IN VITRO ANTI-BACTERIAL ACTIVITY OF TITANIUM OXIDE NANO-COMPOSITES CONTAINING BENZALKONIUM CHLORIDE AND CHLORHEXIDINE GLUCONATE

by

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DECLARATION

I, Abdalla Mohammed Ali Atbayga, declare that the contents of this thesis represent my own unaided work and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

Signed **Date**

ABSTRACT

Newly developed and commercial dental resins which are commonly used nowadays have to be tested for their antimicrobial susceptibility. The purpose of this *in vitro* study was to investigate the antimicrobial activity of a titanium oxide (TiO_2) nano-composite which was prepared with different antibacterial substances and used as restoratives in dentistry to combat certain selected bacteria that are considered the principle causes of some tooth diseases, for example, tooth decay and to prevent unsuccessful dental restoration.

The TiO_2 nano-composite was prepared and divided into four groups: The first group was an untreated TiO_2 nano-composite. The second group was silane-treated TiO_2 nano-composite. The third group was treated TiO_2 nano-composite which was combined with chlorhexidine gluconate (CHxG). The fourth group was treated TiO_2 nano-composite which was combined with benzalkonium chloride (BzCl).

Five of the selected bacteria were grown overnight in Petri dishes. Four of them, namely, *Escherichia coli* (*E. coli*) ATCC 11775, *Staphylococcus aureus* (*S. aureus*) ATCC 12600, *Enterococcus faecalis* (*E. faecalis*) ATCC 29212, and *Pseudomonas aeruginosa* (*P. aeruginosa*) ATCC 10145, were grown on Müller-Hinton Agar (MHA). *Streptococcus mutans* (*S. mutans*) ATCC 25175 was grown on Brain Heart Infusion (BHI) agar. All these bacteria were tested against the TiO_2 nano-composite, and incubated for 24 hours at 37°C, except *S. mutans*, which was incubated separately and exposed to CO_2. It was placed into a CO_2 water-jacketed incubator in an atmosphere of 5% CO_2 for 24 hours at 37°C.

The obtained results showed that neither of the groups of TiO_2 nano-composites, (untreated TiO_2 nano-composite and treated TiO_2 nano-composite) exhibited antimicrobial activity against the pathogens. Only preparations of TiO_2 nano-composites at a concentration of 3 %m/m of both CHxG and BzCl showed antimicrobial activity against *S. aureus*. Antimicrobial activity against *S. mutans*, *E. coli*, *P. aeruginosa*, *E. faecalis* and *S. aureus*, were only realized at a concentration of 10 %m/m for both CHxG and BzCl..

KEYWORDS

Titanium oxide (TiO_2)

Nano-composite

Camphorquinone (CQ)

Urethane dimethacrylate (UDMA)

Silane

Dental resin

Dental restorative materials

Antimicrobial activity

Benzalkonium Chloride (BzCl)

Chlorhexidine gluconate (CHxG)

Carbon dioxide (CO_2)

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DEDICATION

I dedicate this study to my family and my friends.

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GLOSSARY AND ABBREVIATION

ADT	Agar diffusion test
ATCC	American type culture collections
BHI	Broth heart infusion
BzCl	Benzalkonium chloride
CFU	Colony-forming unit
CHxG	Chlorhexidine gluconate
CQ	Camphorquinone
DBS	Dentin Bonding System
DMAEMA	(2-dimethylaminoethyl) methacrylate
FAA	Fastidious anaerobic agar
FAB	Fastidious anaerobic broth
McF	McFarland standard
MHA	Müller-Hinton agar
MHB	Müller-Hinton broth
MIC	Minimum inhibitory concentration
MPTMS	3-(methacryloyloxy)-propyltrimethoxysilane
MRT	Multiple range test
MTA	Mineral trioxide aggregate
MWCNT	Multi-walled carbon nanotubes
ROS	Reactive oxygen species
RPM	Revolution per-minute
SMR	Statistical multiple range
TiO_2	Titanium oxide
TTC	Triphenyltetrazolium chloride
UDMA	Urethane dimethacrylate

YMT	Yeast and mould test
DMAE-CB	Methacryloxylethyl cetyl ammonium chloride
DMAE-BC	Methacryloxylethyl benzyl dimethyl ammonium chloride
DMAE-m-CBC	Methacryloxylethyl m-chloro benzyl dimethyl ammonium chloride

CLARIFICATION OF BASIC TERMS

Dental restorations: or dental fillings, dental restorative materials specially fabricated and designed for use to restore the function, integrity and morphology of missing tooth structure (Christian *et al*., 2006).

Dental resin composites: materials consisting of two or more components, commonly, dental resin composites contain organic, inorganic fillers incorporated into a system that would induce polymerization. Synthetic resins mainly evolved to be used as dental restorative materials (Cramer *et al*., 2011).

Nano-TiO2 particles: titanium filler particles of less than 100 nanometers in diameter exhibiting new or enhanced size-dependent properties compared with microparticles of the same substance (Roy *et al*., 2005).

Nano-composites: a multiphase solid material incorporating filler nano-particles into a resin matrix of standard material; or a material that results from the intimate mixture of two or more nano-phase substances (Chau *et al*., 2008).

Dentine bonding systems (DBS): are certain resin materials used in dentistry to make dental composite filling materials adhere to both dentin and enamel and always contains methacrylates with some volatile carrier and solvent like acetone (Imazato, 2003).

Benzalkonium chloride (BzCl): also known as alkyldimethylbenzylammonium chloride is a quaternary ammonium surfactant and it acts as a biocide, widely used in dental applications as antibacterial agent (Houari & Di Martino, (2007).

Chlorhexidine gluconate (CHxG): biocide is effective on both Gram-positive and Gram-negative bacteria, although less so on Gram-negative bacteria. Its bacteriostatic mechanism causes membrane disruption. It is also useful against fungi and enveloped viruses, though this has not been extensively investigated (Sena *et al*., 2006).

Multi-walled carbon nanotubes (MWCNT): consist of multiple layers of graphite rolled in on themselves to form a tube shape. There are two models which can be used to describe the structures of multi-walled nanotubes. In the first model a sheet of graphite is arranged in concentric cylinders. In the second model, a single sheet of graphite is rolled in around itself,

resembling a scroll of parchment or a rolled newspaper. The interlayer distance in multi-walled nanotubes is close to the distance between graphene layers in graphite, which are about 3.4 Å (Lee *et al*., 2005).

Müller-Hinton agar (MHA): is a microbiological growth medium that is commonly used for antibiotic susceptibility testing. It is also used to isolate and maintain *Neisseria* and *Moraxella* species (Sipert *et al*., 2007).

Table 1: Chemical compositions of Müller-Hinton agar

Ingredients	gram/litre
Beef, dehydrated infusion from	300
Casein hydrolysate	17.5
Agar	17
Starch	1.5
pH at 25°C	7.3±0.1

Brain heart infusion (BHI): is a highly nutritious general-purpose growth medium for fastidious microorganisms, such as *streptococci*, *pneumococci* and *meningococci*. It is made by the recuperation of nutrients from boiled cattle hearts and brains. Soluble factors are released into the broth during the boiling procedure. The broth can then be turned into powder for easy distribution (Joshi & Eley, 1988).

Table 2: Chemical compositions of brain heart infusion broth

Ingredients	Gms / Litre
Calf brain, infusion from	200.000
Beef heart, infusion from	250.000
Proteose peptone	10.000
Dextrose	2.000
Sodium chloride	5.000
Disodium phosphate	2.500
pH at 25°C	7.4±0.2

The agar diffusion test (ADT) is a means of measuring the effect of an antimicrobial agent against Gram-positive and Gram-negative bacteria grown in culture. The bacteria in question are swabbed uniformly across a culture plate. A filter-paper disk, impregnated with the compound to be tested, is placed on the surface of the agar. The compound diffuses from the filter paper into the agar. The concentration of the compound will be highest next to the disk, and will decrease as the distance from the disk increases. If the compound is effective against bacteria at a certain concentration, no colonies will grow where the concentration in the agar is greater than or equal to the effective concentration. This is the zone of inhibition. Thus, the size of the zone of inhibition is a measure of the compound's effectiveness: the larger the clear area around the filter disk, the more effective the compound (Moreau *et al*., 2011).

Dilution tests are used to determine the minimal concentration of an antimicrobial agent required for inhibiting or killing a microorganism. Serial dilutions of the antimicrobial are inoculated with the organism and incubated. The terms "broth" or "tube" and "agar" or "plate" are added to the term "dilution test" to indicate tests performed in liquid and agar media, respectively (Ericsson & Sherris, 1971).

McFarland standards (McF): are used in microbiology as a reference to adjust the turbidity of bacterial suspensions so that the number of bacteria will be within a given range (Ahmad *et al*., 2009).

Table 3: Percentage transmittance and corresponding absorbance of McFarland standard (at wavelength of 600 nm), compared to that of $BaCl_2$ and H_2SO_4

McFarland Standard No	0.5	1	2	3	4
1.0% barium chloride (mL)	0.05	0.1	0.2	0.3	0.4
1.0% sulfuric acid (mL)	9.95	9.9	9.8	9.7	9.6
Approx. cell density (1×10^8 CFU/mL)	1.5	3.0	6.0	9.0	12.0
% Transmittance	74.3	55.6	35.6	26.4	21.5
Absorbance	0.132	0.257	0.451	0.582	0.669

Fastidious anaerobic broth (FAB): is a liquid medium used for cultivation of anaerobic microorganisms. This medium is very rich in nutrients from the selected peptones (casein peptone 10.0 g/L, meat peptone 5.0 g/L). L-cystine 0.5 g/L and sodium thioglycollate 0.5 g/L reduces the electrical potential (E_h) of the medium. Agar decreases oxygen tension and resazurin is the oxidation and reduction indicator (Vianna *et al*., 2004).

Streptococcus mutans (*S. mutans*): is a facultatively anaerobic, Gram-positive coccus-shaped bacterium commonly found in the human oral cavity and is a significant contributor to tooth decay (Clarke, 1924).

Escherichia coli (*E. coli*): is a Gram-negative, rod-shaped bacterium that is commonly found in the lower intestine of warm-blooded organisms (endotherms). Most *E. coli* strains are harmless, but some types can cause serious food poisoning in humans, and are occasionally responsible for product recalls. The harmless strains are part of the normal flora of the gut and can benefit their hosts by producing vitamin K_2, and by preventing the establishment of pathogenic bacteria within the intestine (Reid *et al*., 2001).

Enterococcus faecalis (*E. faecalis*): formerly classified as part of the Group D *Streptococcus* system, a Gram-positive, commensal bacterium inhabiting the gastrointestinal tracts of humans and other mammals. It is among the main constituents of some probiotic food supplements. Like other species of genus *Enterococcus*, *E. faecalis* can cause life-threatening infections in humans, especially in the hospital environment, where the naturally high levels of antibiotic resistance found in *E. faecalis* contribute to its pathogenicity. *E. faecalis* has frequently been found in root canal-treated teeth in prevalence values ranging from 30% to 90% of the cases. Root canal-treated teeth are about nine times more likely to harbor *E. faecalis* than cases of primary infections (Molander *et al*., 1998; Rôças *et al*., 2004).

Staphylococcus aureus (*S. aureus*): known as golden staph and Oro staphira is a facultative anaerobic Gram-positive coccal bacterium. It is frequently part of the skin flora found in the nose and on the skin, and in this manner about 20% of the human population are long-term carriers of *S. aureus. S. aureus* is the most common species of *staphylococci* to cause *Staphylococcal* infections (Ogston, 1984).

Pseudomonas aeruginosa (*P. aeruginosa*): is Gram-negative and it is a common bacterium that could cause disease in humans and animals. It is found in soil, water, skin flora, and most man-made environments throughout the world. It thrives not only in normal atmospheres but also in hypoxic atmospheres, and has thus colonized many natural and

artificial environments. It uses a wide range of organic material for food. In animals, this versatility enables the organism to infect damaged tissues or those with reduced immunity (Balcht & Smith, 1994).

Biosafety levels: are the levels of the bio-containment precautions required to isolate dangerous biological agents in an enclosed facility. The levels of containment range from the lowest bio-safety level 1 to the highest at level 4. These levels have been specified by the Centres for Disease Control and Prevention (CDC), US (Richmond & McKinney, 1999).

The National collection of type culture (NCTC): recognised internationally to supply reference bacterial cultures of medical and scientific world-wide to support academic and health institutions.

Trypticase soy agar (TSA) is a general purpose medium which provides enough nutrients to grow a wide variety of microorganisms. It is widely used in many applications including: culture storage, counting, isolation of pure cultures or simply general culture.

Minimum inhibitory concentration (MIC) is the lowest concentration of antimicrobials that will prevent the growth of microorganisms after overnight incubation. It is important to confirm resistance of microorganisms to antimicrobial agents and also to monitor the activity of new antimicrobial agents (Andrews, 2001; Turnidge *et al*., 2003).

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

With the development and enhancement of nano-technology, nano-materials have widely been applied in various manufacturing industries such as instrument manufacturing, and in the scientific fields such as medicine and dentistry. In dentistry, the nano-materials are considered as direct restoratives (Imazato *et al*., 1994). Essentially, dental restorative treatment depends on many factors. Of these, the most important is the prevention or elimination of bacteria in the oral environment which may cause endodontic infections and periodontal disease. Moreover, the principal modality for infectious process is through restorative treatments during which the infected tissues are removed and replaced with a variety of inert materials (Wright *et al*., 1992).

Extensive investigations on the placement and replacement of dental restorations have been performed in various research studies (Weerheijm *et al*., 1999; Mjör *et al.*, 2000; Deligeorgi *et al*., 2001). However, it can be concluded that primary caries has been proportionately found to be the principal cause for the provision of incipient restorations of composite materials. Thus recurring caries are the principal reason for failure of restorations and therefore justify the replacement of most dental restoratives (Deligeorgi *et al*., 2001).

Presently it is recognised that bacteria are the principal reason for the occurrence of secondary caries; hence the development of dental materials that should inhibit bacterial activity at the dental composite interface may be the most effective route for reducing or even eliminating the recurring caries (Sarrett, 2005; Ahn *et al*., 2009). Numerous studies have been conducted to firstly, ascertain the effectiveness of various compositions of organic components incorporated into the matrix of the dental restorative materials and secondly, to incorporate substances that *per se* have antimicrobial activity. These substances may be of metallic, ionic or bioinorganic nature. In the ensuing text previous studies to evaluate these incorporations are detailed.

While the clinical performance of dental composite materials has highly developed in terms of restoration durability and aesthetics, it has only been lately that increased attention has been paid to achieve antibacterial properties in these composite materials (Imazato, 2003). For instance, the incorporation of antimicrobial agents, such as chlorhexidine (CHX), in the

resin matrix has been found to be effective against various microorganisms (Imazato *et al*., 2003; Welch *et al*., 2010).

In order to produce dental restoratives with antibacterial activities, an antibacterial monomer such as methacryloyloxydodecylpyridinium bromide (MDPB) has been developed. The world's first antibacterial adhesive system with antibacterial properties came in recent years on the market for commercial use (Imazato, 2009).

For glass-ionomer cements used for restorative therapy, the approaches to provide antibacterial property have included the incorporation of CHX. Incorporation of 1% (w/w?) CHX diacetate was found to be most favourable to give adequate antibacterial properties (Takahashi *et al*., 2006; Imazato, 2009).

Compounds of restorations consist of two main composites: dentin bonding systems to be utilized in the dental cavity before placing the restorative filling materials and the resin composites for fillings. The antibacterial characteristics of these two composites should impede the harmful influences caused by bacteria. The antibacterial efficacy of composites for fillings is fundamentally relevant to inhibition of plaque accumulation on the surface of the restorative and tooth around the restorative materials. In terms of ultimate disinfection of the cavity, the antibacterial efficacy of the dentin bonding system (DBS) should inactivate the bacterial growth which invades the adhesive interface of the cavity (Imazato *et al*., 2003).

Dental nano-composite materials are becoming widely acceptable due to their convenience of application, aesthetics, conservative preparation requirement and the generalization of adhesive mechanisms (Mjör, *et al*., 1999). However, Xiao *et al*. (2008) found that cariogenic bacteria such as *Lactobacillus casei* (*L. casei*), *S. mutans*, *S. aureus* and *Actinomyces viscosus* (*A. viscosus*) can more easily propagate and accumulate on the surfaces of cured composite restoratives in the oral cavity than on the surface of restorations made with various chemicals such as methacryloxylethyl cetyl ammonium chloride (DMAE-CB). Furthermore, it has been shown that many dental restorative materials may positively influence the growth of bacteria in the oral environment (Imazato *et al*., 2003). Consequently, several other studies have been carried out in recent years due to the high frequency of recurring caries after restorative treatments and because of the unsuccessful performance of dental restoratives which resulted from the growth of oral bacteria (Mehdawi *et al*., 2009; Vargas-Reus *et al*., 2012). In addition, much attention has been given to the curative effects revealed by direct filling restoratives (Mjor, 1996; Wilson *et al*., 1997).

In a survey undertaken by Tobias, (1988), it was found that none of the currently available dental restoratives provide complete seal of the cavity wall. The result is that micro-spaces are present at the interfaces and cariogenic bacteria can easily permeate through these spaces. Thus, dental restorative composite materials that exhibit antibacterial properties are much needed to improve the longevity of dental restoratives and their usage. The problems associated with bacterial microleakage at the materials' interfaces and the cavity walls were only investigated from the early 1950s era. Fisher, (1966) and Schouboe & MacDonald, (1962) demonstrated that bacteria may remain viable underneath fillings which have not come into contact with antiseptic for long periods of time. Therefore, the continued presence of bacteria underneath restorations is considered a major concern in the development of recurring dental caries (Trowbridge, 1981; Brannstrom, 1984; Brännström, 1986). It has been proposed that the presence of the bacteria may be eliminated if the cut dentine surfaces were perfectly sterilized with antimicrobial solutions before restoring the cavity (Brännström & Nyborg, 1971; Brännström & Nyborg, 1973). These antimicrobial solutions should be harmless to the dental pulp and surrounding tissue (Brännström, 1986).

Since bactericidity cannot be demonstrated on a newly restored cavity wall (Mjör, 1974; Mjör, 1977), the application of antibacterial agents as cavity cleaners has been suggested (Eriksen & Leidal, 1979). Furthermore, for the efficacy of the air-water spray solution, it has been shown that spraying these solutions removed bacteria from an experimentally infected caries-free cavity surface *in vitro* (Vlietstra *et al*., 1980). It is therefore acceptable to tentatively justify the use of cavity cleaning solutions for removal of bacteria from the cavity walls. Consequently, there is a need to provide dental restorative composites that have antibacterial properties which would be an effective inhibitor of bacterial growth (Beagrie & Smith, 1978). In addition, it has been noted that a systematic evaluation of the antibacterial properties of dental materials used as base and lining materials has been performed (Mjör, 1977). The results of this study supported the view that the pulpal infections observed under experimental cavities *in vivo* were not merely due to the chemical irritancy of the restorative materials, but were largely a consequence of bacterial microleakage at the interface of the restorative materials and the cavity wall, furthermore, the antibacterial properties of the materials affected the extent of the bacterial microleakage (Tobias *et al*., 1988).

TiO_2 composites are extensively used in teeth restorations and implantology because of its well-known excellent performance, but it is considered to be among dental restorative materials that could be affected by bacterial accumulation (Del Curto *et al*., 2005; Leonhardt & Dahlén, 2007). Nevertheless, bacterial infections associated with TiO_2 still need to be diagnosed and eliminated. In some investigations, TiO_2 has been investigated for its

antibacterial activity by using different methods of testing and against different types of organisms in order to achieve TiO_2 nano-composites that have antimicrobial properties (Ribeiro & Ericson, 1991; Yeung *et al*., 2009). Presently, there are considerable problems experienced with current available dental restorative composites. As many as half of all dental restoratives are replacements, and the main reason for replacement is reoccurring of secondary caries (Bernardo *et al*., 2007; Sarrett, 2005), similar problems commonly occur in orthodontic application. It was found that adhesives used for fixing the bracket on the enamel indeed enhance the adhesion and accumulation of oral bacteria (Ahn *et al*., 2009; Welch *et al*., 2010)

1.2 Literature review

Various investigations have been carried out to illustrate the antimicrobial activity of dental restorative materials that contain metals. Matsunaga *et al*. (1985) demonstrated the microbicidal effect of TiO_2 nano-composites. The bactericidal activity of TiO_2 nano-particles was based on the photocatalytic reactivity under UV–illumination (Kikuchi *et al*., 1997). The illumination of TiO_2 leads to the generation of reactive oxygen species (ROS) which oxidize membrane lipids of the bacteria and cause disruption of the outer and cytoplasmic membranes of the bacteria by lipid peroxidation, leading to the death of the cells (Maness *et a*l., 1999; Arora *et al.,* 2012)

Arora *et al*. (2012) reported that, since the microbicidal ability of TiO_2 nano-particles depends on the photo response and photocatalytic reactivity, the improvement of photocatalysis should enhance the killing of bacteria, viruses and fungi. The manufacturing of nano-composites to circumvent the photocatalytic reactivity of the TiO_2 due to charge recombination and increasing the photo response of TiO_2 would permit nano-composites to be used for sterilization with sunlight, in a most energy-effective way. Yao *et al*. (2008) and Mo *et al*. (2007) suggested that, multiple additions of other antibactericidal composites can be added to TiO_2 particulate matter such as nano-particles, nano-films and nano-rods so as to create various TiO_2 nano-composites. These additions may include doping with metal and/or non-metal ions, compositing with a polymer or creation of core–shell magnetic nano-particles (Blake *et al*., 1999; Fu *et al*., 2005; Liu *et al*., 2007; Mahltig *et al*., 2007).

Tobias (1988) reported that the antimicrobial action of dental restorative materials can be investigated with different methods and they revealed that the agar diffusion inhibitory test (ADT) is the most frequently used method for evaluating the antibacterial properties of dental materials. It is difficult to compare the results of the many subsequent investigations as various bacterial strains and growth media have been utilized by the different authors as follows:- Turkheim (1953) found that most dental materials proved to be bactericidal whilst setting, that is as long as a chemical reaction was proceeding, and afterwards, zinc oxide (ZnO) was significantly antiseptic and in composition with eugenol had long lasting, highly bactericidal properties. McCue *et al*. (1951) found that the sensitivity for Gram-positive organisms to the bacteriostatic effect of the filling materials was higher than for Gram-negative organisms although the addition of low concentrations of fluorides to resin and zinc phosphate cement did not totally inhibit the growth of the microorganism tested. Both Mangi *et al*. (1959) and Updegraff *et al*. (1971) noted that the duration of bacteriostatic activity for silicate, amalgam and acrylic materials was decreased rapidly when leaching in water and also that the composition of the nutrient agar was a major factor in the determination of

antimicrobial activity. Dahl (1978) noted that saliva at times contained some bacteria which were resistant to the bactericidal effect of the various cement compositions. Thus, the composition of the bacterial flora *in vivo* influences the degree of antibacterial activity. Ørstavik & Hensten-Pettersen (1978) investigated the antibacterial activity *in vitro* of twelve resin based and one silicate dental restorative material against five species of bacteria. Although all materials showed some degree of antibacterial activity when fresh, the activity was strongly decreased (in some cases totally abolished) after setting and storage. The data suggested that the materials act more strongly on some strains than others.

Consequently, selective antibacterial activity of dental materials may also influence the bacterial composition of plaque, thus, modulating the development of recurrent carious lesions near restorations *in vivo*. Glassman & Miller (1984) investigated the antibacterial properties of one conventional and three high copper (Cu) containing amalgams. It was concluded that amalgam alloys possess antibacterial properties, some more than others. It was recommended that future research should be directed toward the production of an amalgam that would display good clinical performance and at the same time contain antibacterial properties. Tobias (1988) investigated the antibacterial activity of several different types of dental restorative materials *in vitro* using the ADT against six microorganisms isolated from plaque. All freshly mixed materials showed some degree of antibacterial activity, though the effects varied amongst bacteria. A ranking order was obtained for each material and each material exhibited some reduction of effect, but only after 72 hours of culturing. Most important in this study, a substantial trend towards a negative correlation was demonstrated between the antibacterial activity of the materials *in vitro* and their pulpal irritancy *in vivo*.

Yoshinari *et al*. (2001) investigated the antibacterial effect of surface modifications to titanium on *Porphyromonas gingivalis* (*P. gingivalis*) and *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*). It was found that microbial plaque accumulation surrounding dental implants may develop into peri-implantitis or peri-implantoclasia, which is presented as inflammation or infection around implants, with accompanying bone loss.

It is important to maintain plaque-free surfaces on both supra- and sub-gingival portions of dental implants to prevent peri-implantitis. There are at least two methods of inhibiting the formation of microbial plaque. The first method is to inhibit the initial adhesion of oral bacteria. The second method is to inhibit the accumulation of oral bacteria, which involves surface antibacterial activity. Microbial colonization and antibacterial activity on metallic and ceramic implant materials have been considered *in vitro* and *in vivo* tests (Gatewood *et al*., 2002). Titanium (Ti) on its own has no antibacterial activity (Leonhardt & Dahlén, 2007), and

there is a probable hazard of plaque formation on titanium implants (Yoshinari *et al*., 2000). Nevertheless, only a few experiments have been conducted to verify if the surface modification to titanium implants would inhibit the accumulation of oral bacteria (Bell *et al*., 1997). The modified surfaces must resist wear, because these are the parts that are brushed as a means of plaque control. Surface modifications using a dry process have been utilized in the medical and dental fields as suitable methods for providing good resistance to wear as well as creating thin and adhesive fine ceramics (Buchanan *et al*., 1987; Miyayama *et al*., 1999).

The initial adherence of oral bacteria on commercially pure titanium (cp-titanium) and surface-modified titanium with a dry process was investigated previously (Yoshinari *et al*., 2000) and showed that comparatively large amounts of *P. gingivalis* and *A. actinomycetemcomitans*, which are periodontopathic bacteria, adhered to polished cp-titanium. These findings indicate that there is a probable hazard of bacterial adhesion to titanium surfaces at the supra- and sub-gingival portions of implants and surface modification to inhibit the adherence of oral bacteria is required. The concluded results showed that some surface modification with a dry process is useful in controlling the initial adhesion of oral bacteria. It is also required to provide antibacterial activity for maintaining plaque-free surfaces on titanium implants exposed to the oral cavity. A recent study that investigated the influence of surface modification to titanium on the colonization of oral bacteria *in vitro* and evaluated the release of fluorine ions and cytotoxicity of the L929 cells on F^+-implanted titanium surfaces, exhibited remarkable antibacterial activity. The antibacterial activity of the surface-modified specimens was demonstrated against *P. gingivalis* and *A. actinomycetemcomitans*. These strains were maintained anaerobically on blood agar plates containing trypticase soy agar (TSA). Polished titanium and surface-modified specimens were placed on the agar. They were then incubated anaerobically in TSB of 0.5 ml with both *P. gingivalis*, and *A. actinomycetemcomitans* of 1×10^6 cells/ml for 48 h at 37°C. Antibacterial activity was expressed as the ratio of colony forming units (CFUs) on each sample to those on the control. Each colonization experiment was performed in triplicate and repeated five separate times. The results showed significant differences ($p<0.01$). F^+-implanted specimens significantly inhibited the growth of both *P. gingivalis*, and *A. actinomycetemcomitans* ($p<0.01$) compared to a polished titanium specimen. Other surface modified samples did not reveal any inhibition of the bacterial growth (Yoshinari *et al*., 2001).

Several other studies have indicated that the possibility of incorporating antibacterial compositions into dental restorative composites could provide many potential advantages to patients. An ideal (quixotic) system would (1) eliminate the recurrence of decay around

margins of restorations, (2) inhibit plaque formation on and around restored surfaces, and (3) reduce the number of bacteria in salivary fluids and the oral cavity (Jedrychowski *et al*., 1983). The antibacterial properties of dental restorative materials have been measured previously by Shay *et al*. (1956) and McCue *et al*. (1951).

The most frequently used noble metals in antimicrobial applications are silver (Ag) and gold (Au). For example, gold-capped TiO_2 nano-composites have a strong oxidizing ability and showed a 60–100% killing efficacy of *E. coli* (Fu *et al*., 2005). Likewise, Ag has long been studied and recognized for its potential as an antimicrobial agent, with Ag ions and nano-particles having been shown capable of killing bacteria, viruses, and fungi (Sheel *et al*., 2008). Lately, Ag–TiO_2 nano-composite powders, Ag–TiO_2 nano-films, and Ag-deposited Ag–TiO_2 nano-composite films were all shown to exhibit enhanced photocatalytic reactivity and bactericidal activity compared to TiO_2 nano-particles and TiO_2 nano-films. For instance, Zhang & Chen (2009) used a one-pot sol–gel approach to produce 10 nm sized TiO_2 nano-composites with a high Ag-loading ability. The nano-composites showed a complete inhibition of *E. coli* growth at Ag concentrations of only 2.4 µg/ml. The compositing of Ag into TiO_2 films has been met with similar success. Liu *et al*. (2008) used the Ag doping of a TiO_2 nano-film to kill Ag-resistant *E. coli* when the nano-composite films were UV light-irradiated. In this instance, the bacterial survival rate on the nano-composite was only 7.0%, compared to 53.7% on UV light–irradiated pure TiO_2 nano-films (Liu *et al*., 2008). Similarly, silicon catheters coated with Ag–TiO_2 nano-films with embedded nano-composites demonstrated a self-sterilizing effect, with a 99% sterilization of *E. coli*, *P. aeruginosa* and *S. aureus* after UV illumination (Yao *et al*., 2008). A similar doping of Ag–TiO_2 nano-films with Ag–TiO_2 nano-composite particles led, under solar light conditions, to a photocatalytic killing of *E. coli* that was 6.9-fold more effective than with TiO_2 nano-films, and 1.35-fold more effective than with Ag-TiO_2 nano-films (Akhavan, 2009). Finally, UV–illuminated platinum nano-particles embedded in a TiO_2 nano-film demonstrated an increase in the photocatalysis-driven killing of *Micrococcus lylae* cells, compared to UV-illuminated pure TiO_2 nano-films (Wang *et al*., 2005).

Miller *et al*. (1890) showed that Au exhibited a bactericidal action on agar plates. They also demonstrated that mercury (Hg), Ag and Cu were able to arrest the growth of various mixed organisms of carious dentine. It was found that not only freshly mixed fillings but also pieces of 'old half-worn-out fillings' often retarded bacterial growth.

A recent study investigated the accumulations of dental plaque generated from bacteria and tested the antibacterial activity of some metal and metal oxide nano-particles (cupric oxide CuO), cuprous oxide (Cu_2O), Ag, ZnO, TiO_2, tungsten oxide (WO_3), Ag and CuO composite

and Ag and ZnO composite. TiO_2 was among those metals which are usually used in the dental field. TiO_2 was tested against some bacterial pathogens such as *Prevotella intermedia* (*P. intermedia*), *P. gingivalis*, *Fusobacterium nucleatum* (*F. nucleatum*) and *A. actinomycetemcomitans* that are associated with dental implantation infections and were evaluated under anaerobic conditions. Time-kill assays were performed to investigate the antibacterial properties. The antibacterial activity obtained from this assay was in descending order as TiO_2 was almost the least infected compared to the other dental alloys tested. Time-kill assay with titanium demonstrated a significant decrease in bacterial growth with all facultative strains and within 4 hours. This study confirmed that coating titanium surfaces of dental alloys with antibacterial nano-particles would lead to an increased ratio of successful implants (Vargas-Reus *et al*., 2012).

Leonhardt & Dahlén (2007) found that many metals and alloys used as dental materials express an antibacterial effect mediated by release and dissolution of metal ions (Berry *et al*., 1992; Glassman & Miller, 1984). The ion release is dependent on factors such as the composition and treatment of the material, the electrolyte composition and various biomechanical conditions (Brune, 1988). The ionization process is mediated through oxidation of metals by atmospheric oxygen in water, termed corrosion. In bio-systems, microbial corrosion, may participate and microorganisms can influence the corrosion of metals by altering the microenvironment, for example, by releasing metabolic products and by consuming oxygen, thereby taking an active part in the dissolution process. The antibacterial effect of metals is dependent on the release of metal ions that interfere with the bacterial metabolism (Williams, 1981).

Ti is successfully used as a dental implant material (Adell *et al*., 1981) and has been reported to have a low toxicity and to be most resistant to corrosive forces in the body environment (Albrektsson *et al*., 1981). However, the metal does not remain totally passive and small amounts can be released into electrolyte-like saliva. However, the amount of release is lower than the intake of this element from food and drink (Brune, 1986). Numerous studies have demonstrated this presence of Ti in tissues associated with titanium implants in animals and patients (Woodman *et al*., 1983; Ducheyne *et al*., 1984; Meachim & Williams, 1973; Agins *et al*., 1988).

Factors that can influence the *in vivo* release of Ti from implants are the specific surface area, the surface condition and the presence of biological substances such as proteins (Ducheyne *et al*., 2004). During the corrosion of Ti, particles of TiO_2 are formed (Kasemo 1983; Solar *et al*., 1979) suggesting that the presence of Ti in tissues adjacent to titanium implants can be explained by the fact that TiO_2 needles formed on the surface were broken

off and dissolved *in vivo.* Certain treatment procedures can remove the oxide needles prior to surgery, resulting in a tissue free of Ti.

The properties of Ti that interfere with bacteria are somewhat contradictory. Joshi & Eley (1988) were not able to show any inhibitory activity on bacteria *in vitro* of TiO_2 or of a titanium abutment cylinder. Leonhardt *et al*. (1995) found no conclusive differences in plaque composition *in vivo* on Ti, hydroxyapatite and amalgam surfaces, respectively. In contrast, other studies (Berry *et al*., 1992; Bundy *et al*., 1980) have found antibacterial activity of ions from dental implants, including Ti, on various oral bacteria. Furthermore, it has been claimed that a titanium-peroxy gel possesses both antibacterial and anti-inflammatory properties *in vitro* (Tengvall *et al*., 1990). Whether such a titanium peroxy gel is formed at all *in vivo* remains, however, to be proven. Many metals and alloys are used as dental restorations, and their effect on oral bacteria would presumably interfere with bacterial acquisition and plaque formation on such surfaces.

After testing Ti against particular Gram-negative and Gram-positive oral bacteria which can be found inside the oral cavity, the results showed that in the experiments focusing on the survival over time of *Streptococcus sanguis* (*S. sanguis*), *Streptococcus mitis* (*S. mitis*)*, Actinomyces naeslundii* (*A. naeslundii*)*, Haemophilus parainfluenzae* (*H. parainfluenzae*)*, Fusobacterium* spp. and *P. intermedia* in the presence of the test beads of Ti, amalgam, Cu, tin (Sn) and glass, the most striking antibacterial effects found were for Cu and amalgam. Gram-positive bacterial species, e.g. *S. sanguis*, *S. mitis* and *A. naeslundii*, all followed a similar pattern, implying that they did not survive for more than 24 hours. In the presence of Cu and amalgam beads, the bacteria survived for shorter times, no longer than 6 hours. The antibacterial effect of Cu and amalgam differed significantly (p<0, 0001) from that of Ti.

Gram-negative bacteria, e.g. *H. parainfiuenzae, Fusobacterium* spp. and *P. intermedia* exhibited a shorter survival time than that of the Gram-positive bacteria. As a result, only a few cells remained viable in the presence of the different test granules after 6 hours.

The Cu and amalgam particles also exerted a significantly (p<0,001) higher antibacterial effect compared to Ti on the Gram-negative species: *H. parainfiuenzae* showed significant differences (p<0,001) in its susceptibility to Cu and amalgam. With respect to *P. intermedia*, no significant difference was obtained for the test materials. It should be noted, though, that only 4 strains of *P. intermedia* were tested. The adsorption experiments showed that Sn and Cu killed more cells of *S. sanguis* by less than 1%, Ti and amalgam by less than 5% and 10% respectively, while glass beads showed the highest adsorbing ability (15%). The reduction of the *P. intermedia* cell count was less than 1% for amalgam and Sn, about 3% for

glass, less than 5% for Ti, and about 7% for Cu. For *S. sanguis* as well as *P. intermedia* a statistically significant reduction ($p<0.0001$ and $p<0.001$, respectively) of the antibacterial effect exerted by the metals was observed in the presence of 50% human inactivated serum. However, *S. sanguis* and *P. intermedia* showed similar viability rates whether glass beads were present or not. The presence of serum had a significant effect on *S. sanguis*, resulting in a 9.2% survival rate after 24 hours compared to <0.01% in citrate-phosphate-buffer (CP) only. No increase of *S. sanguis* count was found although almost 100% of the baseline viable count remained after 6 hours. No effect on serum was found for *P. intermedia.* The respective concentrations of the elements in the amalgam-incubated buffer were: (Hg) <0.02% (mg/ml), Ag 0.11% (mg/ml), Cu 411% (mg/ml) and Sn 304% (mg/ml). In the Ti, Sn- and Cu-incubated buffers, the respective concentrations were <10, 366 and 751 (mg/L) (Leonhardt & Dahlén, 2007).

Previously obtained *in vitro* investigations (Joshi & Eley, 1988; Bundy *et al*., 1980) showed that TiO_2 exhibited some antibacterial activity against *S. mutans*. Other studies on the influence of metals on the growth of oral bacteria are of limited value because they were also performed on single bacterial strains (Nunez *et al*., 1976).

Extensive investigation has been performed on many materials including metal and alloys for use as implants to restore lost teeth (Williams, 1986). Joshi & Eley (1988) studied the effects of TiO_2 and metallic materials such as nickel (Ni), Hg and cobalt on seven bacterial strains commonly found in dental plaque. *Streptococci* were grown in a CO_2-enriched atmosphere and obligate anaerobes were cultured in an anaerobic cabinet in an atmosphere of N_2 - 80%, CO_2 - 10%, H_2 - 10%. The remaining organisms were grown aerobically. All cultures were incubated at 37°C. BHI medium was used. The results which were obtained with the agar diffusion showed no zones of inhibition around the implant with any of the tested organisms on BHI agar.

The heavy metals and their salts have been considered to be bactericidal and amalgam, Au foil, and zinc (Zn) phosphate cement also exhibited bacteriostatic properties. Recently, chlorhexidine glucoate (CHxG) received attention for their antibacterial properties. These antibacterial compounds have been used in combination with mouth-rinses and dentifrices and have reduced plaque (Addy *et al*., 1974; Bay, 1978). When added to dental cements, CHxG inhibited growth of bacterial colonies (Schwartzman *et al*., 1980) and demonstrated inhibitions of bacteria by various CHxG concentrations. It should be observed that the composite resin without CHxG produced a small degree of antibacterial effectiveness (Jedrychowski *et al*., 1983).

The doping of TiO_2 nano-particles with metals and non-metals has been shown to be an effective way of increasing the photocatalytic reactivity of TiO_2. The applications for doped TiO_2 nano-composites range from antimicrobial coatings on textiles, the inactivation of endospores, solid-surface antimicrobial coatings to aqueous system-based biocides (Lee *et al*., 2005; Liu *et al*., 2007; Kangwansupamonkon *et al*., 2009). Another practical application of TiO_2 nano-composites has been the use of (Sn^{4-})-doped TiO_2 nano-films on glass surfaces, so as to confer a self-cleaning function.

In line with this, Sayılkan *et al*. (2009) showed that Sn^{4-}-doped TiO_2 nano-films on UV-illuminated glass surfaces had an antibacterial effect against both Gram-negative *E. coli* and Gram–positive *S. aureus*, whereas the TiO_2 films alone had no antibacterial effect (Yu *et al*., 2005).

The grafting of Multi-walled carbon nanotubes (MWCNTs) into TiO_2 was used to inactivate bacterial endospores under UV light conditions, indicating a biocidal efficiency lethal dose (LD_{90}) of 90% for the inactivation of *Bacillus cereus* endospores. Under the same conditions, pure TiO_2 nano-particles showed no significant biocidal capabilities (Lee *et al*., 2005).

Venkatasubramanian *et al*. (2008) compared the antibacterial and photocatalytic reactivities of W^{4+}, Nd^{3+}, and Zn^{2+}-doped TiO_2 nano-composites. The antibacterial activities of the nano-composites were rated as follows: $W^{4+} > Nd^{3+} > Zn^{2+}$ > pure TiO_2 nano-particles. It is believed that tungsten has the greatest effect on photocatalytic reactivity and antimicrobial activity due to its capability to reduce the band gap of TiO_2 and to assist in charge separation, which makes it a highly photoresponsive and photocatalytically reactive substance, band gap being considered as an energy range in a solid where no electron states can exist.

Studies conducted by Liu *et al*. (2007) showed iron (Fe)-doped TiO_2 nano-composites to have a higher capacity for the UV photocatalytic disinfection of *E. coli* (20% survival) than did pure TiO_2 (40% survival).

As an alternative, iron oxide – silicon dioxide – TiO_2 core - corona – shell nanoparticles showed less photocatalytic reactivity than the pure TiO_2. However, the ability to recycle such nanoconjugate constructs on the basis of their magnetic core outweighed the relative disadvantage of their lesser photocatalytic reactivity (Yao *et al*., 2009; Arora *et al*., 2010).

Schwartzman & Caputo (1982) found that the addition of CHxG to polycarboxylate cements substantially increased their antimicrobial action. The antimicrobial action increased in direct

proportion to the incubation period. The CHxG content did not significantly affect the physical properties of the cement.

Jedrychowski *et al*. (1983) determined the antibacterial and mechanical properties of a composite resin and glass ionomer material to which different concentrations of chlorhexidine (CHX) were added. The general effects of the addition of CHX were a marked increase in the antibacterial activity of the materials. However, although increases in concentration increased the inhibition, the increases were not proportional. Also, the addition of small concentrations of CHX increased the antibacterial activity without compromising the mechanical properties.

Xiao *et al*. (2008) studied the oral bacteria *L. casei*, *S. mutans*, *S. aureus* and *A. viscosus* to determine how easily these bacteria increase and accumulate on the surfaces of cured composite restoratives. In their study, three types of quaternary ammonium salt monomers, (methacryloxylethyl cetyl ammonium chloride [DMAE-CB], methacryloxylethyl benzyl dimethyl ammonium chloride [DMAE-BC] and methacryloxylethyl m-chloro benzyl dimethyl ammonium chloride [DMAE-m-CBC]), were evaluated *in vitro* for their antimicrobial activity against these bacteria. The *in vitro* susceptibility test was performed using the broth dilution method. It was demonstrated that all examined strains were highly susceptible to the three monomers, from 1.2 to 4.8 µg/mL. The time-kill revealed that DMAE-CB achieved 99.44% killing at 19.2 µg/mL against *S. mutans* within 1 minute and 100% killing in 10 minutes of test. These results suggest that the quaternary ammonium salt monomers DMAE-CB could be used as antibacterial agents for incorporation with dental restorative materials.

Amongst the problems that are commonly associate with current research on introducing antibacterial properties through the use of nano-particles, is the time needed to obtain satisfactory results (Applerot *et al*., 2009; Liu *et al*., 2009).

Wan *et al*. (2011) investigated the antibacterial effects stimulated by poly(quaternary ammonium) functionalized TiO_2 nano-particles. To compare the effects of surface modifications, poly(quaternary ammonium) and polyacrylate sodium functionalized nano-particles were examined into two groups separately, they were in the size range of 1-20 nm, each examined nano-particle colloidal dispersions was at concentration of 1.5 mg/mL. Luria Broth (LB) nutrient medium was used, *E. coli* solution was used to test the antimicrobial properties and strains were grown in LB medium at 37°C for 24 hours and then washed twice using deionized water before analysis. Cell solution was diluted as needed in different experiments. In order to evaluate antibacterial effect, cell solutions were mixed with nano-particle colloidal dispersions at different concentrations as 10 µL *E. coli* stock solution with

100 µL of each of the nano-particle colloidal dispersions for 10 minutes. A small portion of the mixture was then cultivated on a LB agar plate using fully sterile techniques. The plates were incubated for 16 hours at 37°C and the number of CFUs was recorded. The results showed that, poly(quaternary ammonium) and polyacrylate sodium functional groups were responsible for the induced antibacterial effect. 99.999% of *E. coli* killed in 10 minutes.

Leung *et al*. (2005) studied the release of CHX from dental composite materials which produced by incorporation of 80 wt% of a strontium fluoroaluminosilicate glass dispersed in methacrylate monomers. The monomers contained 40–100 wt% of a 10 wt% chlorhexidine diacetate (CHxA) in hydroxyethylmethacrylate (HEMA) solution and 60–0wt% of a 50/50 mix of urethane dimethacrylate (UDMA) and triethyleneglycol dimethacrylate (TEGDMA).

Concentrations of 40%, 60%, 80% and 100% HEMA solution were incorporated with a 50/50 w/w mix of TEGDMA and UDMA (Rohm, Tsutta, Oi-cho, Kameoka, Kyoto, Japan) and 1 %m/m each of camphorquinone (CQ) and dimethyl-p-toluidine (DMPT) (Sigma-Aldrich, the old Brickyard, Gillingham, Dorset, UK). Strontium fluoroaluminosilicate dental glass of approximately 5 mm diameter was then added in a powder to liquid ratio 4:1 by weight. Composites were prepared as above with HEMA solution levels of 50%, 60%, 70%, 80%, 90% and 100% of the monomer and CHxA at 10 wt% of the HEMA. Eight discs (10 mm diameter and 1.6 mm thick) of each formulation were prepared. A light cure for each side was used for 1 min. At 1 hour post cure, these eight discs were placed upright into two pots each containing 5ml of distilled water and incubated for 1, 2, 4, 24, 48, 96, 168 and 336 hours at 37°C, the samples were then removed and their mass and volume were also assessed. The antibacterial properties of the three previous used experimental composites and three commercial materials were evaluated. It was found that, most polymerised samples released no detectable monomer at any time period. The sample with 100% HEMA was the only exception as the monomer which released 1% of its initial HEMA content in the first 24 hours. All samples, however, exhibited an initial fast CHxA release over the first 4 hours.

Due to the need for antibacterial dental composites, many attempts were exerted to produce dental restorative materials with high quality and good resistance to bacteria. For instance, another study by Imazato *et al*. (1994) investigated the previous attempts to produce dental composites with antimicrobial properties via incorporation of antimicrobial substances such as CHX, but obstacles prevented the realization of this aim due to release of the inhibitory agents from the composites. Some of these obstacles or problems could include toxic influences, effect on mechanical properties and loss of effectiveness. MDPB was newly synthesized by incorporation of antibacterial activities and the methacryloyl group. The

monomer was combined with resin composite to achieve non-releasing antimicrobial composites. The incorporated composite with MDPB was tested *in vitro* against seven strains of streptococci (*S. mutans*, *S. sobrinus*, *S, oralis*, *S. mitis*, *S. sanguis*, *S. gordonii* and *S. salivarius*) and BHI broth was used. The effectiveness of the incorporation of MDPB on the mechanical properties of resin composite was evaluated. After anaerobic incubation for 24 hours at 37°C it was reported that MDPB demonstrated antibacterial activity against all streptococci.

Tanagawa *et al*. (1999) determined that dental resin composites provided with antimicrobial properties could be beneficial for eliminating the secondary caries frequently observed around restorations. Three types of silver-based antimicrobial chemicals trademarks, namely, Novaron, Amenitop and AIS were used in different amounts (40, 30 and 400 μ/mL consecutively). Antimicrobial substances were added and incorporated into TEGDMA-UDMA-based light-activated resin composite, and then the antimicrobial action of these composites was tested. Results showed that, dental composites incorporated with 5 wt% of Novaron and 7 wt% Amenitop greatly inhibited the microbial growth of *S. mutans*, while dental composites incorporated with more than 10 wt% of AIS did not inhibit the growth.

Although various strains of bacteria have been isolated from dental plaque related to dental caries, there is evidence that some bacteria such as lactobacilli, *S. sobrinus* and *S. mutans* are considered as the most common? human dental pathogens and a principal cause of dental disease (Loesche, 1986). The possibility of having infectious dental composite restoratives which tend to produce dental plaque accumulation by oral bacteria whether *in vivo* or *in vitro* has been reported as a high infection risk and solutions depend on additions of antimicrobial agents as described by several studies (Ribeiro & Ericson, 1991; Del Curto *et al*., 2005; Jaramillo *et al*., 2012).

Additions of antibacterial agents such as CHxG to restorative composites or methacrylate monomer appeared to affect mechanical properties (Marks *et al*., 1976; Jedrychowski *et al*., 1983; Addy & Handley, 1981) to overcome such disadvantages of restoratives which may release agents. Imazato & McCabe (1994) improved non-releasing antibacterial restorative composites, though small, by incorporation of a new synthesized monomer such as MDPB which has antimicrobial actions against oral bacteria, specifically against streptococci. However, the effect of addition of MDPB on the curing conduct of bisphenol a glycidyl-dimethacrylate Bis-GMA-based composites was investigated. Degree of cure, depth of cure, light effect and surface hardness of the composites incorporating 0.4% or 0.5% MDPB were measured via penetrometer. It was observed that, composites incorporated with MDPB showed substantially greater degree of cure than the control ($p<0.05$). The light effect of

MDPB composites expressed less than the control ($p<0.05$) and the obtained data showed that, incorporation of small amounts of MDPB did not negatively affect the composites when it was added to the Bis-GMA-based composites which confirms that the improvement of the new composites incorporated into Bis-GMA-based composites would give beneficial effectiveness.

Due to the recurrent dental caries which has been associated with the inability of dental restorative materials to seal the cavity, it would be advantageous for a restorative system to have components which are antimicrobial and also inhibit acid formation. Grobler *et al*. (1996) investigated growth inhibition on Aelitebond restorative etchant (Uni-Etch) and the rest of the restorative system on five species of oral bacteria. The Aelitebond restorative system used in the field of dentistry was found to be antimicrobial and has an etchant (Uni-Etch) that contains 32% phosphoric acid with 1% BzCl. The Aelitebond bonding resin contains (Bis-GMA), (UDMA), (TEGDMA) and (HEMA). The etching component contains BzCl.

BzCl and CHxG have been used as antimicrobial agents for a long time, fundamentally in medical applications (Barlow *et al*., 1995; Pereira & Siqueira-Júnior, 1995; Sampath *et al*., 1995; Barnes, 1995; Pons *et al*., 1992). The manufacturers of the Aelitebond restorative system have incorporated BzCl in the etchant for its antimicrobial properties. It was noticed that 1-2% BzCl containing etchants have the same remaining antimicrobial property as CHxG containing etchants, but in an acidic medium it is more stable. Moreover, different concentrations of phosphoric acid were found not to affect the bacteria inhibitory reaction (Chan & Hui, 1992; Chan & Lo, 1994). In this study two tested dental composites (Aelitebond restorative etchant and the rest of the restorative system) against five oral organisms showed significant differences ($p<0.05$) only between the controls and the Uni-Etch composite for 3 species [*Veillonella parvula* (*V. parvula*)*, S. sanguis, A. naeslundii*]. The results showed that the component containing 1% was mainly responsible for the antimicrobial activity of the Aelitebond system.

The use of CHxG and BzCl played a significant role as antibacterial substances. Thomas *et al*. (2005) evaluated the antibacterial activity of these substances on *P. aeruginosa*. The minimum inhibitory concentrations (MICs) for BzCl and CHxG were determined. The residuals of CHxG and Hibiscrub Liquid contain CHxG, considered as an antimicrobial agent influenced the susceptibility of *P. aeruginosa* to these biocides and a number of antibiotics. Results showed that none of the strains of *P. aeruginosa* exhibited raised MIC, since CHxG was less sensitive than the parent strain to CHxG or BzCl in either method.

Houari & Di Martino (2007) studied the antibacterial effects of CHxG and BzCl on bacterial biofilm formation against four strains of *E. coli*, *Klebsiella pneumoniae* (*K. pneumoniae*), *P. aeruginosa* and *Staphylococcus epidermidis* (*S. epidermidis*). The conducted procedures were as follow: Biofilm formation and planktonic growth were tested in microtiter plates in the presence of antiseptics. For *E. coli* in the presence of CHxG or BzCl, for *K. pneumoniae* in the presence of CHxG and for *P. aeruginosa* in the presence of BzCl, biofilm development and planktonic growth were affected at the same concentrations of antiseptics. For *P. aeruginosa* in the presence of CHxG and *K. pneumoniae* in the presence of BzCl, planktonic growth was significantly inhibited by a fourfold lower antiseptic concentration than biofilm development. For *S. epidermidis* in the presence of antiseptics at the MIC, a total inhibition of biofilm formation was observed. For *S. epidermidis* exposed to CHxG at $\frac{1}{2}$, $\frac{1}{4}$ and $\frac{1}{8}$ MIC, or to BzCl at $\frac{1}{8}$, $\frac{1}{16}$ and $\frac{1}{32}$ MIC, biofilm formation was increased from 11.4% to 22.5% without any significant effect onto planktonic growth.

Numerous studies have reported on the antibacterial effects of dental restorative materials. In the majority of experiments, the restoratives were tested against both, Gram-negative and Gram-positive bacteria without any attempt to correlate these tests with some organisms considered to be a fundamental cause of dental caries (Shay *et al*., 1956).

Hill & Boester (1934) observed that most of the commercial cements were sufficiently germicidal for clinical purposes. These cements were tested against *S. aureus* after allowing the specimens to set for 24 hours to permit the elimination of the free phosphoric acid.

Kinnear (1935) studied the antibacterial properties exhibited by various dental restorative materials against *Streptococcus viridans* (*S. viridans*) and *S. aureus*. It was observed that Ag cements and Cu cements were the only filling materials used extensively by the dentist that gave appreciable antiseptic action.

McCue *et al*. (1951) investigated the antibacterial properties of commercial dental restorative materials against *E. coli* and *Micrococcus pyogenes* (*M. pyogenes*) *var. aureus*. It was observed that the materials, listed in order of their decreasing bacterial action, were silicate cement, Cu amalgam, Au foil, Zn phosphate cement, Cu cement, acrylic, Ag amalgam and inlay Au.

Qvist *et al*. (1977) reported that the ability of six different intermediary base materials to prevent bacterial entrance beneath silicate cement fillings was investigated *in vivo* in primary molars. After an observation period of 1 month, bacteria were found on the pulpal wall in two

out of 10 cavities beneath Fluoritec and four out of 10 cavities beneath Durelon solid mixed, De Trey phosphate cement solid or creamy mixed. Beneath the intermediary base materials Dycal, zinc oxide-eugenol cement, Dropsin and Durelon creamy mixed, no bacteria were found. In cavities filled with silicate cement or silver amalgam only, bacteria were observed in nine out of 10 and in five out of 10 cavities, respectively. Babin *et al*. (1978) reported that cements which exhibited antibacterial activities after setting for about two weeks were those that contained Zn, Cu or Hg.

Ørstavik (1985) tested nine available commercial dental amalgams for antibacterial activities *in vitro*. Bactericidal test on salivary bacteria, growth inhibition test on *S. mutans* OMZ 176, and a time-dependent bactericidal test on *S. mutans* were used. All amalgams exhibited some antibacterial actions. Dispersalloy and Revalloy were strongly antibacterial in all tests; ANA 2000 and Sybraloy killed *S. mutans* but were less potent in the salivary test and in the growth inhibition experiments. The copper amalgams, Neo-Silbrin and Cupromuc, were the most active in the salivary test but less inhibitory in the growth curve experiments. Spheraloy, Indiloy, and Amalcap showed intermediate activity in the salivary bactericidal test but were relatively weak in the growth inhibition studies. Analysis of Hg, Ag, and Cu in media from the growth inhibition studies showed release of Hg from the copper amalgams and, particularly, from Revalloy; Indiloy gave off Ag, whereas Neo-Silbrin, Cupromuc, Sybraloy, and ANA 2000 released more Cu than the other alloys. Meeker *et al*. (1986) attributed the antibacterial influence of calcium hydroxide liners to the phenolic component.

McComb & Ericson (1987) investigated the antibacterial activity of innovative, commercial lining cements. A liner which contains calcium hydroxide and polymerized by glass-ionomer lining cement (GC lining cement) and visible light (Prisma VLC Dycal) were compared with two more established lining cements (Advanced Formula II Dycal and Life). Antibacterial activity and hemolysis-like agar change at 24, 48, and 72 hours were measured on blood agar plates inoculated with *S. mutans*, *L. casei* subsp. *rhamnosus*, and chewing-stimulated saliva. Prisma VLC Dycal did not affect bacteria or agar. The glass-ionomer lining cement, with an acidic pH at setting, had the most pronounced effect on agar and all test bacteria. Even after 48 hours setting, it inhibited growth of *S. mutans*. The control lining cement (AFII Dycal) showed antibacterial activity toward both specific bacteria as well as some activity against the salivary bacteria. The material Life showed only partial inhibition of bacterial growth. For all lining cements, the hemolytic-like agar change correlated with antibacterial influences. The surface pH of the freshly-set cements containing calcium hydroxide was alkaline. It would seem that a simple correlation between high surface pH and antibacterial

activity among these cements does not exist. Also, further biological characterization of new lining cements is required to direct their appropriate clinical use.

Other investigations have tried to enhance the antibacterial properties of dental materials by adding substances, with reputed antibacterial effects, to the original material. Colton & Ehrlich (1953) added antibiotics to dental cements and direct filling resins. The cavity walls remained sterile up to five weeks. Beagrie & Smith (1978) studied the development of germicidal polycarboxylate cements. The most effective cement was one incorporating 5-F-oxine and chlorhexidine acetate. Growth inhibition lasted up to six weeks.

Many subsequent investigations have also determined that microorganisms are the principal aetiological factors in dental pathologies such as dental caries, periodontitis and pulpitis (Sipert *et al*., 2005). During treatments these organisms are removed by irrigation or intracanal medications. Thus, even after these procedures have been performed, bacteria might still be found in the oral environment for instance inside the root canal system (Byström & Sundqvist, 1983). The disease can persist or re-emerge again (Ørstavik *et al*., 1981). Thus procedures such as root fillings play an essential role in the control of re-infection by entombing residual organisms through the antimicrobial activity of orthodontic materials, for example, endodontic sealers (Grossman, 1980). A second factor involved in the outcome of treatments, for example, root canal treatment, is the healing potential of tissues damaged by pulp/periapical pathology and root canal treatment procedures. Hence, the stimulus for healing depends on the absence of irritating agents originating from bacterial metabolic products, or of chemical origin from sealing materials (Leonardo *et al*., 2003; Ørstavik *et al*., 2004). Thus, for the healing process to occur, the materials employed should not damage periapical tissues, and it would be an advantage if these materials could stimulate the deposition of hard tissue, thereby promoting biological sealing (Leonardo *et al*., 2000; Ørstavik, 2007).

Facultative bacteria have previously been used to evaluate the antimicrobial activity of dental restorative materials. Various test methods have been employed in the evaluation of the antimicrobial activity of dental materials. For instance, in the diffusion methods (Sipert *et al*., 2005) tested in triplicate, Cement, Mineral Trioxide Aggregate (MTA) Angelus, Portland cement, sealers Sealapex, EndoRez and Fill Canal for antimicrobial activity using an agar diffusion method. This investigation was performed on double-layered plates, in which the base layer was made of 10.0 mL of sterilized MHA poured in 20×100 mm sterilized Petri plates. Five wells (one for each material) were made by removal of agar at equidistant points and then filled immediately by sealers/cements after being mixed according to the manufacturer's instructions. Fill Canal, Sealapex, MTA and Portland cement were poured

inside the wells whilst EndoRez was injected into them. The strains used for analysis were *Candida albicans* (*C. albicans*) ATCC 10231*, E. faecalis* ATCC 29212, *E. coli* ATCC 25922, *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 27853, *Micrococcus luteus* (*M. luteus*) ATCC 9341 and *S. epidermidis* ATCC 12228. After activation from stock culture, microorganisms were maintained in MHB until used. Overnight cultures of the bacteria were used. All the microbial strains were grown at 37°C for 24 hours in MHB and then seeded into 15.0 mL of the MH agar, to produce a turbidity of 0.5 McF (*cf* List of Terms, p. xvi), which corresponds to a concentration of 1×10^8 CFU/mL. This broth was used as the second layer. The seeded agar was added over the plates immediately after the insertion of the sealers/ cements. The plates were maintained at room temperature for 2 hours for pre-diffusion of the materials and then incubated at 37°C for 24 hours. The same procedure was conducted in a plate without the addition of bacterial seeding. A total number of twenty two plates were used. Each bacterium was tested in triplicate and one plate was used without strain seeding.

Aliquots of 10.0 mL of 2, 3, 5-triphenyltetrazolium chloride (TTC) gel, prepared with 1.0% MH agar were added and the plates were incubated again at 37°C for 30 minutes (Leonardo *et al*., 2000). This procedure is useful to differentiate areas of microbial growth (red areas) diffusion zones. The zones of inhibition around the wells were then measured with a millimetre ruler with accuracy of 0.5 mm.

Fill Canal, Sealapex, MTA and Portland cement all showed evidence of inhibition. Conversely, EndoRez did not demonstrate any antimicrobial activity. The strain of *E. coli* was not inhibited by MTA and Portland cement whilst Fill Canal and Sealapex inhibited the growth of all tested strains (Sipert *et al*., 2005).

Presently recurrent dental caries and accumulation of bacteria on restoratives are more evident than before (Abo El Naga & Yousef, 2012). Moreover, both recurrent caries and bacterial accumulations considered as complex diseases and their treatments need a period of time to insure that secondary caries which could lead to restoration failure will not occur again. Therefore, these concerns necessitated conducting several investigations and attempts to provide dental restorative materials as TiO_2 nano-composites with antibacterial properties such as CHxG. Ribeiro & Ericson (1991) studied the antimicrobial activities of two specimens of glass ionomer cements, luting cement Aqua Cem (AC) and a restorative material Chem Fil II (CF) [De Trey, Surrey, England] which contained various concentrations of CHxG, from 0 to 10.3% w/w obtained from a 20% stock solution (Hibitane, ICI, Macclesfield, England) by using two experimental methods, a broth culture test and an ADT. AC and CF were obtained as powders (25 mg) and mixed with 25 µL CHxG. The mixture was tested *in vitro* against four oral bacteria. *Lactobacillus casei* (*L. casei*), *S. mutans*

KPSK2, *S. mutans* 10449 and *S. sobrinus* were used. The samples were incubated overnight (95% N_2 and CO_2) at 37°C. It was found that, CHxG which was added to glass-ionomer cements was a composition with increased antibacterial effects compared to the glass-ionomer cement without CHxG. Cements without incorporation of CHxG did not prevent bacterial growth. A small amount which varied from 0 to 10.3% (w/w) of incorporated CHxG was released from the cements. The retrogradation of the cements showed that the material could be advantageous to increase the antibacterial activity as a varnish-link CHxG carrier.

The bacterial elimination was considered one of the main objectives of the endodontic treatments, which in turn promotes the normal healing process of the periodontal tissues (Byström & Sundqvist, 1981). However, facultative bacteria such as *E. faecalis*, *S. aureus*, and even *C. albicans* are considered to be the most resistant species in the oral cavity and one possible cause of root canal treatment failure (Gomes *et al*., 1996; Vianna *et al*., 2004). It is important to decrease the growth of the bacteria in the oral environment and reduce the prevalence of bacteria which is high in root canals prepared without irrigating solutions (Goldman *et al*., 1981; Peters *et al*., 2002). An endodontic irrigant should ideally exhibit powerfull antimicrobial activity and disinfect the root canal space, and have no cytotoxic effects (Harrison, 1984).

CHxG is widely used as a mouth-wash in the prevention and treatment of periodontal diseases such as dental decay and played a significant role to eliminate the microbial plague on dental restoratives. CHxG has been suggested as an irrigating solution or intracanal dressing in endodontic therapy. Vianna *et al*. (2004) investigated the *in vitro* antimicrobial activity of CHxG gel and CHxG liquid in three different concentrations 0.2% (w/v), 1.0% (w/v), and 2.0% (w/v). The antimicrobial property of both irrigating solutions has been tested against endodontic pathogens such as *E. faecalis* ATCC 29212, *C. albicans* ATCC 3736 and *S. aureus* ATCC 25923. All the strains were grown on 5% sheep blood–BHI agar plates for 48 hours at 37°C and *P. gingivalis, P. endodontalis* and *P. intermedia* were isolated from the root canal infections and determined by using conventional biochemical test. These three strains were sub-cultured on 5% sheep blood-Fastidious Anaerobic Agar (FAA) plates for 48 hours in a controlled atmosphere (10% CO_2, 10% H_2 and 80% N_2) at 37°C. The solutions were prepared 24 hours before starting the tests. Natrosol 1% (w/v) and sterile saline 0.89% (w/v) were used as controls in these *in vitro* experiments. The performed methodology of the use of CHxG as (Vianna *et al*., 2004) investigated was adapted by (Gomes *et al*., 2001). The time needed for each irrigant to produce total microbial inhibition growth was recorded for each period of the experiments.

The results showed a good performance of CHxG, whereas CHxG liquid in all concentrations and the 2.0% (w/v) CHxG gel eliminated the facultative bacteria of *E. faecalis* and the aerobic bacteria of *S. aureus and C. albicans* in 1 minute or less. Only 15 seconds were needed for all tested CHxG solutions to kill the Gram-negative strictly anaerobic bacteria *P. gingivalis, P. endodontalis*, and *P. intermedia.* CHxG liquid, in all concentrations, killed all bacteria in 30 seconds or less, while CHxG gel took from 22 seconds [2% (w/v) CHxG gel] to 2 hours [0.2% (w/v) CHxG gel].

A good example of the use of antibacterial additives to dental materials for the production of restorative materials with antimicrobial properties is the development of the commercially available system by Imazato (2009). Imazato developed the world's first antibacterial adhesive system used and the MDPB-containing primer was successfully commercialized. MDPB is potentially viable to different restorative materials since immobilization of the antibacterial component, by polymerization of MDPB enables no retrogradation in mechanical properties of cured composites and exhibition of inhibitory effects against growth of bacteria on their surfaces (Yoshikawa *et al*., 2007).

For glass-ionomer cements used for dental restorative therapies, the approaches to provide antibacterial properties have been attempted by incorporation of CHX. Additions of different ratios of chlorhexidine were found to be perfect solution to give antibacterial properties (Takahashi *et al*., 2006).

MDPB is a polymerizable bactericide, and the antibacterial composition is immobilized after the resinous materials incorporating MDPB are cured (Imazato & McCabe 1994; Imazato *et al*., 1994; Imazato *et al*., 1995). The composition immobilized prevents any leaching from resin matrix (Imazato *et al*., 1998). Therefore, incorporation of MDPB has a significant advantage that mechanical properties of the restorative materials can be remained stable for a long period of time under wet conditions (Imazato *et al*., 1999). The ability to control bacteria would be advantageous to reduce the hazard of further demineralization and cavitation, since dental caries is a frequent infectious disease and the growth inhibition of cariogenic bacteria is the essential principle (Imazato, 2009).

A considerable attention for TiO_2 because it has so many potential applications, for instance, it uses as chemical sensors, as a glass coating material for antifogging and self-cleaning, and as a biomedical material. TiO_2 is also cost effective and chemically stable, with good optical properties, thermal stability, high refractive index, and a deficiency of absorbance of visible light. Many studies have shown that TiO_2 can act as a bioactive material by offering strong interfacial bonding to living tissue (Roether *et al*., 2002; El Fray & Boccaccini, 2005).

The surfaces of fillers are generally treated with methacrylate-functionalised silanes, such as the frequently used a MPTMS to produce covalent links between the inorganic filler particles and the organic resin matrix by free radical copolymerisation. The silane adhesive bonding agent ensures success in dental composites. There is ample evidence indicating that fillers weaken the composite material in the absence of an adhesive silane bonding agent (Zandinejad *et al*., 2006). The particles would act as stress concentrators and cause the different types of material to segregate (Anusavice, 2003).

Filler particles must be chemically bonded to the matrix because if they are not bound they detach from the matrix under tension strength. As a result, the filler does little to improve properties of the composites (Davis, 2003). It also reduces the overall strength and toughness of composites because the filler particles act more like inconsistencies in the composites. Stress is only transferred between the reinforcement and the matrix when effective bonding has occurred between them.

Based on the literature review it is deduced that TiO_2 nano-composites used in dental restorations should be further investigated. And in this study the combinations of the antibacterial agents (CHxG - BzCl) with the TiO_2 nano-composites could play a significant role to eliminate or reduce the occurrence of the secondary caries and the faller of restoratives.

1.3 Statement of the problem

Dental nano-composites would perform better if they have antibacterial properties because they would resist bacterial growth that causes secondary caries. This would possibly be achieved by incorporating antibacterial substances into the composition.

There are several studies on the bacterial effects of dental restorative materials in direct contact with the teeth and the surrounding tissues either anterior or posterior, mainly for dental prosthetic reasons. Furthermore, some of the previous studies (Leung *et al*., 2005; Imazato, 2009; Jandt & Sigusch, 2009; Busscher *et al*., 2010) have extensively been performed to eliminate or reduce the influence of the bacteria on the restoratives and more research is required.

1.4 Hypothesises of the study

1.4.1 Null hypothesis (H_0)

TiO_2 nano-composites are not bactericidal and did not kill the bacteria within 24 hours.

1.4.2 Alterative hypothesis (H_A)

TiO_2 nano-composites are antibacterial and killed the bacteria within 24 hours.

1.5 Aim and Objectives

1.5.1 Aim:

The main purpose of this *in vitro* study was to investigate the antibacterial activity of TiO2 nano-composites to be used in dental applications by separately incorporating antibacterial substances to the resin mixture during the mixing process.

1.5.2 Objectives

- To produce dental nano-composites with antimicrobial activity to be used in dental restorations.
- To compare the antimicrobial activity of different antimicrobial agents incorporated into a nano-composite.
- To measure the inhibition zones produced by different combinations of the antimicrobial agents and nano-composites against *S. mutans*, *E. coli*, *P. aeruginosa*, *E. faecalis* and *S. aureus*.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Experimental design of the study

This *in vitro* study was performed as follow:

- TiO_2 nano-composite treated with silane was prepared and divided into four groups; each as required.
- Antibacterial substances (CHxG and BzCl) were separately incorporated into the TiO_2 nano-composites during mixing the compounds, and in different percentages as shown in (Table 2.1); each one was added according to the TiO_2 nano-composite groups.
- The selected bacteria were prepared and divided into four groups according to the TiO_2 nano-composite groups and then tested against each other.
- Results were collected and inhibition zones were measured and recorded.

Table 2.1: Summary of the synthesis of the TiO_2 nano-composite materials

	Nano-TiO_2	UDMA	MPTMS	Benzalkonium chloride (BzCl)	Chlorhexidine gluconate (CHG)
1	10%	90%	None	None	None
2	10%	90%	Treated	None	None
3	10%	87%	Treated	3%	None
4	10%	87%	Treated	None	3%
5	10%	85%	Treated	5%	None
6	10%	85%	Treated	None	5%
7	10%	83%	Treated	7%	None
8	10%	83%	Treated	None	7%
9	10%	80%	Treated	10%	None
10	10%	80%	Treated	None	10%

2.2 Materials

TiO_2 of 80 nm particle size was sourced from Evonik, Krefeld, Germany. UDMA, CQ, DMAEMA, MPTMS, BzCl as a solid and CHxG as a 20% (m/v) solution were obtained locally through Sigma Aldrich, Johannesburg, South Africa. The bacteria were obtained from Davies Diagnostics, Johannesburg, South Africa.

2.3 Synthesis of TiO_2 nano-composite

2.3.1 Treatment of nano-TiO_2

The treatment of TiO_2 nano-composite was performed by mixing MPTMS in xylene in the presence of 2% (wt/v) n-propylamine used as catalyst and then nano-TiO_2 was mixed with MPTMS by adding 2.5% m/v of MPTMS to 1 g of nano-TiO_2. Acetic acid was added to the mixture until a pH of 3.3 was reached. This mixture was sonicated for 10 minutes, stirred for 1 hour with a mechanised stirrer and then centrifuged for 10 minutes at 10,000 rpm in order to separate the silanised nano-TiO_2 from the solution. Before and after centrifuging, the mixture was washed with ethanol. The mixture was then placed in an oven at 80°C for 12 hours to dry. The silanized-TiO_2 is referred to as treated TiO_2 from here onwards.

2.3.2 Preparation of resin composite (Table 2.1)

The composite mixture was prepared by adding 0.5 gm of each CQ as an initiator and DMAEMA as an accelerator to 99 gm of UDMA. The mixture was then mechanically stirred for 30 minutes.

Of this mixture 90 grams were added to 10 gm of treated TiO_2 and mechanically stirred in a glass vessel for about 30 minutes. Antimicrobial agents CHxG and BzCl were independently added during the mixing of the composite. The mixtures were then casted into a plastic template, light-cured until solidification was reached and then sterilized by alcohol.

2.3.3 Incorporation of antimicrobial agents, chlorhexidine gluconate (CHxG) and benzalkonium chloride (BzCl).

CHxG and BzCl were incorporated into the mixture of the composite in different concentrations, and left until well molded. TiO_2 nano-composites were then divided into four groups as each concentration was added separately, for instance, 3% (m/m), 5% (m/m), 7% (m/m) and 10% (m/m) of CHxG and BzCl respectively.

2.4 Dental nano-composite materials used in evaluation of microbicidal activity

TiO_2 nano-composites are used particularly as direct dental restoratives on teeth inside the oral cavity. In this study the TiO_2 nano-composite prepared in different ways and introduced in this *in vitro* study for evaluating their antimicrobial activities, were prepared following manufacturers' instructions and mentioned as follows:

1. Untreated TiO_2 nano-composite.
2. Treated TiO_2 nano-composite.
3. Treated TiO_2 nano-composite mixed with three concentrations of BzCl, 3% (m/m), 5% (m/m), 7% (m/m) and 10% (m/m) separately.
4. Treated TiO_2 nano-composite mixed with three concentrations of CHxG, 3% (m/m), 5% (m/m), 7% (m/m) and 10% (m/m) separately.

2.5 Selection of the bacteria

Facultative bacteria used in this *in vitro* study were *Streptococcus mutans* ATCC 25175*, Escherichia coli* ATCC 11775, *Pseudomonas aeruginosa* ATCC 10145, *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 12600. These selected bacteria were chosen because they considered as standard for antibacterial tests. A standard Gram-stain was performed on all cultures and these were found to be pure.

2.6 Medium selected for this study

Two types of commonly used media were used with the selected bacteria to evaluate the antimicrobial activity of the TiO_2 nano-composites. BHI was only used with *S. mutans* and MHA was used with the other bacteria, *E. coli, S. aureus, E. faecalis* and *P. aeruginosa*.

MHA is an antimicrobial susceptibility testing medium which may be used in internationally recognized standard procedures, so that was the reason to use it with some of the selected bacteria. *S. mutans* is a difficult bacterium to grow, therefore, it had to be grown in a different medium such as BHI Agar, and because *S. mutans* needs to grow in a CO_2.

CO_2 was only used with *S. mutans* and not with the other bacteria because incubation in a CO_2 enriched atmosphere is not recommended because of its pH effect on the medium.

2.7 Methodology

2.7.1 Testing of specimens

In this *in vitro* study some procedures were conducted to investigate the effect of various dental nano-composites prepared mainly with TiO_2, on some selected bacteria such as *E. coli*, *S. aureus*, *E. faecalis*, *P. aeruginosa* and *S. mutans*.

TiO_2 nano-composites that were used in this *in vitro* study were mainly manufactured and treated by incorporating and reinforcing TiO_2 nano-composites with some other chemical substances in different percentages and in a fixed ratio by weight of these materials as mentioned below.

Initially, the TiO_2 nano-composites were service sterilized by quickly submerging in 70% alcohol and immediately air dried in order to prevent any contamination which may affect the results. Filter papers (Whatman papers) were saturated with 2% CHxG solution and then incubated at 37°C for 15 - 20 minutes for drying before using as a control test. The control test was performed in this *in vitro* study with all the samples of the nano-composites and the bacteria in each experiment which played a significant role in providing more accurate and reliable results. A control test was performed without adding the antibacterial substances into the nano-composites in order to compare the results with the others which had antibacterial substances.

All five strains were prepared and grown according to the standard conditions and procedures. Antimicrobial testing of the TiO_2 nano-composites was performed using the Bauer disk diffusion test (Bauer *et al*., 1966) with MHA and BHI plates. Appropriate colonies selected from an overnight culture of previously mentioned bacteria not older than 24 hours and standardized to a turbidity equivalent of 0.5 McFarland standards. BzCl was added to the mixture of TiO_2 nano-composite in concentrations of 3 %m/m, 5 %m/m, 7 %m/m and 10 %m/m respectively. In separate tests CHxG was added to the mixture of TiO_2 nano-composites in concentrations of 3 %m/m, 5 %m/m, 7 %m/m, and then 10 %m/m respectively according to procedures.

All samples were performed in triplicate and equidistantly in each plate and then the experiments were divided into four groups corresponding to the nano-composite materials used in this study. Each group contained one of the TiO_2 nano-composites and were tested *in vitro* for its antimicrobial activity separately against each one of the five strains respectively. For instance, untreated TiO_2 nano-composite was cultivated with each

bacterium separately and incubated for 24 hours (overnight) at 37°C. Treated TiO_2 nano-composite was cultivated with each bacterium for 24 hours at 37°C.

Treated TiO_2 nano-composite incorporated with concentration of 3% (m/m) of BzCl was cultivated with each one of the bacteria separately for 24 hours at 37°C, and repeated using treated TiO_2 nano-composite with concentration of 5% (m/m), 7% (m/m) and then with 10% (m/m) of BzCl respectively. Thereafter, treated TiO_2 nano-composite incorporated with concentration of 3% (m/m) of CHxG cultivated with each one of the bacteria for 24 hours at 37°C, and treated TiO_2 nano-composite was repeated with concentrations of 5% (m/m), 7% (m/m) and 10% (m/m) of CHxG independently.

All the nano-composite specimens were tested against all the bacteria independently by following the same procedures except *S. mutans* was exposed to 5% CO_2 inside a CO_2 water-jacketed incubator for 48 hours at 37°C.

After the incubation process and an appropriate period of time, all samples were taken out of the incubators and the results were recorded and photos of the samples were taken. The measurements were performed accurately by use of a ruler.

2.7.2 Statistical analysis of the results

In this study all tests were performed in triplicate, including the preparation of the nano-composite materials in order to confirm the obtained findings. This was qualitative descriptive study and statistical analysis was therefore not applicable.

CHAPTER THREE

RESULTS

3.1 Results

The results of this study are summarized in Table 3.1 and Table 3.2. The obtained results showed that untreated TiO_2 nano-composite and treated TiO_2 nano-composite had no antibacterial activity (Figures 3.1 and 3.2).

Figure 3.1: Antimicrobial activity of untreated TiO_2 nano-composite tested against S. mutans. It is evident that the untreated TiO_2 does not possess antimicrobial activity.

Figure 3.2: Antimicrobial activity of treated TiO_2 nano-composite tested against S. mutans. It is evident that the treated TiO_2 does not possess antimicrobial activity.

Treated TiO_2 nano-composite which contained 10% (m/m) BzCl and treated TiO_2 nano-composite that contained 10% (m/m) CHxG gave antibacterial inhibition zones (Figures 3.3 - 3.12) and produced significant degrees of antibacterial effectiveness against *S. mutans, E. coli*, *P. aeruginosa*, *E. faecalis* and *S. aureus*. This confirmed that BzCl and CHxG are effective antimicrobial substances when they are added to the nano-composites. Furthermore, the inhibition zones were clearly observed around all the treated nano-composite specimens, except with *P. aeruginosa* (Figure 3.5, Figure 3.10). The latter showed a small inhibition zone because it resists the influence of the antibacterial substances whereas CHxG is less effective with some Gram-negative bacteria. However, both of these antibacterial substances, CHxG and BzCl, clearly appeared an antibacterial effectiveness at concentrations of 10% (m/m).

Figure 3.3: Antimicrobial activity of a preparation of treated TiO_2 nano-composite composite with 10% (m/m) CHxG tested against *E. coli*. It is evident that the preparation possesses antimicrobial activity.

Figure 3.4: Antimicrobial activity of a preparation of treated TiO_2 nano-composite composite with 10% (m/m) chlorhexagluconate tested against *E. faecalis*. It is evident that the preparation possesses antimicrobial activity.

Figure 3.5: Antimicrobial activity of a preparation of treated TiO_2 nano-composite composite with 10% (m/m) chlorhexagluconate tested against *P. aeruginosa*. It is evident that the preparation possesses antimicrobial activity.

Figure 3.6: Antimicrobial activity of a preparation of treated TiO_2 nano-composite composite with 10% (m/m) chlorhexagluconate tested against *S. aureus*. It is evident that the preparation possesses antimicrobial activity.

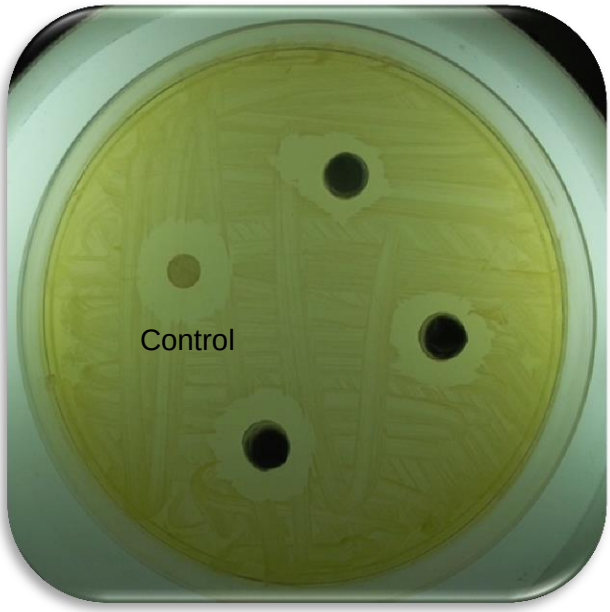

Figure 3.7: Antimicrobial activity of a preparation of treated TiO_2 nano-composite composite with 10% (m/m) chlorhexagluconate tested against *S. mutans*. It is evident that the preparation possesses antimicrobial activity.

Figure 3.8: Antimicrobial activity of a preparation of treated TiO_2 nano-composite composite with 10% (m/m) benzalkonium chloride against *E. coli*. It is evident that the preparation possesses antimicrobial activity.

Figure 3.9: Antimicrobial activity of a preparation of treated TiO_2 nano-composite composite with 10% (m/m) benzalkonium chloride tested against *E. faecalis*. It is evident that the preparation possesses antimicrobial activity.

Figure 3.10: Antimicrobial activity of a preparation of treated TiO_2 nano-composite composite with 10% (m/m) benzalkonium chloride tested against *P. aeruginosa*. It is evident that the preparation possesses antimicrobial activity.

Figure 3.11: Antimicrobial activity of a preparation of treated TiO_2 nano-composite composite with 10% (m/m) benzalkonium chloride tested against *S. aureus*. It is evident that the preparation possesses antimicrobial activity.

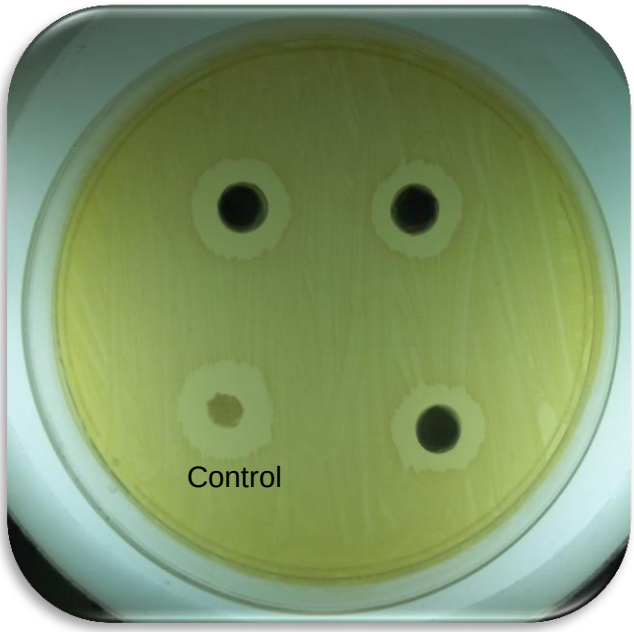

Figure 3.12: Antimicrobial activity of a preparation of treated TiO_2 nano-composite composite with 10% (m/m) benzalkonium chloride tested against *S. mutans*. It is evident that the preparation possesses antimicrobial activity.

The only bacteria that showed a resistance at 10% (m/m) is *P. aeruginosa*, which confirms that this type of bacteria could easily accumulate on the restorative materials on teeth inside the oral cavity. However, treated TiO_2 nano-composites containing 3% (m/m) of both CHxG and BzCl showed no microbial inhibition at all. Furthermore, treated TiO_2 nanocomposites that contained 5% (m/m) concentration of CHG and BzCl could not resist the bacterial growth and the bacteria were easily grown except with *S. aureus*, which produced small inhibition halos around the nano-composites (Figure 3.13, Figure 3.14). Also with a concentration of 7% (m/m) of both CHxG and BzCl, no difference could be observed and the results were almost the same as for the concentration of 5% (m/m).

Figure 3.13: Antimicrobial activity of a preparation of treated TiO_2 nano-composite composite with 5% (m/m) chlorhexadine gluconate tested against *S. aureus*. It is evident that the preparation possesses antimicrobial activity.

Figure 3.14: Antimicrobial activity of a preparation of treated TiO_2 nano-composite composite with 5% (m/m) benzalkonium chloride tested against *S. aureus*. It is evident that the preparation possesses antimicrobial activity.

In contrast, although treated TiO_2 nano-composites which contained 10% (m/m) BzCl and CHxG concentrations were antibacterial resins and although treated TiO_2 nano-composites that contained 5% (m/m) of the same antibacterial agents inhibited the bacterial growth with one of the bacteria, the untreated TiO_2 nano-composites and treated TiO_2 nano-composites without the antimicrobial additives did not inhibit the bacterial growth of any of the bacteria tested and had no significant antimicrobial activities at all. Moreover, these dental nano-composites had no ability to resist the bacterial impacts.

Table 3.1: Tabulated antimicrobial activity of a preparation of treated TiO_2 nano-composite composite with various compositions of benzalkonium chloride tested against pathogens.

Concentrations	*E. coli*	*E. faecalis*	*S. aureus*	*P. aeruginosa*	*S. mutans*
3 m/m%	–	–	–	–	–
5 m/m%	–	–	+	–	–
7 m/m%	–	–	+	–	–
10 m/m%	+	+	+	+	+

Table 3.2: Tabulated antimicrobial activity of a preparation of treated TiO_2 nano-composite composite with various compositions of chlorhexidine gluconate tested against pathogens. + indicates antimicrobial activity and – indicates no antimicrobial activity.

Concentrations	*E. coli*	*E. faecalis*	*S. aureus*	*P. aeruginosa*	*S. mutans*
3 m/m%	–	–	–	–	–
5 m/m%	–	–	+	–	–
7 m/m%	–	–	+	–	–
10 m/m%	+	+	+	+	+

Table 3.3: Inhibition zones mm), as an average of three measurements, of TiO_2 nano-composites against the selected bacteria

Concentrations of CHxG and BzCl	*S. mutans*	*E. coli*	*E. faecalis*	*S. aureus*	*P. aeruginosa*
0% (m/m) CHxG	0	0	0	0	0
0% (m/m) BzCl	0	0	0	0	0
3% (m/m) CHxG	0	0	0	0	0
3% (m/m) BzCl	0	0	0	0	0
5% (m/m) CHxG	0	0	0	0.6	0
5% (m/m) BzCl	0	0	0	0.8	0
7% (m/m) CHxG	0	0	0	0.7	0
7% (m/m) BzCl	0	0	0	0.9	0
10% (m/m) CHxG	1.5	1.4	1.5	1.6	1
10% (m/m) BzCl	1.6	1.5	1.6	1.7	1

CHAPTER FOUR

DISCUSSION

4.1 Discussion

The use of resin-based dental materials in the last century was a revolution in restorative dentistry field. Composites are extensively used in aesthetics since they possess a good appearance and are stable in the oral environment (Jandt *et al*., 2009).

Antimicrobial materials resist bacteria and eliminate, reduce or even avoid the biofilm formations on the composite materials. There are different approaches to achieve this. In general, antimicrobial properties of biomaterials could be achieved by introducing agents such as silver (Stobie *et al*., 2008) or one or more antibiotics into the composite materials (Popat *et al*., 2007). Bacteria are subsequently killed on contact with the materials or through leaching of the antimicrobial agents.

There are many examples of antimicrobial biomaterials used as implants or in pure research (Secinti *et al*., 2008; Tang *et al*., 2007) and the number of studies addressing antimicrobial biomaterials outside the field of dental materials has lately increased rapidly. In the oral environment, significant examples of microbes present are acid producing bacteria, such as *S. mutans* or anaerobic bacteria. A common problem with dental composites is the failure of the resin dentin interface, although new and enhanced bonding systems have helped to decrease the problem. If the interface fails, bacteria as mentioned above have the ability to penetrate the gap, which could result in secondary caries. Therefore, necessity exists to have dental composites with antimicrobial properties.

A number of attempts have been made to provide antimicrobial resin-based dental composites. Ti particles and Ag were introduced into dental composites, respectively, to introduce antimicrobial properties and improve biocompatibility of the dental composites (Jandt *et al*., 2002). In another investigation two types of silver-supported antibacterial materials, 5 w/t % Novaron (N-5) and 7 w/t % Amenitop (AM) were incorporated into dental composites (Yoshida *et al*., 1999). These composites prevented the growth of *S. mutans* after immersion in water for 6 months. This indicated relatively long permanent antimicrobial effects of these composites. No or very little release of Ag ions was observed for the dental composites. Compressive strength and flexural strength were positively affected by N-5 after

storage in water, while there was a considerable difference in both mechanical parameters for the AM containing composites.

Dental composite materials containing 1% (w/w) quaternary ammonium polyethylenimine (PEI) nano-particles were investigated for their antimicrobial activities (Beyth *et al*., 2006). The antibacterial properties of these composites were depended on contact mechanism rather than on leaching. Introducing the PEI nano-particles did not significantly affect on the mechanical properties of the composites. The antimicrobial effects persisted for at least 30 days.

Alkylated ammonium chloride derivatives have been incorporated into dental composites and a reinforcement of the antimicrobial properties was reported for these composites (Kim & Shim, 2001). Therefore, the same study found that alkylated ammonium chloride derivatives with a greater chain length between the acryl (or methacryl) functional groups and the ammonium reduced some of the mechanical properties of the composites.

CHxA was mainly introduced as incorporated antimicrobial agent into dental composite materials (Leung *et al*., 2005). When raising the HEMA content of these composites, the light cure polymerization rates decreased. Furthermore, water sorption induced swelling and rates of diffusion controlled the release of CHxA from the set materials increased. These composites revealed an eliminating or delay in biofilm formation compared to conventional composites but some compositions exhibited polymer leakage.

Since TiO_2 nano-composite is commonly used in many biomedical applications such as dental fillings and dental restorations and because dental caries and periodontal diseases are still related to the most prevalent infectious diseases, bacterial infections associated with these restoratives are still a source of serious concern which could cause possible complications and poor performance of these materials as restoratives. Moreover, as TiO_2 nano-composite is increasingly used in the dental field, especially for restorations, the prevalence of bacterial infection increases. Since microorganisms and their by-products on the restoratives are considered as the principle and major cause of dental complaints, treatment is primarily associated with the elimination of bacteria from infected restorative materials.

The investigations of antibacterial activity of available dental nano-composite materials have been evaluated, in addition to the discussions on several studies, to achieve antibacterial dental nano-composites (Updegraff *et al*., 1971; Tobias, 1988). Commercially available TiO_2 nano-composite which has no antibacterial effect after being cured, might clarify why TiO_2

nano-composite accumulates more plaque than other filling materials. Therefore, many attempts were made to provide antibacterial properties involving modulations to the resin composites and filler components (Yoshinari *et al*., 2001; Zhao *et al*., 2009; Leonhardt & Dahlén, 2007).

Due to formidable concerns, considerable effort has been made to eliminate or at least reduce the bacterial influence on dental nano-restoratives such as TiO_2 nano-composite. Therefore, further modifications are necessary to achieve good nano-composites without ignoring their mechanical property for tooth restoration and considering that they might have direct contact with the surrounding tissue of the teeth. One important approach is to improve and promote the antimicrobial capability of the dental restorative materials by adding some antimicrobial substances such as CHxG and BzCl in order to improve the nano-composites' performance.

Some studies have investigated and focussed on the effectiveness of CHxG and BzCl and their behaviour against bacteria (Jedrychowski *et al*., 1983; Vianna *et al*., 2004) which confirm the significant role of these antibacterial substances even if they are used with dental nano-composite resins.

TiO_2 nano-restorative infections around and on dental nano-restoratives continue to be a big concern in most clinical studies. Extensive investigations have recently been made, but TiO_2 nano-composites with antibacterial activities are still scarce and need to be widely used with a good enough performance. Consequently, the present *in vitro* study was performed to corroborate the antibacterial activity of TiO_2 nano-composite with two different antibacterial agents (CHxG and BzCl) in order to prevent and eliminate the growth of the bacteria on the TiO_2 nano-composite and provide the restorative composites with antibacterial properties. TiO_2 nano-composite was prepared in four different groups, (1) Untreated TiO_2 nano-composite; (2) Treated TiO_2 nano-composite with Silane; (3) Treated TiO_2 nano-composite mixed with four concentrations of BzCl 3%, 5%, 7% and 10% (m/m) independently; and (4) Treated TiO_2 nano-composite mixed with four concentrations of CHxG 3%, 5%, 7% and 10% (m/m) independently. These groups of TiO_2 nano-composite resins were tested against five facultative organisms: *S. mutans* ATCC 25175*, E. coli* ATCC 11775, *P. aeruginosa* ATCC 10145, *E. faecalis* ATCC 29212 and *S. aureus* ATCC 12600, which have the ability to grow inside the oral cavity. All TiO_2 nano-restoratives were incubated with the facultative strains for 24 hours at 37°C except for *S. mutans* ATCC 25175*;* it was incubated for 48 hours at 37°C and exposed to an atmosphere of 5% CO_2 by incubating it in a water-Jacketed incubator.

The obtained results have shown that untreated or treated TiO_2 nano-composite resins with silane, without incorporating any of the antibacterial substances, have no antibacterial activities.

The incorporation of CHxG and BzCl with TiO_2 nano-composites appeared to provide antibacterial behaviour and increased the antibacterial activity of TiO_2 nano-composites. When CHxG and BzCl were incorporated at a concentration of 10% (m/m), they showed clear inhibition zones around the nano TiO_2 restoratives, but in concentrations of 5% and 7% (m/m) both CHG and BzCl showed small inhibition zones around TiO_2 nano-composite when tested against *S. aureus* ATCC 12600 only, while the incorporations of the antibacterial substances did not show any inhibition halos against the other facultative strains. At concentration of 3% (m/m) both antibacterials, CHxG and BzCl, did not resist the bacterial growth at all with any of the strains.

Since bacteria induced problems associated with dental composites are important, it is surprising how little currently available dental composites actually possess antimicrobial properties. The antimicrobial properties of its corrosion products and the Ag content of dental amalgam are have been studied extensively (Beyth *et al*., 2007) and that leaves much to be accomplished in this respect for dental composites. There are often problems with incorporation of antimicrobial agents into dental composites, such as decrease of the antimicrobial activities with time or reduced capability of the composites to light cure. Nevertheless, antimicrobial composites would help to solve or alleviate some of the main clinical problems associated with these resin-based restorative materials. Therefore, one could expect that future dental composites will address this issue and will contain some antimicrobial properties and that this will be a question of future intensive studies.

The goal of *in vitro* antimicrobial susceptibility testing is to provide a reliable predictor of how an organism is likely to respond to an antimicrobial agent. A number of antimicrobial susceptibility testing (AST) methods are available to determine bacterial susceptibility to antimicrobials and the selection of a method is based on many factors such as practicality, flexibility, automation, cost, reproducibility, accuracy and individual preference. It is also essential that AST methods provide reproducible results in day-to-day laboratory use and that the data be comparable with those results obtained by an acknowledged “gold standard” reference method.

We considered the use of the disk diffusion methodology because it is a “gold standard”. Furthermore is straightforward to perform, is reproducible and does not require expensive equipment. Its ease in modifying the test antimicrobial material disks greatly assists in a new

materials design environment. It can identify a subset of new materials to be further investigated. That was the main aim of this study, to determine if different nano-composite materials with added antimicrobial agents at optimum concentrations have any antimicrobial activity.

A liquid culture method such as the broth dilution method can be used for further testing to determine minimum inhibitory concentrations (MICs) once designed material has been indentified and found to be a suitable material for further investigation.

This study confirmed that TiO_2 nano-composites prepared and provided with antibacterial substances such as CHxG and BzCl may prevent the growth of bacteria and may be beneficial as an additive in dental restorations. However, further studies need to be performed to obtain enough information for the development of a nano-composite that can be used clinically.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

Four nano-composites prepared with TiO_2 were studied for their antibacterial activity as they are broadly used in dentistry as dental restoratives. BzCl and CHxG were added independently to the composites of the restorative materials. The addition of certain concentrations of these antibacterial substances increased the antibacterial activity without changing the physical handling properties of these nano-composites.

Lower concentrations of BzCl and CHxG were incorporated into the nano-materials, for instance 3% (m/m), and they all gave negative results in all the experiments and the bacteria were grown easily. Furthermore, the incorporation of 5% and 7% (m/m) concentration of both antibacterial substances CHxG and BzCl also gave negative results except with *S. aureus*, for which it was positive and there were clear inhibition zones around the nano-composites. With addition of 10% (m/m) it showed positive results as clear inhibition zones appeared around the TiO_2 nano-composites and killed the bacteria.

5.2 Summary and recommendation

The commercial TiO_2 nano-composites which are widely used in dentistry as restorative materials are not antibacterial and could not resist the growth of the bacteria that might accumulate on the dental restoratives as a result. Therefore, the only advisable and recommendable solution would be to incorporate some antibacterial substances which can work as antimicrobial agents. BzCl and CHxG can be used in order to prevent the bacterial growth and support their performances and functions as direct restoratives on teeth as crowns, bridges, fillings, veneers and cusps.

The risk of these nano-composite resins causing bacterial infection *in vivo* can be partly approximated by assessing the antibacterial activity of the substances which are released to these nano-composites *in vitro*. The antibacterial substances that were incorporated into the TiO_2 nano-composites used in this *in vitro* study have been shown to be antibacterial agents at sufficient concentrations. The potencies of these substances are quite varied and therefore the risk of bacterial effects depends on their ability to spread through the restoratives.

The ability to combine antimicrobial components in dental restorative materials would provide extra potential benefits to dental restoratives. Further studies using different concentrations and organisms would provide a better indication on which concentration would be the most effective in designing new TiO_2 nano-composites. In conclusion, at least 5% of both antibacterial chemicals mentioned previously should be used in order to provide effective protection against the bacteria.

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