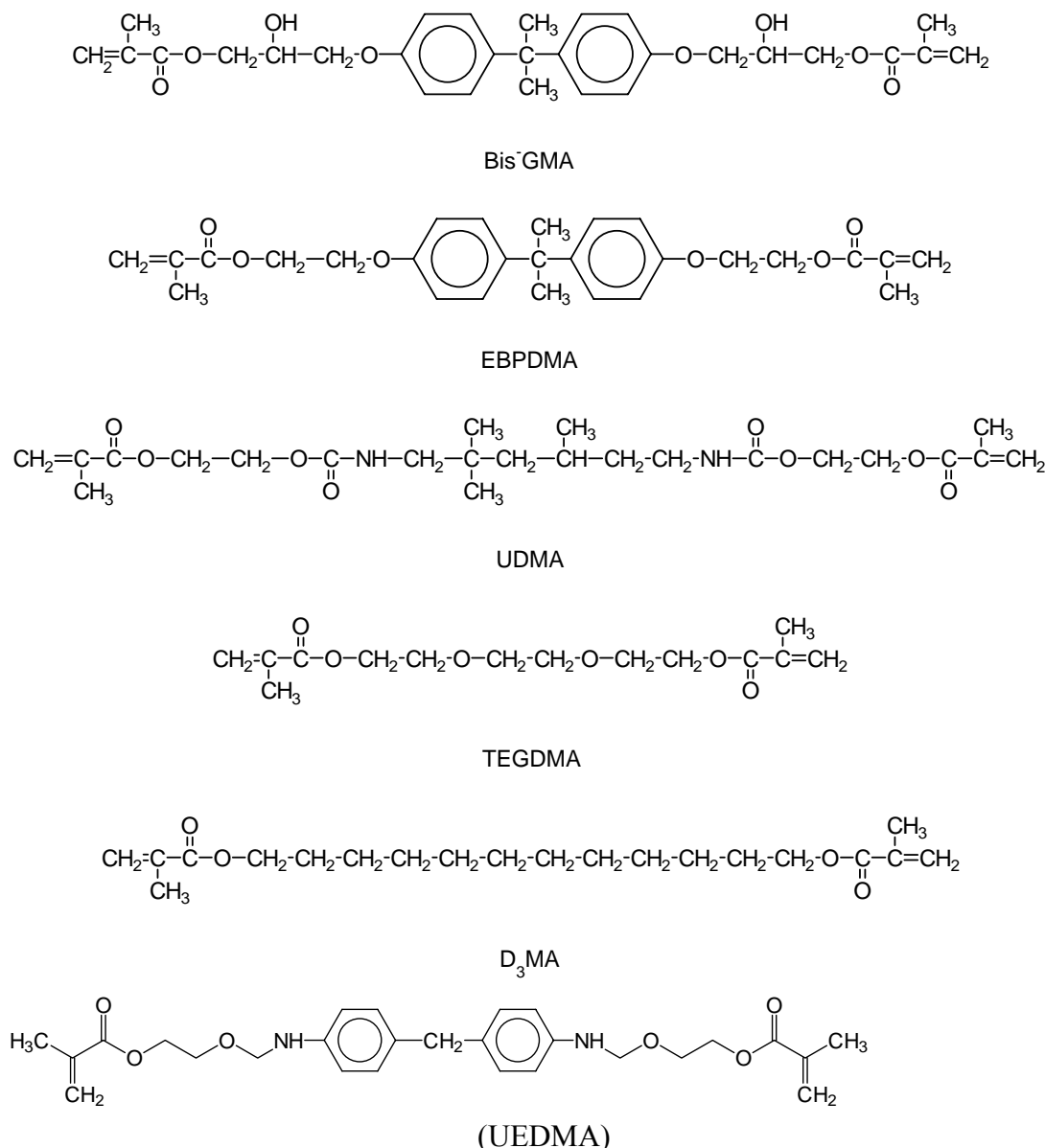
Bis-GMA

EBPDMA

UDMA

TEGDMA

D₃MA

(UEDMA)

Fig.18. Resin monomers used in current dental composites. [36]

2.5.2. Composites in dentistry

Resins first used as composite matrices were developed by Rafael Bowen, who brought them to the commercial dental market more than four decades ago. Composite restorative materials represent a substantial improvement in direct aesthetic restorations. The simplicity of their manufacturing and the esthetic properties of composites naturally led to their exploitation as filling materials. Further favorable properties include improved wear resistance, polymerization shrinkage reduction, improved mechanical properties and higher abrasion resistance than acrylates. Composite resins used in dentistry are composed of a mixture of hard inorganic particles bound to a much softer resin matrix by a bonding agent (silane). [35]
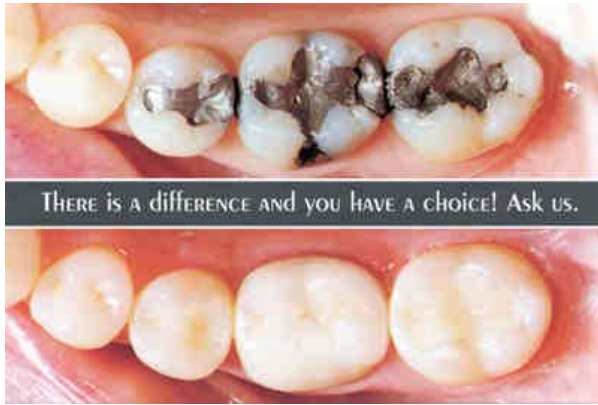
Fig.19. Composite fillings versus amalgam fillings. Source: www.atlantadentist.com/white_fillings.jpg

2.5.2.1.Matrix (organic phase)

The resin matrix binds all the individual components together but at the same time it is inherently responsible for the shrinkage and heat production upon curing. The basic used monomers for commercial purposes are predominantly dimethacrylates bis-GMA a UDMA. They have favorable physical and chemical properties, and longevity in the oral cavity. [1]

2.5.2.2.Filler (dispersed phase))

It was discovered in 1905 that plastics and elastomers can be improved by adding small inorganic filler particles. Fillers are important for various reasons like resistance improvement, manipulation properties improvement, light beam absorption, reduction of the heat expansibility factor, and minimization of polymer shrinkage. The fillers should in general be resistant to the chemical environment in the oral cavity, colorless, non-toxic, and have the same refractive index as the polymer matrix, relatively hard and have an enhancing effect on the matrix phase. Usually, the matrix exhibits viscoelastic behavior and may split under long-term stress. Fillers reduce the level of this splitting during the period of stress application.

Fillers used in dental composites can be manufactured by grinding, crushing, precipitation, or condensation. Commercially used composite resins can be divided into four groups, as presented in Table.1:

Type	Filler size	Filler volume fraction
Conventional	8 – 100 μm	54%
Microfilled	1 μm	25%
Hybrid	0,04 – 50 μm	64%
Ceramic resin bound materials	0,04 – 50 μm	75%

Table 1. Commercially used composite resins. [37]

Microfilled and hybrid composites are the ones used most commonly in dental practice.[37]
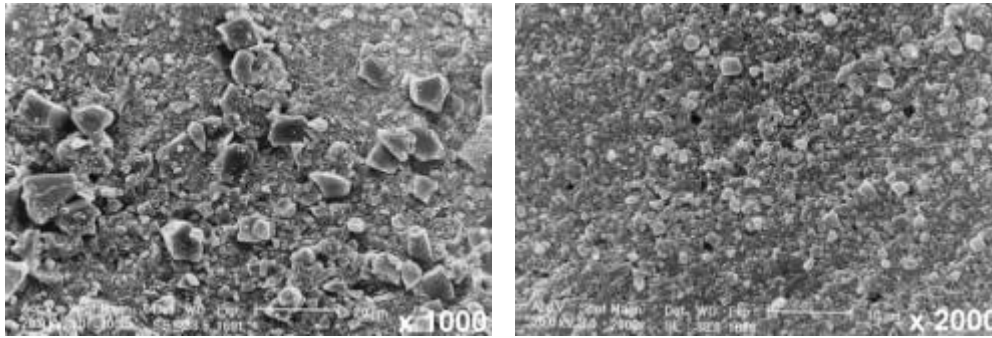
*Fig.20.a) Hybrid composite surface, b) Microfilled composite surface; Source:
146.164.26.9/.../artigos/artigo10109/index.html*

In order to achieve a hydrolytically stable bond between inorganic filler particles and the polymer matrix, it is frequently necessary to use surface active agents. These substances are most commonly on the base of bipolar binding agent, usually an organosilane. Stable adhesive bonding of filler to resin is necessary for the resistance and longevity of the composite. [1]

The plaque deposition onto the inorganic phase is less significant than onto the organic phase (resin matrix). This is why the commonly used organic phase UDMA resin is the subject of research in this thesis.

2.5.3. Dental device finishing

A laboratory or dentist perform use finishing procedures such as polishing of dental composites for both aesthetical purposes to achieve required gloss and to improve oral hygiene by reducing surface roughness. After the curing process, the denture bases can be polished with rotary instruments in order to produce as smooth a surface as possible, this depends on the skill of the attendant and the composite character - microfilled composites are polished easily.

This may however produce grooves or irregularities on the surfaces, thus promoting bacterial retention. This was confirmed by examining the materials by magnification, where a regular pattern of grooves shows on their surfaces, most probably introduced by the instruments used for polishing. It turned out by research of the contact angle and hydrophobicity of polished and unpolished materials that in the presence of filler, polishing increased the surface roughness, thus making polished materials more prone to colonization than unpolished ones, the materials in this thesis were not polished. [1] The influence of polishing on the extent of adhesion is species-specific. [6] Both surface free energy and roughness of the material are important factors in microbial adherence and colonisation. A negative change in interfacial free energy, which thermodynamically favors adhesion, has been shown for interactions of oral streptococci with solid substrata.

Composite material surfaces are also treated by polymer coatings to protect them against the effect of water and to remove any unevenness and microcracks.[38]

2.6.   BACTERIOCIDE ADDITIVES

Efforts to prevent bacterial colonisation of dental biomaterials have focused on the modification of the polymer surfaces to induce bactericidal properties and at the same time preserve the bulk mechanical properties of the device.

Composites with antibacterial activity may be useful to decrease the frequency of secondary caries around restorations and other diseases of the oral cavity. Information in the area of antimicrobial restorative materials in general has been limited both in the number of materials evaluated and in the variety of oral microorganisms tested.

In general, bacteriocide effect can be obtained by two types of mechanisms. Releasing or migrating concepts contain an antimicrobial agent within their mass or on their surface of which the purpose is to migrate partly or completely into the environment, where they exercise their inhibitory action. Non-migrating concepts contain within their mass or on their surface a compound that acts antimicrobial when the target microorganism comes into contact with the antimicrobial surface. [39] Immobilization of antimicrobial agents on suitable substrates serves to lower the ability of groups on these agents to penetrate the cell membrane, thus reducing their toxicity to the host, facilitating their eventual elimination. [40]

There exist several different approaches in antibacterial material development in order to combat biomaterial-centered infection. The following section examines the most prominent ones and compares them.

2.6.1. Antimicrobial Chemical Release

Continuous release of antimicrobial agents (mostly antibiotics) from biomaterials is one approach to prevent microbial infection on the material. Antiseptics are also used but are restricted since they are not selective in their toxicity and might damage host cells as well. The problem with this approach is that after some time 'sub-inhibitory' concentrations of antimicrobial agents released into the surrounding tissue or fluids may lead to the development of resistant strains of microbes. This approach is especially pronounced in implants which are intended to be used for longer time periods.

Partial solution to the problem is to use more than one antibacterial compound, thus broadening the spectrum and reducing the chance of resistance build-up. [41] The leaching of the antimicrobials from the materials has several disadvantages, however, especially the decrease in carrier material mechanical properties over time, short-lived effectiveness and possible toxicity to human health. [42] Therefore new approaches attempting to immobilize the antibacterial agent in composites have been suggested.

2.6.2. Immersion

The clinical success of this approach has been varied. Although immersion is one of the most straightforward methods for loading antimicrobial agents into medical devices, biomaterials generally have a limited affinity for these agents, and the majority of the drugs will be adsorbed on the surface and not diffused into the depths of the polymer matrix. In general, studies have illustrated that the immersion of polymers in antimicrobial solutions, although reducing early-onset colonization of devices, would be unlikely to prevent biofilm formation in long-term implants. For an optimum effect, loading of medical devices by immersion in aqueous antibacterial solutions should be restricted to medical devices that are hydrophilic in nature, i.e. possess a hydrophilic (hydrogel) coating allowing absorption of the aqueous solution. Immersion of hydrophobic medical devices in aqueous antibacterial solutions will result in a weak and limited surface attachment of antibacterial agent to the device with poor clinical effects. However, the method is very effective in preventing bacterial intrusions immediately following the implantation of biomaterial. [41]

2.6.3. Surface Coatings

Specific antimicrobial coatings may be applied onto a material to provide device protection from infection. Drug loading is enhanced by pre-coating the surface with a tie layer, wherein the interaction between the antimicrobial agent and the tie layer is facilitated by electrostatic interactions. [41]
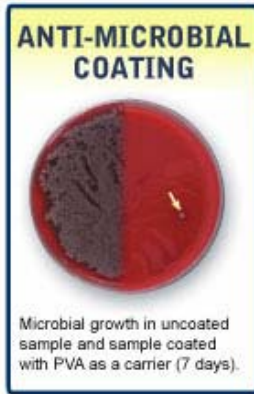

Fig.21. Antibacterial coating. Source: www.bacterin.com/images/coatings_img01.jpg

2.6.4. Matrix Loading

The loading of antimicrobial agents into biomaterials by immersion or coating technologies has the advantage of being relatively simple. However, the limited mass of drug that can be incorporated may be insufficient for a prolonged antimicrobial effect, and the release of the drug following clinical insertion of the device is rapid and relatively uncontrolled. A means of reducing these problems is by direct incorporation of the antimicrobial agent into the polymeric matrix of the medical device at the polymer synthesis stage and/or at the manufacture stage.
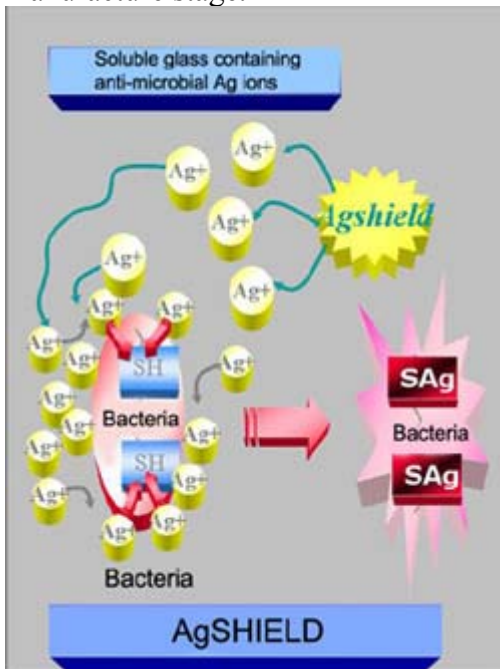

Fig.22. The activity of Ag ions contained in an antibacterial glass. Source: zmdu.hnet.net.cn/image/sjwf_2.jpg

A principal disadvantage to be considered of the direct incorporation of antimicrobial agents into the matrix is the reduction in mechanical properties of the host polymer. The mechanical

properties of the polymer are essential to ensure the optimal performance of the medical device so the potential hazards of incorporation of antimicrobial (and related) agents must be considered. [41]

2.6.5. Drug Polymer Conjugates

Permanent coatings covalently incorporated into the structure of material surface serve antimicrobial purposes well and are long lasting. Covalent attachment of drug to the surface of polymer has received comparatively little attention in contrast to the other methods of drug incorporation into biomaterial structure. The covalent linkage of an agent to a monomer prior to polymerization provides a method of producing perhaps the most resilient drug-polymer material. Drug-polymer conjugates have been found to provide significant reductions in bacterial adherence. There are certain limitations to this approach, such as the selection of therapeutic agents with chemistry that is compatible with the synthetic reaction scheme, and there is a greater expense associated with the synthetic process. [41]

2.6.6. Functionalization by Particle Bombardment

Gamma radiation and glow discharge techniques act by introducing new functional groups onto the polymer surface, and the newly created functional groups may possess intrinsic antimicrobial activity. Antimicrobial substances may also be linked covalently to the functional surface groups to provide increased antimicrobial activity. A photochemical approach to coatings has been demonstrated to cause significant reductions in bacterial attachment to polymers and this could bring further exciting benefits. However, to ensure patient safety, it is recommended that anti-adherent coatings should be combined with site-specific delivery of antimicrobial agents. [41]

2.7. DENTAL MATERIALS WITH ANTIBACTERIAL EFFECT

2.7.1. Non - silver bacteriocides in dental materials

On the search for an antibacterial material with optimum properties, many scientists have discovered more or less potent bacteriocides useful in a variety of applications, some of which are listed in the following.

Metal ions - Heavy metal ions have a strong antibacterial action, but their properties include toxicity to all cells, they are therefore excluded from applications that could directly influence human health.

Polycations - It has been reported that polycations exhibit antibacterial properties, i.e. interact with and disrupt bacterial cell membranes. A number of polymers with antibacterial properties were developed for this purpose, including soluble and insoluble pyridinium-type polymers involved in surface coating.

MDPB - A new monomer, methacryloyloxydodecylpyridinium bromide (MDPB), was synthesized by combining an antibacterial agent and methacryloyl group. The monomer was incorporated into a resin composite to develop a non-releasing antibacterial composite that showed no release of the incorporated monomer but still exhibited antibacterial properties. [42] MDPB has an antibacterial activity before being polymerized, after composite

incorporating MDPB was cured, no elution of the antibacterial components was observed from the material, even after 90 days' immersion in water or other solvents. [43, 44]

However it was found that the amount of MDPB incorporated into the monomer compositions of composites should be limited to less than 0,4% to completely prevent agent release, and this limitation leads to poor reproducibility of anti-plaque effects when the surface is polished to expose filler particles (unpublished observation). Therefore a greater density of bacteriocide immobilized on the surface is considered to be essential for obtaining reliable effects, hence an increase in the amount of incorporation of MDPB without causing any addition of unreacted species needs to be established. In order to resolve this problem, an incorporation of MDPB into a prepolymerized resin filler, which was highly polymerized by heat before loading to composites, was designed. Specimens with 2,83% MDPB showed satisfactory mechanical properties, incorporation of MDPB at higher concentrations not only resulted in the decrease of mechanical properties, but also gave greater viscosity to render the material difficult to be handled. Furthermore, a primary type composite containg MDPB in the matrix showed rapid discoloration, so the color stability is another property to be examined. [42]

Chlorhexidine - Attempts to produce resin composites with antibacterial properties by incorporation of an antibacterial agent such as chlorhexidine have been reported, but problems can arise due to release of the inhibitory agent from the composite. Although initially strong, its antibacterial effect did not last for long periods. Incorporation of antibacterial agents such as chlorhexidine into a methacrylate monomer or restorative materials affects their mechanical properties. Moreover, the release of these antibacterial agents resulted in further changes in the physical properties of the materials. Such problems may include toxic effects, influence on mechanical properties, and loss of effectiveness.
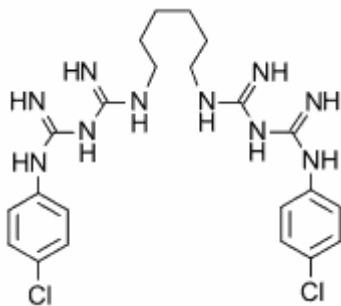

Fig.23. The structure of chlorhexidine. Source: content.answers.com/.../5/5b/Chlorhexidine.png

PEI - The objective of another study was to evaluate clinically the safety and efficacy of alkylated polyethylenimine (PEI) nanoparticles in composite resin restorative materials . In an in vitro study, addition of a small percent (1% w/w) of nanoparticles did not affect significantly the flexural strength of the commercial materials. The mechanical properties of the new composites were close to those of the original composite, but exerted a strong antibacterial activity upon contact that lasted for at least six months. [42]

As compared with conventional antibacterial agents of low molecular weight, the advantages of polymeric antibacterial agents are that they are non-volatile, chemically stable, can be chemically bound within the polymer carrier via active groups for improved integration in the composite, and are difficult to penetrate through the skin.The composite resin materials incorporated with PEI nanoparticles maintained antibacterial activity over 1 month without leaching out and no alteration of the original mechanical properties. [33]

Bacteriocins - Another approach suggests administration of bacteriocins (small bacterially produced peptide antibiotics), that interact with a sensitive cell and interfere with its multiplication, metabolism or viability. [25] Further examined antibacterial agents include Kuwanon G, isolated from the ethyl acetate fraction of methanol extract of Morus alba [45]; flavones as methanolic extracts from plants exhibited antibacterial activity, for example Panduratin A showed significant inhibitory activity against *S.mutans* [46, 47].

OAIS - Another antimicrobial agent, 3-(trimethoxysilyl)-propyldimethyloctadecyl ammonium chloride, was immobilized on silica. Interaction between this material termed OAIS and various oral bacterial species were studied. The results indicated that OAIS adsorption of these oral bacteria was dependent on the degree of hydrophobicity of their surfaces. A major component of this adherence was hydrophobic cell-surface proteins. [40]

All of the previously mentioned bactericidal agents have a problem or limitation that more or less excludes them from being exploited in antibacterial dental material development. An alternative exists in the usage of colloidal silver incorporated into dental materials. Silver has been exploited as a potent antibacterial since ancient times. Its incorporation into polymers might present a solution in finding an antibacterial dental material.
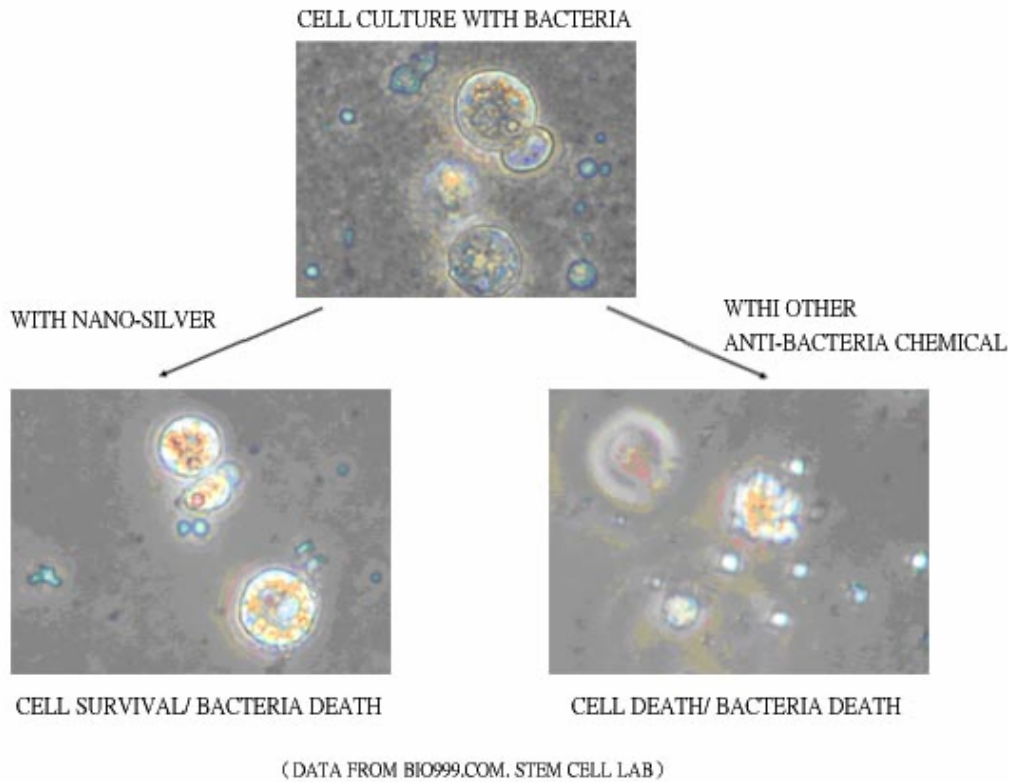

Fig.24. Nanosilver cultured stem cells survive while bacteria are eliminated. Other antibacterials also cause stem cell death. Source: www.bio999.com/nanosilver/image/Enan00_pic01.jpg

2.7.2. Silver containing agents

Colloidal silver particles and various silver salts have been proven to be effective antibacterialů agents. The size of the colloidal silver particles ranges generally between 15 - 5 nm. The smaller the particle size, the grater the ratio of surface area to volume and the greater the area available for reactions. For example 1 gram of pure solid silver in the form of a sphere has a surface are approximately 11 cm^2, whereas 1 gram of silver nanoparticles averaging 10 nm in diameter has a surface area of 6 x 105 cm^2. [48] Metal particles are

highly prone to oxidation due to their high surface energy and affinity for oxygen which can lead to the complete oxidation of the particles. Upon becoming a colloid, silver takes on a positive ionic charge, unless its interactions with the atmosphere are minimized.
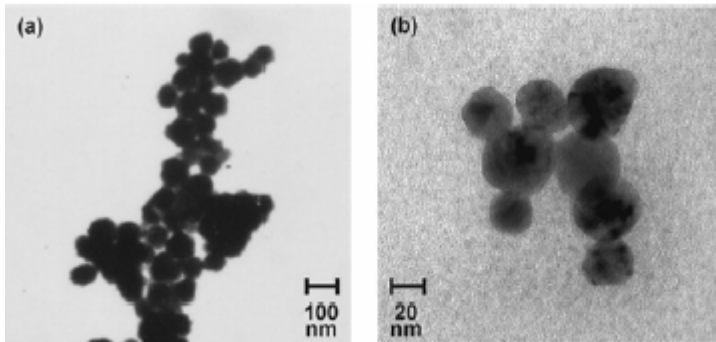

Fig.25.a),b) Silver nanoparticles. Source: www.nature.com/.../v5/n4/images/nmat1615-f1.jpg

Colloidal silver nanoparticles exhibit bioactivity that make them useful in medicine or building bionano-architectures (such as interfacing DNA with magic number Ag55 particles, as shown on Fig.26), a property that cannot be predicted from the properties of bulk (macroscopic) silver. [49]
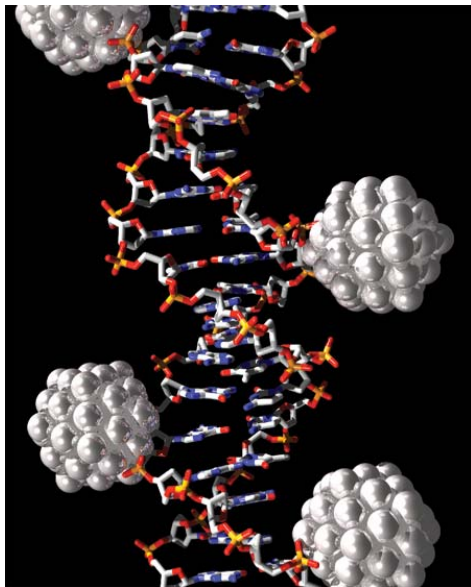

Fig.26. Nano-silver interface with DNA.[76]

2.7.3. Antibacterial activity mechanism

The antibacterial activity of silver has been known for centuries, it is one of the most universal antimicrobial substances, combined with low human toxicity. Silver and its salts have been used medically and in controlling bacteria and other organisms in water.[21] Nanotechnology enables us to expand the surface area of silver particles markedly. The antibacterial properties are related to the total surface area of the nanoparticles. Smaller particles with a larger surface to volume ratio provide a more efficient means for antibacterial activity. [50] The exact mechanism of the antimicrobial effect of silver is still not confirmed, several hypotheses have, however, been published.[51]

2.7.3.1. Silver ion - cell interaction

Silver ions are highly toxic for microorganisms, some recent papers propose that the mechanism of the antibacterial action of silver ions is closely related to their strong interaction with thiol (sulfhydryl, -SH) groups in both functional and structural proteins of the bacterial cell, although other target sites remain a possibility. Research indicates that silver inhibits glucose, succinate and lactate oxidation (dehydrogenation), possibly by means of an uncoupling effect. It has been demonstrated that at low concentrations, silver does not enter the cell. Rather, it is adsorbed onto the bacterial surface just as silver tends to adsorb to other surfaces, thus silver ions immobilize dehydrogenation because respiration occurs across the cell membrane in bacteria rather than across the mitochondrial membrane as in eucaryotic cells. [21] In comparison to other heavy metal ions, silver is probably the most useful as it combines a high antimicrobial activity with a remarkable low human toxicity. [39] It has been found that Ag^+ ions exhibit a much stronger activity than Cu^{2+} ions, Zn^{2+} ions, Mn^{2+} ions and commercial silver-modified material Novaron. [52]
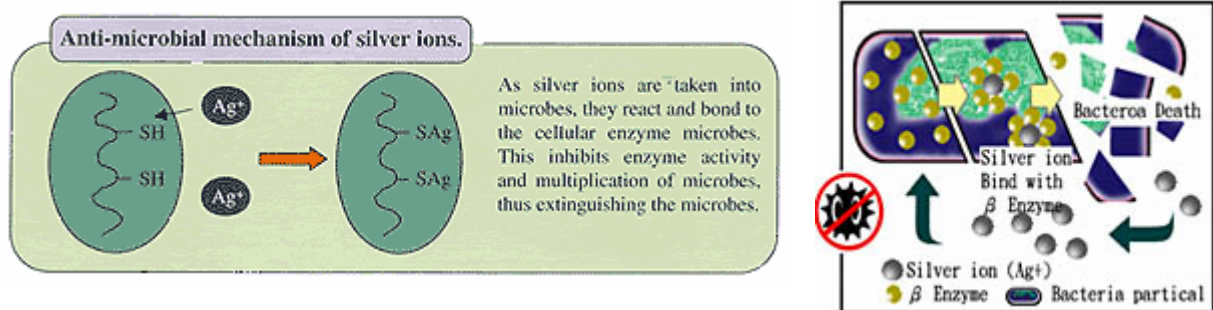

Fig.27.a),b) Antimicrobial mechanism of silver ions. Source a: www.marubeni-sunnyvale.com/images/Anti-microb;b www.bio999.com/nanosilver/image/Enan00_pic01.jpg

Silver works in a number of ways to disrupt critical functions in a microorganism. The silver cation binds strongly to to electron donor groups containing sulphur, oxygen or nitrogen. Biological molecules generally contain these components in the form of negatively charged side groups such as thio- (sulfhydryl), amino-, imidazole, carboxylate and phosphate groups or other charged groups distributed throughout microbial cells. This binding reaction alters the molecular structure of the macromolecule, rendering it worthless to the cell. Silver simultaneously attacks multiple sites within the cell to inactivate critical physiological functions such as cell wall synthesis and translation, protein folding and function, and electron transport, which is important in generating energy for the cell. Silver ions act by displacing other essential metal ions such as Ca^{2+} or Zn^{2+}. The binding of silver to bacterial DNA may inhibit a number of important transport processes such as phosphate and succinate uptake and can interact with cellular oxidation processes as well as the respiratory chain. [39]

There may be multiple correct answers to the question of the mechanism; another study proposes three different mechanisms depending on type of microorganism. i) In Gram-negative bacteria - plasmolysis (cytoplasm of bacteria separated from its cell wall) was observed. ii) In *S. aureus*, the synthesis of bacterial cell wall was inhibited. iii) Nanosilver particles may induce metabolic disturbance in *M. tuberculosis*. Antimicrobial mechanisms of nanosilver were different according to the species of bacteria. It follows therefore that silver nanoparticles will be available as a good antibiotic alternative due to their nonselectivity. [51] Another study claims that the inhibition of bacterial growth was dependent on the interference of DNA function by the binding of silver ions along the helical chain. [22] Without one or more of the aforementioned functions pertrubed by the presence of silver, the

bacterium is either inhibited from growth, or, more commonly, the microorganism is killed. [53]

The antimicrobial activity of silver nanoparticles was further confirmed, when the damage on the researched cells was photographed by a microscope, showing formation of "pits" in the cell wall of the bacteria, while the silver nanoparticles were found to accumulate in the bacterial membrane. A membrane with such a morphology exhibits a significant increase in permeability, resulting in death of the cell. These nontoxic nanomaterials, which can be prepared in a simple and cost-effective manner, may be suitable for the formulation of new types of bactericidal materials. [30]
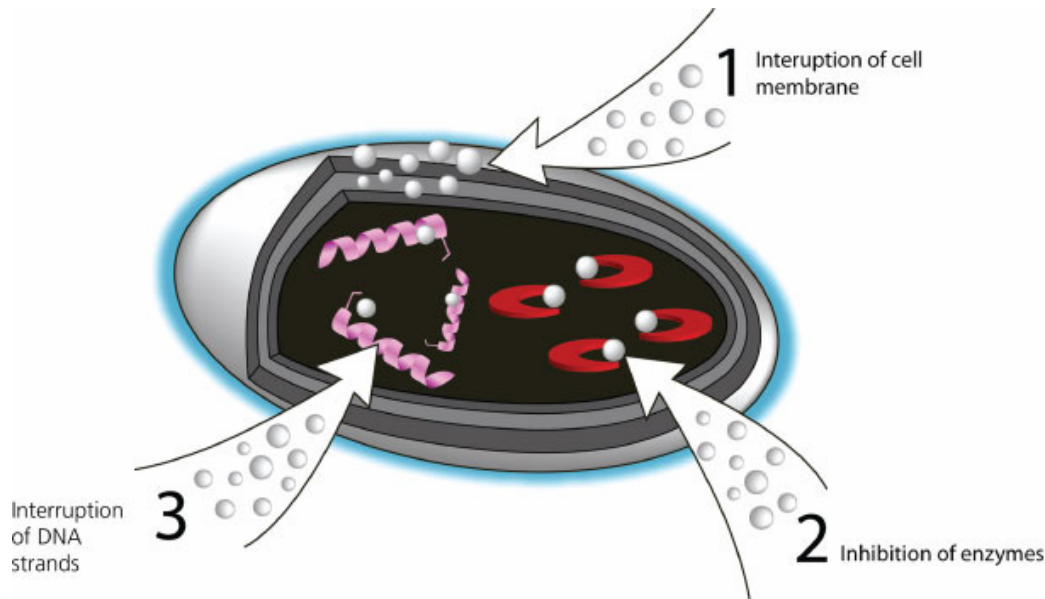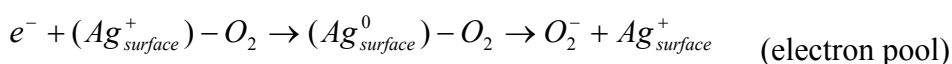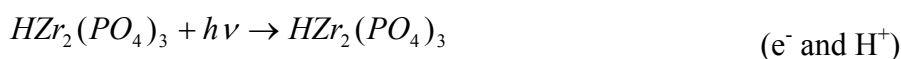

Fig.28, Antibacterial mechanisms of silver. Source: www.labnews.co.uk/cms_images/Image/Sept_pics/

Because silver affects so many different functions of the microbial cell, it is nonselective, resulting in antimicrobial activity against a broad spectrum of medically relevant microorganisms including bacteria, fungi and yeasts. Silver is also more efficient than traditional antibiotics because it is extremely active in small quantities. [53]

2.7.3.2. Oligodynamic effect

Recently, master batches containing Ag-substituted zirconium phosphate ceramics $(Ag_xH_{1-x}Zr_2(PO_4)_3)$, called Novaron (Toagosei Co. Ltd., Japan), have been brought onto the European market. Kourai et al. (1994) found that the bactericidal activity of zirconium phosphate ceramics containing silver ions was extremely enhanced by white light irradiation and oxygen. When $HZr_2(PO_4)_3$ was irradiated, Ag-surface+ would work as an electron pool and O_2 molecules adsorbed on this site would be reduced to O_2^- radicals:

$$HZr_2(PO_4)_3 + h\nu \rightarrow HZr_2(PO_4)_3 \qquad \text{(e}^- \text{ and H}^+\text{)}$$

$$e^- + (Ag_{surface}^+) - O_2 \rightarrow (Ag_{surface}^0) - O_2 \rightarrow O_2^- + Ag_{surface}^+ \qquad \text{(electron pool)}$$

This mechanism in which oxygen is changed into active oxygen through the catalytic action of silver is the so-called oligodynamic effect. However, the mechanism of photogeneration of superoxide radicals and the role of silver ion is not quite clear. [39]

2.7.4. Silver containing bacteriocides

Silver particles - Nano-sized silver particles show high antimicrobial and bactericidal activity against Gram-positive and Gram-negative bacteria, including highly multiresistant strains such as methicillin-resistant Staphylococcus aureus. The antibacterial activity of silver nanoparticles is dependent on the size of the silver particles, the smaller the particles (larger surface to volume ratio), the greater antibacterial effect. A very low concentration of silver (as low as 1.69 µg/mL Ag) exhibits antibacterial performance. [54]

Silver/polymer colloidal composites - Synthesis of silver/polymer colloidal composites from surface functional polymer microspheres had been presented as an Ag/polymer system where Ag nanoparticles were deposited uniformly onto surface functional porous polymer microspheres. They showed excellent antibacterial performance and no yellow discoloration upon long-term storage, the MIC of silver was only approx. 2 ppm.[55]

A simple method of fabricating highly potent dual action antibacterial composites consisting of a cationic polymer matrix and embedded silver bromide nanoparticles is on-site precipitation of AgBr, used to synthesize the polymer/nanoparticle composites. The synthesized composites have potent antibacterial activity toward both gram-positive and gram-negative bacteria. The materials form good coatings on surfaces and kill both airborne and waterborne bacteria. Surfaces coated with these composites resist biofilm formation.. These composites are potentially useful as antimicrobial coatings in a wide variety of biomedical and general use applications. [56]

Fillers incorporating silver - Silver ions can also be implanted in fillers, that are in turn incorporated in dental composites. For example, SiO_2 filler with 0.05 ppm Ag^+ demonstrates antibacterial activity against oral streptococci. The antibacterial effect is due to the Ag ion release from the filler. [22] Another method proposed is preparing antibacterial silver-containg silica glass by the sol-gel method. [57] YDA filler is an antibacterial agent that is currently in commercial dental use, it may help in the development of antibacterial dental materials, such as composite resin, glass-ionomer or temporary cement. [58]

Commercial silver-supported antibacterials - Nonreleasing antibacterial materials including silver ions in inorganic oxides, such as crystal structure of ceramics or silica gel particles, as carriers have been developed. They exhibit strong antibacterial activity against gram-positive and negative bacteria, yeast and molds. These materials may resolve problems such as short term durability of antibacterial activity and degradation. [59]

Antibacterial dental materials were produced by incorporating commercial inorganic silver-supported materials such as Novaron and Amenitop into resin composites. Novaron is a zirconium phosphate ceramic containing silver ions in the crystal structure that exhibits strong antimicrobial activity against gram positive and gram negative bacteria, yeasts and molds. Amenitop containing composites were sufficiently bactericidal but there was a significant difference in compressive and flexural strength after immersion in water.

The results indicated that a composite resin incorporating silver-supported materials like Novaron (min 5 wt%) may be clinically useful due to its longterm inhibitory effect and favorable mechanical properties. [59, 60, 61] The bactericidal activity of Novaron was approximately proportional to the intensity of irradiation by visible light. The bactericidal active materials are superoxide and hydrogen peroxide formed by certain photochemical reactions on the surface of Novaron particles.[52] After immersion in water for 6 months, the Novaron composites inhibited the growth of *S.mutans* with no significant difference in compressive and flexural strength.[62] Also 2% nano-silver antibacterial agent FUMAT

T200-4 incorporated in a denture base resin had sufficient antibacterial properties and no significant influence on the mechanical properties of the denture base resin.[63]

Antibacterial resin - The development of an antibacterial dental resin using the silver methacrylate monomer as an antibacterial substance was tried. It was dissolved into MMA at a concentration of 84 μmol/l, and it was suggested that the concentration was sufficient for the appearance of antibacterial activity. The resin obtained was transparent and colorless, and there was no esthetic problem. The compressive and bending strength of the resin were not significantly different compared with those of PMMA. [64]

Leaching out silver nanoparticles could have a negative effect on the material, so the use of silver immobilized in the material is more appropriate, although possibly less effective.

2.7.5. Possible problems, limitations of Ag-modified antibacterials

2.7.5.1. Discoloration

When Ag-containing samples are stored for a long time, their color turns yellow or dark yellow. This phenomenon causes a serious problem in final applications. Therefore no discoloration is one of the primary requirements. Samples containing conventional colloidal Ag nanoparticles changed eventually from white to dark yellow after 6 month storage at 40°C. This is assumed to stem from a secondary reduction reaction of the ionized Ag resulting in new Ag particles in the sample. [51]

2.7.5.2. Effect on human health

A toxic side effect resulting from a buildup of silver in the bloodstream is known as Argyreia, a blueish-grey discoloration of the skin. Medical science recognizes Argyreia as specifically caused by intensive long-term exposure to silver compounds such as silver nitrate, silver sulfate, silver sulfadiazine, etc. not from microparticles of ionic silver. To date, no medical study conducted on colloidal silver has indicated that it poses a threat to human health. It does not appear to be readily absorbed by the human body. Excess silver deposited in the stomach is precipitated as silver chloride by the stomach bile and almost immediately excreted through the feces.

Fig.29.a),b) Argyreia. Source: www.doh.state.fl.us/pharmacy/alert2.htm

2.7.5.3. Bacterial resistance

The general effectivity of silver is due to the fact that pathogens cannot mutate to avoid its antimicrobial effect. The development of resistance to antimicrobial silver would be

extremely rare because an organism would have to undergo simultaneous mutations in every critical function within a single generation to escape its influence. Spontaneous mutation is rare, occuring in only 1 per 105 divisions, so the probability of multiple dependent mutations occuring in the same generation of microbes is extremely small. [53] This problem is, however, common with antibiotics; bacteria carrying mutations causing antibiotic resistance pose a threat to human health, as they do not react to conventional treatment and may horizontally transfer this trait to other bacteria. Possible ways of resistance gene transfer are depicted below.
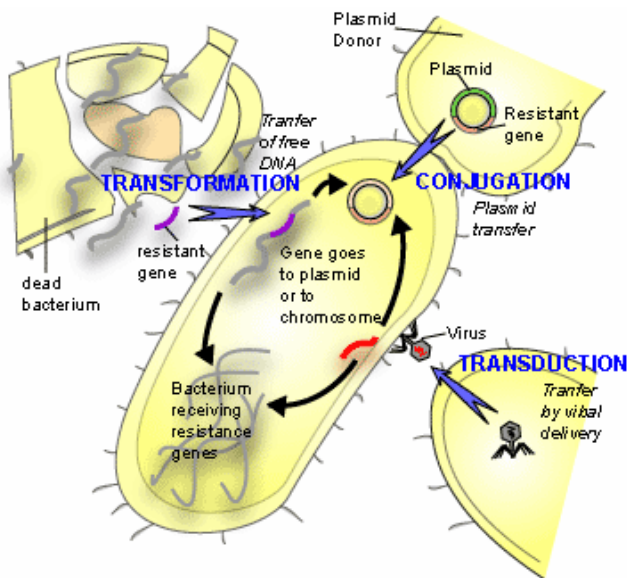

Fig.30. Horizontal resistance gene transfer. Source: www.scq.ubc.ca/.../08/HorizontalTransfer

2.7.5.4.Alteration of mechanical properties

Mechanical properties, such as favorable tensile and flexural properties must be maintained in antibacterial dental materials. Tensile modulus, strength, and elongation at break may decrease with incorporation of silver and an increase in silver concentration. [64] The concentration of nano-silver necessary to achieve sufficient bactericidal activity is so low, however, that no major mechanical property changes are expected.

A number of reports described experiments in which composite resins were impregnated with antibacterial agents such as antibiotics, silver ions, iodine and quaternary ammonium compounds, and gradually released them. However, release of antibacterial agents into the surrounding milieu at various releasing rates has several disadvantages: a decrease in the mechanical properties of the carrier material over time, short-term effectiveness, and possible toxicity if the release is not properly controlled. [42] This is why an approach with an immobilized antibacterial agent is preferable.

3. EXPERIMENTAL – MATERIALS AND METHODS

3.1. LIST OF CHEMICAL AGENTS, EQUIPMENT USED

3.1.1. Chemical agents used:

Biuret agent	VUT Brno
BHIB, BHI Agar	HiMedia Laboratories, India
IMA VLGP containing Petri dishes	Biotechnologické lab.Slezsko, s.r.o.
Glucose	Léčiva Praha
Commercial composite Adoro	Ivoclar, USA
Ceramic discs	VUT Brno
UDMA based resin (initiation system diethylaminomethacrylate and camphorquinone)	
	ADM, a.s.,
Biocide additive 2	
Biocide additive 1	

3.1.2. Equipment used:

Anaerostat (37°C, 5%CO_2)	InCu Safe, Sanyo, Schoeller
Inoculation box with UV lamp	Aura Mini, Bioair
Pressure cooker	Tefal
Ultrasound bath	Bandelin, Sonorex Digitee
Confocal laser scanning microscope	Lext OLS 3000, Olympus, Japan
Spectrophotometer (540 nm)	Helios δ, Thermospectronic
Curing machine	Targis Power, Ivoclar
Metal mold + transparent foil	VUT Brno

3.2. MODEL MICROORGANISMS AND THEIR CULTIVATION

Streptococcus mutans has been proven to be a primary colonizer and the most cariogenic of oral strains, therefore it serves as a model organism in this thesis. The selected strain was an oral cavity isolant *Streptococcus mutans* P2093 (Sbírka mikroorganismů, Brno).
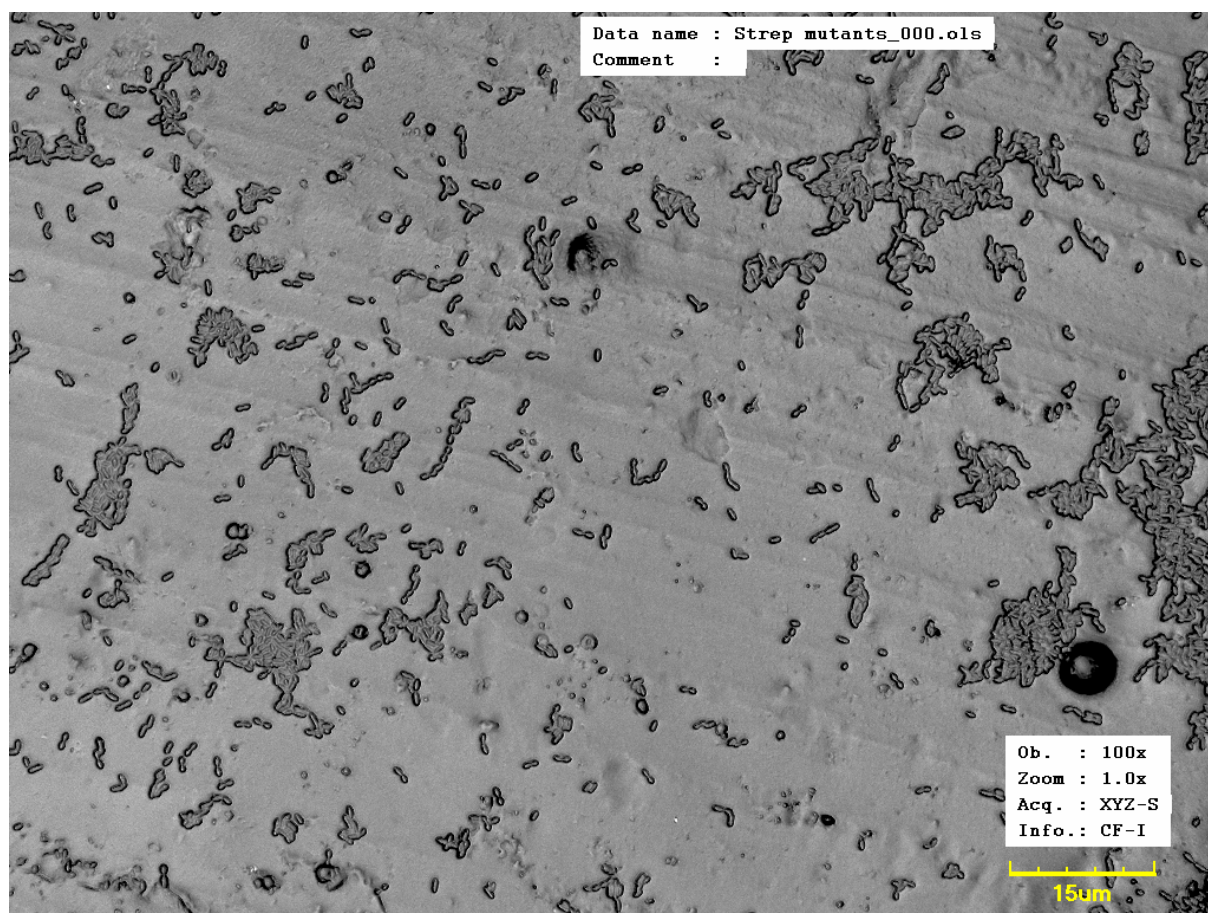
Fig.31. S.mutans growing on composite polymer disc

3.2.1. Nutrition broths

BHIB is a nutrition medium used for cultivating pathogenic cocci and other nutritionally demanding microorganisms from blood samples and clinical material. Blood agar IMA VLAC and IMA VLGP poured onto sterile Petri dishes was used for the cultivation of the microorganisms (Laboratoře lékařské mikrobiologie, Třinec).

Fig.32. Blood agar and liquid medium used for S.mutans cultivation

3.2.2. Cultivation, growth conditions

The model microorganisms were grown in a CO_2 incubator at 37ºC in a 5 % carbon dioxide environment on blood agar Petri dishes. They were inoculated under sterile conditions every seven days onto fresh plates. Microorganisms were withdrawn under sterile conditions from the blood agar plate into the liquid BHIB, after 24 hours 200 µl of bacterial suspension was re-inoculated into fresh BHIB. Measurements were conducted 18 hours after inoculation into fresh BHIB, this choice of time is explained further.

3.3. QUANTIFICATION OF BACTERIAL COLONIZATION, ANTIBACTERIAL ACTIVITY ASSESSMENT

3.3.1. Biuret method

The amount of bacteria adhering to the hard polymer surfaces was quantified by a spectrophotometric assay called the Biuret method. (Biuret agent was prepared by dissolving 1,5 g $CuSO_4.5H_2O$ + 6 g $C_4H_4O_6KNa.4H_2O$ in 500 ml H_2O. 300 ml 10% NaOH was added to the solution and distilled water to make 1000 ml.)

When assessing the bacterial colonization in suspension, 1 ml of the tested suspension was mixed with 4 ml of Biuret agent and 1 ml distilled water. Upon a 10 second shaking period and 30 minute incubation, absorbance against a blank at 540 nm was measured.

Quantifying bacterial colonization of discs involves an extra step. The disc covered with bacteria is placed into 2ml of distilled water and the bacteria are removed by a 5 minute ultrasound bath. Again, 4 ml of Biuret agent are added and absorbance is measured following a 10 s shaking and 30 minute incubation period.

3.3.2. Calibration curve

The protein content is in both cases derived from the callibration curve using bovine serum albumin as a standard. The exact protein mass of the adhering bacteria can therefore be easily calculated.
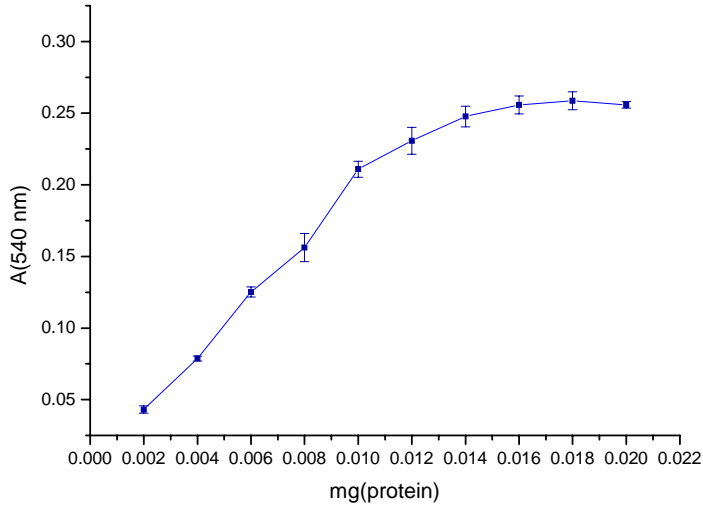

Fig.33. Calibration curve

3.4. DENTAL MATERIALS USED

3.4.1. Adoro commercial dental composite material

Adoro from Ivoclar Vivadent is a newly developed, microfilled, light/heat-curing veneering composite for full coverage veneers and partial veneers.

Fig. 34. Adoro final product on display at Ivoclar

3.4.2. Ceramics

Ceramic discs serve as a referential material, kindly provided by the University of Technology in Brno.

Fig. 35. A final ceramic dental product.

3.4.3. An UDMA based resin

A resin used as a matrix in composites was kindly provided by ADM, a.s. (Brno), it is used in their product Dentapreg, and served as a basis for producing antibacterial discs .

3.4.4. Biocide additive modified resins

Silver modified discs were created by manually mixing antibacterial additives into an UDMA-based resin matrix at different concentrations.

3.5. DENTAL MATERIAL DISC PRODUCTION

The material was placed in a metal mold lubricated in beeswax dissolved in ethanol between a layer of see-through foil and metal. It was cured in the Vectris curing machine at the VUT Brno for 20 minutes at 60°C (to improve the degree of conversion) and 460 nm. After curing, the discs were removed from the mold, sterilized by heat in a pressure cooker or under UV light for 15 minutes and used for further measurements.

1) Biocide additive 2, a very fine black powder, was thoroughly manually mixed into an UDMA-based commercially exploited resin.

2) Biocide additive 1, a fine white powder, was thoroughly mixed into the resin in 4 different concentrations. This material was studied this closely due to its good aesthetics and promising antibacterial properties, as depicted in the following. The higher the biocide additive 1 concentration, the more opaque the disc was.

3) A composite with no antibacterial agents added, serves partially as a reference of the current dental composite market.

4. RESULTS, DISCUSSION

In experiments involving cells, care has to be exercised to maintain exactly the stipulated experiment procedures in order to ensure that the results are reproducible. This includes

growth conditions and medium composition, and also the actual manipulation with the bacteria. The measurements were repeated in larger sets in several independent experiments, nevertheless, the errors in these biological experiments are quite high due to the „randomness" of nature that cannot be influenced to a greater degree in any manner.

4.1. SUBSTRATE STRUCTURE

In the following, there are several views of the investigated discs´ surfaces presented.
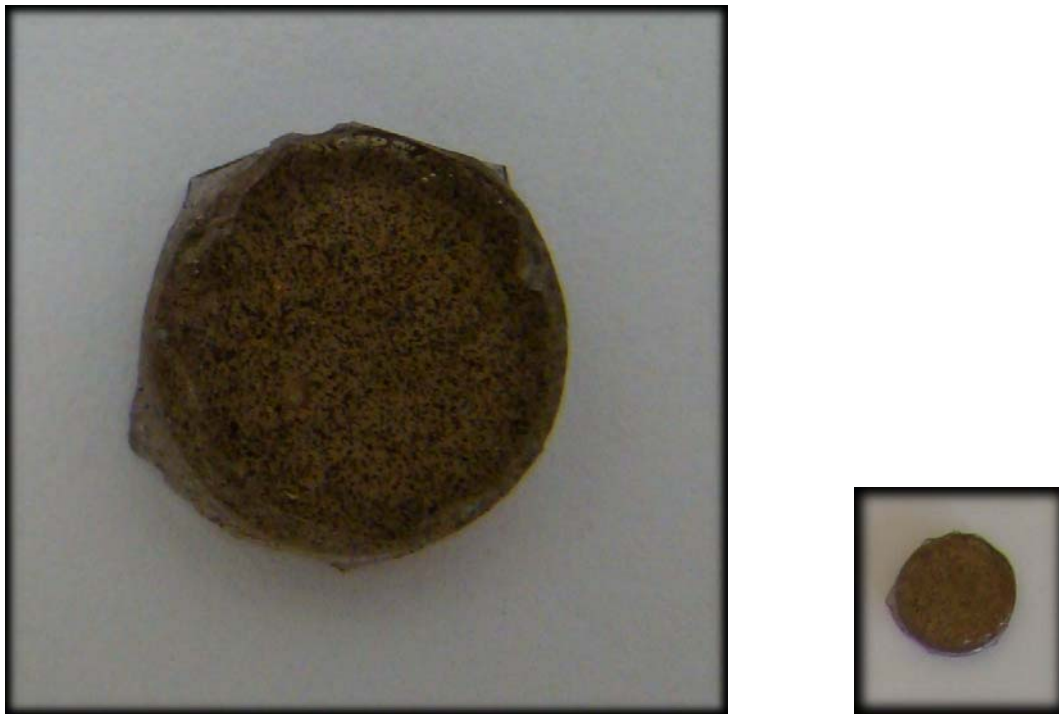
4.1.1. Biocide additive 2 modified disc

Fig. 36. . Biocide additive 2 incorporated into an UDMA-based resin.
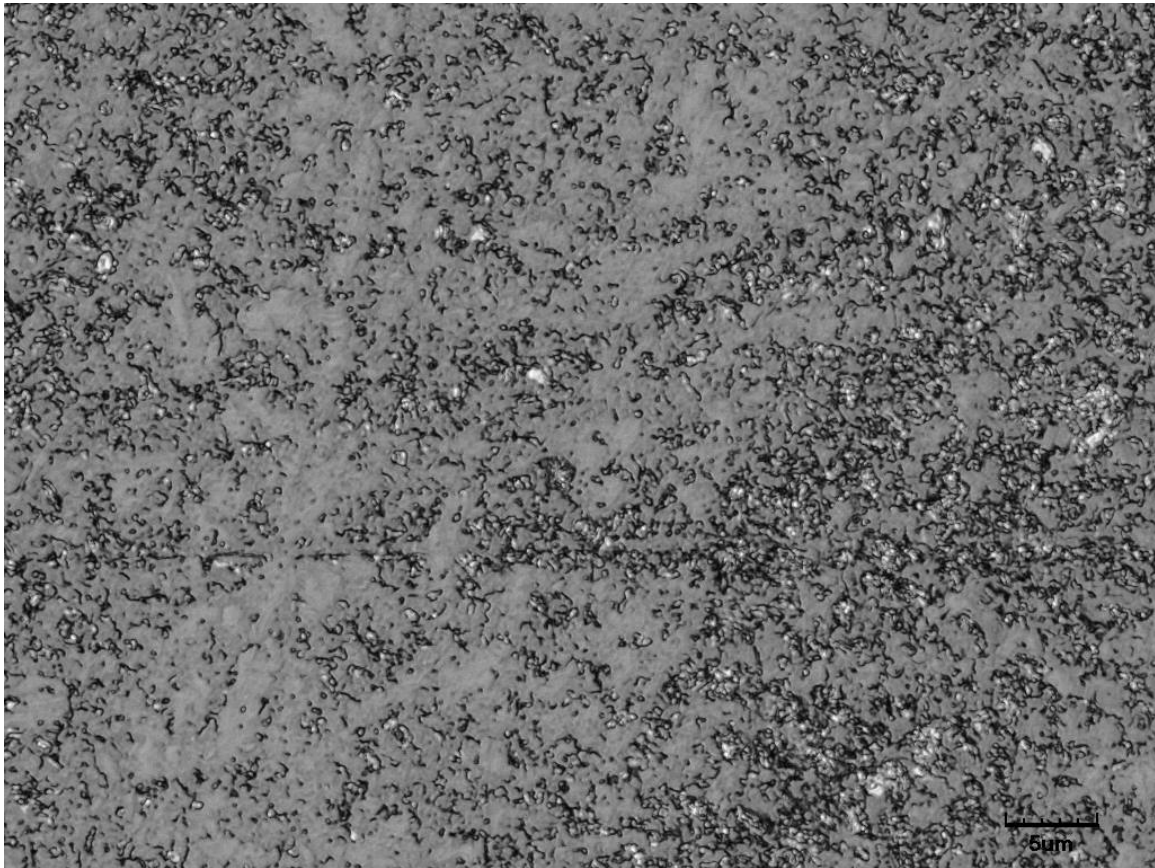
Fig. 37. The biocide additive 2 modified disc surface under a confocal microscope. There are frequent, but small irregularities visible on the surface.

4.1.2. Biocide additive 1 modified discs

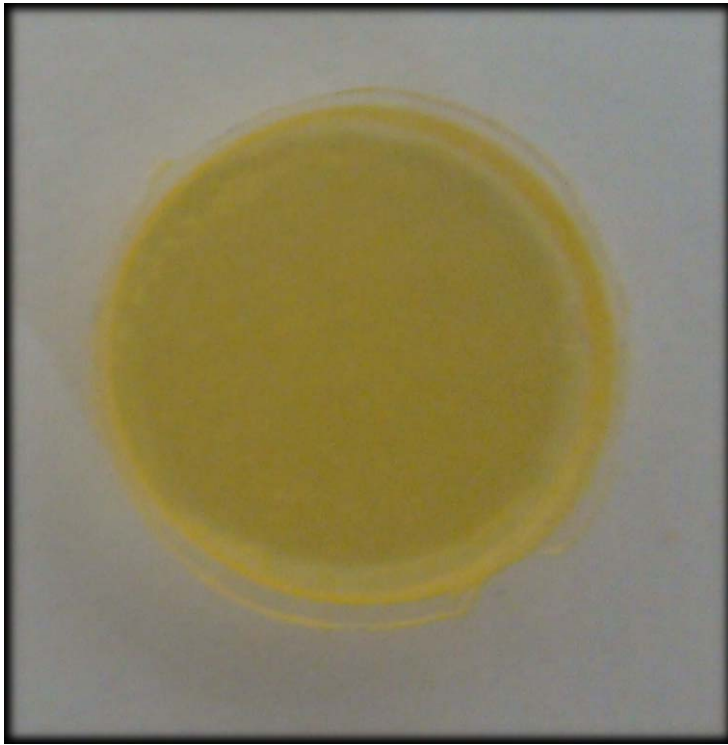*Fig. 38. A disc modified by biocide additive 1, concentration 1*

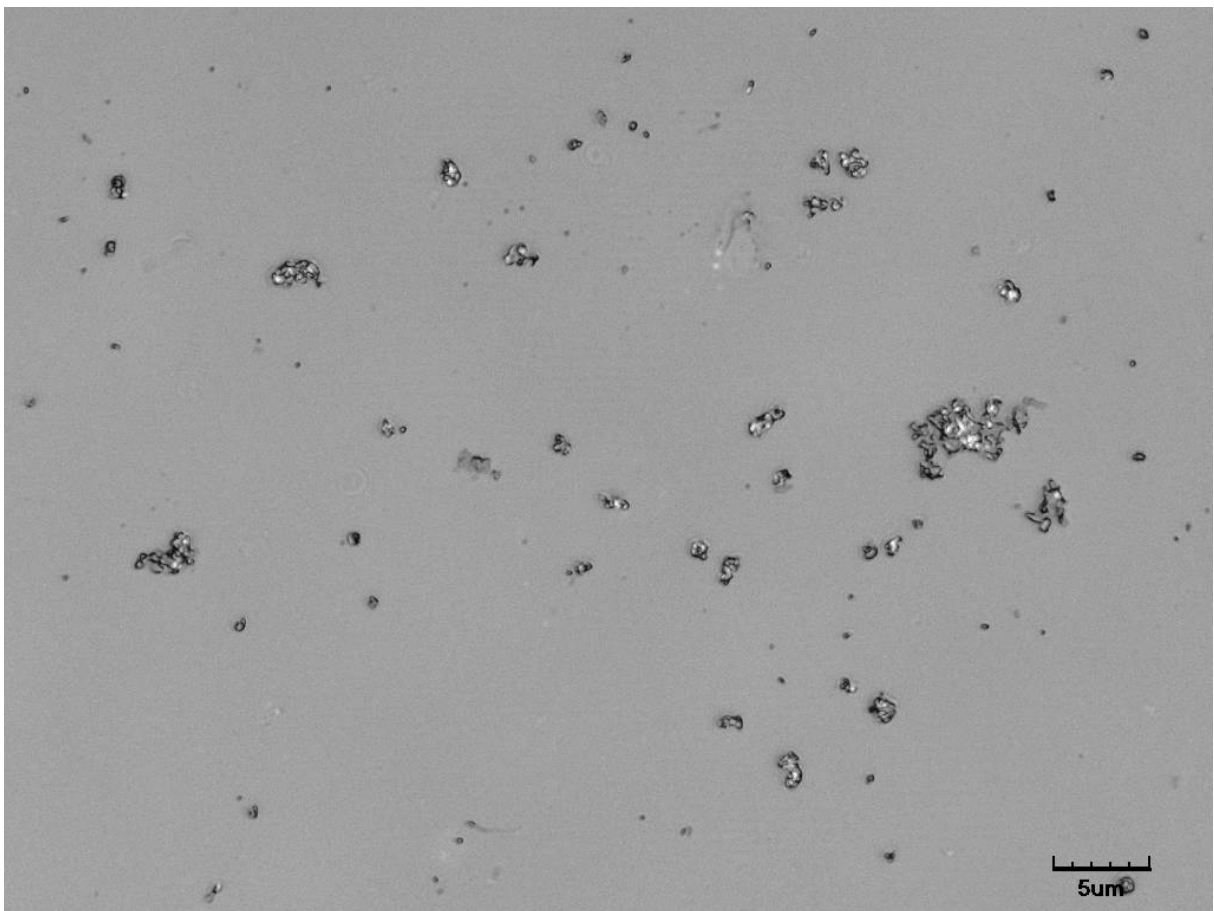*Fig. 39. A disc modified by biocide additive 1, concentration 2*

Fig. 40.The surface of a modified disc, with clearly visible clusters of biocide additive 1 incorporated into the smooth resin.

Fig. 41. A disc modified by biocide additive 1, concentration 3

Fig. 42 A disc modified by biocide additive 1, concentration 4

Fig. 43. UDMA-based resin with no additives

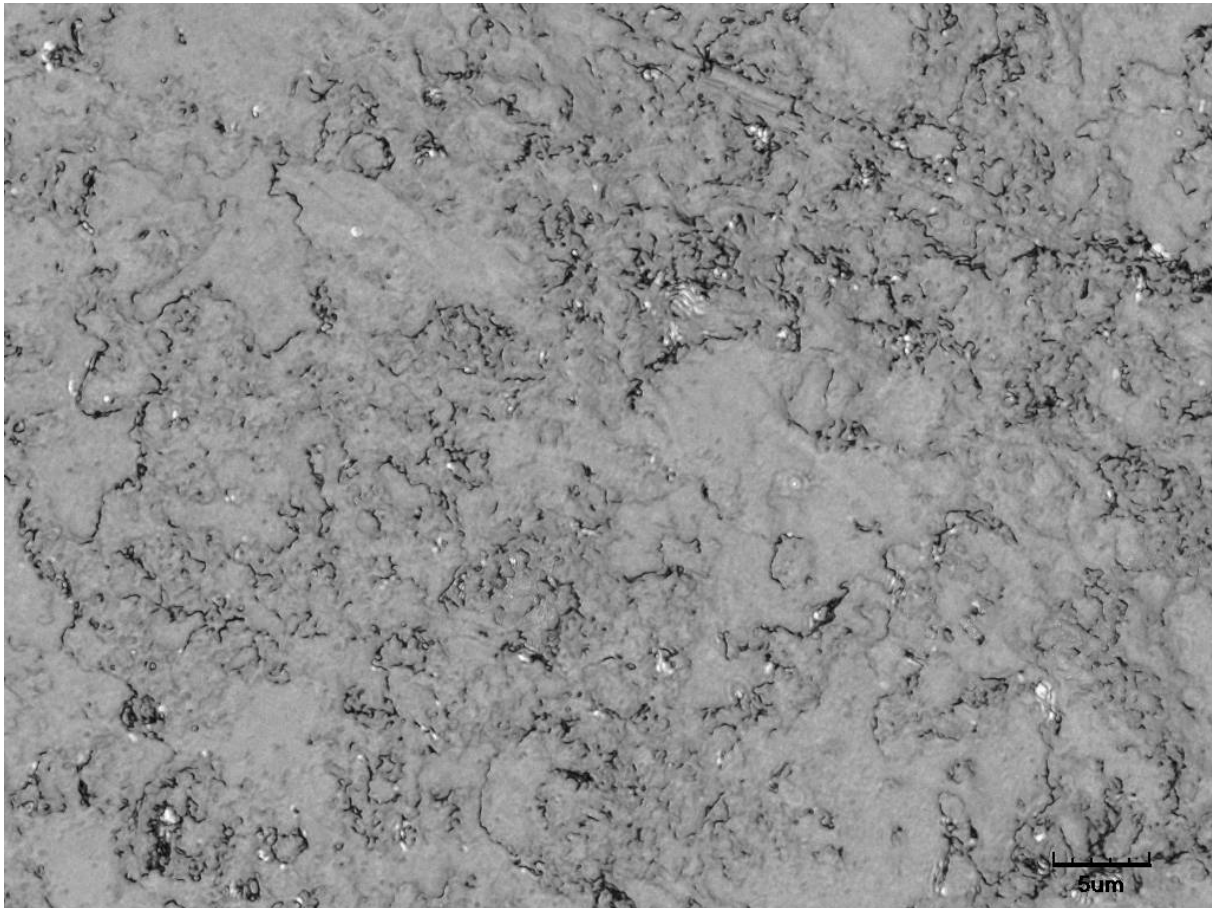### 4.1.3. Commercial composite Adoro

Fig. 44. Adoro disc

Fig. 45. Adoro disc surface under confocal microscope. The irregularities are clearly distinguishable.

4.2. EFFECT OF SURFACE ROUGHNESS ON BACTERIAL ADHERENCE

In addition to the chemical composition of the material surface, its roughness has to be considered a key factor in affecting the bacteria adherence. In this thesis, the main purpose is stating, whether the differences in surface roughness between the various materials were sufficient enough to influence the results. The following material profile results were obtained using a confocal microscope at the University of Technology in Brno. The height of the peaks/crevices was measured in μm in several randomly chosen plains on the disc.

4.2.1. Commercial dental composite (Adoro)

The commercial composite Adoro had an average peak/valley distance of 0.841 μm. This is a little less than the average diameter of *S.mutans* bacteria.
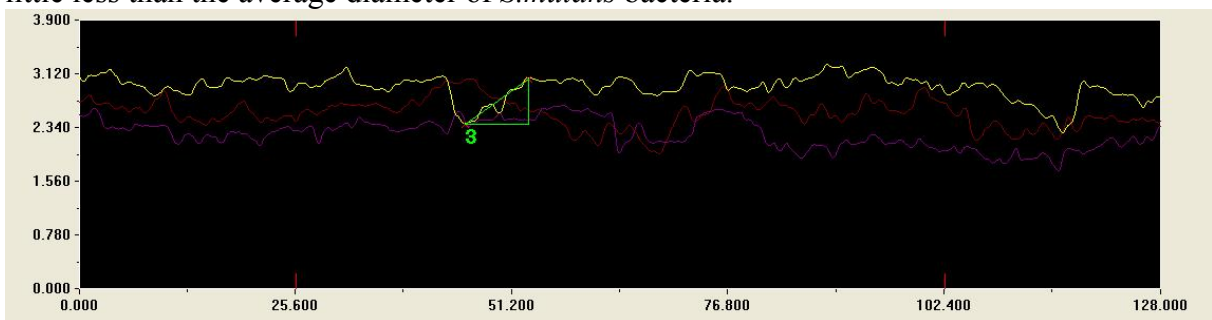

Fig. 50. The profile image of an Adoro disc.

It is probable, that such a surface roughness enables the microorganisms to colonize it more easily compared to a smooth surface.

4.2.2. Roughness of the resin discs

The average peak/valley distance was only 0.04 µm which was significantly less compared to the commercial dental composite. It is reasonable to assume that the attachment to this surface is quite difficult for microorganisms, which seems to be confirmed by the results shown in Section 6.6.
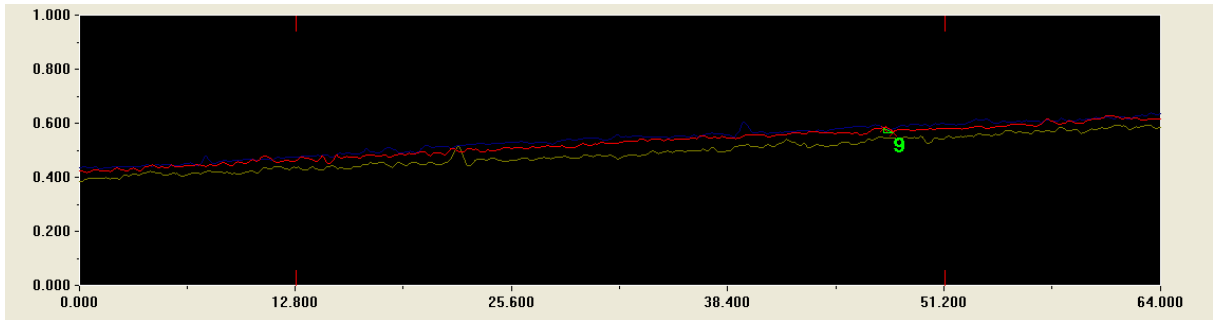

Fig. 51. The profile image of an UDMA-based resin disc.

4.2.3. Biocide additive 1 modified resin discs

The peak/valley distance was around 0.3 µm for all biocide additive 1 concentrations. This was similar to values obtained for the neat resin discs.
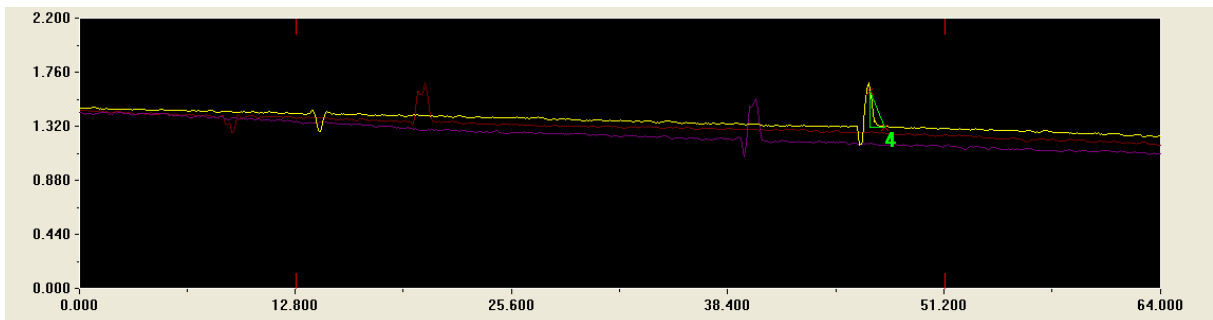

Fig. 52. The profile image of an biocide additive 1, concentration 1 disc.
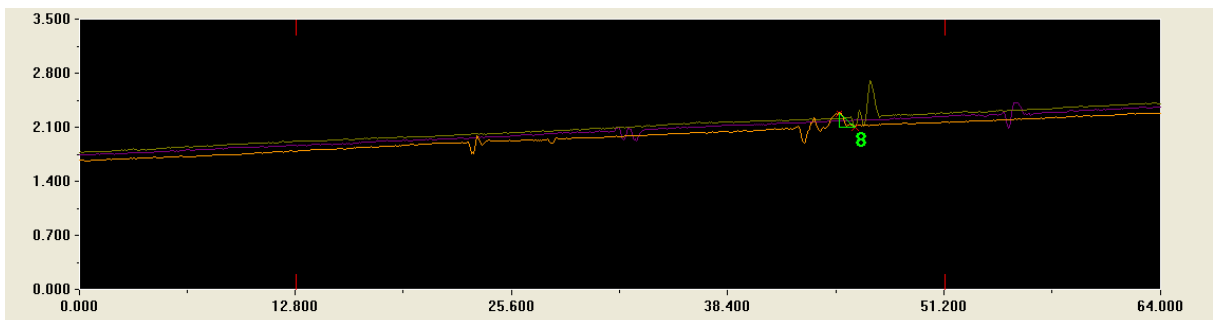

Fig. 53. The profile image of an biocide additive 1, concentration 2 disc.
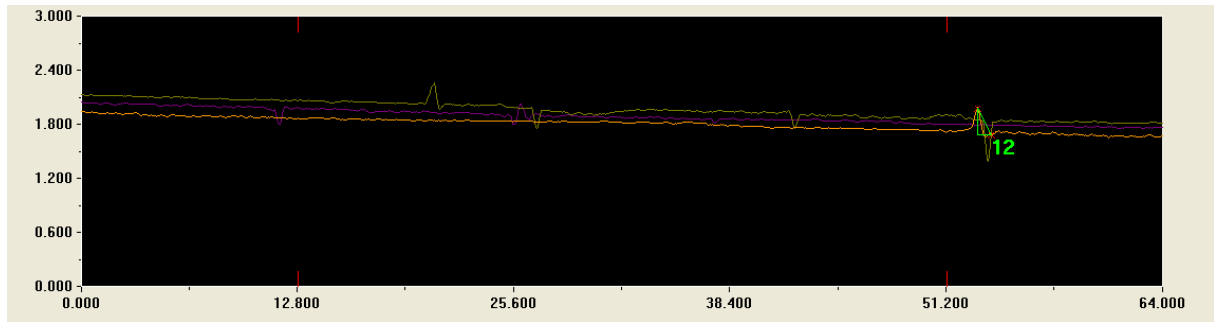
Fig. 54. The profile image of an biocide additive 1, concentration 3 disc.
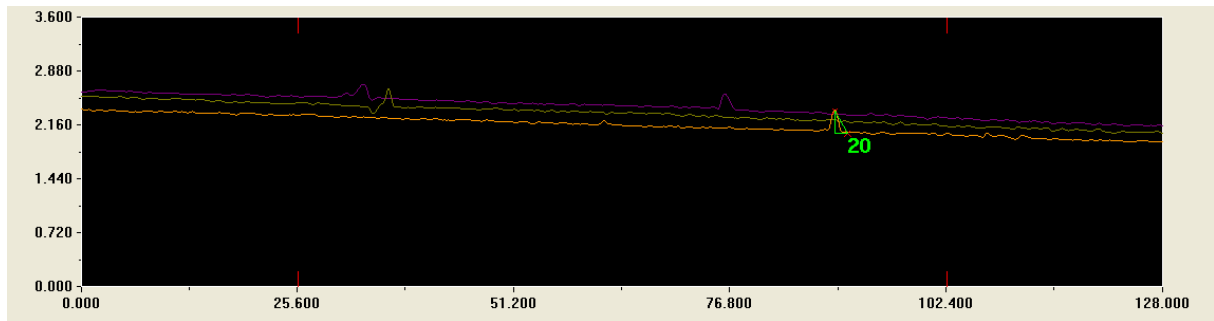

Fig. 55. The profile image of an an biocide additive 1, concentration 4 disc.

From the roughness profiles it seems that the dispersed silver powder forms the peaks on the surface, hence, the surface roughness is given by the particle size of the biocide additive 1 agent.

4.2.4. Biocide additive 2 modified resin discs

The profile image of the Biocide additive 2 + resin mix is a bit different. 0.421 µm is the height average, here suggesting increased roughness when compared to the biocide additive 1-modified materials above.
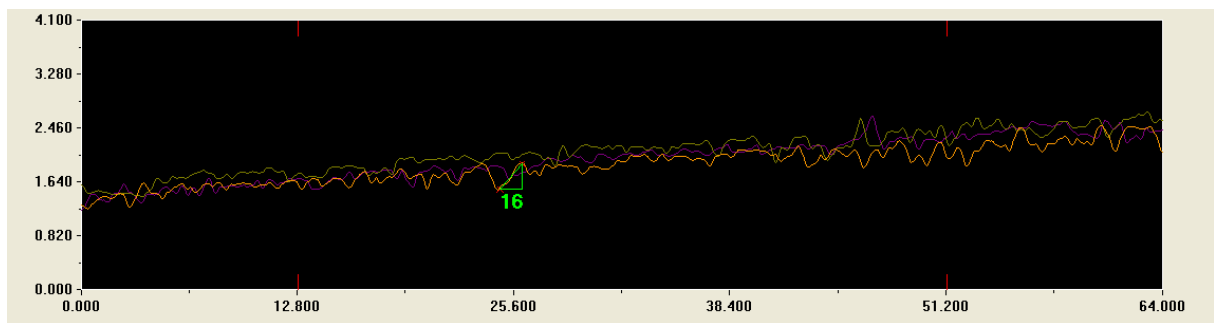

Fig. 56. The profile image of a disc modified by biocide additive 2.

4.3. S. MUTANS GROWTH CURVE

Assessing the kinetics of bacterial growth on a reference material is important prior to carrying out further measurements to select the optimum growth period, preferably at the peak of the growth curve that yields maximum yet consistent absorbance values at 540 nm. One must also differentiate between the growth curve for bacteria in the BHI broth medium and those colonizing the solid surface of the discs, which involves more complicated physico-chemical interactions.

The bacterial growth curves are plotted in Figs. 46 a) and b) for both cases. It was found that the optimum growth period is 18 hours in both cases. Hence, the 18 hours growth period was used in all measurements.
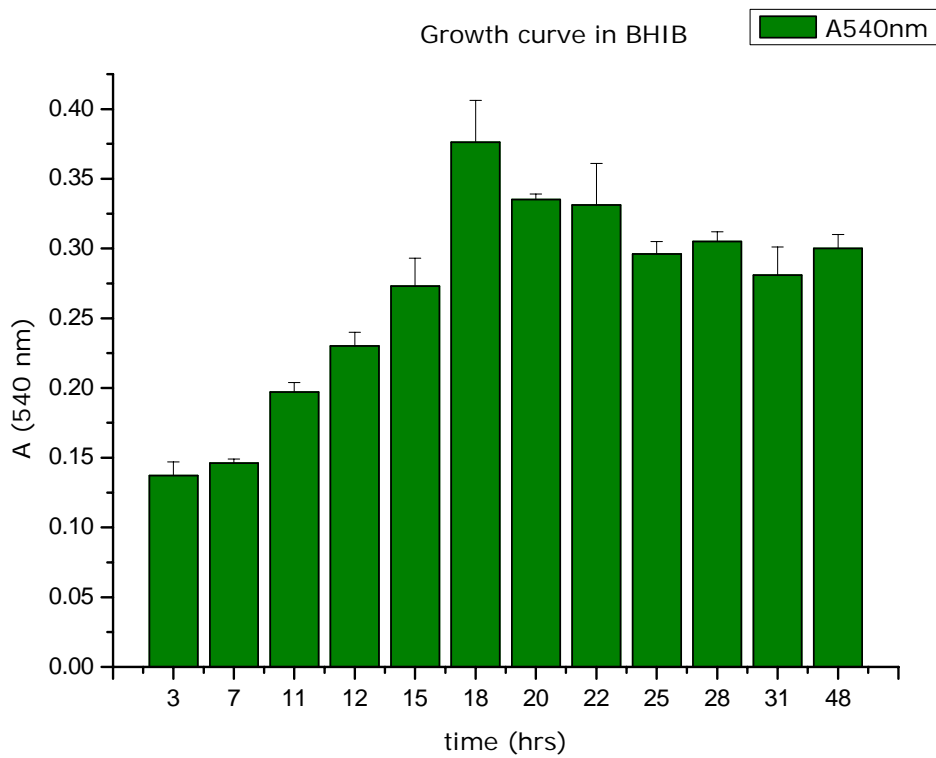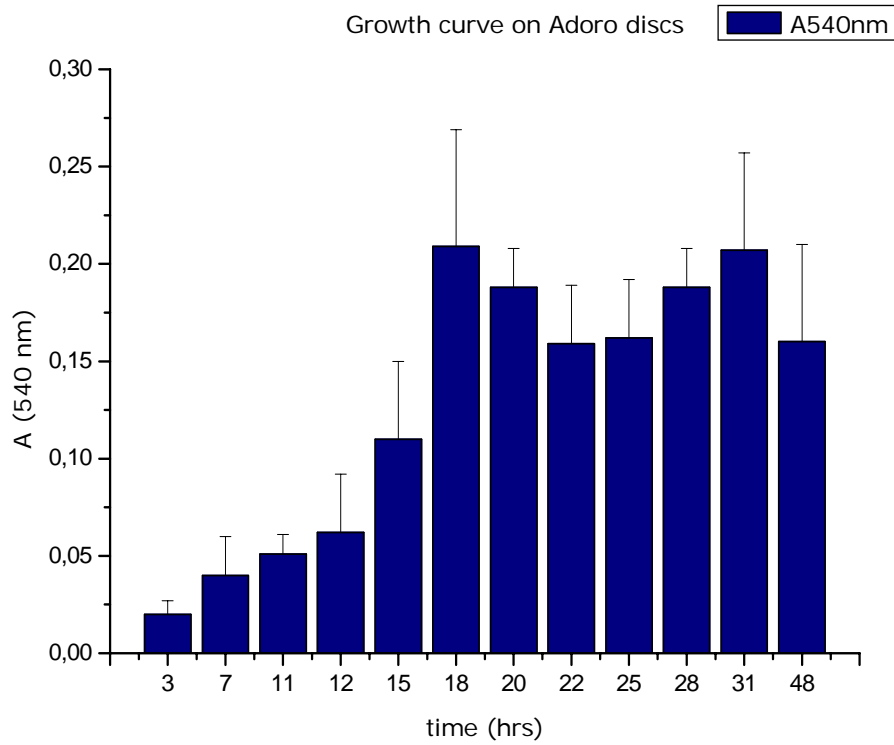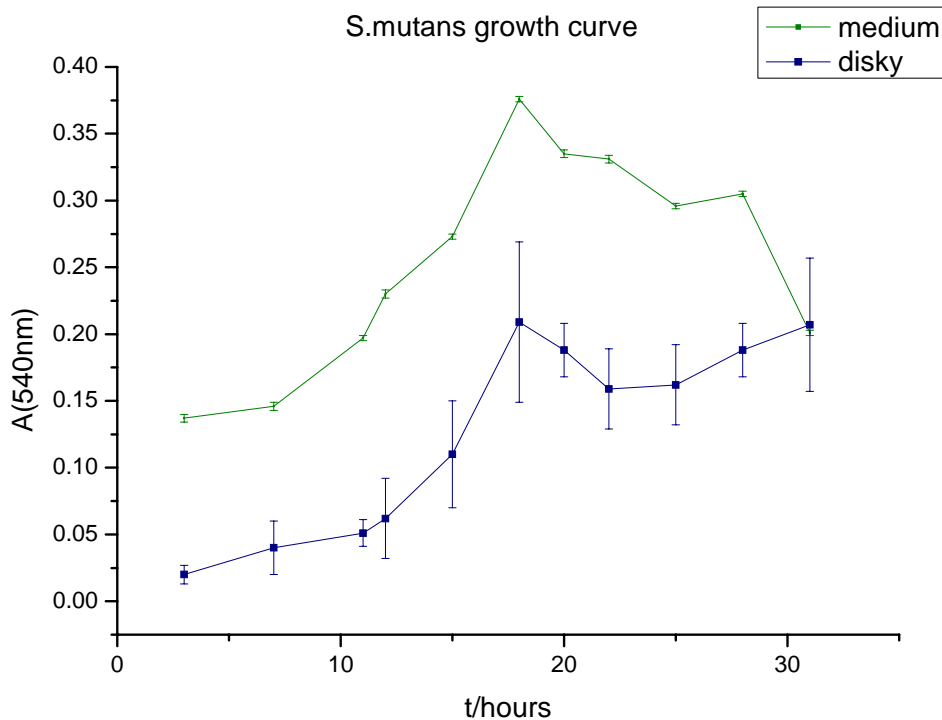
Fig. 46.a) Growth curve on Adoro discs; b) planktonic growth in BHIB; c) comparison of both

The growth curves in both medium and on the discs exhibit typical stages of bacteria growth including primary phase of exponential growth where the bacteria have more or less unlimited nutrient access, followed by a stationary phase indicating that the colony had used up most and eventually all present nutrients, no further significant growth is observed. The final phase of cell death is not depicted due to the fact that the Biuret method used quantifies all proteins present on the disc or in the solution, it does not differentiate between live and dead matter.

The end of the exponential growth phase represents the maximum possible amount of proliferating bacteria, in both cases this is after an 18 hour growth period. The growth curves are quite similar, but vertically shifted, varying more with increasing time. The 18 hours is clearly the optimum for both growth on the discs and in the BHIB medium. All measurements were therefore carried out after an 18 hour incubation.

Standard deviation of the data measured in BHIB media were much smaller compared to the growth on the discs. This was associated with the extra manipulation steps used in the case of bacteria growth on the discs resulting in an enhanced scatter in the measured amount of adhering bacteria on the discs. The discs used for this experiment were standard commercial composite discs (Adoro, Ivoclar AG, Liechtenstein), which are not expected to have antibacterial properties.

Assessing bacterial adherence in the liquid medium is a straightforward procedure, where the only manual manipulation step is extracting 200μl via analytical pipette from the initial test tubes. This is why the errors are quite insignificant in comparison to those in disc adherence measurements. There, several steps where losses/errors can accumulate exist. These include:

1) initial disc quality - The disc is produced in a mold and cured in a foil wrapping. Removal from the mold and wrapping causes microscopic holes and irregularities on the surfaces, providing crevices that are easier for bacteria to colonize than a smooth surface. Surface roughness is a key factor influencing bacterial adherence.

2) extraction from test tube - Prior to the actual measurement, several steps must be taken. The first one is extracting the disc from the test tube containing the liquid medium and bacteria by forceps. This must be done carefully in order not to manually scrape off adhering bacteria. Unfortunately, some always are removed due to the nature of this procedure. The researchers must make sure, that the forceps are always used in a similar manner to ensure the amount of scraped off bacteria does not vary much.

3) rinsing - Another step before the actual measurement is rinsing the extracted disc in distilled water to ensure that bacteria are truly adhering to the disc and the measured value is not just a result of free bacteria sedimenting to the bottom of the test tube onto the disc. The disc gripped in the forceps must be rinsed carefully in order not to wash off all the bacteria. Since there is no protein pellicle on the surface of the polymer, the connection between the bacteria and surface is not as firm as in vivo.

4) ultrasound bath - the time was optimized to 5 minutes in order to ensure that the bacteria are thoroughly removed, yet the surface of the material is not damaged.

5) Biuret method - the measurement is carried out in the following manner: Biuret´s agent is added into the test tube containing disc and distilled water (now containing proteins after ultrasound bath), thoroughly mixed for 10 s and absorbance is measured after 30 minutes. The disadvantage of the method lies in the fact that this must be done precisely, because the absorbance has a slight tendency to fluctuate when the time is increased.

Since all experiments are being carried out in vitro, there is of course the problem of predicting what will happen in vivo. For one, the periods in which bacterial growth is studied in the in vitro experiments are most commonly less than 72 hours (3 days). The life expectancy of dental restoratives is much higher than this - approx. five years and recent research is aiming to increase this time. What will the effects of permanent immersion in saliva, the constant change of its composition, repeated brushing of teeth and mastication on the activity of the bactericidal dental restorative be? This could be partially simulated by carrying out the experiments in a PBS (phosphate buffered saline) environment and mechanical toothbrushing simulation.

The mode in which whole saliva proteins are adsorbed onto the surfaces of restorative materials depends on the type of material. This initial conditioning salivary coat plays an important role in bacterial adsorption to restorative surfaces. Biofilm formation on various restorative materials differs, indicating that the initial biofilm accumulation on the material involves several specific processes. The fact that there is such variation in biofilm coats in the oral cavity has a significant impact on oral ecology and the progression of dental diseases. [19] This means that any potentially bacteriocide agent incorporated into the materials must be nonselective. With regards to the mechanisms suggested in various papers, silver could meet this requirement.

Also the dental material´s storage in the oral environment must be of taken into account. When studied without a surface pellicle, water storage significantly increases adhesion of *S.mutans* to polymers. The highest numbers of adhered bacteria were observed on saliva-coated materials. The contact angle measurements conducted with a denture base

polymer showed a decrease in water contact angles, i.e. an increase in surface wettability after water storage. Low contact angles are an indication of high free surface energy whereas high contact angles indicate low surface free energy. [65]

4.4.   CERAMICS - REFERENCE MATERIAL

Ceramics was chosen as a referential material due to the fact that its properties bear a close resemblance to the human tooth. The discs were manufactured in different sizes, so the results were recalculated in order to correspond with the height and diameter of the polymer discs used in the measurements. The resulting absorbance value for ceramics was 0.077. This value is depicted as a reference in the disc colonization results.
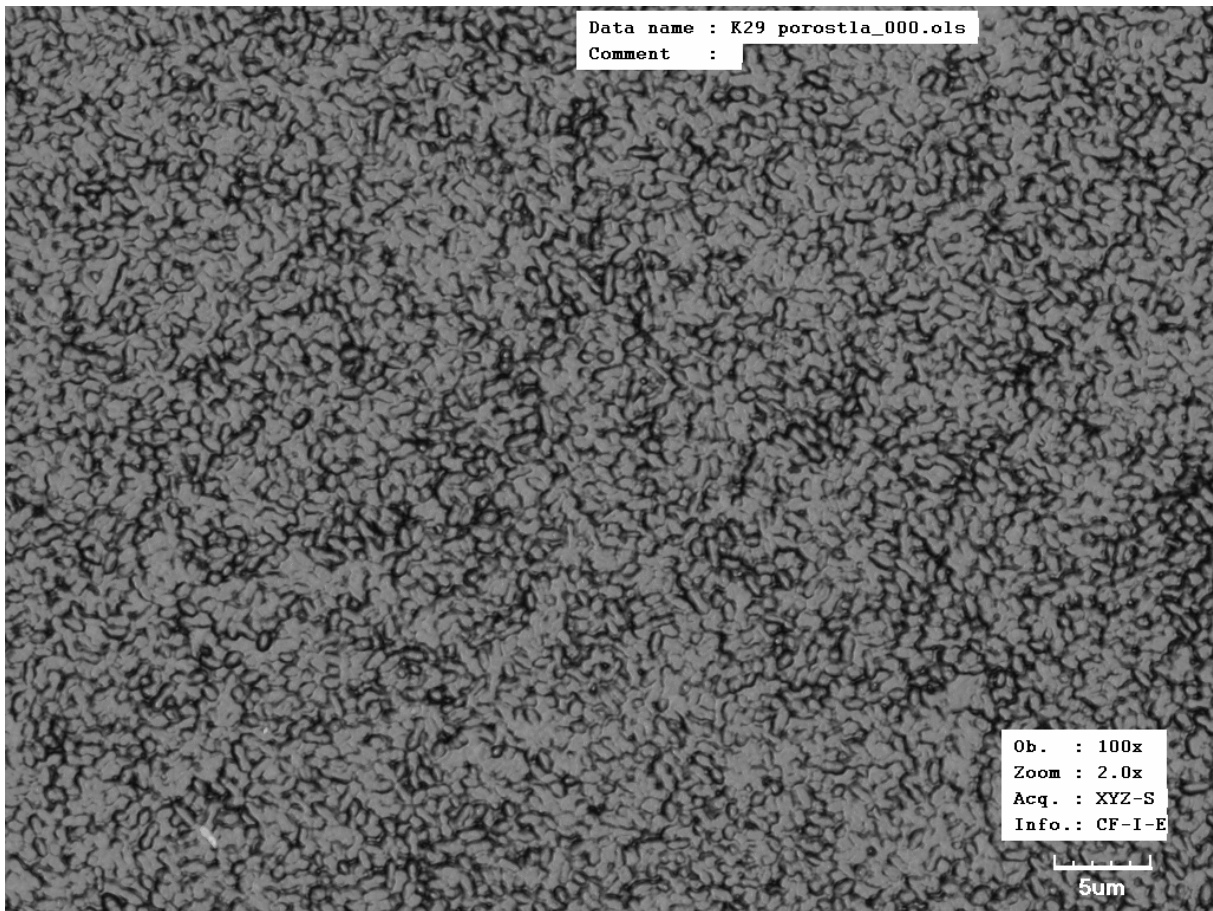

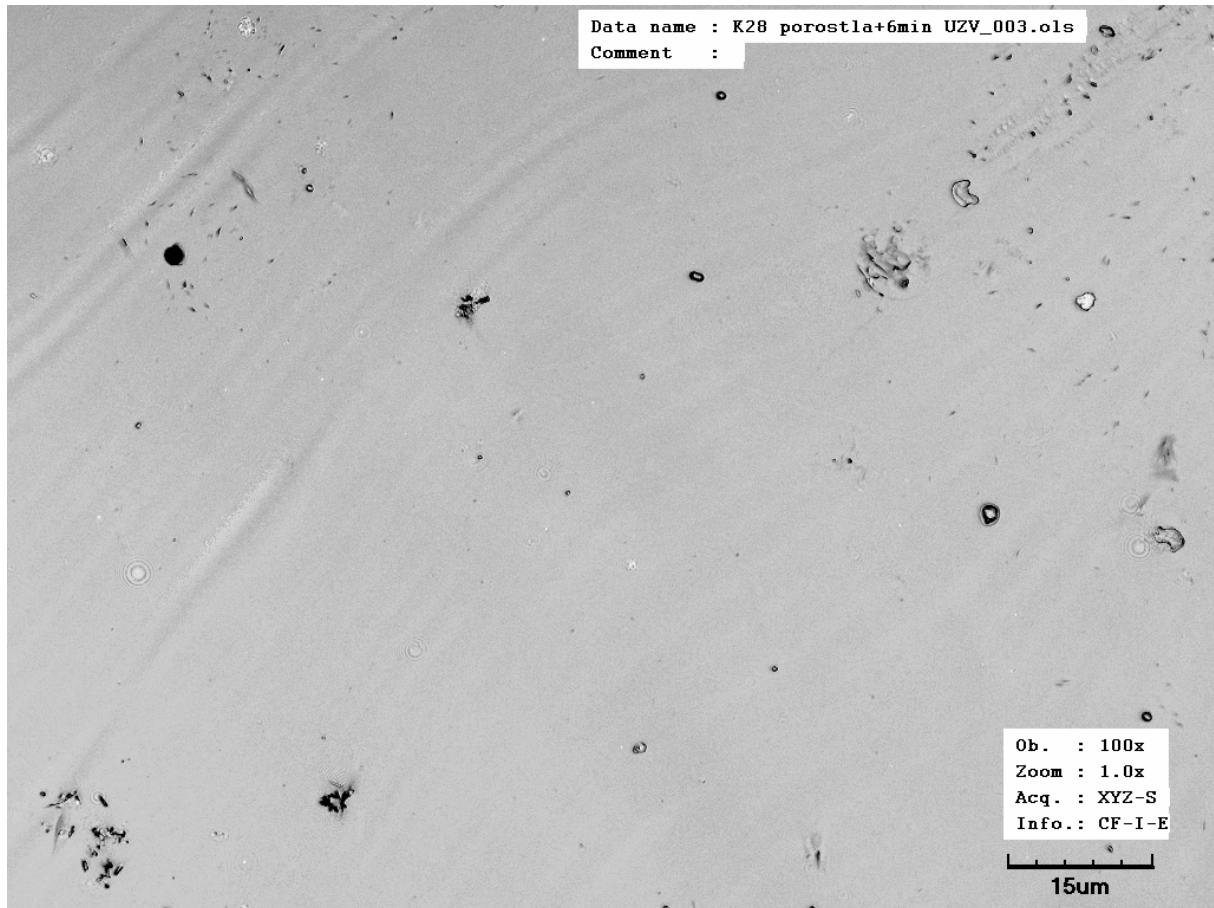*Fig. 47. S.mutans colony on the surface of a ceramic disc.*

Fig. 48. S.mutans has been completely removed after 5 minutes in an ultrasound bath.

4.5. EFFECTIVENESS OF BACTERIOCIDE ADDITIVES INVESTIGATED

First, the effectiveness of the bacteriocide additives in reducing bacteria growth has been determined. In these tests, 0.1g of the various antibacterial materials, such as biocide additive 1, biocide additive 2 and 3 were mixed in test tubes containing 5 ml of BHIB, into which a standard amount of *S.mutans* was inoculated. With respect to the results of the optimum time of growth, absorbance at 540 nm was checked by the Biuret method, where each specimen was first calibrated to 0 with a blank (containing material, growth medium, Biuret agent, but no bacteria). The results are shown in the following Fig.49:
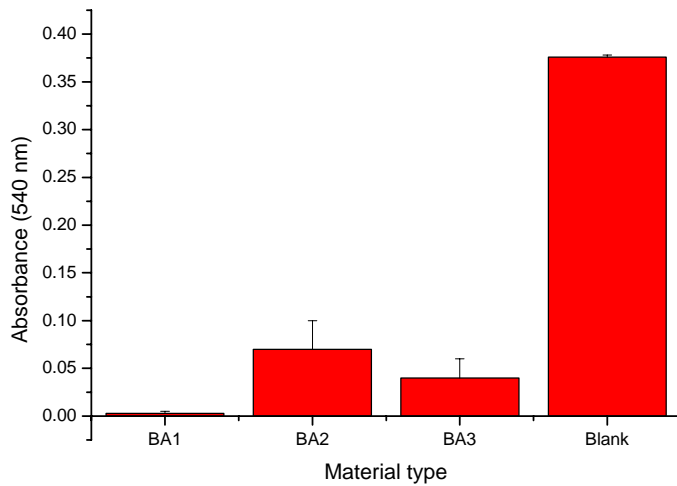
Fig. 49. Comparison bacteriocide additive effects

In comparison to the blank specimen without any antibacterial material, there was a pronounced reduction of bacterial growth apparent as a result of the bacteriocide additive. Considering the amount of bacteria in the blank a base (100%), the biocide additive 1 resulted in 98% decrease of bacteria growth, the biocide additive 2 and 3 addition, respectively, reduced the growth of bacteria by 87% and 91%, respectively. Therefore, the experiment proceeded to the next step, where the three biocide additives have been incorporated into a commercially used urethane-dimethacrylate resin currently used in the production of commercial dental composites.

4.6. BACTERIAL GROWTH ON BACTERIOCIDE MODIFIED RESIN DISCS

Two sets of measurements were carried out. Bacterial growth on a series of 1) unpolished discs sterilized by heat, 2) unpolished discs sterilized by UV light was determined. Parallel to these experiments, the planktonic growth was measured to determine the influence on free unadhered bacteria.
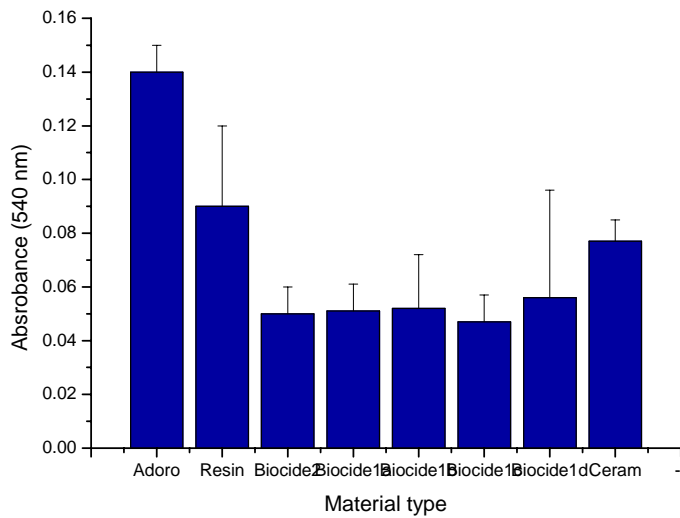
4.6.1. Unpolished UV sterilized discs

Fig. 57. Absorbance corresponding to bacterial colonization on UV sterilized polymer discs.

The results presented above (Fig.57) confirmed the hypothesis that all silver-containing antibacterial additives inhibit the growth and proliferation of bacteria on the cured resin discs in comparison with reference materials without these additives statistically isignificantly. The concentration of biocide additive 1 in the resin did not seem to have a significant influence on tha amount of adhering bacteria. No statistically significant difference has been found for the bacterial growth on resin discs containing chemically immobilized biocide additive 2 and freely dispersed biocide additive 1. The bacteria growth was reduced in average by 63% adding biocide additives.
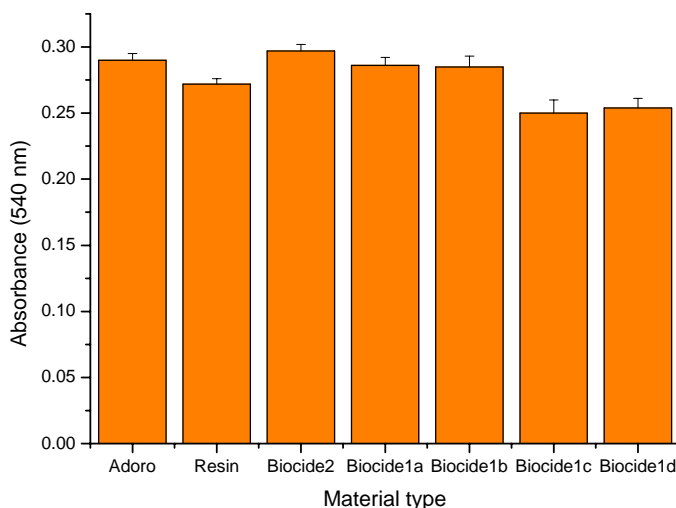

Fig. 58. Absorbance corresponding to bacterial growth in test tubes containing BHIB and antibacterial discs.

No statistically significant reduction in planktonic bacteria growth has been observed (Fig.58) indicating that the type of material does not influence growth of free bacteria. The fact that freely floating bacteria away from the disc surface were not inhibited leads to the

conclusion that silver compounds do not leach out of the material. This is an important result, because in a potential commercial application, the leaching of antibacterial compounds leading to loss of favorable mechanical properties would mean a significant drawback.

4.6.2. Unpolished heat sterilized discs
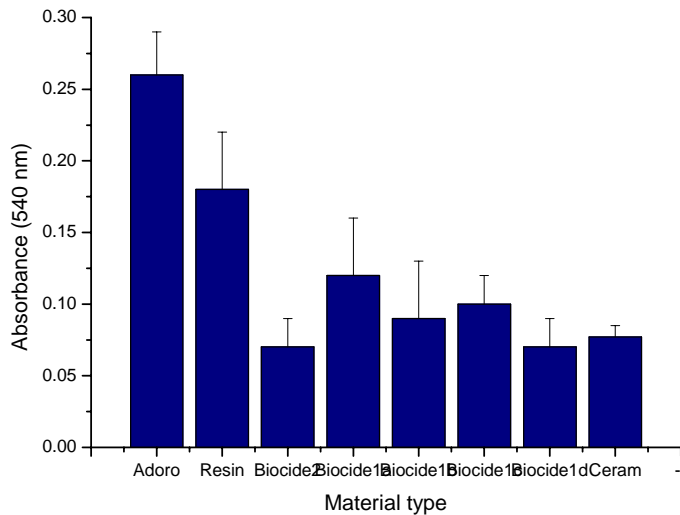
Fig. 59. Bacterial colonization on heat sterilized polymer discs.

Results on bacterial growth on UV sterilized discs show significant difference compared to the heat sterilized discs of the same composition. The total amount of bacteria was greater on the heat sterilized discs for all the materials investigated. At the same time, the bacterial growth was significantly inhibited on all uv-sterilized silver-containing materials similarly to the heat sterilized materials. The greatest reduction has been obtained for the . Biocide additive 2 modified resin where the bacteria growth has been reduced by 78% compared to un-modified commercial dental composite and more than 60% compared to un-modified resin.

Fig. 60. Amount of free bacteria in the liquid medium.

Similarly to the heat sterilized discs, planktonic growth was independent of type of the disc material suggesting that no silver leaching-out has occured.
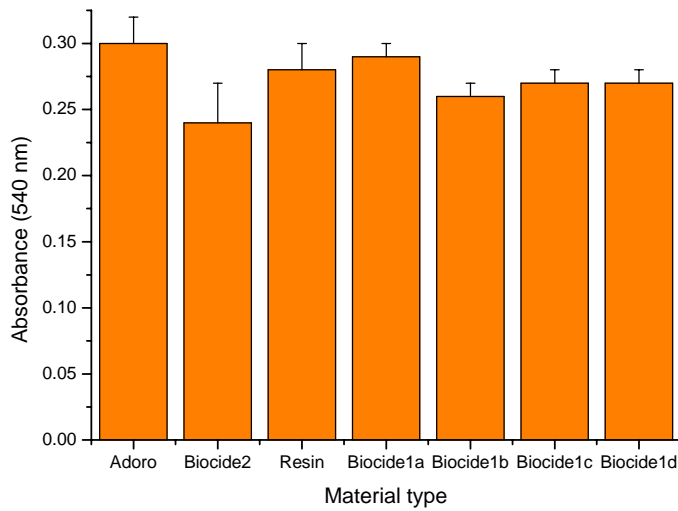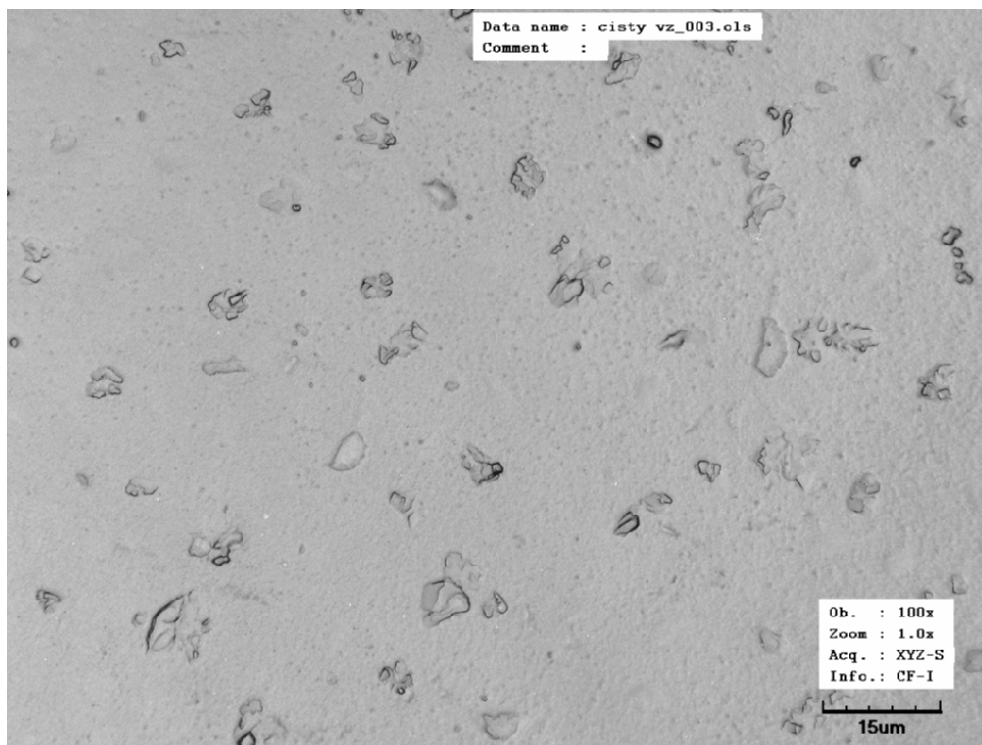

Fig. 61. The holes created by the removal of the foil cover are clearly seen on the surface of an Adoro disc.

5. CONCLUSION

In this Thesis, the effect of silver containing compounds on the adherence and growth of *Streptoccocus mutans* onto the surface of resin based dental materials has been investigated. Dental ceramics and a commercial dental filling composite have been used as reference materials. Bacteria were cultivated using standard conditions and their growth curves in the nutrition broth were established and based on these results, a growth period of 18 hours was selected for the following experiments. Series of urethane-di-methacrylate resins modified with commercial biocide additive 1 and developmental compound biocide additive 2 has been prepared.

Surface roughness of the substrates was measured using a confocal laser scanning microscope suggesting a small but significant effect of the silver additive on the degree of surface roughness. The Biuret method has been used to quantify the amount of bacteria grown on the surface of cured discs made of the various dental materials. Addition of silver compounds to the dental resin resulted in a decrease of bacterial growth by 65% to 97% in comparison to the reference commercial material in the case of both heat and UV sterilized substrates. Bacteriocide modified resin exhibited smaller bacterial growth than dental ceramics which is considered the state-of-the-art today. The incorporation of antibacterial additives such as biocide additive 1 into a commercial resin based dental material could lead to materials retaining favorable properties while reducing the organic plaque deposition and, thus, improving oral health.

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