THE ANTI-BIOFILM EFFECTS OF BISPHOSPHONATES AND BIOACTIVE GLASS ON THE PERIODONTOPATHOGEN *AGGREGATIBACTER ACTINOMYCETEMCOMITANS*

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March 2015

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HELSINGFORS UNIVERSITET
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Title

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Subject

Industrial Pharmacy

Level	Month and year	Number of pages
Master's Thesis	March 2015	85

Abstract

Periodontitis is a globally significant disease which destroys the attachment tissues and alveolar bone of teeth, eventually leading to tooth loss. Biofilms, the most intrinsic lifestyle of bacteria, play a pivotal role in the occurrence of this disease. Periodontal biofilms can be treated with topically administered chlorhexidine and strain-specific antibiotics. However, these antimicrobials do not offer solutions for periodontal attachment tissue and alveolar bone loss. Some therapeutical alternatives for these conventional treatments have been investigated. In numerous studies, periodontitis is treated successfully (increased attachment and/or alveolar bone levels) with topically and systemically administered bisphosphonates. Furthermore, a topically administered bone graft substitute (bioactive glass) has shown to improve periodontal parameters. In addition, bioactive glass has known antimicrobial and anti-biofilm effects. Moreover, a few bisphosphonates have shown antimicrobial activity against some bacterial strains. Hence, both bisphosphonates and bioactive glass are promising materials for dental applications, also raising interest in their combination. Indeed, it could be hypothesized that this combination product could simultaneously treat both the underlying cause (biofilms) and consequences (alveolar bone and attachment tissue loss) of periodontitis.

Open research questions remain for the combination product. Is the anti-biofilm effect enhanced when bioactive glass is combined with bisphosphonates? Moreover, do bisphosphonates have intrinsic anti-biofilm properties? These questions are investigated in this thesis, which is a continuation of a recent doctoral dissertation. In this dissertation, a clodronate-bioactive glass combination product was studied by applying it into periodontal pockets. However, anti-biofilm effects were not assessed. In this thesis, a close examination is carried out on these effects, utilizing relevant biofilm models.

The aims of this work were to investigate anti-biofilm effects of bisphosphonates (alendronate, clodronate, etidronate, risedronate and zoledronate) (i) alone, administered as solutions and (ii) combined with bioactive glass S53P4. Optimization of the used assay methods (96-well plate assay, Static Biofilm method) was performed. The anti-biofilm effects of bisphosphonate solutions were screened in the 96-well plate assay using a model organism *Staphylococcus aureus* Newman and a periodontopathogen A*ggregatibacter actinomycetemcomitans* ATCC 33384. After this, experiments were conducted with bisphosphonate-bioactive glass combinations. The experiments were performed with a single-specie (*A. actinomycetemcomitans* ATCC 33384) dental biofilm model based on the Static Biofilm method. The model mimics conditions encountered by periodontal bacteria in the oral cavity. In this part, bisphosphonate particle sizes were measured to determine a suitable control material. In addition to bacterial experiments, pH measurements were carried out to gain an insight to a possible anti-biofilm mechanism.

Bisphosphonates administered as stand-alone compounds did not have an effect on either the Gram-positive model organism (*S. aureus* Newman) or the Gram-negative periodontopathogen (*A. actinomycetemcomitans* ATCC 33384). In contrast, most combinations of bisphosphonate-bioactive glass revealed a statistically significant increase in anti-biofilm effect on *A. actinomycetemcomitans* ATCC 33384. The combinations were compared to a control composed of inert glass and bioactive glass. In these assay conditions, the risedronate-bioactive glass-combination was the most effective (significant statistical difference, $p < 0.05$). Other combinations also reduced biofilms (significant statistical differences, $p < 0.05$), with the exception of clodronate-bioactive glass, where the change was not statistically significant. The most effective combinations (containing risedronate and etidronate) subjected the biofilms to a period of low pH. Conversely, the least effective combination (clodronate-bioactive glass) rapidly became alkaline, similarly to the control compounds (inert glass and bioactive glass). Thus, anti-biofilm efficacy could be connected to lowered pH. This observation is supported by recent literature where *A. actinomycetemcomitans* has been deemed highly sensitive to acidity. However, establishing the anti-biofilm rank order of bisphosphonate-bioactive glass combinations would benefit from experiments with equal bisphosphonate particle sizes.

Keywords

A. actinomycetemcomitans, *S. aureus*, bisphosphonate, bioactive glass, biofilm, periodontitis

Where deposited

Division of Pharmaceutical Chemistry and Technology

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HELSINGFORS UNIVERSITET
UNIVERSITY OF HELSINKI

Tiedekunta	Osasto
Farmasian tiedekunta	Farmaseuttisen kemian ja teknologian osasto

Tekijä

Anna Katariina Hiltunen

Työn nimi

Bisfosfonaattien ja bioaktiivisen lasin vaikutukset *Aggregatibacter actinomycetemcomitans* -parodontiittipatogeenin biofilmeihin

Oppiaine

Teollisuusfarmasia

Työn laji	Aika	Sivumäärä
Pro gradu -tutkielma	Maaliskuu 2015	85

Tiivistelmä

Parodontiitti on kansainvälisesti merkittävä sairaus, joka tuhoaa hampaan kiinnityskudoksia ja alveoliluuta. Tämä johtaa lopulta hampaan irtoamiseen. Biofilmit, bakteereille ominaisin olomuoto, ovat tärkeässä roolissa parodontiitin synnyssä. Parodontiitin biofilmejä voidaan hoitaa topikaalisesti annostellulla klooriheksidiinillä ja bakteerikannan mukaisesti valituilla antibiooteilla. Kuitenkaan nämä mikrobilääkkeet eivät tarjoa ratkaisuja parodontiitin aiheuttamaan kiinnityskudos- ja alveoliluukatoon. Terapeuttisia vaihtoehtoja näille perinteisille hoitokäytännöille onkin tutkittu. Lukuisissa tutkimuksissa parodontiittia on hoidettu onnistuneesti (kasvaneet alveoliluu- ja/tai kiinnityskudostasot) topikaalisesti ja systeemisesti annostelluilla bisfosfonaateilla. Myös topikaalisesti annostellulla luun täytemateriaalilla (bioaktiivinen lasi) on saavutettu parodontaalistatuksen kohentumista. Tällä materiaalilla on myös todettu olevan mikrobien ja biofilmien vastaista tehoa. Myös eräät bisfosfonaatit ovat osoittaneet tehoa muutamia mikrobikantoja vastaan. Näiden seikkojen vuoksi sekä bisfosfonaatit että bioaktiivisen lasi ovat lupaavia materiaaleja hammassovelluksissa, ja niiden yhdistäminen onkin herättänyt kiinnostusta. Voidaankin esittää hypoteesi, että tällä yhdistelmävalmisteella voidaan hoitaa sekä parodontiitin juurisyytä (biofilmit) että seuraksia (alveoliluu- ja kiinnityskudoskato).

Yhdistelmävalmisteeseen liittyy kuitenkin avoimia kysymyksiä. Voidaanko biofilmien vastaista vaikutusta parantaa yhdistämällä bioaktiivinen lasi bisfosfonaattien kanssa? Onko bisfosfonaateilla itsellään biofilmien vastaista tehoa? Näitä kysymyksiä tutkitaan tässä pro gradu -tutkielmassa, joka on jatkoa tuoreelle väitöskirjalle. Kyseisessä väitöskirjassa tutkittiin klodronaatista ja bioaktiivisesta lasista muodostettua yhdistelmävalmistetta parodontaaliseen ientaskuun annosteltuna. Työssä ei kuitenkaan käsitelty näiden aineiden vaikutuksia biofilmeihin. Tässä pro gradu -tutkielmassa näitä vaikutuksia tutkittiin relevantteja biofilmimalleja hyödyntämällä.

Tämän työn tavoitteina oli kartoittaa bisfosfonaattien (alendronaatti, etidronaatti, klodronaatti, risedronaatti ja tsoledronaatti) vaikutuksia biofilmeihin (i) yksin liuoksena annosteltuna ja (ii) yhdessä bioaktiiviseen lasin S53P4 kanssa annosteltuna. Käytettyjä menetelmiä (96-kuoppalevy- ja Staattinen biofilmimenetelmä) optimoitiin. Bisfosfonaattiliuoksien vaikutuksia malliorganismin (*Staphylococcus aureus* Newman) ja parodontiittipatogeenin (A*ggregatibacter actinomycetemcomitans* ATCC 33384) biofilmeihin tutkittiin 96-kuoppalevymenetelmällä. Tämän jälkeen siirryttiin bisfosfonaatti-bioaktiivinen lasi-yhdistelmien tutkimiseen. Näitä tutkittiin yhdestä kannasta (A*. actinomycetemcomitans* ATCC 33384) koostuvalla biofilmimallilla, joka pohjautuu Staattiseen biofilmimenetelmään. Tämä malli kuvastaa olosuhteita, jotka parodontiittibakteerit kohtaavat suussa. Tässä osassa suoritettiin myös bisfosfonaattien hiukkaskokomittaus sopivan kontrollimateriaalin valitsemiseksi. Bakteeritöiden lisäksi tutkielmaan sisällytettiin pH-mittauksia, joilla selvitettiin mahdollisia biofilmien vastaisia mekanismeja.

Yksikään bisfosfonaattiliuos yksinään annosteltuna ei osoittanut tehoa Gram-positiiviseen malliorganismiin (*S. aureus* Newman) eikä Gram-negatiiviseen parodontiittipatogeeniin (*A. actinomycetemcomitans* ATCC 33384). Sen sijaan, useimmat bisfosfonaatti-bioaktiivinen lasi-yhdistelmät osoittivat tilastollisesti merkittäviä biofilmien (*A. actinomycetemcomitans* ATCC 33384) vastaisia vaikutuksia. Yhdistelmiä verrattiin kontrolliin, joka koostui inertistä- sekä bioaktiivisesta lasista. Risedronaatti-bioaktiivinen lasi-yhdistelmä oli tehokkain (tilastollisesti merkittävä ero, $p < 0.05$) näissä koeolosuhteissa. Myös useimmat muut yhdistelmät vähensivät biofilmien määrää merkittävästi (tilastollisesti merkittävät erot, $p < 0.05$). Vain klodronaatti-bioaktiivinen lasi-yhdistelmän vaikutus ei ollut tilastollisesti merkittävä. Huomattiin, että tehokkaimmat yhdistelmät (etidronaatti- ja risedronaatti-bioaktiivinen lasi) altistivat biofilmit matalan pH:n ajanjaksolle. Sen sijaan vähiten aktiivisella yhdistelmällä (klodronaatti-bioaktiivinen lasi) ja kontrollinäytteellä pH muuttui nopeasti emäksiseksi. Täten, biofilmin vastaiset vaikutukset voivat olla yhteydessä mataliin pH-arvoihin. Myös tuoreessa kirjallisuudessa on havaittu *A. actinomycetemcomitans*:n olevan erittäin herkkä happamuudelle. Jotta yhdistelmävalmisteet voitaisiin asettaa biofilmien vastaiseen paremmuusjärjestykseen, tulisi jatkotutkimus suorittaa samalla bisfosfonaattien hiukkaskoolla.

Avainsanat

A. actinomycetemcomitans, *S. aureus*, bisfosfonaatti, bioaktiivinen lasi, biofilmi, parodontiitti

Säilytyspaikka

Farmaseuttisen kemian ja teknologian osasto

Muita tietoja

Ohjaajat: Professori Anne Juppo ja Dosentti Adyary Fallarero

ACKNOWLEDGEMENTS

This thesis was performed in the laboratory of Pharmaceutical Biology (Division of Pharmaceutical Biosciences) in co-operation with the laboratory of Industrial Pharmacy (Division of Pharmaceutical Chemistry and Technology), in the Faculty of Pharmacy, University of Helsinki.

First of all, I would like to thank Professors Pia Vuorela and Anne Juppo for offering and enabling this work. I would like to express my sincere gratitude to my supervisor Docent Adyary Fallarero for excellent guidance and endless support. I would also like to thank Doctor Malena Skogman for support and teaching me how to work with bacteria. A great praise goes to Doctor Kirsi Rosenqvist for the thesis idea and for advice she gave me in the beginning.

I would like also thank the people I have worked with for making a working environment so comfortable: Dominik, Elina, Krista, Leena, Maarit, Sabina, Sonja and Terttu.

I am grateful to Osmo Antikainen and Heikki Räikkönen for technical support with Flashsizer, as well as to Pirkko Pussinen for providing and introducing one of the utilized bacterial strains.

I would like to thank my parents, Timo and Bibbi and my sister Elina for all the enduring support over the years.

My greatest appreciation is dedicated to Karri whose endless support, patience and constructive criticism encouraged me during the thesis, and also over the studies.

This thesis was performed in the context of the Academy of Finland project "Fabricating bacterial biofilms via artificial nano(bio)components" (ArtFilm).

Helsinki, May 5th 2015

Anna Katariina Hiltunen

LIST OF ABBREVIATIONS

A	Alendronate
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BAG	Bioactive glass S53P4
BP	Bisphosphonate
C	Clodronate
CFU• mL^{-1}	Colony forming units per milliliter
E	Etidronate
FM	Fluorescence microscopy
FPPS	Farnesyl pyrophosphate synthase
HTS	High throughput screening
IG	Inert glass
IV.	*Intravenalis*; intravenous administration
logR	Logarithmic Reduction
MRSA	Methicillin-resistant *Staphylococcus aureus*
MW	Molecular weight
PBS	Phosphate buffered saline
P.O.	*Per os*; oral administration
R	Risedronate
RAU	Relative absorbance units
RT	Room temperature
SD	Standard deviation
SDS	Sodium lauryl sulphate
S/B	Signal-to-background ratio
S/N	Signal-to-noise ratio
TSA	Tryptic soy agar
TSA_{D5}	Tryptic soy agar supplemented with 5% defibrinated sheep blood
TSB	Tryptic soy broth
TSB-YE/Glc	TSB supplemented with 0.6% (w/v) yeast extract and 0.8% (w/v) glucose

w/v	Weight/volume
Z	Zoledronate

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1 INTRODUCTION

Periodontitis is a significant disease both worldwide (Jin et al. 2011) and in Finland (Current Care Guidelines 2010). It destroys the attachment tissues of teeth, eventually leading to tooth loss (Giannobile 2008). This tissue destruction is due to an infection-induced host response inflammation. Biofilms, the most common microbial lifestyle (Tortora et al. 2014a) present in diverse environments (Tortora et al. 2014f), play an important role in the occurrence of this infection (Giannobile 2008). Biofilms are matrix-enclosed microbes displaying higher tolerance against external threats than single-cell bacteria (Costerton et al. 1999). Periodontitis has been treated successfully with bisphosphonates (Rocha et al. 2001; Takaishi et al. 2001; El-Shinnawi and El-Tantawy 2003; Takaishi et al. 2003; Rocha et al. 2004; Palomo et al. 2005; Reddy et al. 2005; Jeffcoat et al. 2007; Veena and Prasad 2010; Pradeep et al. 2012; Sharma and Pradeep 2012a and 2012b) and bioactive glasses (Mengel et al. 2003; Mengel et al. 2006; Chacko et al. 2014). However, bisphosphonate-bioactive glass combinations have not been widely investigated. Such combinations are a focal element of this study, which is a continuation of Dr. Kirsi Rosenqvist's recent doctoral dissertation (Rosengvist 2014). This dissertation focused on the use of a clodronate-bioactive glass combination product in the treatment of periodontitis. A clinical trial and a physicochemical characterization of the combination product were performed and a potential beneficial effect in protection against dental infections was suggested. However, specific studies regarding anti-biofilm effects of the combination product were not carried out. The anti-microbial (Stoor et al. 1998; Leppäranta et al. 2008; Munukka et al. 2008; Drago et al. 2013; Coraça-Huber et al. 2014; Gergely et al. 2014; Romanò et al. 2014) and anti-biofilm (Coraça-Huber et al. 2014; Drago et al. 2014) effects of solely bioactive glass (S53P4) against many strains are known in literature. In addition, alendronate, clodronate (Kruszewska et al. 2002) and risedronate (Kruszewska et al. 2012) have shown antimicrobial activity against some bacterial strains. Similar effects against periodontal biofilms would offer additive benefits for the combination product in addition to known positive bone construction effects of bioactive glass (S53P4) (Virolainen et al. 1997). In addition to clodronate, this thesis extends the anti-biofilm property investigation to include also four other bisphosphonate

compounds (alendronate, etidronate, risedronate and zoledronate). In particular, a close examination is carried out on specific anti-biofilm effects, using relevant biofilm models.

The experimental part of the thesis is divided into three parts: the first aims at investigating whether bisphosphonates have intrinsic anti-biofilm effects as stand-alone compounds. The second part studies the bisphosphonates together with bioactive glass S53P4 (BAG) in the combination product, using a single-specie dental biofilm. A well-known biofilm former organism (*Staphylococcus aureus* Newman) was used in initial tests. *Aggregatibacter actinomycetemcomitans* ATCC 33384, a reference organism known to be a late colonizer in dental plaque, was employed for both parts. For the first two parts, there was an additional objective of optimizing experimental methods in the study of *A. actinomycetemcomitans* ATCC 33384. In the third part, pH measurements were utilized to examine mechanistic anti-biofilm actions of bisphosphonates and bisphosphonate-BAG combinations on biofilms.

2 BACTERIAL BIOFILMS

2.1 Definition

Bacteria can exist as single cells or in a multicellular state (Costerton et al. 1995). Single cells float or swim in a liquid (Tortora et al. 2014f), and they are called planktonic cells (Costerton et al. 1995). Cells that exist in a multicellular, community-based lifestyle are called biofilms. In the field of microbiology, planktonic cells have traditionally been the point of interest. The significance of biofilms surged during the late 1900's, when Costerton et al. (1978) established a biofilm theory which remains valid to the present day. This led to increased research activity within the field. Biofilms are sessile, structured bacterial communities surrounded by a self-produced polymeric matrix attached to a living or non-living surface (Costerton et al. 1999). In addition to this, biofilms also differ phenotypically from their planktonic counterparts (Costerton et al. 1995). It is estimated that in nature, 99% of bacteria exist in biofilms, making them the most common bacterial lifestyle outside laboratory conditions (Tortora et al. 2014a). Biofilms can consist of a single or several microbe species (Tortora et al. 2014c).

2.2 Formation of biofilms

Biofilm formation includes four main phases. At the initial phase, the planktonic bacteria are attached to some abiotic or biotic surface (Phase 1, Figure 1) (Otto 2013). The attachment to abiotic surfaces occurs via hydrophobic and electrostatic forces. In the attachment to biotic surfaces, e.g. strain-specific surface-anchored proteins display an important role. Attachment is followed by bacterial aggregation and proliferation (Phase 2). Upon attachment, the bacteria begin to produce extracellular polymeric substances, which surrounds the bacterial community, leading to a complex that can eventually be considered a mature biofilm (Phase 3) (Otto 2008). A mature biofilm may exhibit a mushroom-like structure with intermittent channels. The channels offer nutritional access to lower bacterial layers (Otto 2013) and an excretion route of toxic waste (Tortora et al. 2014c). At the final phase, bacteria are detached from the mature biofilm and returned into the planktonic phase (Phase 4) (Otto 2013). After detachment, bacteria can disseminate to other sites for further colonization. Convective dissemination occurs with

the aid of water flow in nature and many industrial settings. In the human body, this dissemination can occur via the lymphatic system or the blood stream (Otto 2013).

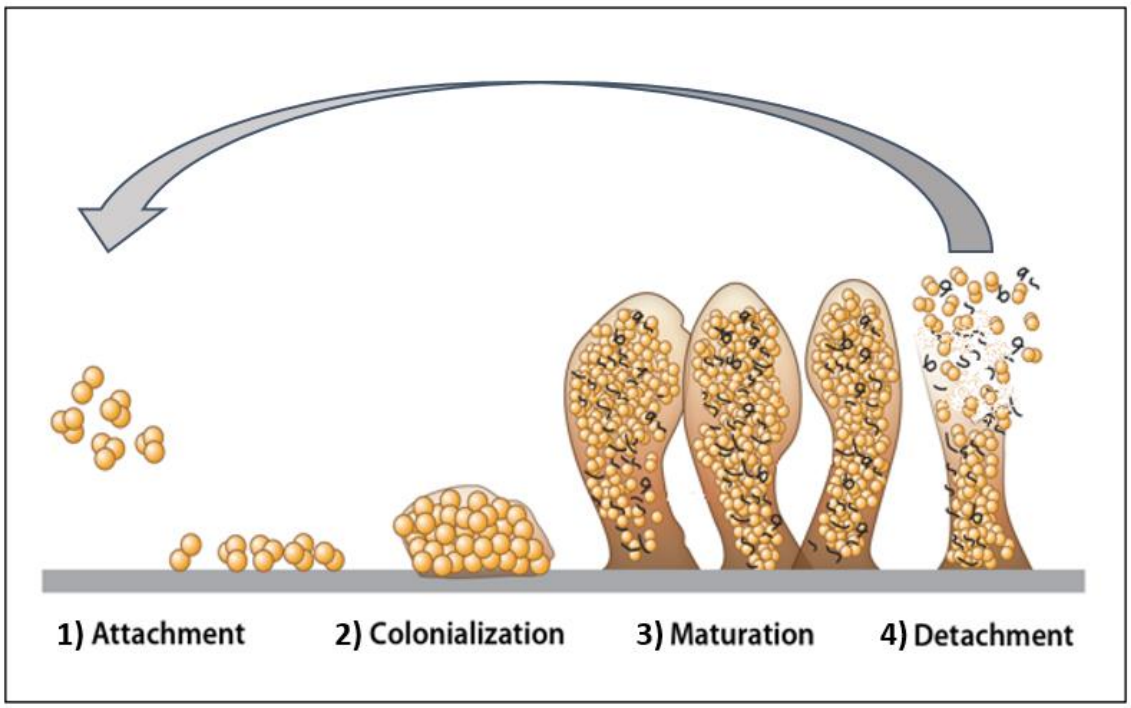


Figure 1. Biofilm evolution described through different phases (modified from Otto 2013).

2.3 Structure of biofilms and functional characteristics

The self-produced matrix (often called slime) is a barrier between bacteria and their external environment (Steinberger and Holden 2005) enclosing the bacterial cells (Costerton et al. 1999). The matrix consists of extracellular polymeric substances (Steinberger and Holden 2005). Matrix composition varies between and even within species, but its main components are lipopolysaccharides, extracellular DNA and proteins. The matrix serves as a barrier inhibiting entrance of harmful agents (Costerton et al. 1999) but also protects the biofilm from dehydration and prevents the nutrients from escaping out of the cell (Tortora et al. 2014a).

Biofilms have an inherently high tolerance against external threats: humidity, osmotic pressure, mechanical stress (Otto 2013), heat, UV radiation (Cos et al. 2010), disinfectants (Tortora et al. 2014d), antimicrobial agents and the host immune system (Costerton et al. 1999). For example, the required antimicrobial agent concentration for

biofilm destruction has been shown to be up to 1000-fold in comparison to planktonic bacteria (Tortora et al. 2014c). A few explanations for this tolerance have been suggested (Costerton et al. 1999). One hypothesis suggests that antibiotics fail to penetrate into their targets inside biofilms since the matrix inhibits the diffusion of antibiotics. According to a second explanation, antibiotic susceptibility is dependent on bacterial metabolic state. Bacteria in biofilms are in different metabolic states due to the differences in nutrient availability. Hence, in some biofilm regions, bacteria are deprived and live in a slow growing, metabolically inactive state. Bacteria in this state are not as susceptible to antibiotics, which predominantly inhibit the processes of metabolically active cells (Otto 2006). These phenotypically different subpopulation members are called persister cells, and they are particularly tolerant against antimicrobial therapies (Lewis 2010). Finally, it is likely that the high tolerance results from a combination of the presently suggested and other, yet unknown mechanisms.

Within a host organism, biofilms release antigens and the host system responds with antibodies (Costerton et al. 1999). However, the antibodies are not necessarily capable of destroying biofilms, but can cause collateral damage to surrounding tissues. Moreover, phagocytosis of biofilms is more difficult than of planktonic cells (Tortora et al. 2014b). When biofilm infection is treated with antibiotic therapy, it usually removes the symptoms caused by released planktonic bacteria, but fails to eliminate the underlying root cause biofilm (Costerton et al. 1999). Cases have been reported where biofilms have survived despite several years of aggressive antibiotic therapy (Stoodley et al. 2011). After discontinuation of an antibiotic regimen, the remaining biofilm can cause recurrent infections (Costerton et al. 1999). As a result, biofilms need to be surgically removed via revision surgery. This, in turn, increases the likelihood for the occurrence of a new infection due to the repeated use of medical devices during hospitalization (i.e. cathethers, implants, valves, etc.). This clearly leads to a negative infection cycle that can be very difficult to overcome.

One presently investigated biofilm destruction method involves the inhibition of quorum sensing. Quorum sensing is a phenomenon where independent bacteria communicate with each other with a signaling chemical (Tapiainen et al. 2010). This signaling chemical is called an inducer (Tortora et al. 2014e). When inducers are diffused into the surrounding medium, they attract other bacteria, which start to release inducers of their own. This

communication has a pivotal role in biofilm formation (Tapiainen et al. 2010). Upon inhibition of quorum sensing, the remaining planktonic bacteria, incapable of forming biofilms, are easier to eradicate with antibiotics. Thus, a future prospect is to combine a quorum sensing inhibitor and an antibiotic in order to increase the performance of the regimen.

2.4 Clinical relevance of biofilms and connection to diseases

It is estimated that 70% of bacterial infections are biofilm-mediated (Tortora et al. 2014c). This is not surprising, considering the protection that biofilms offer for bacterial survival in the host (Otto 2013). Biofilms can colonize indwelling medical devices such as joint and bone prostheses, catheters and artificial heart valves (Costerton et al. 1999; Otto 2013). Other biofilm-mediated infections are e.g. otitis media, osteomyelitis, cystic fibrosis, dental caries and periodontitis (Costerton et al. 1999). Periodontitis will be discussed in detail in Chapter 3. Biofilms can also be the causes for prolonged otitis media and urethritis (Tapiainen et al. 2010). As previously described, biofilm infections are often difficult or impossible to eradicate with antibiotics (Parsek and Singh 2003). Furthermore, biofilm infections are challenging to diagnose since the sampling and recovery of biofilms from tissues is difficult (Tapiainen et al. 2010). On the other hand, bodily biofilms can also be beneficial, e.g. in protection from invading pathogens (Tortora et al. 2014f). This dual nature exists also from an industrial perspective. While biofilms can cause severe problems by clogging industrial pipelines and machines, their controlled growth can be utilized in e.g. biosensors, bioremediation applications (pollutant detoxification and degradation) (Rodriguez et al. 2012) and for efficient energy production. In nature, biofilms in plants provide protection from invading soil pathogens (Rudrappa et al. 2008) and act as nutrition for aquatic animals (Tortora et al. 2014f).

2.5 Relevant biofilm-forming microorganisms

2.5.1 Staphylococcus aureus as a model organism

Staphylococcus aureus (S. aureus) is a Gram-positive bacterium which forms biofilms (Otto 2013). One fifth of the Finnish population has *S. aureus* colonizing their nasal cavities (Lumio 2013). *S. aureus* causes osteomyelitis (Jabra-Rizk et al. 2006), as well as skin, wound and post-surgery infections (Lumio 2013). Moreover, it causes sepsis upon invasion in the bloodstream (Lumio 2013). It is regarded as a main source of nosocomial infections (Jabra-Rizk et al. 2006). *S. aureus* is associated with many biofilm-mediated infections which are tolerant to antimicrobial treatment. Together with *Staphylococcus epidermis*, they are responsible for more than half of prosthetic device-associated infections (Fluckiger et al. 2005). Methicillin-resistant *S. aureus* (MRSA), also resistant to other penicillins and kefalosporines, causes infection epidemics in hospitals (Lumio 2013).

2.5.2 Aggregatibacter actinomycetemcomitans as a dental pathogen

Aggregatibacter actinomycetemcomitans (A. actinomycetemcomitans, previously *Actinobacillus actinomycetemcomitans*) is a periodontal pathogen (Kesić et al. 2009) causing localized aggressive periodontitis i.e. juvenile periodontitis and endocartitis (Bhattacharjee et al. 2011). It is a Gram-negative (Mandell and Socransky 1981; Zambon et al. 1983), capnophilic (Bhattacharjee et al. 2011), microaerophilic, nonmotile (Mandell and Socransky 1981), facultatively anaerobic (Bhattacharjee et al. 2011) rod (Mandell and Socransky 1981). This fastidious and slowly growing organism can be grown on blood and chocolate agar (Kesić et al. 2009). Bacterial colonization starts after 48–72 hours of incubation. The incubation temperature is between 20 to 42º C.

A microaerophilic bacterium requires oxygen, but as concentrations lower than in the ambient air (Tortora et al. 2014c). A facultative anaerobe is a bacterium that can grow both with and without oxygen. When oxygen is not available, such organisms utilize fermentation or anaerobic respiration for energy production. Capnophiles are bacteria that grow better at elevated concentrations of carbon dioxide. Low-oxygen, high-carbon

dioxide conditions required by *A. actinomycetemcomitans* can be created with carbon dioxide incubators, but also with a simple candle jar technique (Figure 2), which is a long-known method in microbiology.

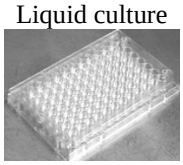
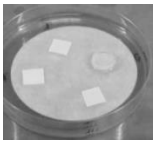
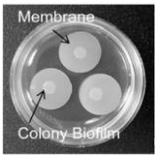
Figure 2. *Aggregatibacter actinomycetemcomitans* ATCC 33384 is incubated on TSA_{D5} (tryptic soy agar supplemented with 5% defibrinated sheep blood) plates in candle jars (microaerophilic conditions).

In this technique, cultures are transferred into a sealed, air-tight container with a lit candle and incubated (Tortora et al. 2014c). The ensuing combustion process consumes oxygen and generates carbon dioxide. The candle stops burning when the air of the container reaches a lowered oxygen concentration of ca. 17%. Consequently, elevated carbon dioxide levels of ca. 3% are acquired. Many pathogenic bacteria require such conditions.

2.6 Biofilm investigation methods

Most of the antimicrobial compounds and their research methods have been developed for planktonic bacteria, despite the facts that most bacteria live in biofilms, and that infections are usually due to biofilms, not planktonic bacteria (Skogman 2012). The unique features of biofilms require the development of reliable and specific research methods, which differ from those optimized for planktonic bacteria (Cos et al. 2010). Since characteristics of biofilms and planktonic bacteria are totally different, investigation methods for planktonic bacteria cannot be conducted for biofilms (Charaf et al. 1999; Donlan and Costerton 2002). Thus, anti-biofilm and anti-planktonic effects of compounds should be tested using distinct protocols. There is an urgent need for reliable anti-biofilm test methods (Sandberg et al. 2008). In addition to reliability, the method should be rapid, simple, reasonable in terms of cost and compliant to automation from the perspective of screening large compound libraries. Biofilms can be grown with liquid and solid-state growth methods. The choice of method is based on the research question under interest (Buckingham-Meyer et al. 2007). The main types of solid and liquid assays are presented in Table 1.

Table 1. Comparison of biofilm models (modified from Blomqvist 2014). Rotating Disk reactor figure from (Biosurface Technologies Corporation 2015). Colony Biofilm Model figure from Jo and Kim (2013).

	Biofilm model	**Culture**	**Substratum**	**Fluid shear**	**Material examination**	**Advantages**
Liquid culture 	**CDC Biofilm Reactor**	Continuous	Various	High	Possible	ASTM Standard Method
Liquid culture 	**Rotating Disk Reactor**	Continuous	Various	Moderate	Possible	ASTM Standard Method
Liquid culture 	**Drip Flow Biofilm Reactor**	Continuous	Various	Low	Possible	ASTM Standard Method
Liquid culture 	**MBEC Assay**	Batch culture	Plastic pegs	Gentle	Yes, but limited by well space	Rapid, HTS, ASTM Standard Method
Liquid culture 	**Well plate assays**	Batch culture	Plastic	No/Low	Yes, but limited by well space	HTS, fairly cheap, user-friendly
Solid culture 	**Static Biofilm method**	Batch culture	Various	No	Possible	Inexpensive, removable coupons, versatile
Solid culture 	**Colony Biofilm model**	Continuous	Polycarbonate filter paper	No	Possible	Antibiotic penetration through biofilms can be examined

2.6.1 Assays performed in liquid cultures

ASTM International (American Society for Testing and Materials) is one of the largest international organizations developing and providing standard methods (ASTM International 2015). There are five ASTM standardized methods for growing (and treating in MBEC Assay) biofilms (Table 1): CDC Biofilm Reactor (ASTM International Standard E2562-12), Rotating Disk Reactor (ASTM International Standard E2196-12), Drip Flow Biofilm Reactor (ASTM International Standard E2647-08), MBEC Assay (ASTM International Standard E2799-11) and Single Tube Disinfection (ASTM International Standard E2871-13). These methods are primarily standardized for *Pseudomonas aeruginosa,* and can be roughly divided into two culture modes: continuous and batch cultures. Continuous culture reactors include the Drip Flow Biofilm Reactor, the Rotating Disk Reactor and the CDC Biofilm Reactor. In these cases, a continuous flow of the medium ensures that fresh nutrients are always present (Merritt et al. 2005). In batch cultures, there is a fixed volume of nutrients, and bacteria may encounter nutritional shortage or exhaustion. The MBEC Assay and the widely applied well plates are batch cultures.

In the CDC Biofilm Reactor, biofilms are formed on coupons inserted into rods. In addition to the continuous flow of nutrients, biofilms are subjected to high flow shear from the rotation of a baffled stir bar. The Rotating Disk Reactor is based on coupons inserted into disks, which experience medium flow shear. With the Drip Flow Biofilm Reactor, biofilms can be formed on coupon surfaces that are inserted into reactor channels. In these conditions, biofilms experience a continuous flow of dripping nutrients with a low fluid shear caused by gravity at an inclination angle of 10°. After biofilm formation, the efficacy of different anti-biofilm compounds can be examined.

The MBEC (Minimum Biofilm Eradication Concentration) Assay is a standardized screening tool, which allows simultaneous testing of many compounds or compound concentrations. The plastic device consists of a lid with 96 pegs and a corresponding receiver plate with 96 wells. Biofilms are formed on the pegs with gentle mixing, without flow of nutrients into or out of an individual well (batch conditions). The formed biofilms are transferred to a new receiver plate for compound efficacy testing.

Well plate assays were utilized in this thesis (with 96-well plates). These cheap and user-friendly well plates are commonly used in the field of biofilms for quick compound screening (Peeters et al. 2008; Coenye and Nelis 2010). As in the MBEC Assay, simultaneous testing of many compounds and concentrations is possible. Another clear benefit is that compound anti-biofilm effects can be investigated with two modes (pre- and post-exposure) (Sandberg et al. 2008). The compound can be applied at the same time as the biofilm-forming bacterial suspension (pre-exposure), or after the formation (post-exposure). The purpose of the pre-exposure assay is to investigate whether the compound can prevent biofilm formation. This is usually possible using smaller concentrations of antibiotics than in post-exposure tests. In contrast, the aim of the post-exposure assay is to examine whether the compound can act on pre-formed biofilms. This generally requires higher concentrations of antibiotics. Cryopreserved ready-made biofilms in 96-well plates and well-plate handling automation can be used to facilitate compound screening (Sandberg et al. 2008).

The different assay methodologies are utilized to mimic the fluid dynamics of the target environment. This is important since flow shear has an effect on formed biofilm tolerance and functional behavior (Buckingham-Meyer et al. 2007). Biofilms formed under high shear conditions (turbulent flow) are denser, more stable (Pereira et al. 2002) and more resistant to chemical insults when compared with those formed in low shear conditions (laminar flow) (Buckingham-Meyer et al. 2007) or planktonic cells (Eginton et al. 1998).

2.6.2 Assays performed in solid-state cultures

In solid-state methods, biofilm growth surfaces are not immersed in liquid (Table 1) (Oja et al. 2014). They are less frequently used, and include the Colony Biofilm model and the Static Biofilm method (Table 1). In the Colony Biofilm model, inoculated polycarbonate membrane filters are transferred regularly onto new agar. Thus, it is a continuous culture. Instead, the Static Biofilm method consists of biofilms grown on a single agar plate for the entire incubation time (batch culture) (Oja et al. 2014). The biofilms are grown in the absence of fluid shear (Buckingham-Meyer et al. 2007; Oja et al. 2014). For this reason, the method can be considered suitable for mimicking infections e.g. in the ear or the skin (Oja et al. 2014). The Static Biofilm method is deemed very versatile, because it offers

the possibility to investigate many strains, coupon materials and compounds. It represents a robust, simple and economic option, which can be utilized in any basically equipped microbiological laboratory without the need of sophisticated instrumentation (Charaf et al. 1999).

2.6.3 Probes for biofilm quantification

The anti-biofilm effect of a compound can be evaluated by staining biofilms with different indicators (probes). Probe effects, e.g. fluorescence or absorbance, can be measured. Biofilm viability, biomass and matrix level are examples of attributes that can be inferred from the measurements (Skogman 2012).

For detection of live and dead bacteria in fluorescence microscopy (FM), bacteria are stained with fluorescent probes. SYTO® 9 and propidium iodide are fluorescent nucleic acid stains emitting green and red light, respectively (Molecular Probes 2004). When biofilms are stained with their combination, SYTO® 9 labels all bacteria, with both intact and damaged membranes. In contrast, propidium iodide penetrates only into bacteria with damaged membranes, concurrently causing a reduction of SYTO® 9. Thus, live bacteria with intact cell membranes show as green, whereas dead bacteria with damaged membranes display a red color.

Resazurin staining is used to detect viable cells (Sandberg et al. 2009). Resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide) is itself a non-fluorescent blue redox dye, but can be reduced to pink resorufin, which possesses a highly fluorescent ability (Guerin et al. 2001). This reduction occurs when cellular metabolic activity is present. Given enough time, the resorufin is further reduced to colourless hyrdoresofurin without a fluorescent ability (Sandberg et al. 2009). Hence, resazurin use requires that dye concentration and incubation time are optimized for each bacterial strain. Optimal exposure times of the dye vary between strains, with excessively short exposure yielding statistically poor results ($Z' < 0.4$). In contrast, overly long incubation times decrease the quality of assay due to resorufin reduction, leading to a weak fluorescence signal (decreased Z'-factor and signal-to-background (S/B) ratio). This phenomenon is intrinsic for rapidly metabolizing bacteria, e.g. *S. aureus*. Another disadvantage of resazurin staining is the high bacterial concentration required ($> 10^7$ CFU• mL^{-1}) for the assay to generate a fluorescent signal.

Due to this high detection limit, another quantification method should be used instead at low bacterial concentrations.

Crystal violet staining is widely used to quantify biomass (Sandberg et al. 2009). This probe functions by absorbance. Its limitations include the fact that it measures only the biomass, and does not distinguish dead cells from live ones. Hence, it offers less information than resazurin. It is also more laborious compared to the resazurin staining. However, (non-invasive) resazurin and crystal violet staining can be performed in sequential workflow (Skogman et al. 2012). With this combination, basic information of the biocide mechanism of action (bactericidal or detergent-like) can be gained. For example, bactericidal effects of compounds can be detected as a decrease of resazurin reduction and crystal violet stained mass. Detergent-like actions can be predicted by a registered decrease of crystal violet stained biomass that is associated to an absence of a corresponding decrease in resazurin reduction. Furthermore, when staining is performed sequentially in the same plate, the consumption of tested compounds, reagents, consumables and time can be reduced. Both methods can be seen as a first level filter to detect promising active hits from large chemical libraries. They are also relatively cheap and fast to carry out.

After the first-level screening, more specific information of bactericidic behavior is often desired. This involves a specific investigation into the biofilm matrix structure, which is outside the scope of resazurin and crystal violet methods. The biofilm matrix of *Staphylococcus aureus* can be for instance stained using a wheat germ agglutinin-Alexa Fluor 488 fluorescent conjugate (Skogman et al. 2012). An antibiotic candidate can lead to a reduction of viability and biomass, but this does not conclusively signify anti-biofilm effectiveness. For example, Skogman et al. (2012) reported a case, where penicillin G caused a reduction of *S. aureus* viability and biomass. However, it simultaneously led to an increased level of the matrix, indicating a likely compensatory mechanism to protect the remaining viable cells from further destruction. Matrix level measurements are pivotal since infections can easily reappear, if the matrix has not been entirely eradicated.

3 PERIODONTITIS

3.1 Physiology of teeth

The visible part of the tooth is called a crown (Figure 3) (Honkala 2009a; Honkala 2009c). The crown is covered by enamel. At the cervix of the tooth, the enamel becomes a thin film called cementum, which covers the root. The cementum is coated by parallel collagenous periodontal ligaments, which are attached to alveolar bone of the jaw.

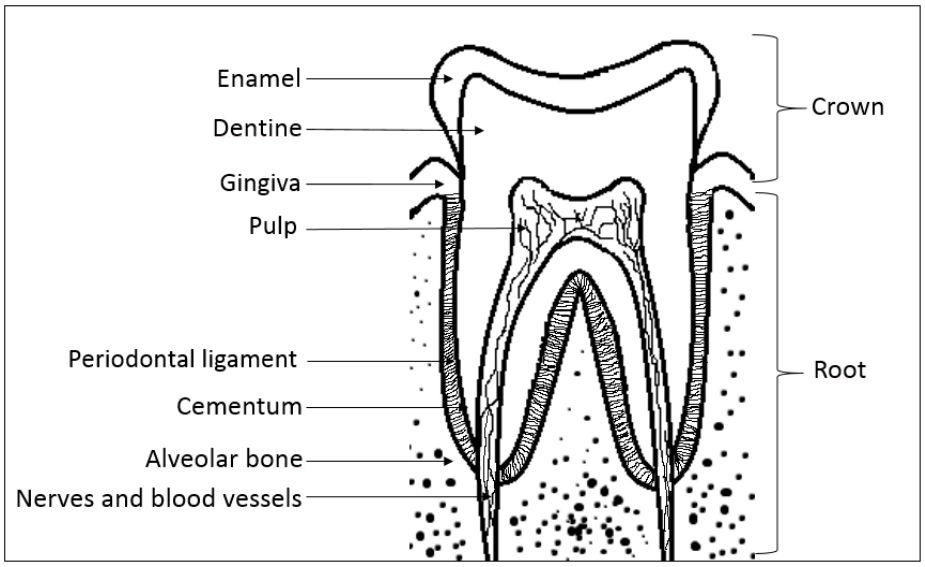


Figure 3. A dissection schematic of tooth structure.

The dentine, the main component of the tooth, is located beneath the enamel (Honkala 2009c). The structure of the dentine is softer than the enamel. The hardness of the dentine is roughly equivalent to other bones in the body, whereas the enamel, mainly composed of minerals, is the hardest tissue in human body. Under the dentine is the pulp, which includes root canals and the medulla. Blood vessels and nerves enter the pulp via holes located on the root tips.

The attachment tissues of teeth are called periodontium (Honkala 2009b). The periodontium consists of gingiva, periodontal ligaments, cementum and alveolar bone. The cervix of the tooth is surrounded by the gingiva. The gingiva and the tooth surface form a V-shaped gingival pocket. The signs of a healthy periodontium include that the gingiva is firm, has a pink color and is tightly attached to the surface of teeth.

3.2 Oral microbiology and biofilm formation

Oral biofilm formation includes several steps. In the first step, a pellicle consisting of glycoproteins and proteins is formed onto the tooth surface (Figure 4) (Darveau et al. 1997). These glycoproteins and proteins originate from saliva and crevicular fluid. The pellicle formation starts to occur within a couple of hours from brushing teeth. Pellicles enable adhesion of pioneer colonizers, such as *Streptococcus gordonii*, *Streptococcus sanguinis*, *Streptococcus mitis* (Peterson et al. 2011), *Streptococcus oralis* and *Actinomyces naeslundii* (Sánchez et al. 2011). Pioneer colonizers are then followed by early colonizers, such as *Streptococcus mutans* and *Veillonella* spp. (Peterson et al. 2011). These are accompanied by attachment of intermediate, bridging microorganisms (*Fusobacterium nucleatum*) and late colonizers (*Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*) (Sánchez et al. 2011), which are primarily anaerobic Gram-negative bacteria and responsible for periodontitis (Peterson et al. 2011).

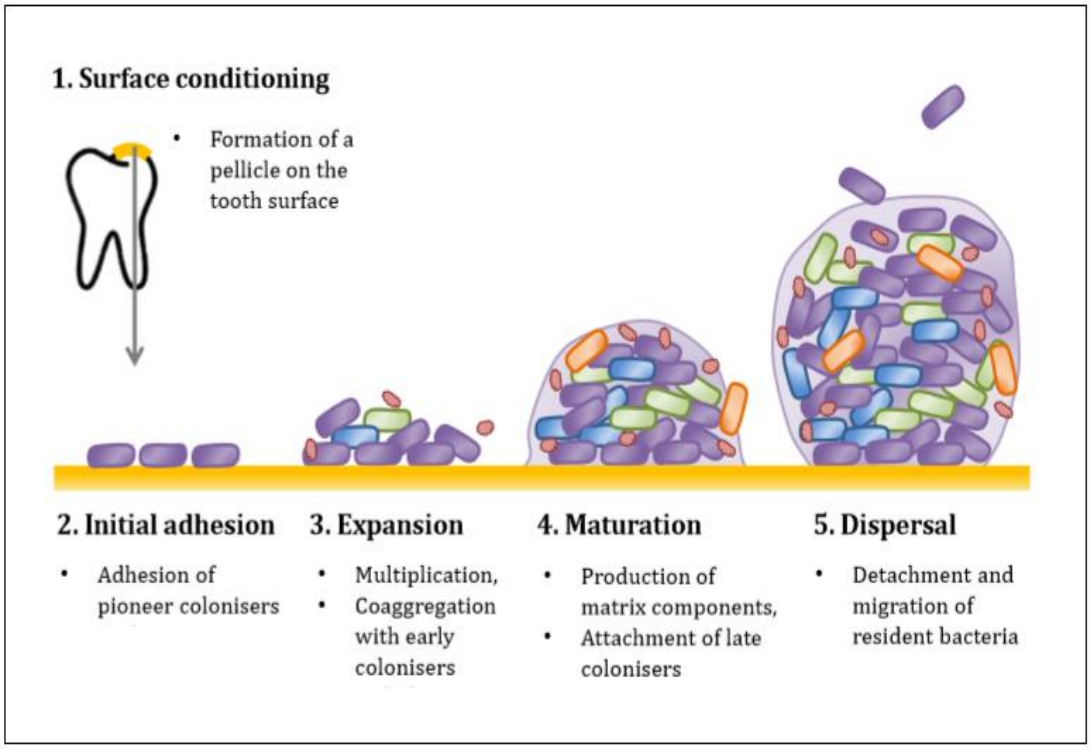


Figure 4. The formation steps of oral biofilms (modified from Clais 2014).

3.3 Pathology of periodontal disease

Periodontitis is a significant disease, both worldwide (Jin et al. 2011) and in Finland, with a remarkable economic load (Current Care Guidelines 2010). It is estimated that 64 % of the over 30 year-old toothed Finnish population have periodontitis (Knuuttila 2004). It is a chronic infection-induced inflammatory disease in the oral cavity, which destroys the attachment tissues of the teeth (Giannobile 2008).

Periodontitis is caused by an inflammation of the teeth supporting tissues (Giannobile 2008). The inflammation is initiated by Gram-negative microbial biofilm infection. When the inflammation extends from the gingiva into the bone and periodontal ligaments, it causes irreversible loss of their attachment (Figure 5) (Buencamino et al. 2009). Usually, periodontitis shows up in the molars of the maxilla (upper jaw) or in the incisors of the mandible (lower jaw) (Hiiri 2009a).

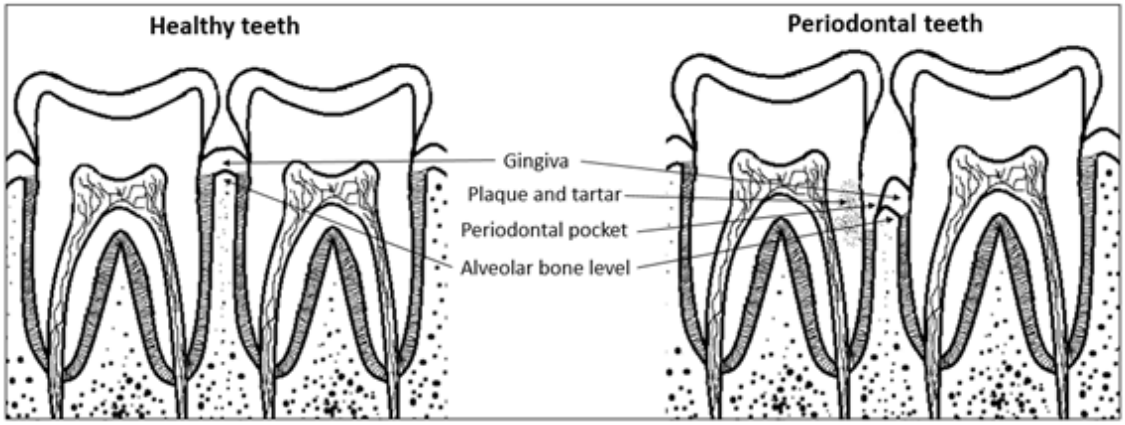


Figure 5. Characteristics of healthy and periodontal teeth.

There are a variety of risk factors associated to periodontitis. These are: an insufficient oral hygiene (Axelsson and Lindhe 1981), smoking (Bergström 2006), *diabetes mellitus* (Taylor 2001), immunodeficiency (Holmstrup and Glick 2002), ageing (Schätzle et al. 2003), the male gender (Kocher et al. 2005), low social-economic position (Klinge and Norlund 2005), genetic predisposition (Kinane and Hart 2003), menopause (Buencamino et al. 2009) and presence of periodontal pathogens (van Winkelhoff et al. 2002; Demmer et al. 2008).

Accumulated plaque i.e. bacterial mass on the surface of teeth, gum-line and between teeth eventually hardens to dental calculus (Hiiri 2009b). Plaque is mineralized by the

calcium- and phosphorus-ions of saliva, forming the calculus (Meurman et al. 2011). Furthermore, the porous calculus increases subsequent bacterial attachment (Könönen 2012a). Calculus and plaque buildup may cause gingivitis, a reversible inflammation of gingiva (Figure 6) (Hiiri 2009b). Gingivitis affects 74% of the Finnish adult population. This, if untreated, may result in non-reversible periodontitis (Giannobile 2008; Buencamino et al. 2009).

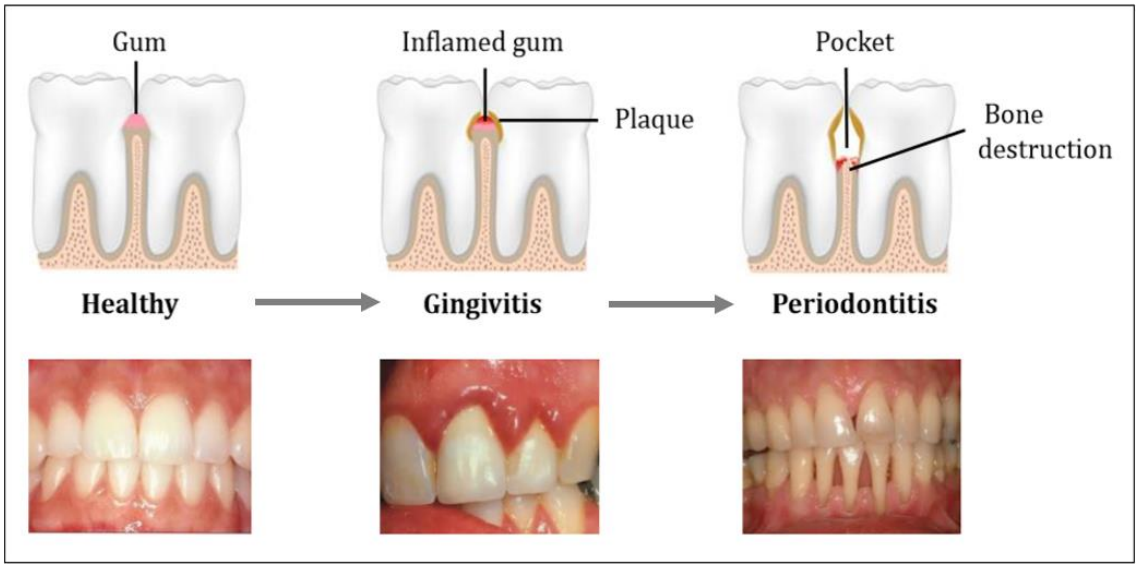


Figure 6. Progression from healthy teeth into gingivitis and periodontitis (modified from Clais 2014).

Inflammation loosens the tissue connection between tooth and gingiva, deepening the periodontal pocket (Meurman 2003; Meurman et al. 2011). The deeper periodontal pockets enable bacterial plaque to intrude deeper, making cleaning more difficult. There is no clear threshold between gingivitis and periodontitis. While the two involve similar microbes, the extent of anaerobic bacteria is larger in periodontitis due to the deeper pockets. The periodontal attachment tissue loss is primarily due to host response rather than direct bacterial damage (Graves and Cochran 2003). The host response refers to defense mechanisms used against exogenous microorganisms. The host response is initiated by bacterial lipopolysaccharides, which are present in the outer membrane of Gram-negative bacteria as a main component (Giannobile 2008). They trigger macrophages to release proinflammatory cytokines, which stimulate the bone resorption and tissue attachment loss with proteolytic enzymes.

Periodontopathogens are usually Gram-negative and strictly or facultatively anaerobic, or microaerobic (Könönen et al. 2007). Pathogens associated in periodontitis are *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis, Tannerella forsythia* (van Winkelhoff et al. 2002; Demmer et al. 2008), *Treponema denticola* (Demmer et al. 2008), *Fusobacterium nucleatum*, *Micromonas micros*, *Prevotella intermedia* (van Winkelhoff et al. 2002) and possibly *Campylobacter rectus* and *Eikenella corrodens* (Demmer et al. 2008). *B. forsythus* and *P. gingivalis* are rarely present in people without periodontitis (van Winkelhoff et al. 2002). Some periodontal bacteria such as *A. actinomycetemcomitans* and *Prevotella nigrescens* can be transmitted to a child from infected parents (Honkala 2009d). Adulthood transmission (*P. gingivalis*) is also possible, even though the normal flora tends to repel the new bacteria. Saliva, mouth mucosa and plaque are relevant transmission routes.

The symptoms of periodontitis are increased periodontal pocket depth, redness, swelling and bleeding of gingiva especially during brushing teeth (Current Care Guidelines 2010). Visual observations include gums that are pulled away from teeth and teeth that do not fit together (Buencamino et al. 2009). Moreover, increased mobility of teeth, migrated teeth, formed gaps, loss of a tooth and discharge are symptoms of advanced periodontitis (Current Care Guidelines 2010). Persistent halitosis (Buencamino et al. 2009) and bad taste in the mouth can be consequences of periodontitis (Könönen 2012b). The disease can also be symptomless, particularly among smokers (Current Care Guidelines 2010) because of constricted blood vessels (Hiiri 2009b). Periodontitis does not usually cause pain, which can later emerge in a stage of abscess (Meurman et al. 2011).

Periodontitis can be investigated by measuring periodontal pocket depth and bleeding on probing (Current Care Guidelines 2010). Increased pocket depth is defined as a minimum of 4 millimeters measured from the gingival margin to the gingival pocket base. In addition, X-ray imaging can be used to examine the loss of alveolar bone ($\geq$ 2 mm).

3.4 Treatment of periodontal disease

In addition to the possible tooth loss, periodontitis has a systemic impact on the body. It can cause systemic inflammation (Paraskevas et al. 2008) and increases the risk for cardiovascular diseases (Pussinen et al. 2007) such as atherosclerosis and coronary heart disease (Mustapha et al. 2007; Friedewald et al. 2009). Management of periodontitis improves the glycemic control of type II diabetic patients (Teeuw et al. 2010). Periodontitis can also show as a first clinical sign of HIV-infection (Holmstrup and Westergaard 1994). Thus, control strategies for periodontal disease are essential for health maintenance.

First, a good oral hygiene is pivotal in the prevention of gingivitis and periodontitis (Current Care Guidelines 2010). A powered toothbrush is more effective to reduce plaque than a manual toothbrush (Robinson et al. 2005; Yaacob et al. 2014). Brushing combined with floss (Terézhalmy et al. 2008) or interdental brushes (Slot et al. 2008) removes plaque significantly better than brushing alone. Interdental brushes remove plaque significantly better when compared to floss (Slot et al. 2008).

Early diagnosis of periodontitis is crucial so that tooth loss can be prevented (Buencamino et al. 2009). After diagnosis, mechanical debridement (scaling and root planing) is performed (Current Care Guidelines 2010). Scaling and root planing cleans dental calculus and bacterial plaque from infected periodontal pockets, and is performed manually or with the aid of an ultrasonic cleaning device. Advanced periodontitis may require gingival surgery, which removes infected tissue and offers access to deep periodontal pockets. Smoking worsens the treatment prognosis, so patients are encouraged to quit smoking. Patient compliance to better domestic oral hygiene is important. Topical chlorhexidine and strain-specific systemic antibiotic therapy are needed in many cases. The aims of the treatment are reduction of inflammation and pocket depths, prevention of further alveolar bone loss and regeneration of alveolar bone and increased attachment. These periodontal parameters have been affected positively with topical or systemic bisphosphonates (Rocha et al. 2001; Takaishi et al. 2001; El-Shinnawi and El-Tantawy 2003; Takaishi et al. 2003; Rocha et al. 2004; Palomo et al. 2005; Reddy et al. 2005; Jeffcoat et al. 2007; Veena and Prasad 2010; Pradeep et al. 2012; Sharma and Pradeep 2012a and 2012b).

4 BISPHOSPHONATES

Bisphosphonates (BPs) are used to treat various bone diseases with excessive bone resorption such as osteoporosis, Paget's bone disease, osteolytic metastases and hypercalcemia of malignancy (Terveysportti: Lääkkeet ja hinnat 2015). Bisphosphonates can be administered orally *(per os;* p.o.) or intravenously (*intravenalis;* iv.). The bisphosphonates of this thesis along with approved doses and indications are presented in Table 2.

Table 2. Indications and approved doses of bisphosphonates used in the thesis (Hillilä 2007; Terveysportti: Lääkkeet ja hinnat 2015).

Bisphosphonate	**Indication**	**Dosing regimen**
Alendronate	Osteoporosis in men and in post-menopausal women, and in osteoporosis associated with the long-term glucocorticoid treatment	10 mg p.o. daily or 70 mg p.o. once a week
Etidronate	Not approved in Finland	400 milligrams p.o. daily for 14 days every three months
Clodronate	Osteolytic bone metastases and hypercalcemia associated with cancer	1600–3200 mg p.o.
Risedronate	Osteoporosis in men and in post-menopausal women	35 mg p.o. once a week
Zoledronate	[1)] Paget's bone disease, osteoporosis in men and in post-menopausal women, and in osteoporosis associated with the long-term glucocorticoid treatment [2)] in cancer to prevent bone incidence and to treat hypercalcemia	[1)] 5 mg infusion iv. once a year [2)] 4 mg infusion iv. every 3.–4. week

The optimal treatment durations are not defined, and are evaluated according to gained risks and benefits (Terveysportti: Lääkkeet ja hinnat 2015).

4.1 Structure and classification

Bisphosphonates are analogues of inorganic pyrophosphates (Rodan 1998). Pyrophosphates are endogenous in blood serum, regulating mineralization of bones and chelating calcium (Russell 2007). Bisphosphonates have a P–C–P-backbone instead of the P–O–P found in pyrophosphates (Figure 7) (Rodan 1998; Mitsuta et al. 2002). Hence,

they have two phosphate (PO_3) groups covalently linked to the same carbon (Otomo-Corgel 2007). P–C–P-bonds render bisphosphonates resistant to metabolic enzymatic hydrolysis (Castrén-Kortekangas et al. 1997). The basic structure is similar for all bisphosphonates, but two side chains (R_1 and R_2), attached to the central carbon, vary between them (Table 3). The R_1 chain influences bisphosphonate affinity to hydroxyapatite of the bone. A hydroxyl (–OH) R_1 group (present in most bisphosphonates), rather than a halogen atom (present in clodronate), increases this affinity (Russell 2007).

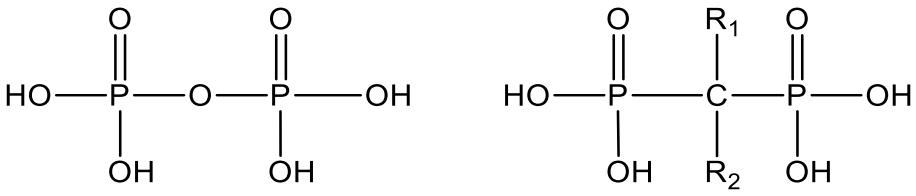
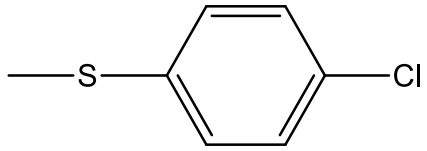
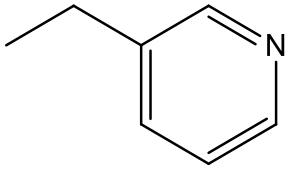
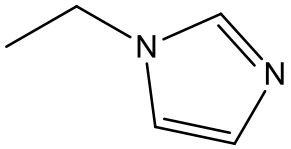


Figure 7. Structure of pyrophosphate (left) and the general structure of bisphosphonates (right) (according to Favus 2010).

Bisphosphonates are divided into three groups according to their structure (Ganapathy et al. 2012). The first group includes tiludronate, clodronate and etidronate, whose side chains do not include nitrogen (Table 3). Tiludronate differs from clodronate and etidronate due to its cyclic side chain. The second group includes alendronate, ibandronate, olpadronate and pamidronate due to the nitrogen-involvement in their side chains. Furthermore, side chains are not cyclic in the second group. The third group includes risedronate and zoledronate. They have cyclic nitrogen-containing side chains.

Table 3. Bisphosphonate classification and their side chains (modified from Ganapathy et al. 2012). The compounds with * are in clinical use in Finland (Terveysportti: Lääkkeet ja hinnat 2015).

Group	Compound	R_1 side chain	R_2 side chain
1. Non-nitrogen-containing BPs	Clodronate*	—Cl	—Cl
	Etidronate	—OH	—CH_3
	Tiludronate	—H	—S—  —Cl
2. Alkyl-amino BPs	Alendronate*	—OH	—$(CH_2)_3$—NH_2
	Ibandronate*	—OH	—CH_2—CH_2N(CH_3)—$(CH_2)_4$—CH_3
	Olpadronate	—OH	—$(CH_2)_2N(CH_3)_2$
	Pamidronate*	—OH	—CH_2—CH_2—NH_2
3. Hetero-cyclic-nitrogen BPs	Risedronate*	—OH	
	Zoledronate*	—OH	

Another, simpler way is to divide bisphosphonates into two groups according to nitrogen-containing side chain R2 (alendronate, ibandronate, olpadronate, pamidronate, risedronate and zoledronate) and non-nitrogen-containing side chain R2 (clodronate, etidronate and tiludronate) bisphosphonates (Table 4) (van Beek et al. 2003). This division also shows in the pharmacological effects of the compounds.

4.2 Pharmacological effects

Bisphosphonates have a very selective pharmacological effect targeting bone tissue (Fleisch 2003). This macro-level effect is due to their inherent high affinity to solid phase calcium phosphate crystals (hydroxyapatite) of bone. On the bone they attach to hydroxyapatite, preventing them from forming, aggregating and slowing their dissolution.

At the micro-level, bisphosphonates prevent the function of osteoclasts (van Beek et al. 2003). The mechanism of action varies according to whether the bisphosphonate contains nitrogen or not. The non-nitrogen-containing bisphosphonates are incorporated metabolically into the phosphate chain of adenosine triphosphate (ATP), inhibiting protein synthesis and resulting in osteoclast apoptosis. The nitrogen-containing bisphosphonates inhibit enzyme farnesyl pyrophosphate synthase (FPPS) in the mevalonate pathway (also known as HMG-CoA reductase pathway), causing inhibition of the prenylation of small GTP-binding proteins in osteoclasts (Luckman et al. 1998). This leads to cytoskeleton disruption and apoptosis. Bisphosphonates are able to interfere with these processes since osteoclasts internalize bisphosphonates when they are adsorbed to the bone surface (Russell 2007). Nitrogen-containing bisphosphonates (also called aminobisphosphonates) are more potent than non-nitrogen-containing compounds (Table 4) (van Beek et al. 2003).

Table 4. The pharmacological potency factors of different bisphosphonates compared to etidronate (Roldán et al. 1998; Watts 1998).

Bisphosphonate	Potency factor	Mechanism of action
Non-nitrogen containing BPs		Incorporation into ATP results in osteoclast apoptosis
Etidronate	1	
Clodronate	10	
Tiludronate	10	
Nitrogen containing BPs		Inhibition of the enzyme FPPS results in osteoclast cytoskeleton disruption and apoptosis
Pamidronate	100	
Alendronate	100–1,000	
Olpadronate	500–1,000	
Ibandronate	1,000–10,000	
Risedronate	1,000–10,000	
Zoledronate	10,000 +	

The destruction of osteoclasts slows the resorption of bone into the bloodstream, meaning that calcium and phosphate do not secrete from the bone (Hillilä 2007). Overall, the bisphosphonate mechanism of action is inhibition of bone resorption, increase of bone mineral density and decrease of bone turnover (Fleisch 2003).

Typical side effects of oral bisphosphonates are gastrointestinal when taken orally (Fleisch 2003). Aminobisphosphonates cause these side effects more frequently (Hillilä 2007). Compounds administered in high doses, such as etidronate, can inhibit normal bone mineralization (Fleisch 2003). Furthermore, severe side effects such as erosion of the esophagus (Hillilä 2007) and osteonecrosis of the jaw (Buencamino et al. 2009) have occurred. The negative effects on the esophagus can be prevented by taking tablets in an upright position and avoiding a prone position for at least half an hour (Terveysportti: Lääkkeet ja hinnat 2015). The tablet should be swallowed as a whole to prevent damage to oral mucosa with a sufficient amount of water. In most cases of the osteonecrosis of the jaw, bisphosphonates had been intravenously administered as high doses in the treatment of cancer (Buencamino et al. 2009). Afflicted patients also possessed other predisposing risk factors.

4.3 Pharmacokinetics

Bisphosphonates can be administered orally and intravenously (Terveysportti: Lääkkeet ja hinnat 2015). When taken orally, it is intrinsic for bisphosphonates to have low bioavailability (0-10%) due to poor absorption from the gastrointestinal tract (Fleisch 2003). The absorption may have inter- and intra-subject variability (Hillilä 2007). Oral bisphosphonates should be taken without a meal (Terveysportti: Lääkkeet ja hinnat 2015), as presence of food decreases the absorption of bisphosphonates significantly (Fleisch 2003). The tablets are recommended to be taken at least 30 minutes before the first meal of the day, other medications or drinks (excluding water) (Terveysportti: Lääkkeet ja hinnat 2015). Coffee, orange juice and some medicines are known to prevent absorption (Hillilä 2007). Bisphosphonates can react with divalent cations (Ca^{2+}, Mg^{2+}, Fe^{2+}, Al^{3+}) (Terveysportti: Sfinx-Pharao 2015) forming near inabsorbable complexes (Hillilä 2007).

Right after the absorption from the gastrointestinal tract, 20–80% of absorbed bisphosphonate rapidly and selectively distributes to the bone mineral (Fleisch 2003). The remaining compound is rapidly eliminated via urine excretion (Fleisch 2003) and does not go through metabolic alterations (Hillilä 2007). The elimination of the remaining bisphosphonate depends of the metabolic activity of the bone tissue, since the compound is released when bone is resorbed (Fleisch 2003). The bone-incorporated compound may therefore experience a long terminal half-life. The following terminal half-lives have been determined: 480 hours for risedronate, 146 hours for zoledronate and an estimate of over 10 years for alendronate (Terveysportti: Lääkkeet ja hinnat 2015). For etidronate, 165 days has been determined in animal studies (Genpharm Inc. 2006), while for clodronate an excact terminal half-life has not been established, other than the fact that it is very long (Castrén-Kortekangas et al. 1997).

4.4 Bisphosphonates in periodontitis

Bisphosphonates have been tested in humans and in animals against naturally occurring periodontitis. They have also been widely tested against experimentally induced periodontitis in animals (Goya et al. 2006). The experimental periodontitis is caused e.g. by a ligature around a tooth. It has been proved in previous studies that systemic

administration of bisphosphonates is effective in periodontitis, e.g. alendronate in beagle dogs (Reddy et al. 1995), alendronate in monkeys (Brunsvold et al. 1992; Weinreb et al. 1994), alendronate in humans (Rocha et al. 2001; El-Shinnawi and El-Tantawy 2003; Rocha et al. 2004; Jeffcoat et al. 2007), alendronate in rats (Menezes et al. 2005; Moreira et al. 2014), clodronate in rats (Alencar et al. 2002), etidronate in humans (Takaishi et al. 2001; Takaishi et al. 2003), risedronate in rats (Shoji et al. 1995) and risedronate in humans (Palomo et al. 2005). Because of the local nature of periodontitis, it would be justifiable to prefer local treatment, minimizing systemic effects (Mitsuta et al. 2002).

Topically administered bisphosphonates have been tested in the treatment of periodontitis in humans and rats. Improved periodontal parameters (increased alveolar bone and/or attachment tissue levels) were demonstrated upon application of alendronate in the treatment of patients with chronic periodontitis (Veena and Prasad 2010; Sharma and Pradeep 2012a), aggressive periodontitis (Sharma and Pradeep 2012b), chronic periodontitis together with type 2 *diabetes mellitus* (Pradeep et al. 2012), bone resorptive lesions (Reddy et al. 2005) and experimental periodontitis in rats (Binderman et al. 2000; Yaffe et al. 2000). Improved bone parameters were also registered with clodronate in rats (Mitsuta et al. 2002; Liu et al. 2004), risedronate in rats (Adachi et al. 1994; Igarashi et al. 1996), olpadronate in rats (Goya et al. 2006) and tiludronate in rats (Furlaneto et al. 2014).

5 BIOACTIVE GLASS

5.1 Definition

Bioactive glasses are synthetic silica-based bioactive materials with different compositions used as bone graft substitutes (Välimäki and Aro 2006). The first bioactive glass was 45S5 Bioglass® developed by Larry Hench in the 1960s (Hench 2006). Bioactive glass S53P4 (BAG), used in this thesis, is a III class medical device of BonAlive Biomaterials Ltd. (BonAlive® Biomaterials Ltd. 2015). Its composition by weight is: SiO_2 53%, Na_2O 23%, CaO 20%, P_2O_5 4%. BAG is indicated in the treatment of bone defects (Figure 8) caused by bone tumors, trauma and chronic infections, e.g. osteomyelitis and chronic sinusitis. It has osteoconductive (BonAlive® Biomaterials Ltd. 2014), osteoproductive (Virolainen et al. 1997) and antibacterial properties (Stoor et al. 1998; Leppäranta et al. 2008; Munukka et al. 2008; Drago et al. 2013; Coraça-Huber et al. 2014; Drago et al. 2014; Gergely et al. 2014; Romanò et al. 2014). Osteoproduction refers to the ability to promote migration, replication and differentiation of osteogenic cells and bone matrix production (Virolainen et al. 1997). In contrast, osteoconductivity facilitates new bone growth on and between the BAG granules (BonAlive® Biomaterials Ltd. 2015).

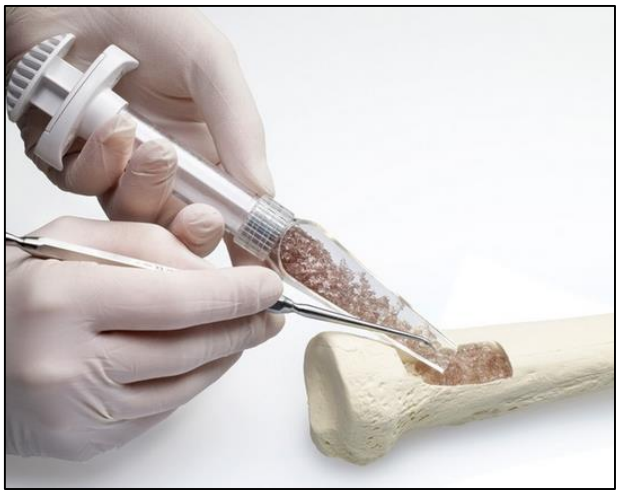


Figure 8. BAG granules can be used to fill bone cavities. (BonAlive® Biomaterials Ltd. 2015).

BAG is activated in aqueous solutions (BonAlive® Biomaterials Ltd. 2014). Clinical applications utilize sterile physiological saline. Ions (Ca^{2+}, Na^{+}, $PO^{4(3-)}$ and Si^{4+}) are released into the surrounding fluid (Stoor et al. 1998). A silica-gel layer is formed on the glass surface (BonAlive® Biomaterials Ltd. 2014). The silica gel is formed by polycondensation of hydrated silica groups. This works as a platform for calciumphosphate precipitation. The calciumphosphate crystallizes to a hydroxyapatite surface, which bonds to bone. This hydroxyapatite resembles the mineral phase of natural bone, resorbs slowly and is replaced by natural bone over several years. The slow dissolution of BAG allows the natural bone to regenerate properly.

5.2 Antibacterial properties of bioactive glasses

BAG is known to have antibacterial (Stoor et al. 1998; Leppäranta et al. 2008; Munukka et al. 2008; Drago et al. 2013; Coraça-Huber et al. 2014; Gergely et al. 2014; Romanò et al. 2014) and anti-biofilm effects (Coraça-Huber et al. 2014; Drago et al. 2014). Because of this ability, its use is beneficial in the treatment of osteomyelitis (Romanò et al. 2014) and in chronic frontal sinusitis (Peltola et al. 2006). The antibacterial properties of bioactive glasses are thought to have several possible root causes.

One antibacterial mechanism theory suggests that high calcium or alkali concentrations cause a disturbance of the bacterial membrane potential (Munukka et al. 2008). However, a more frequently suggested antibacterial mechanism involves elevated pH and osmotic pressure (Gubler et al. 2008). High pH and osmotic pressure are considered unfavorable for bacterial adhesion and proliferation, reducing infection occurrence possibility (Drago et al. 2014). BAG releases ions (Ca^{2+}, Na^{+}, $PO^{4(3-)}$ and Si^{4+}) when in contact with an aqueous solution (Stoor et al. 1998). In the first phase, sodium ions are released from the bioactive glass surface, inducing pH elevation via NaOH formation (Gubler et al. 2008). With a slower onset, ions (remaining Ca^{2+}, Na^{+}, $PO^{4(3-)}$ and Si^{4+}) are released sustainably (Gubler et al. 2008) increasing osmotic pressure (Munukka et al. 2008). According to Zhang et al. (2008) and Rosenqvist et al. (2014), elevation of pH is increased when the bioactive glass particle size is decreased, due to increased surface area per volume. Sodium-containing bioactive glasses have faster bactericidic effects than sodium-free glasses (Gubler et al. 2008). The antibacterial effect of BAG has been investigated widely

against many microbial species, whereas anti-biofilm effects have received less attention. The following paragraphs only deal with the microorganisms that are relevant from the perspective of this thesis.

Stoor et al. (1996 and 1998) have investigated the antibacterial effect of BAG against planktonic *A. actinomycetemcomitans*. In their experiment (1998) they used 1667 mg/mL BAG (particle size ≤ 45 µm), which led to the bacteria losing their viability within one hour. In another study (1996), they evaluated the antibacterial effects of BAG discs on *A. actinomycetemcomitans*. The BAG discs were placed on agars right after microbe plating and no growth inhibition was observed. Interestingly, another study (Waltimo et al. 2006) found that the antibacterial efficacy of BAG against *A. actinomycetemcomitans* could be boosted by pre-culturing the bacteria with bone powder. With hydroxyapatite or decalcified bone powder, a similar boost was not noted. Furthermore, they hypothesized that the mineralized collagen of regular bone powder may act as a catalyst for BAG dissolution into a solvent, thus increasing pH and osmolarity.

BAG has been tested against planktonic- and biofilm cultures of *S. aureus* using different BAG concentrations and particle sizes. Drago et al. (2013) tested concentrations of 400 and 800 mg/mL (particle size 500–800 µm) against planktonic MRSA, and detected bacteria to lose viability within 72 hours with both concentrations. In addition, Drago et al. (2014) tested three BAG formulations against MRSA biofilms grown on titanium discs. The tested formulations were putty, powder (particle size ≤ 45 µm) and granules (particle size 500–800 µm) with concentrations of 400 mg/mL. BAG significantly reduced biofilm biomass while the tested formulations did not show marked efficacy differences. Coraça-Huber et al. (2014) compared the effectiveness of two different BAG particle sizes (≤ 45 µm and 500–800 µm) against planktonic *S. aureus* and *S. aureus* biofilms grown on titanium discs, with concentrations 250 mg/mL and 1000 mg/mL, respectively. They observed that samples of particle size ≤ 45 µm were more effective against planktonic cells and biofilms than the larger size of 500–800 µm. Furthermore, pH elevation was faster for the smaller particle size. They suggest that the smaller particle size is more active due to increased surface area per volume. Increased surface area leads to increased contact between the BAG and the aqueous environment, which indicates a greater release of the ions (Zhang et al. 2008).

In some studies, BAG has been compared to other bioactive glasses or materials from the antimicrobial perspective. Leppäranta et al. (2008) and Munukka et al. (2008) compared BAG (particle size ≤ 45 µm) to other bioactive glasses. They screened several clinically relevant planktonic anaerobic (Leppäranta et al. 2008) and aerobic (Munukka et al. 2008) bacterial strains and observed that BAG was the most effective out of the tested bioactive glasses: it effectively inhibited the growth of even the most resistant pathogens. BAG also inhibited bacterial growth with lower concentrations than any other bioactive glass.

Finally, Gergely et al. (2014) compared the antibacterial effect of BAG and an antibiotic, gentamicin. Gentamicin was embedded in polymethylmethacrylate beads. These beads were used in the experiment because they act as a current "gold standard" treatment of osteomyelitis, in combination with the appropriate impregnated antibiotic. However, they are not biodegradable, so another surgery is required for their removal. In contrast, removal is not necessary for biodegradable bioactive glass. They used BAG as granules (2.0–3.15 mm diameter) and in powdered form. Planktonic *S. aureus* was one of the tested strains, and there was no significant difference between BAG and the beads. In contrast, other tested strains were more susceptible to the gentamicin-containing beads than BAG.

5.3 Bioactive glasses in periodontitis

The applicability of bioactive glasses has been expanded to dentistry, e.g. to treat hypersensitive teeth (Forsback et al. 2004; Tirapelli et al. 2011). In other dental applications, bioactive glasses are interesting due to their ability to mineralize dentine (Forsback et al. 2004) and to function as an antimicrobial agent (Stoor et al. 1998). Bioactive glasses can be used in the treatment of periodontitis as a bone-augmenting agent, as well (Stoor et al. 1996). Improved periodontal parameters (increased attachment and alveolar bone levels) in humans have been gained with the 45S5-bioactive glass (Mengel et al. 2003; Mengel et al. 2006) and with its granulated form, PerioGlas® (Chacko et al. 2014). PerioGlas® is in clinical use for treatment of oral or dental osseous defects (NovaBone Products 2015).

5.4 Combination of bioactive glass and bisphosphonates

The last decade has seen an emergent interest towards bioactive glass combination products (Rosenqvist et al. 2013). One research topic focuses on bioactive glass bone growth ability enhancement with other compounds, such as bisphosphonates. Srisubut et al. (2007) have presented promising results in rats by combining these two components. In their study, local administration of alendronate-bioactive glass combination indicated significantly higher bone formation than bioactive glass administered alone. Local clodronate-BAG has also been tested in patients with periodontitis (Rosenqvist K, unpublished observation 2014). For each patient, two periodontal pockets were treated: one with the combination and the other with solely BAG. The amounts and grades of the combination ingredients were based on their earlier study, where they compared 100, 200 and 300 mg of clodronate combined with 1 gram of different particle sizes of bioactive glass (Rosenqvist et al. 2014). They chose 200 mg of clodronate and 1 gram BAG with particle size 500–800 µm for their further studies. This combination decreased the sensitivity symptoms of the patients slightly more than BAG alone (Rosenqvist K, unpublished observation 2014). Correspondingly, in their earlier study (Rosenqvist et al. 2014), pH increase of solely bioactive glass was more radical than the clodronate-BAG-combination. During physicochemical property characterization, it was observed that a combination product of clodronate-BAG enhanced bioactivity via longer-lasting and more extensive ion exchange, compared to BAG (Rosenqvist et al. 2013). Conversely, if excessive calcium clodronate is precipitated due to a large amount of clodronate, the hydroxyapatite formation of BAG is decreased (Rosenqvist et al. 2014). As a result, dental applications require that the amount of clodronate is sufficient for ion exchange enhancement, but not excessive so that hydroxyapatite formation is retained.

6 AIMS OF THE STUDY

The experimental part of the thesis is divided into three parts. Anti-biofilm effects of 5 bisphophonates (alendronate, clodronate, etidronate, risedronate and zoledronate) were investigated [1)] alone in solution and [2)] together with bioactive glass, i.e. as a part of the combination product. In addition, [3)] pH measurements were included to examine a possible anti-biofilm mechanism of bisphosphonates and bisphosphonate-BAG combinations.

The aim of the first part was to clarify if bisphosphonates have intrinsic effects against biofilms. This was investigated with pre- and post-exposure tests performed in 96-well plates. The second part investigated whether a bisphosphonate-bioactive glass combination has better anti-biofilm effects compared to bioactive glass alone. Here, the Static Biofilm method was utilized. The periodontopathogen *Aggregatibacter actinomycetemcomitans* ATCC 33384 was chosen for both of these anti-biofilm experiments, while *Staphylococcus aureus* Newman was tested only in the first part for assay optimization. During the first two parts, optimization of experimental methods in the study of *A. actinomycetemcomitans* ATCC 33384 was performed. According to the best knowledge of the author, bioactive glass has not been investigated against *A. actinomycetecomitans* biofilms. The purpose of the third part was to establish, whether potential anti-biofilm effects are connected with a significant alteration in acidity or basicity through a clinically relevant period of exposure.

7 MATERIALS AND METHODS

7.1. Materials and bacterial strains

7.1.1 Materials

Tryptic soy agar (TSA; Lot. BCBM4691V and BCBJ5358V), tryptic soy broth (TSB; Lot. BCBM6802V), dextrose (Lot. 031M0140V), Tween® 20 (Lot. SZBE0630V), resazurin sodium salt (molecular weight (MW) = 251.14 g/mol; Lot. MKBL3135V), crystal violet solution (2.3% weight/volume (w/v); Lot. 091M4364), rifampicin (MW = 822.94 g/mol; Lot. SLBH2676V), penicillin G sodium salt (MW = 356.37 g/mol; Lot. BCBB8234V), chlorhexidine dihydrochloride (MW = 578.37 g/mol; Lot. BCBN2790V), etidronic acid monohydrate (Table 5; Lot. BCBC7777V) and sodium chloride ACS (Lot. 109K0076) were purchased from Sigma-Aldrich (Steinheim, Germany). Alendronate sodium trihydrate (Table 5; Lot. 1029/P/02001) and zoledronic acid monohydrate (Table 5; Lot. 120822) were acquired from Kemprotec Limited (Carnforth, United Kingdom). Risedronic acid monohydrate (Table 5; Lot. LC24373) was from AK Scientific (Union City, California, USA), while clodronate disodium tetrahydrate (Table 5; Lot. T07/009 Ph. Eur.) was obtained from PharmaZell GmbH (Raubling, Germany). Bacto™ Yeast Extract (Lot. 8231161) was produced by Becton, Dickinson and Company (Le Pont de Claix, France). Bioactive glass S53P4 (BAG; granule size: 500–800 µm; Lot. BG-05/13 and BS-16/13/9) was purchased from BonAlive Biomaterials Ltd. (Turku, Finland). Aseptically collected defibrinated sheep blood was purchased from Bio Trading (Lot. 14297 SG85, 14325 SG93 and 15009 SG21). LIVE/DEAD® BacLight™ Bacterial Viability Kit L7007 (Lot. 1562297) was from Molecular Probes by Life Technologies (Eugene, Oregon, USA). Phosphate buffered saline (PBS; Lot. 4MB127) was from Lonza (Verviers, Belgium). Sodium hypochlorite (14% Cl_2 in aqueous solution; Lot. 14G100514) was from VWR Chemicals (Fontenay-sous-Bois, France). Etax Aa (minimum 99.5 weight-%; Lot. 13202) was from Altia Oyj (Rajamäki, Finland).

Table 5. The bisphosphonates used in the experiments. The purities of all five bisphosphonates were ≥ 95%.

Bisphosphonate	CAS	Molecular formula	Molecular weight
Alendronate sodium trihydrate	121268-17-5	$C_4H_{12}NNaO_7P_2 \cdot 3\ H_2O$	325.12 g/mol
Clodronate disodium tetrahydrate	22560-50-5	$CH_2Cl_2\ Na_2O_6P_2 \cdot 4\ H_2O$	360.92 g/mol
Etidronic acid monohydrate	25211-86-3	$C_2H_8O_7P_2 \cdot H_2O$	224.04 g/mol
Risedronic acid monohydrate	105462-24-6	$C_7H_{11}NO_7P_2 \cdot H_2O$	301.13 g/mol
Zoledronic acid monohydrate	165800-06-6	$C_5H_{10}N_2O_7P_2 \cdot H_2O$	290.10 g/mol

7.1.2 Bacterial strains

The strain *Aggregatibacter actinomycetemcomitans* ATCC (American Type Culture Collection) 33384 was kindly donated by Dr. Pirkko Pussinen from the Institute of Dentistry, University of Helsinki, Finland. *Staphylococcus aureus* Newman was similarly donated by Docent Pekka Varmanen from the Faculty of Veterinary Medicine, University of Helsinki, Finland.

7.2 Biofilm trials performed in 96-well plates

Anti-biofilm effects of bisphosphonates as stand-alone compounds were first investigated in susceptibility trials. The trials were performed in 96-well plates, which is one of the most widely used systems for quick assessment of anti-biofilm activity in a convenient-to-use format. Tested compounds included five bisphosphonates: alendronate, clodronate, etidronate, risedronate and zoledronate.

7.2.1 Bacterial culturing and biofilm formation by Staphylococcus aureus Newman

The strain *Staphylococcus aureus* Newman was stored on a TSA plate at a temperature of 4°C. Pre-cultures were initiated by taking a full 1 µl-loop of colonies from the TSA plate (less than 1 month old) and transferring them into a Falcon tube containing 3 milliliters of TSB. The pre-cultures were incubated under aerobic conditions at 37°C with 200 rpm shaking for 12–16 hours (Skogman et al. 2012). Liquid cultures were prepared by diluting the pre-cultures 1:1000 in TSB and incubated under aerobic conditions at 37°C with 200 rpm shaking for 3–5 hours to reach an exponential growth (usually a concentration of 10^8 CFU• mL^{-1}). The bacterial concentration was estimated with Varioskan™ Flash Multimode Reader (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) by measuring spectrophotometric turbidity at 595 nm. A spectrophotometer passes light through a cell suspension and measures unscattered light (Tortora et al. 2014c). Turbidity i.e. optical density increases when more cells are present in the cell suspension. The bacterial concentration was then confirmed via determination of viable colony forming units per milliliter (CFU• mL^{-1}) by performing 10-fold serial dilutions in TSB. Four sequential dilutions (5 drops, à 10 µl) were plated onto quarters of TSA on a Petri dish (VWR, Radnor, Pennsylvania, USA). TSA plates were incubated under aerobic conditions at 37°C for 16–18 hours. After incubation, the plate count was perfomed, where viable colony forming units (CFU) were determined. The concentration (CFU• mL^{-1}) was calculated according to Equation 1 (Tortora et al. 2014c).

$$CFU \bullet mL^{-1} = \frac{Average\ of\ number\ of\ colonies\ x\ \frac{1}{dilution}}{Volume\ plated\ (mL)} \qquad (1)$$

For forming biofilms, the exponentially grown cultures were diluted 1:100 in TSB in order to contain approximately 10^6 CFU• mL^{-1}. Biofilms were grown on sterile, flat-bottomed Nunclon™ Δ surface 96-well polystyrene plates (Nunc™, Roskilde, Denmark).

7.2.2 Bacterial culturing and biofilm formation by Aggregatibacter actinomycetemcomitans ATCC 33384

The strain of *Aggregatibacter actinomycetemcomitans* ATCC 33384 was stored at a temperature of -80°C in milk. The strain was recovered by spreading 20 µl of the bacterial

stock suspension onto a TSA plate supplemented with 5% defibrinated sheep blood (TSA_{D5}) (Paino et al. 2011). The plates were incubated in microaerophilic conditions at 37°C for 3 days. These microaerophilic conditions were accomplished by maintaining the bacteria in an airtight container with a candle inside ("candle jar"). This ensemble typically provides conditions that are known to be favorable for the growth of this microorganism (Paino et al. 2011): richer with carbon dioxide (3%) and lower with oxygen (17%) than in the atmosphere (Tortora et al. 2014c).

After the bacterial colonies had clearly formed on the plates under the conditions described above, 4 full 1 µl-loops of colonies from the plate were scraped into 1 mL of TSB-YE/Glc (TSB supplemented with 0.6% (w/v) yeast extract and 0.8% (w/v) glucose) (Paino et al. 2011) to gain an optical density of 1 (approximately 10^9 CFU• mL^{-1}). The optical density values were measured with Varioskan™ Flash Multimode Reader at 595 nm and confirmed by determining viable CFU• mL^{-1} (see 7.2.1.) on TSA_{D5}. Serial dilutions were performed with TSB-YE/Glc (Figure 9). The plates were incubated in microaerophilic conditions at 37°C for 3 days.

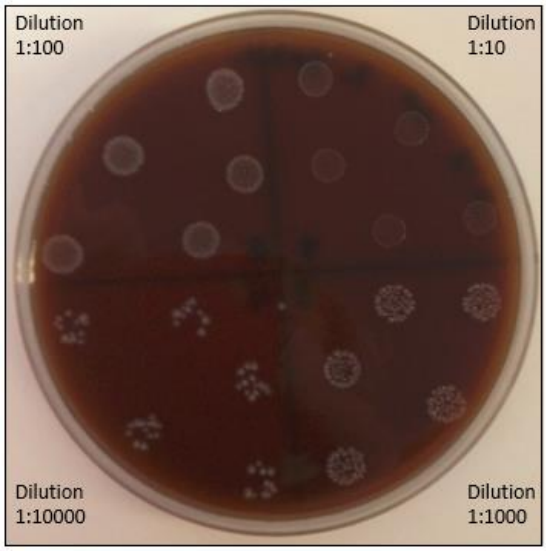


Figure 9. *A. actinomycetemcomitans* ATCC 33384 grown on TSA_{D5} plate using 4 serial dilutions for CFU• mL^{-1} determination.

For biofilm formation, the exponentially grown cultures were diluted 1:100 with TSB-YE/Glc to be approximately 10^7 CFU• mL^{-1}. Biofilms were grown on sterile, flat-bottomed polystyrene 96-well plates.

The suitable *A. actinomycetemcomitans* ATCC 33384 biofilm formation time was determined by growing biofilms 24 and 48 hours at 37°C in microaerophilic conditions. The formed biofilms were stained with crystal violet. Planktonic suspension was removed and rinsed with 200 µl of Milli-Q-water (Sandberg et al. 2008). Then, 170 µl of crystal violet stain (2.3% w/v) was added and allowed to stain for 5 minutes at room temperature (RT) and removed. The Milli-Q-water washing step was perfomed another two times. The remaining dye was solubilized in 200 µl of 96% ethanol for one hour (at RT). Absorbance was measured at 595 nm using Varioskan^{TM} Flash Multimode Reader.

7.2.3. Exposure of biofilms to the tested compounds or control antibiotics

Bisphosphonates, penicillin G and rifampicin were dissolved in water in stock solutions of 3 mM, 4 mM and 100 µM, respectively. However, taking into account the diluting effect of the bacterial suspension (180 µl), the final concentrations of the compound solution (20 µl) encountered by bacteria were 10-fold smaller. Thus, final concentrations for the tested compounds were 300 µM, 400 µM and 10 µM, respectively. The antibiotics penicillin G and rifampicin served as positive controls for *S. aureus* Newman and *A. actinomycetemcomitans* ATCC 33384, respectively.

The tested compounds were measured in two different modes: pre- and post-exposure. The workflows are presented in Figure 10 (for *S. aureus* Newman) and in Figure 11 (for *A. actinomycetemcomitans* ATCC 33384). In the pre-exposure assay, 20 µl of compounds were dispensed at the same time with 180 µl of bacterial suspensions (10^6 CFU• mL^{-1} for *S. aureus* Newman and 10^7 CFU• mL^{-1} for *A. actinomycetemcomitans* ATCC 33384). The plates were incubated under aerobic conditions at 37 °C, with 200 rpm for 18 hours (*S. aureus* Newman) (Skogman et al. 2012) or in microaerophilic conditions at 37 °C for 24 hours (*A. actinomycetemcomitans* ATCC 33384). In the post-exposure assay, 200 µl of bacterial suspension was added per well (10^6 CFU• mL^{-1} for *S. aureus* Newman and 10^7 CFU• mL^{-1} for *A. actinomycetemcomitans* ATCC 33384), and biofilms were first allowed to form for 18 hours (*S. aureus* Newman) (Skogman et al. 2012) or 24 hours (*A. actinomycetemcomitans* ATCC 33384). After this incubation period, planktonic suspension was removed (Skogman et al. 2012). 20 µl of compound and 180 µl of TSB (*S. aureus* Newman) or 180 µl TSB-YE/Glc (*A. actinomycetemcomitans* ATCC 33384)

were then pipetted onto the formed biofilms and allowed to have an effect on the biofilms for an additional 24 hours.

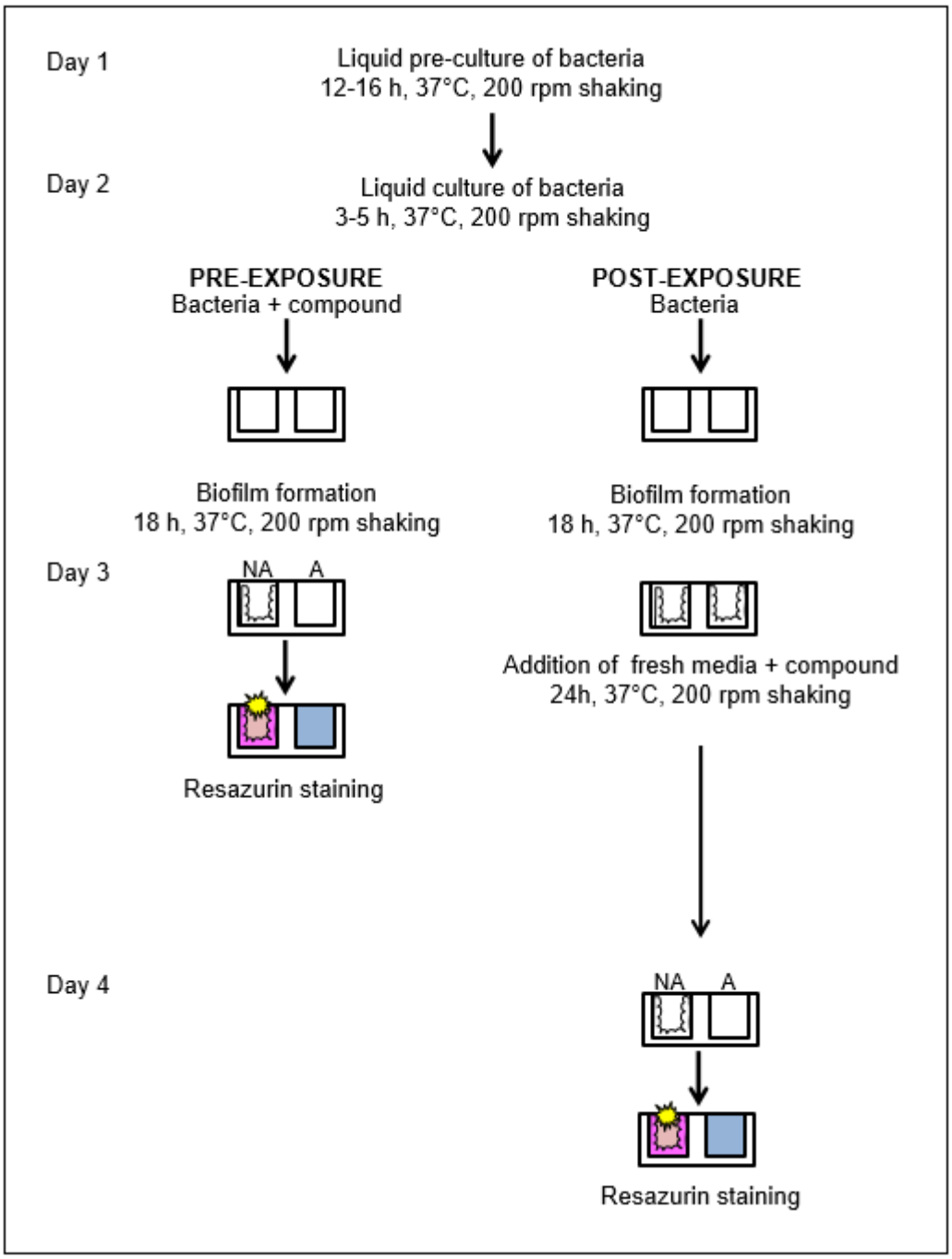


Figure 10. The workflow for testing non-active (NA) and active compound (A) against *S. aureus* Newman (modified from Skogman et al. 2012). A yellow star indicates fluorescence.

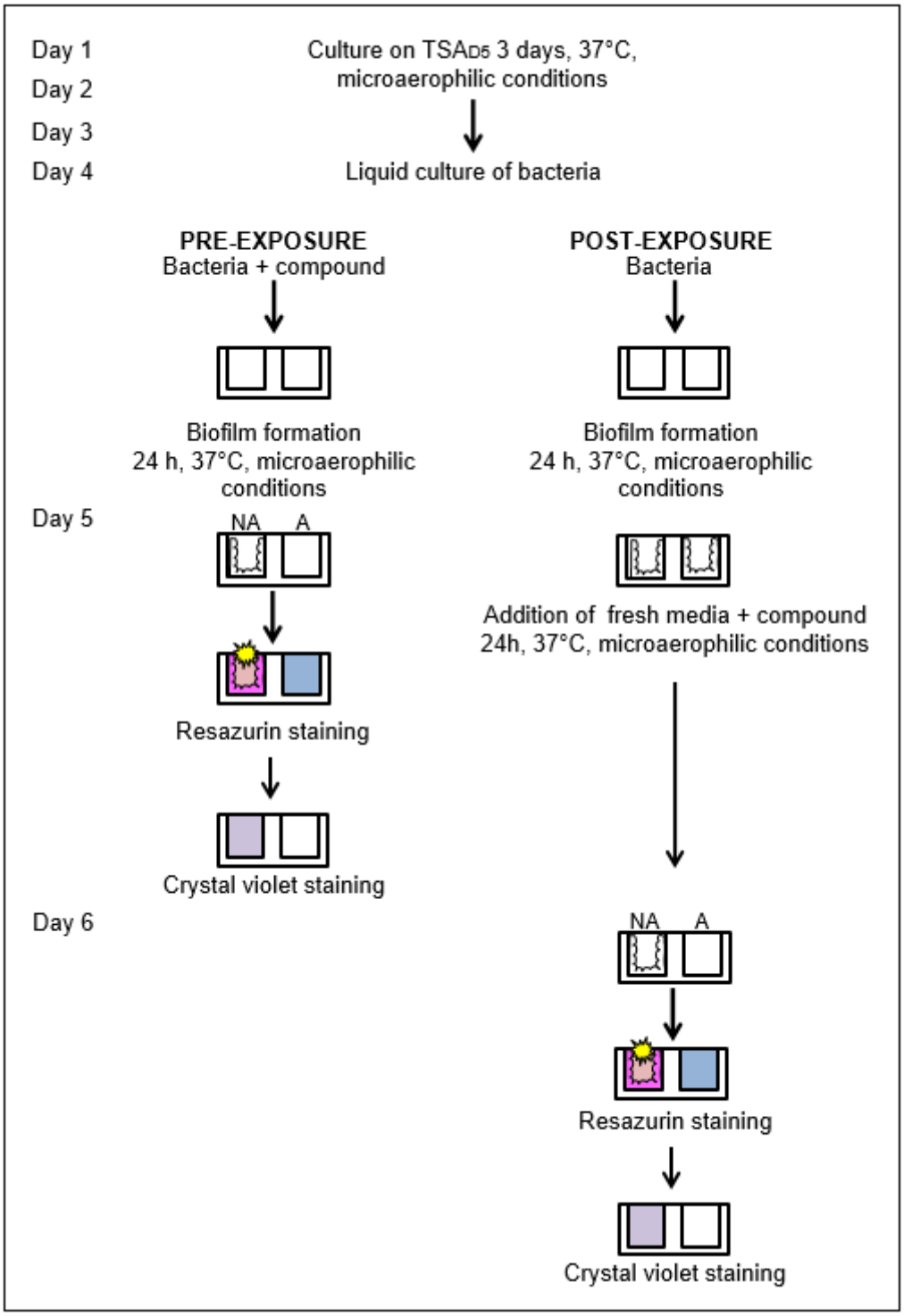


Figure 11. The workflow for testing non-active (NA) and active compound (A) against *A. actinomycetemcomitans* ATCC 33384 (modified from Skogman et al. 2012). A yellow star indicates fluorescence.

7.2.4 Quantification of biofilm viability

After exposure to the compounds or control antibiotics, viable biofilms were stained. First, planktonic suspension was removed. Then, biofilms were stained with 200 µl of 20 µM resazurin in PBS. The incubation time of *S. aureus* Newman (Figure 12) was 20 minutes (at RT, 200 rpm, in the darkness). The concentration of resazurin solution and the incubation time were based on Sandberg et al. (2009). For *A. actinomycetemcomitans* ATCC 33384, the same concentration of resazurin was used, but the incubation time was chosen to be 60 minutes in microaerophilic conditions (at RT, in the darkness), based upon optimization. In optimization trials, different incubation times and conditions were compared. The incubation was first performed in aerobic conditions (at RT, in the darkness) (45, 60, 85, 120 and 240 minutes). After that, incubation was performed in microaerophilic conditions (at RT, in the darkness) (60 minutes). Fluorescence caused by resazurin reduction was monitored by utilizing excitation and emission wavelengths of 560 and 590 nm, respectively. Measurements were made using the Varioskan™ Flash Multimode Reader.

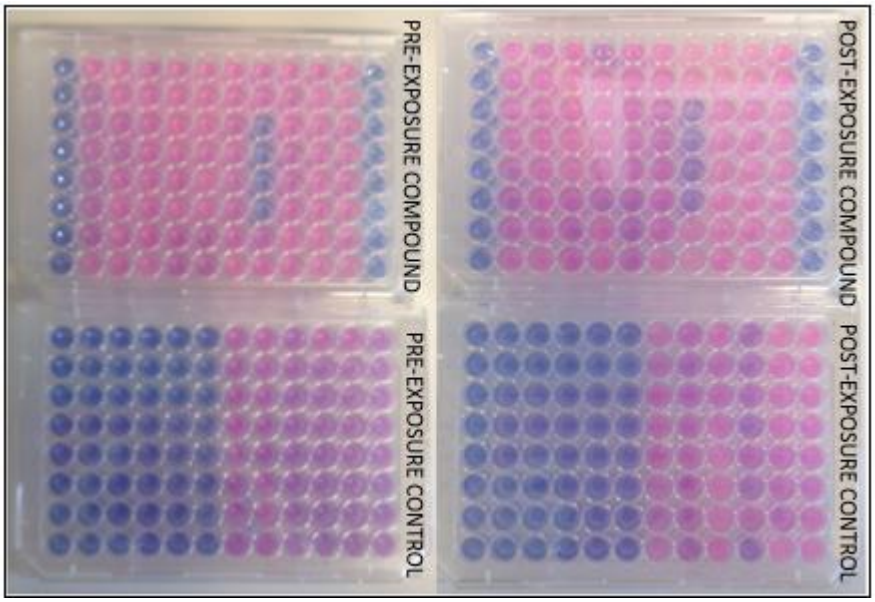


Figure 12. Resazurin stained *S. aureus* Newman on 96-well plates. The compound plates above and the control plates (no compounds added) below. The blue color indicates medium or non-bacterial metabolic activity and the pink color indicates bacterial metabolic activity.

Performance of screening assays can be estimated by means of statistical parameters. These include a signal window coefficient (Z'-factor; Equation 2) (Zhang et al. 1999), signal-to-noise ratio (S/N; Equation 3) (Sandberg et al. 2008) and signal-to-background ratio (S/B; Equation 4) (Zhang et al. 1999). The active compound can be distinguished from non-active compounds in well plate assays with the aid of a threshold value (hit limit; HL; Equation 5) (Zhang et al. 1999).

SD_{min} and SD_{max} indicate standard deviations of minimal signal and maximal signal, respectively. X_{min} and X_{max} describe the mean of the minimal signal and maximal signal, respectively. The maximal signal is the signal of positive control wells including only bacterial suspension, while the minimal signal is the signal given by negative control wells, which include only medium.

$$Z' = 1 - \frac{(3\ x\ SD_{max} + 3\ x\ SD_{min})}{|X_{max} - X_{min}|} \tag{2}$$

$$\frac{S}{N} = \frac{X_{max} - X_{min}}{({SD_{max}}^2 + {SD_{min}}^2)^{1/2}} \tag{3}$$

$$\frac{S}{B} = \frac{X_{max}}{X_{min}} \tag{4}$$

$$HL = X_{max} - 3\ \times SD_{max} \tag{5}$$

7.2.5 Quantification of biofilm biomass

After resazurin staining, *A. actinomycetemcomitans* ATCC 33384 biofilms were also stained with crystal violet, since this method had earlier been reported as typical for biomass quantification of biofilms (Sandberg et al. 2008). In addition, this was a useful strategy to confirm feasibility of the performed redox staining (resazurin). The resazurin stain was removed and rinsed with 200 µl of Milli-Q-water. Then, 170 µl of crystal violet stain (2.3% w/v) was added and allowed to stain for 5 minutes at RT and removed. The Milli-Q-water washing step was perfomed another two times. The remaining dye was

solubilized in 200 µl of 96% ethanol for one hour (at RT). Absorbance was measured at 595 nm using Varioskan^{TM} Flash Multimode Reader.

7.3 Biofilm trials performed with the Static Biofilm method

The combinations of bioactive glass and bisphosphonates were investigated in a dental biofilm model, using the Static Biofilm method as described in Oja et al. (2014). This method was chosen because of its ability to mimic oral conditions. In practice, this requires the absence of fluid shear stress and a small fluid volume. The same bisphosphonates were included as in previous experiments: alendronate, clodronate, etidronate, risedronate and zoledronate. Inert glass beads served as a negative control. In the Static Biofilm method, biofilms typically develop on the underside surfaces of coupons which are placed on a filter paper covered agar (Charaf et al. 1999; Oja et al. 2014). The protocol was modified from Oja et al. (2014) in three specific points: [1)] a second layer of filter paper was added in susceptibility trials, [2)] the filter papers were remoistened with undiluted medium in susceptibility and optimization trials, [3)] for practical reasons, compound covered coupons were not immersed into the medium prior to sonication in susceptibility trials, as detailed below. The main steps of the Static Biofilm method are presented in Figure 13.

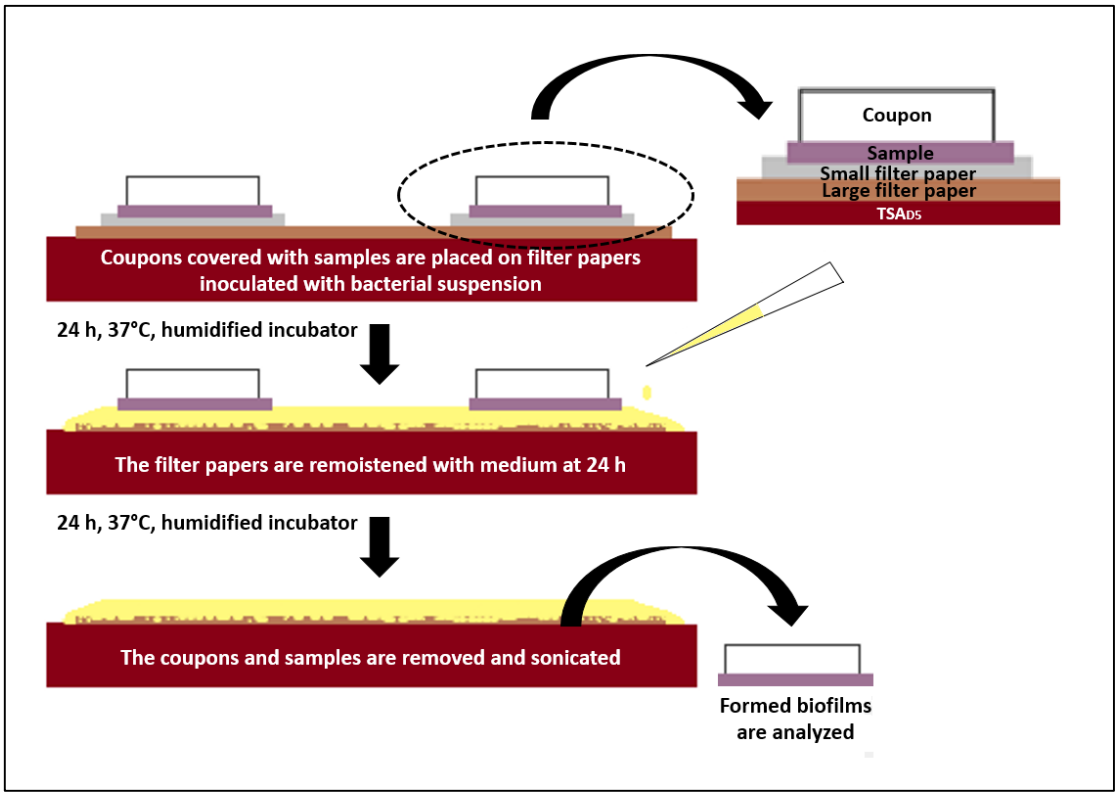


Figure 13. Workflow of the Static Biofilm method utilized in this investigation. Biofilms are drawn in yellow (modified from Oja et al. 2014).

In the publication of Oja et al. (2014) only glass coupons were used. In contrast, the present study also experimented with other materials that were more relevant for the dental application. This was based on an optimization study, which is described in subsection 7.3.2.

7.3.1 Cleaning of coupons used as surfaces

Cleaning of coupons was performed twice, right before and after their usage. The cleaning was performed according to an ASTM protocol (ASTM International Standard E2196-12), but sodium hypochlorite treatments were added to avoid contamination, as described below.

Before use, coupons were soaked for 30 minutes in 14% sodium hypochlorite. This was followed by a 10 minute sonication in 70% ethanol in a water bath. Finally, coupons were soaked in Etax Aa and dried in a laminar hood.

After use, coupons were visually inspected for damages. After that, they were sonicated for 30 seconds in a 1% SDS-water solution. SDS (sodium lauryl sulphate) is a tencide. Then, the coupons were rinsed with MQ-water until no bubbles were present and sonicated in MQ-water for 5 minutes. The coupons were rinsed a further three times with MQ-water. Next, coupons were soaked for 30 minutes in 14% sodium hypochlorite to ensure full eradication of the remaining biofilms. Before storing, coupons were briefly dipped into Etax Aa to rinse the sodium hypochlorite and let to dry in a laminar hood.

7.3.2 Coupon optimization

Before proceeding to susceptibility trials performed with the Static Biofilm method, suitable materials for biofilm formation were assessed. In this optimization study, glass, plexi glass and hydroxyapatite coupons were compared without any samples to clarify how *A. actinomycetemcomitans* ATCC 33384 bacteria attach onto different materials. The purpose was to determine whether hydroxyapatite or plexi glass coupons (more relevant for dental application) could be used instead of glass coupons, which were used in Oja et al. (2014). Hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] is the mineral component of dentine and bones (Pryor et al. 2009), and both hydroxyapatite and plexi glass are used as materials in dental implants. Thus, a quick comparative screen of *A. actinomycetemcomitans* ATCC 33384 attachment on 3 different coupon materials (hydroxyapatite, plexi glass, glass) were studied.

The pre-culture of *A. actinomycetemcomitans* ATCC 33384 was prepared as previously described in subsection 7.2.2. A sterile Whatman filter paper (70 mm diameter, Qualitative Grade 2, GE Healthcare, Little Chalfont, United Kingdom) was placed on a TSA_{D5} plate (Oja et al. 2014). The filter paper was inoculated with 1.5 mL of 10^7 CFU• mL^{-1} pre-culture dilution. The sterile coupons [borosilicate glass (0.4 cm height, 1.27 cm diameter), plexiglass (acrylic; 0.4 cm height, 1.27 cm diameter) and hydroxyapatite (0.25 cm height, 1.27 cm diameter), BioSurface Technologies Corporation, Montana, USA] without compound samples were placed on this paper. The plate was incubated at 37°C for 48 hours under humidified microaerophilic conditions. After 24 hours of incubation, the filter paper was remoistened with 1.5 mL of undiluted TSB-YE/Glc. The coupons

were immersed into medium to remove planktonic suspension, and sonication for the biofilm quantification phase (7.3.5) was performed.

7.3.3 Preparation of the tested samples

The combination samples were formed by mixing 50 mg of bisphosphonate or inert glass beads (Figure 14) and 500 mg of BAG and 450 µl of saline 0.9% (w/v) for each coupon, in the same manner as performed by Rosenqvist et al. (2014). Each prepared sample was then transferred to a filter paper in order to evaporate excessive saline. The coupons were covered with the samples using a spatula. Furthermore, suitability of inert glass beads (Jencons Ltd., Bedfordshire, UK) as a negative control material was evaluated by combining 550 mg of the beads with 450 µl of saline.

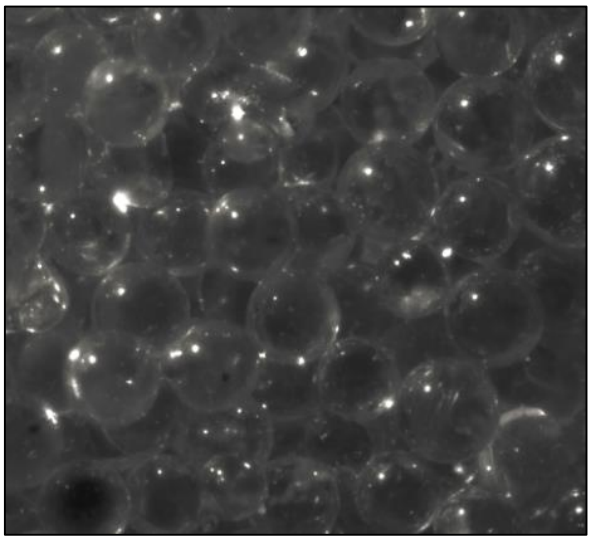


Figure 14. Flashsizer image of inert glass beads used as a negative control in the Static Biofilm method.

7.3.4 Bacterial culturing and biofilm formation by Aggregatibacter actinomycetemcomitans ATCC 33384

The pre-culture of *A. actinomycetemcomitans* ATCC 33384 was prepared as previously described in subsection 7.2.2. A sterile filter paper (70 mm diameter) was placed on TSA_{D5} plates (Oja et al. 2014). Smaller sterile filter papers per each coupon (25 mm diameter, cut from 70 mm diameter) were placed on the larger ones. The function of the

large filter paper was to prevent coupons from sticking to the agar and to act as a barrier material, enabling nutrients to diffuse from the agar to the coupon surface (Oja et al. 2014). The purpose of the smaller filter paper was to facilitate the scraping of samples into Falcon tubes in the later biofilm disaggregation phase. The double-layered filter papers were inoculated with 1.5 milliliter of 10^7 CFU• mL^{-1} pre-culture dilution (Oja et al. 2014). The sterile coupons covered with a sample were placed on the smaller filter papers, so that the samples lie between the coupons and the smaller filter papers (Figure 15). The biofilms develop on the filter papers, on the samples and on the underside of the coupons. The plates were incubated at 37°C for 48 hours under humidified microaerophilic conditions. After 24 hours of incubation, the filter paper was remoistened with 1.5 mL of undiluted TSB-YE/Glc.

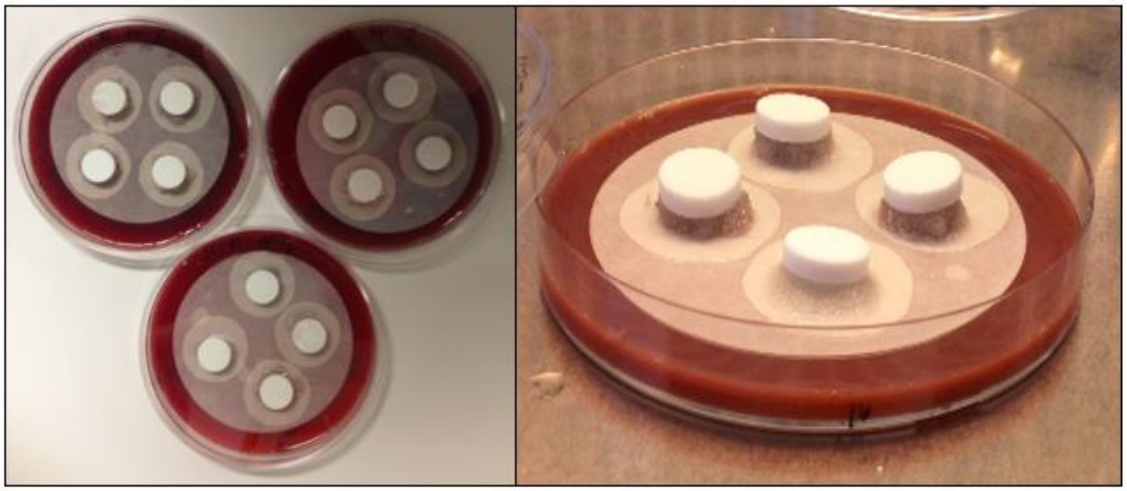


Figure 15. The Static Biofilm method using *A. actinomycetemcomitans* ATCC 33384.

7.3.5 Quantification of biofilms

After 48 hours of incubation, the coupons and samples were transferred into Falcon tubes containing 1 mL of 0.5% (w/v) Tween® 20 in TSB-YE/Glc, as in Oja et al. (2014). The tubes were sonicated in Ultrasonic Cleaner 3800 (Branson Ultrasonics, Danbury, Connecticut, USA) in a water bath for 5 minutes at 25°C, 35 kHz. A 20 second vigorous mixing of the tubes was performed with Vortex mixer SA8 (Stuart, Stone, United Kingdom) just before and after sonication. Serial dilutions of the resulting suspensions were prepared and plated onto TSA_{D5} plates to determine viable colony forming units per

milliliter (CFU• mL^{-1}) as described in the subsection 7.2.1. The plates were incubated under microaerophilic conditions at 37°C for 3 days. The bacterial attachment on coupons is expressed on a $\log_{10}$ scale, and compound anti-bacterial effects can be expressed as logarithmic Reduction of the bacterial burden (logR-values, Equation 7) (Buckingham-Meyer et al. 2007).

$$logR = log_{10}\langle(CFU/ml)_{control}\rangle - log_{10}\langle(CFU/ml)_{compound}\rangle \quad (7)$$

where $\langle\cdot\rangle$ denotes averaging over samples.

7.4 Fluorescence microscopy

7.4.1 Biofilm formation and exposure to compounds

Bacterial culturing by *A. actinomycetemcomitans* ATCC 33384 was performed as previously described in subsection 7.2.2. The cultures were then diluted 1:100 to be approximately 10^7 CFU• mL^{-1} in TSB-YE/Glc to form biofilms. Round coverslips (13 mm diameter, VWR International, Helsinki, Finland) were used as substrates for biofilm formation for the imaging. The coverslips were first cleaned by immersing them into 14% sodium hypochlorite, then into Etax Aa and finally into sterile water. After that, they were let to dry in a laminar hood. Biofilms were then grown onto the coverslips, which were placed on sterile, flat-bottomed Nunclon™ Δ surface 24-well polystyrene plates (Nunc™, Roskilde, Denmark). In treated wells, 100 µl of compound solution was dispensed simultaneously with 900 µl of bacterial suspension, while in untreated biofilms, 1000 µl of bacterial suspension was added. The biofilms were treated with 250 µM chlorhexidine and 100 µM rifampicin stock solutions, which were prepared by dissolving them into water. The final concentrations encountered by the bacteria were 25 µM and 10 µM, respectively. The plates were incubated in microaerophilic conditions at 37 °C for 24 hours.

As a parallel procedure, a 96-well plate pre-exposure test was performed to support data gained from FM. *A. actinomycetemcomitans* ATCC 33384 culturing, biofilm formation and biofilm exposure to the compounds were performed as described in the subsections

7.2.2 and 7.2.3. Biofilms were exposed to the same antibiotics and concentrations as in the former paragraph, and incubated in microaerophilic conditions at 37 °C for 24 hours. Following the exposure, viable biofilms were stained. First, the planktonic suspension was removed. Then, biofilms were stained with 200 µl of 20 µM resazurin in PBS for 60 minutes in microaerophilic conditions (at RT, in the darkness). Fluorescence caused by resazurin reduction was measured with Varioskan™ Flash Multimode Reader, using excitation and emission wavelengths of 560 and 590 nm, respectively.

7.4.2 Staining of biofilms for fluorescence microscopy

For fluorescence microscopy (FM) studies, bacteria were stained with fluorescent probes. In LIVE/DEAD® BacLight™ Bacterial Viability Kits, green-fluorescent nucleic acid probe SYTO® 9 and the red-fluorescent nucleic acid probe propidium iodide are combined (Molecular Probes 2004). The spectral characteristics and their ability to penetrate into healthy bacterial cells vary between these probes. The SYTO® 9 probe usually labels all bacteria in a population, with both intact and damaged membranes. Instead, propidium iodide can only penetrate into bacteria with damaged membranes, causing a reduction in the SYTO® 9 stain fluorescence when both dyes are present. Thus, bacteria with intact cell membranes are labeled fluorescent green, whereas bacteria with damaged membranes are labeled fluorescent red. The excitation/emission maxima for these dyes are about 480/500 nm for SYTO® 9 and 490/635 nm for propidium iodide, when the background remains virtually nonfluorescent.

After 24 hours of incubation (24-well plate), the planktonic suspension was removed. Biofilms were washed with saline (0.9% w/v) and stained with LIVE/DEAD® BacLight™ Bacterial Viability Kit L7007. The probe mixture was prepared by dissolving 1.5 µL of Component A (1.67 mM SYTO® 9 nucleic acid stain and 1.67 mM propidium iodide in DMSO) and 1.5 µL of Component B (1.67 mM SYTO® 9 nucleic acid stain and 18.3 mM propidium iodide in DMSO) in 1 mL of sterile water. From this solution, 34.5 µl was dispensed per well (final concentrations of SYTO® 9 and propidium iodide were 5 and 30 µM, respectively). Biofilms were incubated with the probe solution at RT, in the darkness for 15 minutes. Next, the coverslips were carefully removed with tweezers and mounted with BacLight™ mounting oil on a Superfrost® Plus microscope glass slide

(25 x 75 x 1.0 mm, Thermo Scientific, Braunschweig, Germany) upside down. Images were acquired with a fluorescence microscope EVOS® FL Imaging System AMF4300 (Life Technologies™, Eugene, Oregon, USA) using two light cubes (RFP and GFP to detect the propidium iodide and SYTO® 9 emission, respectively), and a 40X (Inf Plan Fluor 40X, 0.75NA/0.72WD, coverslip corrected) objective. Images with the independent channels were captured and the one containing the overlay of both channels was automatically created by the microscope software.

7.5 Particle size determination

The particle sizes of bisphosphonates and inert glass beads were evaluated with Flashsizer FS3D (Intelligent Pharmaceutics Ltd., Helsinki, Finland), a 3D surface-imaging device (Soppela et al. 2011). It utilizes a technique where white light is illuminated from different angles to create 3D surfaces. This rapid technique can be used to determine particle sizes. In addition, the technique offers information regarding particle shape and roughness. A few cubic centimeters of compounds were transferred on a plate and measured. The particle sizes are expressed as D10-, D50- and D90-fractile values, meaning that 10%, 50% and 90% of particles (respectively) are smaller than the presented size range.

7.6 pH measurements

For pH measurements, 300 µM bisphosphonate solutions in TSB and TSB-YE/Glc were prepared to mimic the conditions which *S. aureus* Newman and *A. actinomycetemcomitans* ATCC 33384 encountered in the 96-well plate test, respectively. To imitate the conditions in the Static Biofilm assay, 50 mg of bisphosphonate or inert glass beads, 500 mg of BAG and 450 µl saline were combined. As control, 550 mg of inert glass beads and 450 µl saline were mixed. pH values were measured at the following points in time: 0, 30, 60, 90, 120, 240, 360, 480 minutes, 24 hours, 48 hours and 72 hours. As an additional control, the values were also measured from media (TSB, TSB-YE/Glc and saline) at time points 0, 24, 48, 72 hours. The measurement apparatus 744 pH Meter Ω (Metrohm AG, Herisau, Switzerland) was calibrated daily.

7.7 Data processing and statistical analysis

Data processing and statistical analysis was performed with Microsoft Excel 2013 software and GraphPad Software (Prism, version 5.0 for Mac). For paired comparisons of the gathered data, an unpaired t-test with Welch's correlation was utilized. $p < 0.05$ was considered statistically significant.

8 RESULTS AND DISCUSSION

On the market, there is a wide range of antimicrobial agents with proven efficacy against planktonic bacteria (Skogman 2012). However, no effective anti-biofilm agents are yet available for clinical use. This problem could be overcome with proper anti-biofilm testing. As presented in the subsection 2.6, there are five ASTM standardized methods for biofilm studies, but these methods are only optimized for the strain *Pseudomonas aeruginosa.* Thus, establishing anti-biofilm testing protocols could allow testing of antimicrobials already on the market, widening their indications.

In this thesis, a focal topic was periodontal biofilms. They are treated with topically administered chlorhexidine and strain-specific antibiotics (Current Care Guidelines 2010). However, these antimicrobials do not offer solutions for periodontal attachment tissue and alveolar bone loss. In this thesis, a therapeutical approach composed of bioactive glass and bisphosphonates is further investigated. These two compounds were combined, because it was hypothesized that this combination could simultaneously treat both the underlying root cause (biofilms) and consequences (alveolar bone and attachment tissue loss) of periodontitis. For bisphosphonates, other potential beneficial attributes considering periodontitis have also been presented. Some bisphosphonates are known to possess antimicrobial and anti-inflammatory effects in addition to their positive bone effects. This could potentially be beneficial considering periodontal treatment. Antimicrobial effects of clodronate against *Pseudomonas aeruginosa*, alendronate against *P. aeruginosa*, *Candida albicans* and *Escherichia coli* (Kruszewska et al. 2002), risedronate against *P. aeruginosa and E. coli* have been reported (Kruszewska et al. 2012). Alendronate treatment in rat decreased periodontal pathogen levels *in vivo* (Menezes et al. 2005). In the treatment of inflammatory diseases such as periodontitis, the anti-inflammatory effect could be beneficial. It has been proved that clodronate has inflammation suppressing effects (Österman et al. 1995; Mönkkönen et al. 1998). In contrast, N-containing bisphosphonates have shown effects that are rather pro- than anti-inflammatory (Mönkkönen et al. 1998; Makkonen et al. 1999; Pecherstorfer et al. 2000).

The purpose of this work was to investigate anti-biofilm effects of bisphosphonates as stand-alone compounds and combined with bioactive glass. A late colonizer *A. actinomycetemcomitans* ATCC 33384 was chosen to mimic dental biofilms. For such

biofilms, there are no validated testing methods, as the methods validated by ASTM do not apply to this organism. Furthermore, they are mainly based on biofilm reactors requiring a vast amount of compounds, and therefore they are not suitable for pre-testing or screening. Thus, a methodological problem of dental biofilm research was encountered.

This situation calls for developing and standardizing new biofilm methods that can be more widely applied to other biofilm-mediated conditions. Therefore, effort was invested into optimizing a new dental biofilm model. Because a dental pathogen (*A. actinomycetemcomitans* ATCC 33384) was not optimized for studies, the experiments were started with model organism (*S. aureus* Newman), which was already optimized in 96-well assay conditions. This way, assay settings were built piece by piece, by changing one component at a time.

8.1 Effect of bisphosphonates on bacterial biofilms formed in liquid cultures in 96-well plates

8.1.1 Effect of bisphosphonates on Staphylococcus aureus Newman biofilms

First, a preliminary bisphosphonate anti-biofilm assessment on model organism (*S. aureus* Newman) was performed in 96-well plates. This non-dental bacterium was used as a model organism because it is well known, widely used in first-tier screening studies and already optimized for this assay type (Sandberg et al. 2008; Sandberg et al. 2009; Skogman et al. 2012). Moreover, this strain is frequently used for an assay condition optimization and validation. In this experiment, exposed biofilms were stained with resazurin to detect biofilm viability. Resazurin concentration and incubation time were based on Sandberg et al. (2009). The effects of bisphosphonates on biofilm viability (at 37°C, in aerobic conditions) are presented in Figure 16. Penicillin G, at a very high concentration (400 µM) was used as a control. It prevented 99.9% of biofilm viability but destroyed only 74.6% of formed biofilms. The chemotolerance of *S. aureus* biofilms to penicillin G has been demonstrated earlier (Skogman et al. 2012; Manner S, unpublished observation, 2015) indicating that the test succeeded. In contrast, none of the

bisphosphonates had any significant effect on biofilms when compared to the threshold value (hit limit).

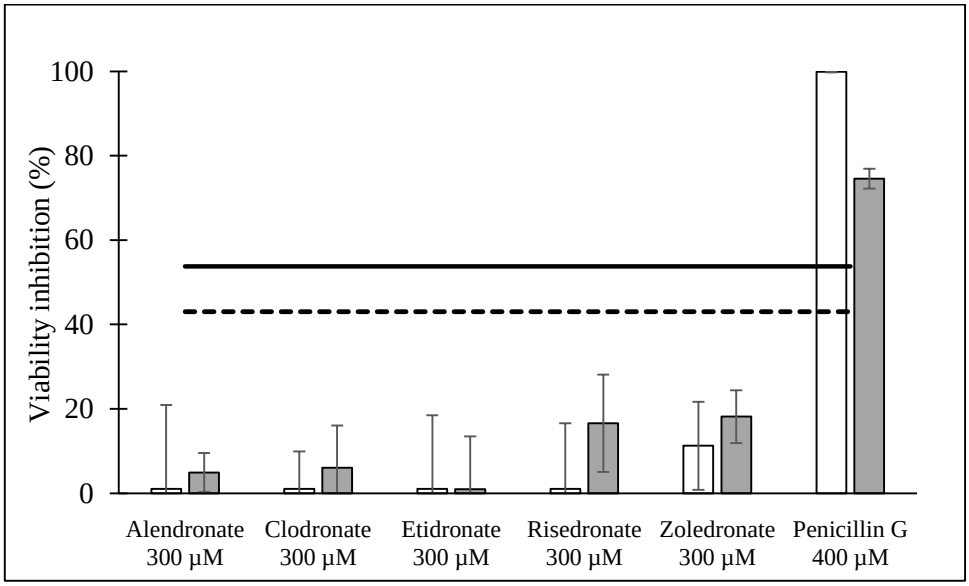


Figure 16. *S. aureus* Newman biofilm viability when exposed to the bisphosphonates. The results of pre-exposure are drawn in white and post-exposure in gray. The hit limits are indicated with the dotted line for pre-exposure and with the solid line for post-exposure assay.

Each compound treatment was performed in six wells, except penicillin G which was applied in only four wells. The assay quality was appropriate ($Z' > 0.5$; $S/N > 5.5$; $S/B > 9$). In conclusion, bisphosphonates do not seem to have an effect on the Gram-positive model organism (*S. aureus* Newman). The results are similar to Kruszewska et al. (2002 and 2012), where antimicrobial activity of alendronate (2002), clodronate (2002) and risedronate (2012) against *S. aureus* ATCC 6538P was tested without any effect.

8.1.2 Effect of bisphosphonates on Aggregatibacter actinomycetemcomitans ATCC 33384 biofilms

Next, the same 96-well plate assay was conducted by changing the strain to a more relevant dental pathogen (*A. actinomycetemcomitans* ATCC 33384), which is a late colonizer in dental biofilms. Culturing conditions for this strain were adopted from Paino et al. (2011). These included the composition of medium (TSB-YE/Glc; TSB supplemented with 0.6% (w/v) yeast extract and 0.8% (w/v)), TSA_{D5} plates (TSA plate

supplemented with 5% defibrinated sheep blood) and incubation in microaerophilic culture conditions (at 37°C, in candle jars).

Before proceeding to bisphosphonate susceptibility testing with this strain, it was pivotal to optimize biofilm formation time and resazurin staining conditions. Optimization of the formation time was performed by growing biofilms 24 and 48 hours (at 37°C in microaerophilic conditions) to assess the growth trend of biofilm biomass. This was carried out with crystal violet staining. Crystal violet was used because it does not require any strain-specific optimization. The relative absorbance units (RAU) of the measurement are presented in Figure 17. Crystal violet staining of other *A. actinomycetemcomitans* strains has been previously reported by Paino et al. (2011) and Massa et al. (2014), but not in 96-well plates.

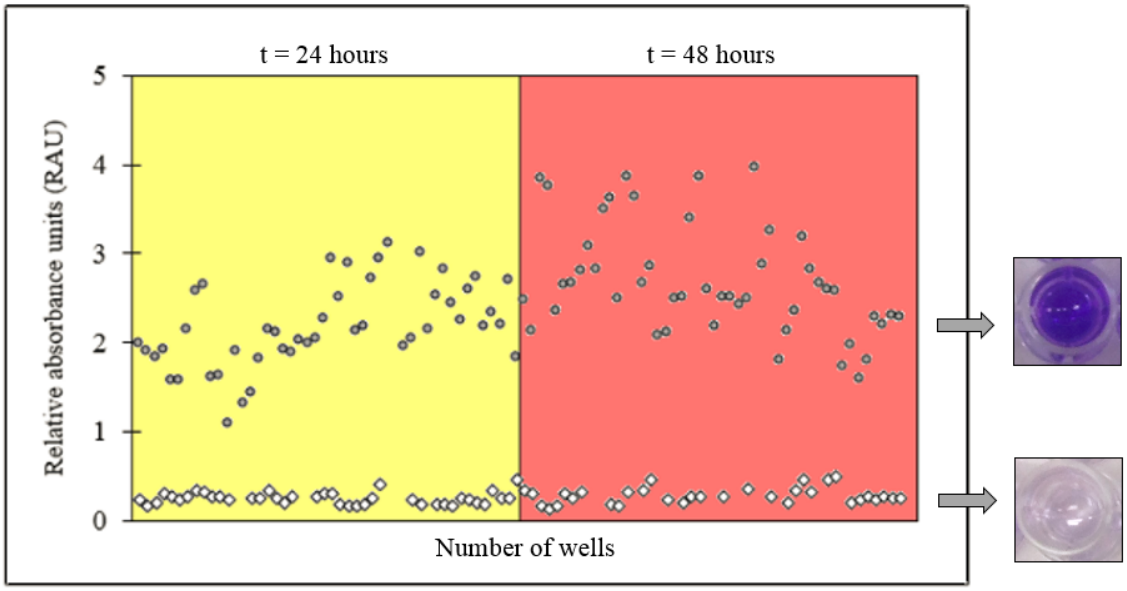


Figure 17. *A. actinomycetemcomitans* ATCC 33384 biofilm biomass after 24 hours (left) and 48 hours incubation (right). Biofilms are indicated with the grey circles and TSB-YE/Glc-control with the white squares. The photos of biofilms (above) and TSB-YE/Glc (below) on the right were taken at the end of the staining process.

Biomass formation was clearly detected after 24 hours. Thus, there was a significant signal in bacteria containing wells (grey circles) when compared to the background signal (white squares). Biofilm *Z'*-values were 0.16 and 0.12 for 24 hours and 48 hours, respectively. Based on this, a convenient biofilm formation time was chosen to be 24 hours. The averages of biofilm relative absorbance units were 2.2 and 2.7 for 24 hours

and 48 hours, respectively. Other assay quality parameters were also appropriate (S/N > 3.8; S/B > 8.5).

As previously indicated, biomass quantification does not provide a complete view on how antibiotics affect biofilms. For instance, in cannot be conclusively stated whether the remaining biofilms are still alive since biofilm biomass measurement does not distinguish between live and dead organisms. Hence, it is essential to measure the amount of living cells in susceptibility trials. This is enabled by redox probes such as resazurin, but they typically require strain-specific optimization. Resazurin staining of other *A. actinomycetemcomitans* strains has been carried out by Paino et al. (2011) and Sreenivasan et al. (2003) (not in 96-well plates), but these experiments did not offer suitable information concerning stain incubation times. Thus, the next step was to optimize the resazurin incubation time and conditions for *A. actinomycetemcomitans* ATCC 33384.

Suitable resazurin stain incubation time and conditions were optimized by comparing incubation in aerobic conditions (at RT, in the darkness for 45, 60, 85, 120 and 240 minutes) and in microaerophilic conditions (at RT, in the darkness for 60 minutes). The optimization trials were performed first in aerobic conditions since these conditions were previously used for *S. aureus* Newman. Z'-values of these incubation experiments, indicating assay performance, are presented in Table 6. All of the incubation times provided sufficient result quality in terms of Z'-values. Therefore, the convenient time of 60 minutes was selected for a further optimization trial, which was conducted in microaerophilic conditions (for 60 minutes), more relevant for *A. actinomycetemcomitans* ATCC 33384. While every incubation time-condition combination indicated suitable test quality parameters (Z' > 0.5; S/N > 9; S/B > 3.7), 60 minutes in microaerophilic conditions (at RT, in the darkness) was chosen for further trials.

Table 6. The resazurin staining performance expressed as Z'-values for *A. actinomycetemcomitans* ATCC 33384.

Minutes	Z'-value in aerobic conditions	Z'-value in microaerophilic conditions
45	0.70	
60	0.65	0.67
85	0.69	
120	0.67	
240	0.71	

For an additional confirmation that biofilms were indeed formed under the chosen conditions, FM imaging of LIVE/DEAD® BacLight™ stained biofilms was carried out. Fluorescence microscope imaging of other *A. actinomycetemcomitans* biofilm strains on different disk surfaces (using the same staining agents) has been reported by Massa et al. (2014) and Sánchez et al. (2011 and 2014), indicating that such imaging could be feasible also in this case. Thus, untreated and treated biofilms (at 37°C, in microaerophilic conditions) were stained with LIVE/DEAD® BacLight™ probe (Figure 18).

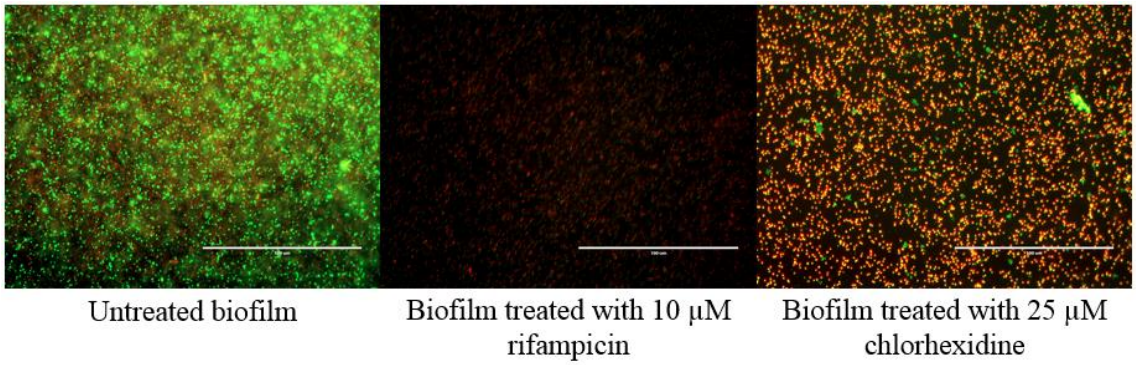


Figure 18. The overlay FM images taken from untreated and pre-treated *A. actinomycetemcomitans* ATCC 33384 biofilms. The scale bars indicate a distance of 100 µm.

Biofilms for FM were grown in the same conditions as the ones where crystal violet and resazurin stainings were performed. A simple visual observation reveals the existence of living biofilms in the untreated stained wells. Living cells, indicating green fluorescence (SYTO® 9 stain), are predominant in the untreated (control) biofilms and are not quenched by dead cell-indicating red fluorescence (propidium iodide stain). On the other hand, biofilms pre-treated with 10 µM rifampicin and 25 µM chlorhexidine have the

opposite behavior where propidium iodide reduces SYTO® 9 in damaged cells, causing red fluorescence. This is a further confirmation that *A. actinomycetemcomitans* ATCC 33384 biofilms are formed under the established conditions and that such biofilms are susceptible to the known antimicrobials. The results are addionally substantiated by viability inhibition values measured in similar pre-exposure conditions (at 37°C, in microaerophilic conditions) (Table 7).

Table 7. Viability of *A. actinomycetemcomitans* ATCC 33384 biofilms when exposed to the control antibiotics of FM assay.

Compound	Hit limit (%)	Viability inhibition (%)	SD
Rifampicin 10 µM	42.86	99.51	0.32
Chlorhexidine 25µM	42.86	99.98	0.14

After establishing suitable assay conditions, the final step was to study the strain susceptibility to bisphosphonates. Compound-exposed biofilms were stained with resazurin to detect biofilm viability. The optimized conditions (24 hours of biofilm formation, 60 minutes of resazurin staining time in microaerophilic conditions) were utilized. Bisphosphonate effects on biofilm viability are presented in Figure 19 (at 37°C, in microaerophilic conditions). The control compound rifampicin almost completely (~100%) prevented biofilm viability and destroyed 72.6% of formed biofilms. In contrast, none of the bisphosphonates had any significant effect on biofilms when compared to the threshold value (hit limit). For each bisphosphonate, treatment was performed in six wells. Rifampicin was applied in four wells. The assay quality was appropriate ($Z' > 0.5$; $S/N > 6.3$; $S/B > 3.9$).

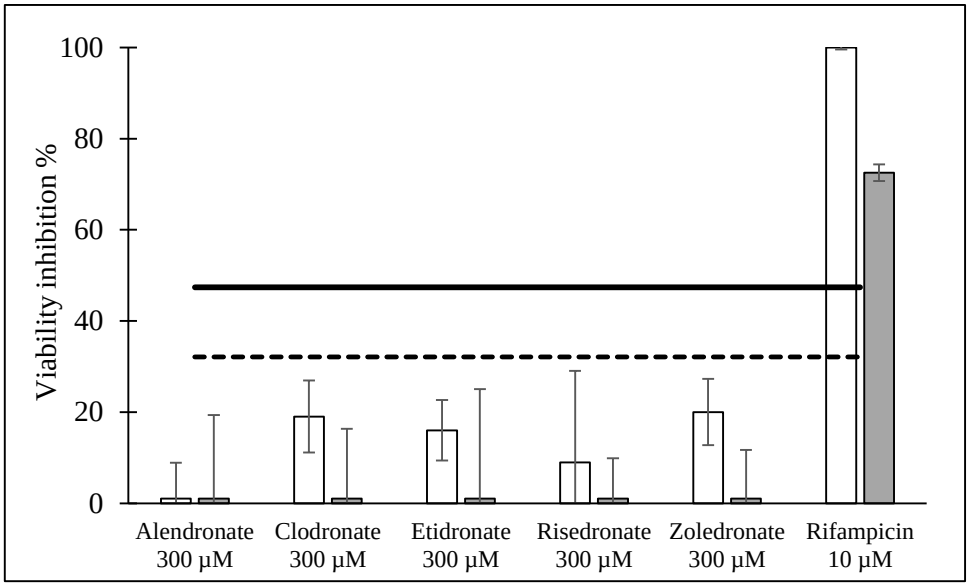


Figure 19. *A. actinomycetemcomitans* ATCC 33384 biofilm viability when exposed to the bisphosphonates. Pre-exposure results are drawn in white and post-exposure results in gray. The hit limits are indicated with the dotted line for pre-exposure and with the solid line for post-exposure.

The results gained from resazurin staining were verified with crystal violet staining (results not presented). In conclusion, bisphosphonates do not appear to have an effect either on the Gram-positive model organism (*S. aureus* Newman) or the Gram-negative dental biofilm organism (*A. actinomycetemcomitans* ATCC 33384).

8.2 Effect of bisphosphonates in combination with BAG on *Aggregatibacter actinomycetemcomitans* ATCC 33384 in a single-specie dental biofilm model

Considering the objectives of this work, 96-well plate assays alone do not offer sufficient information for a number of reasons. First, 96-well plates are not ideal for solid samples. Thus, bioactive glass was not compatible with this assay. A further drawback is that the influence of planktonic bacteria cannot be ignored from the wells, introducing a potential result bias. Moreover, the medium is quickly exhausted due to the limited well space (Merritt et al. 2005). Finally, biofilms in 96-well plates are formed on polystyrene, which are not relevant formation surfaces for dental biofilms.

The surface material issue can be circumvented by growing biofilms on coupons, which can be installed in either liquid cultures of biofilm reactors, or in solid-state cultures with

the Static Biofilm method. The drawback of biofilm reactors is that flow shear is significant. Assuming such conditions would be questionable for the present target environment, especially for periodontal pockets. Another similar issue is the small, ca. 1 mL volume of saliva in the mouth. Methods containing large amounts of medium are not suitable in properly reflecting oral conditions.

For a meaningful study of the bisphosphonate-BAG combination against dental biofilms, a different model needed to be introduced. This led to introduction of the single-specie dental biofilm model (*Aggregatibacter actinomycetemcomitans* ATCC 33384). This model was based on the Static Biofilm method, originally introduced by Charaf et al. (1999) and further developed by Oja et al. (2014). Here, relevant biofilm formation substrates, e.g. plexi glass and hydroxyapatite, could be utilized in low shear conditions relevant to the oral space. Additional strengths of this method include its reliability, simplicity and the fact that it requires only basic laboratory supplies (Charaf et al. 1999). The Static Biofilm method can be considered ideal for studying many kinds of treatments against biofilms.

The drawbacks of this method are that it is quite laborious and time consuming. This is highlighted especially when a number of different treatments are tested, leading to poor suitability for high throughput screening. Hence, use of the Static Biofilm method is limited to study biofilms growing in specific locations (Oja et al. 2014). Still, it offers a simple and inexpensive tool for biofilm formation studies on different materials or in biocide susceptibility testing.

Before starting bisphosphonate-BAG testing, it was pivotal to identify a relevant substrate material for dental applications, on which *A. actinomycetemcomitans* ATCC 33384 biofilms could be formed. In addition to the relevance of hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] as the mineral component of dentine and bones (Pryor et al. 2009), both hydroxyapatite and plexi glass are employed as materials in dental implants. These two materials were compared to the reference substrate (glass). Thus, a quick comparative screen of *A. actinomycetemcomitans* ATCC 33384 attachment was carried out on 3 different coupon materials (hydroxyapatite, plexi glass, glass) during 48 hours at 37°C in humidified microaerophilic conditions. The attached biofilms were quantified with $\log_{10}$ of viable CFU counts (Figure 20) as in Oja et al. (2014).

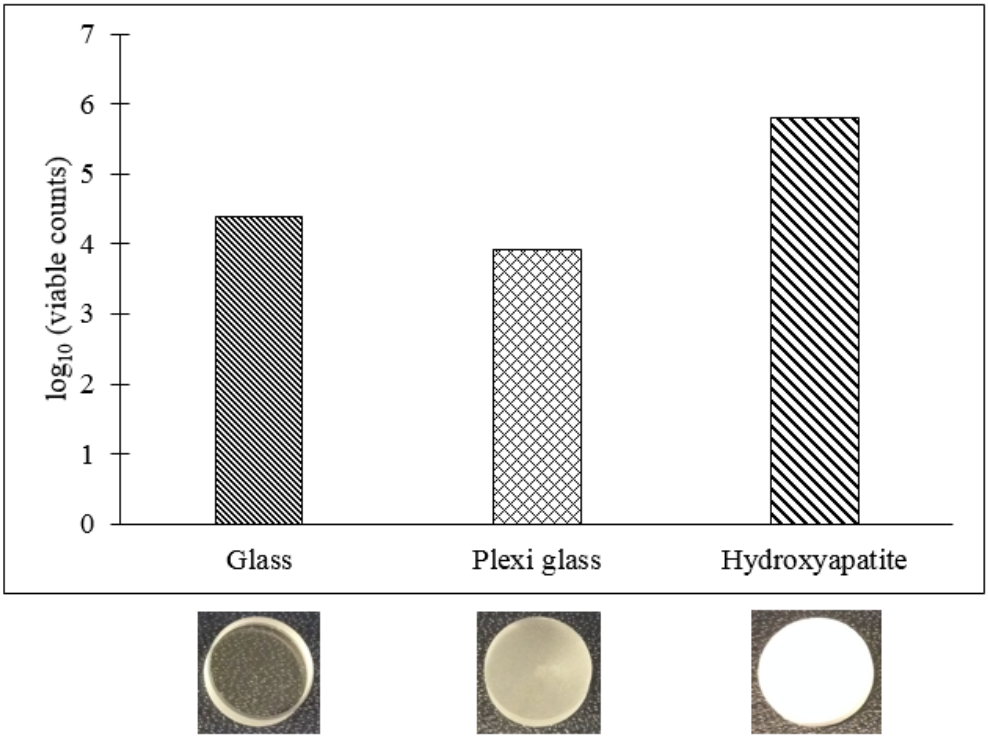


Figure 20. *A. actinomycetemcomitans* ATCC 33384 attachment on the different coupon materials (glass, plexi glass and hydroxyapatite) after 48 hours.

In the conditions of the assay, bacteria were able to attach to all three surfaces. Highest viable counts were measured on the hydroxyapatite surface. Other *A. actinomycetemcomitans* strain biofilms have been grown on saliva-coated hydroxyapatite surfaces by Sánchez et al. (2014) in 24-well plates. They obtained similar biofilm attachment values ($\log_{10}$ ca. 6) after 48 hours of incubation, which are in agreement with present results. Thus, hydroxyapatite coupons were chosen for further studies.

To test the bisphosphonate-BAG samples in this model system, additional aspects needed to been taken into account. One of them was the selection of a proper (negative) control material for bisphosphonates to be combined with BAG. Such a material is required to be inert (no intrinsic antimicrobial activity) and also to possess a similar particle size to bisphosphonates. The particle size equality is pivotal since small particles have higher surface area per volume, indicating increased attachment area for bacteria. One promising control candidate was inert glass. Commercially available inert glass is non-reactive and does not display any biological activity. However, different particle sizes are offered by suppliers. Therefore, the particle sizes of alendronate, etidronate, risedronate and

zoledronate and inert glass were measured with Flashsizer FS3D, and the values of the inert glass were compared to the values of the bisphosphonates (Table 8). The values of clodronate were acquired from Rosenqvist et al. (2013). The particle sizes are expressed as D10-, D50- and D90-fractile values, which denote that 10%, 50% and 90% of particles (respectively) are smaller than the presented size range. According to the D50-values (representing the median particle size), the acquired inert glass was deemed preliminarily suitable for the dental biofilm model. However, a verification of bacterial attachment to the inert glass beads was required. Attachment was tested with a combination of 550 mg of inert glass and 450 µl of saline on hydroxyapatite coupons. The $\log_{10}$ values were 7.75 (SD = 0.18), which is expectedly a notable increase over the coupons. Thus, inert glass beads were chosen to be used as control in this two-component system.

Table 8. The particle sizes of bisphosphonates and their control (inert glass beads) evaluated with Flashsizer FS3D.

Compound	D10 (µm)	D50 (µm)	D90 (µm)
Alendronate	45	95	185
Clodronate	80	160	230
Etidronate	50	100	155
Risedronate	20	35	50
Zoledronate	45	80	125
Inert glass beads	55	105	200

According to the measurements, risedronate particles were clearly the smallest, while the differences between other compounds were less significant. These differences in particle size between the tested compounds present an additional source of uncertainty for the following experiments. On one hand, low particle size may facilitate higher bacterial attachment (large surface area per volume). On the other hand, if bisphosphonates indeed participate in the production of anti-biofilm effects, a smaller particle size may accentuate this effect. To reduce this uncertainty, it would be optimal to test bisphosphonate samples of similar particle size. To acquire a similar partice size, e.g. a drug filter sieve could have been utilized. However, small compound amounts restricted this opportunity.

With the developed dental biofilm model, the anti-biofilm effects of bisphosphonate-BAG combinations were studied on *A. actinomycetemcomitans* ATCC 33384 biofilms during 48 hours at 37°C in humidified microaerophilic conditions (Figure 21).

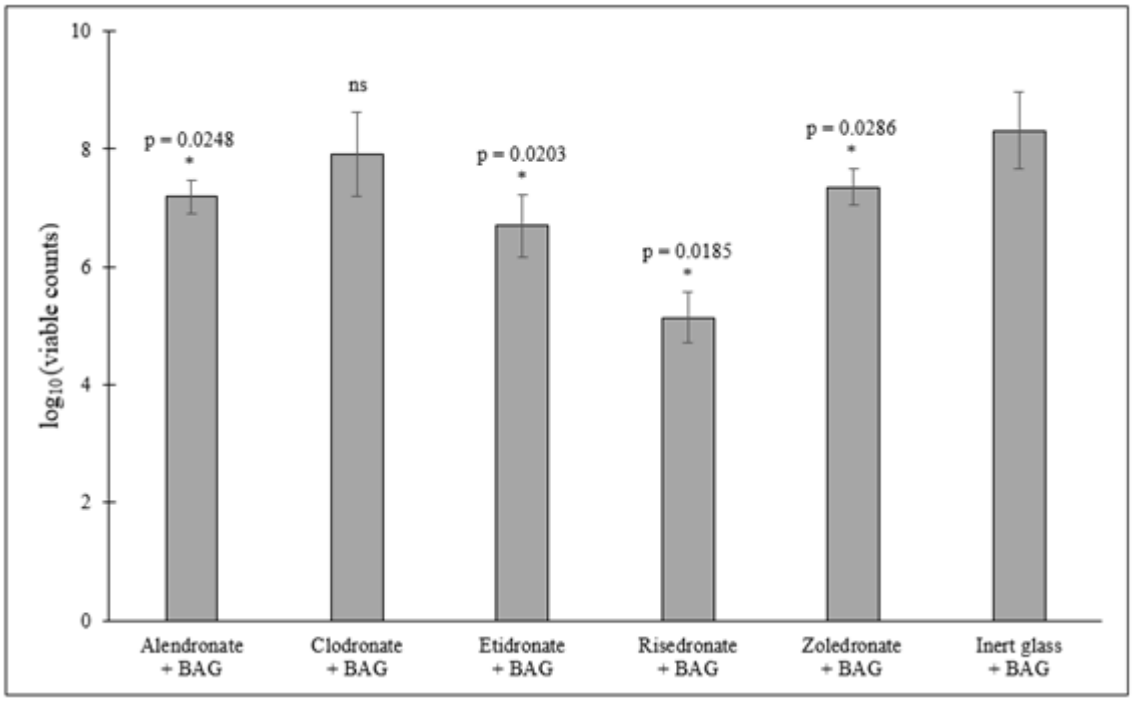


Figure 21. *A. actinomycetemcomitans* ATCC 33384 attachment on coupons covered with two-component samples. The samples were composed of 50 mg of bisphosphonates or inert glass beads (control) and 500 mg of BAG. The probability values were obtained by unpaired t-tests with Welch's correction using GraphPad Software. Statistically significantly differences ($p < 0.05$) are marked with * and *ns* indicates not significant difference.

It was observed that inert glass-BAG-treated coupons possessed more viable counts than untreated hydroxyapatite coupon (Figures 20 and 21). Again, the increased surface area is assumed to be more prone to bacterial attachment. However, the inert glass-BAG-treated control coupons possessed more viable counts than all bisphosphonate-BAG-treated coupons. Logarithmic Reduction (logR)-values, representing the reduction in bacterial viability (Equation 7), are presented in Table 9. The risedronate-BAG-combination was the most active combination in reducing the bacterial biofilm formation (statistically significant difference, $p < 0.05$). Other combinations also reduced biofilm viable counts (statistically significant differences, $p < 0.05$), with the exception of clodronate-BAG, where no statistically significant changes were detected. The experiment was performed with triplicates.

Table 9. The logR-values of bisphosphonate-BAG-combinations, indicating the difference between the control and the compound samples.

Combination	logR
Alendronate + BAG	1.16±0.03
Clodronate + BAG	0.56±0.15
Etidronate + BAG	1.73±0.11
Risedronate + BAG	3.24±0.06
Zoledronate + BAG	0.99±0.03

A. actinomycetemcomitans ATCC 33384 has not been previously used in the Static Biofilm method. Attachment studies with the following strains have been reported: *Staphylococcus aureus* (Charaf et al. 1999; Buckingham-Meyer et al. 2007; Oja et al. 2014), *Staphylococcus epidermis* (Oja et al. 2014), *Escherichia coli* (Oja et al. 2014) and *Pseudomonas aeruginosa* (Charaf et al. 1999; Buckingham-Meyer et al. 2007; Oja et al. 2014). Thus, both Gram-positive and Gram-negative strains have been tested previously. However, previous studies utilize only aerobic bacterial cultivation conditions. Hence, both the strain (*A. actinomycetemcomitans* ATCC 33384) and growing conditions (microaerophilic) used in this method were novel aspects of this thesis. Another method for attachment studies is to grow biofilms on coupons by incubating them with bacterial suspension in well plates (Sánchez et al. 2014).

Previous efficacy testing of compounds to eradicate biofilms formed on coupons biofilms has been tested. In one method, the biofilms were growth with the aid of the Static Biofilm (Charaf et al. 1999; Buckingham-Meyer et al. 2007; Blomqvist 2014). In another method, biofilms were formed on coupon surfaces by incubating coupons with bacterial suspension in well plates (Coraça-Huber et al. 2014; Drago et al. 2014). After biofilm formation, the coupons are removed and exposed to compounds. Hence, the method used in this thesis differs from these aforementioned, since the compounds were applied in the beginning of the incubation period. This pre-exposure mode in the Static Biofilm method is also a novel aspect of the present work.

8.3 Mechanistic insights of the actions of bisphosphonates and bisphosphonate-BAG-combinations on bacterial biofilms

Changes in pH may create a hostile environment for bacterial growth and can therefore be one reason associated to the antimicrobial effects of certain compounds or materials. This is a possible case with the bisphosphonate-BAG-combination. First, pH changes caused by the stand-alone bisphosphonates were tested. The same conditions were used as in the 96-well plate assays: 300 µM bisphosphonate solutions in TSB and TSB-YE/Glc. These mimic the conditions which *S. aureus* Newman (Figure 22) and *A. actinomycetemcomitans* ATCC 33384 (Figure 23) encountered in 96-well plate, respectively (subsections 7.2.1, 7.2.2, 7.2.3).

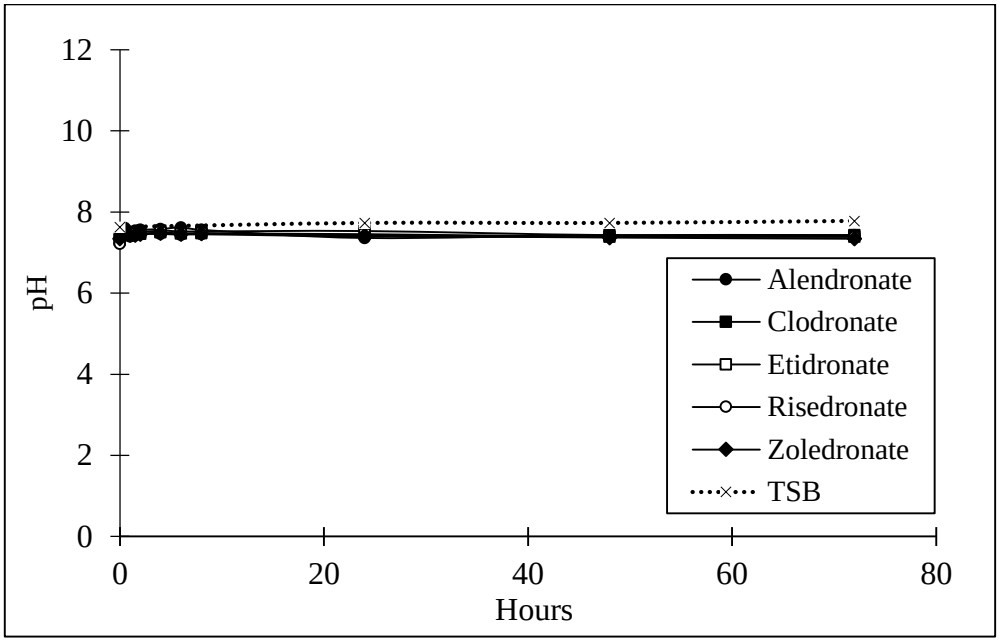


Figure 22. pH-time series of 300 µM bisphosphonate solutions in TSB.

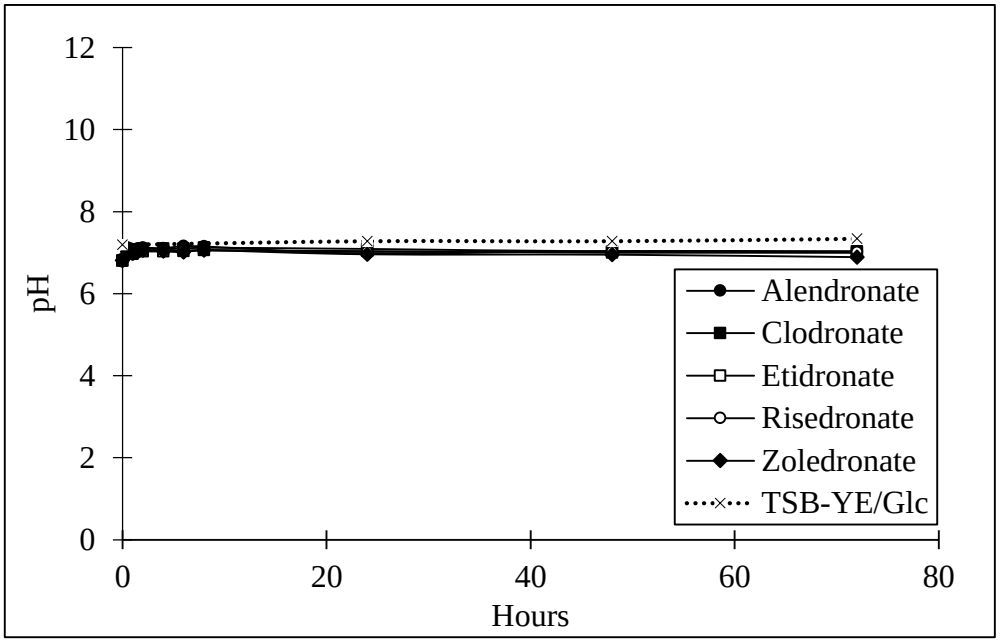


Figure 23. pH-time series of 300 µM bisphosphonate solutions in TSB-YE/Glc.

As shown in Figures 22 and 23, the pHs of media (TSB and TSB-YE/Glc) remained close to the initial pH value 7 during the entirety of both measurements. The presence of bisphosphonates did not change this behavior. This strongly suggests that bisphosphonates do not have an intrinsic pH-altering effect.

Next, pH changes were measured in the same conditions as *A. actinomycetemcomitans* ATCC 33384 encountered in the dental biofilm model. This included a combination of 50 mg of bisphosphonates or 50 mg of inert glass beads combined with 500 mg of BAG and 450 µl of saline (Figure 24). As control, 550 mg of inert glass beads and 450 µl of saline were mixed. BAG as a stand-alone compound in saline has been observed to result in a pH increase to 9-10 during the first 72 hours (Rosenqvist et al. 2013).

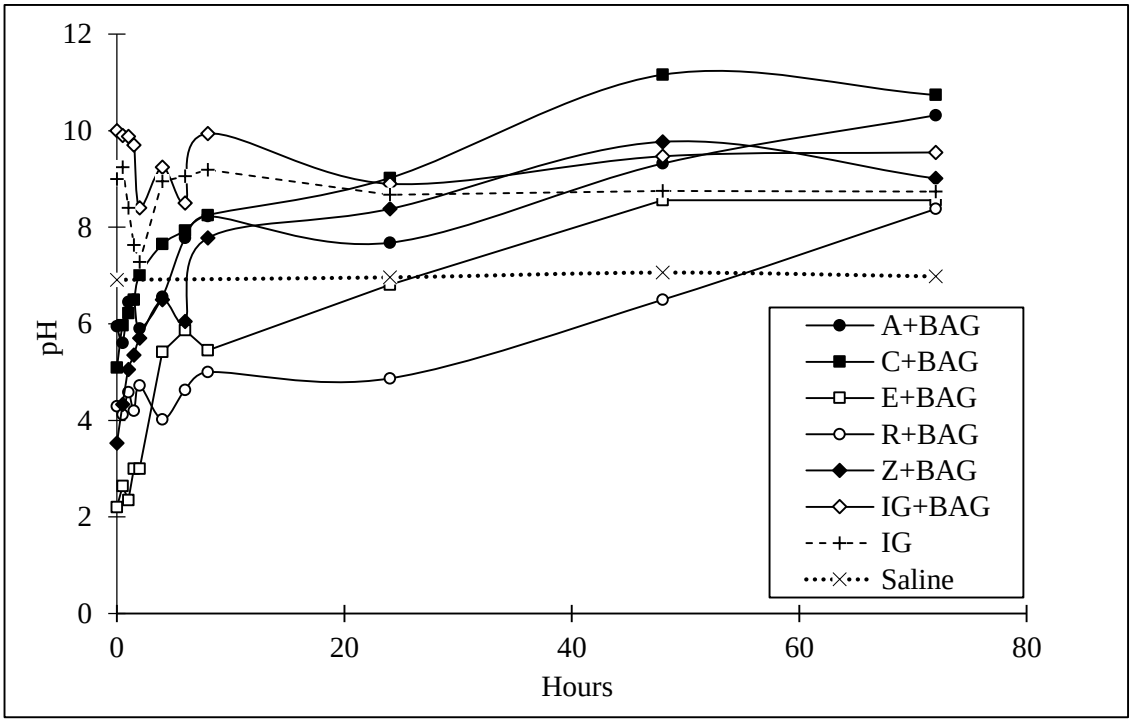


Figure 24. pH-time series of combinations. ● = 50 mg alendronate and 500 mg BAG, ■ = 50 mg clodronate and 500 mg BAG, □ = 50 mg etidronate and 500 mg BAG, ○ = 50 mg risedronate and 500 mg BAG, ♦ = 50 mg zoledronate and 500 mg BAG, ◊ = 50 mg inert glass beads and 500 mg BAG, + = 550 mg inert glass beads, x = saline.

Due to the limited amount of available compounds, the pH measurements were performed only once. Furthermore, sample volumes were small, which caused difficulties in pH measurement stabilization. Increased compound amounts would provide better stability for the measurement probe. Saline pH remained neutral during the entire measurement.

When pH measurements and logR values are considered together, some interesting observations can be made. Both BAG (Rosenqvist et al. 2013) and inert glass show an intrinsic tendency to raise the medium (saline) pH. However, it was noted that the bisphosphonates disrupt this process with a notable, temporary increase in acidity. This increase, in turn, is strongly compound-specific. Etidronate caused a brief but radical pH drop, while the moderate drop induced by risedronate was much longer lasting. These compounds also led to the greatest reduction in biofilm viability. In contrast, the least active compound, clodronate, caused only a mild decrease in pH. The decrease was also short-lived, as the medium became alkaline after only a couple of hours.

With these observations, it is reasonable to hypothesize that low pH is connected with decreased bacterial viability in the present case. Indeed, this is not surprising, considering the documented sensitivity of *A. actinomycetemcomitans* (Bhattacharjee et al. 2011) to acidity. They reported that this organism starts to lose its viability even in the mildly acidic pH. In their study, the drop of medium pH (from 7 to 6) reduced bacterial viability with logR of 2. An interesting aspect would be how the lowered pHs caused by the most effective bisphosphonate-BAG-combinations affect the base pH of crevicular fluid (7.5–8.7) (Bickel et al. 1985). This analysis could provide information on whether these combinations have adverse effects on oral tissues.

It is pivotal to note that the different bisphosphonate particle sizes may have an impact on pH behavior. A smaller particle size may induce a more radical pH change, causing a more hostile environment for bacteria. Thus, for determining the rank order for bisphosphonate-BAG combinations, additional tests with similar bisphosphonate particle sizes should be performed.

Despite the evident correlation between pH decrease and lowered biofilm viability, it is still possible that differences in the chemical structure of bisphosphonates are also influential. However, this factor cannot be evaluated in the present study. Considering the molecular weights and functional groups of the bisphosphonates, no clear correlation with biofilm viability is established.

9 CONCLUSIONS AND FUTURE PERSPECTIVES

The research objectives of this work were to investigate anti-biofilm effects of bisphosphonates alone and combined with bioactive glass, and to optimize the methods which were utilized. First, the anti-biofilm effects of five 300 µM bisphosphonates (alendronate, clodronate, etidronate, risedronate and zoledronate) solutions were assessed on a model organism (*S. aureus* Newman) in 96-well plates. It was observed that the compounds did not have any intrinsic anti-biofilm effect. Testing in 96-well plates was conducted with a relevant periodontal strain (*A. actinomycetemcomitans* ATCC 33384). Several assay condition optimization trials were performed before proceeding to susceptibility trials. This optimization comprised testing of suitable biofilm formation time (24 h), resazurin probe incubation time (60 min) and resazurin incubation conditions (microaerophilic). The following susceptibility tests confirmed the lack of intrinsic anti-biofilm activity on the strain.

After 96-well plate assays, bisphosphonate-BAG tests were introduced. These were performed with a single-specie (*Aggregatibacter actinomycetemcomitans* ATCC 33384) dental biofilm model, which was based on the Static Biofilm method. Again, assay condition optimization was required. Hence, different coupon materials for biofilm growth were compared, which led to the use of hydroxyapatite coupons in further investigations. Particle size measurements were performed to determine a suitable material for bisphosphonate replacement in control samples (inert glass). Utilizing the optimized method, the susceptibility of biofilms to bisphosphonate-BAG samples was investigated. In these assay conditions, the risedronate-BAG-combination (statistically significant difference, $p < 0.05$) was the most effective. Other combinations also reduced biofilm viability (statistically significant differences, $p < 0.05$), with the exception of clodronate-BAG, where the change was statistically insignificant. However, it is notable that the bisphosphonate particle sizes were different. Risedronate particles were clearly the smallest, while the differences between other compounds were less significant. Efficacy of the bisphosphonate-BAG combinations could be connected to temporal changes in pH. This observation is supported by recent literature where *A. actinomycetemcomitans* has been deemed highly sensitive to acidity. However,

establishing the rank order of bisphosphonate-BAG combinations would require experiments with equal bisphosphonate particle sizes.

This project included some novel aspects. For the first time, *A. actinomycetemcomitans* ATCC 33384 was tested in 96-well plate assay, stained with resazurin, crystal violet and imaged with fluorescence microscopy. It was also introduced as a new strain for the Static Biofilm method. Previous susceptibility testing of biocidic compounds with the method has only been performed by using samples in liquid form. Here, solid samples were included as a novel feature in the field. Usability improvements, such as the double filter paper layer, were developed for the method. Furthermore, new coupon materials were introduced.

All of the experiments were performed using mono-species models. Thus, future studies could introduce multi-species biofilms to reflect the periodontal biofilm status in a more realistic manner. These would comprise components of all biofilm stages: pioneer, early, intermediate and late colonizers. The Static Biofilm assays were performed only by using a single bisphosphonate concentration. Different concentrations could be investigated to clarify the concentration-response relationship. During this thesis, only perfunctory knowledge of the mechanism of action was obtained with pH measurements. Other mechanistic studies can be implemented in the future to supplement the information provided by the present pH measurements. One such example is to expose *A. actinomycetemcomitans* ATCC 33384 to a range of pH-controlled solutions. This could potentially reveal other, additive explanations for anti-biofilm activity. In addition, e.g. proteomics can be utilized to explore the mechanism of action further.

For *in vivo* animal models, there is little information available regarding the effects of combination products to periodontal status parameters, e.g. alveolar bone level. Such models could further clarify how the promising bisphosphonate-BAG combinations affect periodontal parameters as well as periodontopathogen levels in infection models. At the same time it could also be investigated whether the lowered pHs caused by bisphosphonate-BAG-combinations are not harmful to oral tissues. It should be noted that in addition to anti-biofilm studies, combination effects on osteoclast and osteoblast behavior should be studied for a more comprehensive understanding of the subject.

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