UNIVERSITÀ DEGLI STUDI DI NAPOLI FEDERICO II

DOTTORATO DI RICERCA IN

MEDICINA CLINICA E SPERIMENTALE

CURRICULUM IN SCIENZE ODONTOSTOMATOLOGICHE

XXXI Ciclo
(Anni 2015-2018)

Coordinatore: Prof. Francesco Beguinot

TESI DI DOTTORATO

ANTIMICROBIAL EFFECT OF NEW RESTORATIVE DENTAL MATERIAL INCORPORATING SILVER NANOPARTICLES

RELATORI

Chiar.mo
Prof. Sandro Rengo
Chiar.mo
Prof. Gianfranco Peluso

CANDIDATO

Dott. Raffaele Conte

UNIVERSITY OF NAPLES FEDERICO II

PH.D. PROGRAM IN

CLINICAL AND EXPERIMENTAL MEDICINE

CURRICULUM IN ODONTOSTOMATOLOGICAL SCIENCES

XXXI Cycle
(Years 2015-2018)

Chairman: Prof. Francesco Beguinot

PH.D. THESIS

ANTIMICROBIAL EFFECT OF NEW RESTORATIVE DENTAL MATERIAL INCORPORATING SILVER NANOPARTICLES

TUTORS

Prof. Sandro Rengo
Prof. Gianfranco Peluso

PH.D. STUDENT

Dr. Raffaele Conte

Scopo di ogni attività dell'intelletto è ridurre

il mistero a qualcosa di comprensibile

(Albert Einstein)

INDEX

SUMMARY

ABSTRACT

Secondary or recurrent caries are dental lesions originated at the margins of an existing restoration, and are considered the most common reason for restoration failure. [1] Usually, these lesions are histologically similar to the primary caries and can be difficult to detect unless somewhat advanced, resulting in a considerable loss of tooth structure. Over the past decades, resin-based dental materials have been used in restorative dentistry for their excellent esthetics and improved mechanical performance. [2] However, they represent potential sources of carbon and energy for microorganisms including oral bacteria and fungi residual in the dental cavity. In addition, cariogenic bacteria can infiltrate the restoration-tooth margins compromise the restoration's longevity. [3] Because caries at the restoration margins is a main reason for restoration failures, it would be highly desirable for the composite and bonding agent to possess antibacterial capabilities.

Novel antibacterial dental materials were developed by introducing quaternary ammonium monomers, including 12-methacryloyloxydodecylpyridinium bromide (MDPB), dimethylaminohexadecyl methacrylate (DMAHDM), and dimethylaminododecyl methacrylate (DMADDM). [4] [5] [6] [7] [8] These monomers can form covalent bonds with the polymer matrix and be immobilized in the resin-based materials, representing a non-released, contact-killing agent. [4] Several other antimicrobial formulations were also developed, including a methacryloxylethylcetyl dimethyl ammonium chloride (DMAE-CB) containing adhesive, quaternary ammonium

polyethylenimine (PEI) nanoparticles for antimicrobial dental composites, antibacterial glass ionomer cements, and antibacterial nanocomposites and bonding agents incorporating a quaternary ammonium dimethacrylate (QADM). [9] [10] [11]

Quaternary ammonium acrylate (QAM) resins possess positively-charged quaternary amine N^+ which can interact with the negatively-charged membrane of bacteria, leading to membrane disruption and cytoplasm leakage. [10] It is postulated that long-chained quaternary ammonium compounds can be especially effective by inserting into the bacterial membrane, resulting in physical disruption and bacteria death. [12] [13] [14]

Aside from the antibacterial monomers added to the resin matrix, an alternative approach is to add silver nanoparticles. Indeed, silver (Ag) is known for its antimicrobial activity against a diverse group of bacteria and has been used for many years as an antimicrobial substance in the medical field. [15] Composite containing Ag particles with long-lasting antibacterial activity have been manufactured and observed to inhibit *S. mutans* growth [16]. In addition, resins containing Ag nanoparticles were able to inhibit biofilm viability. [17,18] Although the restorative materials had significant evolvement in the past few decades, the high rates of treatment failure suggest that the current restorative approaches are not yet optimized and have a potential for improvement.

The aim of this work is to synthesize and evaluate new bioactive and antibacterial composite materials based on photo-activated Bis-GMA/TEGDMA matrix, containing an hydrotalcite-like compound intercalated with Ag nanoparticles as filler.

We have obtained a dental resin with improved physical and biological properties and, in addition, able to release low amount of silver in a controlled and tunable way for a long period of time.

In contrast to the conventional and resin-modified glass-ionomers, our CR-Agx were able to release silver ions when intraoral pH values drop below the critical pH of 5.5, counteracting the demineralization process of the tooth surface. The caries protective effect of these materials may be related to the material's ability to release adequate amounts of silver ions for sustained periods of time and during acidic attack.

RIASSUNTO

Le carie secondarie sono lesioni che si originano ai margini di restauri dentali e sono tra le cause più comuni di fallimenti terapeutici. Questo tipo di lesioni sono istologicamente simili alle carie primarie e sono difficili da distinguere da queste ultime, se non in stadi avanzati.

Durante gli ultimi decenni le resine dentali sono state ampiamente utilizzate in odontoiatria restaurativa per le loro eccellenti caratteristiche estetiche e le loro performance meccaniche. In contrasto, sono una potenziale risorsa di carbonio per i batteri residui nelle cavità dentali. Tali microorganismi cariogeni possono infiltrarsi nei margini tra dente e restauro e comprometterne la longevità. A causa di ciò sono stati sintetizzati nuovi materiali dentali antibatterici tramite introduzione di monomeri di ammonio quaternario come, ad esempio, bromuro di 12-metacrilossidodecilpiridinio (MDPB), dimetilamminoesadecil metacrilato (DMAHDM) e dimetilamminododecil metacrilato (DMADDM). Questi monomeri formano legami covalenti con la matrice polimerica e sono immobilizzati nella resina, rappresentando un agente battericida da contatto. Sono state inoltre sviluppate altre formulazioni antimicrobiche come, ad esempio, adesivi contenenti cloruro di metacrilossietilcetil dimetilammonio (DMAE-CB), nanoparticelle di polietilenimmina da inserire in compositi dentali con attività antibatteriche, GIC (glass ionomer cements) antibatterici e nano compositi contenenti ammonio quaternario dimetilacrilato (QADM). Tali resine posseggono ammine quaternarie cariche positivamente capaci di interagire con le cariche negative della membrana batterica provocandone la distruzione. Inoltre, composti con ammonio quaternario a lunga catena sono particolarmente

efficaci grazie alla capacità di inserirsi all' interno della membrana batterica.

Oltre alla presenza di monomeri antibatterici aggiunti alla matrice della resina, un approccio alternativo è quello di utilizzare nanoparticelle di argento. Infatti l'argento è dotato di attività antimicrobica contro un ampio spettro di batteri. Compositi contenenti nanoparticelle di argento con attività antibatterica a lungo termine sono stati sintetizzati e utilizzati contro *S. mutans* e come agenti anti-biofilm.

In generale, anche se i materiali restaurativi hanno avuto significanti miglioramenti negli ultimi decenni, gli elevati tassi di fallimento indicano che gli attuali approcci restaurativi non sono ancora ottimizzati ed hanno margini di miglioramento.

Lo scopo di questo lavoro è quello di sintetizzare e valutare nuovi materiali compositi bioattivi basati su una matrice foto attivata di Bis-GMA/TEGDMA contenente compositi idrotalcite simili intercalati con nanoparticelle di argento come riempitivo.

Abbiamo quindi ottenuto resine dentali con caratteristiche fisiche e proprietà biologiche migliorate, capaci di rilasciare basse quantità di argento in maniera controllata per un lungo periodo di tempo.

A differenza delle resine convenzionali, il nostro CR-Ag è in grado di rilasciare ioni argento quando i valori di pH intra-orale sono al di sotto del pH critico di 5.5, contrastando il processo di demineralizzazione della superficie del dente. Quindi, l'effetto carie protettivo di questi materiali è in relazione all' abilità del materiale di rilasciare quantità adeguate di ioni argento per un tempo sostenuto durante l'attacco acido.

CHAPTER 1 . DENTAL CARIES

1.1 ETIOPATHOGENESIS OF DENTAL CARIES

Dental caries is defined as a chronic, diet o microbial, site-specific disease of dental hard tissues, caused by shifts from protective factors favoring tooth remineralization to destructive factors leading to demineralization. [19] When sugars or other fermentable carbohydrates are ingested, the resulting fall in dental plaque Ph caused by organic acids increases the solubility of calcium hydroxyl apatite in the dental hard tissues and demineralization occurs as calcium is lost from the tooth surface (Figure 1).

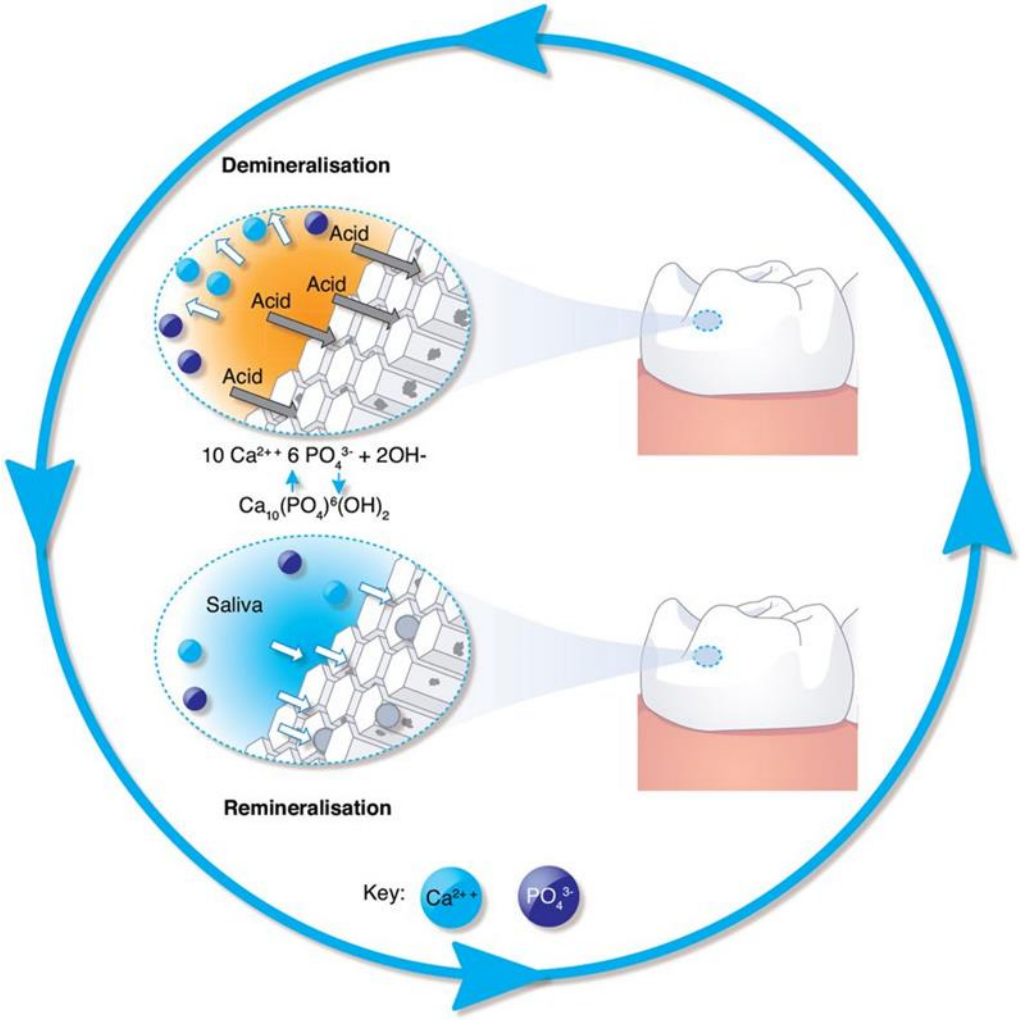


Figure 1. The process of tooth remineralization. Adapted from Dodds et al. [20]

The pH of dental plaque is a key factor in the balance between acid demineralization of the teeth and the remineralization of the initial caries lesion. Plaque pH falls each time acid accumulates in the plaque due to bacterial acid production following the consumption of fermentable carbohydrates – mainly sugars – in foods and drinks. Conversely, plaque Ph rises when the acids are washed away or neutralized by saliva, which contains the important buffer, bicarbonate.[20]

In healthy teeth, the loss of minerals is balanced by the reparative mechanisms of saliva. [20]When the saliva pH or the plaque pH is below a 'critical value' of about 5.5, the saliva or plaque becomes unsaturated with respect to tooth mineral. [20] As a result, tooth enamel can begin to dissolve. However, when the pH is above this value, the saliva and plaque are supersaturated with respect to tooth mineral. The calcium and phosphate ions in saliva then start to repair any damaged mineral crystals in the enamel, starting the process of remineralization.

Thus, acidic conditions contribute to bringing phosphate and hydroxyl ions below saturation levels, allowing the solid hydroxyapatite crystals of the tooth mineral to dissolve. If above saturation levels, the chemical reaction will move towards remineralization and any damaged crystals will be repaired by the acquisition of ions from the solution.

The World Health Organization (WHO) affirms that dental caries qualifies as a major public-health problem, owing to its high prevalence in all regions of the world, with the greatest burden of disease being on disadvantaged and socially marginalized populations.

The initiation and development of the disease is the result of the interaction of four main etiological factors (Figure 2):

- the tooth structure;

- the oral bacteria in dental plaque;
- the dietary, salivary and genetic influences.
- the time

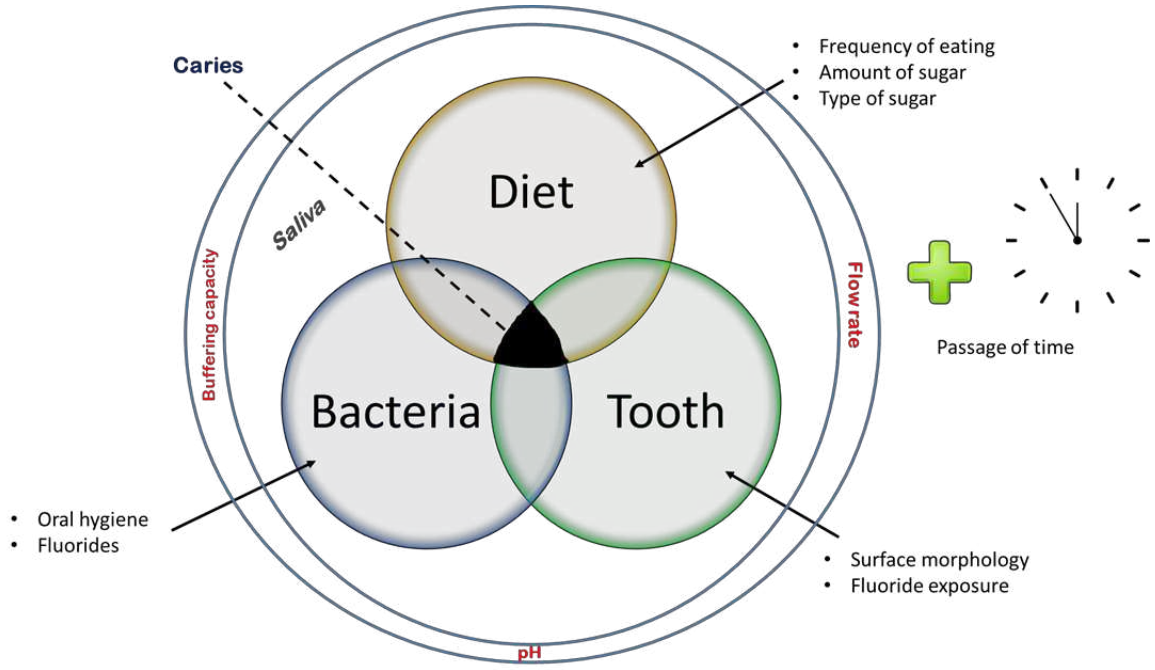


Figure 2. Diagram that describes the multifactorial etiology of dental caries. Adapted from Mathur et Dhillon. [21]

The plaque and the dietary factors are interdependent on each other for the causation of damage. The tooth becomes the 'platform' for this interaction, as well as the 'victim' in the interaction of the other two factors.

It is indeed true that multiple factors have to act in concert with each other to produce the disease, but not necessarily at the same time (Figure 3).

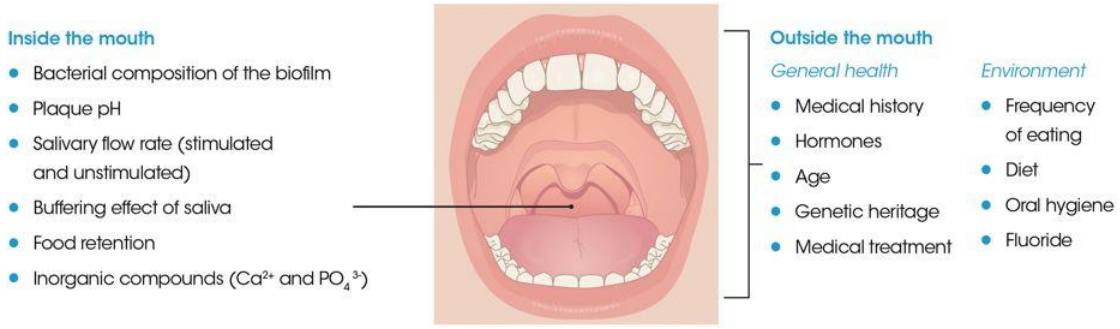


Figure 3. Factors affecting the development of dental caries. Adapted from Dodds et al. [20]

Environmental factors, such as behavioral habits, [22] may also influence the development of dental decay. Low socio-economic status is a non-biological risk factor which is often related to educational level, the perception of the individual about his/her own health, life style, dietary composition, and access to dental care. [23]

Moreover, caries development is a dynamic process that consists of rapidly alternating periods of tooth demineralization and remineralization, which, if net demineralization occurs over sufficient time, results in the initiation of specific caries lesions at certain anatomical predilection sites on the teeth. It is important to balance the pathological and protective factors that influence the initiation and progression of dental caries. Protective factors promote remineralization and lesion arrest, whereas pathological factors shift the balance in the direction of dental caries and disease progression (Figure 4).

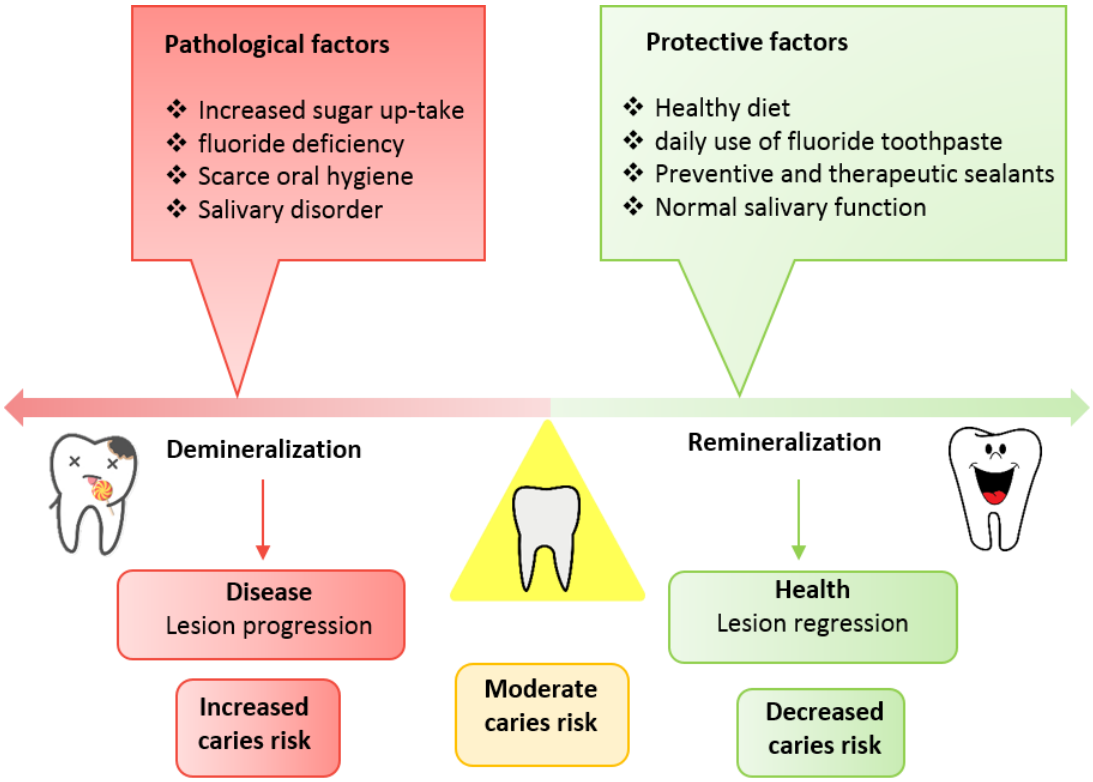


Figure 4. Balancing pathological and protective factors in dental caries. A focus on optimizing the protective factors will promote remineralization and shift the dynamic balance of the caries process in the direction of health and lesion arrest. A failure to mitigate the effects of the pathological factors will promote demineralization and shift the dynamic balance in the direction of disease initiation and disease progression. Adapted from Pitts N.B. et al.[24]

The oral microbiota

The oral cavity is a unique environment that support the presence of up 500 species of microorganisms (including viruses, fungi, protozoa and bacteria). [25] Unlike oral epithelium, the morphology of the tooth makes many areas inaccessible to physiological clearance mechanisms. Thus, a tooth becomes an ideal place for the stubborn adherence for many of these species. [26] Organisms that are capable of adhesion, adhere to the salivary pellicle on the tooth and form a convenient arena for the

subsequent aggregation of other organisms that are incapable of initial adhesion.

According to the 'ecological plaque hypothesis' (Figure 5), the pathogenesis of dental caries is related to a disturbance in two types of homeostasis / physiological equilibrium that exist in an oral cavity:

1. Disruption of microbial homeostasis in the 'biofilm'.
2. Disruption of mineral homeostasis between the tooth and the 'oral fluid'.

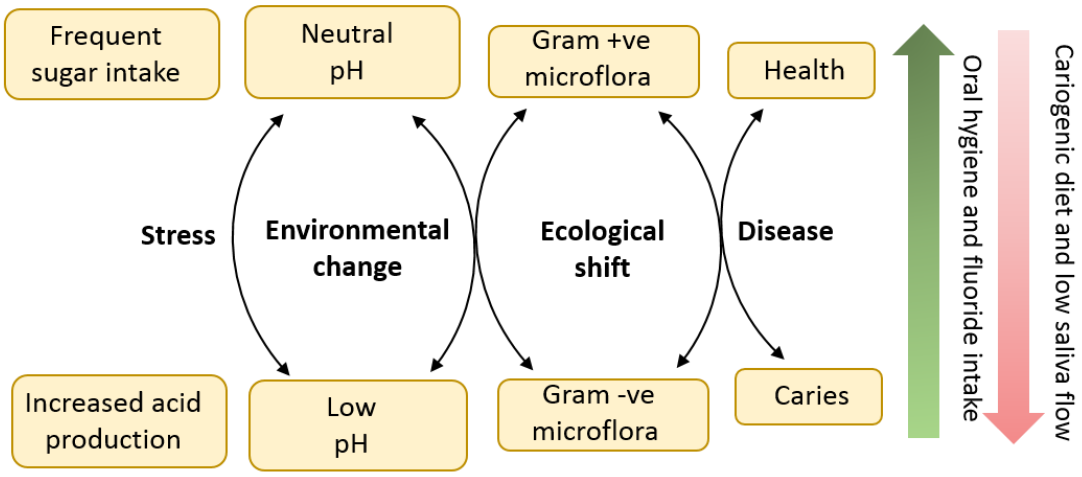


Figure 5. Ecological plaque hypothesis. An increased frequency of fermentable sugar intake results in the biofilm spending more time at a low Ph, which will select for bacteria that grow preferentially under acidic conditions. The growth of bacteria associated with sound surfaces is then disadvantaged, which over time results in an increase in the proportions and activity of cariogenic species at a site and a heightened risk of caries. This risk is raised in individuals with impaired saliva flow and sugar-rich diet, but it is reduced in those with appropriate oral hygiene and exposure to fluoride. Adapted from Pitts N.B. et al. [27]

The dental biofilm is a population or community of bacteria living in organized structures attached to a tooth surface, embedded in a matrix of extracellular polymeric substances produced by microbes. [9] The formation of dental biofilm is a multiple-stage process (Figure 5).

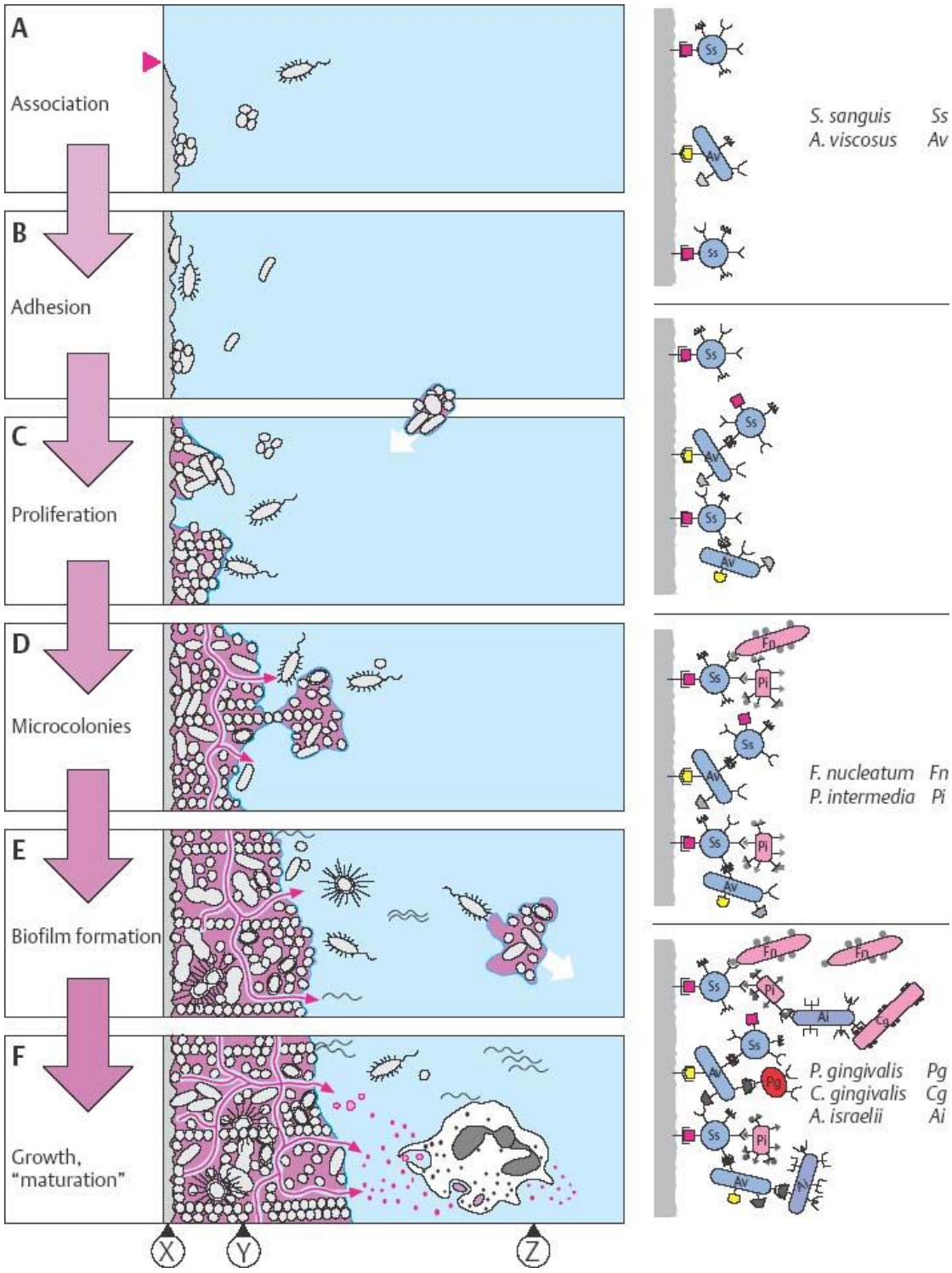


Figure 6. Stages of dental biofilm formation. Within minutes after completely cleansing the tooth surface, a pellicle forms from proteins and glycoproteins in saliva. **A** Association: Through purely physical forces, bacteria associate loosely with the pellicle. **B** Adhesion: Because they possess special surface molecules that bind to pellicle receptors, some bacteria become the "primary colonizers," particularly streptococci and actinomyces. Subsequently, other microorganisms adhere to the primary

colonizers. **C** Bacterial proliferation ensues. **D** Microcolonies are formed. Many streptococci secrete protective extracellular polysaccharides (e. g., dextrans, levans).**E** Biofilm ("attached plaque"): Microcolonies form complex groups with metabolic advantages for the constituents. **F** Plaque growth—maturation: The biofilm is characterized by a primitive "circulatory system." The plaque begins to "behave" as a complex organism! Anaerobic organisms increase. Metabolic products and evulsed cell wall constituents (e. g., lipopolysaccharides, vesicles) serve to activate the host immune response. Bacteria within the biofilm are protected from phagocytic cells (PMN) and against exogenous bacteriocidal agents. (Adapted from Wolf HF. Biofilm-plaque formation on tooth and root surfaces. In:Wolf HF, Rateitschak KH (eds). Periodontology, ed 3. Stuttgart: Thieme, 2005:24) [28]

The process starts with an initial formation of salivary pellicle, a combination of active proteins and glycoproteins from saliva and gingival crevicular fluid. After four hours, the aerobic species of the genera *Streptococcu*s, *Capnocytophaga*, *Veillonella* or *Actinomyces* (known of as initial and early colonizers) adhere to the tooth proteic film, forming a first layer of biofilm. This process requires the presence, on the bacterial cell surface, of specific (e.i statherins, mucins, agglutinins, alpha-amylase and prolin rich proteins) that act like chains between tooth and early colonizers. A second layer of biofilm develops through a process known as coaggregation or coadhesion, in which other microorganisms attach the first colonizers through adhesion of their respective cell surfaces . [29] Middle colonizers, such as *Fusobacterium nucleatum*, and late colonizers, like *Lactobacillus* spp., contribute to this second layer formation.

The first step is reversible adhesion mediated by electrostatic and hydrophobic forces. The second step is irreversible adhesion caused by a time-dependent shift to a higher binding affinity state, which involves multiple on the bacterial surface and polymer matrix. Division of the

attached bacterial cells produces microcolonies. Confluent growth results in the formation of plaque biofilm, which increases in complexity with time.

The presence of dental-caries-associated streptococci in the mouth of nearly all adults indicates that dental caries is probably the most ubiquitous bacterial infectious disease of humans. [30] Although other oral microorganisms can be cariogenic, *mutans streptococci* have unique biochemical features that make them efficient at accumulating and producing carious surfaces.The characteristics that make *mutans streptococci* particularly efficient at causing dental caries include: [31]

1. production of large amounts of lactic acid at a rapid rate;
2. tolerance to extremes of sugar concentration, ionic strength and Ph;
3. production of the enzymes dextranases and fructanases capable of metabolizing extracellular polysaccharides, which contribute to the acid production and constitute a substratum in the periods with less oxygen supply;
4. production of insoluble glucans that contribute to biofilm complexity and impede salivary protection.

However, any acidogenic species, including the *mutans streptococci*, aciduric non-*mutans streptococci*, *Bifidobacterium*, *Lactobacillus, Actinomyces*, and *Scardovia*, may contribute to disease development. [32] [33] [34] [35]

In particular,*Lactobacillus spp.* is the cariogenic bacteria responsible of the progression of carious lesions. *Lactobacillus spp*. are not able to quickly attach to hard surfaces and live in niches with low Ph. *Lactobacillus spp.* tolerate acid environments, since these bacteria contain the agmatine pathway, which helps neutralize their cytoplasm pH. [36] Similarly,

Veillonella spp. are present at all stages of caries progression under high-glucose conditions and appear to be implied in acid production. Interestingly, *Veillonella alcalescens* and *S. mutans* can act in synergy producing more acid. [25]

In addition, Tanner AC et al. (2011) [34] propose *Scardovia wiggsiae* as a clear initiator agent of early childhood caries, and *Bifidobacterium spp*. as important microorganisms of tooth decay in root caries lesions. The yeast Candida spp. have also been involved in the carious process, since they may be present in acidogenic environments. [25] Indeed, excess sugar may promote the growth and multiplication of *Candida albicans*.

Oral immunity is the balance system used by the human body to control the microorganisms present in oral tissues. The mouth is a path of entry and exchange with the environment, and is therefore subject to constant fluctuations that must be controlled by the immune system. The main barriers against microorganism are saliva, dental tissues and immunological components.

Dental tissues have no immunological capacity to test and respond to the degradation and colonization of microorganisms, due to their inert nature. [37] During caries infection, oral bacteria degrade enamel and dentin and trigger an innate immune response in the dental pulp through the diffusion of bacterial by-products into dentin tubules. This response may eliminate the insult and block the route of infection when accompanied by dentin neo-formation within tubules and/or at the pulp–dentin interface. Pathogen invasion may result in excessive and deleterious pulp immune response, irreversible acute inflammation, tissue necrosis, and microbe dissemination through blood vessels.

Cariogenic diet

Observations in humans and animals have shown clearly that frequent and prolonged oral exposure to certain carbohydrates and sugars, especially sucrose, are fundamental to caries activity that serves as a substrate for microorganisms of the oral cavity. Indeed, the fermentable carbohydrates are the main class of compounds that markedly influence the ecology and health of the mouth, because can be broken down to acids by acid genic bacteria. Sucrose can be converted by bacterial enzymes (glucosyltransferases, GTF and fructosyl transferases, FTF) into glucans and fructan, which can be used to consolidate attachment or act as extracellular nutrient storage compounds. The frequent consumption of dietary carbohydrates is consequently associated with a shift in the proportions of the microflora of dental plaque. In addition, the oral microflora synthesizes extracellular polysaccharides that play a key role in dental plaque formation and in the production of organic acids.

Stimulation of saliva flow results in an increase in the washing out of acids (and sugars), and also an increase in the amount and concentration of bicarbonate buffer and of remineralizing ions. On the other hand, 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 waterier saliva secretion, consuming meat produces saliva with a higher mucoid substance content. These parameters return back to normal approximately 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 occurrence of caries.

Oral health is directly related to diet and nutrition and dietary advice is recommended by dentists for certain 'at risk' groups in the community. [38] Whilst proteins from food debris in the mouth can be important in bacterial generation of malodor.

Today the world faces two kinds of malnutrition, one associated with hunger or nutritional deficiency and the other with dietary excess. Urbanization and economic development result in rapid changes in diets and lifestyles, which may be reflected by a higher risk of dental caries development. A study developed in Scotland confirms a lower prevalence of dental caries in the rural areas, mainly justified by the fact that adolescents may practice a better and healthier diet when compared with adolescents living in urban areas. [39] Market globalization has a significant and worldwide impact on dietary excess leading to chronic diseases such as obesity, diabetes, cardiovascular diseases, cancer, osteoporosis and oral diseases. Diet and nutrition affects oral health in many ways. Nutrition, for example, influences cranio-facial development, oral cancer and oral infectious diseases. Dental diseases related to diet include dental caries, developmental defects of enamel, dental erosion and periodontal disease.

The nutrition transition is a relevant example on how common risks influence public health, including oral health. The public health community involved with oral health should gain an understanding of the

health effects of these complex developments in order to prevent or control oral diseases.

Clearly, food can have also effect on the resident microorganisms of the oral cavity. High intake of sweetened baked goods has been shown to be a determinant of caries prevalence in children with moderate to high salivary counts of *S. mutans*. [40] In 2002 a WHO/FAO Expert Consultation on Diet, Nutrition and the Prevention of Chronic Diseases recommended that the intake of free sugars should provide ≤10% of energy intake. [41] Moreover, in March 2015, the WHO published a new guideline for intake of sugars for adults and children [42] and made a strong recommendation for a reduced intake of free sugars throughout the life course. A strong recommendation was also made for both children and adults that the intake of free sugars should be reduced to ≤10% of total energy intake. The WHO also made a conditional recommendation for a further reduction of the intake of free sugars to <5% of total energy intake. It was also stated that for countries with a low intake of free sugars that levels of intake should not be increased and that higher intakes of free sugars might jeopardize the quality of the diet by providing energy without nutrients. The WHO guideline stated that increasing or decreasing the intake of dietary free sugars was associated with parallel changes in body weight and that the relation exists irrespective of the quantity of sugar either as amount measured or percent contribution to energy intake. The quantitative recommendations were therefore based on evidence relating to the association of dental caries and free sugars.

The teeth

The tooth is the hardest structure in the oral cavity and represents, in humans, about the 20% of all the oral cavity surface. Primary teeth start to form between the sixth and eighth weeks in utero, while permanent teeth begin to form in the twentieth week. Erupted teeth are located in a cavity of the bone called alveolus where a complex specialized ligament, the periodontal ligament, supports them. Each tooth is divided in an upper portion, the crown, and a bottom one, the dental root, completely included in the dental alveolus. [43] These area are separated through a boundary zone called neck (Figure7).

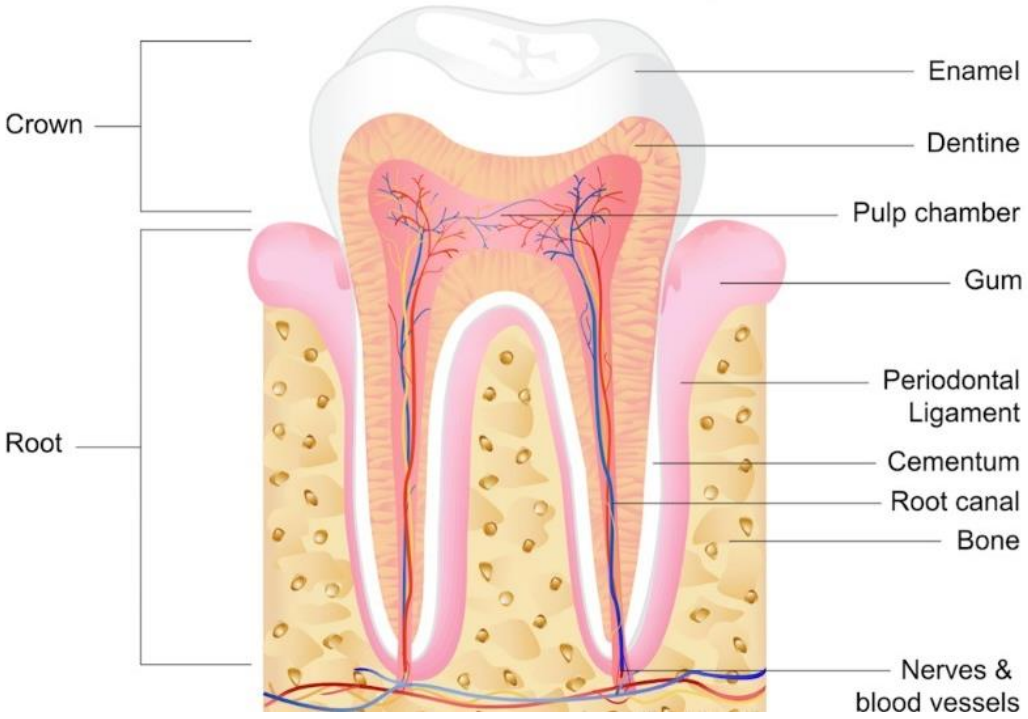


Figure 7. Divisions and tissues of the tooth.

Teeth are composed of four tissues: enamel, dentin, cementum (the hard mineralized tissues) and pulp (the soft tissue)[44],[45].

- Enamel is the hardest tissue in the body, made up of 96 wt% inorganic materials; the main part is composed by carbonated hydroxyapatite crystals, while sodium, magnesium, chlorine, carbonate, potassium and fluoride represent trace elements. The enamel formation process, the Amelogenesis, consists in hydroxyapatite crystals precipitation and growth, initiated by the secretory activity of the ameloblasts (enamel forming cells) into the extracellular space adjacent to the dentino-enamel junction [44]. However, enamel shows an acellular and avascular structure without the capability to regenerate or repair itself, but with the ability of remineralize. Demineralization and remineralization can occur without loss of tooth structure when proper nutrition and oral care are respected. [43]
- Dentin is an hydrated tissue that consists of approximately 50 vol% of carbonated hydroxyapatite (Hap) minerals, 30 vol% of collagen and noncollagenous molecules and 20 % of water [44]. The 90 wt% of the organic phase in dentin is composed of collagen type I. Dentinogenesis, the biological process that lead to the formation of dentin, involves a chain of different mechanisms such as cell differentiation and interactions, the synthesis of an organic matrix, and the eventual formation of mineral crystals in this extracellular matrix. The process is induced by odontoblasts that differentiate from ectomesenchymal cells of the dental papilla following an organizing influence that emanates from the inner dental epithelium. Thus the dental papilla is the formative organ of dentin and eventually becomes the pulp of the tooth. There are three types of dentin: primary, secondary and tertiary. Primary dentin

forms during tooth eruption while secondary dentin grow throughout the life of the tooth. Tertiary dentine, also known as reparative dentin, forms as a response to irritation and trauma (e.g. erosion and dental caries) [43].

- Cementum is a mineralized avascular connective tissue, similar in composition to bone. It is composed of 65 wt% of Hap minerals, 23 wt% of organic matrix and 23 %wt of water. The organic substance consists in proteoglycans and glycoproteins for the amorphous part, and in collagen fibers for the structured one. Cement is secreted during cementogenesis by cementoblasts, which are cells that share a similar morphology with odontoblasts. This tissue cannot be repaired. [44]
- Dental pulp is a mucous connective tissue contained in the pulp chamber, limited by dentin. Dental pulp and dentin have the same embryonic origin but the pulp is mainly composed by amorphous gelatinous ground substance, rich in glycoproteins, proteoglycans and glycosaminoglycans (mostly hyaluronic acid) and few fibroblasts. The dental pulp is the best cell source for tissue engineering, since it is a rich reservoir of stem cells residing in various areas (mainly in root) and with numerous plasticity characteristics.

In the case of a caries lesion, the histopathology of dental caries can be divided into two stages: the enamel stage, characterized by ultrastructural and white spot lesions, and the dentin stage, where tubular sclerosis and reactionary dentin are involved. Specifically, the initial stage of dental caries starts as a not visible lesion of enamel, resulting from the biofilm

activity. The process consists in the direct dissolution of the outer enamel surface due to an enlargement of the intercrystalline spaces because of the partial dissolution of the individual crystal peripheries, followed by the development of irregularities such as pits and focal holes. [46] White spot lesions can advance to clinically detected cavitated lesions Cavitation occurs because of external forces that lead to the collapse of the outer surface, which in turn leads to a discontinuity or break in the surface. This stage of the disease is irreversible and requires operative intervention to restore function and to arrest the caries process. When the caries lesion reaches dentin, pulp-dentin complex deposits mineral within the dentinal tubules, during a process called “tubular sclerosis” or “translucent dentin” (due to its translucent aspect when examined by transmitted light microscopy). [37]Tubular sclerosis in dentin is visible before the enamel lesion extends to the enamel-dentin junction. Another defense mechanism of the pulp-dentin complex, indicative of a dental disease, is the formation of reactionary dentin by surviving odontoblast cells at the pulp-dentine interface and in peritubular/intratubular location. [47]

Time

The time factor has an important role in the manifestation of clinical signs of the development of carie lesions. [48] Time factor was added by Newbrun to the primary etiological factors identified by Keyes, since these need to be present for a certain period of time, so that the progressive demineralization of enamel may develop. [49]

Secondary etiological factors

Fluorides

Research has shown that fluoride is most effective in dental caries prevention when a low level of fluoride is constantly maintained in the oral cavity. The goal of community-based public health programmes, therefore, should be to implement the most appropriate means of maintaining a constant low level of fluoride in the oral cavity.[50] Fluorides can be obtained from fluoridated drinking-water, salt, milk, mouth rinse or toothpaste as well as professionally applied fluorides, or from combinations of fluoridated toothpaste with either of the other two fluoride sources. [50] Fluoride is being widely used on a global scale, with much benefit. Millions of people worldwide use fluoridated toothpaste. Recent local studies have shown that affordable fluoridated toothpaste is effective in caries prevention and should be made available for use by health authorities in developing countries. The WHO Global Oral Health Programme is currently undertaking further demonstration projects in Africa, Asia and Europe in order to assess the relevance of affordable fluoridated toothpaste, milk fluoridation and salt fluoridation. [51] [52] There is clear evidence that long-term exposure to an optimal level of fluoride results in diminishing levels of caries in both child and adult populations.[48] However, populations in many developing countries do not have access to fluorides for prevention of dental caries for practical or economic reasons. [48] There are some undesirable side-effects with excessive fluoride intake. Experience has shown that it may not be possible to achieve effective fluoride-based caries prevention without some degree of dental fluorosis, regardless of which methods are chosen

to maintain a low level of fluoride in the mouth. The public health administrators must seek to maximize caries reduction while minimizing dental fluorosis.

Saliva

Human saliva is part of the mechanisms of natural or innate immunity of the oral cavity. Its viscosity makes the adhesion of microorganisms difficult. In addition, salivary flow exerts a cleaning function, provides antibacterial proteins, enables high buffering capacity and helps neutralize acids. [53] The two major functions of saliva are:

1. Protection of the oral and peri-oral tissues exerting functions of lubrication, dilution of sugars after food and drink intake, antimicrobial and cleansing activity, degradation of some bacterial cell walls and inhibition growth, buffering (neutralizing) acid production and controlling plaque Ph with bicarbonate, remineralization of enamel with calcium and phosphates, tissue repair.
2. Facilitating eating and speech exerting function of food preparation (enhancing chewing, the clearing of food residues and swallowing), digestion (food breakdown with enzymes), enhancing taste, enabling speech by lubricating the moving oral tissues.

Normally, the daily production of saliva ranges between 0.5 and 1.0 liter and is composed of more than 99% water and less than 1% solids. In particular, the solid content consists of desquamated epithelial cells, white blood cells, bacteria, yeasts, fungi and viruses. Total salivary proteins constitute 0.3% of the volume and are essential elements for microorganism's interactions.

The whole saliva is derived predominantly from three paired major salivary glands: the parotid, submandibular and sublingual glands, but also from the minor salivary glands in the oral mucosa (Figure8). [54]

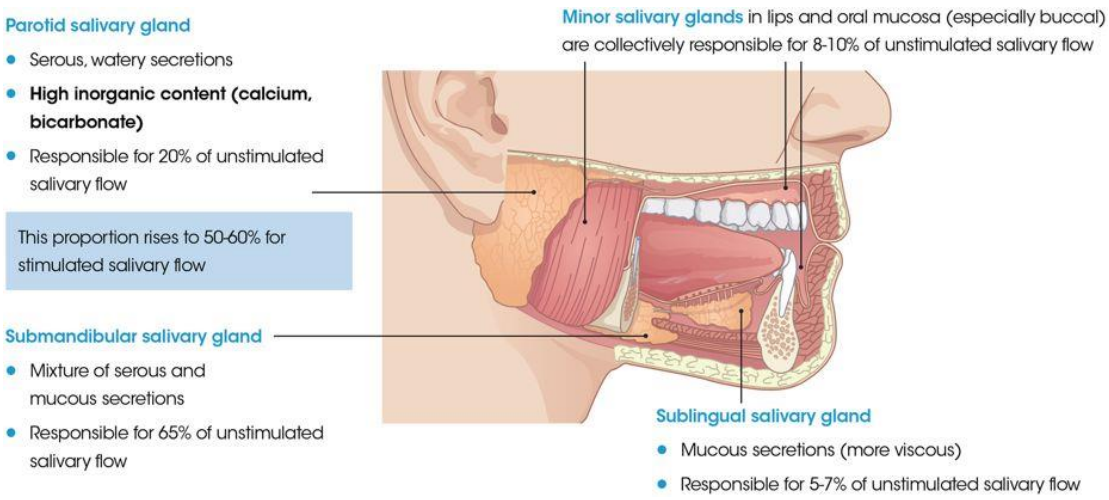


Figure 8. Salivary glands and saliva function. Adapted from Dodds et al. [20]

The stimulated saliva is produced in response to a mechanical, gustatory, olfactory, or pharmacological stimulus, contributing to around 40-50% of daily salivary production. In addition, without exogenous or pharmacological stimulation, there is an unstimulated secretion of saliva useful to cover, moisturize, and lubricate the oral tissues.

Other important functions of saliva consists in the protection of the teeth by neutralization of acids by buffering actions, the saliva maintains supersaturated calcium phosphate concentration with regard to hydroxyapatite, and also by participating in enamel pellicle formation. Furthermore, saliva components participate in mucosal coating and antimicrobial defense as well as digestive actions. Thus, saliva plays a major role in oral health and changes affecting salivary function, it may also compromise hard and soft oral tissues structure and functions. [55]

The oral cavity is constantly exposed to many different kinds of substances, some of which influence the caries process to a great extent. An important function of saliva is therefore the dilution and elimination of substances introduced into the oral cavity, through a physiological process usually referred to as salivary clearance or oral clearance. In patients with reduced quantity of saliva the mechanistic and cleaning properties of this fluid in the mouth are impaired. With regard to prolonged oral clearance, a low oral sugar clearance inevitably increases the risk of caries development. Concerning this relation, the unstimulated flow rate has been found to be diagnostically more important than the stimulated one.

Oral hygiene

There is a strong correlation between oral hygiene and the prevalence of dental caries. [56] Good oral hygiene habits help to prevent the development of caries by reducing the build-up of dental plaque. [57]

The composition of the dental plaque varies not only from individual to individual, but also upon the location of the oral cavity and tooth surface. Control of bacterial plaque through proper hygiene, performed by each individual and complemented with the intervention of a dental professional are key preventive primary measures for the improvement of oral health and disease prevention, including dental caries. [48]

1.2 DENTAL CARIES THERAPY

Dental caries is regarded as a disease that will affect most people in the world to some extent during their lifetime. This inevitability of caries developing, at least historically, was a strong stimulus to the development and promotion of preventive measures. [58] Therefore, the prevention of caries has been, and still is, a major goal for the dental profession. [59] [60] It may present different connotations according to the target population. For caries-free people, which means person without prior caries experience, the definition of prevention follows the classical precepts of avoiding the development of a disease. On the other hand, for person presenting previous experience of dental caries, preventive measures imply in avoiding the development of new lesions and/or the reactivation of previous ones.

In this contest, with a clear understanding of the etiology of dental caries and the risk factors that lead to and facilitate the spread of this disease, the prevention guidelines should also be multifocal, concentrating on topics that can affect the risk of disease, such as dietary counseling (limit sugary foods and drinks to mealtimes and avoid carbonated, sugared beverages and juice), oral hygiene, and delivery of fluoride, including community-based options (water fluoridation), self-administered modalities (fluoride toothpaste and supplements), and professional applications (fluoride varnish). [61]

Water fluoridation is a community-based intervention that optimizes the level of fluoride in drinking water, to protect pre-eruptive and post-eruptive teeth especially in countries with drinking waters with an inadequate amount of fluoride. [62]

Fluoride toothpaste is an important way to deliver fluoride to the surface of the tooth and to reduce dental caries in both primary and permanent teeth. [63] Finally, fluoride varnish is a professionally applied, sticky resin of highly concentrated fluoride, effective in preventing caries in children at high risk of all ages. [64] Application of fluoride varnish is even more effective when coupled with counseling. [65]

Failure to identify and prevent dental disease has consequential and costly long-term adverse effects. Indeed, untreated caries in permanent teeth was the most prevalent condition evaluated across all medical conditions, with a global prevalence of 35% for all ages combined, with 2.4 billion people affected.

Dental caries therapeutic approach has changed during the time, following the scientific knowledge about caries etiology, and the availability of new dental materials. In the late ninetieth century, G.V. Black introduced a classification of dental caries, as well as established the principals of tooth preparation, based on his understanding of the nature of the disease. Black's theories required the complete removal of all areas of demineralized tooth structures and their subsequent reconstruction through inert restoration [66]. The phrase, "extension for prevention," is still famous in the dental community today and represents Black's idea that dentists should incorporate more grooves and pits than those currently exhibiting decay as a preventive measure against those grooves and pits developing tooth decay in the future. [67] [68] Nevertheless, 'Black's Classification of Caries Lesions' (Figure 9), based on the most common carious lesion sites and size, is still in use today:

- Class I Caries affecting pit and fissure, on occlusal, buccal, and lingual surfaces of molars and premolars, and palatal of maxillary incisors.
- Class II Caries affecting proximal surfaces of molars and premolars.
- Class III Caries affecting proximal surfaces of centrals, laterals, and cuspids.
- Class IV Caries affecting proximal including incisal edges of anterior teeth.
- Class V Caries affecting gingival 1/3 of facial or lingual surfaces of anterior or posterior teeth.
- Class VI Caries affecting cusp tips of molars, premolars, and cuspids.

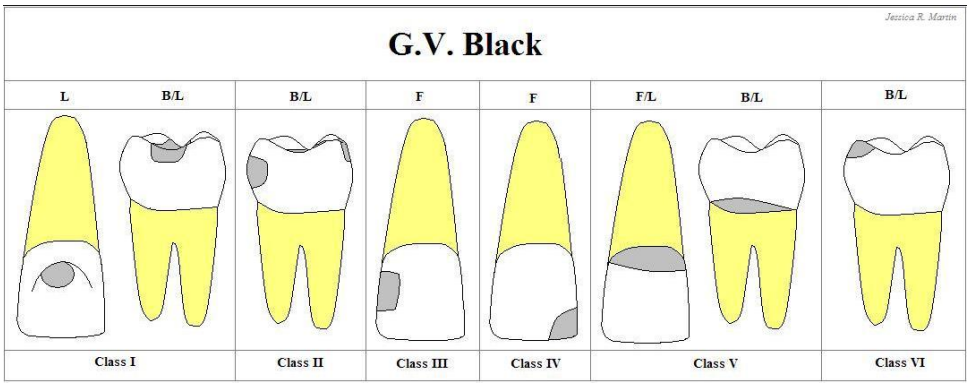


Figure 9. G.V. Black Classification of Restorations. Image adapted from https://commons.wikimedia.org/wiki/File:GV-BLACK.JPG by Jessica R. Martin

This classical therapeutic approach includes extrinsic dental interventions, such as tooth filling, tooth extraction and implantation of an inert, artificial (metal, ceramic) substitute. Although the “extension for prevention” was a widely accepted model, some authors raised many arguments about it, provoking unpleasant and/or adverse side-effects [69] [70]. In the 1904, Slagle introduced the concept of “extension for retention”. This concept focused more on the “anchorage” or retention of

the restorative material inside the prepared cavities after careful evaluation of occlusal forces. Nowadays, modern dentistry is based on the concept of “prevention of extension” that is applied in the “minimally invasive dentistry”(MID). [71] The main purpose of MID is to achieve as much conservation of dental tissue as possible, taking advantage of modern dental materials. [72] MID includes early detection of dental caries, assessment and management of caries-risk, remineralization of early caries lesions, only restoring cavitated lesions, restriction of the excavation to the caries-infected areas and using adhesive-based technologies. [73] [74] Instead, it should follow the extent of a carious lesion and only eliminate caries-infected tissue with the preservation of both caries-affected and sound tissues. [71]

Indeed, caries-affected dentin can remineralize due to the presence of viable odontoblasts in the inner layer and of the collagen network still capable of binding calcium and fluoride ions .In particular, MID caries treatment is carried out removing only the dentine affected by caries at external level (dentin decomposed) and preserving the one at inner levels (demineralized dentin). Such tissue is able to remineralize using particular biomaterials. Moreover, significant improvement of amalgam alloys and introduction of bonded amalgam restorations have modified the cavity preparation for amalgam to be more conservative of tooth structure. [75]

Such approach decreases the risk of more complex interventions of conventional prostheses for many years .

CHAPTER 2. SECONDARY CARIES

2.1 ETIOPATHOGENESIS OF SECONDARY CARIES: THE ROLE OF MICROLEAKAGE

Secondary caries (or recurrent caries) denotes caries of the tooth at the margin of existing restoration. Although the “recurrent” term typically is used in North America, the term “secondary caries” is used more commonly in European languages for caries develops after initial caries has been removed and replaced by a restorative material. The phenomenon has been known since the early days of restorative dentistry, and it was the basis for the “extension for prevention” concept of G.V. Black’s principles of cavity preparation. [67] During the Black’s period, an obvious solution to prevent recurrent caries was to place the cavosurface margin in a location accessible to the toothbrush. Conversely, the present-day concept of MID is based on the removal only of the dentine affected by caries at the external level to minimize the risk of developing recurrent caries.

Secondary caries may develop rapidly around and below a broken restoration, or slower and more localized on the enamel along the cavosurface margin. (Figure 10) [76] A third type of secondary caries is called “cavity wall caries (Figure 10).

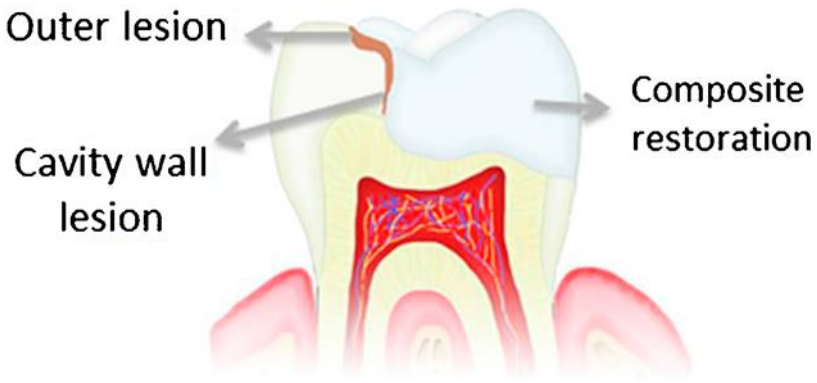


Figure 10. Histopathology of secondary caries lesions next to restoration. Secondary caries may present as an outer lesion, a cavity-wall lesion or as a lesion consisting of both an outer and cavity-wall lesion.

On the basis of the *in vitro* study of Hals and Nernaes [77] secondary caries lesions consist of two regions: the outer lesion and the cavity wall lesion. The outer lesion has a progressing front parallel to the outer surface of the tooth surface and is histologically similar to a primary lesion, a localized process of both demineralizations of enamel and dentin and enzymatic and bacterial degradation of dentin. [19] The wall lesion develops at the interface between restoration and tooth and progresses perpendicularly to the tooth-restoration interface.

Any site along the cavosurface margin will demineralize if the local conditions change to an acidic environment, that depends on the biomass of specific cariogenic bacteria on the more or less polished restoration surface, or rather to the intermediary salivary glycoproteins that first form a pellicle to this surface. [78] Therefore, a minimum critical amount of mature biofilm is required to create an acidic environment, and there are few alternative hypotheses regarding the origin of the acidic environment. Indeed, it has been discussed whether secondary caries would initiate on the external surface and progress to the gap between tooth structure and

restoration and/or would (also) be started within this interface by the diffusion of bacteria or their products. (Figure 11) [79] [80] [81] [5]

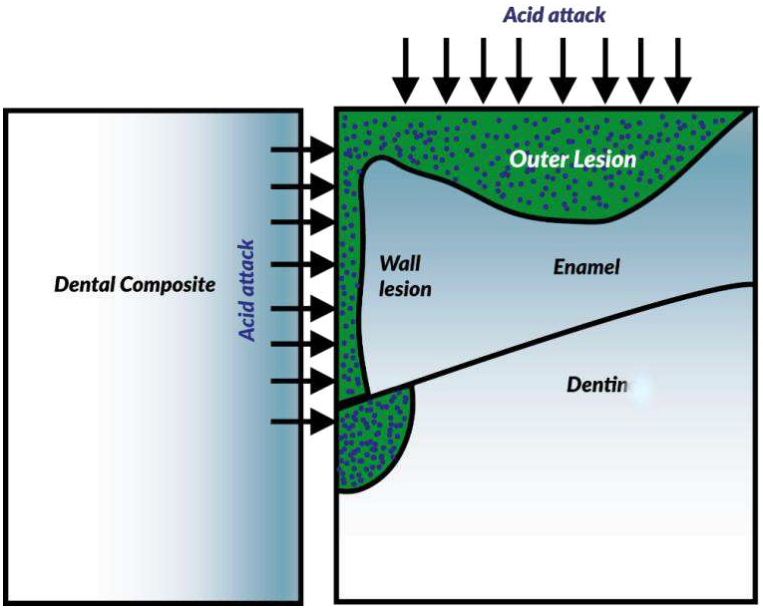


Figure 11. Schematic representation of injuries related to the secondary caries lesions and external wall. Image adapted from Kuper et al. [82]

According to the hydrodynamic flow theory, when the marginal areas of a restoration deteriorate with time, this may result in the existence of a gap or defect at the cavity wall. Subsequently, a biofilm can establish itself in this defect along the tooth-restoration interface and secondary caries can develop within the gap. This theory recognizes the not bonded tooth-restoration interface as a sensitive site, subjected to opening and closing forces that create a hydrodynamic flow. The dissolution products move with this flow allowing a new acid attack. [83] Consistent with this theory, it is clear that a wall lesion can develop in any gap, but the wider the gap, the higher the risk that it will occur.

Recently, several alternative theories have been proposed to explain the gaps/lesion wall relationship. [84] In particular, the "theory of micro-infiltration" indicates, as the cause of the demineralization and the consequent lesion wall, the passage of small amounts of fluid into the

gaps of the tooth/restoration interface. Bacteria carried with the fluid are able to reach the sensible site and cause pathogenicity. In addition, the "theory of macro-infiltration" suggests the necessity of a stable settlement of the biofilm in the gap to have considerable demineralization and lesion development. Gap's dimensions are expected to be from 225 micrometers to 400 micrometers to allow bacteria settlement. [83] [85] [86]

In this contest, several microleakage studies attest to the existence of this "weak link" or "microspace" between the tooth and the restoration. [77] Nelsen et colleagues in 1952 reported that droplets with a diameter up to 44 µm developed along the restoration cavosurface margins in extracted teeth upon rapidly freezing and thawing. [87] The authors defined the phenomenon "*marginal percolation*" attributed to differences in thermal expansion of the tooth and the restorative material. However, only in 1961, it was reported the use of the term "*marginal leakage*", [88] while the catchy term "microleakage*"*, still currently used, was published in 1966. [89] However, numerous variables influence the microleakage experiment outcomes such as the source and type of teeth or tooth specimens, the choice of storage substrate and time, the type of intra- or extra-coronal restoration, including the location of the cavosurface margin and angulation of cavity walls. [90] [91] [92] [93] The handling, placement technique and polishing of the restorative material also influence the extent of observable microleakage. [94] Moreover, the characteristics of the dye or tracer, such as its diameter, and the chemical properties of the solute and solvent as a function of concentrations and exposure time appear also to play a central role. [95] [96] Most tracers have a molecular radius of less than one nm, and it is not improbable that the microleakage in many cases is simply a manifestation of capillary action phenomena.

The minuscule molecular dimension of dyes allows penetration into inter- and intra-prismatic microporesin enamel considered to be 1–30 nm wide, as well as in the peri- or inter-tubular dentin having 0.8–2.5 µm wide dentinal tubules.

Therefore, as a consequence of a microleakage, there may be the seepage of saliva, which drains the bacterial cells into the treated tooth. These cracks also act as an ecological niche for the growth of anaerobes. [97] However, a general belief is that the cariogenic biofilm for primary and secondary caries are similar, and consist mainly of *Streptococcus mutans*, *Lactobacilli* and *Actinomyces naeslundii*, [98] [99] [100] although a contrary opinion based on observations made in an in situ experiment has been proposed. [101]

In addition, the individual's risk for secondary caries is also modified by the saliva quantity and qualities, which in particular comprise the salivary buffering ability. Patients with xerostomia for whatever reason experience more secondary caries, as a reflection of a higher risk of all forms of caries. In this contest, the oral hygiene habits of the patient are one of the primary factors that determines if secondary caries develops.

However, despite efforts to reduce the effects of caries, population-based studies reveal that the prevalence of caries remains stubbornly high. An example of this is seen within the United Kingdom population, where 84% of dentate adults were found to have at least one restoration. Of these adults each had, on average, 7.2 filled teeth. Analysis of the survival of dental restorations from within a large database of dental treatments within UK dental practice reveals that further intervention is required: [102]

• within 11% of fillings after 1 year of placement

•within 20% of fillings after 3 years of placement

•within 50% of fillings after 10 years of placement.

Reasons for this can include marginal defects, secondary caries, fracture of the restoration or adjacent tooth substance and, in the case of tooth-colored restorations, unacceptable appearance. [103] Although the recent innovation on dental restoration materials, the percentage of replacement restorations in adults still accounted for about 50% (with a range of 45 to 55 percent), remaining constant since to 2001. [104] [103] This percentage is higher for amalgam than for resin-based composite restorations. Moreover, the percentage of replaced restorations because of the diagnosis of recurrent caries is much higher in general dental practice than in controlled clinical trials, in which recurrent caries represents 2 to 3 percent of the failures. In particular, the ratio of restoration replacement to primary restorations in general dental practice has been reported to be as high as 80:20 for resin-based composite restorations and 70:30 for amalgam restorations. [105] More recent studies indicate that this ratio is about 50:50 for restorations in permanent teeth. [83] Many factors affect this ratio, including the age of the population studied and the position of the restoration. [106] In addition, all factors that enhance the accumulation of biofilm mass or impede biofilm removal may be considered as risk factors for secondary caries. It is probably the reason why the location of the restoration played a major role in the occurrence of secondary caries. [107] Cervical composite restorations (class V) were the least affected by secondary caries, which obviously may be related to the fact that many class V studies were set up to evaluate the clinical effectiveness of adhesives in non-carious cervical lesions in patients with low caries risk and good oral hygiene. The highest overall incidences were

found in the posterior region, although there are few studies that reported the vulnerability of anterior restorations (classes III and IV). [107] However, also the gingival margins of all types of Class II through Class V restorations are more prone to develop secondary caries, due to the possible contamination from gingival fluid and saliva leaking between the matrix and the cavosurface margin, especially if a rubber dam is not used. Moreover, corrosion and biodegradation products originating from the restorative material may influence the biofilm, [108] even if this correlation is complex and not yet fully understood. [109]

2.2 DIAGNOSIS AND MANAGEMENT OF SECONDARY CARIES

Accurate detection of secondary lesions is crucial for estimating the true burden of the disease and allocating appropriate treatments. Currently, there is no standard to be recommended for performing such detection, with dentists using a variety of methods, with the even greater heterogeneity of subsequent treatment decisions.

Several conventional and newer methods are available to detect secondary lesions. [110] Most of the techniques used for detecting primary caries have also been used to detect secondary caries and artificial caries-like lesions adjacent to restorations. Visual or visual-tactile examinations, often combined with bitewing radiography, are still the most common. [111] [112]

Traditionally, secondary caries lesions were assessed via tactile examination. This method seemed to be specific (specificity increased even further if only clearly detectable ditches were regarded as lesions) but insensitive. In clinical terms, only few secondary lesions would be detected, while the risk of false-positive detections was not drastically decreased compared with, for example, visual detection. For clearly cavitated secondary lesions, the tactile assessment might well be a useful method, as both sensitivity and specificity are presumably increased. However, the presence of marginal ditching, staining, discoloration of the dental tissues and gaps at the tooth restoration interface are unreliable predictors for secondary caries. [76] [113] [114] [115] Therefore, visual detection of secondary caries is a challenge for the dentist [116] and may be confused with microleakage, that can be visualized as a line of stain

around the restoration, or with residual (arrested) caries, which can show a grey discoloration involving the restoration.

Radiographic assessment is regularly performed to screen for proximal primary or secondary caries lesions. Secondary caries at proximal or gingival locations in restorations are diagnosed by X-rays radiography with a variable angle in relation to the lesion. [117] However, the radio-opacity of restorative materials are radiopaque may hide the lesion completely or partially. [118] The risk stemming from such non detection largely depends on the progression speed of such lesions, which is so far not fully understood. The visual and radiographic assessment might be complementary when nonproximal and proximal surfaces are checked, respectively. Laser fluorescence-based instruments have been developed as an adjunct to visual lesion detection, not causing any radiation and allowing easy reexamination and monitoring of lesions and their activity. Overall accuracy was similar to that of radiographic detection, which makes it a potential alternative, especially in children. Quantitative light-induced fluorescence (QLF) generates images of the analyzed areas, with presumable carious tissues being less fluorescent than sound areas or restoration materials. [119] However, the value of this method for detecting secondary lesions might be limited in clinical routine since QLF is currently available for visible (nonproximal) surfaces. Moreover, given the fact that even on these visually assessable surface, QLF led to false-positive detections in nearly 4 of 10 cases, there should be severe doubts toward the suitability of this method for the outlined purpose. Moreover, the burnout that frequently occurs at the cervical margin also makes the interpretation difficult. [120]

Marginal defects and staining around the restoration are not predictive for secondary caries, [114] [121] [122] and are likely the main factors that lead to misinterpretations and possible overtreatment. For example, black and brown marginal staining can be misinterpreted as initial lesions and are more often detected in tooth-colored resin restorations than in amalgam restorations. [76] [91] Therefore, the diagnosis of recurrent caries lacks consistency, and the diagnostic variations among clinicians are impressive. [123] These differences reflect the subjective disparities that characterize the subsequent treatment.

To date, the clinical diagnosis of secondary caries invariably has resulted in the replacement of the restoration affected, on the basis of the often proclaimed advice: “in doubt, take it out”. However, the new guidelines recommend to repair and refurbish any localized defects at restoration margins, including clinically diagnosed secondary caries, rather than performing a total replacement. In the era of minimally invasive operative dentistry, the replacement of restorations should be preferably the last alternative for patients with a defective restoration, based on the available evidence for monitoring, refurbishment and repair of restorations. [124] For secondary caries, diagnostic criteria should reflect the best options for management based on the presence of cavitation and lesion activity, ensuring the best health outcome for the patient. [125] In this contest, the treatment has to take into account the extent of the lesion, examining three characteristics of carious lesions and specifically softening of the tissues, discoloration and wetness of the lesions. Subsequently, a small part of the resin-based composite material adjacent to the stained margins are removed and, if the defects did not extend deep into the tooth-restoration interface, the cavities are considered

suitable for repair using a conventional restorative technique. [126] Dental teaching programs related to localized defects on restorations, including secondary caries, indicate that repair of the restoration is adopted frequently as an alternative to total replacement. [83] The majority of dental schools consider repair a definitive measure and reported that an acceptable life span of repaired restorations is four years. [83]

2.3 PREVENTION OF SECONDARY CARIES BY SILVER

The oral hygiene habits of the patient are the primary factor that determines if secondary caries develops, not whether the restoration along the cavosurface margin can be considered as 'excellent', 'adequate' or 'deteriorated'. The prevention and preservation approach is significant as the caries development is a slow process. Hence, preventing early carious lesions by the removal of biofilm as well as the application of silver or placement of sealants is advised. [127] Nowadays, silver ions (Ag^+) are one of the most effective methods to control bacterial growth in a variety of medical applications including the prevention of caries disease.

Since ancient times, the silver ion has been known to be effective against a broad range of microorganisms. [15] For instance, vessels made of Ag have been used for water disinfection and food preservation since the time of the Persian kings [128]. Later, the Phoenicians, Greeks, Romans, and Egyptians adopted this practice. In 1869, Raulin described, for the first time, the antimicrobial activity of silver against *Aspergillus niger*. [128] It is currently accepted that Ag^+ is responsible for the antibacterial properties although they are relatively reactive. The binding of silver ions in the form of insoluble precipitates (AgCl), or during the interactions with proteins (e.g., albumin), causes a significant decrease of its antibacterial efficacy.

The dynamic development of nanotechnology in recent years has provided new challenges for fabricating silver nanoparticles (AgNPs). In general, silver atoms (Ag^0) on the surface of AgNPs, interacting with molecular oxygen or with other redox-active compounds, can be oxidized to silver oxide. [129] [130] [131] [132] [133] [134] The oxidation of silver oxide allows the release of silver ions in the environmental, and biological media

carrying out its antibacterial action. Therefore, AgNPs can also be used as a source of Ag^+ through the release process.

Due to the oligodynamic effect, the antibacterial activity of Ag^+ is directly proportional to its environmental concentration. Jung and colleagues compared the antibacterial activity of silver ions obtained in various ways and showed that silver ions produced in an electrolytic way are better antibacterial agents than those obtained by dissolving the silver compounds. [57]

Despite numerous studies conducted over the last decade, there are still considerable gaps in our knowledge about the specific mechanisms of antibacterial\antibiofilm action of silver ions. Several proposals have been developed to explain the inhibitory effects of Ag^+ ions on bacteria. Furthermore, the precise basis of their antibacterial activity has yet to be defined. This is mainly due to the pleiotropic effects of nano-silver on bacterial cells, which suggests multiple mechanisms of action on several cellular targets (Figure 12).

The most noticeable are:

(i) interaction with the bacterial cell envelope (destabilization of the membrane—loss of K^+ ions and a decrease of ATP level);

(ii) interaction with molecules inside the cell (e.g., nucleic acids and enzymes);

(iii) the production of reactive oxygen species (ROS).

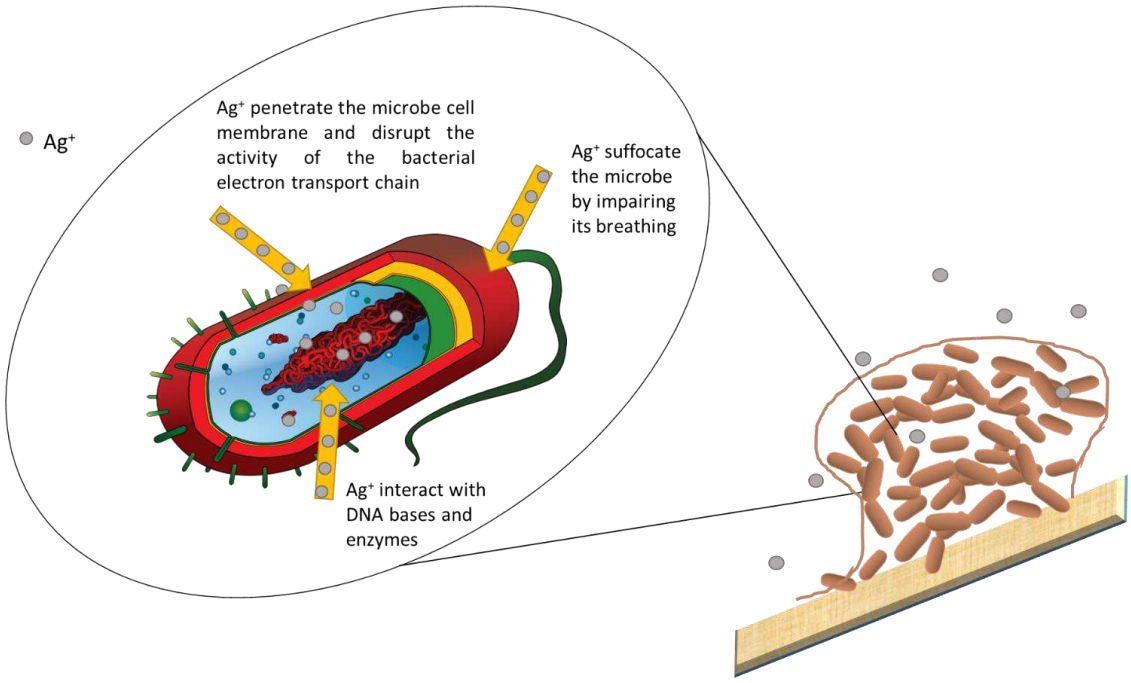


Figure 12. Schematic representation of the silver nanoparticle mechanism of action on the biofilm forming a microbial cell. Ag^+ adhere to the microbial cell surface and results in membrane damage and disruption of electron transport chain; Ag^+ penetrate inside the microbial cells and affect cellular machinery interacting with DNA bases and enzymes.

The positive charge of Ag^+ interacts with the negative charge of the microbial cell wall leading to disruptions in the structural morphology of the microbial cell. [135] [136] [137] [138] Jung et al. proved that the accumulation of Ag^+ in the bacterial cell envelope is followed by the separation of the cytoplasmic membrane from the cell wall in both Gram-positive and Gram-negative bacteria. [57] According to reference Jung et al., carboxyl groups (–COOH) in glutamic acid and phosphate groups in teichoic acid are mostly responsible for the binding of silver ions. However, the thickness and composition of the microbial wall is also one of the crucial factors in deciding the potency of the antimicrobial Ag. [139] The presence of a 30-nm-thick negatively charged peptidoglycan layer in gram-positive bacteria such as *Staphylococcus aureus* makes it less vulnerable to the silver compared to gram-negative bacteria such as *E. coli*

where the peptidoglycan layer is a scanty 3–5 nm. [140] [141] The thickness of the peptidoglycan layer and its negative charge renders the Ag^{+} ions inactive, thereby making the gram-positive bacteria highly resistant to the antimicrobial therapy. [142] [143] The gram-negative bacteria, on the other hand, have lipopolysaccharides in their cell membranes which protect the microbe from the chemical attacks and help maintain the structural integrity of the membrane. The negative charge on lipopolysaccharides promotes the adhesion of AgNPs to the membrane, thereby making the bacteria highly susceptible to the antimicrobial therapy. [144] The morphology and charge on the microbial membrane, therefore, render the gram-negative bacteria highly susceptible even at a lower antibiotic concentration. [145]

Silver ions possess a strong affinity towards the sulfur-containing proteins in the microbial cell wall. [146] These interactions are sufficient to irreversibly disassemble the structural integrity of the microbial lipid bilayer adversely affecting its permeability. [147] This restrains the microbial cell to regulate the membrane-based transport activity that, in particular, impairs the uptake and release of PO_4^{-2} ions and K+ ions. [148] Owing to the malfunctioning of the cell membrane, the loss of vital nutrients, cellular contents, and ATP is also inevitable leading to the cellular necrosis and cell death. [149]

The Ag^{+} ions intercalate between the purine and pyrimidine base pairs, disrupt the intermolecular H-bonds leading to the collapse of the double helical structure of DNA. [150] [151] The replication phenomenon is also impaired because the DNA of the silver-treated cells gets transformed from a relaxed state to the condensed state. This leads to the inhibition of cell division and reproduction in bacteria *S. aureus*. [152]

The biocidal mechanism of silver involves the damaging of the microbial membrane to penetrate the cell followed by the generation of ROS (reactive oxygen species) eventually impairing the cellular machinery. In order to develop a natural survival strategy against the host, some bacteria develop biofilms by adhering to a surface. [153] [154] It is also one of the contributing factors towards the development of antibiotic resistance and, hence, being difficult to control, augments the severity of the infection.

The cariogenic biofilm of secondary caries is similar or identical to that of primary caries. There are some reports targeting the biofilm formation in bacteria without affecting the viability of the mammalian cells. [155] For example, AgNPs capped with CMT (carboxymethyl tamarind polysaccharide) are known to inhibit the growth and biofilm formation of both gram-positive bacteria, *Bacillus subtilis*, and gram-negative bacteria, *E. coli* and *Salmonella typhimurium*, at concentrations much lower than the minimum inhibitory concentration (MIC) by altering the locating and expression of bacterial some cytoskeletal proteins. [156] [157] Nano-silver (average particle diameter 25.2 ± 4 nm) was found to effectively prevent the formation of *P.* reduction in the number of colony-forming units), suggesting that it could be used for the prevention and treatment of biofilm-related infections. [158] A decrease in the bacterial mass in biofilms of *E. coli*, *P. aeruginosa,*and *Serratia proteamaculans* was observed when the AgNP concentration was between 5 and 10 micrograms per milliliter. [159]

This raises the intriguing possibility of treating infections caused by biofilm-forming bacteria with Ag^{+}. In this contest, silver-containing dental materials (i.e. resins, fillers, adhesives, etc.) represent an alternative to

reduce the risk of caries acting as a delivery system to increase the silver levels in the proximity of restoration.

2.4 SILVER IN DENTAL MATERIALS

Silver was adopted for caries management and oral care in the early 1900s taking advantage of its disinfectant and antibacterial properties. Indeed, during the 19th century, silver has been gained a central role in tooth restoration as one of the main components in dental amalgams. However, its use in amalgams has been reduced since 1930 as they were progressively substituted by esthetic polymer-based resins. [160] With the evolution of nanotechnology, the interest in silver has been renewed, and several promising new technologies are currently under development, especially in dental materials. Indeed, AgNPs have been demonstrated a very high antimicrobial effect, in comparison with several antimicrobial molecules, in prosthetic materials, [161] adhesives, [162] [163] and implants, [164] in promoting caries arrestment [165] and preventing biofilm formation. [166] However, to be generally accepted as replacements for the traditional antibacterial agents, new material-formulation must be safe, or safer than the existing product, and more effective. [167] Additionally, any therapeutic agent should not compromise the integrity of the dental materials. AgNPs have shown a good biocompatibility [161] [168] and a synergistic action with several types of antibiotics [169] [170] [171] [172]. It appears, in fact, that bacteria are far less likely to acquire resistance against silver nanoparticles than other conventional and narrow-spectrum antibiotics because metals

may act on a broad range of microbial targets, and many mutations should occur for microorganisms to resist their antimicrobial activity. [173]

One of the major challenge for AgNPs involves their bacterial uptake (penetrability), taking into account that the penetrability of ions and nanoparticles are different. Indeed, AgNPs are able to penetrate through cell membranes more readily respect to ions, resulting in very high antimicrobial activity, [174] which is especially important since microorganisms in biofilms are more resistant to antimicrobial agents than planktonic pathogens. [175]

Specifically in dentistry, the preparation of nanoparticles must take into account the biofilm architecture and the mechanistic aspects of AgNPs. The nanoparticle's properties may affect its efficiency and interfere with its mechanism of action. Important aspects to be mentioned in this connection: (i) the diffusion of nanoparticles in biofilm exhibits an inverse relationship between effectiveness and size; nanoparticles over 50 nm are not able to penetrate the biofilm due to the relative self-diffusion coefficients in the biofilm, and this decrease exponentially with the square of the nanoparticle diameter. [176] In addition, (ii) charged nanoparticles do not diffuse easily through the biofilm, probably because the presence of phosphoryl and carboxyl groups on the surface of the bacteria, which gives the cell surface an electronegative character. [177]

The size and shape of AgNPs may also affect their bactericidal activity. Materials with a particle size of less than 10 nm have been shown to be the most effective against bacteria, [178] [179] while triangular NPs may be more bactericidal compared to those with spherical or needle-like morphology. [173] Indeed, according to Cheng et al., [17] [180] AgNPs ranging from 2 to 5 nm were able to penetrate on dentinal tubules,

representing a good possibility of inactivating residual bacteria on dentine. (Figure 13)

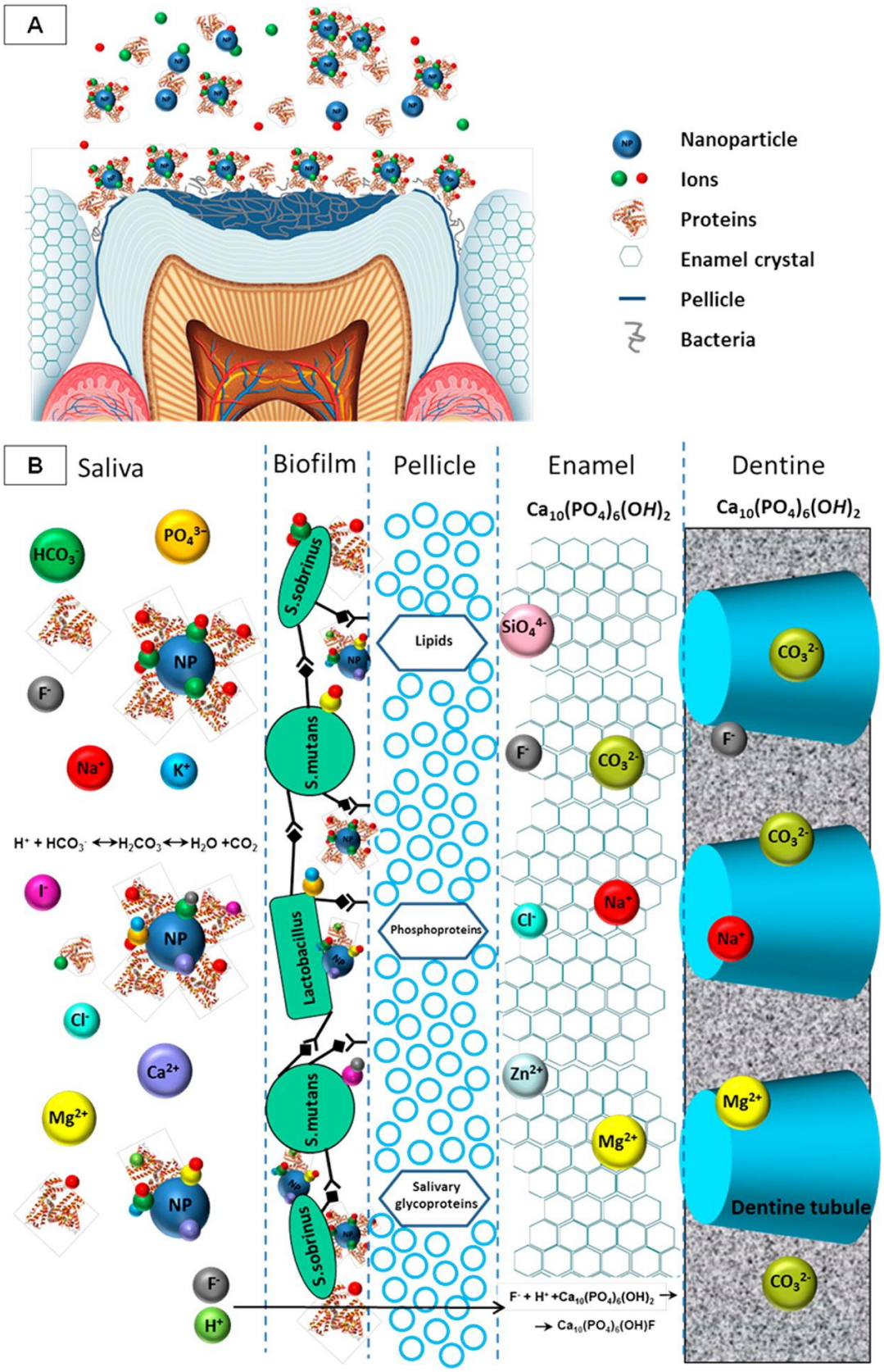


Figure 13. (A) Diagram shows the presence of NPs (isolated particles or agglomerates) in saliva and the structure of dental tissues. The pellicle covers the superficial layer of enamel, and the oral biofilm develops on the pellicle surface. The characteristic hexagonal shape of the enamel crystallites is apparent and also the presence of the dentinal tubules in the underlying tissue of dentine. The NP–ion–protein complexes do not adhere directly to the tooth surfaces, but adhesion occurs either to the pellicle layer or the developing biofilm. (B) Schematic diagram of the oral environment, oral biofilm, and dental mineralized tissues showing the distribution of NPs and ions. Natural saliva normally contains a range of ions and proteins. In the presence of NPs, NP–ion–protein complexes are formed. Oral conditions promote particle agglomeration that results in particle sedimentation onto the dental surfaces. The pellicle has a globular structure and its proteinaceous layer facilitates the adherence of the early colonizing species necessary for the oral biofilm development. The oral biofilm and pellicle act as diffusion/permeation barriers to NPs preventing them from reaching the enamel–pellicle interface. Certain ions (F^{-}, Cl^{-}, SiO_4^{4-}, Zn^{2+}) are more abundant near the external surface of enamel, while others (Na^{+}, Mg^{2+}, CO_3^{2-}) are found at higher concentrations near the dentino–enamel junction. The most commonly ions found in dentine are F^{-}, Na^{+}, Mg^{2+}, and CO_3^{2-}.
Adapted from Besinis et al. ACS Nano, 2015, 9 (3), pp 2255–2289 [181]

These characteristics promoted AgNPs incorporation into dental materials, such as acrylic resin, root canal fillings, implants, composite resin and adhesive systems.

Dentures, mostly constituted by poly(methyl methacrylate) (PMMA) acrylic resin [179], have their inner surface considerably rough, [182] and this roughness, allied to other factors (e.g., poor hygiene, xerostomy, and HIV infection), contributes to the emergence of denture stomatitis. [183] [184] This pathology, characterized by red focal area, mostly localized in palatal mucosa, is present in 50–70% of complete denture wearers, [185] [186] and it is frequently associated with Candida species colonization.

These fungi colonize denture surfaces forming a biofilm, [187] which acts as a key-factor to denture stomatitis development. [188]

The treatment of denture stomatitis is based on topical or systemic antifungical drugs, for example, fluoconazole and nystatin. [189] [190] However, this infection is often persistent, since antifungical resistance has been reported in Candida biofilms. [188] Moreover, it has been observed that Candida species present in biofilms are less susceptible to antifungical drugs than planktonic cells. [191] [192] Another problem related to denture stomatitis is that many geriatric prosthetic wearers present difficulties on keeping the denture clean, due to their reduced motor dexterity, memory loss, and cognitive impairment. [193]

Considering the aforementioned factors, denture stomatitis represents a challenge for dentistry, and methods for its prevention, should be encouraged. Accordingly, AgNPs have been satisfactorily incorporated into polymers used as tissue conditioners and as denture base. [194] [195] The action mechanisms of AgNPs-incorporated polymers is still unclear, since some authors attribute the antimicrobial effectiveness to the silver ions release [196] [197] and others to the direct contact between the material and the microorganisms. [198]

Acosta-Torres et al. [199] developed a PMMA containing 1 μg/mL of AgNPs and they compared this new compound to unmodified PMMA. It has been observed that PMMA-AgNPs specimens showed significantly less *Candida albicans* adherence compared to PMMA, demonstrating the antifungical potential of AgNPs incorporated to acrylic resin. Besides that, they evaluated the activity of mouse fibroblasts and human lymphocytes, and it has been shown that PMMA-AgNP compound does not present

cytotoxity or genotoxicity. These results suggest that the novel acrylic resin incorporated with AgNPs could be developed as a denture base.

In a study performed by Monteiro et al. [200] AgNPs were incorporated in a commercial acrylic resin, in different concentrations (0.05%, 0.5%, and 5% of AgNPs, by mass). The authors evaluated the mechanical properties of the modified resin, as well of the unmodified one (0% of AgNPs). Thereunto, the flexural strength test was performed, and it was observed that all the groups presented very similar flexural resistance values, suggesting that AgNPs incorporation does not affect the mechanical properties of acrylic resin.

When dentures are ill-fitted is recommended recovering his base with tissue conditioners, which are easily degradable with time and occasionally susceptible to microbial colonization. [201] Thus, AgNPs incorporation could also be profitable in this material and not only in dentures base.

Accordingly, Nam [193] has incorporated AgNPs into a commercial tissue conditioner, in the following concentrations: 0.1%, 0.5%, 1.0%, 2.0%, and 3.0%. Their inhibitory effect was evaluated against Staphylococcus aureus, Streptococcus mutans, and *Candida albicans* after 24 h and 72 h. The authors have reported that the modified tissue conditioner presented antimicrobial properties even at lower concentrations, that is, 0.1% (for *S. mutans* and S. aureus) and 0.5% (for *C. albicans*).

Several studies have demonstrated that bacteria are the main etiologic agent of pulpal infection and periradicular lesion formation. [202] [203] The microbiota of infected root canals is polymicrobial and is dominated by Gram-negative anaerobes. [204] [205] It has been demonstrated that

the presence of residual bacteria in root canal is connected with significantly higher rates of treatment failure. [206]

Since elimination of bacteria in root canals is the key to treatment success, [207] endodontic materials should ideally provide some antimicrobial activity, [208] [209] in order to improve the prognosis of endodontically treated teeth. [210] Various materials have been used as root canal fillings, among which gutta-percha is one of the most used. [207] This material has been proved to present slight antibacterial property, provided by the zinc oxide in its components; however, this does not provide to gutta-percha an effective bactericidal potential. [210]

Accordingly, Iranian researchers have introduced nanosilver-gutta-percha, as an attempt to improve the antibacterial effect of gutta-percha. The new material, which is standard gutta-percha coated with AgNPs, has demonstrated significant effect against *Enterococcus faecalis*, *Staphylococcus aureus*, *Candida albicans*, and *Escherichia coli*.

Besides that, Shantiaee et al. [211] have tested the biocompatibility of this new material, by comparing the cytotoxicity of nanosilver-coated gutta-percha and normal gutta-percha on mouse fibroblasts. In this study, after 24 hours, nanosilver-coated gutta-percha presented cytotoxicity similar to normal gutta-percha and, after one week, it reached the lowest level of cytotoxicity among the tested materials.

Other important step in the endodontic treatment is the chemomechanical debridement of pulpal tissue and pathogenic bacteria. In this stage, irrigant solutions should be used, for dissolving tissue and disinfecting the root canal system. [212] For this purpose, sodium hypochlorite (NaOCl) has been used for more than 70 years, and it remains as one of the most common solutions. [213] However, if NaOCl

passes beyond the apex, it is extremely toxic to the periapical tissues. [214]

In this context, Lotfi et al. performed a study comparing the antibacterial effect of NaOCl and AgNP solution against *Enterococcus faecalis*, which is a bacterium often isolated from failed endodontic treatment cases. [214] Authors have observed that there were no significant differences among 5.25% NaOCl and 0.005% AgNPs, suggesting that this solution, in a remarkably lower concentration, possesses the same bactericidal effect as 5.25% NaOCl; hence, it could be used as a new intracanal irrigant.

Another important endodontic material is the mineral trioxide aggregate (MTA), used in many indications such as perforations sealing, external/internal root resorption repair, and apexification. [215] [216] In spite of being a material of wide application, the antimicrobial properties of MTA are controversial, and they seem to be limited. [217] [218]

Aiming to improve its antimicrobial potential, Samiei et al. [219] modified MTA by adding AgNPs, at 1% weight. Its effect against oral bacteria and fungi species was assessed. Results have showed that AgNPs-containing MTA possesses higher antimicrobial effect against *Enterococcus faecalis*, *Candida albicans*, and *Pseudomonas aeruginosa*, compared to unmodified MTA.

Although AgNP is a promising antimicrobial, there are only a few studies employing it in endodontic materials. And considering that endodontic treatment success is highly connected to the bacteria elimination, researches involving AgNPs incorporation to root canal filling materials and intracanal irrigators should be encouraged.

Titanium (Ti) implants, widely used in dentistry, usually present infection around their surface, which remains one of the most important

complications in implantology. [220] [221] Several measures have been proposed to avoid bacterial contamination, such as implant disinfection and aseptic surgical protocols; nevertheless, bacterial invasion often occurs after surgery. [222]

In order to prevent biofilm formation over implants surface, antibacterial coatings have been developed; however, most of them present poor long-term antibacterial action and also the possibility of generating resistant strains after prolonged use. [223] In this context, AgNPs incorporation to implant surface has been suggested, [224] [222] since it would be possible to produce coatings with long-term antibacterial properties by controlling Ag release.[225]

In study performed by Zhao et al., [225] AgNPs were incorporated into titania nanotubes (TiO2-NTs) on Ti implants, in a process involving silver nitrate immersion and ultraviolet radiation. The antibacterial effect against *Staphylococcus aureus* was assessed, and results have shown inhibition of planktonic bacteria during the first several days. Moreover, AgNPs-coating Ti implants have presented ability to prevent bacteria adhesion for up to 30 days, which are considered sufficient time to prevent post-infection in early stages.

In a similar study, Flores et al. [226] have evaluated the antibacterial activity of AgNPs against *Pseudomonas aeruginosa*. It has been reported that the number of total cells found on AgNP-modified implants represents only 20% of those attached to unmodified surfaces. This data suggests that the incorporation of AgNPs on Ti implants is an efficient method to protect implant surface against pathogen colonization.

As important as the antibacterial potential is the biocompatibility of these modified implants. Aiming to evaluate this property, Lu et al. [227] have

tested Ti implants incorporated with different concentrations of AgNPs (0.5, 1, 1.5, 2 M). For all the tested concentrations, osteoblasts started to adhere on the coatings after 1 day of culture and spread well until 7 days of culture. However, after this, the inhibitory effect of 1 M Ag on cell proliferation became significant, suggesting that AgNP coatings with low amounts of silver were more favorable for osteoblasts growth.

In order to prevent or reduce biofilm accumulation over composite and in the restorations margins, were developed antimicrobial restorative materials through the incorporation of AgNPs to composite resins [228] [83] and adhesive systems. [83] These materials are multiphase substances composed of an organic polymer matrix, filler particles, coupling agent (silane), and the initiator-accelerator of polymerization, and AgNPs incorporation is based on the modification in the filler components. [229]A research developed by Cheng et al. [230] reported the effect of AgNPs incorporation, at different concentrations, to a composite resin, in order to investigate its mechanical properties and biofilm formation. In this study, composites were synthesized with AgNPs at 0.028, 0.042, 0.088, and 0.175%. Mechanical properties of composites with AgNPs at 0.028% and 0.042% were similar to those with no AgNPs. Besides that, counts of colony forming units for total streptococci and *S. mutans*, using AgNPs at 0.042%, were 75% smaller than the control group without AgNPs. These data suggest that AgNPs incorporation to composite resins enables good mechanical properties and notable antimicrobial potential, even at low concentration. In order to evaluate the influence of AgNPs incorporation on bond strength to the dental substrate, Melo et al. [231] added AgNPs, at 0.1% by mass, to an adhesive system. The results have shown that AgNPs did not compromise the bond strength and that it

decreased metabolic activity on biofilm, compared to the control group without AgNPs. In this study, it was also observed a reduction of the number of total microorganisms, total streptococci, and mutans streptococci. Li et al. [232] performed a study incorporating of AgNPs, at 0.05% by mass, to an adhesive system, aiming to assess bacterial inhibition provided by this antimicrobial. It has been reported that AgNPs reduced the number and acid lactic production on biofilm over and away to the adhesive surface, evidencing that AgNPs-containing adhesives enable long-distance antibacterial potential. Another important aspect to be assessed is the biocompatibility of AgNPs-containing restorative materials. Accordingly, Zhang et al. [83] have studied the effects of AgNPs incorporation, at 0.05% by mass, to a primer and an adhesive, regarding human gingival fibroblast viability. It has been shown that AgNPs addition did not affect the cytotoxicity of primer and adhesive tested, evidencing the clinical applicability of this antimicrobial. Based on above mentioned studies, it is possible to say that the antibacterial effects of AgNPs-containing restorative materials might decrease the development of recurrent caries, to increase the longevity of tooth restorations, and to be effective in decreasing the formation of bacterial biofilms on teeth and restorations, without compromising mechanical properties and cytotoxicity of composite resins and adhesive systems.

CHAPTER 3. INNOVATIVE DENTAL RESTORATIVE MATERIALS

3.1 RESTORATIVE DENTAL MATERIALS

Dental structure, compromised by trauma or dental caries, can be direct restored using biocompatible synthetic materials (i.e. esthetic resin-based composites, ion-release glass ionomer cements, etc.). [233] [234]

Although the restorative materials had significant evolvement in the past few decades, the high rates of treatment failure suggest that the current restorative approaches are not yet optimized and have a potential for improvement. [235] [236]

In fact, dental restorative materials placed in oral cavity are subjected to aggressive attack by bacteria, that biodegrade material components leading to the impairment of the marginal integrity, and development/progression of secondary caries. [237] [238] [239]

Currently, four classes of direct-placement restorative materials exist:

a. amalgam;
b. composites;
c. glass ionomers;
d. resin ionomers.

Advantages and disadvantages of each class are summarized in Table 1.

Factor	Amalgam	Glass ionomers	Resin ionomers	Composites
Cavity preparation	Sound tooth structure to be removed for material manipulation	Adhesive bonding allows removal of less tooth structure	Adhesive bonding allows removal of less tooth structure	Adhesive bonding allows removal of less tooth structure
Restoration use	Especially posterior teeth	Nonload bearing areas	Nonload bearing areas	Esthetic zone
Clinical conditions	Wide range tolerance	Well controlled field of operation	Well controlled field of operation	Well controlled field of operation
Resistance to fracture	Brittle, chips at the edge	Low	Low to moderate	Moderate
Durability	Good to excellent	Good in nonload bearing ; Poor in load bearing	Moderate to good in nonload bearing	Good in small to moderate restorations
Wear resistance	High	Low on occlusal surfaces	Low on occlusal surfaces	Moderate
Moisture tolerance during placement	Moderate	Very low	Very low	Very low
Leakage	Moderate	Low	Low with proper bonding	Low with proper bonding
Recurrent decay	Similar to other materials	Similar to other materials	Similar to other materials	Dependant on tooth-material bond
Esthetics	Poor	Good	Good	Excellent
Fluoride releas	No	Yes	Yes	No
Placement time compared to amalgam	1X	2X	2X	2X
Material cost compared to amalgam	1X	30% more	30% more	30% more
Failure rate (%)	2.2	7.6	3.1	3.5
Approx. life of restoration (years)	10	4	2	7
Potential environmental impact	Yes	Not known	Not known	Not known
Operator skills	Material predictable and forgiving, dentist comfortable with usage	Material unforgiving, dentists experience required while using	Material unforgiving, dentists experience required while using	Material unforgiving, dentists experience required while using

Table 1. Direct placement restorative materials- brief overview

Dental amalgam

Dental amalgam is a dental filling material served as an excellent and versatile restorative material for many years, despite periods of controversy. Indeed, the combination of reliable long-term performance in load bearing situations and low cost is unmatched by other dental restorative material.

Currently, dental amalgam is a mixture of metals, consisting of liquid mercury (approximatively 50% by weight) and a powdered alloy composed of silver (40–70%), tin (12–30%,) and copper (12–24%). It may also include indium 0–4%, palladium 0.5% and zinc up to 1%.

Besides being prepared easily, dental amalgam is relatively inexpensive compared to most other materials used in dental treatment, and the longevity of dental amalgam restorations is relatively high. [240] It is the only dental material known for marginal-sealing capacity due to the corrosion products released from dental amalgam restorations. [241] It also tolerates a wide range of clinical placement conditions such as wet fields (for zinc-free products).

Today, dental amalgam is used in the following situations:

1. in individuals of all ages,
2. in stress-bearing areas and in small-to-moderate sized cavities in the posterior teeth,
3. when there is severe destruction of tooth structure and cost is an overriding consideration,
4. as a foundation for cast-metal, metal-ceramic, and ceramic restorations,
5. when patient commitment to personal oral hygiene is poor,

6. when moisture control is problematic with patients, and
7. when cost is an overriding patient concern.

It is not used when:

1. esthetics are important, such as in the anterior teeth and in lingual endodontic-access (root canal) restorations of the anterior teeth,
2. patients have a history of allergy to mercury or other amalgam components, and
3. a large restoration is needed and the cost of other restorative materials is not a significant factor in the treatment decision.

However, toxicity of dental amalgam due to mercury has always been a concern. In fact, people with amalgam have higher concentrations of mercury in various tissues (including blood, urine, kidney, and brain) than those without amalgam. Also, a small proportion of individuals may manifest allergic reactions to these restorations.

However, current evidence does not preclude the use of amalgam in dental restorative treatment. The choice should be based on patient characteristics such as primary or permanent teeth, pregnancy, the presence of allergies to mercury, and the presence of impaired renal clearance.

Dental composites

Dental composites were developed as an aesthetic alternative to dental amalgam and were intended for restorations of anterior teeth, as they were unable to withstand masticatory loads on posterior teeth. However, these restorative materials suffered from various issues:

1. low abrasion resistance;
2. poor mechanical strength;

3. color instability.

Nowadays, composite materials have become a versatile material class suitable for filling of all restoration classes, as well as the material of choice for direct restorative treatment.

Glass ionomers

Glass Ionomer Cements (GICs) (also referred to as polyalkanoate cements or Aluminosilicate-polyacrylic acid cements), glassy powder based on acid soluble calcium fluoroaluminosilicate and polyacrylic acids with copolymers in liquid form used for dental fillings and luting cements, capable of stabilize teeth calcium-deficient carbonated hydroxyapatite by ion exchange. [242] The carboxylic groups of GICs replace the phosphate ions of the teeth hydroxyapatite surface to establish ionic bonds with calcium ions derived from the partially dissolved crystals. [243] GICs interfere with subgingival biofilm formation, decreasing the irritation of the periodontal tissues. [244] Examples of commercialized GICs are Fuji IX GP, Ketac N100, Dyract Extra and Wave. [83]

Resin-modified glass ionomer cements (RMGICs), formed starting from GICs through acid-base reaction and free radical polymerization mechanisms. RMGICs have highly packed filler composition (~69%), of which approximately two-thirds are nano-fillers useful to improve thermo-mechanical properties and to set a controlled release of active principles. [245]

Resin

Acrylic resins, a group of related thermoplastic or thermosetting polymeric substances derived from acrylic acid, methacrylic acid or other acrylates

that are widely diffused to fabricate dentures due to their excellent biocompatibility, aesthetical properties and easy handling. [246] However, acrylic polymers are susceptible to wear and shrinkage due to the uncomplete conversion of the monomers during polymerization reaction and these phenomena can lead to poor marginal seal and fractures. [247]

Composite resins, fillers for tooth cavities made of synthetic monomers able to form the resin matrix (e.g. dimethacrylate), reinforcing fillers (e.g. radiopaque glass, quartz or silica), chemical agents (to promote the polymerization reaction) and silane. [248] Composite resins are the most commercialized materials for restoration, as shown by the widespread utilization of triethylene glycol dimethacrylate(TEGDMA) or urethane dimethacrylate (UDMA) blended with bisphenol-glycidyl methacrylate (bis-GMA). [83]

Inorganic fillers, such as silicon dioxide, quartz in its crystalline state, aluminium oxide (Al2O3), titanium dioxide (TiO2), zinc oxide (ZnO) and zirconium oxide (ZrO2), that are easy to handle but not immune from marginal degradation during time and the consequent gap formation between the tissue/material interface. [249]

Fluoroapatites, phosphate minerals formed by fluoride binding to calcium hydroxyapatite (HA) used as fillers able to conduct enamel mineralization and interfere with bacterial metabolism and dental plaque acidogenicity. [83] Such materials can also remineralize dentin and help prevent secondary caries. [83]

Giomers, restorative materials derived from composites and GICs, constituted by a glass core coated with 3 semi-permeable layers that protect the durability and aesthetics of the glass, while allowing ions to

travel freely between the glass core and the oral environment. Such materials are often fluoride modified. Giomers are widely used in toothpaste, topic gels, mouth rinse, and fluoridated water. [83]

Table 2 recaps the characteristics of the described dental materials.

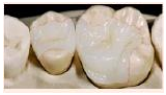
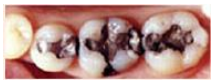
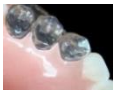
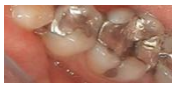
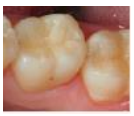
Material	Description	Uses	Appearance	Risk/benefits	Ref.
Porcelaines 	Ceramic elements that are bonded into place.	Fillings, Crowns, Inlays.	Tooth coloured.	Brittle. Well tolerated. No harmful.	[250]
Amalgams 	Alloys of mercury with silver and other metals. Harden by chemical reaction.	Fillings.	Silver coloured.	Well tolerated. Rare sensitivity and allergies. No harmful.	[251]
Acrylic resins 	Thermoplastic substances able to cross-link	Dentures.	Transparent.	Excellent biocompatibility.Easy handling.	[246]
Composite resins 	Mixtures of glass filler and acrylic that harden by chemical reaction.	Fillings, Sealants.	Tooth coloured.	Well tolerated. Exposure to a small amount of estrogen-like materials.	[248]
Inorganic Fillers 	Metal oxides of various elements.	Fillings.	Colour of the metal used.	Easy to handle. Can have marginal degradation during time.	[249]
Fluoroapatites 	Phosphate minerals formed by fluoride binding to calcium hydroxyapatite.	Fillings.	Opaque.	Well tolerated. Rare sensitivity and allergies. No harmful.	[83]
Glass ionomers 	Divided in Glass Ionomer Cements, Resin-modified Glass Ionomer Cements and Giomers.	Fillings, Sealants, Cements for crown and bridges.	Opaque.	Well tolerated. No harmful. Low resistance to wear and fracture.	[242]

Table2. Restorative materials

However, no restorative materials were immune from marginal degradation during time and/or gap formation between the tissue/material interfaces. For this reason, there is a need of innovative restorative materials able to prevent the recurrence of caries and to repair and/or regenerate the defected dental tissue.

3.2 NANOTECHNOLOGICAL APPROACH FOR BIOACTIVE DENTAL MATERIALS: LDH AND SILVER NANOPARTICLES

Science and technology in the 21st century rely heavily on the development of new materials, which are expected to respond to the environmental changes and manifest their own functions according to the optimum conditions. [252] Great attention has recently emerged around the composites in which layered fillers are dispersed at a nanometric level in a polymeric matrix. Such composites possess unusual properties, very different from their microscale counterparts.

New smart dental materials exhibiting antibacterial and antibiofilm function were developed. These light activated, nanofilled restorative materials were able to release silver ions when intraoral pH values drop below the critical pH of 5.5, counteracting the demineralization process of the tooth surface. The caries protective effect of these materials may be related to the material's ability to release adequate amounts of silver ions for sustained periods of time and during acidic attack. For example, LDHs loaded with fluoride (LDH-F) were incorporated into commercial light-activated restorative material Bis-GMA/TEGDMA dental resin.

Layered double hydroxide nanoparticles (LDH) are one of the best nanocarriers due to their excellent biocompatibility, low toxicity, low cost, ease of preparation, biodegradability, pH dependent stability, and moreover their drug release rate can be tuned by changing the environmental conditions. [253] A dental resin with improved physical and biological properties and, in addition, able to release low amount of fluoride in a controlled and tunable way for a long period of time was obtained using visible-light cured composites based on photo-activated

Bis-GMA/TEGDMA matrix, containing a filler based on an hydrotalcite-like compound intercalated with fluoride ions. Such composite have no initial toxic fluoride 'burst' effect and levels of fluoride release remain relatively constant over time. This type of delivery is obtained by a matrix-controlled elution and elicits the beneficial effects of low amount of fluoride on hDPSC differentiation.

Smart composites containing ACP (amorphous calcium phosphate) were also developed. ACP is one of the most soluble of the biologically important calcium phosphates, exhibiting the most rapid conversion to crystalline hydroxyapatite (HAP). ACP, when integrated into specially designed and formulated resins to make a composite material, has an extended time release nature to act as a source for calcium and phosphate which will be useful for preventing caries. ACP has been evaluated as a filler phase in bioactive polymeric composites. In fact, active restorative materials that contain ACP as filler encapsulated in a polymer binder may stimulate the repair of tooth structure because of the releasing of significant amounts of calcium and phosphate ions in a sustained manner. Moreover, ACP have excellent biocompatibility, and the ACP containing composites release calcium and phosphate ions into saliva milieus, especially in the oral environment caused by bacterial plaque or acidic foods, that can be deposited into tooth structures as apatitic mineral, which is similar to the hydroxyapatite (HAP) found naturally in teeth and bone. ACP at neutral or high pH remains as ACP. When low pH values (at or below 5.8) occur during a carious attack, ACP converts into HAP and precipitates, thus replacing the HAP lost to the acid. So, when the pH level in the mouth drops below 5.8, these ions merge within seconds to form a gel. In less than 2 minutes, the gel becomes

amorphous crystals, resulting in the release of calcium and phosphate ions [254] [255]. This response of ACP containing composites to pH can be described as smart.

Layered double hydroxide (LDH)

Hydrotalcites, also defined anionic clays or layered double hydroxide (LDH), are magnesium aluminium-hydroxycarbonate naturally occurring in lamellar warped form. They were discovered in Switzerland in 1842, but their molecular formula, $Mg_6Al_2(OH_{16})(CO_3) \cdot 4H_2O$, was published for the first time only in 1915 by Manasse. In 1942, Feitknecht introduced the term "double-layer compound" assuming that the synthesized compounds with hydrotalcite structure are constituted of intercalated hydroxide layers. This hypothesis was confirmed by Allmann and Taylor, who demonstrated that the cations are co-located in the layers while carbonate anions are intercalated together with water molecules. Overall, the structure of hydrotalcite is comparable to brucite, ($Mg(OH)_2$), where each Mg^{2+} ion is bound to six OH^- ions with octahedral coordination. In particular, the octahedra share an angle, forming layers organized on top of each other and bound together by hydrogen bonds. If part of Mg^{2+} ions is substituted by trivalent ions of suitable size, such as Al_3^+, layers become positive and this charges are balanced by intercalated anions (the most common is carbonate). Water molecules in interlayers are binded to layers and anions by hydrogen bonds. (fig.14) [256] Various combinations of LDH are obtained changing metals during synthesis processes. Below are listed the most used:

- DivalentMetals = zinc (Zn^{2+}), cobalt(Co^{2+}), nickel (Ni^{2+}), manganese (Mn^{2+})
- Trivalent Metals =chromium (Cr^{3+}), iron (Fe^{3+}), vanadium (V^{3+}), cobalt (Co^{3+}) e gallium (Ga^{3+})[257].

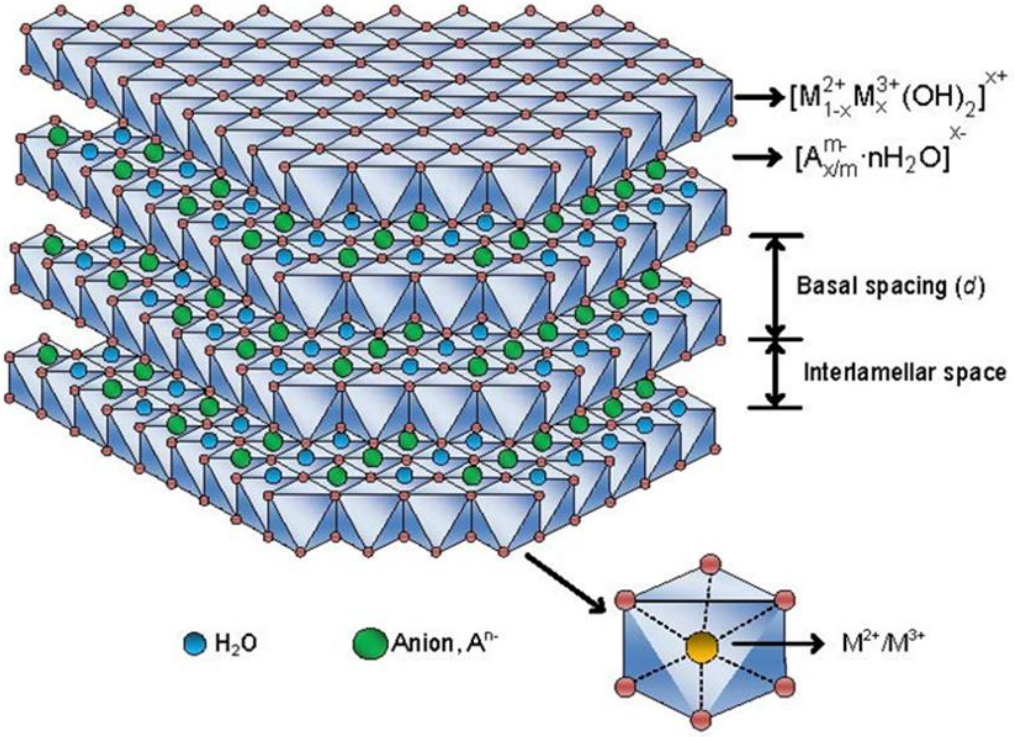


Figure 14. Schematic representation of LDH structure (Reproduced from Tronto et al., 2013[258]).

The loading of anions interposed between lamella sheets is carried out by two simple methods: ion-exchange or co-precipitation. [256] LDH/polymer/anion complexes were firstly evaluated for pharmaceutical application as delivery carrier for anti-cancer drugs. [257],[83] In fact, the incorporated negative charged molecules can be drugs, bioactive anions or nucleic acids, substances able to elicit a therapeutic action. Moreover, potential fillers can be composed also of nanosized LDHs (NPs-LDH). Several studies have demonstrated nano-LDHs biocompatibility and activity. In fact, observations on Human Osteosarcoma Cells confirmed

LDH biocompatibility and defined its intracellular trafficking. In particular, microscopy analysis showed the size dependence of the cellular uptake of LDH nanoparticles. Specifically, 50 nm NPs-LDH followed lysosomal degradation pathway, whereas 100 nm NPs-LDH were not degraded and were probably exocytosed from the Golgi. [259]

Mouse Motor Neuron uptake studies confirmed the importance of LDH nanoparticles dimensions in cellular localization. Two different LDH-NPs were analyzed using fluorescein isothiocyanate labeling: 20 nm CO_3-LDH and 180 nm NO_3-LDH. Confocal laser microscopy results showed that 20 nm LDHs-NPs were localized in nucleus, while 180 nm LDH-NPs in the cytoplasm [260]. The release kinetics is a further characteristic that can be optimized in loaded LDHs. Different chemical compositions of LDHs exhibit distinct adsorption and release kinetics of loaded drugs or anions. [261],[262] Indeed, other crucial design parameters are the pH of the solution containing the anions to load and that of the fluid in which their release occurs. Strongly basic pH leads to a wide presence of OH^- ions that competes with anions during anionic exchange in loading/release process while acid pH triggers LDH layers degradation, releasing the anion in few minutes. For example, LDH release capability in acid conditions is used in enteral administration (gastric fluid pH is 1.2). [83] LDHs release capability was also investigated according to their concentration in resin in order to modulate the release kinetics. Hypothetically, concentration optimization must be related with appropriate LDH delamination, to guarantee anionic exchangers accessibility and to confer a right degree of rigidity to the resin. Usually, nano-filler concentration inside the resin varies from less than 3% w/w to 20% for larger size ones. [263] Indeed, Rojas and co-workers demonstrated that low hydrotalcites concentrations induce an

uncontrolled release, due to excessive layers slip. In fact, low hydrotalcites concentrations compressed resins in platelets showed an initial release without classical burst effect and uncontrolled kinetics. [264] Finally, LDHs as fillers were also exploited for industrial composites due to their capability to improve the mechanical properties (rigidity and flexibility) of the material in which they are inserted. In addition, industrial LDH composites are also able to protect the material from degradation triggered by UV rays, since they are absorbed between the layers. [265] Such feature is interesting in dentistry due to the widespread use of light-curing resins in restoration.

For all these properties, LDH has been chosen as filler in our experimental dental adhesive resin as well as to exploit the possible silver antibacterial activity.

The experimental design of the thesis was planned in order to obtain a new dental adhesive with improved bioactivity and adequate mechanical properties. The project idea was to take advantage from a delivery of low amount of silver for a long period of time in order to obtain strong antibacterial effect avoiding cytotoxicity of high silver concentration. This goal can be obtained by using silver nanoparticles loaded Layered double hydroxide. Such antimicrobial device can give continuous protection against bacteria and microbes by not allowing them to grow on the surface avoiding biofilm formation. For another, at the present, very few researches have been done in the field of antibacterial dental adhesive preparation using layered double hydroxide.

CHAPTER 4. MATERIALS AND METHODS

Preparation of layered double hydroxide (LDH)

The LDH in the nitrate form [Mg0.65Al0.35(OH)2](NO3)0.350.68H2O (LDH-NO3) was prepared by ammonia precipitation to exclude CO32– ions from the interlayer region. In a typical preparation, a mixed metal nitrate solution (1 M) containing the stoichiometric requirement ratio of Mg2+ and Al3+ ions, was added dropwise to a solution of ammonia (1 M) with vigorous stirring at ambient temperature (25–27°C). During the addition, the solution's pH was adjusted to 9.0±0.5 with 1 M NaOH. The resulting white precipitate was aged for 24 hours at room temperature with continuous stirring. The solid obtained was separated from the supernatant by centrifugation, washed several times with decarbonated, deionized water until the pH of the wash was neutral, and dried in an oven at 60°C.

Anion exchange of MgAl-($NO3^-$) LDH by citrate ($C_6H_5O_7^{3-}$)

The citrate ion exchanged MgAl-LDH was performed by the de-intercalation of the nitrate ions from the as prepared LDH by treating it with a salt–acid mixed solution as reported by Liu et al. [266] In this condition, the inorganic citrate anion ($C_6H_5O_7^{3-}$) readily replaces the weak nitrate ion. 1 g of the MgAl-LDH sample was added into 60 ml of aqueous solution containing 8.8 g of tri-sodium-citrate (0.03 mol) and was stirred for 18 hours at room temperature. The solid was separated from the supernatant by centrifugation, washed several times with deionized water and anhydrous ethanol, and dried in an oven at 60°C for 24 h.

Preparation of Ag nanoparticle deposited Mg-Al LDH

Monodisperse Ag nanoparticle supported LDH was prepared by the reduction of an aqueous silver salt solution by intercalated citrate ions.

The mechanism of reaction could be expressed as follows: [267] [268]

$4Ag^{+} + C_6H_5O_7Na_3 + 2H_2O \rightarrow 4Ag^{o} + C_6H_5O_7H_3 + 3Na^{+} + H^{+} + O_2\uparrow$.

In particular, 500 ml of 0.170 g (1 mmol) of $AgNO_3$ solution in double-distilled water was heated to boiling at 90°C. Then, 0.5 g of citrate exchanged LDH was added and stirred for 1 hour, in the dark. The reaction was complete at room temperature and the resulting precipitate was collected by repeated centrifugation, washing with water and finally dried at room temperature in a desiccator.

Preparation of resin containing Ag-LDH

The Ag-LDH (with mass fraction of 0.5, 1, and 5%) was added into a commercial light-activated restorative material (CR) provided by Kerr s.r.l. (Italy), which consisted of bisphenol-A glycidyldimethacrylate (Bis-GMA), tri-ethylene glycol dimethacrylate (TEGDMA), camphorquinone (CQ), ethoxylated bisphenol A dimethacrylate (EBPADMA) and glass fillers. The samples are coded CR-Agx, where x is the percentage by weight of the inorganic solid Ag-LDH in the commercial resin. CR composite resin was used as a control. Specimen disks 14 mm in diameter and 1 mm thick were fabricated using steel molds. The composite obtained were cured by photo-polymerization using a visible light curing unit (Optilux 380, distributed through KERR, USA; irradiated diameter: 11 mm) with an

irradiation time of 120 s. During the experiment, the light intensity was maintained at 550 mW/cm^2.

Before incubation with bacteria, all the CR-Agx resins were gas sterilized using ethylene oxide.

Characterization and evaluation

X-Ray Powder Diffraction (XRPD)

XRPD patterns were recorded, in reflection, with an automatic Bruker diffractometer (equipped with a continuous scan attachment and a proportional counter), using the nickel filtered Cu Ka radiation (l = 1.54050 A°) and operating at 40 kV and 40 mA, step scan 0.058 of 2u and 3 s of counting time.

Fourier Transform Infrared Analysis (Ft-Ir)

Infrared absorption spectra were obtained by a Bruker spectrophotometer, model Vertex 70, with a resolution of 4 cm^{-1} (32 scans collected).

Dynamic-Mechanical Analysis

Dynamic-mechanical properties of the samples were performed in triplicate with a dynamic mechanical thermo-analyzer (TA instrument-DMA 2980). The samples were tested by applying a variable flexural deformation in dual cantilever mode. The displacement amplitude was set to 0.1%, whereas the measurements were performed at the frequency of 1 Hz. The range of temperature was -50 to 150 °C, scanning rate of 3 °C/min.

Silver release test

Weighed disks of all samples were placed at 37°C under magnetic stirring in physiological medium and artificial saliva medium (SAGF, 15 ml). [269] SAGF was prepared from calculated amounts of chemicals supplied by Sigma-Aldrich (Milan, Italy), according to the procedure described in the literature. [270] [271] After the time intervals (every hour for 8 h, then every day for 10 d, and then every week for 3 wk), the free silver ion concentration (ppm) were quantified using an inductively coupled plasma mass spectrometer model ELAN DRC II (Perkin Elmer-Sciex, Norwalk, CT, USA), operating with high-purity argon (99.999%, White Martins - Praxair, Bauru, São Paulo, Brazil). Each specimen was inserted in a polypropylene Falcon tube (Becton Dickinson, Franklin Lakes, New Jersey, USA) to a final volume of 10 mL (20-fold dilution) with a solution containing 2% HNO_3. Analytic calibration standards were prepared at a concentration ranging between 0 and 100 μg/L in the same diluent. After the specimens had been prepared, they were injected directly into the device, and the results were expressed in μg/L. The determination of each test solution was performed in triplicate (n=9).

Bacterial strains and growth conditions

Pseudomonas aeruginosa PAO1 (ATCC® BAA-47™), and *Staphylococcus aureus* (ATCC® 25923) were purchased by American Type Culture Collection (ATCC, Italy), and growth as supplier's instructions.

In addition, saliva from 3 volunteers (without active caries or periodontal disease) was used to produce salivary bacteria. Oral bacterial sample was collected using a sterile cotton bud and dissolved into 500μl phosphate

buffered saline (PBS) buffer (0.12M NaCl, 0.01M Na2HPO4, 5mM KH2PO4 [pH 7.5]). An aliquot (100 µl) of bacterial suspension was spread on Nutrient Broth (NB, Oxoid, Basingstoke, Hants, UK) agar plate, and growth overnight at 37°C. Bacterial colonies with distinct morphology were cultured on NB liquid medium and subjected to biochemical tests specific for their identification and characterization(API®/ID32, bioMérieux, Grassina, Italy). In particular, *Streptococcus spp.*, *Bacteroidesfragilis*, and *Staphylococcus epidermidis* were identified and grown at 37°C in non-selective NB.

Minimum inhibitory concentration (MIC) determination

Bacterial strains were grown overnight (18 h) and sub-cultured in 5 mL of sterile NB broth to log phase (OD600 0.7–1.0). Then, individual wells of sterile, 96-well flat-bottom polystyrene TCPs were filled with 100 µL of diluted culture (at OD600 0.1), and CR-Agx were added. After a 48-h incubation, inhibition of bacteria growth was assessed by measuring the OD600 using a microplate reader (Cytation 3, ASHI, Italy). CR was used as negative control while ampicillin (50 µg/ml) was used as positive control. The MIC90 was defined as the concentration required to inhibit the growth of 90% of microorganisms and normalized on the basis of the total silver content.

Determination of biofilm activity using the tissue culture plate method (TCP)

This assay was performed to determine the ability of silver released by CR-Agx disks to inhibit biofilm activity. The assay is based on colorimetric measurements of the crystal violet incorporated by sessile cells. [272]

[273] Briefly, individual wells of sterile, 96-well flat-bottom polystyrene TCPs were filled with 180 µl of a single bacterial species (1×10^6/ml). After culturing for 24 h, CR (used as negative control) or CR-Agx disks were added. The cell culture plates were then incubated statically at 37°C in a humid atmosphere for 48 – 72 h, until a mature biofilm was obtained. After incubation, the media were removed and the disks were washed three times with 200 µl sterile PBS to remove non-adherent bacteria. The wells were air dried for 45 min and 200 µl per well of a 0.1% (*v*/*v*) crystal violet solution in water were added for 30 min. The wells were then washed five times with 300 µl of sterile PBS to remove excess stain. The dye incorporated by the adherent cells was solubilized with 200 µl of 96% (*v*/*v*) ethanol. The absorbance of each well was measured at 570 nm using a microplate reader (Cytation 3, ASHI, Italy). Experiments were carried out in triplicate.

LDH assay

Biofilm viability was measured by lactate dehydrogenase (LDH) assay (Sigma). Biofilm was obtained for all bacterial strain as previously described. After culturing for 24 h, CR (used as negative control) or CR-Agx disks were added, and LDH release into the surrounding medium measured after 12, 24 and 48 h, according to the manufacturer's protocol. Absorbance values were corrected with CR. LDH data were expressed as a percentage of the total LDH released from cells into the culture medium.

Long term antibacterial and antibiofilm activity

The evaluation of the long term bactericidal properties was performed by collecting the supernatant of bacterial growth in presence of CR or Cr-Agx resins after 22 days. For the analysis of long term antibiofilm activity,

biofilm of each bacterial strain was incubated in presens of CR or CR-Agx resins for 22 days. An aliquot (100 µl) of bacterial suspension from both bacterial and biofilm inoculum was spread on NB agar plate, and growth overnight at 37°C.A single colony of each bacteria strains was inoculated separately in nutrient broth and grown overnight at 37°C. After adjusting to an optical density equivalent to 10^8 cells per ml in PBS, sequential tenfold dilutions were added to tubes containing equal volumes of the extracts. The effect of the materials' extracts on bacterial growth was assayed by colony forming units (CFU) on nutrient agar plates after 24 h of growth.

Statistical Analysis

All quantitative data are presented as the mean ± SD. Each experiment was performed at least 3 times. Student's *t* test was used for the silver release. Statistical analyses were performed by 1-way analysis of variance (ANOVA) with Bonferroni's post hoc test.

CHAPTER 5. RESULTS AND DISCUSSION

INCORPORATION OF LDH-F INTO THE DENTAL RESIN

MECHANICAL PROPERTIES

The mechanical properties were investigated in a wide range of temperatures by performing a dynamic mechanical analysis able to detect either the elastic modulus and the tan δ in the investigated range of temperature. Fig.15 shows the elastic modulus for the pristine resin and the resin containing 0.5, 1, 5, and 10 wt.% of Ag-LDH (CR-Agx).

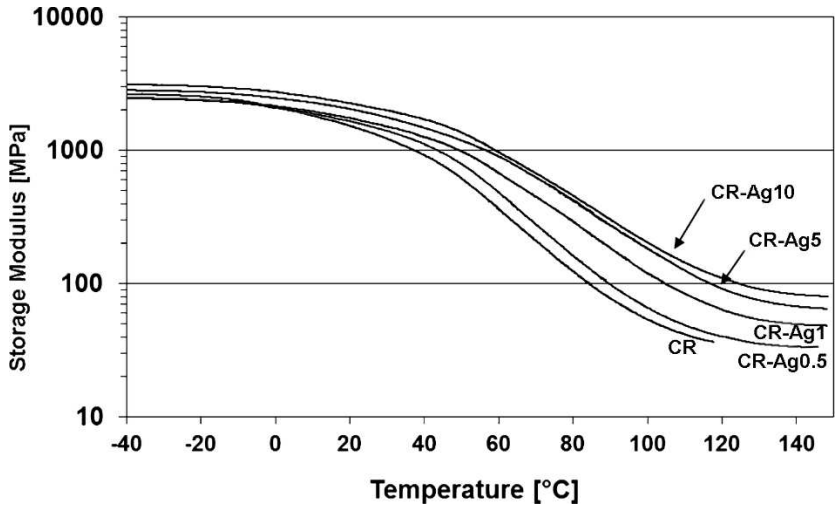


Figure 15. Storage modulus (MPa) versus temperature (°C) of: CR, CR-Ag0.5, CR-Ag1, CR-Ag5, and CR-Ag10.

The study of the mechanical properties in a wide temperature range demonstrated that the values of the elastic modulus of the CR-Agx increased compared with the resin CR. This increase, which was evident after the glass transition temperature, was observed at different temperatures and for different compositions. The comparison of the storage moduli at three different temperatures (0°C, 37°C, 50°C) and the values of the glass transition temperatures are reported in Fig 16 (a and b)

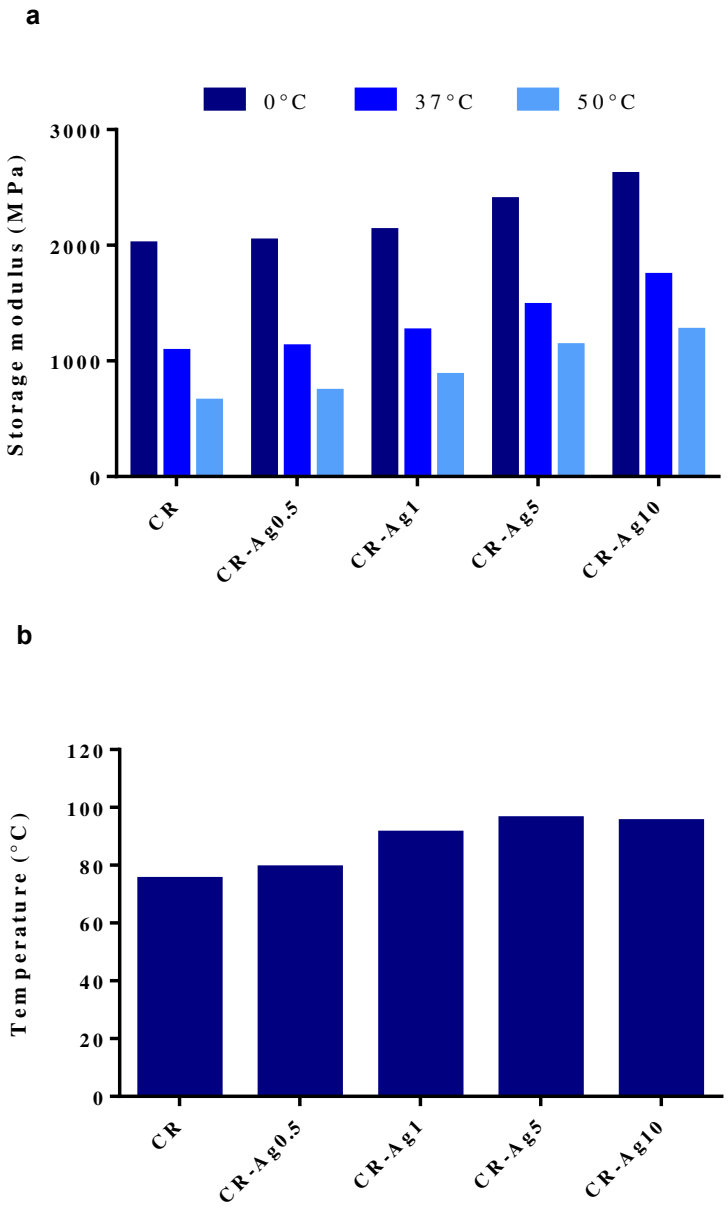


Figure 16: (a) Storage moduli (MPa) at 0°C, 37°C and 50°C and (b) glass transitiontemperature (°C) for the pristine resins and its composites.

We observed that the storage moduli of the composite resins are consistently higher than the pristine resin and the increase is particularly relevant at 37°C, the body temperature. The observed reinforcement

increases on increasing the inorganic silver concentration. As expected, as shown in many composite systems, the deformation at breaking of the composite resin was found slightly lower than the pristine resin. However, since the stress is increasingly higher in the composites, the toughness remained almost unchanged.

Silver release

Cumulative silver release from CR-Ag0.5, CR-Ag1, CR-Ag5, and CR-Ag10 was evaluated for 35 days at 37 °C in artificial saliva medium (SAGF), and at different pH (pH7.4 and pH 4.5 simulating the two most common *in vivo* conditions). Silver release was determined using Inductively Coupled Plasma - Mass Spectrometry (ICP-MS), an analytical technique that performs elemental analysis with excellent sensitivity. This technique can be applied to solutions, solids and gases, and employs argon plasma, as the ionisation source, and a mass spectrometer to separate the produced ions. During ICP-MS analysis the investigated material is transferred by an argon flow into inductively coupled plasma in which an effective temperature results in atomisation and ionisation of the material. Subsequently, the ions are extracted into a mass spectrometer, using which the elemental composition of the material is determined. ICP-MS, as an analytical technique, has many advantages in the laboratory usage for trace metals identification:

- almost all elements can be determined and identified,
- combination of high sensitivity and low background signal provide a very low detection limit and
- rapid analysis, which is a result of high-speed operation of the mass spectrometer (quadrupole)

As shown in Figure 17 and Figure 18, the analyses were performed every hour for 8 h, then every day for 10 days, and then weekly until the end of the experiment. The results demonstrated a time-dependent increase in the silver content for all tested resins, at both pH analyzed.

The major result was obtained with CR-Ag5 and CR-Ag10: a silver concentration of 9.20±0.44 and 11.23±0.35 ppm respectively was determined at pH 7.5, while at pH 4.5 the concentration reached was of 15.25 ± 0.36 and 17.24 ± 0.39 ppm respectively.

On the contrary CR-Ag0.5 and CR-Ag1 released very low concentration of silver at both pH and for all time point tested.

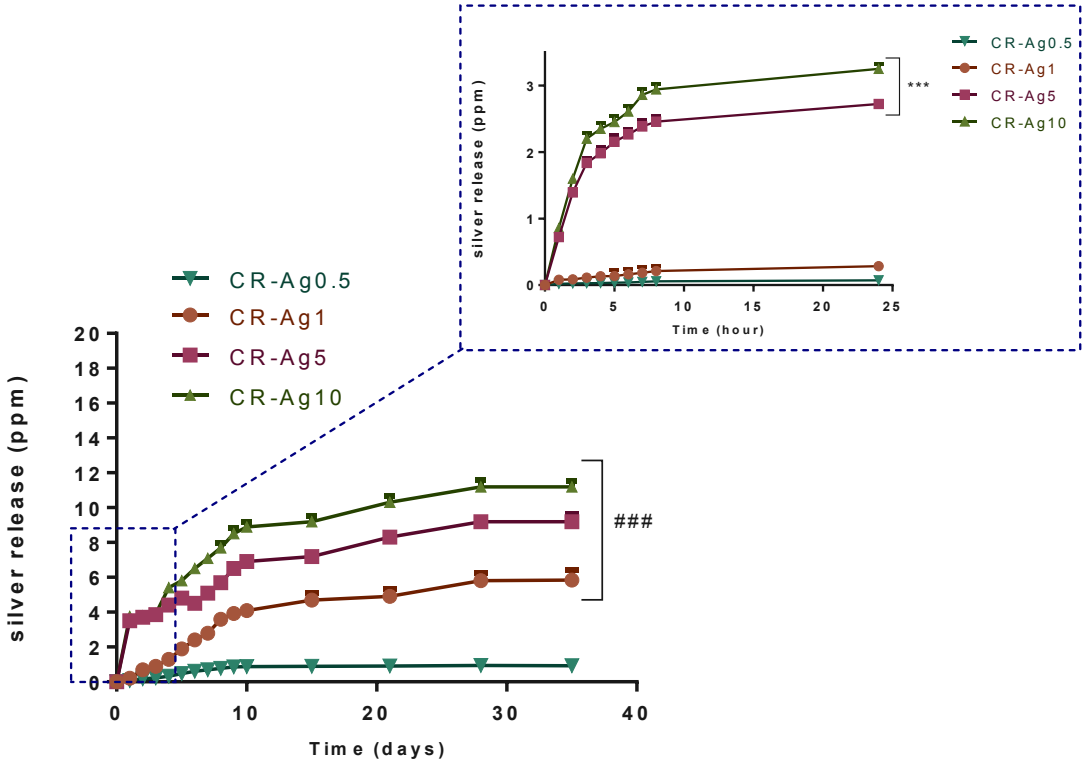


Figure 17: Release amounts of silver in artificial saliva at pH 7.4. Samples were put in mineral medium with composition similar to saliva (SAGF) for 35 d. The bars represent means ± standard deviation (n = 12). Statistically significant variation: ### $p<$ 0.001 for CR-Ag1, CR-Ag5 and CR-

Ag10*versus*CR-Ag0.5 at all-time points tested; ***p< 0.001 for CR-Ag5 and CR-Ag10*versus*CR-Ag0.5 and CR-Ag1 at all-time points tested.

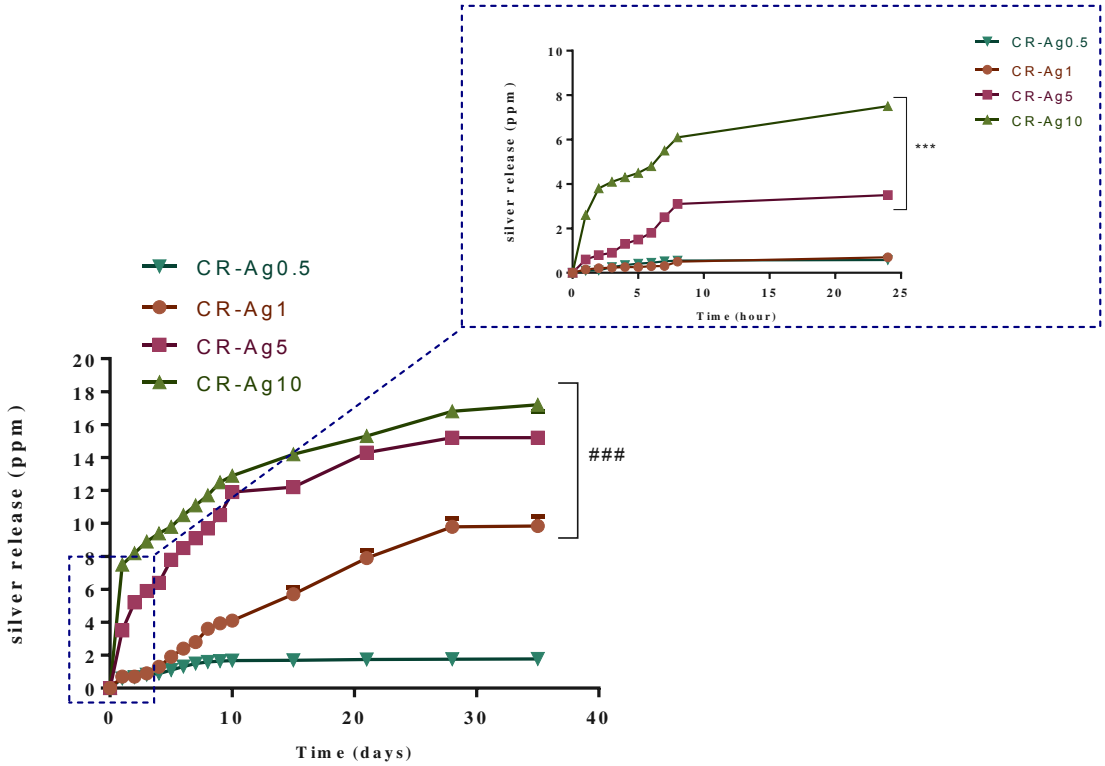


Figure 18: Release amounts of silver in artificial saliva at pH 4.5. Samples were put in mineral medium with composition similar to saliva (SAGF) for 35 d. The bars represent means ± standard deviation (n = 12). Statistically significant variation: ### p< 0.001 for CR-Ag1, CR-Ag5 and CR-Ag10*versus*CR-Ag0.5 at all-time points tested; ***p< 0.001 for CR-Ag5 and CR-Ag10*versus*CR-Ag0.5 and CR-Ag1 at all-time points tested.

Bacterial strains

Dental caries is the destruction of hard tissue of teeth due to due to acidogenic and aciduric abilities of several strain of bacteria having cariogenic potential. [274]

Bacterial strain selected for this thesis were *Pseudomonas aeruginosa* PAO1, and *Staphylococcus aureus*, two opportunistic pathogenic bacteria frequently associated with peri-implant disease and implant failure. [275] [276] [277] [278]

- ***Pseudomonas aeruginosa***

Pseudomonas aeruginosa PAO1, a non-periodontal microbial specie, is one of the most important Gram-negative bacteria causing biofilm-associated infections, particularly in immuno-compromised persons, and is a well-established model organism to study biofilm development. Indeed, it has a strong tendency to form biofilm [279] [280] extremely resistant to antibiotics. [281] [282] Their biofilm formation involves an initial attachment to a solid surface leading to the formation of micro-colonies that during the time, differentiate into exopolysaccharide-encased, mature biofilms. As reported by Canullo et al. PAO1 was identified in patients affected by peri-implant disease at the level of both peri-implant sulcus, gingival sulcus of the adjacent teeth and the connection and abutment at the inner portion of each implant. [283] Moreover, studies of patients with mechanical ventilation in intensive care units showed that the *P. aeruginosa* present in the oral cavity may cross easily different anatomical sites, such as the nose, the paranasal sinuses, colonizing the lungs and promoting pulmonary infections. [284] [285] [286] *P. aeruginosa* is also associated with mandibular osteomyelitis by intraosseous dissemination [287] and to necrotic lesions of the oral mucosa of immunosuppressed patients. [288]

- ***Staphylococcus aureus***

Staphylococcus aureus is a multi-drug resistant bacterium, [289] [290] [291] that usually harbors in the nasal passages and ears of patients, [292] and is associated with medical device-related infections. It grows on catheters and chronic wounds as biofilm [293] and is not only a significant cause of many localized and systemic infections such as osteomyelitis, [294] and chronic rhinosinusitis, [294] but also has a strong connection to dental implant infections. [295] [296] Indeed, several studies have demonstrated that *S. aureus*has high affinity for titanium surfaces and can be found in peri-implant lesions [297] as well as in therapy-resistant cases of periodontitis. [298] Moreover, *S. aureus* is a putative pathogen of many oral diseases, such as oral mucositis, [299] endodontic infections [300] and even dental caries. [301]

In addition, saliva from three healthy volunteers (without active caries or periodontal disease) were collected using a sterile cotton budand dissolved in phosphate buffered saline (PBS) buffer to produce salivary bacteria. Bacterial communities in the oral cavity contain species that promote health states, while others contribute to disease. [302] Recent studies have shown that poor oral hygiene and/or the presence of specific microorganisms in the oral cavity may be associated with periodontitis, respiratory and intestinal diseases. [303] [304] [305] In addition, the salivary microbiota has been used in different human epidemiological studies [306] and has been proposed as a diagnostic marker for oral cancer, [307] periodontal disease [308] and dental caries. [309]

The oral saliva suspension was spread into a Nutrient Broth (NB) agar plate and incubated at 37 °C over night. Bacterial colonies with distinct morphology were picked, subsequently cultured on NB liquid medium and subjected to biochemical tests specific for their identification and characterization.

Biochemical identification methods such as the Biomerieux produced Analytical Profile Index (API) systems are very useful in identifying bacteria at a species level. API is a test system of physiological tests for fast identification of microorganisms. First invented in the 1970s, API introduced standardized, miniaturized versions of established tests like the citrate utilization test, the Voges-Proskauer testor the indole test and combined them within small and easy to handle test strips. [310] Over the time test strips adapted for the testing of different groups of microorganisms were developed. The API®/ID32 kit used includes 15 identification systems that cover more than 600 bacteria species.

Indeed, API®-tests are defined series of physiological tests in a micro-format that are used for the identification of microorganisms. Every single test represents a physiological reaction, for example the activity of an enzyme and/or the utilization of a metabolite (figure 19).

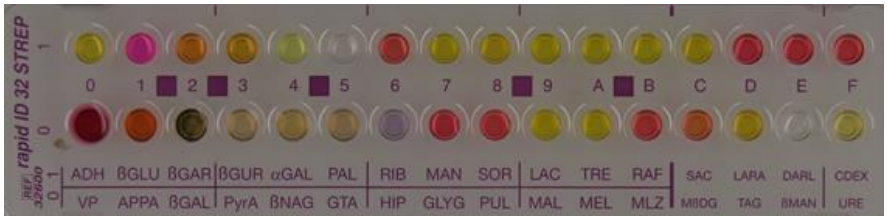


Figure 19. Example of API®/ID32 obtained.

The tests are interpreted by color change or turbidity and recorded as positive (+), negative (-) or weak (+/-). The identification of the bacteria species was made comparing the results obtained with those recorded in the API database.

As reported in table 3, *Streptococcus spp.*, *Bacteroides fragilis*, and *Staphylococcus epidermidis* were identified

Table 3. Interpretation of API/ID32 of saliva isolated bacteria from three healthy volunteers

Legend:

URE: Urease/Urea hydrolysis; ADH (Arg): Arginine dihydrolase; αGAL: α Galactosidase; βGAL: β Galactosidase; βGP: β Galactosidase 6-phosphate; αGLU: α Glucosidase; βGLU: β Glucosidase; αARA: α Arabinosidase; βGUR: β Glucoronidase; βNAG: β-N-acetyl-β-glucosaminidase; MNE: Fermentation of D-Mannose; RAF: Fermentation of Raffinose; GDC: Glutamate decarboxylase; αFUC: α Fucosidase; NIT: Reduction of nitrate; IND: Indole production; PAL: Alkaline Phosphatase; ArgA: L-Ariginine Arylamidase; ProA: Proline Arylamidase; LGA: Leucyl Glicyn Arylamidase; PheA: Phenylalanine Arylamidase; LeuA: Leucine Arylamidase; PyrA: Pyrrolidonyl Arylamidase; TyrA: Tyrosine Arylamidase; AlaA: Alanine Arylamidase; GlyA: Glycine Arylamidase; HisA: Histidine Arylamidase; GGA: Glutamyl-glutamate Arylamidase; SerA: Serine Arylamidase

	ID test																												
	URE	ADH (Arg)	αGAL	βGAL	βGP	αGLU	βGLU	αARA	βGUR	βNAG	MNE	RAF	GDC	αFUC	NIT	IND	PAL	ArgA	ProA	LGA	PheA	LeuA	PyrA	TyrA	AlaA	GlyA	HisA	GGA	SerA
Streptococcus spp.	-	+/-	-	-	+/-	-	-	-	-	-	+	-	-	-	-	-	+	+	-	-	+	+	-	+	+	+	+	-	+
Bacteroides fragilis	-	-	+	+	-	+	+	-	-	+	+	+	+	+	-	-	+	+	-	+	-	+/-	-	+	+	-	-	+	-
Staphylococcus epidermidis	+	+	-	+	-	+/-	+	-	+	-	+/-	-	-	-	+	-	+	-	-	+/-	-	+	-	+	+	+	+/-	-	-

- *Streptococcus species*

Streptococcus spp. is commonly found in the oral cavity and considered as one of the early colonizers of oral surfaces. The bacteria produce extracellular polysaccharide in response to dietary sucrose that firmly attaches the cells to surfaces and contributes to the biofilm matrix. Streptococci represent the majority of the total microbial species detected in dental caries. [31] In particular, among the isolates of other *Streptococcus* species, *S. mutans* isolates have a greater ability to form biofilm. [311] *S. mutans* appears to be important in the initiation of dental caries since its activities lead to colonization of the tooth surface, dental plaque formation and demineralization. Montanaro et al. [312] demonstrated that *S. mutans* adhesion and colonization on restorative material surfaces can also occur in the absence of specific saliva proteins and in a period as short as 4 h. In addition, *S. epidermidis* has greater potential to cause infections in patients with immune-compromised state, intravenous drug abusers, AIDS patients, immuno-suppressive therapy patients and premature new born. [313] [314]

- *Bacteroides fragilis*

Bacteroides fragilis is an opportunistic periodontopathogen, as many reports have documented the isolation of *B. fragilis* from dental plaque or periodontal pockets of patients with periodontitis. [315] [316]

- *Staphylococcus epidermidis*

Staphylococcus epidermidis is the most frequently encountered member of the coagulase-negative staphylococci on human epithelial surfaces, and

due to its paucity of virulence factors is less invasive than its coagulase-positive relation, *Staphylococcus aureus.* [317] However, in recent years *S. epidermidis* has become a frequent and important nosocomial pathogen, particularly in immunocompromised patients. [318] [319] Many strains of *S. epidermidis* are able to produce biofilms and readily colonize implanted medical devices, in particular intravascular devices, cerebrospinal fluid shunts, intraocular lenses, prosthetic joints and heart valve replacements. Colonization of such medical devices may progress to infections that manifest as subacute or chronic in nature. *S. epidermidis* bacteremia is predominantly caused by entry of the bacteria through colonized intravascular medical devices and removal of the device is recommended as an integral part of patient treatment. [320]

Antimicrobial performances of CR-Agx

The antimicrobial activity of CR-Agx materials was analyzed against above reported microorganisms (*Pseudomonas aeruginosa* PAO1, *Staphylococcus aureus, Streptococcus spp.*, *Bacteroides fragilis*, and *Staphylococcus epidermidis*). Table 4 reported, for each sample tested, the minimum inhibitory concentration required to inhibit the growth of 90% of microorganisms (MIC90).

The results obtained revealed that only CR loading high concentration of Ag (CR-Ag5 and CR-Ag10) were able to inhibit both Gram positive and negative bacteria growth. In particular, CR-Ag10 showed the higher antimicrobial activity, ranging from 2.8 ± 0.3 to 3.9 ± 0.5 μg ml^{-1}.

Lower Ag concentration does not affect bacterial growth.

Recently Hwang et al. [321] reported that chemically derived silver nanoparticles in the size range 10 to 25 nm are effective antimicrobial agents. Earlier studies show that the interaction stage of Ag nanoparticles in *E. coli* and found that at initial stage of the interaction of AgNPs adhere to bacterial cell wall subsequently penetrate the bacteria and kill bacterial cell by destroying cell membrane. AgNPs may pass through the cell wall of bacteria to oxidize the surface proteins on the plasma membrane and consequently disturb cellular homeostasis. [322] [323]

	MIC90[a] (µg ml^{-1})				
Samples	*Pseudomonas aeruginosa* PAO1	*Staphylococcus aureus*	*Streptococcus spp.*	*Bacteroides fragilis*	*Staphylococcus epidermidis*
CR-Ag0.5	>5000	>5000	>5000	>5000	>5000
CR-Ag1	>1000	>1000	>1000	>1000	>1000
CR-Ag5	4.9 ± 0.5	5.4 ± 0.5	5.1 ± 0.4	6.0 ± 0.6	5.5 ± 0.4
CR-Ag10	3.2 ± 0.2	2.8 ± 0.3	3.4 ± 0.4	3.6 ± 0.6	3.9 ± 0.5
CR (negative control)	>10000	>10000	>10000	>10000	>10000
Ampicillin (positive control)	0.8 ± 0.06	0.5 ± 0.04	0.4 ± 0.05	0.5 ± 0.07	0.4 ± 0.03

a The MIC90 values were normalized with respect to the silver content

Table 4. Antimicrobial activity (MIC 90) of LDH-Ag nanocomposites, LDH and positive control against selected microorganisms.

Anti-biofilm activity of AgNPs

The dose-dependent ability of CR-Agx to inhibit the activity of biofilms formed by the human pathogens *Pseudomonas aeruginosa* PAO1, *Staphylococcus aureus, Streptococcus spp.*, *Bacteroides fragilis*, and *Staphylococcus epidermidis*was determined under *in vitro* conditions. The results showed that, for all the tested bacterial strains, the inhibition of biofilm activity was observed only for higher concentration of Ag (CR-Ag5

and CR-Ag10), while no effect was observed with both CR-Ag0.5 and CR-Ag1 (figure 20).

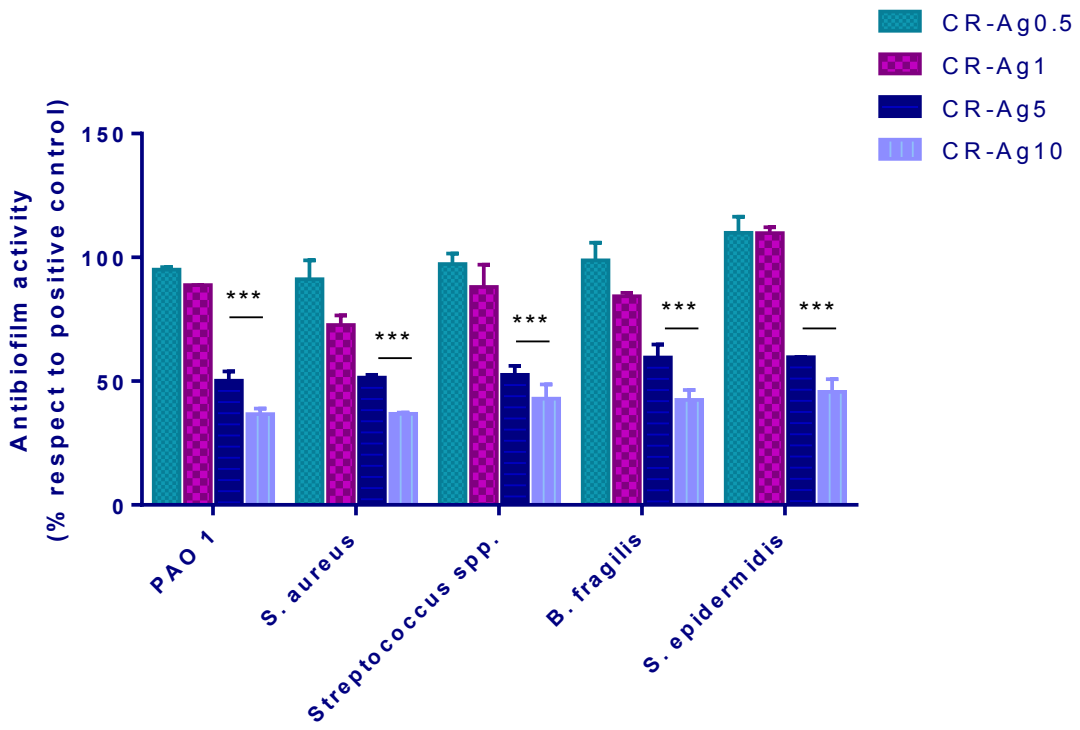


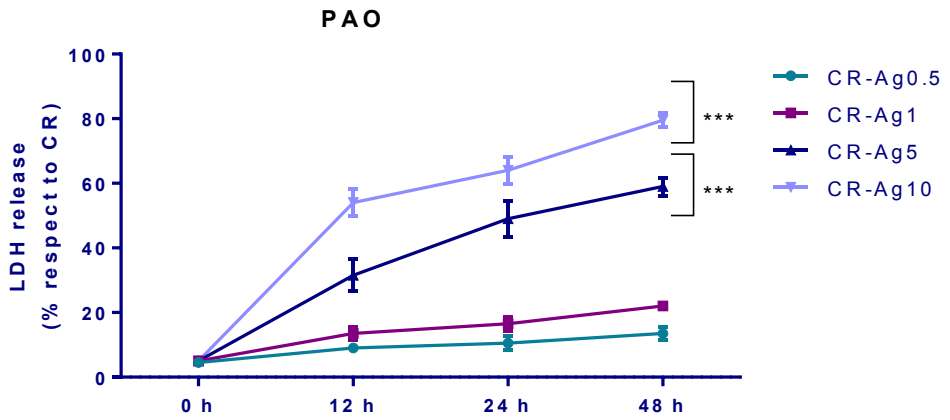
Figure 20. Antibiofilm activity of CR-Agx materials against selected pathogenic bacteria. The bars represent the means ±s.d. (n =6). Statistically significant variations *** p<0.001 for CR-Ag5 and CR-Ag10 *versus* CR-Ag0.5 and CR-Ag1.

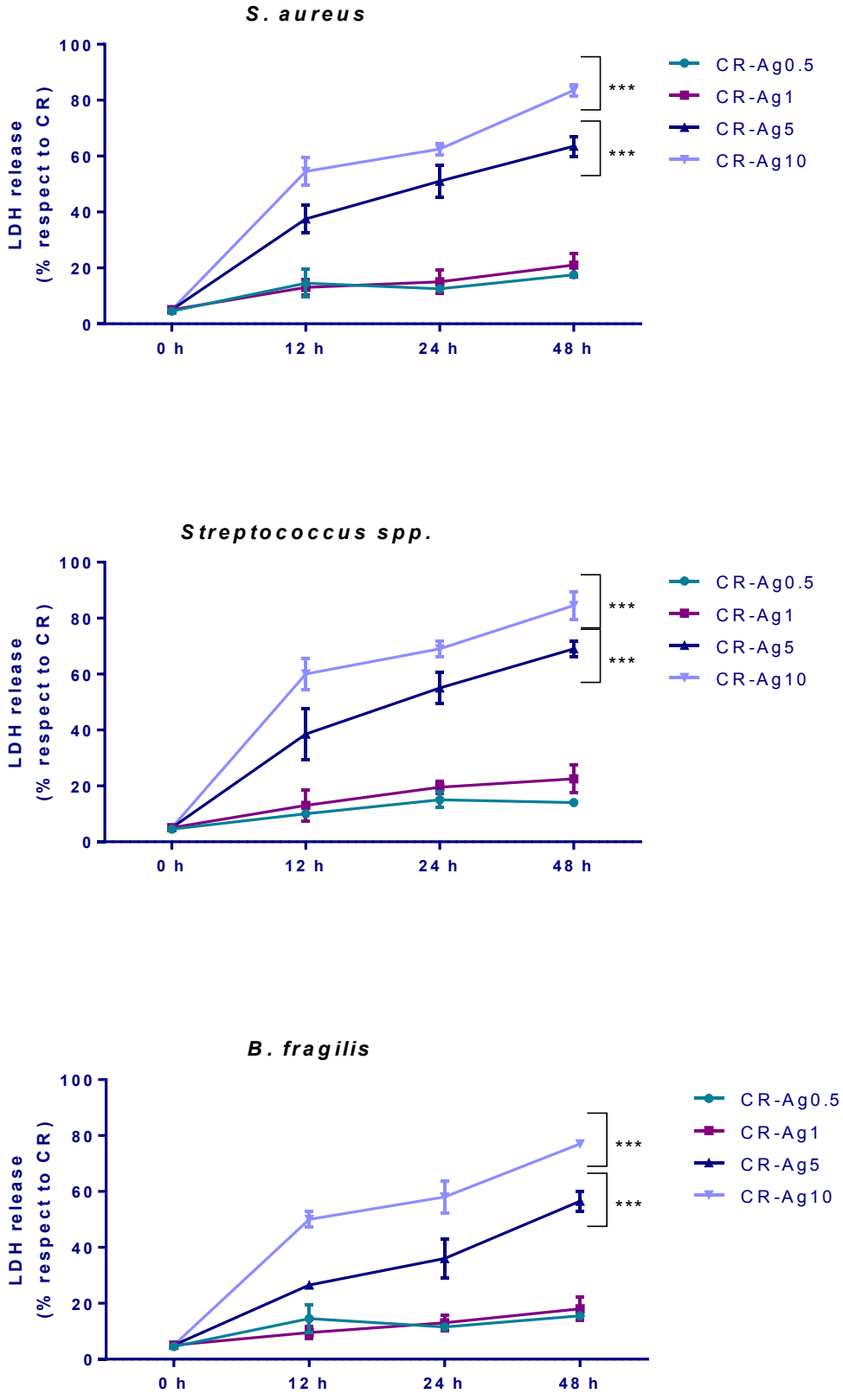
Kalishwaralal et al. [273] reported that anti-biofilm activity of biologically synthesized AgNPs against *P. aeruginosa* and *S. epidermidis* biofilms and found that 100 nM of AgNPs resulted in a 95% to 98% reduction in biofilm formation. Ansari et al. [324] demonstrated that the colonies were grown without AgNPs, the organisms appeared as dry crystalline black colonies, indicating the production of exopolysaccharides, which is the prerequisite for the formation of biofilm, whereas when the organisms were grown with AgNPs, the organisms did not survive. Thus, when the

exopolysaccharide synthesis is arrested, the organism cannot form biofilm. However, different signaling mechanisms could be involved in cell survival and biofilm formation. Indeed, Chaudhari et al. [325] reported that AgNPs derived from *B. megaterium* showed enhanced quorum quenching activity against *S. aureus* biofilm and prevention of biofilm formation, and they suggested that AgNPs might be involved in neutralizing these adhesive substances thus preventing biofilm formation.

Effect of CR-Agx on biofilm viability

To determine the effect of CR-Agxon biofilm viability, LDH activity, a reliable marker for determining cell status, was determined. The effects of CR-Agxon LDH activities of selected bacteria are shown in Figure 21.




S. aureus
LDH release
(% respect to CR)
100
80
60
40
20
0
0 h
12 h
24 h
48 h
***
***
CR-Ag0.5
CR-Ag1
CR-Ag5
CR-Ag10
Streptococcus spp.
LDH release
(% respect to CR)
100
80
60
40
20
0
0 h
12 h
24 h
48 h
***
***
CR-Ag0.5
CR-Ag1
CR-Ag5
CR-Ag10
B. fragilis
LDH release
(% respect to CR)
100
80
60
40
20
0
0 h
12 h
24 h
48 h
***
***
CR-Ag0.5
CR-Ag1
CR-Ag5
CR-Ag10

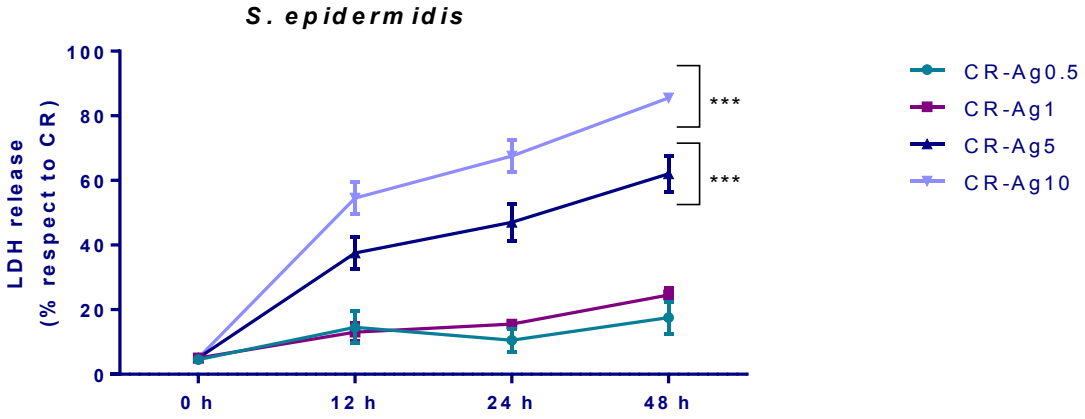


Figure 21: Evaluation of CR-Agx on biofilm viability. Results are expressed as % LDH release by bacteria into the culture medium. The bars represent the means ±s.d. (n =6). Statistically significant variations *** p<0.001 for CR-Ag5 and CR-Ag10 *versus* CR-Ag0.5 and CR-Ag1.

Our data demonstrated that both CR-Ag5 and CR-Ag10 resins were able to decrease the viability of the biofilm already after 24 h of incubation.

Long Term Antibacterial Activity

The long term bactericidal and antibiofilm properties of CR-Agx against the selected bacterial strains were determined by measuring the colony forming unitper ml after 22 days of incubation.

As shown in figure 22, a significant bacteria inhibition after culture with CR-Ag5 and CR-Ag10 was observed compared to CR, CR-Ag0.5 and CR-Ag1 resins.

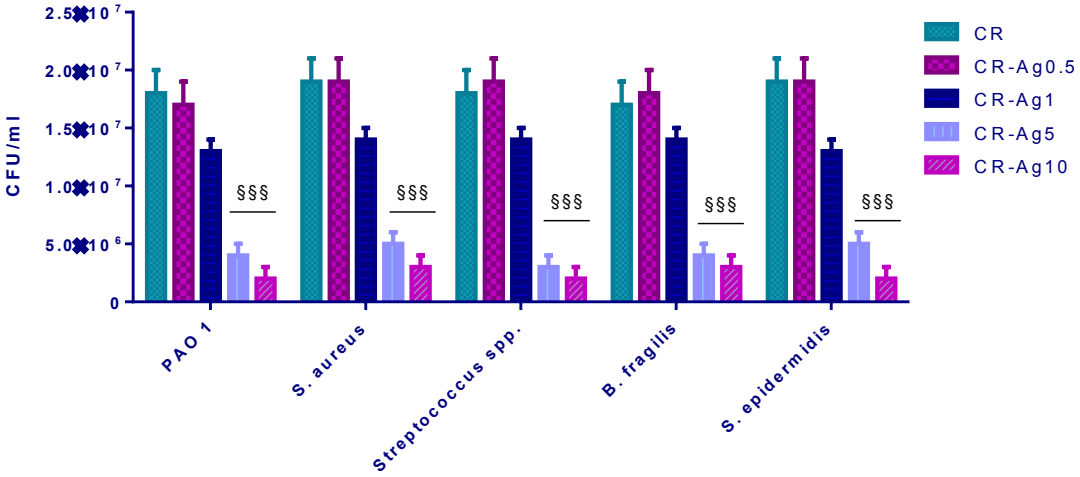


Figure 22. Colony Forming Units per ml for all bacterial strain after 22 days of culture with resins.The bars represent the means ±s.d. (n = 12). Statistically significant variations §§§ $p<0.001$ for CR-Ag5 and CR-Ag10 *versus* CR, CR-Ag0.5 and CR-Ag1.

In addition, figure 23 shown the long term antibiofilm activity measured after 22 days of incubation in presence of selected biofilm.

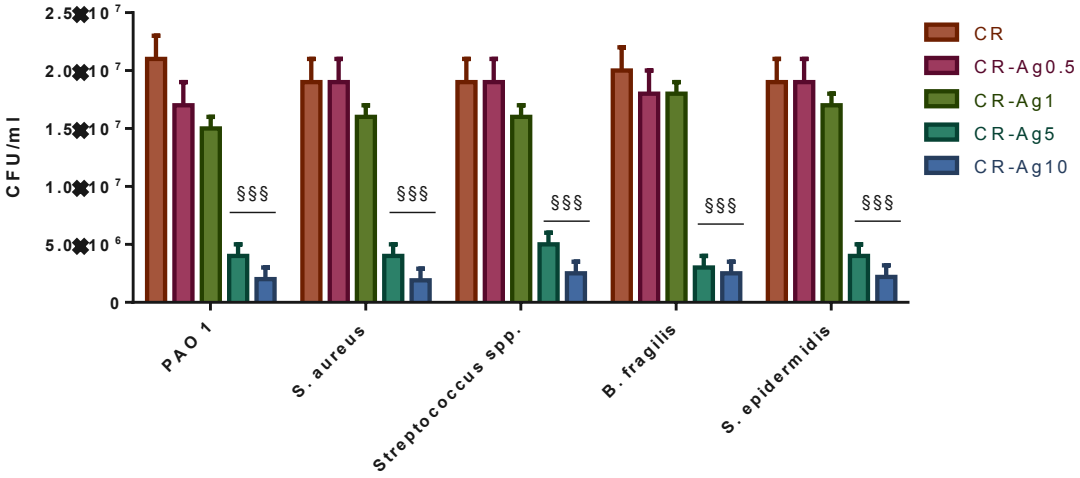


Figure 23. Colony Forming Units per ml for all bacterial biofim after 22 days of culture with resins.The bars represent the means ±s.d. (n = 12). Statistically significant variations §§§ $p<0.001$ for CR-Ag5 and CR-Ag10 *versus* CR, CR-Ag0.5 and CR-Ag1.

This results confirmed that the slow and continuous release of Ag^+ from the CR-Ag5 and CR-Ag10 is able to elicit both a good antibacterial and antibiofilm activity also after 22 days of incubation.

CONCLUSION

Secondary caries are caries of the tooth at the margin of existing restoration and are considered the most common reason for restoration failure. This pathology develops rapidly around and below a broken restoration, while is slower and more localized on the enamel along the cavosurface margin.

In this thesis was discussed the formulation, preparation and characterization of novel LDH based restorative dental materials intercalated with silver nanoparticles. Such composites have no initial toxic Ag 'burst' effect and the levels of silver release remain relatively constant over time.

In particular, such long-term controlled delivery of micromolar amounts of silver gives to the modified-hydrotalcite restorative dental resin a strong antibacterial effect. Moreover, LDH-Ag are able to release silver ions when intraoral pH values drop below the critical pH of 5.5, counteracting the demineralization process of the tooth surface.

LDH-Ag, then, thanks to its physic-chemical characteristics and its inherent biocompatibility, satisfy the clinical practical aspects of restorative dentistry and acts as an ideal filler able to reduce bacterial infiltration and secondary caries development.

As future perspective, it should be interesting to continue this study with in vivo analysis to confirm the effect of silver release kinetic.

AKNOWLEDGMENTS

I would like to thank all those people who made this thesis possible. First of all, my gratitude goes to my mentor, Prof. Gianfranco Peluso, for the continuous support at my PhD research activities and his invaluable motivation and knowledge, who supported me during these three years. Moreover, I would like to thank my PhD coordinator Prof. Sandro Rengo for his academic support and Dr. Orsolina Petillo for her precious help.

A special thank is for my tutor, Dr. Anna Calarco, the person who first trained me into the research world and that is always helpful and supportive. Thanks to her and Prof. Peluso I grew up in a fantastic research group composed of Dr. Anna Di Salle, Dr. Anna Valentino, Dr. Francesca Di Cristo, Dr. Ilenia De Luca, Dr. Mauro Finicelli. All together we turn the working time spent in laboratory into real "scientific passion". Moreover, I had the possibility to collaborate with qualified researchers as Dr. Pierfrancesco Cerruti, Dr. Bartosz Tylkowski and Dr. Valentina Marturano.

My gratitude is also extended to the biologic laboratory of Dr. Sabrina Margarucci and Dr. Maria D'Apolito.

Finally, I would like to thank my wife Iolanda, my parents Michele and Rosa, my sister Mariarca, my parents in law (Carmine and Nunzia) and the acquired family (Giusy, Sara and Enzo) who believed in me and strongly supported my choices.

REFERENCES

1. Li Y, Carrera C, Chen R, et al. (2014) Degradation in the dentin-composite interface subjected to multi-species biofilm challenges. *Acta Biomater* 10: 375-383.
2. Zimmerli B, Strub M, Jeger F, et al. (2010) Composite materials: composition, properties and clinical applications. A literature review. *Schweiz Monatsschr Zahnmed* 120: 972-986.
3. Bourbia M, Ma D, Cvitkovitch DG, et al. (2013) Cariogenic bacteria degrade dental resin composites and adhesives. *Journal of dental research* 92: 989-994.
4. Imazato S, Ma S, Chen JH, et al. (2014) Therapeutic polymers for dental adhesives: loading resins with bio-active components. *Dent Mater* 30: 97-104.
5. Imazato S (2009) Bio-active restorative materials with antibacterial effects: new dimension of innovation in restorative dentistry. *Dent Mater J* 28: 11-19.
6. Cheng L, Weir MD, Zhang K, et al. (2012) Dental plaque microcosm biofilm behavior on calcium phosphate nanocomposite with quaternary ammonium. *Dent Mater* 28: 853-862.
7. Wang S, Zhang K, Zhou X, et al. (2014) Antibacterial effect of dental adhesive containing dimethylaminododecyl methacrylate on the development of Streptococcus mutans biofilm. *Int J Mol Sci* 15: 12791-12806.
8. Zhang K, Wang S, Zhou X, et al. (2015) Effect of antibacterial dental adhesive on multispecies biofilms formation. *J Dent Res* 94: 622-629.
9. Li F, Chen J, Chai Z, et al. (2009) Effects of a dental adhesive incorporating antibacterial monomer on the growth, adherence and membrane integrity of Streptococcus mutans. *J Dent* 37: 289-296.
10. Beyth N, Yudovin-Farber I, Bahir R, et al. (2006) Antibacterial activity of dental composites containing quaternary ammonium polyethylenimine nanoparticles against Streptococcus mutans. *Biomaterials* 27: 3995-4002.
11. Xie D, Weng Y, Guo X, et al. (2011) Preparation and evaluation of a novel glass-ionomer cement with antibacterial functions. *Dent Mater* 27: 487-496.
12. Li F, Weir MD, Xu HHK (2013) Effects of quaternary ammonium chain length on antibacterial bonding agents. *Journal of dental research* 92: 932-938.
13. Zhang K, Cheng L, Weir MD, et al. (2015) Effects of quaternary ammonium chain length on the antibacterial and remineralizing effects of a calcium phosphate nanocomposite. *International journal of oral science* 8: 45-53.
14. Zhang K, Cheng L, Wu EJ, et al. (2013) Effect of water-ageing on dentine bond strength and anti-biofilm activity of bonding agent containing new monomer dimethylaminododecyl methacrylate. *J Dent* 41: 504-513.
15. Castellano JJ, Shafii SM, Ko F, et al. (2007) Comparative evaluation of silver-containing antimicrobial dressings and drugs. *Int Wound J* 4: 114-122.
16. Tanagawa M, Yoshida K, Matsumoto S, et al. (1999) Inhibitory effect of antibacterial resin composite against Streptococcus mutans. *Caries Res* 33: 366-371.
17. Cheng YJ, Zeiger DN, Howarter JA, et al. (2011) In situ formation of silver nanoparticles in photocrosslinking polymers. *J Biomed Mater Res B Appl Biomater* 97: 124-131.
18. Cheng L, Weir MD, Xu HH, et al. (2012) Antibacterial amorphous calcium phosphate nanocomposites with a quaternary ammonium dimethacrylate and silver nanoparticles. *Dent Mater* 28: 561-572.

19. Zero DT, Fontana M, Martínez-Mier EA, et al. (2009) The Biology, Prevention, Diagnosis and Treatment of Dental Caries. *The Journal of the American Dental Association* 140: 25S-34S.
20. Dodds M, Roland S, Edgar M, et al. (2015) Saliva A review of its role in maintaining oral health and preventing dental disease. *Bdj Team* 2: 15123.
21. Mathur VP, Dhillon JK (2018) Dental Caries: A Disease Which Needs Attention. *The Indian Journal of Pediatrics* 85: 202-206.
22. Fejerskov O (2004) Changing paradigms in concepts on dental caries: consequences for oral health care. *Caries Res* 38: 182-191.
23. Bastos JL, Gigante DP, Peres KG, et al. (2007) [Social determinants of odontalgia in epidemiological studies: theoretical review and proposed conceptual model]. *Cien Saude Colet* 12: 1611-1621.
24. Pitts NB, Zero DT, Marsh PD, et al. (2017) Dental caries. *Nature Reviews Disease Primers* 3: 17030.
25. Noorda WD, Purdell-Lewis DJ, van Montfort AM, et al. (1988) Monobacterial and mixed bacterial plaques of Streptococcus mutans and Veillonella alcalescens in an artificial mouth: development, metabolism, and effect on human dental enamel. *Caries Res* 22: 342-347.
26. Usha C, R S (2009) Dental caries - A complete changeover (Part I). *Journal of conservative dentistry : JCD* 12: 46-54.
27. Pitts NB, Zero DT, Marsh PD, et al. (2017) Dental caries. *Nat Rev Dis Primers* 3: 17030.
28. Dhir S (2013) Biofilm and dental implant: The microbial link. 5-11 p.
29. Theilade E (1986) The non-specific theory in microbial etiology of inflammatory periodontal diseases. *J Clin Periodontol* 13: 905-911.
30. Taba M, Jr., Kinney J, Kim AS, et al. (2005) Diagnostic biomarkers for oral and periodontal diseases. *Dental clinics of North America* 49: 551-vi.
31. Hamada S, Slade HD (1980) Biology, immunology, and cariogenicity of Streptococcus mutans. *Microbiological reviews* 44: 331-384.
32. Ruoff KL (1991) Nutritionally variant streptococci. *Clin Microbiol Rev* 4: 184-190.
33. Ma C, Chen F, Zhang Y, et al. (2015) Comparison of Oral Microbial Profiles between Children with Severe Early Childhood Caries and Caries-Free Children Using the Human Oral Microbe Identification Microarray. *PLOS ONE* 10: e0122075.
34. Tanner AC, Kent RL, Jr., Holgerson PL, et al. (2011) Microbiota of severe early childhood caries before and after therapy. *J Dent Res* 90: 1298-1305.
35. Mantzourani M, Gilbert SC, Sulong HNH, et al. (2009) The Isolation of Bifidobacteria from Occlusal Carious Lesions in Children and Adults. *Caries Research* 43: 308-313.
36. Arena ME, Manca de Nadra MC (2001) Biogenic amine production by Lactobacillus. *J Appl Microbiol* 90: 158-162.
37. Bleicher F (2014) Odontoblast physiology. *Exp Cell Res* 325: 65-71.
38. Moynihan P, Petersen PE (2004) Diet, nutrition and the prevention of dental diseases. *Public Health Nutr* 7: 201-226.
39. Levin KA, Nicholls N, Macdonald S, et al. (2015) Geographic and socioeconomic variations in adolescent toothbrushing: a multilevel cross-sectional study of 15 year olds in Scotland. *J Public Health (Oxf)* 37: 107-115.

40. Meyer F, Enax J (2018) Early Childhood Caries: Epidemiology, Aetiology, and Prevention. *International Journal of Dentistry* 2018: 7.
41. Organization WH (2003) Diet, nutrition and the prevention of chronic diseases. *World Health Organ Tech Rep Ser* 916: i-viii, 1-149, backcover.
42. (2015) WHO Guidelines Approved by the Guidelines Review Committee. *Guideline: Sugars Intake for Adults and Children*. Geneva: World Health Organization

43. Nelson SJ, Ash, M. M. (2010) Wheeler's Dental Anatomy, Physiology and Occlusion *Saunders Elsevier*.
44. Shahmoradi M, Bertassoni LE, Elfallah HM, et al. (2014) Fundamental Structure and Properties of Enamel, Dentin and Cementum. 2: 511-547.
45. Galler KM, D'Souza RN (2011) Tissue engineering approaches for regenerative dentistry. *Regen Med* 6: 111-124.
46. Holmen L, Thylstrup A, Ogaard B, et al. (1985) A scanning electron microscopic study of progressive stages of enamel caries in vivo. *Caries Res* 19: 355-367.
47. Goldberg M, Kulkarni AB, Young M, et al. (2011) Dentin: structure, composition and mineralization. *Front Biosci (Elite Ed)* 3: 711-735.
48. Matalon S, Peretz B, Sidon R, et al. (2010) Antibacterial properties of pit and fissure sealants combined with daily fluoride mouth rinse. *Pediatr Dent* 32: 9-13.
49. Feng QL, Wu J, Chen GQ, et al. (2000) A mechanistic study of the antibacterial effect of silver ions on Escherichia coli and Staphylococcus aureus. *Journal of Biomedical Materials Research* 52: 662-668.
50. Aoba T (2004) Solubility properties of human tooth mineral and pathogenesis of dental caries. *Oral Dis* 10: 249-257.
51. Ahovuo-Saloranta A, Forss H, Walsh T, et al. (2013) Sealants for preventing dental decay in the permanent teeth. *Cochrane Database Syst Rev*: Cd001830.
52. Loyola-Rodriguez JP, Garcia-Godoy F (1996) Antibacterial activity of fluoride release sealants on mutans streptococci. *J Clin Pediatr Dent* 20: 109-111.
53. Fábián TK, Hermann P, Beck A, et al. (2012) Salivary defense proteins: their network and role in innate and acquired oral immunity. *International journal of molecular sciences* 13: 4295-4320.
54. de Almeida Pdel V, Gregio AM, Machado MA, et al. (2008) Saliva composition and functions: a comprehensive review. *J Contemp Dent Pract* 9: 72-80.
55. Featherstone JD (2008) Dental caries: a dynamic disease process. *Aust Dent J* 53: 286-291.
56. Vanobbergen J, Martens L, Lesaffre E, et al. (2001) Assessing risk indicators for dental caries in the primary dentition. *Community Dent Oral Epidemiol* 29: 424-434.
57. Jung WK, Koo HC, Kim KW, et al. (2008) Antibacterial activity and mechanism of action of the silver ion in Staphylococcus aureus and Escherichia coli. *Appl Environ Microbiol* 74: 2171-2178.
58. Holbrook WP, Arnadottir IB, Kay EJ (2003) Prevention. Part 3: prevention of tooth wear. *Br Dent J* 195: 75-81.
59. Warren E, Pollicino C, Curtis B, et al. (2010) Modeling the long-term cost-effectiveness of the caries management system in an Australian population. *Value Health* 13: 750-760.

60. Ladewig NM, Camargo LB, Tedesco TK, et al. (2018) Management of dental caries among children: a look at the cost-effectiveness. *Expert review of pharmacoeconomics & outcomes research* 18: 127-134.
61. Sicca C, Bobbio E, Quartuccio N, et al. (2016) Prevention of dental caries: A review of effective treatments. *Journal of clinical and experimental dentistry* 8: e604-e610.
62. Singh KA, Spencer AJ (2004) Relative effects of pre- and post-eruption water fluoride on caries experience by surface type of permanent first molars. *Community Dent Oral Epidemiol* 32: 435-446.
63. dos Santos AP, Nadanovsky P, de Oliveira BH (2014) A systematic review and meta-analysis of the effects of fluoride toothpastes on the prevention of dental caries in the primary dentition of preschool children. Evid Based Dent. 2014; 1: 5. *Evid Based Dent* 15: 67.
64. Affairs ADACoS (2006) Professionally applied topical fluoride: evidence-based clinical recommendations. *J Am Dent Assoc* 137: 1151-1159.
65. Weintraub JA, Ramos-Gomez F, Jue B, et al. (2006) Fluoride varnish efficacy in preventing early childhood caries. *Journal of dental research* 85: 172-176.
66. Dalli M, Colak H, Mustafa Hamidi M (2012) Minimal intervention concept: a new paradigm for operative dentistry. *J Investig Clin Dent* 3: 167-175.
67. Black GV (1936) G. V. Black's Work on operative dentistry with which his Special dental pathology is combined. Published in four volumes. Chicago, London: Medico Dental Publishing Company; H. Kimpton. 4 v. p.
68. Black GV (1920) A work on special dental pathology devoted to the diseases and treatment of the investing tissues of the teeth and the dental pulp including the sequelae of the death of the pulp; also, systemic effects of mouth infections, oral prophylaxis and mouth hygiene. Chicago: Medico-Dental Publishing Co. xxviii, 489 p. p.
69. Christensen GJ (2005) How to kill a tooth. *J Am Dent Assoc* 136: 1711-1713.
70. Conway DI, McMahon, A.D. , Robertson, D. , Macpherson, L. M. D. (2014) Epidemiology of Dental Diseases. *Handbook of Epidemiology*
2321-2353.
71. Peters MC, McLean ME (2001) Minimally invasive operative care. I. Minimal intervention and concepts for minimally invasive cavity preparations. *J Adhes Dent* 3: 7-16.
72. Walsh LJ, Brostek AM (2013) Minimum intervention dentistry principles and objectives. *Aust Dent J* 58 Suppl 1: 3-16.
73. Ericson D (2007) The concept of minimally invasive dentistry. *Dent Update* 34: 9-10, 12-14, 17-18.
74. Frencken JE, Peters MC, Manton DJ, et al. (2012) Minimal intervention dentistry for managing dental caries - a review: report of a FDI task group. *Int Dent J* 62: 223-243.
75. Bonsor SJ (2011) Bonded amalgams and their use in clinical practice. *Dent Update* 38: 222-224, 226-228, 230.
76. Mjor IA, Toffenetti F (2000) Secondary caries: a literature review with case reports. *Quintessence Int* 31: 165-179.
77. Hals E, Nernaes Å (1971) Histopathology of in vitro Caries Developing around Silver Amalgam Fillings. *Caries Research* 5: 58-77.

78. Song F, Koo H, Ren D (2015) Effects of Material Properties on Bacterial Adhesion and Biofilm Formation. *J Dent Res* 94: 1027-1034.
79. Sarrett DC (2005) Clinical challenges and the relevance of materials testing for posterior composite restorations. *Dent Mater* 21: 9-20.
80. Totiam P, Gonzalez-Cabezas C, Fontana MR, et al. (2007) A new in vitro model to study the relationship of gap size and secondary caries. *Caries Res* 41: 467-473.
81. Cenci MS, Pereira-Cenci T, Cury JA, et al. (2009) Relationship between gap size and dentine secondary caries formation assessed in a microcosm biofilm model. *Caries Res* 43: 97-102.
82. Kuper N (2015) Secondary caries, mind yhe gap! *Ipskamp Drukkers, Enschede, The Netherlands*.
83. American Dental Association Council on Scientific Affairs. Professionally applied topical fluoride: evidence-based clinical recommendations. J Am Dent Assoc. 2006 Aug;137(8):1151-9.
84. Marsh PD (2010) Microbiology of dental plaque biofilms and their role in oral health and caries. *Dent Clin North Am* 54: 441-454.
85. Jorgensen KD, Wakumoto S (1968) Occlusal amalgam fillings: marginal defects and secondary caries. *Odontol Tidskr* 76: 43-54.
86. Pimenta LA, Navarro MF, Consolaro A (1995) Secondary caries around amalgam restorations. *J Prosthet Dent* 74: 219-222.
87. Nelsen RJ, Wolcott RB, Paffenbarger GC (1952) Fluid exchange at the margins of dental restorations. *J Am Dent Assoc* 44: 288-295.
88. Swartz ML, Phillips RW (1961) In vitro studies on the marginal leakage of restorative materials. *J Am Dent Assoc* 62: 141-151.
89. Going RE (1972) Microleakage Around Dental Restorations: A Summarizing Review. *The Journal of the American Dental Association* 84: 1349-1357.
90. Gale MS, Darvell BW (1999) Dentine permeability and tracer tests. *J Dent* 27: 1-11.
91. Dennison JB, Sarrett DC (2012) Prediction and diagnosis of clinical outcomes affecting restoration margins. *J Oral Rehabil* 39: 301-318.
92. Heintze SD (2013) Clinical relevance of tests on bond strength, microleakage and marginal adaptation. *Dent Mater* 29: 59-84.
93. Schmid-Schwap M, Graf A, Preinerstorfer A, et al. (2011) Microleakage after thermocycling of cemented crowns--a meta-analysis. *Dent Mater* 27: 855-869.
94. Dietschi D, Argente A, Krejci I, et al. (2013) In vitro performance of Class I and II composite restorations: a literature review on nondestructive laboratory trials--part I. *Oper Dent* 38: E166-181.
95. Hilton TJ (2002) Can modern restorative procedures and materials reliably seal cavities? In vitro investigations. Part 2. *Am J Dent* 15: 279-289.
96. Mente J, Ferk S, Dreyhaupt J, et al. (2010) Assessment of different dyes used in leakage studies. *Clin Oral Investig* 14: 331-338.
97. Mo SS, Bao W, Lai GY, et al. (2010) The microfloral analysis of secondary caries biofilm around Class I and Class II composite and amalgam fillings. *BMC Infect Dis* 10: 241.
98. Fitzgerald RJ, Adams BO, Davis ME (1994) A Microbiological Study of Recurrent Dentinal Caries. *Caries Research* 28: 409-415.

99. Gonzalez-Cabezas C, Li Y, Gregory RL, et al. (1999) Distribution of three cariogenic bacteria in secondary carious lesions around amalgam restorations. *Caries Res* 33: 357-365.
100. González-Cabezas C, Li Y, Gregory RL, et al. (2002) Distribution of cariogenic bacteria in carious lesions around tooth-colored restorations. *American journal of dentistry* 15: 248-251.
101. Thomas RZ, van der Mei HC, van der Veen MH, et al. (2008) Bacterial composition and red fluorescence of plaque in relation to primary and secondary caries next to composite: an in situ study. *Oral Microbiol Immunol* 23: 7-13.
102. Burke FJ, Lucarotti PS (2009) How long do direct restorations placed within the general dental services in England and Wales survive? *Br Dent J* 206: E2; discussion 26-27.
103. Eltahlah D, Lynch CD, Chadwick BL, et al. (2018) An update on the reasons for placement and replacement of direct restorations. *J Dent* 72: 1-7.
104. Deligeorgi V, Mjor IA, Wilson NH (2001) An overview of reasons for the placement and replacement of restorations. *Prim Dent Care* 8: 5-11.
105. Mahmood S, Chohan AN, Al-Jannakh M, et al. (2004) Placement and replacement of dental restorations. *J Coll Physicians Surg Pak* 14: 589-592.
106. Qvist V, Qvist J, Mjor IA (1990) Placement and longevity of tooth-colored restorations in Denmark. *Acta Odontol Scand* 48: 305-311.
107. Mjor IA (1985) Frequency of secondary caries at various anatomical locations. *Oper Dent* 10: 88-92.
108. Hahnel S, Henrich A, Rosentritt M, et al. (2010) Influence of artificial ageing on surface properties and Streptococcus mutans adhesion to dental composite materials. *J Mater Sci Mater Med* 21: 823-833.
109. Ionescu A, Brambilla E, Wastl DS, et al. (2015) Influence of matrix and filler fraction on biofilm formation on the surface of experimental resin-based composites. *J Mater Sci Mater Med* 26: 5372.
110. Signori C, Gimenez T, Mendes FM, et al. (2018) Clinical relevance of studies on the visual and radiographic methods for detecting secondary caries lesions - A systematic review. *J Dent* 75: 22-33.
111. Boston DW (2003) Initial in vitro evaluation of DIAGNOdent for detecting secondary carious lesions associated with resin composite restorations. *Quintessence Int* 34: 109-116.
112. Ando M, Gonzalez-Cabezas C, Isaacs RL, et al. (2004) Evaluation of several techniques for the detection of secondary caries adjacent to amalgam restorations. *Caries Res* 38: 350-356.
113. Kidd EA (2001) Diagnosis of secondary caries. *J Dent Educ* 65: 997-1000.
114. Kidd EA, Beighton D (1996) Prediction of secondary caries around tooth-colored restorations: a clinical and microbiological study. *J Dent Res* 75: 1942-1946.
115. Rudolphy MP, van Amerongen JP, Penning C, et al. (1995) Grey discolouration and marginal fracture for the diagnosis of secondary caries in molars with occlusal amalgam restorations: an in vitro study. *Caries Res* 29: 371-376.
116. Nedeljkovic I, Teughels W, De Munck J, et al. (2015) Is secondary caries with composites a material-based problem? *Dent Mater* 31: e247-277.

117. Gordan VV, Riley JL, 3rd, Geraldeli S, et al. (2012) Repair or replacement of defective restorations by dentists in The Dental Practice-Based Research Network. *J Am Dent Assoc* 143: 593-601.
118. Kursun S, Dinc G, Oztas B, et al. (2012) The visibility of secondary caries under bonding agents with two different imaging modalities. *Dent Mater J* 31: 975-979.
119. Gonzalez-Cabezas C, Fontana M, Gomes-Moosbauer D, et al. (2003) Early detection of secondary caries using quantitative, light-induced fluorescence. *Oper Dent* 28: 415-422.
120. Clark TD, Mjor IA (2001) Current teaching of cariology in North American dental schools. *Oper Dent* 26: 412-418.
121. Kidd EA, Joyston-Bechal S, Beighton D (1994) Diagnosis of secondary caries: a laboratory study. *Br Dent J* 176: 135-138, 139.
122. Foster LV (1994) Validity of clinical judgements for the presence of secondary caries associated with defective amalgam restorations. *Br Dent J* 177: 89-93.
123. Brouwer F, Askar H, Paris S, et al. (2016) Detecting Secondary Caries Lesions: A Systematic Review and Meta-analysis. *J Dent Res* 95: 143-151.
124. Wilson N, Lynch CD, Brunton PA, et al. (2016) Criteria for the Replacement of Restorations: Academy of Operative Dentistry European Section. *Oper Dent* 41: S48-s57.
125. Baelum V (2010) What is an appropriate caries diagnosis? *Acta Odontol Scand* 68: 65-79.
126. Mjor IA, Gordan VV (2002) Failure, repair, refurbishing and longevity of restorations. *Oper Dent* 27: 528-534.
127. Heidmann J, Holund U, Poulsen S (1987) Changing criteria for restorative treatment of approximal caries over a 10-year period. *Caries Res* 21: 460-463.
128. Clement JL, Jarrett PS (1994) Antibacterial Silver. *Metal-Based Drugs* 1: 467-482.
129. McShan D, Ray PC, Yu H (2014) Molecular toxicity mechanism of nanosilver. *Journal of food and drug analysis* 22: 116-127.
130. Reidy B, Haase A, Luch A, et al. (2013) Mechanisms of Silver Nanoparticle Release, Transformation and Toxicity: A Critical Review of Current Knowledge and Recommendations for Future Studies and Applications. *Materials (Basel, Switzerland)* 6: 2295-2350.
131. Unrine JM, Colman BP, Bone AJ, et al. (2012) Biotic and abiotic interactions in aquatic microcosms determine fate and toxicity of Ag nanoparticles. Part 1. Aggregation and dissolution. *Environ Sci Technol* 46: 6915-6924.
132. Yang X, Gondikas AP, Marinakos SM, et al. (2012) Mechanism of silver nanoparticle toxicity is dependent on dissolved silver and surface coating in Caenorhabditis elegans. *Environ Sci Technol* 46: 1119-1127.
133. Bone AJ, Colman BP, Gondikas AP, et al. (2012) Biotic and abiotic interactions in aquatic microcosms determine fate and toxicity of Ag nanoparticles: part 2-toxicity and Ag speciation. *Environ Sci Technol* 46: 6925-6933.
134. George S, Lin S, Ji Z, et al. (2012) Surface defects on plate-shaped silver nanoparticles contribute to its hazard potential in a fish gill cell line and zebrafish embryos. *ACS Nano* 6: 3745-3759.

135. Tripathi DK, Tripathi A, Shweta, et al. (2017) Uptake, Accumulation and Toxicity of Silver Nanoparticle in Autotrophic Plants, and Heterotrophic Microbes: A Concentric Review. *Front Microbiol* 8: 07.
136. Phanjom P, Ahmed G (2017) Effect of different physicochemical conditions on the synthesis of silver nanoparticles using fungal cell filtrate of Aspergillus oryzae (MTCC No. 1846) and their antibacterial effect. 045016 p.
137. Patil SV, Borase HP, Patil CD, et al. (2012) Biosynthesis of silver nanoparticles using latex from few Euphorbian plants and their antimicrobial potential. *Appl Biochem Biotechnol* 167: 776-790.
138. Dibrov P, Dzioba J, Gosink KK, et al. (2002) Chemiosmotic mechanism of antimicrobial activity of Ag(+) in Vibrio cholerae. *Antimicrob Agents Chemother* 46: 2668-2670.
139. Strużyńska L (2017) Chapter 7 - The Application, Neurotoxicity, and Related Mechanisms of Silver Nanoparticles. In: Jiang X, Gao H, editors. *Neurotoxicity of Nanomaterials and Nanomedicine*: Academic Press. pp. 151-177.
140. Mai-Prochnow A, Clauson M, Hong J, et al. (2016) Gram positive and Gram negative bacteria differ in their sensitivity to cold plasma. *Sci Rep* 6: 38610.
141. Abbaszadegan A, Ghahramani Y, Gholami A, et al. (2015) The Effect of Charge at the Surface of Silver Nanoparticles on Antimicrobial Activity against Gram-Positive and Gram-Negative Bacteria: A Preliminary Study. *Journal of Nanomaterials* 2015: 8.
142. Idelevich EA, Hoy M, Görlich D, et al. (2017) Rapid Phenotypic Detection of Microbial Resistance in Gram-Positive Bacteria by a Real-Time Laser Scattering Method. *Frontiers in microbiology* 8: 1064-1064.
143. David MZ, Dryden M, Gottlieb T, et al. (2017) Recently approved antibacterials for methicillin-resistant Staphylococcus aureus (MRSA) and other Gram-positive pathogens: the shock of the new. *Int J Antimicrob Agents* 50: 303-307.
144. Liu YY, Chandler CE, Leung LM, et al. (2017) Structural Modification of Lipopolysaccharide Conferred by mcr-1 in Gram-Negative ESKAPE Pathogens. 61.
145. Sperandeo P, Martorana AM, Polissi A (2017) Lipopolysaccharide biogenesis and transport at the outer membrane of Gram-negative bacteria. *Biochim Biophys Acta Mol Cell Biol Lipids* 1862: 1451-1460.
146. Patra JK, Baek KH (2017) Antibacterial Activity and Synergistic Antibacterial Potential of Biosynthesized Silver Nanoparticles against Foodborne Pathogenic Bacteria along with its Anticandidal and Antioxidant Effects. *Front Microbiol* 8: 167.
147. Velusamy P, Kumar GV, Jeyanthi V, et al. (2016) Bio-Inspired Green Nanoparticles: Synthesis, Mechanism, and Antibacterial Application. *Toxicol Res* 32: 95-102.
148. Dakal TC, Kumar A, Majumdar RS, et al. (2016) Mechanistic Basis of Antimicrobial Actions of Silver Nanoparticles. *Front Microbiol* 7: 1831.
149. Jung J, Kasi G, Seo J (2018) Development of functional antimicrobial papers using chitosan/starch-silver nanoparticles. *Int J Biol Macromol* 112: 530-536.
150. Rim K-T, Song S-W, Kim H-Y (2013) Oxidative DNA damage from nanoparticle exposure and its application to workers' health: a literature review. *Safety and health at work* 4: 177-186.

151. Salomoni R, Léo P, Montemor AF, et al. (2017) Antibacterial effect of silver nanoparticles in Pseudomonas aeruginosa. *Nanotechnology, science and applications* 10: 115-121.
152. Asharani P, Sethu S, Lim HK, et al. (2012) Differential regulation of intracellular factors mediating cell cycle, DNA repair and inflammation following exposure to silver nanoparticles in human cells. *Genome Integr* 3: 2.
153. Jiang HS, Yin LY, Ren NN, et al. (2017) Silver nanoparticles induced reactive oxygen species via photosynthetic energy transport imbalance in an aquatic plant. *Nanotoxicology* 11: 157-167.
154. Ansari MA, Khan HM, Khan AA, et al. (2014) Antibiofilm efficacy of silver nanoparticles against biofilm of extended spectrum β-lactamase isolates of Escherichia coli and Klebsiella pneumoniae. *Applied Nanoscience* 4: 859-868.
155. Wang XH, Wang Z, Zhang J, et al. (2016) Cytotoxicity of AgNPs/CS composite films: AgNPs immobilized in chitosan matrix contributes a higher inhibition rate to cell proliferation. *Bioengineered* 7: 283-290.
156. Sanyasi S, Majhi RK, Kumar S, et al. (2016) Polysaccharide-capped silver Nanoparticles inhibit biofilm formation and eliminate multi-drug-resistant bacteria by disrupting bacterial cytoskeleton with reduced cytotoxicity towards mammalian cells. *Sci Rep* 6: 24929.
157. Singh BR, Singh BN, Singh A, et al. (2015) Mycofabricated biosilver nanoparticles interrupt Pseudomonas aeruginosa quorum sensing systems. *Scientific reports* 5: 13719-13719.
158. Martinez-Gutierrez F, Boegli L, Agostinho A, et al. (2013) Anti-biofilm activity of silver nanoparticles against different microorganisms. *Biofouling* 29: 651-660.
159. Radzig MA, Nadtochenko VA, Koksharova OA, et al. (2013) Antibacterial effects of silver nanoparticles on gram-negative bacteria: influence on the growth and biofilms formation, mechanisms of action. *Colloids Surf B Biointerfaces* 102: 300-306.
160. Rueggeberg FA (2002) From vulcanite to vinyl, a history of resins in restorative dentistry. *J Prosthet Dent* 87: 364-379.
161. Nunez-Anita RE, Acosta-Torres LS, Vilar-Pineda J, et al. (2014) Toxicology of antimicrobial nanoparticles for prosthetic devices. *Int J Nanomedicine* 9: 3999-4006.
162. Zhang K, Melo MA, Cheng L, et al. (2012) Effect of quaternary ammonium and silver nanoparticle-containing adhesives on dentin bond strength and dental plaque microcosm biofilms. *Dent Mater* 28: 842-852.
163. Melo MA, Cheng L, Zhang K, et al. (2013) Novel dental adhesives containing nanoparticles of silver and amorphous calcium phosphate. *Dent Mater* 29: 199-210.
164. Godoy-Gallardo M, Rodriguez-Hernandez AG, Delgado LM, et al. (2015) Silver deposition on titanium surface by electrochemical anodizing process reduces bacterial adhesion of Streptococcus sanguinis and Lactobacillus salivarius. *Clin Oral Implants Res* 26: 1170-1179.
165. Santos VE, Jr., Vasconcelos Filho A, Targino AG, et al. (2014) A new "silver-bullet" to treat caries in children--nano silver fluoride: a randomised clinical trial. *J Dent* 42: 945-951.

166. Jia Z, Xiu P, Li M, et al. (2016) Bioinspired anchoring AgNPs onto micro-nanoporous TiO2 orthopedic coatings: Trap-killing of bacteria, surface-regulated osteoblast functions and host responses. *Biomaterials* 75: 203-222.
167. Juillerat-Jeanneret L, Dusinska M, Fjellsbo LM, et al. (2015) Biological impact assessment of nanomaterial used in nanomedicine. introduction to the NanoTEST project. *Nanotoxicology* 9 Suppl 1: 5-12.
168. Murphy M, Ting K, Zhang X, et al. (2015) Current Development of Silver Nanoparticle Preparation, Investigation, and Application in the Field of Medicine. *Journal of Nanomaterials* 2015: 12.
169. Fayaz AM, Balaji K, Girilal M, et al. (2010) Biogenic synthesis of silver nanoparticles and their synergistic effect with antibiotics: a study against gram-positive and gram-negative bacteria. *Nanomedicine* 6: 103-109.
170. Manisha B, Swapnil G, Avinash I, et al. (2010) A New Report on Mycosynthesis of Silver Nanoparticles by Fusarium culmorum. *Current Nanoscience* 6: 376-380.
171. Ruden S, Hilpert K, Berditsch M, et al. (2009) Synergistic interaction between silver nanoparticles and membrane-permeabilizing antimicrobial peptides. *Antimicrob Agents Chemother* 53: 3538-3540.
172. Huang L, Dai T, Xuan Y, et al. (2011) Synergistic combination of chitosan acetate with nanoparticle silver as a topical antimicrobial: efficacy against bacterial burn infections. *Antimicrob Agents Chemother* 55: 3432-3438.
173. Pal S, Tak YK, Song JM (2007) Does the antibacterial activity of silver nanoparticles depend on the shape of the nanoparticle? A study of the Gram-negative bacterium Escherichia coli. *Appl Environ Microbiol* 73: 1712-1720.
174. Park HJ, Park S, Roh J, et al. (2013) Biofilm-inactivating activity of silver nanoparticles: A comparison with silver ions. *Journal of Industrial and Engineering Chemistry* 19: 614-619.
175. Chaloupka K, Malam Y, Seifalian AM (2010) Nanosilver as a new generation of nanoproduct in biomedical applications. *Trends Biotechnol* 28: 580-588.
176. Peulen T-O, Wilkinson KJ (2011) Diffusion of Nanoparticles in a Biofilm. *Environmental Science & Technology* 45: 3367-3373.
177. Abu-Lail NI, Camesano TA (2003) Role of Lipopolysaccharides in the Adhesion, Retention, and Transport of Escherichia coli JM109. *Environmental Science & Technology* 37: 2173-2183.
178. Morones JR, Elechiguerra JL, Camacho A, et al. (2005) The bactericidal effect of silver nanoparticles. *Nanotechnology* 16: 2346-2353.
179. Verran J, Sandoval G, Allen NS, et al. (2007) Variables affecting the antibacterial properties of nano and pigmentary titania particles in suspension. *Dyes and Pigments* 73: 298-304.
180. Cheng L, Zhang K, Melo MAS, et al. (2012) Anti-biofilm Dentin Primer with Quaternary Ammonium and Silver Nanoparticles. *Journal of Dental Research* 91: 598-604.
181. Besinis A, De Peralta T, Tredwin CJ, et al. (2015) Review of Nanomaterials in Dentistry: Interactions with the Oral Microenvironment, Clinical Applications, Hazards, and Benefits. *ACS Nano* 9: 2255-2289.
182. Bulad K, Taylor RL, Verran J, et al. (2004) Colonization and penetration of denture soft lining materials by Candida albicans. *Dent Mater* 20: 167-175.

183. Boscato N, Radavelli A, Faccio D, et al. (2009) Biofilm formation of Candida albicans on the surface of a soft denture-lining material. *Gerodontology* 26: 210-213.
184. Paranhos HF, Silva-Lovato CH, de Souza RF, et al. (2009) Effect of three methods for cleaning dentures on biofilms formed in vitro on acrylic resin. *J Prosthodont* 18: 427-431.
185. Budtz-Jlrgensen E, Mojon P, Banon-Clement JM, et al. (1996) Oral candidosis in long-term hospital care: comparison of edentulous and dentate subjects. *Oral Dis* 2: 285-290.
186. Al-Dwairi ZN (2008) Prevalence and risk factors associated with denture-related stomatitis in healthy subjects attending a dental teaching hospital in North Jordan. *J Ir Dent Assoc* 54: 80-83.
187. Samaranayake LP, Nair RG (1995) Oral Candida infections--a review. *Indian J Dent Res* 6: 69-82.
188. Chandra J, Mukherjee PK, Leidich SD, et al. (2001) Antifungal resistance of candidal biofilms formed on denture acrylic in vitro. *J Dent Res* 80: 903-908.
189. Perezous LF, Flaitz CM, Goldschmidt ME, et al. (2005) Colonization of Candida species in denture wearers with emphasis on HIV infection: a literature review. *J Prosthet Dent* 93: 288-293.
190. Rowan R, McCann M, Kavanagh K (2010) Analysis of the response of Candida albicans cells to Silver(I). *Med Mycol* 48: 498-505.
191. Douglas LJ (2003) Candida biofilms and their role in infection. *Trends Microbiol* 11: 30-36.
192. Monteiro DR, Gorup LF, Takamiya AS, et al. (2009) The growing importance of materials that prevent microbial adhesion: antimicrobial effect of medical devices containing silver. *Int J Antimicrob Agents* 34: 103-110.
193. Nam KY (2011) In vitro antimicrobial effect of the tissue conditioner containing silver nanoparticles. *J Adv Prosthodont* 3: 20-24.
194. Dhir G, Berzins DW, Dhuru VB, et al. (2007) Physical properties of denture base resins potentially resistant to Candida adhesion. *J Prosthodont* 16: 465-472.
195. Abe Y, Ishii M, Takeuchi M, et al. (2004) Effect of saliva on an antimicrobial tissue conditioner containing silver-zeolite. *J Oral Rehabil* 31: 568-573.
196. Kassaee MZ, Akhavan A, Sheikh N, et al. (2008) Antibacterial effects of a new dental acrylic resin containing silver nanoparticles. *Journal of Applied Polymer Science* 110: 1699-1703.
197. Kong H, Jang J (2008) Antibacterial properties of novel poly(methyl methacrylate) nanofiber containing silver nanoparticles. *Langmuir* 24: 2051-2056.
198. Ahn SJ, Lee SJ, Kook JK, et al. (2009) Experimental antimicrobial orthodontic adhesives using nanofillers and silver nanoparticles. *Dent Mater* 25: 206-213.
199. Acosta-Torres LS, Mendieta I, Nunez-Anita RE, et al. (2012) Cytocompatible antifungal acrylic resin containing silver nanoparticles for dentures. *Int J Nanomedicine* 7: 4777-4786.
200. Monteiro DR, Gorup LF, Takamiya AS, et al. (2012) Silver distribution and release from an antimicrobial denture base resin containing silver colloidal nanoparticles. *J Prosthodont* 21: 7-15.

201. Okita N, Orstavik D, Orstavik J, et al. (1991) In vivo and in vitro studies on soft denture materials: microbial adhesion and tests for antibacterial activity. *Dent Mater* 7: 155-160.
202. Bystrom A, Sundqvist G (1981) Bacteriologic evaluation of the efficacy of mechanical root canal instrumentation in endodontic therapy. *Scand J Dent Res* 89: 321-328.
203. Sundqvist G, Figdor D, Persson S, et al. (1998) Microbiologic analysis of teeth with failed endodontic treatment and the outcome of conservative re-treatment. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 85: 86-93.
204. Baumgartner JC, Falkler WA, Jr. (1991) Bacteria in the apical 5 mm of infected root canals. *J Endod* 17: 380-383.
205. Baumgartner JC, Khemaleelakul SU, Xia T (2003) Identification of spirochetes (treponemes) in endodontic infections. *J Endod* 29: 794-797.
206. Sjogren U, Figdor D, Persson S, et al. (1997) Influence of infection at the time of root filling on the outcome of endodontic treatment of teeth with apical periodontitis. *Int Endod J* 30: 297-306.
207. Shantiaee Y, Maziar F, Dianat O, et al. (2011) Comparing microleakage in root canals obturated with nanosilver coated gutta-percha to standard gutta-percha by two different methods. *Iran Endod J* 6: 140-145.
208. Torabinejad M, Hong CU, McDonald F, et al. (1995) Physical and chemical properties of a new root-end filling material. *J Endod* 21: 349-353.
209. Torabinejad M, Hong CU, Pitt Ford TR, et al. (1995) Antibacterial effects of some root end filling materials. *J Endod* 21: 403-406.
210. Kreth J, Kim D, Nguyen M, et al. (2008) The Antimicrobial Effect of Silver Ion Impregnation into Endodontic Sealer against Streptococcus mutans. *Open Dent J* 2: 18-23.
211. Shantiaee Y, Dianat SO, Mohammad Khani H, et al. (2011) Cytotoxicity comparison of nanosilver coated gutta-percha with Guttaflow and normal gutta-percha on L929 fibroblast with MTT assay. *Journal of Dental School Shahid Beheshti University of Medical Sciences* 29: 62-68.
212. Kandaswamy D, Venkateshbabu N (2010) Root canal irrigants. *J Conserv Dent* 13: 256-264.
213. Clarkson RM, Moule AJ (1998) Sodium hypochlorite and its use as an endodontic irrigant. *Aust Dent J* 43: 250-256.
214. Harrison JW, Svec TA, Baumgartner JC (1978) Analysis of clinical toxicity of endodontic irrigants. *J Endod* 4: 6-11.
215. Abbasipour F, Akheshteh V, Rastqar A, et al. (2012) Comparing the effects of mineral trioxide aggregate and calcium enriched mixture on neuronal cells using an electrophysiological approach. *Iran Endod J* 7: 79-87.
216. Sahebi S, Nabavizadeh M, Dolatkhah V, et al. (2012) Short term effect of calcium hydroxide, mineral trioxide aggregate and calcium-enriched mixture cement on the strength of bovine root dentin. *Iran Endod J* 7: 68-73.
217. Stowe TJ, Sedgley CM, Stowe B, et al. (2004) The effects of chlorhexidine gluconate (0.12%) on the antimicrobial properties of tooth-colored ProRoot mineral trioxide aggregate. *J Endod* 30: 429-431.
218. Hernandez EP, Botero TM, Mantellini MG, et al. (2005) Effect of ProRoot MTA mixed with chlorhexidine on apoptosis and cell cycle of fibroblasts and macrophages in vitro. *Int Endod J* 38: 137-143.

219. Samiei M, Aghazadeh M, Lotfi M, et al. (2013) Antimicrobial Efficacy of Mineral Trioxide Aggregate with and without Silver Nanoparticles. *Iran Endod J* 8: 166-170.
220. Darouiche RO (2004) Treatment of infections associated with surgical implants. *N Engl J Med* 350: 1422-1429.
221. Zhao L, Chu PK, Zhang Y, et al. (2009) Antibacterial coatings on titanium implants. *J Biomed Mater Res B Appl Biomater* 91: 470-480.
222. Hardes J, Ahrens H, Gebert C, et al. (2007) Lack of toxicological side-effects in silver-coated megaprostheses in humans. *Biomaterials* 28: 2869-2875.
223. Hendriks JG, van Horn JR, van der Mei HC, et al. (2004) Backgrounds of antibiotic-loaded bone cement and prosthesis-related infection. *Biomaterials* 25: 545-556.
224. Chen W, Liu Y, Courtney HS, et al. (2006) In vitro anti-bacterial and biological properties of magnetron co-sputtered silver-containing hydroxyapatite coating. *Biomaterials* 27: 5512-5517.
225. Zhao L, Wang H, Huo K, et al. (2011) Antibacterial nano-structured titania coating incorporated with silver nanoparticles. *Biomaterials* 32: 5706-5716.
226. Flores CY, Diaz C, Rubert A, et al. (2010) Spontaneous adsorption of silver nanoparticles on Ti/TiO2 surfaces. Antibacterial effect on Pseudomonas aeruginosa. *J Colloid Interface Sci* 350: 402-408.
227. Lu X, Zhang B, Wang Y, et al. (2011) Nano-Ag-loaded hydroxyapatite coatings on titanium surfaces by electrochemical deposition. *J R Soc Interface* 8: 529-539.
228. Kim JS, Kuk E, Yu KN, et al. (2007) Antimicrobial effects of silver nanoparticles. *Nanomedicine* 3: 95-101.
229. Ferracane JL (2011) Resin composite--state of the art. *Dent Mater* 27: 29-38.
230. Cheng L, Weir MD, Xu HHK, et al. (2012) Effect of amorphous calcium phosphate and silver nanocomposites on dental plaque microcosm biofilms. *Journal of Biomedical Materials Research Part B: Applied Biomaterials* 100B: 1378-1386.
231. Melo MAS, Cheng L, Zhang K, et al. (2013) Novel dental adhesives containing nanoparticles of silver and amorphous calcium phosphate. *Dental Materials* 29: 199-210.
232. Li F, Weir MD, Chen J, et al. (2013) Comparison of quaternary ammonium-containing with nano-silver-containing adhesive in antibacterial properties and cytotoxicity. *Dental materials : official publication of the Academy of Dental Materials* 29: 450-461.
233. Demarco FF, Correa MB, Cenci MS, et al. (2012) Longevity of posterior composite restorations: not only a matter of materials. *Dent Mater* 28: 87-101.
234. Berg JH (2002) Glass ionomer cements. *Pediatr Dent* 24: 430-438.
235. Pallesen U, van Dijken JW, Halken J, et al. (2014) A prospective 8-year follow-up of posterior resin composite restorations in permanent teeth of children and adolescents in Public Dental Health Service: reasons for replacement. *Clin Oral Investig* 18: 819-827.
236. Bourbia M, Ma D, Cvitkovitch DG, et al. (2013) Cariogenic bacteria degrade dental resin composites and adhesives. *J Dent Res* 92: 989-994.
237. Breschi L, Mazzoni A, Ruggeri A, et al. (2008) Dental adhesion review: aging and stability of the bonded interface. *Dent Mater* 24: 90-101.

238. Spencer P, Ye Q, Park J, et al. (2010) Adhesive/Dentin interface: the weak link in the composite restoration. *Annals of biomedical engineering* 38: 1989-2003.
239. Khvostenko D, Salehi S, Naleway SE, et al. (2015) Cyclic mechanical loading promotes bacterial penetration along composite restoration marginal gaps. *Dent Mater* 31: 702-710.
240. Roulet JF (1997) Benefits and disadvantages of tooth-coloured alternatives to amalgam. *J Dent* 25: 459-473.
241. Uçar Y, Brantley WA (2011) Biocompatibility of dental amalgams. *International journal of dentistry* 2011: 981595-981595.
242. Baig MS, Fleming GJP (2015) Conventional glass-ionomer materials: A review of the developments in glass powder, polyacid liquid and the strategies of reinforcement. *Journal of dentistry* 43: 897-912.
243. Khoroushi M, Keshani F (2013) A review of glass-ionomers: From conventional glass-ionomer to bioactive glass-ionomer. *Dental Research Journal* 10: 411-420.
244. Rizzante FAP, Cunali RS, Bombonatti JFS, et al. (2015) Indications and restorative techniques for glass ionomer cement. *RSBO (Online)* 12: 79-87.
245. Mitra SB, Oxman JD, Falsafi A, et al. (2011) Fluoride release and recharge behavior of a nano-filled resin-modified glass ionomer compared with that of other fluoride releasing materials. *Am J Dent* 24: 372-378.
246. Price CA (1994) A history of dental polymers. *Australian prosthodontic journal / Australian Prosthodontic Society* 8: 47-54.
247. Bowen RL (1963) Properties of a silica-reinforced polymer for dental restorations. *J Am Dent Assoc* 66: 57-64.
248. Yiu CK, Pashley EL, Hiraishi N, et al. (2005) Solvent and water retention in dental adhesive blends after evaporation. *Biomaterials* 26: 6863-6872.
249. Habib E, Wang R, Wang Y, et al. (2016) Inorganic Fillers for Dental Resin Composites: Present and Future. *ACS Biomaterials Science & Engineering* 2: 1-11.
250. Della Bona A, Kelly JR (2008) The clinical success of all-ceramic restorations. *J Am Dent Assoc* 139 Suppl: 8s-13s.
251. Rathore M, Singh A, Pant VA (2012) The dental amalgam toxicity fear: a myth or actuality. *Toxicology international* 19: 81-88.
252. Badami V, Ahuja B (2014) Biosmart materials: breaking new ground in dentistry. *TheScientificWorldJournal* 2014: 986912-986912.
253. Wang Q, O'Hare D (2012) Recent advances in the synthesis and application of layered double hydroxide (LDH) nanosheets. *Chem Rev* 112: 4124-4155.
254. Zhao J, Liu Y, Sun W-b, et al. (2012) First detection, characterization, and application of amorphous calcium phosphate in dentistry. *Journal of Dental Sciences* 7: 316-323.
255. Zhao J, Liu Y, Sun W-B, et al. (2011) Amorphous calcium phosphate and its application in dentistry. *Chemistry Central journal* 5: 40-40.
256. Zhang K, Xu Z, Lu J, et al. (2014) Potential for Layered Double Hydroxides-Based, Innovative Drug Delivery Systems. *International Journal of Molecular Sciences* 15: 7409.
257. Bi X, Zhang H, Dou L (2014) Layered Double Hydroxide-Based Nanocarriers for Drug Delivery. *Pharmaceutics* 6: 298.
258. Tronto J, Bordonal AC, Naal Z, et al. (2013) Conducting Polymers / Layered Double Hydroxides Intercalated Nanocomposites.

259. Chung H-E, Park D-H, Choy J-H, et al. (2012) Intracellular trafficking pathway of layered double hydroxide nanoparticles in human cells: Size-dependent cellular delivery. *Applied Clay Science* 65–66: 24-30.
260. Li S, Li J, Wang CJ, et al. (2013) Cellular uptake and gene delivery using layered double hydroxide nanoparticles. *Journal of Materials Chemistry B* 1: 61-68.
261. Lv L, He J, Wei M, et al. (2006) Factors influencing the removal of fluoride from aqueous solution by calcined Mg–Al–CO3 layered double hydroxides. *Journal of Hazardous Materials* 133: 119-128.
262. Kameda T, Oba J, Yoshioka T (2015) Recyclable Mg–Al layered double hydroxides for fluoride removal: Kinetic and equilibrium studies. *Journal of Hazardous Materials* 300: 475-482.
263. Sudheer M, Prabhu R, Raju K, et al. (2014) Effect of Filler Content on the Performance of Epoxy/PTW Composites. *Advances in Materials Science and Engineering* 2014: 11.
264. Rojas R, Jimenez-Kairuz AF, Manzo RH, et al. (2014) Release kinetics from LDH-drug hybrids: Effect of layers stacking and drug solubility and polarity. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* 463: 37-43.
265. Peng Y, Wang W, Cao J, et al. (2015) Effects of a layered double hydroxide (LDH) on the photostability of wood flour/polypropylene composites during UV weathering. *RSC Advances* 5: 41230-41237.
266. Liu Z, Ma R, Osada M, et al. (2006) Synthesis, anion exchange, and delamination of Co-Al layered double hydroxide: assembly of the exfoliated nanosheet/polyanion composite films and magneto-optical studies. *J Am Chem Soc* 128: 4872-4880.
267. Iglesias-Silva E, Rivas J, León Isidro LM, et al. (2007) Synthesis of silver-coated magnetite nanoparticles. *Journal of Non-Crystalline Solids* 353: 829-831.
268. Xu H, Suslick KS (2010) Water-Soluble Fluorescent Silver Nanoclusters. *Advanced Materials* 22: 1078-1082.
269. Wandera A, Spencer P, Bohaty B (1996) In vitro comparative fluoride release, and weight and volume change in light-curing and self-curing glass ionomer materials. *Pediatric dentistry* 18: 210-214.
270. Schiff N, Grosgogeat B, Lissac M, et al. (2002) Influence of fluoride content and pH on the corrosion resistance of titanium and its alloys. *Biomaterials* 23: 1995-2002.
271. Kocijan A, Merl DK, Jenko M (2011) The corrosion behaviour of austenitic and duplex stainless steels in artificial saliva with the addition of fluoride. *Corrosion Science* 53: 776-783.
272. Christensen GD, Simpson WA, Younger JJ, et al. (1985) Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. *Journal of clinical microbiology* 22: 996-1006.
273. Kalishwaralal K, BarathManiKanth S, Pandian SR, et al. (2010) Silver nanoparticles impede the biofilm formation by Pseudomonas aeruginosa and Staphylococcus epidermidis. *Colloids Surf B Biointerfaces* 79: 340-344.
274. Parisotto TM, King WF, Duque C, et al. (2011) Immunological and microbiologic changes during caries development in young children. *Caries Res* 45: 377-385.

275. Albertini M, Lopez-Cerero L, O'Sullivan MG, et al. (2015) Assessment of periodontal and opportunistic flora in patients with peri-implantitis. *Clin Oral Implants Res* 26: 937-941.
276. Canullo L, Rossetti PH, Penarrocha D (2015) Identification of Enterococcus Faecalis and Pseudomonas Aeruginosa on and in Implants in Individuals with Peri-implant Disease: A Cross-Sectional Study. *Int J Oral Maxillofac Implants* 30: 583-587.
277. Persson GR, Renvert S (2014) Cluster of bacteria associated with peri-implantitis. *Clin Implant Dent Relat Res* 16: 783-793.
278. Heitz-Mayfield LJ, Lang NP (2010) Comparative biology of chronic and aggressive periodontitis vs. peri-implantitis. *Periodontol 2000* 53: 167-181.
279. Rivas Caldas R, Le Gall F, Revert K, et al. (2015) Pseudomonas aeruginosa and Periodontal Pathogens in the Oral Cavity and Lungs of Cystic Fibrosis Patients: a Case-Control Study. *J Clin Microbiol* 53: 1898-1907.
280. Sadikot RT, Blackwell TS, Christman JW, et al. (2005) Pathogen-host interactions in Pseudomonas aeruginosa pneumonia. *Am J Respir Crit Care Med* 171: 1209-1223.
281. Drenkard E, Ausubel FM (2002) Pseudomonas biofilm formation and antibiotic resistance are linked to phenotypic variation. *Nature* 416: 740-743.
282. Drenkard E (2003) Antimicrobial resistance of Pseudomonas aeruginosa biofilms. *Microbes Infect* 5: 1213-1219.
283. Canullo L, Penarrocha-Oltra D, Covani U, et al. (2016) Clinical and microbiological findings in patients with peri-implantitis: a cross-sectional study. *Clin Oral Implants Res* 27: 376-382.
284. Scannapieco FA, Yu J, Raghavendran K, et al. (2009) A randomized trial of chlorhexidine gluconate on oral bacterial pathogens in mechanically ventilated patients. *Crit Care* 13: R117.
285. Amaral SM, Cortes Ade Q, Pires FR (2009) Nosocomial pneumonia: importance of the oral environment. *J Bras Pneumol* 35: 1116-1124.
286. Heo SM, Haase EM, Lesse AJ, et al. (2008) Genetic relationships between respiratory pathogens isolated from dental plaque and bronchoalveolar lavage fluid from patients in the intensive care unit undergoing mechanical ventilation. *Clin Infect Dis* 47: 1562-1570.
287. Coviello V, Stevens MR (2007) Contemporary concepts in the treatment of chronic osteomyelitis. *Oral Maxillofac Surg Clin North Am* 19: 523-534, vi.
288. Barasch A, Gordon S, Geist RY, et al. (2003) Necrotizing stomatitis: report of 3 Pseudomonas aeruginosa-positive patients. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 96: 136-140.
289. Jones SM, Morgan M, Humphrey TJ, et al. (2001) Effect of vancomycin and rifampicin on meticillin-resistant Staphylococcus aureus biofilms. *Lancet* 357: 40-41.
290. O'Donnell LE, Smith K, Williams C, et al. (2016) Dentures are a Reservoir for Respiratory Pathogens. *J Prosthodont* 25: 99-104.
291. Lewis N, Parmar N, Hussain Z, et al. (2015) Colonisation of dentures by Staphylococcus aureus and MRSA in out-patient and in-patient populations. *Eur J Clin Microbiol Infect Dis* 34: 1823-1826.

292. Smith A, Buchinsky FJ, Post JC (2011) Eradicating chronic ear, nose, and throat infections: a systematically conducted literature review of advances in biofilm treatment. *Otolaryngol Head Neck Surg* 144: 338-347.
293. Hansson C, Hoborn J, Moller A, et al. (1995) The microbial flora in venous leg ulcers without clinical signs of infection. Repeated culture using a validated standardised microbiological technique. *Acta Derm Venereol* 75: 24-30.
294. Lew DP, Waldvogel FA (2004) Osteomyelitis. *Lancet* 364: 369-379.
295. Salvi GE, Furst MM, Lang NP, et al. (2008) One-year bacterial colonization patterns of Staphylococcus aureus and other bacteria at implants and adjacent teeth. *Clin Oral Implants Res* 19: 242-248.
296. Harris LG, Richards RG (2004) Staphylococcus aureus adhesion to different treated titanium surfaces. *J Mater Sci Mater Med* 15: 311-314.
297. Harris LG, Richards RG (2004) Staphylococcus aureus adhesion to different treated titanium surfaces. *Journal of Materials Science: Materials in Medicine* 15: 311-314.
298. Edwardsson S, Bing M, Axtelius B, et al. (1999) The microbiota of periodontal pockets with different depths in therapy-resistant periodontitis. *J Clin Periodontol* 26: 143-152.
299. Passariello C, Puttini M, Iebba V, et al. (2012) Influence of oral conditions on colonization by highly toxigenic Staphylococcus aureus strains. *Oral Diseases* 18: 402-409.
300. Poeschl PW, Crepaz V, Russmueller G, et al. (2011) Endodontic pathogens causing deep neck space infections: clinical impact of different sampling techniques and antibiotic susceptibility. *J Endod* 37: 1201-1205.
301. Kouidhi B, Zmantar T, Hentati H, et al. (2010) Cell surface hydrophobicity, biofilm formation, adhesives properties and molecular detection of adhesins genes in Staphylococcus aureus associated to dental caries. *Microb Pathog* 49: 14-22.
302. Lazarevic V, Whiteson K, Huse S, et al. (2009) Metagenomic study of the oral microbiota by Illumina high-throughput sequencing. *J Microbiol Methods* 79: 266-271.
303. Avila M, Ojcius DM, Yilmaz O (2009) The oral microbiota: living with a permanent guest. *DNA Cell Biol* 28: 405-411.
304. Kuehbacher T, Rehman A, Lepage P, et al. (2008) Intestinal TM7 bacterial phylogenies in active inflammatory bowel disease. *J Med Microbiol* 57: 1569-1576.
305. Raghavendran K, Mylotte JM, Scannapieco FA (2007) Nursing home-associated pneumonia, hospital-acquired pneumonia and ventilator-associated pneumonia: the contribution of dental biofilms and periodontal inflammation. *Periodontol 2000* 44: 164-177.
306. Nasidze I, Li J, Quinque D, et al. (2009) Global diversity in the human salivary microbiome. *Genome Res* 19: 636-643.
307. Mager DL, Haffajee AD, Devlin PM, et al. (2005) The salivary microbiota as a diagnostic indicator of oral cancer: a descriptive, non-randomized study of cancer-free and oral squamous cell carcinoma subjects. *Journal of translational medicine* 3: 27-27.

308. Faveri M, Mayer MP, Feres M, et al. (2008) Microbiological diversity of generalized aggressive periodontitis by 16S rRNA clonal analysis. *Oral Microbiol Immunol* 23: 112-118.
309. Vitorino R, Lobo MJ, Duarte JR, et al. (2005) The role of salivary peptides in dental caries. *Biomed Chromatogr* 19: 214-222.
310. Smith PB, Tomfohrde KM, Rhoden DL, et al. (1972) API system: a multitube micromethod for identification of Enterobacteriaceae. *Applied microbiology* 24: 449-452.
311. Tamura S, Yonezawa H, Motegi M, et al. (2009) Inhibiting effects of Streptococcus salivarius on competence-stimulating peptide-dependent biofilm formation by Streptococcus mutans. *Oral Microbiol Immunol* 24: 152-161.
312. Montanaro L, Campoccia D, Rizzi S, et al. (2004) Evaluation of bacterial adhesion of Streptococcus mutans on dental restorative materials. *Biomaterials* 25: 4457-4463.
313. Otto M, Sussmuth R, Jung G, et al. (1998) Structure of the pheromone peptide of the Staphylococcus epidermidis agr system. *FEBS Lett* 424: 89-94.
314. Vadyvaloo V, Otto M (2005) Molecular genetics of Staphylococcus epidermidis biofilms on indwelling medical devices. *Int J Artif Organs* 28: 1069-1078.
315. Duerden BI, Goodwin L, O'Neil TC (1987) Identification of Bacteroides species from adult periodontal disease. *J Med Microbiol* 24: 133-137.
316. Lacroix J-M, Walker CB (1996) Detection and prevalence of the tetracycline resistance determinant Tet Q in the microbiota associated with adult periodontitis. *Oral Microbiology and Immunology* 11: 282-288.
317. Otto M (2014) Staphylococcus epidermidis pathogenesis. *Methods Mol Biol* 1106: 17-31.
318. Fey PD, Olson ME (2010) Current concepts in biofilm formation of Staphylococcus epidermidis. *Future Microbiol* 5: 917-933.
319. Rogers KL, Fey PD, Rupp ME (2009) Coagulase-negative staphylococcal infections. *Infect Dis Clin North Am* 23: 73-98.
320. Hogan S, Stevens NT, Humphreys H, et al. (2015) Current and future approaches to the prevention and treatment of staphylococcal medical device-related infections. *Curr Pharm Des* 21: 100-113.
321. Hwang IS, Hwang JH, Choi H, et al. (2012) Synergistic effects between silver nanoparticles and antibiotics and the mechanisms involved. *J Med Microbiol* 61: 1719-1726.
322. Nel A, Xia T, Madler L, et al. (2006) Toxic potential of materials at the nanolevel. *Science* 311: 622-627.
323. Sondi I, Salopek-Sondi B (2004) Silver nanoparticles as antimicrobial agent: a case study on E. coli as a model for Gram-negative bacteria. *J Colloid Interface Sci* 275: 177-182.
324. Ansari MA, Maayah ZH, Bakheet SA, et al. (2013) The role of aryl hydrocarbon receptor signaling pathway in cardiotoxicity of acute lead intoxication in vivo and in vitro rat model. *Toxicology* 306: 40-49.
325. Chaudhari PR, Masurkar SA, Shidore VB, et al. (2012) Effect of Biosynthesized Silver Nanoparticles on Staphylococcus aureus Biofilm Quenching and Prevention of Biofilm Formation. *Nano-Micro Letters* 4: 34-39.