Antibacterial measures for biofilm control

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By Marieke van de Lagemaat

University Medical Center Groningen, University of Groningen
Groningen, The Netherlands

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Prof. dr. Y. Ren

Prof. dr. H.J. Busscher

Prof. dr. H.C. van der Mei

Beoordelingscommissie

Prof. dr. M.S. Cune

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Prof. dr. H. He

Aan mijn ouders, broer, zus en Ardan

Paranimfen: Arjen Grotenhuis, Msc

                  Femke van de Lagemaat, Msc

Contents

Chapter 1

General introduction and aim of this thesis

Orthodontic treatment and risks

Orthodontic treatment aims at improving function of the masticatory system, correcting the position irregularities of teeth and achieving better facial esthetics. However, orthodontic treatment also bears a potential oral health risk that can subsequently compromise oral function and dental esthetics. The main adverse effect is the accumulation of dental plaque or oral biofilm around the orthodontic appliances (Travess et al. 2004). Oral biofilm develops naturally on tooth surfaces and it is highly associated with caries and periodontal diseases (Marsh and Bradshaw 1995). Mechanical removal of biofilm by routine oral care is severely hampered by the presence of orthodontic fixed appliances, such as brackets, bands, tads and other auxiliaries due to the increasing crevices and niches introduced in the mouth. Composite bonding resins are prone to bacterial adhesion at the vulnerable bracket-adhesive enamel junction, especially since polymerization shrinkage may yield a gap at the contact interface into which bacteria can easily infiltrate. Temporary devices, such as mini-implants also create retention sites for oral biofilms. In these protecting niches, biofilm is difficult to remove mechanically and can grow undisturbed.

Also, removable clear appliances, such as positioners and aligners, gaining increasing clinical popularities, showed no advantage in terms of oral hygiene control compared with fixed appliances (Chibber et al. 2018). Aligners typically cover entire tooth surfaces and 1 to 2 mm of the gingiva. This extensive oral surface coverage has been shown to limit the flow of saliva, negating saliva's natural cleansing, buffering, and remineralizing properties (Addy et al. 1982). Moreover, the nature cleansing activities of the lips, cheeks, and tongue are interrupted, allowing undisturbed biofilm growth under the appliance (Moshiri et al. 2013). In short, a variety of additional surfaces introduced by orthodontic devices provides a favorable environment for microorganisms to grow in a biofilm mode and survive from mechanical removal.

Problems related to oral biofilm

Biofilm on oral hard and soft tissues can cause enamel demineralization and gingival inflammation (Marsh and Nyvad 2003). Demineralization of enamel, which in its mildest form yields white spot lesions indicative of subsurface decalcification, occurs

in 23-97% of the orthodontic treated patients (Ren et al. 2014). Decalcification can lead to caries and cavities, until restorative treatment is necessary.

Biofilm formed below the gingival margin can lead to inflammation of the gingiva, and in an extreme case periodontitis and tooth loss. Biofilm-related inflammation of soft tissues surrounding temporary devices, such as mini-screws, can cause inflammatory reactions similar to peri-implantitis, especially when it is related to biofilm formed on transgingival parts of the devices. These inflammations are associated with a 30% increase in failure rate of temporary anchorage devices (Miyawaki et al. 2003).

Daily oral care

Manual or powered brushing are still by far the most effective measure for oral hygiene maintenance in orthodontic patients. Manual toothbrushes with a special head design for orthodontics, v-shaped, or triple-headed, are more efficient than brushes with a conventional planar bristle field (Rafe et al. 2006). Powered toothbrushes reduce biofilm and gingivitis more than manual tooth brushing in the short and long term (Yaacob et al. 2014). Powered toothbrushes also promote gingival health more effectively than manual toothbrushes in orthodontic patients (Al Makhmari et al. 2017). In *in vitro* settings powered toothbrushes demonstrated noncontact removal of oral biofilm (Schmidt et al. 2013) up to brushing distances of 6 mm. Mechanisms of hydrodynamic action, passing air-liquid interfaces, and acoustic energy transfer are contributing to this beneficial impact (Busscher et al. 2010). Therefore powered toothbrushes are beneficial for patients with orthodontic appliances with additional crevices and niches that are difficult to reach by manual brushes (Sharma et al. 2015). It has been demonstrated *in vitro* that the structure of biofilm changes after powered brushing in favor of antimicrobials penetration to kill bacteria to a greater depth (He et al. 2014).

Besides mechanical methods of oral hygiene, chemical products for oral care such as toothpastes, mouthrinses and varnishes containing antimicrobial agents assist in the control of oral biofilm. A variety of antimicrobials in toothpastes, mouthrinses and varnishes, are available and contain agents like chlorhexidine, quaternary ammonium compounds, triclosan, essential oils, metal salts and fluoride. Formulations with chlorhexidine, triclosan, and fluoride have demonstrated

significant antibiofilm efficacy *in vivo* (James et al. 2017; Riley and Lamont 2013; Marinho et al. 2003). Fluoride is most commonly used and is applicable in many different formulations and acts as a buffer to neutralize acids produced by bacteria and suppresses their growth (Khoroushi and Kachuie 2017). However, the benefits of fluoride mainly confine to the inhibition of demineralization (Busscher et al. 2010). Mouthrinses with chlorhexidine are considered the gold standard in dentistry with respect to antibacterial effects (Varoni et al. 2012). Chlorhexidine exhibits broad spectrum activity against both Gram-positive and Gram-negative bacteria, yeast, dermatophytes and lipophilic viruses (Beyth et al. 2003; Denton 1991) and is considered effective in helping reduce oral biofilm and gingivitis (James et al. 2017). Patients using chlorhexidine during orthodontic treatment have significantly less white spot lesions (Okada et al. 2016). Synergistic effects of different antibacterial chemicals have been shown in *in vivo* studies. A combination of different chemicals, such as an amine fluoride/stannous fluoride-containing toothpaste and mouthrinse with chlorhexidine showed improved cariostatic effects in an orthodontics-induced caries model compared with conventional fluoride formulation (Øgaard et al. 2001; Øgaard et al. 2017).

Antibiotic resistance

With wide application of antimicrobials worldwide, the development of antibiotic resistance has been a major concern in public health (Van de Belt et al. 1999; Neut et al. 2003; Howard et al. 2003). Oral antibiotics with non-selective antibacterial effect may be very effective, but resistance has emerged in clinical isolates resistant to multiple drugs, including chlorhexidine, such as in methicilin-resistant *Staphylococcus aureus* (Block and Furman 2002) and other oral strains (Saleem et al. 2016). Uncontrolled use of oral health products containing antimicrobial agents may stimulate development of multidrug resistant strains that can retain in oral biofilms left behind after brushing as 100% biofilm removal can never be achieved (Busscher et al. 2010). These resistant strains can act as a source for dissemination and pose a life threatening infection in a host with compromised immunological conditions (Davies 1994). Cell wall deformation plays an important role in understanding the bacterial susceptibility to antimicrobials and probably the development of resistance. An increase in deformation of the bacterial cell wall is accompanied by an increase in

the surface area of the lipid membrane, making it more susceptible for antimicrobials to penetrate. Reliable measurements of nanoscopic cell wall deformation as a result of bacterial adhesion to surfaces can be defined by exploiting surface enhanced fluorescence (Li et al. 2014; Carniello et al. 2018) and is highly important in the understanding bacterial responses to antimicrobials and recommendations for clinical use or the development of alternatives for current antimicrobials.

Prevention and control of oral biofilm

Strategies to prevent and control oral biofilm formation have been extensively studied in clinical research. Antibiofilm activity may be achieved by different mechanisms of action: by preventing bacterial adhesion, by limiting bacterial growth, by disrupting an already established biofilm or by altering the composition and/or pathogenicity of the biofilm (Sanz et al. 2013). One strategy of particular interest in dentistry is modifying dental materials with antimicrobial properties, by mechanisms based either on releasing antimicrobial particles from the material or modifying the material surface with 'contact-killing' features. For orthodontics and dentistry in general, prolonged antimicrobial action is desired, therefore materials that can kill bacteria upon contact are of great clinical relevance.

Polymers containing covalently bonded antimicrobial moieties, such as immobilized quaternary ammonium compounds, possess the unique feature of bacterial 'contact-killing' (Tiller et al. 2001; Imazato 2003). Adhering bacteria are killed upon contact by severe membrane disruption through extremely strong electrostatic attraction (Asri et al. 2014). Bacterial killing upon adhesion to cationic quaternary ammonium surfaces has been shown in many *in vitro* studies. *In vivo* efficacy, however, has only been shown in animal studies (Gottenbos et al. 2003; Imazato et al. 2004; Schaer et al. 2012). Another limitation is that there exists no ubiquitously accepted method to evaluate the efficacy of bacterial contact-killing on these surfaces. Nevertheless, bacterial contact-killing materials with long lasting actions are promising as a non-antibiotic based way to prevent biofilm formation. For clinical applications, it would be even better to incorporate the contact-killing property in a material with other unique features, e.g. 3D printability and mechanical versatility.

Aim of the thesis

The aim of this thesis is to investigate measures for oral biofilm control related to oral biofilm infections.

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Chapter 2

Case example:
Participation of end-users in setup and topic selection for
biomedical research

Marieke van de Lagemaat, Henny C. van der Mei,
Henk J. Busscher, Yijin Ren

Abstract

A case example of the participation of orthodontic end-users in selecting research topics is presented, in which patients, parents and care providers are involved in the set-up and topic selection of a part of this thesis, using a structured questionnaire. The questionnaire addressed different aspect oral biofilm control in orthodontic patients and asked what aspects and new developments would be valued most by them as end-users. All respondents, including patients, parents of patients, orthodontists and paramedics scored highest for 'non-compliance' bacterial-killing adhesives with lasting killing effect. The results demonstrate that end-users can make a valuable contribution for scientists in the selection for societally-relevant research topics, when the main purpose of the research work is to reach its potential end-users and provide benefit for their health and wellbeing. Moreover, public opinion can help scientists to better understand the needs of end-users.

Today, scientists are urged more than ever to demonstrate societal impact and economic value creation of their research work by scientific journals, university boards, research funding agencies, scientific output evaluation committees and the general public. In the early days, the only aspect of interest when measuring the impact of research was the impact on academia and scientific knowledge. Since the 1990s, trust in the value of science for society decreased and a visible trend emerged that evidence should be provided to demonstrate its value for society (Bornmann 2013). The significance of research can be evaluated by its scientific and societal impact. Research can be of low scientific quality while still having a large resonance in society and vice versa. In the academic world, scientific quality of a research work is often indicated by the impact factor of the journal in which it has been published (Eliades and Athanasiou 2001). Ideally, all scientific research should be of high quality and demonstrate considerable societal impact contributing to the well-being of the general public. In reality, however, scientific quality and social impact of a research work shows only a weak correlation (Mostert et 2010). Good scientific research with a well-designed methodology does not necessarily ensure a high societal impact. Relevance to society is an important objective for scientific studies in all fields including biomedical research. Evaluation of research work should therefore not be restricted to its scientific quality alone, but also take into account its impact outside the scientific domain (KNAW;2001).

Societal impact can be divided in three levels, or so called end-users or general (lay) public, healthcare professionals and the private sector (KNAW; 2001). To achieve impact in society there must be some interaction between a research group and the potential end-users of their research work (ERiC; 2010). The public's opinion is increasingly important as it gets a more demanding vote in the selection for research topics, which may eventually influence the policy makers in decisions on funding priorities. Therefore, the interaction between scientists and end-users becomes an important aspect for research, and scientists should be aware of the needs and preferences by end-users (Bouter 2010). Although real societal impact can often only be assessed many years after a research work has been published, the 'productive interactions' between researchers and 'end-users' may be considered as a proxy for further (future) impact (Wit and Merkx. 2010). To this end, a survey can be a suitable method to identify topics of importance as perceived by the end-users.

Here we present a case example on the participation of end-users in setup and topic selection of a research study on prevention of biofilm formation during orthodontic treatment. A questionnaire was developed to measure the needs and expectations of those who would be potentially affected by the outcomes of the research study.

A structured questionnaire with closed questions and pre-formulated answers were used in the survey. Survey respondents included patients, parents of the patients, paramedical personnel including oral hygienists, dental nurses and clinical administrative workers, and orthodontists. Patients, regardless their age, filled in the questionnaire by themselves and in addition, parents of patients younger than 16 (if present at the time of the survey), were also asked to filled in the questionnaire. All respondents were either patients and their families or employees working at the Department of Orthodontics at the University Medical Center Groningen. The survey consisted of two parts, with one part focusing on research topics (A), the other part on clinical applications (B). First, the preferences of the users for a research topic were measured; after that we continued with the preferences on clinical applications on the selected research topic. Respondents had to answer on a likert scale (Likert; 1932). Scoring a 10 indicates that the participant completely agreed, scoring a 0 indicates completely disagree. The questionnaire was explained by one of the researchers, and subsequently, the respondents filled in the questionnaire on their own and handed it in immediately afterwards.

Data were analyzed with the Statistical Package for Social Sciences (Version 16.0, SPSS Inc., Chicago, IL, USA). A one-way analysis of variance (ANOVA) was used to compare the mean scores. A post-hoc Bonferroni test was used for comparisons between the groups. Statistical significance was set at $p < 0.05$.

In total 91 subjects filled in the first part of the questionnaire (Fig. 1A), with an age range between 8 to 58 years. The respondents consisted of 28 patients, 26 parents of patients, 17 orthodontists and 20 paramedics. Figure 1-A shows the results on selection of research topics.

Patients, parents of patients, orthodontists and paramedics all gave a lowest score (mean score 5.6 ± 2.6) to free distribution of toothbrushes at schools. This is probably because toothbrushes in the Netherlands are relatively cheap and highly affordable. A difference can be seen in 'educational websites', on which medics and paramedics scored significantly (p<0.05) higher (mean scores 7.9 ± 1.3 and 7.9 ± 1.0

) than patients and their parents (mean score 6.5 ± 2.3 and 7.0 ± 2.1). 'Efficient E-brush', on the contrary, scored significantly lower by medics and paramedics ($p< 0.01$) than by patients and parents. With a mean score of 8.1 (± 1.5), 'development of bacteria-killing braces', scored the highest among all four topics without any significant differences between the groups ($p > 0.05$) indicating a clear preference by all different end-users for this topic.
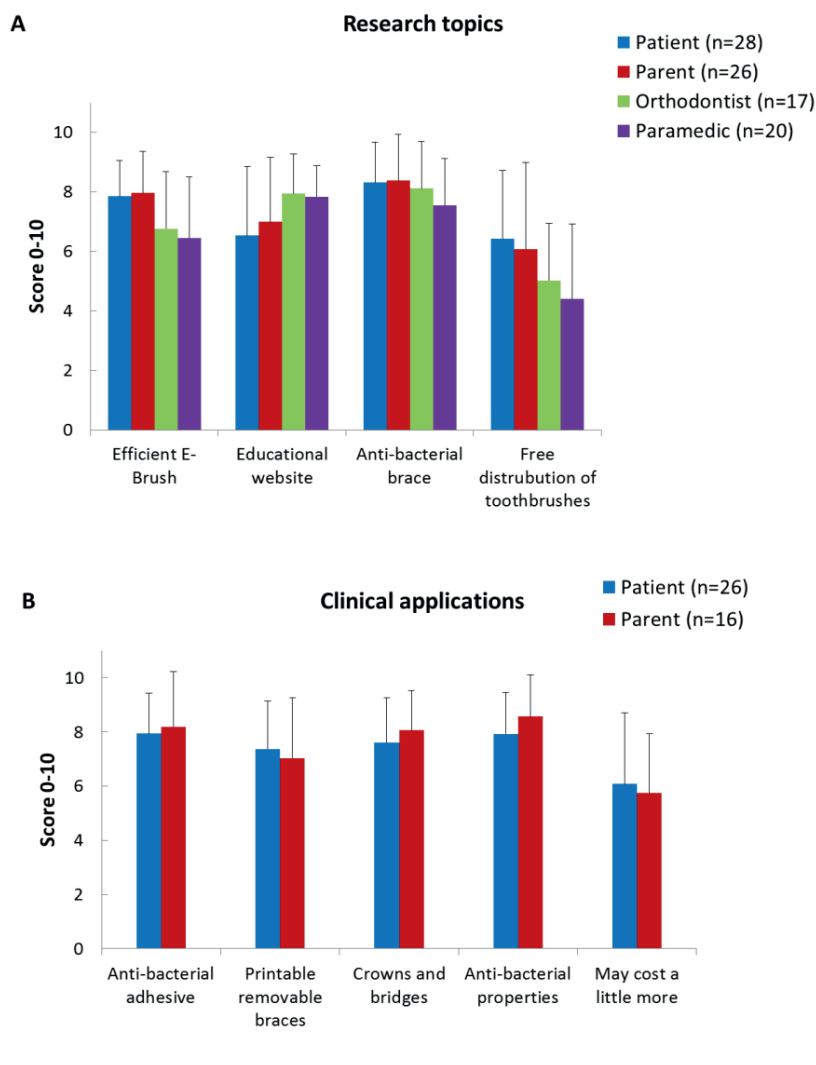

Figure 1. a) Scores on selection of research topics by patients, parents, orthodontists and paramedics. b) Scores on selection of research aims of patients (mean age 16.4 ± 6.4, age range 10-35 years) and parents (mean age 43.5 ± 5.2 age range 36-58 years).

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Bacterial-killing braces can have different applications in dentistry and orthodontics. Accordingly, to find out about preferences for applications by the potential users, 4 different clinical applications and 1 question on cost aspects were presented. 26 patients (mean age 16.4 ± 6.4, age range 10-35 years) and 16 parents (mean age 43.5 ± 5.2, age range 36-58 years) filled in this part of the questionnaire (B). There was no significant differences between the groups although all respondents scored highest for bacterial-killing adhesives and lasting killing effect, with a mean score of 8.0 ± 1.7 and 8.2 ± 1.6 respectively (Figure 1-B). Parents scored higher on the crown and bridge work than patients. This may have to do with a higher awareness of their dental conditions, while patients (mean age 16.4 ± 6.4), at a young age, often have relatively healthy dentitions and are less familiar with dental work options. The respondents scored lower (mean 7.2 ± 1.9) for a 3D printable material and scored the lowest on the extra costs (mean 5.9 ± 2.4). Accidently, two parents indicated their considerations towards a lower score for the 3D printability: '3D' sounded very high-tech, and therefore its application must be quite costly. This argument fits well to the low scores on 'extra costs'. The health care system in the Netherlands has somewhat encouraged the development of a common mentality of the public that health care is expensive and should be 'free'(NOS [internet]).

Although what is presented here is only one case example performed within one academic clinical department, the results reflect clearly the needs perceived by patients, their families and healthcare workers. Remarkably, even patients as young as 8 years old were able to indicate their needs and preferences independently. It is interesting to see that the needs and preferences of patients and parents differ significantly from orthodontists and healthcare workers, indicating false expectations may exist not only in researchers towards end-users, but also in indirect users (healthcare workers) towards direct users (parents and families). Awareness of this may help to improve the quality of care by healthcare workers. Even more interesting is that there exists a high agreement in almost all aspects between patients and parents, indicating patient families are generally more able to think in line with the needs of their family members. This is valuable information for clinicians to consider in their decision-making when more treatment options exist for a patient.

To summarize, we presented a case example on the participation of end-users, including patients, their families and their care providers, in setup and topic selection of a research study using a structured questionnaire. The results demonstrate that

end-users can make valuable contribution for scientists in selection for a research topic, when the main purpose of the research work is to reach its potential end-users and provide benefit for their health and or wellbeing. While the needs and preferences may differ between direct and indirect users, the outcome of this survey indicate clearly that public opinion is worth considering by scientists in the clinical downward translation of their fundamental research work.

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Panel 1

U/je bent: patiënt (leeftijd   ) / ouder /medici / paramedici

vaste apparatuur   wenselijk resultaat   realiteit

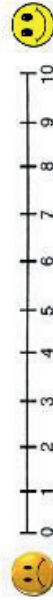uitneembare apparatuur   wenselijk resultaat   realiteit

Het probleem is vooral de groei van orale bacteriën op tandglazuur door slecht of onvoldoende tandenpoetsen. In ernstige gevallen kan dit leiden tot gaatjes. Het is onesthetisch en ongezond. Wat voor soort onderzoek zal je voor stemmen om dit probleem aan te pakken? Hoe hoger de score die je geeft, hoe meer je denkt dat je profiteert van dat onderzoek.

1. Ontwikkeling van een meer efficiënte tandenborstel voor makkelijker tandenpoetsen specifiek voor kinderen
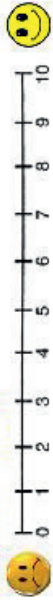
0 1 2 3 4 5 6 7 8 9 10

2. Ontwikkeling van een patiënt educatieve website met video's en foto's voor een goede mondhygiëne

0 1 2 3 4 5 6 7 8 9 10

3. Ontwikkeling van anti-bacteriële beugels zodat bacteriën er niet op kunnen groeien (patiënten moeten goed blijven tandpoetsen natuurlijk).

0 1 2 3 4 5 6 7 8 9 10
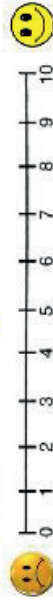
4. Ontwikkeling van een goedkope tandenborstel voor eenmalig gebruik met pasta en voor het gratis verdelen op school voor kinderen

0 1 2 3 4 5 6 7 8 9 10

Hartelijk dank voor uw medewerking!
Prof. dr. Y Ren, Orthodontist, Afdeling Orthodontie UMCG

Panel 2

U/je bent patient / ouder   Uw/jouw leeftijd is:

vaste apparatuur   wenselijk resultaat   realiteit

uitneembare apparatuur   wenselijk resultaat   realiteit

Het probleem is vooral de groei van orale bacteriën op tandglazuur door slecht of onvoldoende tandenpoetsen. In ernstige gevallen kan dit leiden tot gaatjes. Het is onesthetisch en ongezond. In het UMCG gaan we een onderzoeksproject beginnen over de ontwikkeling van anti-bacteriële beugelmaterialen, zodat bacteriën er niet op kunnen groeien. Er zijn verschillende benaderingen om een anti-bacteriële beugelmateriaal te ontwikkelen. We willen graag uw/jouw mening hierover. Hoe hoger de score die je geeft, hoe meer je denkt dat je/een patiënt profiteert van dat onderzoek.

1. Ontwikkeling van een anti-bacteriële lijm voor de "slotjes", waarop bacteriën niet meer kunnen gedijen (bacteriën kunnen heel goed gedijen op de huidige gebruikte lijm).

0 1 2 3 4 5 6 7 8 9 10
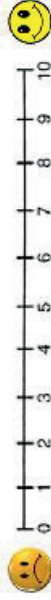
2. Ontwikkeling van een 3D printbare anti-bacteriële beugel, zodat een uitneembare beugel direct op maat geprint kan worden met eventueel wenselijke kleur en/of smaak.

0 1 2 3 4 5 6 7 8 9 10

3. Ontwikkeling van anti-bacteriële materiaal dat niet alleen voor beugels, maar ook voor kronen en bruggen gebruikt kan worden, zodat vele andere tandheelkunde patiënten van verschillende leeftijden hier ook van kunnen profiteren.

0 1 2 3 4 5 6 7 8 9 10

4. De anti-bacteriële eigenschap van het nieuwe beugelmateriaal moet blijven bestaan tot de beugel behandeling klaar is.

0 1 2 3 4 5 6 7 8 9 10
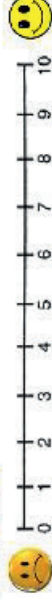
5. Als het nieuwe anti-bacteriële beugelmateriaal aan alle bovengenoemde eisen voldoet, mag het materiaal iets meer kosten (<10%).

0 1 2 3 4 5 6 7 8 9 10
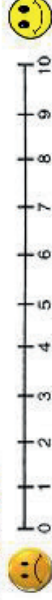
Hartelijk dank voor uw medewerking!
Prof. dr. Y Ren, Orthodontist, Afdeling Orthodontie UMCG

Chapter 3

**Synergy of
brushing mode and antibacterial use
on in vivo biofilm formation**

*Marieke van de Lagemaat, Marije A. Jongsma, Henk J. Busscher, Gesinda I.
Geertsema-Doornbusch, Jelly Atema-Smit,
Henny C. van der Mei, Yijin Ren*

Abstract

Orthodontic, multi-strand retention-wires are used as a generalized model for oral retention sites to investigate whether biofilm left-behind after powered toothbrushing in-vivo enabled better penetration of antibacterials as compared with manual brushing. 2-cm multi-strand, stainless-steel retention-wires were placed in brackets bonded bilaterally in the upper arches of 10-volunteers. Volunteers used NaF-sodium-lauryl-sulphate-containing toothpaste and antibacterial, triclosan-containing toothpaste supplemented or not with an essential-oils containing mouthrinse. Opposite sides of the dentition including the retention-wires, were brushed manually or with a powered toothbrush. Health-care-regimens were maintained for 1-week, after which wires were removed and oral biofilm was collected. When powered toothbrushing was applied, slightly less bacteria were collected than after manual brushing, regardless whether an antibacterial-regimen was used or not. Powered-toothbrushing combined with antibacterial-regimens yielded lower biofilm viability than manual brushing, indicating better antibacterial penetration into biofilm left-behind after powered brushing. Major shifts in biofilm composition, with a decrease in prevalence of both cariogenic species and periodontopathogens, were induced after powered brushing using an antibacterial-regimen. Oral biofilm left-behind after powered brushing *in-vivo* enabled better penetration of antibacterials than after manual brushing.

Introduction

Amount, viability and composition of oral biofilm play a major role in the development of oral pathologies, such as caries, gingivitis and periodontitis. Prevention of biofilm-related oral pathologies can be achieved either by mechanical or chemical removal of biofilm, changing its composition or preventing its formation (Marsh 2012). Mechanical biofilm removal by powered toothbrushing has been demonstrated to be superior to manual brushing (Yaacob et al. 2014). However, complete biofilm removal can never be achieved and after a single self-performed brushing, the amount of oral biofilm can only be reduced by 50–60% (Paraskevas et al. 2006; Van der Weijden et al. 2008), leaving biofilm behind at locations out of reach for mechanical removal such as fissures, buccal pits, posterior interproximal areas and gingival margins, where oral pathologies mostly develop (Sheiham and Sabbah 2010). In orthodontic patients, the number of locations out of reach of mechanical removal is even higher, making orthodontic patients more prone to oral pathologies than non-orthodontic patients (Ren et al. 2014).

The use of antibacterial containing toothpastes or mouthrinses can be a valuable addendum to mechanical biofilm control in order to reduce the viability of biofilm left-behind after brushing (Marsh 2012). However, the general structure and composition of oral biofilm hampers penetration of oral antibacterials through the depth of an entire biofilm (Van Leeuwenhoek 1684). Oral biofilm consists of a large variety of adhering bacteria embedded in an extracellular-polymeric-matrix that acts both as a glue for bacteria as well as a barrier against penetration of antibacterials (Flemming and Wingender 2010; Marsh 2010). Powered toothbrushing of *in vitro* oral biofilm has been demonstrated to impact the structure of biofilm left-behind to create a more open structure, more amenable to antibacterial penetration (He et al. 2014), especially when the bristles of the brush have not been able to touch the biofilm and remove it (Busscher et al. 2010). This more open structure is caused by a high energy transfer from a powered toothbrush into the biofilm through strong fluid flows (Van der Mei et al. 2007), air bubble inclusion (Parini and Pitt 2006) and acoustic waves (Busscher et al. 2010). Accordingly it has been demonstrated in vitro that due to this more 'fluffed-up', open biofilm structure chlorhexidine and cetylpyridinium-chloride penetrate and kill bacteria to a greater depth into biofilm left-behind after powered brushing (He et al. 2014). Also, once oral antibacterials

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have penetrated the biofilm, the biofilm left-behind might act as a reservoir for the oral antibacterial agents ensuring a prolonged action of the agent (Otten et al. 2012). However, the impact of these in vitro findings for the clinical situation has never been demonstrated and could only be speculated upon.

In order to determine whether the improved penetration of antibacterial agents into biofilm left-behind after powered brushing as observed in vitro, also yields clinical benefits, we here aim to compare biofilm formation and composition in vivo on orthodontic, multi-strand retention wires after manual versus powered toothbrushing using a control, NaF-sodium lauryl sulphate-containing toothpaste or an antibacterial, triclosan-containing toothpaste supplemented or not with the use of an essential-oils containing mouthrinse. Orthodontic, multi-strand retention wires are known to be difficult to clean (Levin et al. 2008; Jongsma et al. 2014) and were employed as a generalized model for oral retention sites. Different regimens of oral health care were maintained for 1-week in a group of volunteers, equipped with multi-strand, stainless steel retention wires, after which oral biofilm left-behind after different modes of brushing was evaluated.

Materials and methods

Retention wires, volunteers, inclusion criteria and oral hygiene regimens

In this study, biofilm growth was evaluated on multi-strand, stainless steel retention wires (Quadcat®, PG Supply, Inc., Avon, USA), serving as a model for oral sites that are difficult to reach with a toothbrush. In addition, retention wires are easily removable for evaluation of biofilm formed. Brackets (SPEED System Orthodontics, Cambridge, Canada) were bonded to the buccal side of the first molar and the second premolar bilaterally in the upper arch of 10 healthy volunteers (5 males, age ranging from 24 to 31, 5 females, age ranging from 20 to 37) in agreement with the rules set out by the Ethics Committee at the University Medical Centre Groningen (letter June 23rd, 2011). A power analysis indicated that 10 volunteers would be sufficient to achieve 80% power at an alpha level of 0.0500. The outcome for the sample calculation was bacterial counts in a logarithmic scale which was treated as a continuous variable. The expected difference between groups was set at 0.3, the standard deviation at 0.3, and the correlation coefficient at 0.5. Volunteers were included in the study, provided that they had a healthy and complete dentition, no bleeding upon probing, did not use any medication and were not pregnant. All volunteers were dental students, dentists, orthodontists or dental hygienists. All volunteers granted a written informed consent. Wires with a length of 2 cm were placed between the brackets. The wires were sterilized in 70% ethanol before use and stayed in situ for one week during which the volunteers were instructed to brush twice a day for 2 min with a manual toothbrush (Lactona iQ X-Soft, Lactona Europe B.V., Bergen op Zoom, The Netherlands) on one side of the dentition or with a powered toothbrush (Sonicare DiamondClean®, Philips Nederland B.V., Eindhoven, The Netherlands) on the other side. Proper use of the different toothbrushes was demonstrated to the volunteers. Volunteers were furthermore instructed to use a NaF-sodium lauryl sulphate (NaF-SLS) containing toothpaste without antibacterial claims (Prodent Softmint®, Sara Lee Household & Bodycare, Exton, USA), or a triclosan-containing toothpaste (Colgate Total®, Colgate-Palmolive Company, Piscataway, USA) with antibacterial claims. In addition, the use of the triclosan containing toothpaste was supplemented with the use of an essential-oils containing mouthrinse (Cool Mint Listerine®, Pfizer Consumer Healthcare, Morris Plains, NJ,

USA) (Fig. 1). The oral hygiene products were presented to the volunteers in their original packaging. The order in which the regimens were applied in the different volunteers was determined at random. Volunteers were asked to pick a number corresponding to a certain order of toothpaste/mouthrinse regimens. In between regimens and before the start of the experiment, a washout period of 6 weeks was applied during which only the NaF-SLS containing toothpaste was allowed to be used. The duration of the washout period was based on the results of a pilot study including 5 volunteers that indicated that the composition of the oral biofilm returned to base line values within 5 weeks after use of an antibacterial toothpaste.

Regimens were maintained for 1 week, after which wires were removed and oral biofilm was collected from the wires and the buccal enamel surfaces surrounding the brackets. Enamel biofilms were removed with a sterile cotton swab and in order to obtain enough biofilm for evaluation, the entire buccal enamel surface surrounding the brackets was swabbed. Wires were removed in the morning after breakfast and regular brushing by the volunteers. Wires and cotton swabs containing enamel biofilms were stored in an Eppendorf tube containing 1.0 ml filter sterile reduced transport fluid (RTF) (Syed and Loesche 1972) for transportation from the orthodontic clinic to the laboratory. The collection and evaluation of the biofilm was performed blinded. All samples were given a number and the researchers were not told which type of oral hygiene regimen corresponded with that number. This code was broken for the statistical analysis of the results.
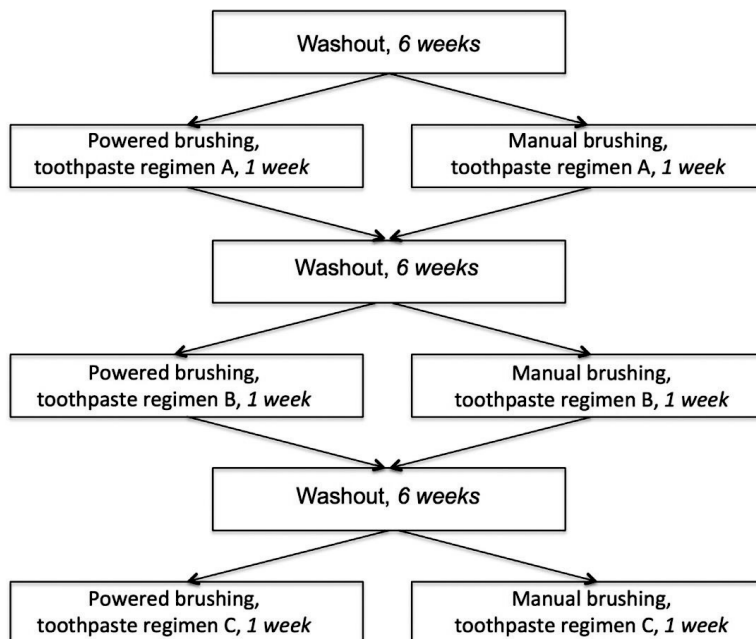
Figure. 1. Schematic representation of the experimental protocol. The study was performed as a split-mouth design. One of the wires, placed on the upper arch of the volunteers, was brushed manually, while the other wire was brushed with a powered toothbrush. The order in which the different toothpaste/mouthrinse regimens were applies was determined at random. The different regimens that were applied consisted of—A NaF-SLS containing toothpaste without antibacterial claims; A triclosan containing toothpaste; A triclosan containing toothpaste combined with a essentals oils containing mouthrinse. During the washout period a NaF-SLS containing toothpaste without antibacterial claims was used by all volunteers.

Upon arrival in the laboratory, retention wires with adhering biofilm and biofilm collected from enamel surfaces were separately sonicated three times for 10 s with 30 s intervals in Eppendorf tubes containing 1.0 ml RTF on ice chilled water, to disperse bacteria. Part of the bacterial dispersions were stored at -80 °C until use for PCR-Denaturing Gradient Gel Electrophoresis (DGGE), while another part was used to determine bacterial number and viability. For enumeration of the numbers of adhering bacteria, bacteria were enumerated in a Bürker-Türk counting chamber, while the percentage viability of the biofilms was evaluated after live/dead staining (*Bac*Light™, Invitrogen, Breda, The Netherlands) of the dispersed biofilms. Live/dead stain was prepared by adding 3 μl of SYTO®9/propidium iodide (1:3) to 1 ml of sterile, demineralised water. Fifteen μl of the stain was added to 10 μl of the undiluted bacterial dispersion. After 15 min incubation in the dark, the number of live and dead bacteria were counted using a fluorescence microscope (Leica DM4000B,

Leica Microsystems Heidelberg GmbH, Heidelberg, Germany) and expressed as a percentage viability. Note that strictly speaking, live/dead staining is not a measure of microbial killing but of membrane damage (Shi et al. 2007; Netuschil et al. 2014). The membrane of live bacteria is permeable to SYTO9, staining both live and dead organisms and yielding green fluorescence. Propidium-iodide can only enter through damaged membranes, where it replaces SYTO9, yielding red fluorescence of dead or damaged cells.

DGGE analysis of in vivo biofilms

After all dispersed biofilms were collected, PCR-DGGE was carried out in order to compare their bacterial composition, as described previously (Jongsma et al. 2014). Briefly, for extraction of DNA, frozen bacterial dispersions were thawed, centrifuged for 5 min at 13,000 × g, washed and vortexed with 200 μl TE-buffer (10 mM Tris–HCl, 1 mM EDTA pH 7.4) and again centrifuged. After DNA extraction, PCR was performed on 100 ng DNA with a T-gradient thermocycler for PCR amplifications. PCR products were analyzed by electrophoresis on a 2.0% agarose gel containing 0.5 μg/ml ethidium bromide. DGGE of PCR products generated with the F357-GC/R-518 primer set was performed, as described by Muyzer et al (Muyzer et al. 1993). The PCR products were applied on 0.08 g/ml polyacrylamide gel in 0.5 × TAE buffer (20 mM Tris acetate, 10 mM sodium acetate, 0.5 mM EDTA, pH 8.3). The denaturing gradient consisted of 30−80% denaturant (100% denaturant equals 7 M urea and 37% formamide). A 10 ml stacking gel without denaturant was added on top. Electrophoresis was performed overnight at 120 V and 60 ºC. Gels were stained with silver nitrate (Zijnge et al. 2006). Each DGGE gel was normalized according to a marker consisting of 7 reference species comprising common bacterial species associated with oral health and disease (Marsh 2006). The reference strains included *Streptococcus oralis* ATCC 35037, *Streptococcus mitis* ATCC 9811*, Streptococcus sanguinis* ATCC 10556, *Streptococcus salivarius* HB, *Actinomyces naeslundii* ATCC 51655, *Lactobacillus* sp.*, Streptococcus sobrinus* ATCC 33478*, Streptococcus mutans* ATCC 10449, *Porphyromonas gingivalis* ATCC 33277 and *Prevotella intermedia* ATCC 49046 (Otten et al. 2012).

Statistical analysis

Data were analyzed with the Statistical Package for Social Sciences (Version 16.0, SPSS Inc., Chicago, IL, USA). A log transformation was used on the data concerning number of bacteria. The distribution of the number of bacteria and the percentage live bacteria were tested for normality. Both number of bacteria and percentage live bacteria were found to be distributed normally. Multiple paired t-test were used to assess pairwise comparisons on the number of bacteria and their percentage viability with brushing modes and oral care regimen as variables. Statistical significance was set at $p < 0.05$.

DGGE gel images were converted and transferred into a microbial database with GelCompar II, version 6.1 (Applied Maths N.V, Sint-Martens-Latem, Belgium). Similarities in bacterial composition of the different biofilms were analysed using a band based similarity coefficient and a non-weighted pair group method with arithmetic averages was used to generate dendograms indicating similarities in composition (Signoretto et al. 2010).

Results

When powered toothbrushing was applied, less bacteria were collected from retention wires than after manual brushing, while enamel surfaces harvested insufficient amounts of biofilm for enumeration, providing a validation for the use of orthodontic, multi-strand retention wires as a model for oral retention sites. Within the regimens involving manual brushing, only the use of an antibacterial, triclosan-containing toothpaste supplemented with an essential-oils containing mouthrinse yielded a significant decrease in the number of bacteria (Table 1). When powered toothbrushing was applied however, significantly less bacteria were collected when using the antibacterial, triclosan-containing toothpaste whether or not supplemented with an essential-oils containing mouthrinse, than when using the NaF-SLS-toothpaste.

Viability of retention wire biofilm was significantly lower after the use of the antibacterial, triclosan-containing toothpaste whether or not combined with an essential-oils containing mouthrinse, when compared to the use of a NaF-SLS-containing toothpaste regardless of the brushing method. Moreover, in case of an antibacterial regimen, biofilm viability was significantly lower after brushing with a powered toothbrush than after manual brushing.

Bacterial composition of biofilms formed on retention wires and enamel under the influence of the different oral hygiene regimens and brushing modes are compared in cluster trees (Fig. 2A and B). Mode of brushing has no influence on the clustering of bacterial composition data, neither on retention wires (Fig. 2A) nor on enamel surfaces (Fig. 2B), as can be seen from the proximity of similarly coloured dots to one another. However, the antibacterial regimens clearly separate from the NaF-SLS regimen, although this is more clear on the retention wires than on enamel surfaces.

These changes in bacterial composition can further be exemplified from the prevalence of the marker strains applied (see Table 2), although it is difficult to find consistent patterns in effects of manual versus powered brushing. However, powered brushing yields a consistent decrease in the prevalence of *P. gingivalis*, both for biofilm collected from retention wires and enamel. Also the prevalence of *S. sanguinis* is consistently lower in case of powered brushing, but this is only the case for biofilm collected from retention wires. On the other hand, the prevalence of *S.*

oralis/S. mitis increases after the use of a powered toothbrush compared to a manual toothbrush. In general, stronger effects of antibacterial regimens on the prevalence of marker stains are seen on retention wires than on enamel surfaces. Prevalences of *S. salivarius, Lactobacillus, S. mutans* and *P. gingivalis* decrease in prevalence on retention wires after use of the antibacterial, triclosan-containing toothpaste and these decreases become more pronounced when use of the antibacterial toothpaste is supplemented with an essential-oils containing mouthrinse. Prevalence of *S. oralis/S. mitis* on retention wires increases after the use of an antibacterial regimen.

Table 1. Number and viability of bacteria retrieved from 1 cm stainless steel retainer wires after manual or powered toothbrushing with a NaF-SLS and an antibacterial, treiclosan-containing toothpaste supplemented or not with the use of an essential-oils containing mouthrinse. The data represent averages ± standard deviations over 10 different volunteers and p-values for the comparisons between different regimes, accounting for a split-mouth design and considering multiple measurements per patient due to the cross-over design (pair-wise comparison).

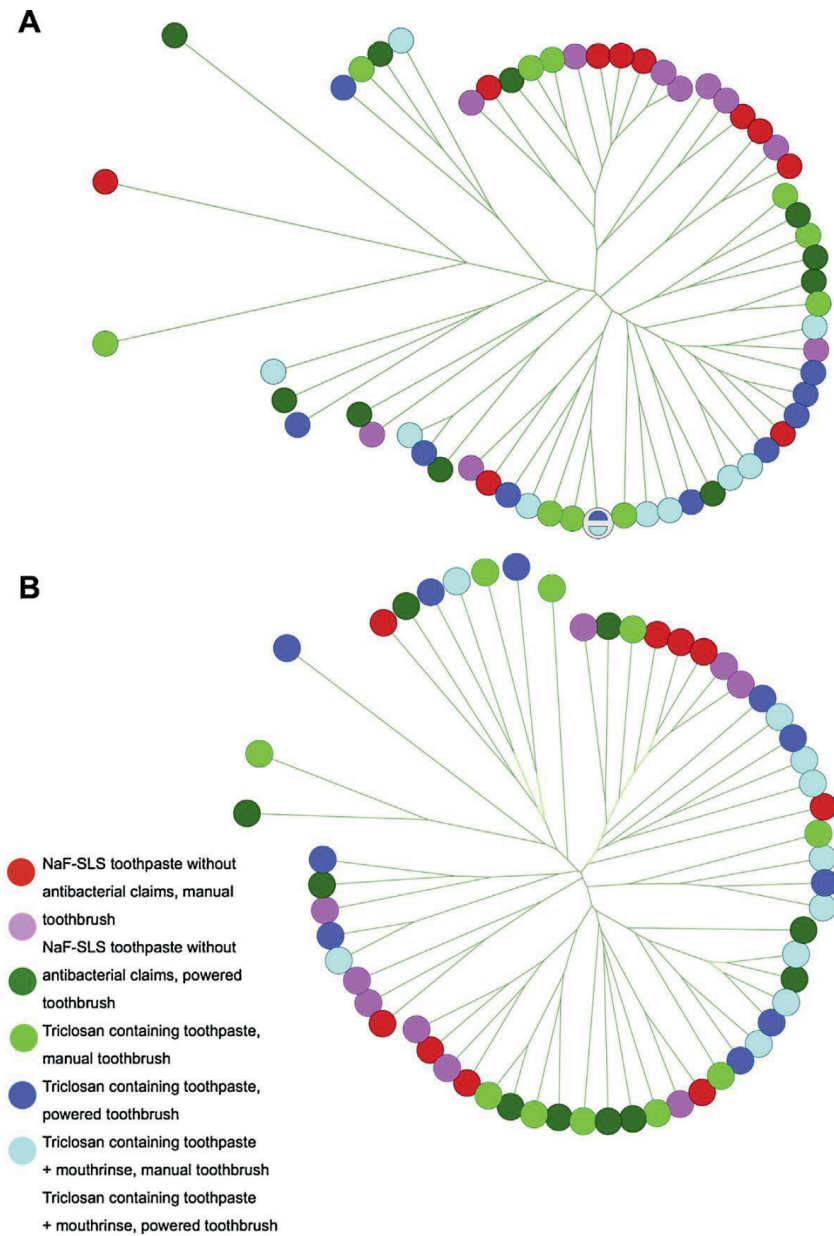| | Average ± S.D | | p-values for number of bacteria | | | | | |
| | | | Manual brushing | | | Powered brushing | | |
	Number of bacteria (Log-units)	%live bacteria	1 NaF-SLS toothpaste	2 Triclosan toothpaste	3 Triclosan toothpaste + mouthrinse	4 NaF-SLS toothpaste	5 Triclosan toothpaste	6 Triclosan toothpaste + mouthrinse
Manual brushing 1 NaF-SLS toothpaste	7.9 ± 0.1	68 ± 12		0.333	0.001	0.333	< 0.001	< 0.001
2 Triclosan toothpaste	7.6 ± 0.2	42 ± 8	< 0.001		0.728	1.000	0.015	< 0.001
3 Triclosan toothpaste + mouthrinse	7.5 ± 0.2	37 ± 5	< 0.001	1.00		0.728	1.000	< 0.001
Powered brushing 4 NaF-SLS toothpaste	7.6 ± 0.1	60 ± 7	0.58	< 0.001	< 0.001		0.015	< 0.001
5 Triclosan toothpaste	7.3 ± 0.3	28 ± 9	< 0.001	0.004	0.228	< 0.001		0.011
6 Triclosan toothpaste + mouthrinse	7.0 ± 0.2	16 ± 4	< 0.001	< 0.001	< 0.001	< 0.001	0.027	
					p-values for % live bacteria			

Figure 2. Clustering trees describing the bacterial compositions of biofilm samples taken from stainless steel retention wires (A) or enamel surfaces (B) in different volunteers using manual or powered toothbrushing in combination with different healthcare regimens. The closer the proximity of similarly coloured dots to one another, the more the composition is alike.

Table 2. Prevalence (%) of marker strains in biofilm samples from stainless steel retention wires or buccal enamel surfaces in different volunteers using manual or powered toothbrushing in combination with different healthcare regimens. 100% indicates that biofilm samples from wires or enamel surfaces in all volunteers contained the indicated marker strain (n = 10 volunteers for all samples).

STRAINS	NaF-SLS toothpaste without antibacterial claims				Triclosan containing toothpaste				Triclosan containing toothpaste + mouthrinse			
	Manual brushing		Powered brushing		Manual brushing		Powered brushing		Manual brushing		Powered brushing	
	Wire	Enamel	Wire	Enamel	Wire	Enamel	Wire	Enamel	Wire	Enamel	Wire	Enamel
S.oralis/S. mitis	20	70	40	50	20	50	50	80	80	40	70	60
S. sanguinis	80	80	20	70	40	60	30	70	60	30	40	50
S. salivarius	30	20	30	10	10	30	10	20	0	10	10	10
A. naeslundii	0	15	0	0	0	10	0	0	0	0	0	0
Lactobacillus	20	20	20	20	10	30	10	10	0	0	0	0
S. sobrinus	30	10	30	30	30	70	30	30	20	40	10	10
S. mutans	30	10	50	0	10	20	10	0	0	0	0	0
P. gingivalis	30	10	10	0	20	10	10	0	0	0	0	0
P. intermedia	0	0	0	0	10	10	0	0	0	0	0	0

Discussion

Stress-relaxation analysis of mechanically compressed biofilms has pointed out that the structure and water content of in vitro biofilm-left behind after powered brushing changes into a direction that makes it more amenable to penetration of chlorhexidine and cetylpiridinium chloride than after manual brushing (He et al. 2014). Here we demonstrate the clinical impact of these in vitro findings. Clinical impact involves a reduction in the viability of in vivo formed biofilms left-behind after powered brushing on retention sites upon the use of an antibacterial triclosan-containing toothpaste with or without supplementation with an essential-oils containing mouthrinse. Thus also clinically, a synergy between mode of brushing and antibacterial-regimen applied exists.

We chose to study in vivo biofilms as formed on orthodontic retention wires after different 1-week regimens of oral health care, as especially multi-strand retention wires possess multiple sites where biofilm is sheltered from mechanical and chemical attack (Jongsma et al. 2013). Therewith retention wires can be considered as a generalized model for biofilm-retention sites in the oral cavity, with as an additional advantage that they are easily replaceable. Biofilm will be more readily left-behind on such retention sites after brushing and in this respect it is telling that in accordance with literature (Praskevas et al. 2006; Van der Weijden et al 2008), biofilm could be collected from retention wires both after manual as well as after powered brushing (see Table 1), but hardly from smooth enamel surfaces. Powered toothbrushing generates a larger energy input into a biofilm than manual toothbrushing, amounting around 0.1 mW for a manual brush and 110 mW for sonic brushing (Veeregowda et al. 2012). Since biofilms have visco-elastic properties, biofilm will first expand due to energy input during powered brushing after which it will detach (Cense et al. 2006; Rmaile et al. 2014; Peterson et al. 2015). However, biofilm left-behind will remain in its expanded, more open state enabling better antibacterial penetration, which explains why in the current study we observe a greater reduction in biofilm viability upon application of antibacterial regimens when using a powered brush versus a manual brush. Note that the use of either one of the brushing methods without the use of an oral antibacterial regimen hardly affected the viability of the biofilm compared to an unbrushed biofilm (Jongsma et al. 2013). This indicates that the decrease in viability is solely attributed to the oral antibacterial

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agents, and not to toothbrushing itself (MacNeill et al.1998). This shows the existence of a synergy between mode of toothbrushing and antibacterial action with clinically demonstrable effects. General long-term (>2 months) benefits of powered toothbrushing and antibacterial regimens have been described in the literature (Stoeken et al. 2007; Cortelli et al. 2013; He et al. 2013; Riley and Lamont 2013). Although our study only extends over a time period of one week, with a relatively small sample size and involving volunteers with a high level of oral hygiene awareness predominantly, we believe that the clinical effects observed can be extrapolated to longer-term effects in the general population, as structural changes in the biofilm are underlying to the mechanisms of enhanced penetration of antibacterials in biofilm left-behind.

Also other clinical studies, not geared towards demonstrating a synergy between mode of brushing and antibacterial use, have shown that oral biofilm formation is reduced after the use of antibacterial toothpastes (He et al. 2013; Riley and Lamont 2013), with minor effects of the supplemental use of an essential-oils containing mouthrinse (Cortelli et al. 2013; Stoeken et al. 2007; Tufekci et al. 2008). However, we saw sizeable further reduction of biofilm viability after supplemental use of an essential-oils containing rinse (Table 1), along with changes in bacterial composition of the biofilm (Fig. 2) that we earlier attributed to adsorption of triclosan to bacterial cell surfaces altering their cell surface hydrophobicity to stimulate removal by hydrophobic ligands (Jongsma et al. 2014).

DGGE analysis shows that the composition of biofilm formed on stainless steel retention wires differs from biofilm formed on enamel (Table 2). Atomic force microscopy has pointed out that bacterial adhesion forces to different materials used in orthodontics, including stainless steel, differ from the ones exerted by enamel surfaces in a strain-specific fashion (Mei et al. 2009). Accordingly this explains (Wessel et al. 2014) why biofilms on different materials have a different bacterial composition, including the enamel and stainless steel surfaces as involved here. Furthermore, the biofilm taken from retention wires will be more mature than biofilm taken from smooth enamel surfaces, as more biofilm will be left-behind after brushing on retention wires than on smooth enamel surfaces on which biofilm has to develop newly after each brushing. The composition of a newly formed biofilm as regularly developing on smooth enamel is thus different than that from a mature

biofilm as in interproximal areas and fissures (Marsh 2004), the latter likely being comparable with biofilm found on the retention wires.

Further enhancing the synergy between powered toothbrushing and oral antibacterials may be a goal of future research, either by changing the design of powered toothbrushes or use of different oral antibacterials. Since oral sites where biofilm is most frequently left-behind, are also most susceptible to disease, this approach may proof to have major impact on oral health.

Conclusions

This study shows that a synergy exists between powered toothbrushing and antibacterial regimen with clinically demonstrable effects, most notably on the viability of biofilm left-behind after brushing, but also with regard to the amount and composition of the biofilm.

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Chapter 4

Reversible cell wall deformation and development of chlorhexidine resistance in *S. mutans versus S. aureus*

Marieke van de Lagemaat Valerie Stockbroekx, Melissa Dijk, Vera Carniello, Joop de Vries, Henny C. van der Mei, Henk J. Busscher, Yijin Ren

Abstract

Oral antimicrobials with non-selective antibacterial efficacy, such as chlorhexidine can be effective in reducing oral biofilm, but bear the risk of inducing resistance in specific strains. Clinically, strains such as *Staphylococcus aureus* have been found resistant to chlorhexidine, while in dental practice oral bacterial strains, including *Streptococcus mutans* have remained largely susceptible to chlorhexidine. The aim of this chapter is to speculate on the mechanisms through which *S. aureus* adapts resistance against chlorhexidine *versus S. mutans* remaining susceptible. Chlorhexidine exposure of adhering bacteria to (sub)-MIC concentrations of chlorhexidine yielded reversible, nanoscopic cell wall deformation in *S. mutans*, but not in *S. aureus*, indicative of loss of intracellular, cytoplasmic pressure in *S. aureus*. Although overall cell surface properties of both strains did not significantly change, propidium iodine staining demonstrated that the *S. aureus* cell membrane was indeed more easily damaged than the *S. mutans* cell membrane. Significantly, metabolic activity of *S. mutans* changed little upon exposure to chlorhexidine, while *S. aureus* metabolic activity became much higher. Concurrently, repeated culturing in presence of chlorhexidine demonstrated that chlorhexidine resistance was easy to induce in *S. aureus*, but not in *S. mutans*. Exact interpretation of these data is difficult. *S. aureus* may adapt a high metabolic activity to survive chlorhexidine attack, e.g. by activating efflux pumps or opening of membrane channels to decrease the intracellular chlorhexidine concentration. This may cause loss of intracellular pressure yielding cell wall deformation, and at the same time stimulate development of chlorhexidine resistance. In *S. mutans*, cell wall deformation was reversible within 15 min after exposure to chlorhexidine, suggesting spontaneous, strong cell wall self-repair. Due to cell wall self-repair, *S. mutans* may be unable to effectively reduce the chlorhexidine concentration in its interior, preventing its survival and development of a resistant progeny.

Introduction

The increasing resistance of bacteria to antimicrobials occurring over in past decades has become a major concern in global, public health (Howard et al. 2013; Roca et al. 2015; WHO.net 2018). Chlorhexidine is a non-selective antimicrobial and widely used in healthcare settings as a disinfectant and antiseptic for the skin, hands and in the oral cavity (Zhang et al. 2013; Okada et al. 2016). Microbial resistance against chlorhexidine has long been considered rare if not impossible (Schlett et al. 2014; Saleem et al. 2016), but has recently been reported in *Staphylococcus aureus*, coagulase-negative staphylococci , *Klebsiella pneumoniae, Pseudomonas aeruginosa*, *Acinetobacter baumanii* and *Candida albicans*. For these strains, the intensity of chlorhexidine use was found proportional with the development of resistance (Block and Furman 2002). Importantly, after acquiring resistance to chlorhexidine, Acinetobacter spp., *K. pneumoniae* and Pseudomonas spp. seem to have a potential for developing cross-resistance to some antibiotics (Kampf 2016). Horizontal gene transfer of chlorhexidine resistance at sub-MIC concentrations of chlorhexidine has been reported in *Escherichia coli* (Jutkina et al. 2018). Based on the threatening development of bacterial resistance against chlorhexidine, restricted use of chlorhexidine to applications with a clear patient benefit and elimination of its use in applications without clear benefit have been suggested (Kampf 2016).

Chlorhexidine carries positively charged groups that can bind to negatively charged bacterial cell surfaces (Neu and Marchall 1990) to cause cell wall damage and catastrophic leakage of intracellular material, eventually resulting in cell death (Gilbert and Moore 2005). However, transcriptomic responses of bacteria to chlorhexidine exposure have also been reported. Exposure of *P. aeruginosa* to 4 μg/ml chlorhexidine yielded downregulation of genes involved in membrane transport, oxidative phosphorylation, electron transport and DNA repair, while multidrug efflux pump genes were upregulated (Nde et al. 2009). These properties provide chlorhexidine with a broad spectrum activity against both Gram-positive and Gram-negative bacteria, yeast, dermatophytes and lipophilic viruses (Beyth et al. 2003; Denton 2001). In appropriately low concentrations, chlorhexidine is safe for use in the oral cavity (James et al. 2017) and in dentistry it has become the "gold" standard in anti-bacterial mouthrinses. Bacterial resistance against chlorhexidine in oral bacteria is still rare, despite it hampered deep penetration in oral biofilms

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(Zaura-Arite et al. 2001) and low concentration presence due to its prolonged substantive presence on oral soft tissues (Beyth et al. 2003).

Therefore, as an aim of this chapter, it seems worthwhile to speculate on the mechanisms through which *S. aureus*, an emerging oral pathogen involved in peri-implantitis (McCormack et al. 2015), adapts resistance against chlorhexidine versus *S. mutans*, a cariogenic oral pathogen (Loesche 1986), remaining susceptible. To this end, nanoscopic deformation of the cell walls of both strains upon exposure to chlorhexidine were determined using surface enhanced fluorescence (Li et al. 2014), as an indicator of cell wall damage and loss of intracellular pressure (Carniello et al. 2018). Cell wall damage at a more microscopic level was studied after propidium iodine staining of chlorhexidine exposed bacteria using fluorescence microscopy, while cell surface hydrophobicities and zeta potentials were measured to assess overall changes to the cell surface. Metabolic activity was monitored from MTT conversion, to evaluate whether responses of the bacteria to chlorhexidine encouraged or discouraged processes in the organisms that required metabolic activity. In addition, bacteria were cultured repetitively under sub-MIC chlorhexidine pressure to determine the ease at which resistant variants could develop. A resistant variant obtained of *S. aureus* was also subjected to the above experiments.

Material and Methods

Bacterial Strains and Growth Conditions

In order to allow measurement of cell wall deformation using surface enhanced fluorescence, two green-fluorescent bacterial strains had to be selected. *S. mutans* UA159 PDM15[GFP] (Deng et al. 2007) and *S. aureus* ATCC 12600[GFP] were grown on Todd Hewitt Broth (THB; OXOID, Basingstoke, England) and Tryptone Soya Broth (TSB; OXOID) agar plates, respectively. THB agar plates were supplemented with 10 µg/mL erythromycin (Sigma-Aldrich, St. Louis, MO, USA) and TSB agar plates with 10 µg/mL tetracycline (Sigma-Aldrich). One colony of *S. mutans* was inoculated in THB supplemented with 10 µg/mL erythromycin and similarly for *S. aureus* in TSB supplemented with 10 µg/mL tetracycline. *S. mutans* UA159 PDM15[GFP] was grown at 37°C with 5% CO_2 and *S. aureus* under aerobic conditions. After 24 h, these

precultures were inoculated in 200 mL of the appropriate growth media without antibiotics and cultured for 16 h at 37°C.

Bacterial cultures were harvested by centrifugation (5 min, 5000 g, 10°C) and washed twice with adhesion buffer (2 mM potassium phosphate, 50 mM KCl and 1 mM $CaCl_2$, pH 7.0*)*. After washing, bacteria were resuspended in adhesion buffer with THB or TSB (1:30) to maintain metabolic activity. The bacterial suspension was sonicated (3 x 10 s, 30 W) in an ice-water bath (Vibra Cell Model 375, Sonics and Materials Inc., Danbury, CT, USA) and the bacteria were enumerated using a Bürker-Türk counting chamber and suspensions (3 x 10^8/mL) were diluted in adhesion buffer (containing THB or TSB (1:30)) to the appropriate bacterial concentration for each experiment. All experiments were done in triplicate with different bacterial cultures.

Minimal Inhibitory (MIC) and Minimal Bactericidal Concentration (MBC) of Chlorhexidine

A chlorhexidine containing mouthrinse (Curasept ADS 212, 0.12% (1200 μg/mL), Curaden Benelux Division, Velddriel, The Netherlands) was used as a chlorhexidine source. The mouthrinse contained next to chlorhexidine digluconate also aqua, xylitol, propylene glycol, PEG-40 hydrogenated castor oil, ascorbic acid, aroma, poloxamer 407, sodium metabisulfite, sodium citrate, CI 42090. Bacteria were exposed to twofold dilutions of the mouthrinse in sterile water. Dilutions were added to bacterial suspensions (2 x 10^5/mL in medium) into a 96-well plate and incubated at 37°C for 24 h, under the appropriate conditions. After incubation, the minimal inhibitory concentration (MIC) was taken as the lowest chlorhexidine concentration at which no visible growth was observed. Wells displaying no visible growth were subsequently plated on THB or TSB agar plates and incubated for another 24 h at 37°C. The minimal inhibitory bactericidal concentration (MBC) was taken as the lowest concentration for which no colonies were visible on the agar plates (Wiegand et al. 2008).

Development of Chlorhexidine-Resistant Bacterial Strains

For the development of chlorhexidine-resistant bacterial strains, pre-cultures of *S. mutans* UA159 PDM15[GFP] and *S. aureus* ATCC 12600[GFP] were diluted 1 : 100 in fresh growth medium (THB or TSB) and grown for 4 days with chlorhexidine added at its MIC. After 4 days, the cultures were diluted 1 : 100 in fresh medium with chlorhexidine added at 0.6 µg/mL higher concentration than in the previous step. This procedure was repeated for maximally 28 steps for *S. aureus*, while after the first step *S. mutans* did not show growth anymore, while monitoring the MIC and MBC of the resulting cultures (see above). In order to check whether prolonged culturing under chlorhexidine exposure affected bacterial fluorescence, fluorescence was monitored regularly on agar plates using the *In Vivo* Imaging System (IVIS, Lumina II, Caliper LifeScience, Hopkinton, MA, USA), with an excitation wavelength of 465 nm and emission in a range from 515-575 nm. Cultures after repeated growth in presence of chlorhexidine were stored in a -80ºC freezer, with 7% dimethylsulfoxide added.

Surface Enhanced Fluorescence (SEF)

For SEF, bacteria suspended in adhesion buffer supplemented with growth medium (30 : 1), were injected in a parallel plate flow chamber, possessing a glass top-plate and a polished stainless steel bottom-plate (surface enhanced fluorescence can only be measured on metallic substrata (Lee K et al. 2011)). Fluorescence was measured using a bio-optical imaging system (see above). Images had a field of view of 7.5 × 7.5 cm, while exposure time was set at 10 s, employing a focal ratio of 1. Temperature throughout an experiment was maintained at 20ºC. With the Living Image software package 3.1 (Caliper LifeScience), a user-defined region-of-interest was constructed in each image of 4.0 × 1.0 cm to calculate the average fluorescence radiance (photons s^{-1} cm^{-2} $steradian^{-1}$).

For measurements, background fluorescence in the region-of-interest was measured in a flow chamber filled with adhesion buffer, supplemented with medium. This background fluorescence radiance was subtracted from all fluorescent radiances measured. Next, bacteria were injected into the flow chamber. Sedimentation of all suspended bacteria and their adhesion was allowed in the absence of flow under the

influence of gravity, while acquiring images every 15 min for 3 h, previously found sufficient for complete sedimentation (Li et al. 2014). Subsequently, the flow chamber was filled with 5 mL chlorhexidine solutions at two-fold serial dilutions downward from the MIC, and fluorescent image acquisition continued every 15 min for an additional 2 h. Adhesion buffer supplemented with medium (30 : 1) was used as a control.

Assuming the green-fluorescent protein is evenly distributed intracellularly, the increase in fluorescent radiance, due to adhering bacteria relative to planktonic bacteria was expressed as total fluorescence enhancement (TFE) (Li et al. 2014), according to

$$\text{TFE}(t) = \frac{R(t) - R_0}{R(0) - R_0}$$

(1)

where R(t) is the fluorescence radiance at time t, R(0) is the fluorescence radiance measured for a planktonic suspension and R_0 is the fluorescence radiance of the background.

Bacterial Membrane Damage

To determine the percentage of membrane damage upon chlorhexidine exposure, bacteria were stained with red-fluorescent propidium iodide (Live/Dead Baclight Bacterial Viability, ThermoFisher Scientific, Waltham, MA, USA), that is only able to enter membrane damaged bacteria (Lehtinen, Nuutila and Lilius. 2004). Membrane damage was determined according to a similar protocol as the SEF, but now after incubation with chlorhexidine, 15 μL dead stain 20 mM propidium iodide was added to each well and left for 15 min in the dark after which fluorescence was imaged using a Leica DM4000B fluorescence microscope with a 40x water objective. The corresponding Leica software was used to make 3 images per well. Green-fluorescent bacteria were taken to enumerate the total number of bacteria in an image, while those displaying red-fluorescence as well, were taken as membrane-damaged (Lehtinen, Nuutila and Lilius. 2004). As controls, the full strength mouthrinse (1200 μg/mL chlorhexidine) and 70% ethanol were used.

Metabolic Activity

The influence of the different concentrations of chlorhexidine on bacterial metabolic activity was determined with the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay (Krom et al. 2007). According to a similar protocol as SEF, 3 h bacterial adhesion and 15 min and 2 h exposure to chlorhexidine using a 96 wells plate, MTT solution (0.5 mg/mL thiazolyl blue tetrazolium bromide in phosphate buffer, 10 mg/mL glucose and 0.1 mM menadion) was added to each well and left for 30 min at 37°C in the dark. After 30 min, wells were washed once with water and acid-isopropanol (5% 1 M HCl in isopropanol) was added to the wells for 15 min. After 15 min, 100 µL of the suspension was removed and added to a new 96-wells plate and absorptions were measured at 560 nm with the FluoStar Optima plate reader (BMG Labtech, Offenburg, Germany). All experiments were performed in triplicate with different bacterial cultures. The data were normalized with respect to the metabolic activity after 3 h adhesion, i.e. before chlorhexidine exposure.

Overall bacterial cell surface characterization

To determine whether the overall bacterial cell surfaces were affected by chlorhexidine exposure Microbial Adhesion to Hydrocarbons (kinetic MATH assay) and zeta potentials were measured after different exposure times of the bacteria to chlorhexidine. MATH was carried out as previously described (Lichtenberg et al. 1985). Briefly, bacteria were resuspended in 3 mL adhesion buffer pH 7.0 containing 1:20 hexadecane to an optical density at 600 nm between 0.4 and 0.6 (initial absorbance at time zero [A0]) as photospectrometrically measured (Spectronic 20 Genesys, Spectronic Instruments, Rochester, NY, USA). After vortexing the suspension for 10 s and settling of the bacteria for 10 min, the optical density was measured again (absorbance at time t [At]), and this procedure was repeated for five more times, to allow calculation of the initial rate of bacterial removal from the aqueous phase according to

$$\text{Rate of initial removal} = \lim_{t \to 0} \frac{\mathrm{d}}{\mathrm{d}t} \log\left(\frac{A_t}{A_0} \times 100\right)$$

(2)

where t is vortexing time.

The charge properties of the bacterial surfaces were determined by measuring the electrophoretic mobility using a bacterial suspension (3×10^8 bacteria/mL) in adhesion buffer pH 7.0. Particulate micro-electrophoresis was carried out on a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, United Kingdom). Electrophoretic mobilities were converted into zeta potentials, employing the Helmholtz-Smoluchowski equation (Van der Wal et al. 1997).

Statistical Analysis

Data were statistically analyzed using paired, two tailed Student's t-tests with Microsoft Excel 2010. Significance was established at $p < 0.05$.

Results

The initial MIC of chlorhexidine was 2.4 µg/mL for *S. mutans* UA159 PDM15[GFP] and for *S. aureus* ATCC 12600[GFP]. The MBC of chlorhexidine for *S. aureus* ATCC 12600[GFP] was the same as its MIC, while for *S. mutans* UA159 PDM15[GFP] it was 4.8 µg/mL. No resistance against chlorhexidine could be invoked in *S. mutans* by repeated culturing in presence of chlorhexidine. However, after 28 steps of culturing in presence of chlorhexidine, *S. aureus* ATCC 12600[GFP] had acquired a resistance to chlorhexidine of up to 8 times its initial MIC. A resistant variant of *S. aureus* ATCC 12600[GFP] resistant to 9.6 µg/mL chlorhexidine, was subsequently involved in all further experiments.

SEF demonstrated increased cell wall deformation in all adhering strains upon exposure to chlorhexidine (Fig. 1). For *S. mutans*, deformation as concluded from increased SEF, was most evident at the highest chlorhexidine concentration. Importantly, cell wall deformation, i.e. increased SEF, was reversible in *S. mutans*, but not in *S. aureus*.

Exposure for 2 h to chlorhexidine at 1.2 mg/mL and to 70% alcohol led to 100% cell membrane damaged bacteria in both *S. aureus* ATCC 12600[GFP] (Fig. 2A), its chlorhexidine resistant variant (Fig. 2B) and *S. mutans* UA159 PDM15[GFP] (Fig. 2C). At lower chlorhexidine concentrations however, 40%-50% of all *S. aureus* ATCC

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12600^{GFP} became cell membrane damaged, allowing entry of red-fluorescence propidium iodine into the bacteria. The resistant *S. aureus* variant and the *S. mutans* strain showed hardly any cell membrane damaged bacteria in their population. Cell membrane damage did not result in changes in overall cell surface properties, and all strains remained hydrophilic (low initial removal rates by hexadecane) and kept possessing similarly negative zeta potential prior and after exposure to chlorhexidine (Fig. 2D).

Metabolic activity of the bacteria was measured 15 min and 2 h after exposure to chlorhexidine in polystyrene wells. For both *S. aureus* and its chlorhexidine resistant variant as well as for *S. mutans*, metabolic activity hardly changed upon exposure to chlorhexidine at 15 min after chlorhexidine exposure. However, 2 h after exposure changes in metabolic activity of both *S. aureus* strains had increased five-fold, while metabolic activity change of *S. mutans* remained similarly low as at 15 min.
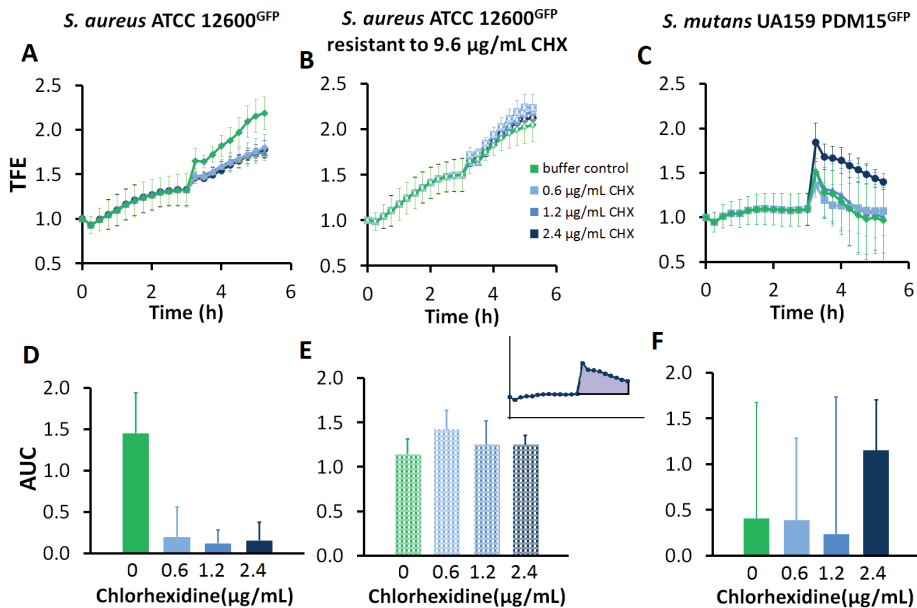
Figure 1. Cell wall deformation in *S. aureus* and *S. mutans* adhering to stainless steel upon exposure to chlorhexidine. (A) Total fluorescence enhancement (TFE) as a function of exposure time to different chlorhexidine concentrations for *S. aureus* ATCC 12600^{GFP}. Stable cell wall deformation due to adhesion only was established in buffer during 3 h after which chlorhexidine was added. (B) Same as (A) for *S. aureus* ATCC 12600^{GFP}, made resistant to 9.6 µg/mL chlorhexidine. (C) Same as (A) for *S. mutans* UA159 PDM15^{GFP}. (D) Increases in total fluorescence enhancement expressed as AUC upon exposure of *S. aureus* ATCC 12600^{GFP} to different chlorhexidine concentrations. Inset represents the area under the curve (AUC) after adding of chlorhexidine (in purple), taken with respect to the

stationary level in TFE observed after 3 h. (E) Same as (D) for *S. aureus* ATCC 12600[GFP], made resistant to 9.6 μg/mL CHX. (F) Same as (D) for *S. mutans* UA159 PDM15[GFP].
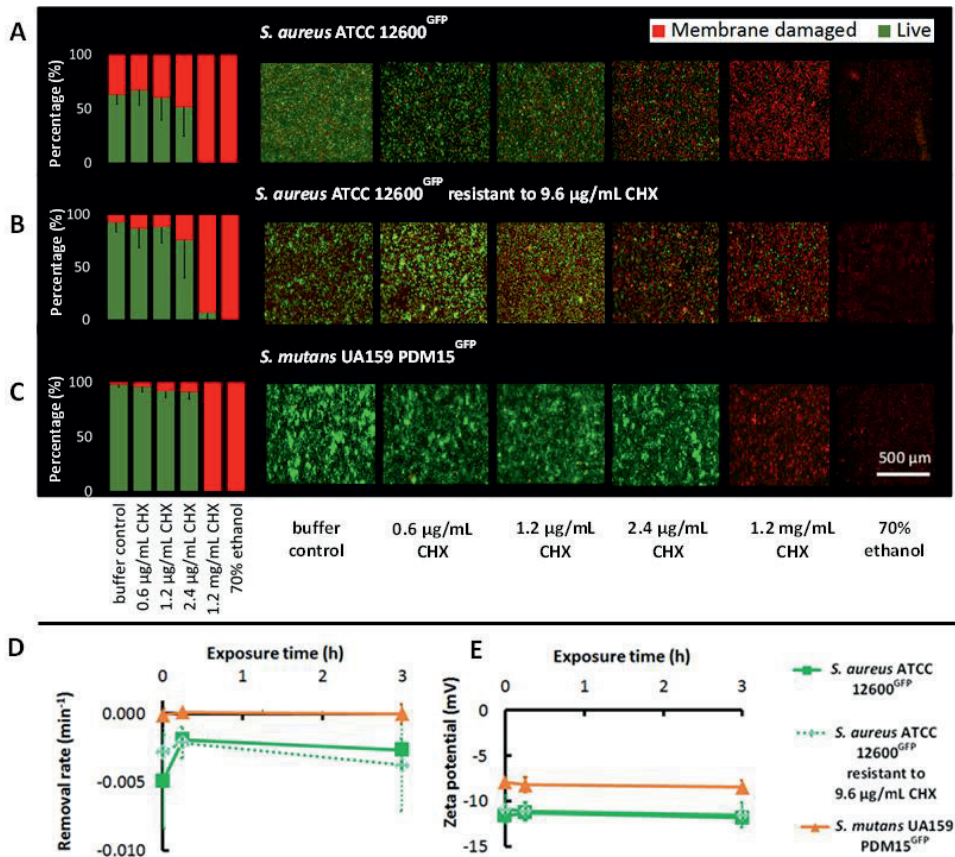
Figure 2. Cell membrane damage and overall cell surface properties in *S. aureus* and *S. mutans* upon exposure to chlorhexidine. (A) Percentage cell membrane damaged bacteria after 2 h exposure time to different chlorhexidine concentrations for *S. aureus* ATCC 12600[GFP]. Cell membrane damaged was inferred from red-fluorescence after staining with propidium iodide. Red-fluorescence prior to exposure to chlorhexidine was taken as a 100% level (B) Same as (A) for *S. aureus* ATCC 12600[GFP], made resistant to 9.6 μg/mL chlorhexidine. (C) Same as (A) for *S. mutans* UA159 PDM15[GFP]. (D) Initial removal rates of bacteria from an aqueous phase (adhesion buffer) by hexadecane as a function of time exposed to 1x MIC chlorhexidine. Error bars represent standard errors over measurements on three different bacterial cultures. (E) Zeta potentials of bacteria in saliva buffer as a function of time exposed to 1x MIC chlorhexidine. Error bars represent standard errors over measurements on three different bacterial cultures.
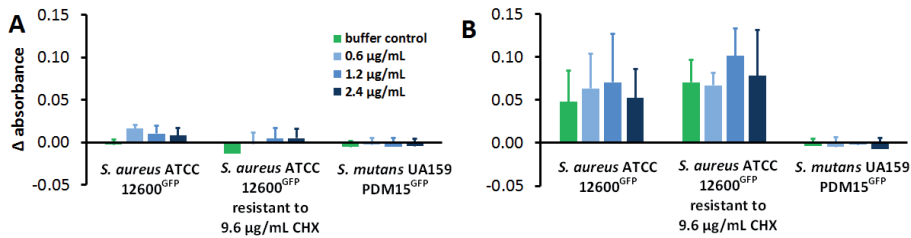
Figure 3. Change in metabolic activity of *S. aureus* ATCC 12600[GFP], its chlorhexidine resistant variant and *S. mutans* UA159 PDM15[GFP] after addition of different concentrations of chlorhexidine, expressed with respect to the metabolic activity measured just prior to chlorhexidine exposure. (A) Metabolic activity of the bacterial strains after exposure to chlorhexidine for 15 min. (B) Metabolic activity of the bacterial strains after exposure to chlorhexidine for 2 h.

Discussion

With increasing use of chlorhexidine for the prevention of nosocomial and community-associated infections and supported by evidence from large randomized clinical trials on the important role of chlorhexidine in reducing the occurrence of MRSA and VRE (Climo et al. 2013; Huang et al. 2013; Miller et al. 2012), concerns have arisen about the potential emergence of chlorhexidine resistant strains (Wang et al. 2008). Here we demonstrate important differences between *S. aureus* and *S. mutans* in their response to chlorhexidine exposure, including development of resistance against chlorhexidine, that may be useful to understand and possibly prevent use of chlorhexidine leading to resistant in specific bacterial strains. To this point, *S. mutans* could not be cultured in presence of chlorhexidine to create a resistant variant, while *S. aureus* could. On the basis of the experiments performed, we will now present a speculative explanation for this difference.

The cell wall of these two Gram-positive bacterial strains is composed of a relatively thick layer of peptidoglycan, designed to resist intracellular turgor pressure and maintain cell shape (Dover et al. 2015). Cell shape is compromised by the adhesion forces a bacterium experiences when it adheres to a surface, which leads to nanoscopic deformation of the cell wall. When the adhesion force experienced, matches the opposing elastic forces from the peptidoglycan layer and the intracellular turgor pressure, cell wall deformation stabilizes (see Fig. 1). Upon exposure to chlorhexidine, cell wall deformation increases in both strains, indicative of the formation of holes in the membrane, leading to leakage of intracellular material

(Gilbert and Moore 2005) and therewith a loss of turgor pressure, leading to ongoing deformation. As an advantage of SEF over microscopic technique, it measures over several millions of bacteria, while microscopic techniques yield visualization of the damage. After exposure to chlorhexidine, numerous spots of indentation on the cell wall, presumable microscopic holes, were found in both *Bacillus subtilis* and *Escherichia coli*. The number of holes increased with exposure time to chlorhexidine and concentration (Cheung et al. 2012). This indicates that at a microscopic level, the ability of propidium iodine to penetrate membrane damaged bacteria (Lehtinen, Nuutila and Lilius. 2004). Also depends on microscopic damage to more outer layers, including the peptidoglycan layer. Permanent changes in the cell wall of the Gram-negative *Pseudomonas stutzeri* affected membrane permeability, while in addition chlorhexidine-resistant *P. stutzeri* were larger with thicker cell walls than susceptible parents (Tattawasart et al. 2000). This is not withstanding, that in our study, gross overall damage to the cell surface could not be concluded from changes in cell surface hydrophobicity and charge.

Opposite as in *S. aureus*, *S. mutans* evidently repairs the holes formed during chlorhexidine exposure, and cell wall deformation returns to the level observed prior to deformation. In *S. aureus*, deformation is ongoing, possibly aided by the switching on of efflux pumps or opening of membrane channels, as observed in *P. aeruginosa* (Nde et al. 2009). Evidence for these energy consuming actions in *S. aureus* can be found in their increased metabolic activity upon chlorhexidine exposure (Fig. 3B). The self-repair of membrane holes at no additional energy expense in *S. mutans* might be simply explained by physico-chemical redistribution of lipids in the deformed membrane. However, due to this spontaneous self-repair and in absence of activation efflux pumps, membrane channel opening or other means to remove intracellular chlorhexidine, *S. mutans* becomes unable to survive intracellular chlorhexidine and dies without the opportunity to develop resistance, as *S. aureus* evidently does.

Concluding, we speculated on differences in mechanisms by which *S. aureus* and *S. mutans* may or may not acquire resistance against chlorhexidine. Important in this respect, is the suicidal self-repair of cell wall damage upon chlorhexidine exposure in *S. mutans*, that should be maintained in order to avoid development of resistant variants, as occurring in *S. aureus*, lacking a similar self-repair mechanism. Considering the large use of chlorhexidine in oral health care, it is important to gain

better understanding of how chlorhexidine-resistance develops, in order to take measures to prevent future development of chlorhexidine-resistance in oral bacteria. Further experiments, using atomic force microscopy or scanning electron microscopy may provide confirmation of the formation of microscopic holes in bacterial cell walls or membranes, as alluded to in the speculative mechanisms described above.

Author contributions

M. van de Lagemaat and V.J.E.O. Stockbroekx contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; M. Dijk, contributed to data acquisition, analysis, and interpretation, critically revised the manuscript; V. Carniello contributed to data acquisition, critically revised the manuscript; H.J. Busscher, H.C. van der Mei and Y. Ren, contributed to conception, design, data analysis, and interpretation, drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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Chapter 5

**Comparison of methods
to evaluate
bacterial contact-killing materials**

*Marieke van de Lagemaat, Arjen Grotenhuis, Betsy van de Belt-Gritter,
Steven Roes,t Ton J.A. Loontjens, Henk J. Busscher,
Henny C. van der Mei, Yijin Ren.*

Abstract

Cationic surfaces with alkylated quaternary-ammonium groups kill adhering bacteria upon contact by membrane disruption and are considered increasingly promising as a non-antibiotic based way to eradicate bacteria adhering to surfaces. However, reliable in vitro evaluation methods for bacterial contact-killing surfaces do not yet exist. More importantly, results of different evaluation methods are often conflicting. Therefore, we compared five methods to evaluate contact-killing surfaces. To this end, we have copolymerized quaternary-ammonium groups into diurethane dimethacrylate/glycerol dimethacrylate (UDMA/GDMA) and determined contact-killing efficacies against five different Gram-positive and Gram-negative strains. Spray-coating bacteria from an aerosol onto contact-killing surfaces followed by air-drying as well as ASTM E2149-13a (American Society for Testing and Materials) were found unsuitable, while the Petrifilm® system and JIS Z 2801 (Japanese Industrial Standards) were found to be excellent methods to evaluate bacterial contact-killing surfaces. It is recommended however, that these methods be used in combination with a zone of inhibition on agar assay to exclude that leakage of antimicrobials from the material interferes with the contact-killing ability of the surface.
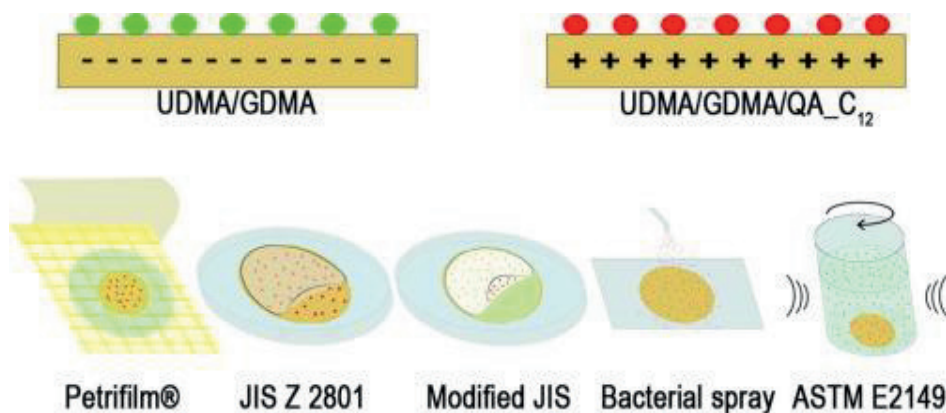
Introduction

Bacterial adhesion and subsequent biofilm formation can be a costly problem in many fields. Examples can be found in e.g. food processing and packaging industry, drinking water systems, in the marine environment, on surfaces exposed to a hospital environment, including dental restorative materials and the surfaces of biomaterials implants and devices. Especially in the biomedical arena, bacterial adhesion can yield life-threatening diseases (Busscher et al. 2012; Hasan, Crawford, Ivanova 2013).

Different types of coatings are being considered as antibacterial or infection-resistant that are either non-adhesive to bacteria such as hydrophobic coatings (Brady 2000; Jansen and Kohnen 1995), polyethylene glycol (PEG) brush coatings (Dalsin and Messersmith 2005; Norde and Gage 2004), hydrogel coatings (Yao et al. 2013), coatings with nanoparticles (Taheri et al. 2014) or antibiotic releasing coatings (Kazemzadeh-Narbat et al. 2010), which are aimed to yield high particle or antibiotic concentrations around a biomaterials implant or device in order to kill the bacteria present (Cado et al. 2013). A drawback of these 'release-killing' materials is, that they all show a high-burst release upon insertion in the human body, followed by a low-level tail-release that can extend to several years. Since the low-level tail-release often yields concentrations insufficient for killing but also far below the minimal inhibitory concentration for growth, tail-release has been associated with the development of antibiotic-resistant strains (Neut et al. 2003; van de Belt et al. 1999).

Polymers containing covalently bonded antimicrobial moieties, such as immobilized quaternary ammonium compounds, possess the unique feature of bacterial 'contact-killing' (Tiller et al. 2001). Provided the cationic charge density (Kugler, Bouloussa, Rondelez 2005; Murata et al. 2007), on the surface is above 1014 positive charges per cm2 and created through alkylated ammonium groups with appropriate alkyl chain lengths (Siedenbiedel and Tiller 2012), adhering bacteria will be killed upon contact by severe membrane disruption through extremely strong electrostatic attraction (Asri et al. 2014). Bacterial killing upon adhesion to cationic quaternary ammonium coated surfaces has been shown in many in vitro studies (Andresen et al. 2007; Asri et al. 2014; Cleophas et al. 2014; Fu et al. 2005; Haldar et al. 2006; Imazato et al. 2003; Klink et al. 2012; Mellouki et al. 1989; Murata et al. 2007; Tiller et al. 2001; Yue et al. 2015), while in vivo efficacy of cationic coatings has been demonstrated in rats (Gottenbos et al. 2003) and sheep (Schaer et al. 2012). Bacterial contact-killing

materials and coatings are increasingly promising as a non-antibiotic based way to eradicate bacteria adhering to surfaces, but largely confine themselves to coatings comprised of quaternarized ammonium compounds with a suitable hydrocarbon tail length. Moreover, cell wall damage may often be so severe leaving little possibilities for adhering bacteria to stay alive in a growth inhibited state, whatever alive may mean for a bacterium (Hammes, Berney, Egli 2011).
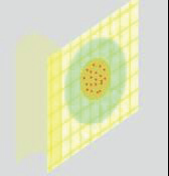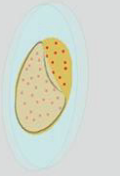
Despite their promise, no ubiquitously accepted method to evaluate the efficacy of bacterial contact-killing of cationic surfaces exists. Often applied methods (see Table 1 for a description of the essential features of these methods) include the ASTM E2149-13a (American Society for Testing and Materials) (ASTM E2149-13a 2013), the JIS Z 2801 (Japanese Industrial Standards) (JIS Z 2801 2010) and the modified JIS method (Necula et al. 2009), spray-coating of bacteria on a surface from an aerosol (Haldar, Weight, Klibanov 2007) and the Petrifilm® assay (Petrifilm®). A comparison of methods to establish bacterial contact-killing on cationic surfaces has never been made however, but is direly needed considering the interest in the topic, that is stimulated by the increasing lack of effective antimicrobials worldwide (Fears and ter Meulen 2014). Therefore the aim of this study was to evaluate and compare five methods frequently used in the current literature with respect to their efficacy to evaluate bacterial contact-killing using different Gram-positive and Gram-negative bacterial strains. As an easy to prepare contact-killing material, quaternary ammonium groups were directly copolymerized into conventional diurethane dimethacrylate/glycerol dimethacrylate (UDMA/GDMA), yielding a fully crosslinked material with demonstrated ability to facilitate contact-killing of a variety of different bacterial strains in absence of leaching antibacterial compounds (Yue et al. 2015). Bacterial strains used were *Escherichia coli, Staphylococcus epidermidis, Enterococcus faecalis, Staphylococcus aureus* and *Streptococcus mutans*, that all occur in a wide range of applications where bacterial adhesion to surfaces can be troublesome. Criteria to demonstrate bacterial death are not trivial unfortunately. Many living bacteria can be unculturable, while sometimes bacteria indicated to be dead by LIVE/DEAD staining appeared culturable (Hammes, Berney, Egli 2011). Hence we used the criteria for cell death as given in the protocols of respective methods evaluated, with taking the ratio of the log reduction in viable organisms observed over the maximal log reduction that could be achieved considering the bacterial challenge applied in a certain method as the final criterion for comparison.

Materials and methods

Preparation of the positively charged, quaternary ammonium containing polymer samples

The preparation of positively charged quaternary ammonium polymer samples was described before in detail (Yue et al. 2015). Briefly, UDMA (52 wt%), GDMA (35 wt%) and quaternary ammonium methacrylate with an alkyl chain length of C_{12} (QA_C_{12}) (13 wt%) were mixed and sonicated at room temperature for 120 min to create a homogeneous solution. Subsequently, after complete dissolving, the photo-initiators camphorquinone (CQ) (0.5 wt% solution) and ethyl-4-dimethylaminobenzoate (EDMAB) (0.5 wt% solution) were added and sonication was performed for another 30 min to dissolve the photo-initiators in the mixture. As a control polymer, the mixture was also prepared without QA_C_{12}. Samples with a diameter of 15 mm and 0.5 mm thick were prepared using a polydimethylsiloxane mold. The mold was filled with the polymer, air bubbles were removed and a glass slide was placed on top of the mold in order to create a smooth surface. Light-curing (Optilux 501, Kerr Dental, Middleton, WI, USA) with an irradiance of around 1000 mW/cm² was performed on both sides for 90 s. After light-curing, samples were washed with isopropanol to remove unreacted monomers. All samples were sterilised by immersion in 70% ethanol followed by air drying. Prior to sterilization, UDMA/GDMA/QA_C_{12} samples were first kept for three days in 200 mL demineralized water per sample at 37 °C, while refreshing the water every 24 h, in order to remove possible antibacterial leachables

Table 1. Summary of the methods compared in this paper for the evaluation of the efficacy of contact-killing materials containing covalently bonded antimicrobial moieties, together with their advantages and disadvantages as perceived, based on the comparisons made in the current paper.

Method	Description	Advantages	Disadvantages	References
Petrifilm®	Bacteria on a contact-killing surface are confined between a transparent film containing nutrients and a staining agent and allowed to grow for a defined time period.	Small fluid volume ensuring contact between bacteria and sample surface. Nutrient availability during the experiment. No additional steps for *in situ* enumeration.	High numbers of bacteria cannot be counted. Antibacterial leachables cannot be excluded.	(Asri et al. 2014; Yue et al. 2015)
JIS Z 2801	A droplet of a bacterial suspension in buffer is placed on a contact-killing surface and left to incubate for a defined time period.	Small fluid volume ensuring contact.	No nutrient availability during the experiment. Antibacterial leachables cannot be excluded. Bacteria have to be dislodged for enumeration.	(Cleophas et al. 2014)
Modified JIS	A bacterially inoculated filter is placed on a contact-killing surface, and placed with the filter side on a nutrient agar.	Small fluid volume ensuring contact. Nutrient availability during the experiment.	Bacteria may reside deep in the filter and not come in contact with the sample. Antibacterial leachables cannot be excluded. Bacteria have to be dislodged for enumeration. High nutrient availability stimulates bacterial growth also on contact-killing surfaces.	(Andresen et al. 2007)
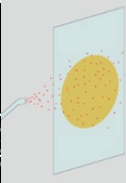
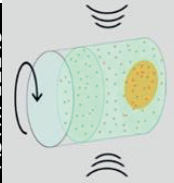| Method	Description	Features	Limitations	References
Bacterial spray	Bacteria are sprayed from an aerosol to a contact-killing surface.	Mimics bacterial contamination of surfaces through transport in air.	Challenge number difficult to determine. Dehydration will contribute to cell death.	(Haldar et al. 2006; Tiller et al. 2001)
ASTM E2149	Incubation of a surface in a bacterial suspension while shaking.	Flexible with regards to shape and size of substratum.	No nutrient availability during the experiment. Antibacterial leachables cannot be excluded. Challenge number unknown and dependent on shaking, i.e. number of collisions between bacteria and sample surface. Enumeration of dead bacteria indirect.	(Fu et al. 2005; Imazato et al. 2003; Klink et al. 2012; Mellouki et al. 1989)

Characterization of the quaternary ammonium polymer samples

X-ray photoelectron spectroscopy (XPS)

Quaternized nitrogen on the sample surface was determined by XPS, as described before (Rouxhet and Genet 2011). Briefly, an XPS (S-probe; Surface Science Instruments, Mountain View, CA), equipped with a monochromatic X-ray source (Al Kα anode yielding 1486.8 eV X-rays), was operated at 10 kV accelerating voltage and 22 mA filament current. The direction of the photoelectron collection angle was set to 35° with respect to the sample surface, and the electron flood gun was set at 10 eV. A survey scan was made with a $1000 \times 250\,\mu m2$ spot and a pass energy of 150 eV. Binding energies were determined by setting the binding energy of the C_{1s} binding energy peak (carbon bound to carbon) at 284.8 eV. Detailed scans of the N_{1s} binding energy peaks over a binding energy range of 20 eV were made using a pass energy of 50 eV. The N_{1s} peak was subsequently decomposed in two fractions at 399.3 and 402.4 eV. The occurrence of a peak at 402.4 eV is indicative for the presence of quaternized nitrogen species (Busscher et al. 2012) and was expressed in atom percentage (at.%) charged nitrogen species by multiplying the peak fraction at 402.4 eV with the total at.% nitrogen.

Cationic charge density using fluorescein staining

The cationic charge density of the sample surfaces was determined using fluorescein staining. To this end, UDMA/GDMA/QA_C_{12} and UDMA/GDMA control samples were immersed in 2 mL 1 wt% fluorescein (disodium salt) solution in demineralized water and shaken at 60 rpm for 10 min. The samples were washed three times with 2 mL demineralized water to remove any dye not complexed with cationic charges. Next, the samples were placed in 2 mL of a 0.1 wt% cetyltrimethylammonium chloride solution in demineralized water and sonicated for 5 min and shaken at 60 rpm for 5 min to desorb complexed fluorescein dye. Subsequently, 200 μL of 100 mM phosphate buffer, pH 8, was added. UV/VIS measurements (Spectra max M2 UV/VIS spectrophotometer) were carried out at 501 nm to yield the concentration of fluorescein dye in the extraction solution [Dye] in M according to

(1) $[Dye]=(Abs501)/(\varepsilon 501 \times L)$

in which Abs501 is the UV absorption at 501 nm, ε501 is the extinction coefficient ($77\,mM^{-1}\,cm^{-1}$ for fluorescein) and L is the length of a polystyrene cuvette (1 cm) traversed by the UV-light beam. The cationic charge density per cm^2 sample surface area was subsequently calculated using:

(2) Charge density=[Dye]×V×N/A

in which V is the volume of the extraction solution (1 mL), N is Avogadro's number (6.023×10^{23}) and A is the surface area of the UDMA/GDMA/QA_C_{12} polymer sample ($3.77\,cm2$).

Assessing leaching of antibacterial components

In order to determine whether after three days immersion of samples in water, antibacterial components leached out in antibacterially active amounts, Brain Heart Infusion (OXOID, Basingstoke, UK) agar plates were inoculated with a suspension of 1×10^8 bacteria per mL of *Enterococcus faecalis* OG1RF with a cotton swab and air dried for 2 min. The washed samples were placed in the middle of the agar plates and incubated for 24 h at 37 °C. The plates were assessed for the development of an inhibition zone surrounding the UDMA/GDMA/QA_C12.

Bacterial strains, grow conditions and harvesting

The bacterial strains used in this study were Gram-negative *E. coli* ATCC25922 and Gram-positive *S. epidermidis* ATCC12228, *E. faecalis* OG1RF, *S. aureus* ATCC12600 and *S. mutans* NS. The bacterial strain was first streaked on a blood agar plate from a frozen stock solution (7 v/v% DMSO) and grown overnight at 37 °C. One colony was inoculated in 10 mL of the appropriate growth medium and incubated at 37 °C for 24 h. The growth media used were: Brain Heart Infusion for *E. coli* and *E. faecalis,* Tryptone Soya Broth (OXOID) for *S. epidermidis* and *S. aureus*, and Todd Hewitt Broth (OXOID) for *S. mutans.* 10 mL of these pre-cultures was used to inoculate a main culture of 200 mL growth medium, which was incubated for 18 h at 37 °C. Bacteria were harvested by centrifugation for 5 min at 6500g and 10 °C three times and subsequently washed with phosphate-buffered saline (PBS; 10 mM potassium phosphate and 150 mM NaCl, pH 7.0). The number of bacteria in suspension were enumerated using a Bürker-Türk counting chamber. Concentrations were adjusted by dilution with PBS to the requirements of the specific methods. The percentage live

Methods for evaluating bacterial killing upon contact with the surface

All evaluation methods were applied in triplicate with separately grown bacterial cultures. In order to provide for a comparable scale valid for all five methods included in this study, we have determined the maximum log-reduction in the number of live or viable bacteria that could be achieved in each method. Next, the actual log-reduction achieved in each method was determined according to

(3) Logreduction= $\log_{10}$(challenge number per cm^2) - $\log_{10}$(viable number per cm^2 on contactkilling surface)

and expressed as a 'percentage contact-killing efficacy' according to

(4) Efficacy = Logreduction / Max logreduction x 100%

Adsorbed protein film

A macromolecular conditioning film was applied on the samples in order to perform the evaluations of contact-killing efficacies under more practically realistic conditions.

For possible oral applications, this involved adsorption of a salivary film from lyophilized human whole saliva (van der Mei et al. 2012), reconstituted in 1.5 mg/mL buffer (2 mM potassium phosphate, 1 mM $CaCl_2$, 50 mM KCl, pH 6.8). Each sample was immersed overnight in 2.5 mL of thus prepared saliva, taken out with a pair of sterile tweezers, and the excess saliva was dripped off before conducting the evaluation of their contact-killing efficacy against the *S. mutans* strain employed, an oral pathogen. Similarly, samples were immersed in 10% fetal calf serum and evaluations performed against an *S. epidermidis* strain, often found on biomaterial implants in the human body.

Petrifilm® method

The Petrifilm® Aerobic Count plate system (Petrifilm® 3M Microbiology, St. Paul, MN, USA) consists of two films: a bottom film containing standard nutrients, a cold-

water gelling agent and an indicator dye that facilitates colony counting and a top film enclosing the sample within the system. The bottom film containing the gelling-agent was first swelled with 1 mL sterile demineralized water for 60 min and transferred to the transparent top film before use. Next, a sterile UDMA/GDMA/QA_C$_{12}$ and UDMA/GDMA sample were placed on the bottom film. 10 μL of different dilutions of bacterial suspensions (10^4, 10^6 and 10^8 bacteria/mL) was pipetted on top of each samples and the top film was closed. Closure of the Petrifilm® system ensured spreading of the bacterial suspension over the surface area of the samples. The closed Petrifilm® system was left to incubate at 37 °C for 48 h after which the numbers of CFUs were counted and used to calculate the percentage contact-killing efficacy.

Japanese Industrial standard method (JIS)

In the JIS Z 2801:2010, samples were placed in a sterile 6-wells plate. 10 µL of a bacterial suspensions in PBS (10^8 bacteria/mL) was pipetted on top of a sterile UDMA/GDMA/QA_C12 and UDMA/GDMA sample. Next, the well plate was covered with sterilized Parafilm® (diameter 15 mm) and left to incubate at 37 °C for 24 h under humidified atmosphere. After incubation, 5 mL 0.1% (v/v) Tween80 in PBS was added to each well, followed by sonication for 30 s and gentle shaking for 2 min in order to dislodge adhering bacteria. The resulting suspension was serially diluted and the numbers of CFUs were determined by plate counting on agar after 24 h incubation at 37 °C, from which the percentage contact-killing efficacy was calculated.

Modified JIS method

In the modified JIS method (Necula et al. 2009), 10 µL of a bacterial suspension in PBS (108 bacteria/mL) was pipetted on sterilized nitrocellulose filters (pore size 0.45 µm and diameter 15 mm) placed on a agar plate. The liquid was absorbed by the agar while the bacteria retained on the filter. A sterile UDMA/GDMA/QA_C_{12} and UDMA/GDMA sample were placed in a 6-wells plate. Next, 20 µL of the appropriate growth medium in 10 mM potassium phosphate buffer (1% v/v), with 50% fetal calf serum added, was pipetted centrally on the surface of a sample after which an inoculated filter was carefully placed on the sample, with filter-side on which the bacteria were retained contacting the sample. The system was left to incubate at 37 °C for 24 h in humidified atmosphere. After incubation, 5 mL growth medium was added to each sample and corresponding filter, sonicated for 30 s and vortexed for 1 min to dislodge adhering bacteria. Finally, the resulting suspensions were serially diluted and the numbers of CFUs were determined by plate counting on agar after 24 h incubation at 37 °C and used to calculate the percentage contact-killing efficacy.

Bacterial spray method

A bacterial suspension with a concentration of 1×10^8 bacteria per mL was sprayed for 2 s onto a sterile UDMA/GDMA/QA_C_{12}, UDMA/GDMA sample and control glass

slide from a distance of approximately 15 cm, placed under an angle of 45° (Haldar et al. 2007). After spraying, surfaces with adhering bacteria were air dried for 2 min and stained for 15 min in the dark with 15 µL live-dead Baclight (BacLight™, Molecular probes, Leiden, The Netherlands) viability stain containing SYTO 9 dye (yielding green fluorescence for live bacteria) and propidium iodide (yielding red fluorescence in cell membrane-damaged bacteria, generally considered to be "dead" bacteria). In order to ensure proper indication of dead organisms, we have used the SYTO 9 propidium iodide stain in a 1:1 concentration, as for some bacterial strains species higher amounts of propidium iodide are required, possibly with longer incubation times, as also described in the manufacturers manual. A fluorescence microscope (Leica DM4000B; Leica Microsystems GmbH Heidelberg, Germany) was used to visualize live and dead adhering bacteria. After enumeration of the number of viable and dead bacteria, the percentage of viable bacteria was determined and expressed as percentage contact-killing efficacy, as defined above.

ASTM method

In the ASTM E2149-13a (ASTM E2149-13a 2013) protocol, 10 mL of a bacterial suspension (3×10^5 bacteria/mL) was added into test tubes together with or without sterile UDMA/GDMA/QA_C_{12} and UDMA/GDMA samples and agitated in an orbital mixer at 200 rpm for 15 min at room temperature (21 °C). After 5, 10 and 15 min 100 µL aliquots were taken of the suspension, serially diluted and the numbers of CFUs were determined by plate counting on agar and used to calculate the percentage contact-killing efficacy. In one experiment, aliquots were taken up to 120 min in order to check whether contact-killing continued after 15 min.

Statistical analysis

Data were analyzed with the Statistical Package for Social Science (Version 16.0, SPSS Inc., Chicago, IL, USA). A Student t-test was used to compare the number of bacteria in log reduction between UDMA/GDMA (control) and UDMA/GDMA/QA_C12 (contact killing material) of each bacterial strain and the mean of all five bacterial strains for each method evaluated. A one-way analysis of variance (ANOVA) was used to compare the killing efficiency on all five bacterial strains between different

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methods on UDMA/GDMA/QA_C_{12}. A Bonferroni test was used for post-hoc multiple comparisons. Statistical significance was set at $p < 0.01$.

Results

Characterization of cationically charged, UDMA/GDMA/QA_C_{12} samples

The cationic charge density of the UDMA/GDMA/QA_C_{12} samples was quantified by fluorescein staining (Fig. 1a) and amounted 2.5×10^{16} N+/cm², well above 10^{14} positive charges per cm², required to kill bacteria upon contact(Kugler, Bouloussa, Rondelez 2005; Murata et al. 2007). XPS yielded a slight increase in the at.% N upon copolymerization with alkylated ammonium into UDMA/GDMA (Fig. 1b), but after copolymerization the N1s photoelectron binding energy peak showed a clear component at 402.4 eV indicative of quaternized nitrogen (compare Fig. 1c and d) and confirming successful incorporation of positively charged quaternized nitrogen in the polymer matrix (Rouxhet and Genet 2011).

	at%N	at%N$_{399.3\ eV}$	at%N$_{402.2\ eV}$
UDMA/GDMA			
	4.69 ± 0.41	4.55 ± 0.43	0.15 ± 0.12
UDMA/GDMA/QA_C$_{12}$			
	5.03 ± 0.39	4.28 ± 0.57	0.72 ± 0.19
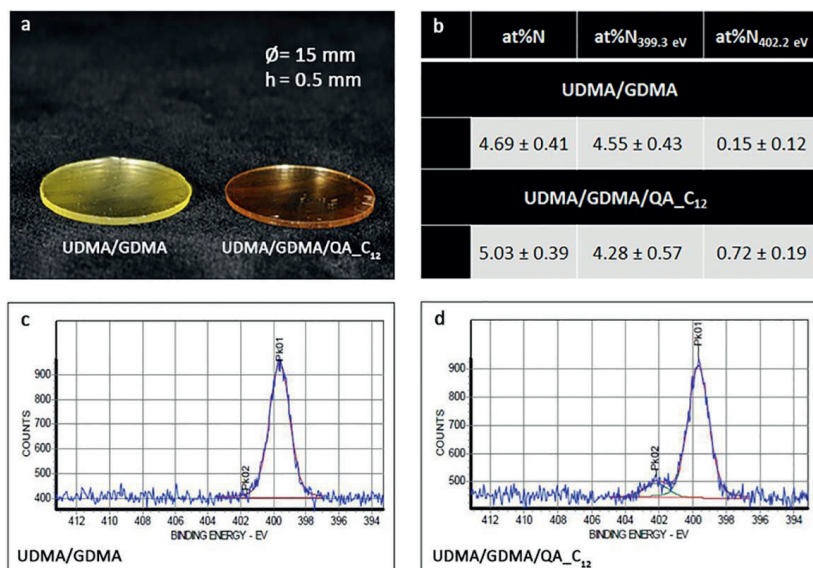
Figure 1. Characterization of cationically charged, UDMA/GDMA/QAC$_{12}$ samples. (a) UDMA/GDMA and UDMA/GDMA/QA_C_{12} sample. (b) Quantitative evaluation (averages with standard deviations over 5 samples) of N1s photoelectron binding energy peaks of UDMA/GDMA and UDMA/GDMA/QA_C_{12}. (c) N1s photoelectron binding energy peak for UDMA/GDMA. (d) N1s photoelectron binding energy peak for UDMA/GDMA/QA_C_{12}, with a clear peak component at 402.4 eV, indicative of the incorporation of quaternary ammonium groups.

In order to exclude that leaching of antibacterial components out of the material prepared would interfere with their contact-killing, all samples were immersed for three days at 37 °C in an excess of demineralized water (200 mL per sample, refreshing the volume every 24 h). No antibacterial efficacy was observed by the lack of an inhibition zone around a disc shaped sample placed on a bacterially inoculated agar plate (Fig. 2). Moreover, only bacteria underneath the sample were killed, inferring contact-killing.
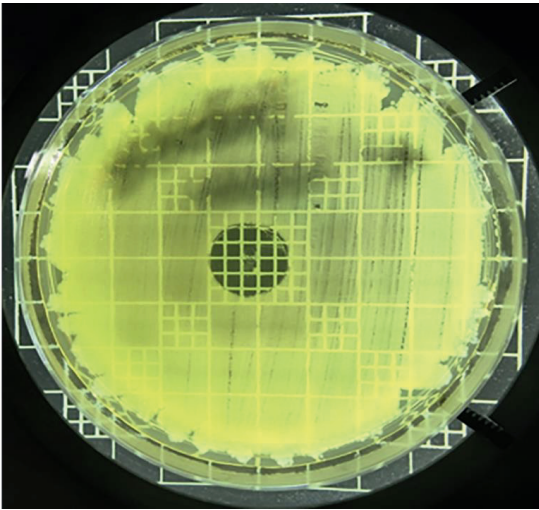

Figure 2. Absence of an inhibition zone around an UDMA/GDMA/QA_C12 sample (diameter 15 mm) on an agar plate inoculated with *E. faecalis* OG1RF indicated absence of leaching of antibacterial components from the samples that might interfere with the evaluation of contact-killing. Note the absence of bacteria underneath the sample, inferring bacterial contact-killing.

Methods for evaluating bacterial killing upon contact with the surface

All methods compared (Table 1) were applied as closely as possible to the instructions given in the authentic literature references or instruction guides. Minor modifications were made to keep the bacterial challenge numbers per cm² in each method approximately equal for better comparison. However, minor variations in bacterial challenge numbers could not be avoided and challenges ranged from 7.5×10^4 CFU cm² for ASTM E2149-13a to 5.8×10^5 CFU cm² for the bacterial spray assay (Table 2). The logarithms of these challenge numbers represent the maximal log reduction that can be achieved in a particular method (see also Table 2).

In Table 2 it can be seen that none of the methods demonstrated contact-killing on control samples of UDMA/GDMA, and maximal log reductions of 0.6 CFU cm−2 were observed for *E. faecalis* OG1RF on UDMA/GDMA in ASTM E2149-13a. Whereas in some methods, minor growth (negative log reductions) in the order of −0.1 CFU cm−2 was observed on UDMA/GDMA samples, major growth was found in the modified JIS method, with abundant nutrient availability, for four out of the five strains used, even up to −1.9 CFU cm−2 for *S. epidermidis* ATCC12228. Accordingly, the modified JIS method was the only method that exhibited growth for *E. coli* ATCC25922 on contact-killing UDMA/GDMA/QA_C12 samples. The bacterial spray method and ASTM E2149-13a showed far lower log reductions than did the Petrifilm® and JIS Z 2801 methods (p < 0.01).

Fig. 3a compares the percentage contact-killing efficacy in the different methods, made directly comparable by defining the contact-killing efficacy in a method as the ratio over the log reduction observed over the maximal log reduction that could be achieved considering the bacterial challenge applied. If the entire bacterial challenge applied was killed, the efficacy of the method was denoted as 100%. Generally, the bacterial spray method and ASTM E2149-13a indicated absence of contact-killing, while the JIS Z 2801 and the Petrifilm® method indicated 100% contact-killing efficacy for four out of the five strains, with the exception of Gram-negative *E. coli* showing less contact-killing. The modified JIS method demonstrated near 100% contact-killing efficacy for *S. epidermidis and S. aureus*, but not for *E. faecalis* and *S. mutans*, while showing growth for the *E. coli* strain.

In the ASTM E2149-13a method, samples are agitated in 10 mL bacterial suspension during 15 min, after which the killing efficacy is determined. Considering the lack of contact-killing observed using ASTM E2149-13a, the method was also carried out with longer agitation times up to 60 min and 120 min, but this did not make any difference with respect to the ability of the method to demonstrate bacterial contact-killing (see Supplementary Fig. 1).

In many potential applications of contact-killing coatings, the coatings attract a film of adsorbed macromolecular components before bacteria adhere. Under clinical conditions in the human body for instance, biomaterial surfaces often become coated with a layer of proteins adsorbed from body fluids before the first bacteria adhere. Hence, to enlarge the practical significance of our comparison, UDMA/GDMA/QA_C12 samples were first provided with a film of adsorbed salivary

proteins and subsequently evaluated against the oral bacterium *S. mutans* NS or a film of proteins adsorbed from 10% fetal calf serum and evaluated against *S. epidermidis* ATCC12228. The killing efficacy of UDMA/GDMA/QA_C12 was not affected by the presence of an adsorbed macromolecular film in none of the methods compared (Fig. 3b).

Table 2. Log reductions actually achieved in the various methods for five bacterial strains.* For each method, the bacterial challenge applied is given per cm2, from which the maximum log reduction that can possibly be achieved is calculated. ± Signs indicate the SD over triplicate experiments with separately cultured bacteria.

Method	Petrifilm®	JIS Z 2801	Modified JIS	Bacterial spray	ASTM E2149
Challenge (CFU cm^{-2})	5×10^5	5×10^5	5×10^5	5.8×10^5	7.5×10^4
Max log reduction possible	5.7	5.7	5.7	5.8	4.9
UDMA/GDMA: log reduction achieved					
E. coli ATCC25922	0.1 ± 0.2	0.4 ± 0.3	-1.5 ± 0.5	0.0 ± 0.0	0.3 ± 0.2
S. epidermidis ATCC12228	-0.1 ± 0.2	0.5 ± 0.2	-1.9 ± 0.5	0.0 ± 0.0	0.4 ± 0.1
E. faecalis OG1RF	0.0 ± 0.0	0.1 ± 0.2	-0.6 ± 0.1	0.0 ± 0.0	0.6 ± 0.4
S. aureus ATCC12600	0.0 ± 0.0	-0.1 ± 0.1	-0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.3
S. mutans NS	0.0 ± 0.0	-0.1 ± 0.1	0.3 ± 0.4	0.1 ± 0.0	0.1 ± 0.3
UDMA/GDMA/QAC_12: : log reduction achieved					
E. coli ATCC25922	1.0 ± 0.7	5.1 ± 1.1[a]	-1.2 ± 1.1	0.0 ± 0.0	0.1 ± 0.2
S. epidermidis ATCC12228	5.7 ± 0.0[a]	5.7 ± 0.0[a]	5.7 ± 0.0[a]	0.2 ± 0.2	0.2 ± 0.1
E. faecalis OG1RF	5.7 ± 0.0[a]	5.7 ± 0.0[a]	4.2 ± 1.4	0.1 ± 0.1	0.1 ± 0.1
S. aureus ATCC12600	5.7 ± 0.0[a]	5.7 ± 0.0[a]	5.7 ± 0.0[a]	0.1 ± 0.0	0.1 ± 0.3
S. mutans NS	5.7 ± 0.0[a,b]	5.7 ± 0.0[a,b]	3.7 ± 1.9[b,c]	0.1 ± 0.0[d]	0.1 ± 0.3[d]

*Negative log reductions indicate bacterial growth. a. Significantly different from UDMA/GDMA tested on the same bacterial strain using the same method. b. Significantly different from UDMA/GDMA tested on all five bacterial strains using the same method. c. Significantly different between JIS Z 2801 and Modified JIS. d. Significantly different from Petrifilm®, JIS Z 2801 and Modified JIS.
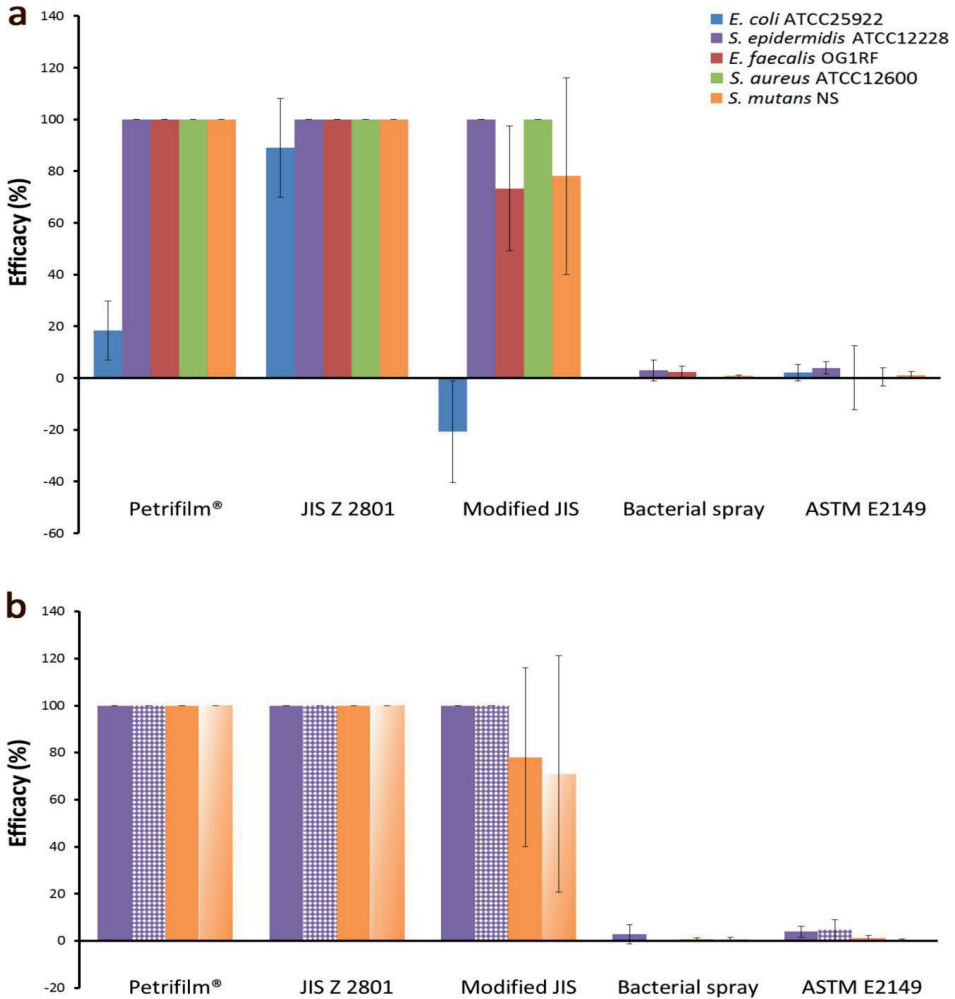
Figure 3. Bacterial contact-killing efficacy (%) achieved in the various methods on UDMA/GDMA/QA_C12 in absence and presence of an adsorbed macromolecular film. Percentage efficacy is defined as the log reduction achieved divided by the maximally possible log reduction. Error bars indicate the SD over triplicate experiments with separately cultured bacteria. (a) Bacterial contact-killing efficacy (%) achieved in the various methods for five bacterial strains in absence of an adsorbed macromolecular film. (b) Bacterial contact killing efficacy (%) achieved in the various methods for two bacterial strains, for uncoated and UDMA/GDMA/QA_C12 with a macromolecular conditioning film. The samples were coated overnight with 10% fetal calf serum (dotted columns) for *S. epidermidis* ATCC12228 or saliva (shaded columns) for *S. mutans* NS.

Discussion

Quaternary ammonium coatings possess the unique feature of killing bacteria upon contact, which offers a promising alternative for antibiotic-based approaches, particularly considering the rise in the number of antibiotic resistant strains and species developing (Fears and ter Meulen 2014; Montali 2006). Unfortunately, no ubiquitously accepted method exists to properly evaluate the efficacy of contact-killing materials or coatings. In addition, results may be obscured by the fact that for many antibacterial materials reported on in the literature, it is not clear whether effects are due to leaching of residual antimicrobial compounds (Andresen et al. 2007; Asri et al. 2014; Irikura, Hasegawa, Takahashi 2003; Mellouki et al. 1989; Pasquier et al. 2007)('release-killing') from a contact-killing material or due to contact-killing itself. Here we have compared five different methods (see Table 1) with respect to their virtues for evaluating the contact-killing efficacy of an established, non-leaching contact-killing coating of quaternary ammonium compounds covalently bound in a UDMA/GDMA resin against five different bacterial strains.

In Table 1 we give an overview of the advantages and disadvantages of each used contact-killing method, as perceived based on the current comparison and discussed below. Two out of the five methods evaluated demonstrated clear contact-killing efficacy against Gram-positive bacteria (Petrifilm® and JIS Z 2801). Efficacies against a Gram-negative bacterium were slightly less compared to Gram-positive strains, presumably due to the possession of a double membrane by Gram-negative bacteria (Hammond et al.2001)(Thoma, Boles, Kuroda 2014; Xue et al. 2012). Bacterial spraying and ASTM E2149-13a yielded no indications of contact-killing, while the modified JIS method yielded growth on a contact-killing UDMA/GDMA/QA_C_{12} surface, although less than on a control UDMA/GDMA material without quaternary ammonium incorporated. Percentage bacterial contact-killing ranked as follow in the different methods: JIS Z 2801 = Petrifilm® > modified JIS > bacterial spray = ASTM E2149-13a. Differences obtained between JIS Z 2801 and Petrifilm® are not significant and the methods are in fact highly comparable. In both methods bacteria are contacted with a contact-killing surface within a very small fluid volume, ensuring contact (see also Table 1). However, the Petrifilm® method may be considered slightly more convenient than JIS Z 2801 because surviving

bacteria are grown into countable colonies during contacting. In JIS Z 2801 bacteria have to be dislodged by sonication after adhering strongly to the sample through electrostatic attraction (Pasquier et al. 2007), which not only constitutes an additional step with respect to the Petrifilm® method, but possibly also explaining the slightly higher contact-killing efficacy in JIS Z 2801 as the forceful sonication required may yield additional killing. The small fluid volumes in which bacteria and contact-killing surfaces are brought together, also constitute a possible danger of these two methods, as extremely small amounts of antimicrobial leachables may easily cause the build-up of a high concentration of antimicrobial compounds to interfere with contact-killing. Therefore it is needed to use JIS Z 2801 and Petrifilm® in combination with an agar zone of inhibition assay to ascertain that there is no release of antimicrobial compounds with demonstrable biological effects. The presence of a balanced amount of nutrients in the Petrifilm® is considered as an advantage, depending on the application of the coatings aimed for.

The modified JIS method (Necula et al. 2009) was developed in order to provide an opportunity to the bacteria to grow during adhesion to a contact-killing surface, which makes it similar in principle to the Petrifilm® method (Table 1). Yet in the modified JIS method, the growth opportunities are too much and easily overshadow contact-killing by the surface: the contact-killing efficacies for two Gram-positive bacterial strains (*E. faecalis and S. mutans*) are clearly smaller compared to results obtained in the Petrifilm® and JIS Z 2801 method, whereas for Gram-negative *E. coli* in the modified JIS method, survivors were able to grow to the extent that "negative killing" was measured. Accordingly, the modified JIS method is only useful when comparing the growth observed on a contact-killing surface with the growth on a non-contact-killing, control surface such as the UDMA/GDMA sample in the current study. Although advocated as an advantage of the modified JIS method that it can be applied in the presence of a pre-adsorbed proteinaceous conditioning film, this advantage is not exclusive to the modified JIS method and can be equally applied in all other methods (see also Fig. 3b).

The bacterial spray method is used in several papers (Haldar, Weight, Klibanov 2007; Haldar et al. 2006; Tiller et al. 2001) and often demonstrated 100% contact-killing amongst others against *S. aureus* and *E. coli* strains, but yielded no worthwhile contact-killing in the current comparison of methods. Self-admitted also

determination of the challenge number of bacteria that actually come into contact with a coating is hard to establish and can easily be over- or underestimated and cause erroneous contact-killing efficacies. Yet, for air-borne bacterial contamination of surfaces, the spray method remains the only one available, although alternatively, air-borne contaminated surfaces might be further analyzed in the Petriflm® assay with greater accuracy. The ASTM E2149-13a method (ASTM E2149-13a 2013) only yielded minor bacterial contact-killing efficacy, although contact-killing efficacies against *E. coli* after 30 min between 50% and 100% have been reported for different contact-killing, cationic coatings (Milovic et al. 2005; Sandrine et al. 2005). Experiments carried out as a function of agitation time in this study demonstrated that killing efficacies did not further increase with time (Suppl. Fig. 1). The advantage of the method is that samples with a variety of shapes and sizes can be used, but the method fails to control the challenge number of bacteria that actually come into contact with a surface. Also bacterial enumeration is indirect, since aliquots are taken from the suspension for CFU analysis, instead of measuring directly on the contact-killing surface. Therewith it is impossible to distinguish between killed and adhering bacteria that are still alive. Originally, ASTM E2149 was designed for testing the antimicrobial activity, i.e. contact-killing of non-leaching, antimicrobial surfaces under dynamic contact conditions. The current comparison of methods to evaluate bacterial contact-killing by antimicrobial surfaces points out that even after several revisions (ASTM E2149-13a 2013) ASTM E2149-13a remains unsuitable to evaluate bacterial contact-killing and will likely be more suitable to evaluate antimicrobial-release coatings rather than contact-killing ones. In line with the conclusions of this paper, the last revision of ASTM2149-13a emphasizes that the test cannot exclude leaching, and recommends additional assays to exclude "release-killing" by leaching of antimicrobials from a sample.

Conclusions

This study reveals that depending on the method used, different efficacies can be obtained in the evaluation of bacterial contact-killing surfaces. We conclude that the Petrifilm® and JIS Z 2801 are preferable methods, with Petrifilm® being most convenient and possibly more reliable due to a balanced amount of nutrients. Like all others, Petrifilm® and JIS Z 2801 need a complementary assay to exclude killing due

to release of antimicrobial compounds, because even a small release of an antimicrobial compound can have a large influence on bacterial killing. The modified JIS method is acceptable, but does not contain balanced amount of nutrients compared to the Petrifilm® and JIS Z 2801 method. Therefore the modified JIS method should only be used with respect to a non-contact killing control. ASTM E2149-13a and bacterial spray methods are not reliable, the main reason being the lack of control over the applied bacterial challenge and actual contact of bacteria with the surface.

Acknowledgements

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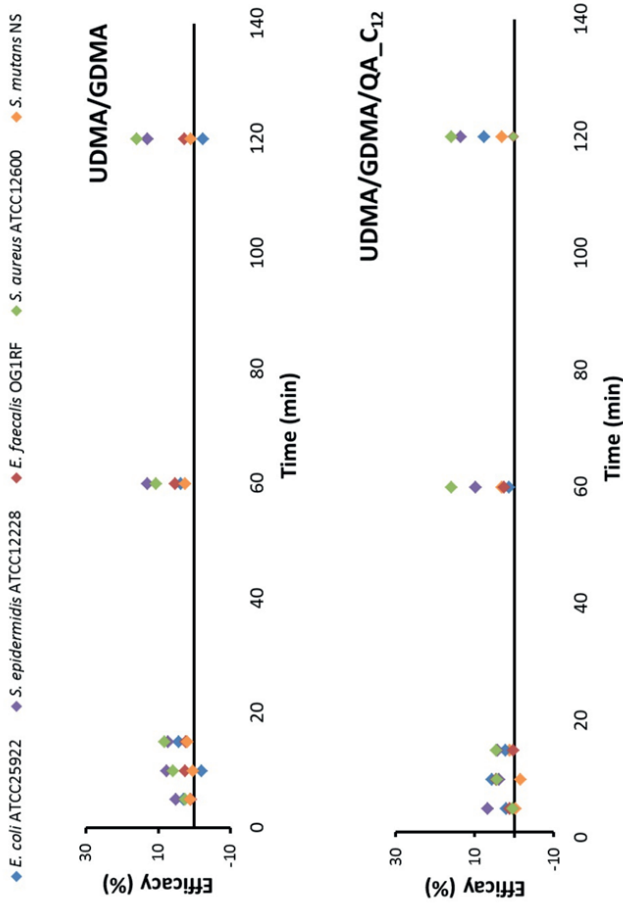
Supplementary material

Figure 1: Contact-killing efficacy (%) achieved in the ASTM E2149-13a method for five bacterial strains as a function of the agitation time of UDMA/GDMA and UDMA/GDMA/QA_C12 samples in the bacterial suspensions. Data points represent triplicate experiments with separately cultured bacteria.

Chapter 6-I

Three dimensional – printable antimicrobial composite resins

Jun Yue, Pei Zhao, Jennifer Y. Gerasimov, Marieke van de Lagemaat, Arjen Grotenhuis, Minie Rustema-Abbing, Henny C. van der Mei, Henk J. Busscher, Andreas Herrmann, Yijin Ren

Abstract

3D printing is seen as a game-changing manufacturing process in many domains, including general medicine and dentistry, but the integration of more complex functions into 3D-printed materials remains lacking. Here, it is expanded on the repertoire of 3D-printable materials to include antimicrobial polymer resins, which are essential for development of medical devices due to the high incidence of biomaterial-associated infections. Monomers containing antimicrobial, positively charged quaternary ammonium groups with an appended alkyl chain are either directly copolymerized with conventional diurethanedimethacrylate/glycerol dimethacrylate (UDMA/GDMA) resin components by photocuring or prepolymerized as a linear chain for incorporation into a semi-interpenetrating polymer network by light-induced polymerization. For both strategies, dental 3D-printed objects fabricated by a stereolithography process kill bacteria on contact when positively charged quaternary ammonium groups are incorporated into the photocurable UDMA/GDMA resins. Leaching of quaternary ammonium monomers copolymerized with UDMA/GDMA resins is limited and without biological consequences within $4-6$ d, while biological consequences could be confined to 1 d when prepolymerized quaternary ammonium group containing chains are incorporated in a semi-interpenetrating polymer network. Routine clinical handling and mechanical properties of the pristine polymer matrix are maintained upon incorporation of quaternary ammonium groups, qualifying the antimicrobially functionalized, 3D-printable composite resins for clinical use.

Introduction

Over the past several years, additive manufacturing techniques, more commonly referred to as "3D printing" (3DP) (Berman 2012), have entered the realm of public awareness by rapidly penetrating a variety of application areas beyond small-scale manufacturing and prototyping. Advancements in 3DP technology have also had an impact on drug delivery systems (Sandler et al. 2014; Ursan, Chiu, Pierce 2013), medical devices, (Chimento, Crane, Jason Highsmith 2011; Rankin et al. 2014) tissue engineering, (Atala, Kasper, Mikos 2012; Mannoor et al. 2013; Miller et al. 2012; Murphy and Atala 2014; Silberstein et al. 2014) dental restorations, (Silva et al. 2011; van Noort 2012) microfluidics, and customized reactionware for chemical synthesis and analysis (Erkal et al. 2014; Symes et al. 2012). 3DP enables the low-cost, bottom-up fabrication of objects with complex geometries that are difficult to produce by traditional fabrication methods. Although 3D printed objects composed of metals, ceramics, polymers,16 and even cell-loaded hydrogels have been realized (Kolesky et al. 2014; Bertassoniet al. 2014; Pataky et al. 2012), the development of materials with integrated functions amenable for 3DP has been slow.

The surface properties of 3D printed materials are especially vital to their implementation in general medicine and dentistry, as nearly all medical devices have an interaction with the human body that occurs initially at the materials surface. Specifically, since many medical device surfaces attract microorganisms, engineering an intrinsic antimicrobial functionality in or onto implantable medical devices can reduce the risk of microbial infections associated with the presence of a foreign material in the human body (Monack, Mueller, Falkow 2004). Device-related infections pose major health threats and are currently the leading cause of failure of implanted devices. It has been estimated that at least 50% of all nosocomial infections are device-related and affect around two million patients each year in the United States alone (Chitnis et al. 2012). Similarly, oral health is severely affected by the formation of infectious biofilms as many patients are unable to maintain sufficient oral hygiene using traditional means as tooth brushing or floss wire, especially when access to oral surfaces is hampered by, for instance, orthodontic appliances. Up to 15% of oral biofilm-related post-treatment complications in orthodontic patients require professional care with annual costs of over 500 million dollars in the United States (Ren et al. 2014). Dental patients in the United States

spend over 20 billion dollars annually to replace failed resin composite restorations that were damaged by bacterial infiltration and resulting secondary caries underneath a restoration (Wall et al. 2014). Thus, motivated by the significant negative consequences of microbial biofilms in oral health and the highly individualized nature of customized intraoral appliances and prostheses calling for an all-digital workflow, we present a polymer design strategy to develop 3D printed, antimicrobial resins for manufacturing intraoral appliances and dental restorations. Design of antimicrobial resins is strongly preferred above surface coating of existing materials, as coating requires an additional step representing time and money in a clinical setting. Moreover, the design strategies presented are unique in the sense that they do not require any additional or even altered routines by the physicians involved. Application is not limited to dental or general medical ones, but can be transferred to other application areas where antimicrobial properties are desired.

Numerous efforts have been undertaken to equip conventional dental restorations with antimicrobial properties. These focused on release of various antibacterial agents such as fluorides (Wiegand, Buchalla, Attin 2007), zinc ions (Aydin Sevinc and Hanley 2010; Osinaga et al. 2003), silver ions (Yamamoto et al. 1996), chlorhexidine (Sandham, Nadeau, Phillips 1992), and antimicrobial peptides (Pepperney and Chikindas 2011).However, the release of antimicrobial agents is always temporal and may impair the mechanical properties of the restorations or exert toxicity on the surrounding tissue if release is not properly controlled. Therefore, a material that functions through the mechanism of killing microorganisms on contact is a much more promising alternative. In several previous studies (Cui et al. 2010; Lu, Wu, Fu 2007; Muñoz-Bonilla and Fernández-García 2012; Tiller et al. 2001; Waschinski et al. 2008), positively charged quaternary ammonium compounds have been covalently grafted onto surfaces to realize contact-killing effects against a variety of bacterial strains. Although their exact killing mechanism is still not fully elucidated, it is generally accepted that the grafted positively charged groups interact with the bacterial cell wall and disrupt the lipid membrane to release cytoplasmic constituents (Muñoz-Bonilla and Fernández-García 2012), which causes cell death albeit through a different mechanism than utilized by positively charged compounds in solution (Gottenbos et al. 2002; Thebault et al. 2009; Waschinski and Tiller 2005). Inspired by these studies, the aim of this study was to develop 3D printable, bacterial contact-killing resins that contain positively

charged moieties and are compatible with stereolithographic, 3DP technologies. In addition, the cytotoxicity of possible leachables from the 3D printable materials developed was investigated using a method fine-tuned to dental application. 3D printable materials possessing complex functions, like the ability to kill adhering bacteria on contact, do not yet exist to the best of our knowledge. This is likely because it requires incorporation of a charged moiety into neutral resin components, minimization of leaching products, and adjustment of the rheological properties of the monomer mixture for 3DP at the same time, none of which is too trivial to achieve.

Materials and methods

Materials

UDMA (mixture of isomers, ≥97%), CQ (97%), EDMAB (≥99%), 2-(dimethylamino) ethyl methacrylate (DMAEMA, 98%), GDMA (mixture of isomers, 85%), HEMA (97%), methacrylic acid (98%), N,N′-dicyclohexylcarbodiimide (99%), 4-cyano-4-(dodecylthiocarbonothioylthio)pentanoic acid (CDTA, 97%), 1-bromooctane (99%), 1-bromodecane (98%), 1-bromododecane (97%), and 1-bromohexadecane (97%) were purchased from Aldrich. 1-bromobutane (99%) and hydroquinone (ReagentPlus, ≥99.5%) were obtained from Sigma-Aldrich. All other chemicals including solvents were used as received.

Synthesis of Quaternary Ammonium Methacrylate

Quaternary ammonium methacrylate (QA_Cn) (see Scheme S3, Supporting Information) with alkyl chain lengths (Cn) in the range of C4−C16 were synthesized: 10 g of DMAEMA (63.6 mmol) and 67 mmol of alkyl bromides ($C_nH_{2n+1}Br$) were dissolved in 100 mL chloroform (He et al. 2011). To inhibit self-polymerization of DMAEMA during quaternization, a small amount of hydroquinone (70 mg, 0.636 mmol) was added to the mixture. The reaction was conducted at 50 °C for 24 h, and then the solvent was evaporated under reduced pressure, followed by three times precipitation in a large excess of hexane. The precipitates were collected, redissolved

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in 20 mL of chloroform, and then passed through a layer of alkaline aluminum oxide to get rid of hydroquinone. The final product was obtained by removal of the solvent to give a white powder (yield: 89%). 1H NMR (400 MHz, CDCl3): δ (ppm) = 0.91 (t, 3H, N+CH2CH2(CH2)n−3CH3), 1.40 (m, (2n−6)H, N+CH2CH2(CH2)n−3CH3), 1.75 (m, 2H, N+CH2CH2(CH2)n−3CH3), 1.93 (s, 3H, CH2C(CH3)COO), 3.49 (s, 6H, CH2N+(CH3)2C2H2), 3.68 (t, 2H, N+CH2CH2(CH2)n−3CH3), 4.15 (t, 2H, COOCH2CH2N+), 4.65 (t, 2H, COOCH2CH2N+), 5.66 and 6.12 (s, 2H, CH2C(CH3)COO).

Synthesis of pQA

First, tertiary amine (TA)-containing polymers were synthesized by RAFT copolymerizations of DMAEMA and HEMA in the presence of the chain transfer agent CDTA (Scheme S4, Supporting Information). In detail, CDTA (0.248 mol), DMAEMA (7.44 mol), HEMA (6.2 mol), and azobisisobutyronitrile (AIBN) (0.0496 mol) were dissolved in 10 mL of N,N-dimethylformamide (DMF) in a Schlenk flask, followed by five "freeze–pump–thaw" cycles to remove oxygen. Then the flask was filled with argon and sealed to allow polymerization at 65 °C for 12 h. Subsequently, the flask was cooled to room temperature and the viscous solution was precipitated in a large excess of diethyl ether to obtain the product in 70% yield. Afterward, quaternization of the above polymers was carried out using tenfold excess of alkyl bromides in relation to the nitrogen component. The reaction was conducted at 70 °C in DMF for 2 d and finally, the reaction mixture was precipitated in a large excess of n-hexane/diethylether (3/1, v/v). To fully remove the unreacted alkyl bromides and monomers, the precipitated polymers were redissolved in ethanol, followed by dialysis against a solvent mixture of ethanol/acetone (10/1) for one week. The final products were obtained after evaporating the solvent remaining in the dialysis bags (cellulose membrane, cut-off molecular weight: 3.5 kD). A 1H NMR spectra (400 MHz, d6-DMSO) of quaternized polymer is shown in Figure S1 (Supporting Information).

Fabrication of QA_Cn-Containing Polymer Network

Homogeneous resin mixtures containing 50 mol% of UDMA, 36 mol% of GDMA, and 14 mol% of QA_C_n monomers were prepared by gentle sonication of the components at room temperature for 60 min. In order to initiate the polymerization under visible light, CQ (photoinitiator) and EDMAB (coinitiator) at an amount of 0.5 mol% in relation to the total amount of double bonds were added and sonication was performed for another 30 min to dissolve the photoinitiator and coinitiator. After that, a stainless steel mold (diameter: 20 mm; thickness: 0.5 mm) was filled with the viscous solutions and placed between two thin glass slides. The photocuring was carried out by vertical illumination of both sides of the polymerization mold at room temperature for 90 s, respectively, using a dental light source (Optilux 501, sds Kerr Sybron Dental Specialties, Middleton, WI, USA) with an irradiance of ≈1000 mW cm−2. After photocuring, the sample surfaces were washed with isopropanol to remove unreacted monomers.

Fabrication of Semi-Interpenetrating Polymer Network Incorporating pQA Polymers

First, homogeneous mixtures containing 18 wt% of UDMA, 55 wt% of GDMA, and 25 wt% of pQA were prepared and then 1 wt% of CQ and EDMAB in relation to the total amount of double bonds was added, followed by sonication of the mixture for 1 h to form a clear solution. Subsequently, photocuring was carried out as described above. After photocuring, the sample surfaces were washed with isopropanol several times to remove unreacted monomers.

3D Printing

Two series of photocurable resin mixtures were prepared for 3D printing, requiring slight adjustment of the resin formulations described in the former two sections. To fit the laser wavelength of the stereolithographic printer (Formlabs Form 1), 1 wt% of bisacrylphosphine oxide photoinitiator (Ir819) (Kolczak et al. 1996) was added to the monomer mixture, instead of CQ and EDMAB. For 3D printing, several specially designed CAD models of dental restorations (i.e., molar teeth and crowns) and

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orthodontic retainers as well as specific dumbbell-shaped test bars for tensile tests and discs for the evaluations of contact-killing efficacies of the materials were sliced and each slice was projected onto the bottom layer of the resin tank of the printer. After transferring the resin mixtures into the resin tank, the printing process was started and a beam of UV light drew the object onto the surface of the liquid. Once a layer was completely traced and cured, the z-stage with the substrate was moved upward by 200 μm, covered with new resin and the next layer was cured. The resolution of the device was ≈300 μm in the XY-plane and 25 μm in the Z-direction. After all layers were printed, the printed objects were removed from the platform and washed with isopropanol to remove the adhering resin liquid. Finally, postprinting photocuring was carried out with models in a UV chamber for another 5 h.

Mechanical Evaluations

Tensile evaluations of dumbbell-shaped specimens were carried out to compare the mechanical properties of the materials fabricated by normal curing and 3D printing. The dimensions of the test geometries were the following: thickness: 2.0 mm; width: 4.5 mm; gauge length: 15 mm. The dumbbell-shaped specimens were clamped vertically between two holding grips and then the crosshead elongated the samples with a speed of 1 mm min−1 until the specimen broke. As such breaking stress and strain% were recorded. Three parallel samples for each formulation were prepared.

X-Ray Photoelectron Spectroscopy

The quaternized nitrogen on the surface was determined by XPS. The instrument (S-probe; Surface Science Instruments, Mountain View, CA) was equipped with a monochromatic X-ray source (Al Kα anode yielding 1486.8 eV X-rays), and was operated at 10 kV accelerating voltage and 22 mA filament current. The direction of the photoelectron collection angle was set to 35° with respect to the sample surface, and the electron flood gun was set at 10 eV. A survey scan was made with a 1000 × 250 μm2 spot and a pass energy of 150 eV. Binding energies were determined by setting the binding energy of the C1s binding energy peak (carbon bound to carbon) at 284.8 eV. Detailed scans of the N1s binding energy peaks over a binding energy range of 20 eV were made using a pass energy of 50 eV. The N1s peak was

subsequently decomposed in two fractions at 399.2 and 401.7 eV. The occurrence of a peak at 401.7 eV is indicative for the presence of charged nitrogen species39 and was expressed in at% charged nitrogen species by multiplying the peak fraction at 401.7 eV with the total at% nitrogen.

Contact Angle Measurements

Water contact angles were measured on selected materials using the sessile drop method. Contact angles were calculated from droplet contours using a home-made contour monitor based on black and white thresholding.

Determination of Monomer Conversion Rates Using Fourier-Transform Infrared Spectroscopy (FTIR)

Polymerization kinetics were determined by FTIR using a Bruker IFS88 instrument. To this end, a small amount of monomer mixture was homogeneously spread between two KBr pellets to form a thin film. Next, the samples were irradiated for defined period of times (0, 2, 4, 6, 8, 10, 15, 20, and 30 s) with the dental light source (Optilux 501) and subsequently the FTIR spectra were recorded. The degree of conversion was obtained from the difference of peak areas at a wavelength of 1638 cm^{-1} (CC stretching vibration) before and after polymerization.

Contact Killing by Photocured Quaternary Ammonium Containing Resins Using 3M Petrifilm Aerobic Count Plates

Gram-positive *S. mutans* NS, an own clinically isolated strain from the human oral cavity, causing dental caries, was used throughout this study. The strain was first streaked on a blood agar plate from a frozen stock and grown overnight at 37 °C. One colony was inoculated in 10 mL Todd Hewitt broth (THB, Oxoid, Basingstoke, UK) and incubated at 37 °C for 24 h. 10 mL of this culture was used to inoculate a main culture of 200 mL THB, which was incubated for 16 h at 37 °C. Bacteria were harvested by centrifugation for 5 min at 6500g and 10 °C and subsequently washed two times with buffer (2×10^{-3}M potassium phosphate, 50×10^{-3} m potassium chloride, and 1×10^{-3}M calcium dichloride, pH 6.8). Bacterial suspension

concentrations of 10^4, 10^5, and 10^6 bacteria mL^{-1} were used to evaluate the contact killing of the coatings using a Petrifilm Aerobic Count plate (3M Microbiology, St. Paul, USA) at different challenge numbers. Contact-killing abilities of QA_C$_{12}$ and pQA_C$_{12}$ containing resins were evaluated in absence and presence of an adsorbed salivary film. To this end, human whole saliva from 20 volunteers of both genders was collected into ice-cooled cups after stimulation by chewing Parafilm and then pooled, centrifuged, dialyzed, and lyophilized for storage. Prior to lyophilization phenyl methyl sulfonyl fluoride was added to a final concentration of 1×10^{-3} M as a protease inhibitor. Lyophilized saliva was reconstituted at 1.5 mg mL^{-1} in buffer. Thus, reconstituted saliva will be referred to as "saliva." Volunteers gave their consent to saliva donation, in agreement with the Ethics Committee at UMCG (Approval No. M09.069162). Resin discs were sterilized using 70% ethanol and both discs with and without quaternary ammonium included were stored overnight in 2.5 mL saliva, taken out with a sterile tweezer, and the excess saliva was dripped off before placing the sample on the Petrifilm system. Bacterial suspension droplets (10^4, 10^5, 10^6 bacteria mL-1) of 5 μL were subsequently added on bare resin discs and discs with an adsorbed salivary conditioning film, corresponding with a bacterial challenge number of 30, 300, and 3000 bacteria cm-2, respectively.

A Petrifilm AC plate consists of two films, a bottom film containing standard nutrients, a cold-water gelling agent, and an indicator dye that facilitates colony counting, and a top film enclosing the sample within the Petrifilm system. The top layer of the Petrifilm was lifted to expose the substrate (plating surface) containing the gelling agent and 1 mL of sterilized demineralized water was added. Then the top film was slowly rolled down and a plastic "spreader" was used for even distribution of the liquid. After keeping the film at room temperature for 1 h to allow gelling, a sample disc was placed in between the two layers, followed by addition of 5 μL of an *S. mutans* NS suspension on the disc surface (diameter: 20 mm; thickness: 0.5 mm). After rolling down the top layer, Petrifilms were kept at 37 °C for 48 h. Finally, the number of colony forming units (naked eye–distinguishable red dots) was counted.

Biofilm Inhibition by Photocured Resins with Quaternary Ammonium Compounds

First, *S. mutans* was suspended in sterile buffer at a concentration of 3×10^8 mL^{-1}. Then, resin discs (for dimensions see above) were incubated with 3 mL of this

bacterial suspension at 37 °C for 5 h, followed by exchange of the buffer with 3 mL of fresh THB medium. Next, the samples were incubated at 37 °C for another 6 d and growth medium was exchanged every 48 h. After that, each sample surface was stained with a mixture of SYTO 9 and propidium iodide dyes (LIVE/DEAD BacLight Bacterial Viability Kits) at room temperature for 15 min in the dark for CLSM observations.

UPLC-MS-Chemical Detection of Leakage

Photocured resin discs constituted of UDMA/GDMA/QA_C_{12} (14 mol%) were immersed in 4 mL of demineralized water at 37 °C for 6 d. Afterward, 200 μL of released medium was collected for UPLC-MS. Real-time UV signals were collected and each detectable peak (limit of detection for QA_C12 was 100×10^{-6} M) was measured by MS to confirm the target molecular weight of 326 g mol^{-1}.

Cell Biological Consequences of Leakage

Mouse fibroblasts NCTC-clone 929 (ATCC CCL-1) were grown in monolayer cultures in Dulbecco's modification of Eagle's medium supplemented with 4.5 g L^{-1} d-glucose (DMEM/HG), 10% (v/v) fetal bovine serum, and 0.2×10^{-3} M ascorbic acid-2-phosphate at 37 °C in a humidified atmosphere with 5% CO_2. At 95% confluence, fibroblasts were passaged using a trypsin-EDTA solution (Invitrogen, Breda, The Netherlands) and thus grown cells were used for cytotoxicity testing. Resin discs (surface area of 1 cm²) were inserted in 100 mL cellular growth medium. Fibroblasts (10 000 cells mL^{-1}) were seeded (1 mL) in a 12-well plate and grown for 24 h. Growth medium was replaced every 24 h up to 6 d and each daily elution medium was used to grow fibroblasts for 24 h according to the above protocol after which cell growth was compared with 24 h growth in native 24 h culture medium using phase contrast microscopy.

Microbiological Consequences of Leakage

In order to determine whether elution media contained any antibacterial monomer or QA_C12, a modified Kirby Bauer47 test was carried out. A streptococcal culture was

used to inoculate THB agar plates with a sterile cotton swab. 10 min after inoculation, 10 μL droplets of elution medium were placed in the center of each plate and plates were left to incubate for 48 h at 37 °C in ambient air, after which the width of the inhibition zones around the elution droplets was examined.

Statistical Analysis

Student's t-test was used to determine the statistical difference between various experimental and control groups. Differences at a level of $p < 0.05$ were considered statistically significant.

Results and Discussion

Synthesis Strategies and General Properties of the Resulting Materials

In the course of stereolithographic printing, a z-stage is moved in a liquid polymer resin tank and layer-by-layer photocuring provides one with a 3D object. Due to the outstanding geometry adaptability, different dental restorations can be easily fabricated in a single process just by changing the computer-aided design (CAD) drawing file. In the context of stereolithographic printing, rapid solidification of photopolymer liquid is an essential prerequisite for successful printing (Liska et al. 2007). In this study, biocompatible diurethanedimethacrylate (UDMA) was selected as the frame component, which can be rapidly cross-linked by visible-light irradiation employing the widely used photosensitizer camphorquinone (CQ) in the presence of the coinitiator ethyl 4-dimethylaminobenzoat (EDMAB) (Figure 1a). To decrease the viscosity of UDMA, glycerol dimethacrylate (GDMA) was added as a cross-linkable "diluent" in proportion of 20−40 wt%. As an antimicrobial additive, we synthesized a series of quaternary ammonium-modified methacrylate monomers with different alkyl chain lengths (n = 4, 8, 12, 16). Due to the presence of polymerizable methacrylate groups, the quaternary ammonium (QA_C_n) groups are covalently introduced into the polymer network by in situ copolymerization with the matrix resin components UDMA and GDMA (see also Figure 1a). Water contact angles on thus prepared polymers increased with increasing chain length for n = 4−16 from 58° to 63°, 64°, and 68°, respectively.

Next, the surface atom compositions of the photocured resins were determined by X-ray photoelectron spectroscopy (XPS) upon incorporating QA_C$_{12}$ in the UDMA/GDMA matrix. As shown in Figure 1b, the appearance of an electron binding energy peak at 401.7 eV is indicative of the presence of quaternized nitrogen and supports the successful incorporation of positive charges into the cross-linked polymer matrix (Rouxhet and Genet 2011). By adjusting the content of positively charged monomers before photocuring, the percentage of surface quaternized nitrogen in the afterward photocured material can be controlled (Figure 1c). Note that whereas the %N varied with increasing amounts of QA_C$_{12}$, the surface percentage of nitrogen at a binding energy of 401.7 eV, indicative of the presence of quaternized nitrogen, increased almost linearly with the QA_C12 feed.

In another novel fabrication strategy that was motivated by minimizing leaching products from the resin, a high molecular weight, antimicrobial cationic polymer was incorporated in a semi-interpenetrating polymer network (SIPN) (Figure 2a), therewith trapping the positively charged macromolecule inside the cross-linked matrix. Such a material was realized in two steps: first, QA_C$_{12}$ monomers were converted into a QA-containing polymer (pQA) and mixed with the frame components for photocuring (Figure 2b). pQA was synthesized by copolymerization of cationic QA_C$_{12}$ monomers and 2-hydroxyethyl methacrylate (HEMA) employing the reversible addition-fragmentation chain-transfer (RAFT) polymerization. The comonomer was selected because it increases the compatibility with the frame components by binding to the ester and urethane groups within the cross-linked matrix through hydrogen bonding (see Scheme S1, Supporting Information). Before incorporation into the final resin, unreacted QA monomers and oligomers were removed through a simple precipitation and dialysis procedure. Analysis of the polymerization kinetics showed that the incorporation of the pQA_C$_{12}$ has no significant influence on the conversion of the matrix resin (Figure 2c). Trapping of pQA_C$_{12}$ (25 wt% pQA_C$_{12}$) in the SIPN resin was confirmed using XPS, showing the presence of 8 at% N401.7 eV, which is higher than obtained when copolymerizing QA_C$_{12}$ within a UDMA/GDMA resin. The thiocarbonylthio group at the end of the polymer chain (Figure 2a) may take part in the free-radical chain transfer reactions during photocuring, leading to the covalent conjugation of pQA_C$_{12}$ to the matrix resin (see Scheme S2, Supporting Information).
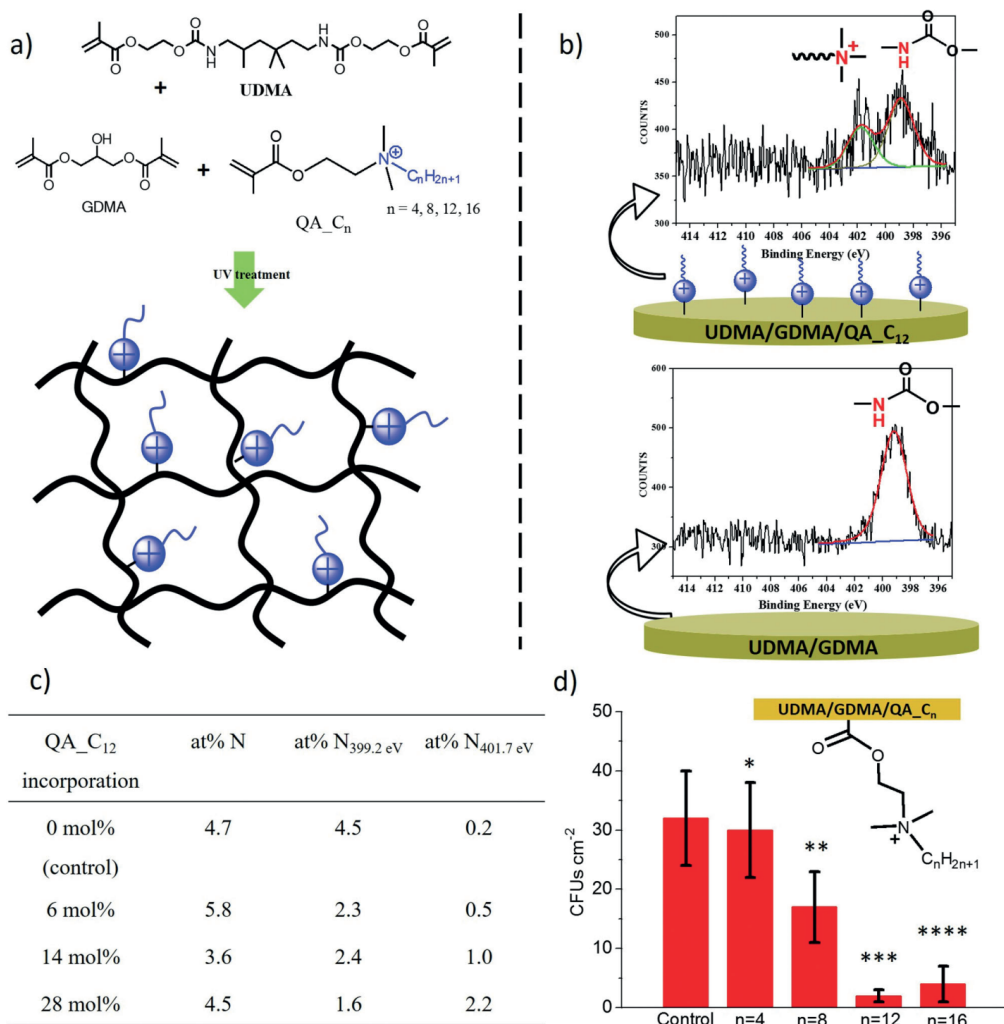
Figure 1. QA_C$_n$ incorporated in a composite resin system. a) Structures of UDMA, GDMA, and QA_C$_n$ monomers, and the incorporation of QA_Cn into the matrix resins; b) N1s electron binding energy peaks for UDMA/GDMA with 14 mol% QA_C$_{12}$ and without QA_C$_{12}$ (control) incorporated; and c) percentage surface nitrogen of UDMA/GDMA/QA_C$_{12}$ resins with different feeds of QA_C$_{12}$ obtained using XPS. The nitrogen peak was decomposed into two components of which the one at 401.7 eV is indicative of the presence of quaternized nitrogen; d) the number of CFUs per unit area (*S. mutans* NS) surviving contact with 14 mol% QA_C$_n$ incorporated resins at a bacterial challenge concentration of ≈30 CFUs cm^{-2}. *p > 0.05, **p < 0.05, ***p < 0.01, and ****p < 0.01 as compared with a control (0 mol% QA_C$_n$).
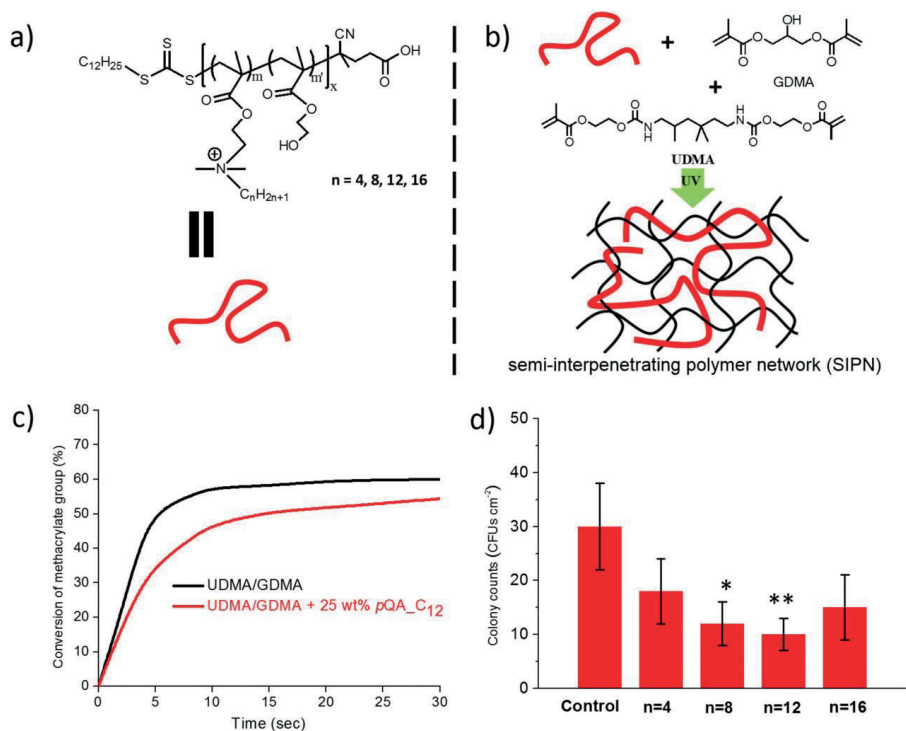
Figure 2. Semi-interpenetrating polymer network with QA_C_n incorporated. a) Chemical structures of QA-containing polymers with different alkyl chain lengths; b) incorporation of pQA polymer into the SIPN resin forming a semi-interpenetrating polymer network; c) kinetics of photopolymerization of methacrylate groups in UDMA/GDMA with and without 25 wt% pQA_C_{12}, as derived from FTIR spectroscopy; and d) the number of CFUs per unit area (*S. mutans* NS) surviving contact with 25 wt% pQA_C_n incorporated in a UDMA/GDMA resin at a bacterial challenge concentration of 30 CFUs cm^{-2}. *$p < 0.01$, **$p < 0.01$ as compared with a control (0 mol% pQA_C_n).

Biological Responses to Positively Charged Monomers opolymerized in a Composite Resin

The bacterial contact-killing activities of solid QA_Cn containing resins were investigated by Petrifilm plate counting of colony forming units (CFUs) of bacteria after adhesion to the resins. To this end, we used Gram-positive Streptococcus mutans, a bacterial strain commonly found in the human oral cavity and a significant contributor to tooth decay (Loesche 1986). The results (Figure 1d) showed that the length of the alkyl chain of the quaternized ammonium group has a significant ($p < 0.05$ for $n = 8$ and $p < 0.01$ for $n = 12$ and $n = 16$) influence on the contact-killing efficacy of the QA_C_n containing resins. Clearly, the killing efficacy increases with increasing alkyl chain length with an optimum for UDMA/GDMA/QA_C_{12}.

UDMA/GDMA/QA_C$_4$ did not show any significant (p > 0.05) streptococcal killing compared to the control. Contrary to several other methods to determine bacterial contact killing, for instance, spray-coating (Haldar, Weight, Klibanov 2007) of a bacterial aerosol onto a surface, the Petrifilm plate counting system allows accurate determination of the bacterial challenge number. Bacterial challenge numbers used in this study range from 30 to 3000 CFUs cm^{-2}. This range comprises both challenge numbers coinciding with bacterial numbers per unit area expected to contaminate biomaterial implants and devices during surgical implantation (Fitzgerald 1979) as well as challenge numbers of bacteria commonly found in early clinical biofilms as averages per unit area in the oral cavity. Table 1 shows that the killing efficacy, i.e., the number of bacteria that are killed upon contact with the material divided by the challenge number, increases with increasing challenge numbers of streptococci for UDMA/GDMA/QA_C$_{12}$, up to >99.99% at a challenge number of 3000 CFUs cm^{-2}. Note virtual absence of streptococcal contact killing by UDMA/GDMA without the QA_C$_{12}$ component (control). These results are in agreement with previous findings employing antimicrobial surfaces modified with quaternized ammonium coatings and polymers that are not 3D printable (Thebault et al. 2009; Tiller et al. 2001; Waschinski and Tiller 2005).

Table 1. The contact-killing efficacy of UDMA/GDMA and of UDMA/GDMA/QA_C$_{12}$ (14 mol%) in absence and presence of an adsorbed salivary conditioning film for different challenge numbers of *S. mutans* NS, obtained using the Petrifilm plate counting system. All data represent triplicate experiments with separate bacterial cultures and individually prepared materials

Material	30 CFUs cm^{-2}	300 CFUs cm^{-2}	3000 CFUs cm^{-2}
In absence of an adsorbed salivary conditioning film			
UDMA/GDMA	< 1%	< 0.1%	< 0.01%
UDMA/GDMA/QA_C$_{12}$	> 99%	>99.9%	>99.99%
In presence of an adsorbed salivary conditioning film			
UDMA/GDMA	< 10%	< 1%	< 0.1%
UDMA/GDMA/QA_C$_{12}$	>99%	> 99.9%	> 99.99%

Since in the oral cavity, materials are continuously bathed in saliva, salivary proteins will always adsorb before bacteria are able to adhere. This raises the important question of whether bacterial contact-killing materials still work after adsorption of a salivary protein film, generally called a "conditioning film." Therefore, streptococci were also allowed to adhere and contact UDMA/GDMA/QA_C_{12} in presence of an adsorbed salivary conditioning film. In Table 1, it can be seen that the presence of a salivary film does not impede streptococcal contact killing. Previously (Asri et al. 2014), the persistence of bacterial contact killing by hyperbranched quaternary ammonium coatings in presence of adsorbed protein films has been attributed to protein displacement underneath adhering bacteria by the pressure developing under the influence of the strong adhesion forces exerted by the positively charged coating upon the negatively charged bacteria (Olsson et al. 2012). In addition, it can be envisaged that bacterial enzymes degrade an adsorbed salivary protein film.

3D Printability and Biological Responses to Positively Charged Monomers Copolymerized in 3D Printed Composite Resin

Once the bacterial contact-killing ability of the quaternary ammonium containing UDMA/GDMA resin in absence and presence of an adsorbed salivary film was established, these materials were employed for 3D printing. For 3D printing, layer-by-layer photocuring is necessary, which possibly affects the tensile strength and bacterial contact-killing efficacy of the printed object. Therefore, we first established that the conversion rate of the methacrylate groups was not affected by the presence of QA_C_{12} at 14 mol%. Figure 3 confirms rapid polymerization (<10 s to reach higher than 55% conversion) in absence and presence of QA_C_{12}, which meets the needs of rapid curing required for stereolithographic printing.

A molar tooth model (Figure 4a, top) and a clear dental splint (Figure 4a, bottom) were successfully fabricated by subjecting a 14 mol% UDMA/GDMA/QA_C_{12} formulation to 3D printing. In order to test the mechanical properties as well as the bacterial contact-killing efficacy of the 3D printed objects, dumbbell-shaped and disc-shaped objects were printed, respectively. Tensile tests of the 3D printed dumbbell-shaped bars were compared to test bars that were fabricated in a polymerization mold by conventional photoillumination. Both test bars exhibited very similar mechanical properties in tensile tests, as reflected in similar breaking stresses and breaking

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elongation values (Figure 4b). Thus, it can be concluded that CAD-sliced layers were fused together well during the layer-by-layer photocuring. Subsequently, the bacterial contact-killing efficacy was investigated employing the Petrifilm plate counting system to examine whether the bacterial contact-killing ability of the material was preserved after 3D printing. From the absence of colony forming units on the 3D printed, UDMA/GDMA/QA_C_{12} disc as compared with the number of colony forming units on the UDMA/GDMA discs without QA_C_{12}, it can be concluded that the bacterial contact-killing ability of the material is preserved during 3D printing (Figure 4c). The long-term contact-killing ability of 3D printed objects was investigated by growing S. mutans biofilms on 3D printed discs. Confocal laser scanning microscopy (CLSM) after live/dead staining of 6 d old streptococcal biofilms on 3D printed UDMA/GDMA/QA_C12 discs showed very little bacteria, that were in addition mostly dead when compared to 3D printed UDMA/GDMA discs without QA_C12 (Figure 4d).

Leaching of unreacted monomer or QA_C12, can not only affect the mechanism of bacterial killing but also the cytotoxicity of the material, although the requirement of absence of cytotoxicity depends largely on the application aimed for. In general, all composite resins demonstrate leakage of antibacterial and slightly cytotoxic components initially after photocuring. The degree of conversion of resin composites is never complete and ≈5%–10% of unpolymerized monomer can be extracted in water (Hansel et al. 1998). In Figure 5a it can be seen, that different components including QA_C12, leach out of UDMA/GDMA/QA_C12 discs in chemically detectable amounts when a volume of 10 mL of water is used as an elution fluid volume, but this does not necessarily imply biological consequences.
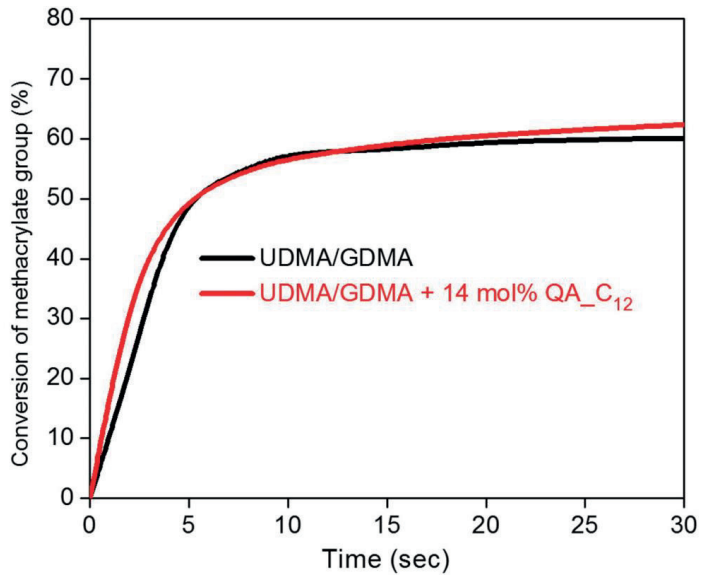
Figure 3. Kinetics of photopolymerization of methacrylate groups in UDMA/GDMA resins with and without 14 mol% QA_C_{12}, as derived from FTIR spectroscopy.
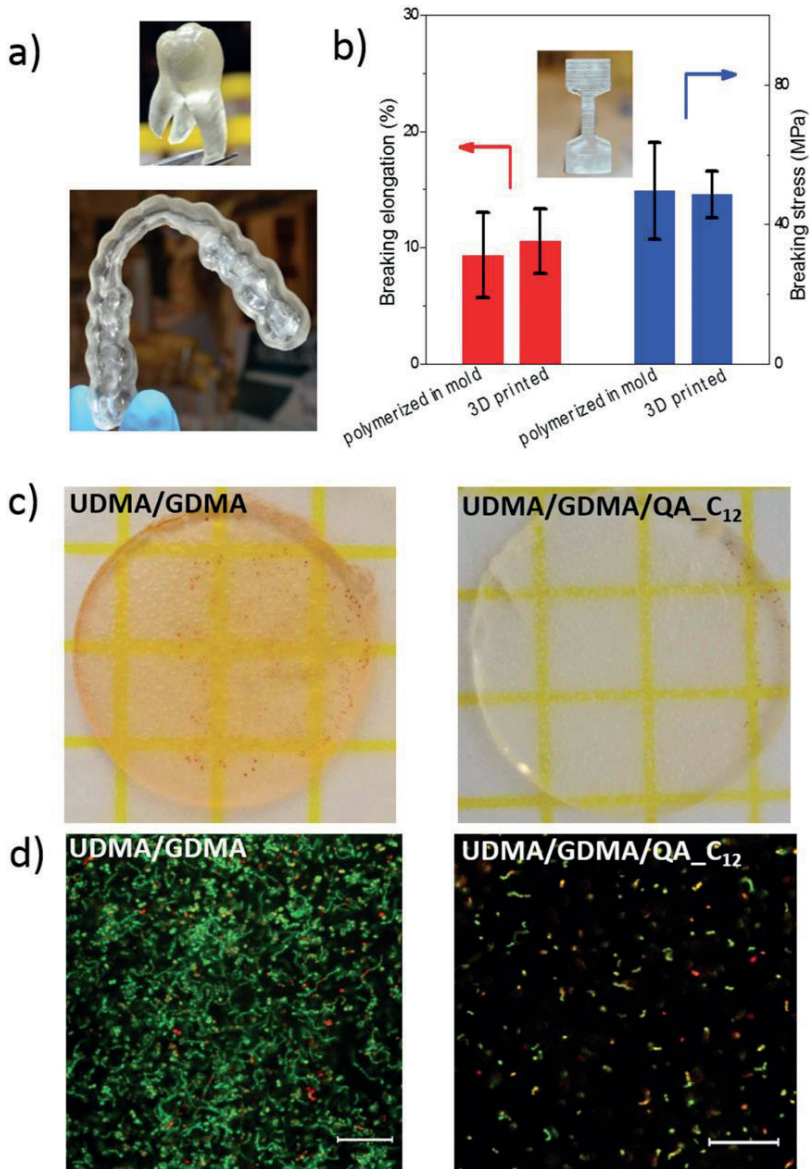
Figure 4. Object examples and properties of 3D printed UDMA/GDMA/QA_C_{12} a) 3D printed model of a molar tooth model (top) and a clear dental splint (bottom), b) tensile properties of 14 mol% UDMA/GDMA/QA_C_{12} 3D printed tensile test bar and a test bar prepared in a polymerization mold by conventional photoillumination, c) comparison of the contact-killing efficacy of 3D printed UDMA/GDMA and UDMA/GDMA/QA_C_{12} discs for *S. mutans* NS in the Petrifilm plate counting system (challenge number equals 30 CFUs cm^{-2}). Presence of CFUs (red dots) on the UDMA/GDMA disc versus the absence of colony forming units on the UDMA/GDMA/QA_C_{12} disc indicates that the bacterial contact-killing ability of the material is preserved upon 3D printing, d) comparison of the long-term contact-killing efficacy of 3D printed UDMA/GDMA and UDMA/GDMA/QA_C12 discs, demonstrated by CLSM after live/dead staining of 6 d old, *S. mutans* biofilms, grown in THB at 37 °C. Live bacteria are green fluorescent, while dead bacteria are red fluorescent. Bar markers indicate 50 μm.

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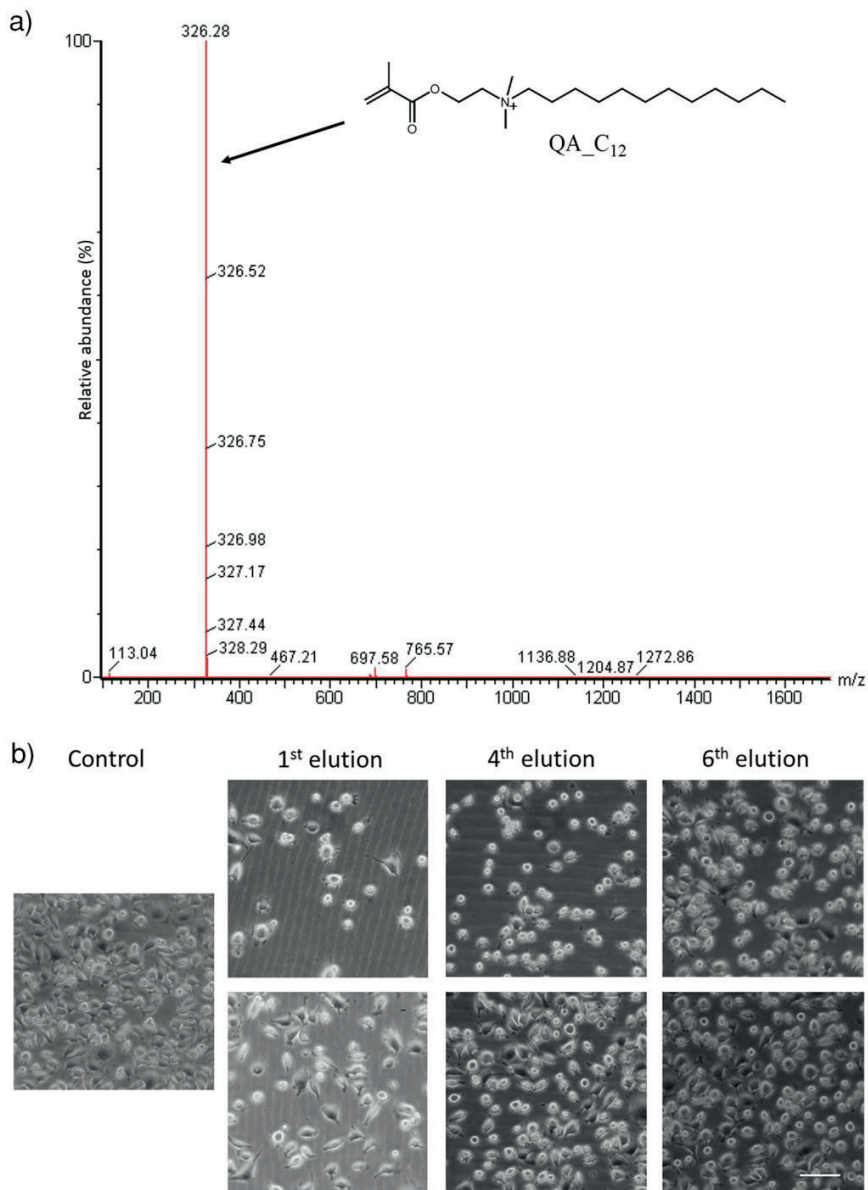
Figure 5. Leaching of QA_C_{12} and effects on cytotoxicity
a) UPLC-MS detection of QA_C_{12} (arrow) leached out of 1 cm² discs containing 14 mol% QA_C_{12} immersed in 10 mL water for 6 d and b) morphology of fibroblast cells after growth in control medium (left panel) and in elution medium. Top row involves UDMA/GDMA discs and bottom row refers to UDMA/GDMA/QA_C_{12} discs. Note initial effects of leached components on the morphology of the cells on both UDMA/GDMA and UDMA/GDMA/QA_C_{12} discs with respect to the control. By comparison with the control, it can be seen that leakage of cytotoxic components, including possibly QA_C_{12}, occurs in cell-biologically negligible amounts within six elutions.

A proper design for experiments that demonstrate a biological significance of the leached amount of QA_C_{12}, is hard to make, as results can be greatly influenced by adjusting the elution fluid volume and the surface area of the material exposed. For maximal sensitivity, ultrahigh performance liquid chromatography−mass spectrometry (UPLC-MS) detection was applied with a small volume of 10 mL (Figure 5a). However, considering application of the present material in the oral cavity, only small surface areas of the material in the order of magnitude of 1 cm^2 will be exposed to saliva. The volume of saliva present in the human oral cavity is several mL's and is swallowed and replaced continuously during the day. Daily, between 1000 and 1400 mL of saliva is secreted by the different glands in the oral cavity (Dawes 2008). Therefore, we based our experiments to investigate a possible biological significance of the amount of QA_C_{12} leached out, on a total 24 h elution fluid volume of 100 mL (representing a tenfold "worst-case scenario"), immersing a UDMA/GDMA/QA_C_{12} disc with a total surface area of 2 cm^2. Note that based on these considerations, our chemical detection assay represents a hundredfold "worst case scenario." Elution fluid was cellular growth medium and was replaced every 24 h, up to 6 d, using each daily volume for experiments. No zones of inhibition were observed around droplets of elution media placed on bacterially inoculated agar plates ("modified Kirby−Bauer test") (Darouiche et al. 1998), demonstrating absence of microbiologically significant amounts of monomer or QA_C_{12} leaching out over the course of minimally 6 d. In order to demonstrate possible cytotoxic effects, fibroblast cells were cultured in the elution media collected during different 24 h intervals. Fibroblasts are routinely used in cytotoxicity demonstration due to their high sensitivity to cytotoxic compounds. In Figure 5b, it can be seen that fibroblasts grow equally well with a similar morphology in 4 and 6 d elution medium as in growth medium. This implies that monomers leach out in cell-biologically significant amounts during the first 24 h of elution in 100 mL medium, but this release is highly temporary as common with composite resins, apart from the fact that we studied release in a tenfold "worst-case scenario."

Biological Responses to Prepolymerized Cationic Polymer Chains Incorporated into a Semi-Interpenetrating Polymer Network

Similar to the small molecule−based QA systems, contact killing with *S. mutans* NS of pQA (25 wt%) containing SIPN resins was evaluated. The pQA_C_{12} containing

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SIPN exhibited the highest contact-killing efficacy toward S. mutans (Figure 2d) compared to the other alkyl chain lengths on the nitrogen center, as did UDMA/GDMA/QA_C$_{12}$. Differences between chain lengths were less pronounced, however, than for UDMA/GDMA/QA_C$_{12}$ (compare Figures 1b and 2d). Subsequently, the UDMA/GDMA/pQA_C$_{12}$ containing SIPN was subjected to more extensive evaluation of its contact-killing ability and streptococcal challenge numbers were increased, while also the ability of the material to kill adhering streptococci upon contact in the presence of an adsorbed salivary film was evaluated (Table 2). Interestingly, bacterial contact killing by UDMA/GDMA/QA_C$_{12}$ in absence of a salivary conditioning film is similar as observed for UDMA/GDMA/pQA_C$_{12}$ (compare Tables 1 and 2), but in presence of a conditioning film, UDMA/GDMA/pQA_C$_{12}$ loses a couple of percentages in contact-killing efficacy despite the higher amount of quaternized nitrogen.

3D Printability and Biological Responses to 3D Printed Prepolymerized Cationic Polymer Chains Incorporated into a Semi-Interpenetrating Polymer Network

Subsequently, 3D objects from pQA_C$_{12}$ containing UDMA/GDMA resin were printed. As shown in Figure 6a, different dental models and appliances were successfully fabricated by stereolithographic printing and their geometry and sizes were in accordance with the predetermined specifications. However, it was found that a material's compatibility with 3D printing was strongly dependent on the viscosity of the printing liquid. Printing was only successful when the content of the high-viscosity UDMA was above 40 wt%. Tensile properties of 3D printed test bars of pQA_C$_{12}$ containing UDMA/GDMA resin were similar as for UDMA/GDMA/pQA_C$_{12}$ fabricated in a polymerization mold (Figure 6b), although the material is considerably more brittle than UDMA/GDMA/QA_C$_{12}$ (compare Figures 4b and 6b). Moreover, also bacterial contact-killing efficacy of 3D printed discs made of pQA_C12-containing UDMA/GDMA resin was fully preserved upon printing (Figure 6c).

UPLC-MS is a suitable method to chemically detect small molecules with a molecular weight of less than 5000 Da, but molecules with a higher molecular weight are difficult to detect. Accordingly, we attempted to detect pQA_C12 from a UDMA/GDMA SIPN resin using UPLC-MS as done for QA_C12 (see Figure 5a), but

we observed no signals from pQA_C12 in water. Therefore, we only carried out assays to establish the absence of biological consequences of possible leaching. As did UDMA/GDMA/QA_C_{12}, neither monomer nor pQA_C_{12} leached out of UDMA/GDMA/pQA_C_{12} in microbiologically significant amounts. However, by comparison with UDMA/GDMA/QA_C_{12}, the first elution from UDMA/GDMA/pQA_C_{12} yielded a higher number of cells (compare Figure 5b with Figure 7). This confirms that leakage of pQA_C_{12} from UDMA/GDMA/pQA_C_{12} is reduced in a cell-biologically significant manner.

Table 2. The contact-killing efficacy of UDMA/GDMA (taken from Table 1) and of 25 wt% UDMA/GDMA/pQA_C_{12} in an SIPN resin in absence and presence of an adsorbed salivary conditioning film for different challenge numbers of *S. mutans* NS, obtained using the Petrifilm plate counting system. All data represent duplicate experiments with separate bacterial cultures and individually prepared materials

Material	30 CFUs cm^{-2}	300 CFUs cm^{-2}	3000 CFUs cm^{-2}
In absence of an adsorbed salivary conditioning film			
UDMA/GDMA	< 1%	< 0.1%	< 0.01%
UDMA/GDMA/pQA_C_{12}	> 99%	>99.9%	>99.9%
In presence of an adsorbed salivary conditioning film			
UDMA/GDMA	< 10%	< 1%	< 0.1%
UDMA/GDMA/pQA_C_{12}	>82%	>97	>98
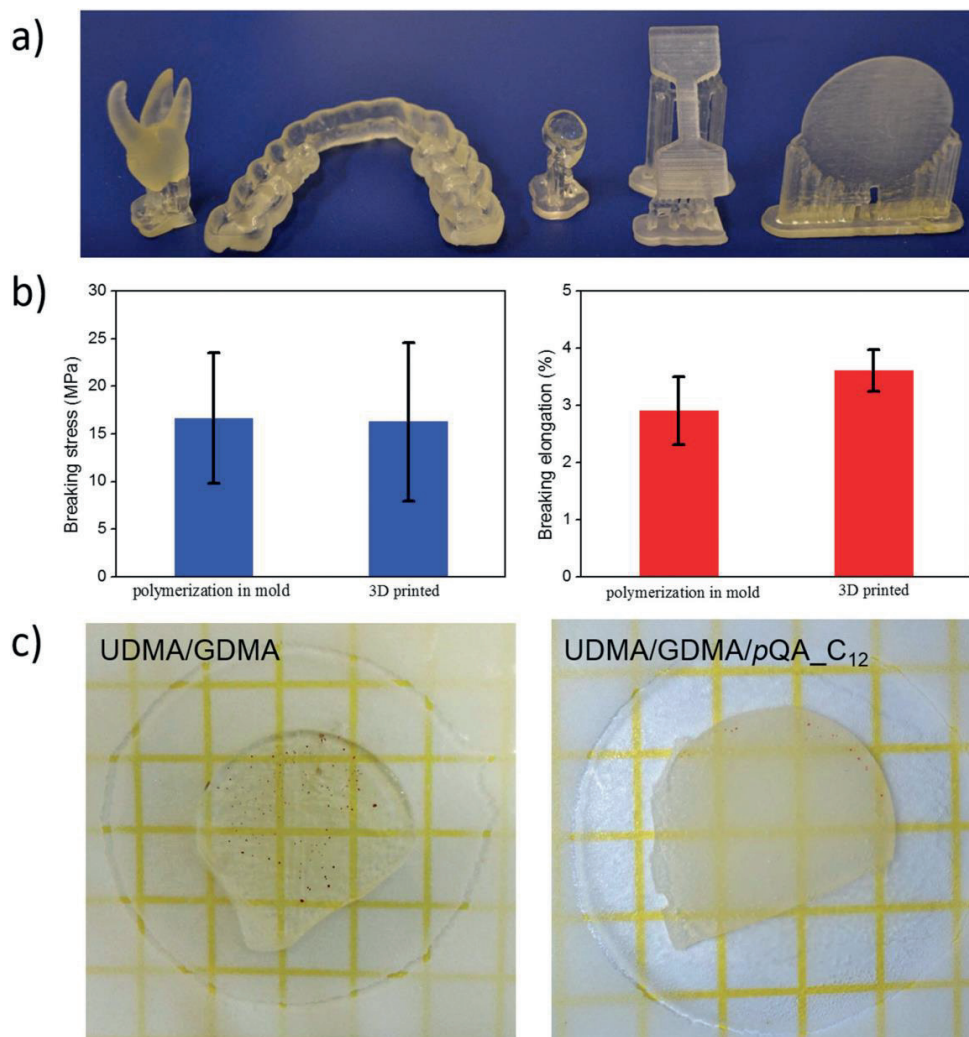
Figure 6. Object examples and properties of 3D printed, 25 wt% pQA_C$_{12}$ in a polymer-incorporated SIPN resin of UDMA/GDMA. a) 3D printed dental appliances or models (from left to right: molar tooth, clear splint, crown, tensile test bar, contact-killing test disc) based on polymerized positively charged compounds incorporated in an SIPN resin; b) tensile properties of 25 wt% pQA_C$_{12}$ in a polymer-incorporated SIPN system of UDMA/GDMA 3D printed tensile test bar and a test bar prepared in a polymerization mold by conventional photoillumination; and c) comparison of the contact-killing efficacy of a 3D printed 25 wt% pQA_C$_{12}$ in a polymer-incorporated SIPN system of UDMA/GDMA disc for *S. mutans* NS in the Petrifilm plate counting system with the one of a UDMA/GDMA disc (challenge number equals 30 CFUs cm^{-2}). Presence of CFUs (red dots) on the UDMA/GDMA disc versus the absence of CFUs on the UDMA/GDMA/QA_C$_{12}$ disc indicates that the bacterial contact-killing ability of the material is preserved upon 3D printing.
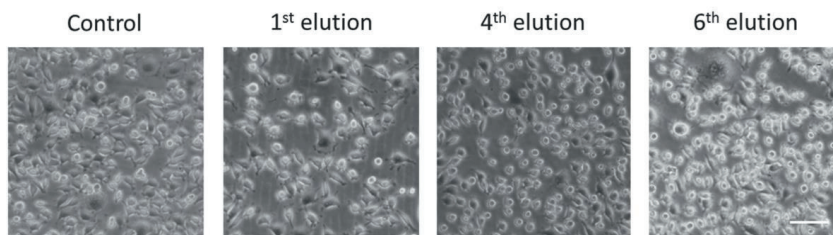
Figure 7. Biological consequences of leaching of pQA_C_{12} from a polymer-incorporated SIPN resin of UDMA/GDMA. Morphology of fibroblast cells after growth in elution medium (see above). Well spread cells are indicating leaching of cytotoxic components including pQA_C_{12} in cell-biologically negligible amounts.

Several other compounds including, for instance, telechelic poly(2-oxazoline), have been used as additives to dental repair materials (Fik et al. 2014), but materials were seldom evaluated for their ability to kill adhering bacteria upon contact in the presence of a salivary conditioning film. A quaternary ammonium methacryloxy silicate added to dental acrylic (Gong et al. 2013) was evaluated in the presence of an adsorbed salivary conditioning film and showed contact killing of an adhering *S. mutans* and *Actinomyces naeslundii* strain. Moreover, 24 h biofilm formation was inhibited after three months water aging of the materials. Our study demonstrates 6 d efficacy of our QA_C_{12} containing resins against bacterial adhesion and growth. Considering that most people brush their teeth to remove oral biofilm twice a day but will inevitably leave biofilm behind at random, difficult to reach sites, the ability of these surfaces to reduce oral biofilm formation over a time period of several days demonstrates that contact killing of adhering bacteria may be a clinically relevant way to prevent oral biofilm-related diseases. None of the contact-killing materials described in the literature are 3D printable, which makes our QA_C_{12} containing resins new, offering many opportunities for developing antimicrobial objects for diverse applications (EPC Application No. 15167409.0-1454).

Conclusion

In summary, we have presented two general strategies to fabricate antimicrobial resins. Positively charged monomers with an appended alkyl chain are responsible for the antibacterial property and were either directly copolymerized with conventional resin components by photocuring or prepolymerized as a linear chain, which was then incorporated into a semi-interpenetrating polymer network by light-induced polymerization. Although both strategies yielded materials with minimal leaching of

bioactives, the latter strategy resulted in polymer resins that exhibited the least leaching of the bioactive positively charged moieties possibly due to the formation of hydrogen- or covalent bonds between the cross-linked network and the antimicrobial polymer. The contact-killing abilities of the antimicrobial resins were even preserved upon coating of the materials with a salivary conditioning film, although incorporation of pQA_C_{12} in a polymer-incorporated SIPN resin yielded slightly less bacterial contact killing than copolymerizing QA_C_{12} directly in the resin. Therewith, the antimicrobial resins prepared can find application in dentistry as an adhesive, luting cement, or composite either to restore cavities or fix orthodontic brackets on teeth. With only little variation, both resin formulations were amenable to 3D printing with the prepolymerized QA polymer strategy yielding a more brittle material that has to be accounted for depending on the specific application aimed for. Complex geometries of oral appliances such as antimicrobial crowns or splints were successfully realized and the 3D printed objects exhibited mechanical properties that were almost identical to conventionally photocured polymer samples. The antimicrobial properties were shown to be caused by bacterial contact killing with the material rather than the release of antimicrobial compounds from the resin. Having optimized the activity and stability of these materials, we have a prototype at hand that is suited for further testing in a clinical setting, including not only dental applications but also, for instance, orthopedic ones like spacers and other polymeric parts used in total hip or knee arthroplasties. Moreover, the approach to developing 3D printable antimicrobial polymers can easily be transferred to other nonmedical application areas, such as food packaging, water purification, or even toys for children. To the best of our knowledge, the resins we developed represent the first report of an antimicrobial, contact-killing 3D printable material (EPC Application No. 15167409.0-1454).

Acknowledgements

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Chapter 6-II

**Media coverage
of a scientific project on 3D printable
antimicrobial composite resin**

*Marieke van de Lagemaat, Henny C. van der Mei,
Henk J. Busscher, Yijin Ren*

Abstract

Our manuscript on the development of a 3D printable antimicrobial composite resin, within the first weeks after being published in Advanced Functional Materials, immediately received tremendous attention from both national and international mainstream media. Even though our research project received enormous attention and positive recognition at the social media platforms, the impact of these reports is a double-edged sword. The public attention of this research project could be largely attributed to recognition by the general public of its potential societal impact that the research outcome might generate but was not based on its true scientifically proven content. Therefore, it remains a challenge for researchers to reach the public with their research project, and properly guide this process so that the right information and interpretation are spread.

In October 2015, our manuscript on the development of a 3D printable antimicrobial composite resin (chapter 6) was published in Advanced Functional Materials (Yue et al. 2015), and immediately received tremendous attention from both national and international mainstream media. The work described in this article presented a novel polymeric material which shows positive surface charge after photo-curing (polymerization) without leaching of any non-polymerized components nor toxicity to human tissue cells. The positive surface charge of the material demonstrated a bactericidal effect when in contact with bacteria that generally have a negative cell surface charge (killing on contact). The material provides sufficient mechanical and favorable physicochemical properties for a variety of clinical applications, including 3D printing (Yue et al. 2015).

The aim of this chapter is to summarize the media coverage on this novel 3D printable antimicrobial composite resin and discuss the double-edge sword effect of social media on scientific work.

During the project in 2014 two Dutch newspapers had already reported the study in, 'De Telegraaf', and 'Dagblad van het Noorden', which are two daily newspapers with the largest circulations in the Netherlands and in the North of the Netherlands, respectively (Figure 1 and 2). With the progress of the study and subsequent publication, various news websites in de The Netherlands and in China reported the outcome with enthusiastic and exciting titles (Figure 3) (Groninger Internet Courant 2014; Nu.nl 2014; RTV Drenthe 2014; Ouderenjournaal 2014; Nationale zorggids 2014; Radio Westerwolde 2014; Lunchradio 2014). The reports on the research project were informative and generally accurate about the functionality of the material and its promising potential for clinical applications. After the online publication (The Wiley Online Library 2015) on October 9th 2015 the attention from the media became tremendous. In different parts of the world, i.e. India, China, Russia, Canada, United States of America, Nigeria and in Europe articles were posted on local websites or reported on radio and television. Direct contacts were sought out with the researchers in the Netherlands and from Germany for live radio interview (BNR eyeopener radio interview 2015), television interview (BBC World Service 2016) etc. In addition, reports were posted in social media platforms like Twitter and Facebook. In the same month, on 26th of October 2015, Google showed 4 pages of hits on '3D printable antimicrobial resins'.

With the increasing number of scientific publications every year, even professionals can only manage to read critically a very small percentage of scientific articles related to their work (Bouter and Knotterneus 2000). Therefore, publications in local journals or on social media have become an important channel to obtain knowledge and updates for professionals and the general public alike (ERiC; 2010). Reports on the world wide web can spread information easily and speedily. On the website of Altmetrics.com a tool can be found which enables an analysis of how different scientific disciplines are represented in modern media types. This website is a specialized search engine and is able to search for scientific articles that are mentioned in "blog posts, tweets, and mainstream media". It offers an interesting possibility to measure the impact of research in the modern media. According to a study for Altmetric scores for papers published in high-ranking journals, Nature, Science and PNAS had an average score of 70 (Wilbertz 2013). The paper of 3D printable antimicrobial composite has a score of 134 points on Altmetrics (Figure 4) indicating a relatively large resonance in the mainstream media.

Even though our research project received tremendous attention and recognition at the social media platforms, the impact of these reports is a double-edged sword. Scientific articles, which are written in jargon language are written by scientist for scientists, and can therefore be difficult to interpret by people outside the scientific domain. This can lead to distortion of the content of the papers. One example was found on the website of 'New Scientist'(New Scientist 2015), where a report stated ; "…of the University of Groningen in the Netherlands have developed an antimicrobial plastic, allowing them to 3D print teeth that also kill bacteria" (Figure 5). This statement by itself is not incorrect, but implied that such a 3D printed tooth is functional and therefore can be used as a replacement for lost teeth or implants, which is entirely misleading. Other news reports had provoking or catchy titles, like: "Never brush again" (Chemie is overal 2015) or "New 3D-printed teeth also kill bacteria" (Dental products report 2015) or "Lost a tooth? Soon your dentist can print another and it keeps your mouth clean as well" (Figures 6). Some of these reports were accurately, while others remained vague about the actual content causing a multiple interpretable message. One report on the UK RuG website (Newsletter from University of Groningen) first posted the report with the title: "Never brush again", changed only later to "New adhesive for braces keep the teeth clean" (Figure 7) (University News UK 2015).

Clips of news reports

Figure 1: Article from a Dutch Newspaper 'De Telegraaf' about braces that kill bacteria, published in May 2014. Title translated in English: "Bacterial killing braces".

DAGBLAD VAN HET
NOORDEN

03 juni 2014 , pag. 4

Beugels doden bacteriën

GRONINGEN Orthodontisten van het UMCG doen onderzoek naar de mogelijkheid beugels en tandprotheses te maken van bacteriedodend materiaal.

De nieuwe protheses en beugels moeten voorkomen dat er tandplaque ontstaat dat zich ophoopt rondom de beugel. Deze plaque kan leiden tot gaatjes en witte vlekken in de tanden.

Ongeveer 40 procent van de jongeren van twaalf jaar en ouder krijgt een beugel, maar ook steeds meer volwassenen kiezen voor een rij rechte tanden. Uit onderzoek blijkt dat ongeveer 60 procent van alle pa-

tiënten uiteindelijk problemen ondervindt in deze twee tot drie jaar durende behandeling als gevolg van tandplaque. Van hen heeft 15 procent zelfs professionele zorg nodig vanwege aangehechte bacteriën.

Deze schade komt het meest voor bij jongeren. Volgens hoogleraar orthodontie Yijin Ren vinden kinderen in deze leeftijd poetsen sowieso lastig. „Met een beugel is dat nog veel lastiger. Bovendien worden composietmaterialen op de tanden gebruikt als lijm voor de brackets of slotjes." De bacteriën hierop kunnen het glazuur aantasten, waardoor niet alleen witte vlekken kunnen ontstaan, maar ook gaatjes. Dit komt

door de ontkalking van de tanden.

Alleen al in Amerika blijkt dat deze nazorg een kostenpost van 500 miljoen dollar met zich meebrengt. „We hebben het dan nog niet eens over groeiende economieën als China, Brazilië en Turkije, waar orthodontie aan een enorme opmars bezig is", stelt Ren. „Wereldwijd wordt er daarom hard gezocht naar nieuwe materialen."

Volgens Ren zal het nog even duren voordat de eerste bacteriedodende beugels beschikbaar komen. Zij wijst er nadrukkelijk op dat tot die tijd voorlichting over mondhygiëne, goed poetsen en regelmatige controle heel belangrijk blijft.

Figure 2: Report from a Dutch Newspaper 'Dagblad van het Noorden' about braces that kill bacteria, published in June 2014. Title translated in English: "Braces kill bacteria".

Figure 3: Report from a Chinese mainstream media website reporting on 3D printable antimicrobial composite published in June 2014. Title translated in English: "Novel antibacterial material may pioneer 3D printing revolution in health care".
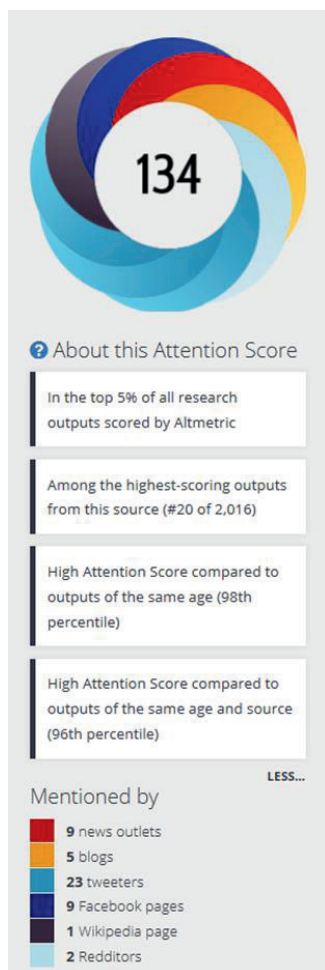
Figure 4: Score on Altmetrics.com in November 2017, of the paper: Yue J, Zhao P, Gerasimov JY, van de Lagemaat, Grotenhuis A, Rustema-Abbing M, van der Mei, Busscher HJ, Herrmann A, Ren Y. 2015. 3D-printable antimicrobial composite resins. Adv Funct Mater. 25(43):6756-67.

Dutch researchers 3D print teeth that can kill 99% of bacteria in the mouth

October 20, 2015 • News

Researchers at the University of Groningen in the Netherlands have developed a 3D printed plastic that is able to destroy 99% of bacteria that come into contact with it without causing any harm whatsoever to humans.

This amazing plastic can have a wide range of applications, but what's most interesting is that it can be used to 3D print teeth and braces.

According to their article "3D-Printable Antimicrobial Composite Resins", the team of scientists managed to embed antimicrobial quaternary ammonium salts inside existing dental resin polymers. The salts are positively charged and so disrupt the negatively charged bacterial membranes, which causes them to burst and die out.

3D printed tooth
Image credit: Herrmann, Ren et al. / www.rug.nl

The mixture was hardened by using ultraviolet light and then the team printed out a whole range of dental objects.

In order to test the plastic's antimicrobial properties, the scientists covered the 3D printed samples with a mixture of saliva and Streptococcus mutans, the bacterium that causes tooth decay, and found that the material had killed over 99% of the bacteria.

Although the scientists are convinced that the applications of the 3D printed plastic could be extended greatly, they do admit that further research and further testing are needed in order to be sure that the material will be strong enough to be used as an actual tooth.

Further tests are also needed to evaluate whether the material would be compatible to have the same effects on bacteria if it were used for other products such as toothpastes or retainers.

"The antimicrobial properties were shown to be caused by bacterial contact killing with the material rather than the release of antimicrobial compounds from the resin. Having optimised the activity and stability of these materials, we have a prototype at hand that is suited for further testing in a clinical setting, including not only dental applications but also, for instance, orthopaedic ones like spacers and other polymeric parts used in total hip or knee arthroplasties," the researchers write.

"Moreover, the approach to developing 3D printable antimicrobial polymers can easily be transferred to other nonmedical application areas, such as food packaging, water purification, or even toys for children. To the best of our knowledge, the resins we developed represent the first report of an antimicrobial, contact-killing 3D printable material."

Figure 5: Media report on 3D printable antimicrobial composite resin, with a provoking title reporting on bacterial killing printed teeth. From: Australian Manufacturing [Internet]. Dutch researchers 3D print teeth that can kill 99% of bacteria in the mouth; [cited 2015 Oct 20]. Available from: http://www.australianmanufacturing.com.au/33028/dutch-researchers-3d-print-teeth-that-can-kill-99-of-bacteria-in-the-mouth

Lost a tooth? Soon your dentist could print you another – and it'll help keep your mouth clean, too.

Figure 6: First sentence of news report from New Scientist [Internet]. 3D printed teeth to keep your mouth free of bacteria; [cited 2015 Oct 16]. Available from: https://www.newscientist.com/article/dn28353-3d-printed-teeth-to-keep-your-mouth-free-of-bacteria

Figure 7: Title of publication in 2015 saying : "Never brush again?" corrected to title saying: "New adhesive for braces keep the teeth clean" From: http://archief.ukrant.nl/wetenschap-onderwijs/wetenschap-wetenschap-onderwijs/nieuwe-beugellijm-houdt-tanden-schoon.html

Figure 8: A 3D printed molar tooth with the newly developed contact-killing material. From: Yue J, Zhao P, Gerasimov JY, van de Lagemaat, Grotenhuis A, Rustema-Abbing M, van der Mei, Busscher HJ, Herrmann A, Ren Y. 2015. 3D-printable antimicrobial composite resins. Adv Funct Mater. 25(43):6756-67.

Misinterpretations in the news may understandably be related to the picture of a 3D printed tooth model from the original publication (Figure 8). Nevertheless, the main reason for reporting and rapid spreading of a distorted message is the lack of fact checking and the current model of 'social media journalism', which can easily result in a vicious circle of reporting 'false' or 'misleading' news (Social Embassy 2014).

To prevent the start of spreading of inaccurate news, the introduction of a plain language summary in addition to the scientific abstract, may aid journalists to formulate a realistic story with the correct conclusions, which eventually will help knowledge transfer to a broader public and encourage better connections between scientific research and its societal impact.

In conclusion, the research project, described in chapter 6, received great public attention, which can be largely attributed to recognition by the general public of the significant societal impact the research outcome may generate. However, it remains a challenge for researchers to reach the public with their research project while guiding this process so that the right information are spread.

In addition we present here a list of the available mainstream media reports, with the title of the report and the link to the website in a chronological order. The search took place in August and September 2017 with Google using the term: "3d printable antimicrobial composite resin" and showed in total 139 hits including a free search.

Links of mainstream media reports

English media

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- The Business Insider [Internet]. This 3-D-printed artificial tooth could eliminate bacteria; [cited 2015 Oct 17]. Available from: http://www.businessinsider.com/this-3-d-printed-artificial-tooth-could-eliminate-bacteria-2015-10?IR=T
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Chapter 7

General discussion

Orthodontic treatment aims at improving function of the masticatory system, correcting dental irregularities and achieving facial harmony and esthetics, ideally without side effects caused by the accumulation of dental plaque or oral biofilm around the orthodontic appliances. In order to avoid biofilm formations during orthodontic treatment different antibacterial measures exist. This thesis describes an investigation of antibacterial measures for oral biofilm related infection, demonstrating that a combination of antibacterial measures would contribute greatly to the management of biofilm. These findings may be of considerable value to clinical practice and the society in general.

Motivating patients to maintain good oral hygiene by mechanical and chemical measures is daily practice for dental and orthodontic professionals. Removal of oral biofilm is important for prevention of dental diseases, but biofilm can never be thoroughly removed, especially not from fissures, buccal pits, interproximal areas, gingival margins and around orthodontic appliances. We adopted an *in vivo* model for such retention sites in the oral cavity using multi- strand wires. Two important findings are clinically relevant. First, a synergy between brushing mode and antibacterial-regimes exists, whereby the use of powered toothbrushes enhances the action of oral antimicrobials. The more 'fluffed-up', open state of the biofilm after powered brushing facilitates antimicrobial penetration. Second, by using different regimens of oral antimicrobials, an evident change in composition of oral biofilm can be achieved. Particularly the use of triclosan combined with an essential oil containing mouthrinse led to a distinct decrease in the prevalence of cariogenic bacteria, such as *Streptococcus mutans* (Loesche 1986) and *Lactobacilli* (Caufield et al. 2015), when compared to a solely Natrium Fluoride-containing toothpaste without antibacterial claims. This reduction in the presence of these cariogenic species might point to a shift in the composition of the adhering oral microbiome in a more healthy direction. After using the antimicrobial regime only one week, this change in composition of the biofilm was already clearly visible (see Chapter 3) and may become stronger after prolonged use of the oral antimicrobials. Especially because the oral microbiota is natural and provides benefits to the host (Marsh 2018), approaches to modify the microbiota to a more healthy and biological equilibrium is logical.

In oral self-care, mouthwash and toothpaste containing antimicrobials are commonly used to assist control of biofilm growth (James et al. 2017; Riley and

Lamont 2013; Marinho et al. 2003). However, extensive use of antimicrobials may result in development of resistant bacterial strains. Concerns about presence of antimicrobial resistant bacterial strains in the oral cavity arise, especially of *Staphyloccocus aureus* (Block and Furman 2002), commonly isolated from infections around implants (McCormack et al. 2015). In light of recent findings that bacteria in dental biofilm with reduced susceptibility to chlorhexidine, can also develop multidrug resistance (Saleem et al. 2016), investigation of mechanisms to develop antimicrobial resistance by oral bacteria may not be overlooked. Using surface enhanced fluorescence, nanoscopic bacterial cell wall changes after exposure of chlorhexidine were demonstrated to be dependent on the bacterial strain, indicating differential response and repair mechanism of *S. aureus* and *S. mutans*. Accordingly, *S. aureus* developed resistance toward chlorhexidine, while our *S. mutans* did not. These findings raise the question about the routine use of chlorhexidine in dentistry and its over-the-counter availability for the patient. Frequent use of chlorhexidine may induce resistance of *S. aureus*, making the treatment with antibiotics when necessary ineffective or even potentially unsafe for medically comprised patients. The precise mechanism behind these different survival strategies is worth exploring in further research. Using atomic force microscopy (AFM), transmission electron microscopy (TEM) and scanning electron microscopy (SEM) cytological evidence may be obtained to help explain the mode of action of bacterial resistance to chlorhexidine, eventually leading to recommendations for clinically safe and effective use of antimicrobials.

Although measures such as tooth brushing and the use of antimicrobials in toothpaste and mouthwashes are effective in maintaining a healthy equilibrium of the oral flora, these measures are dependent on patient compliance, which often declines during a long course of orthodontic treatment (Al-Jewair and Suri 2011). Alternatively, compliance independent pathways for interception of biofilm related problems can be very beneficial. In a survey for patients to identify relevant research topics, all respondents, including patients, parents of patients, orthodontists and paramedics scored highest for 'non-compliance' bacterial-killing adhesives with lasting killing effect. The outcome of this survey demonstrates that the opinion of end-users makes a valuable contribution, not only to establish a sound professional basic clinical relationship between doctors and patients, but also to get a better understanding of their needs. With increasing awareness of the importance of oral

health and recognition of academic research being part of a wider process in healthcare, the public's opinion is becoming a leading factor, influencing the policy makers in decisions on funding priorities and healthcare strategies. Therefore, obtaining public support through interactions between scientists and end-users is an important aspect for research in academia (Bouter 2010).

Development of 'non-compliance' dental and orthodontic materials attracting less biofilms goes back for decades. One of the earliest examples of non-leaching organ silicon quaternary ammonium compounds (QAC) capable of killing microorganisms on contact was reported in the early 1970s (Isquith et al. 1972). Since then, many attempts have been made to develop effective antimicrobial adhesives to prevent biofilm. QAC is a promising antibacterial monomer for clinical applications (Makvandi et al. 2018). Yet, till date antibacterial materials are not commonly available for orthodontic patients. Most research on antibacterial composites were based on laboratory tests, where no unanimously accepted method exists in literature that can reliably evaluate the efficacy of bacterial contact-killing on these surfaces, making comparison between different studies meaningless. We showed that three out of five commonly used methods for evaluating contact-killing, including an established ASTM, are unsuitable. Methods found suitable are Petrifilm® and JIS Z 2801 (Japanese Industrial Standards), provided being used in combination with a zone-of-inhibition-assay to establish absence of antimicrobial leaching, which can potentially interfere with contact-killing. The modified JIS method is acceptable, but does not contain balanced amount of nutrients and should only be used with respect to a non-contact killing control. ASTM (E2149-13a) and bacterial spray methods are not reliable, the main reason being the lack of control over the applied bacterial challenge and actual contact of bacteria with the surface. The identification of suitable assays for evaluating bacterial contact-killing will greatly assist progress in this emerging field and may be valuable to the advancement of clinical downward translation. For future research, methods suitable for studying multi-species biofilm may extend our knowledge of the efficacies of contact-killing materials.

From engineering perspective manufacturing of 3D printable material with the unique feature of contact killing is promising for clinical applications. In orthodontics, 3D printing technology is currently used in aligner systems, where series of 3D printed models are made for producing aligners, in diagnostic set-ups, indirect bracket-bonding sets (Dawood et al. 2015) and removable retainers (van der

Meer et al. 2016). However, a relatively narrow range of biocompatible, 3D printable materials with a limited spectrum of physico-chemical properties still restricts the application of this disruptive technology (Umme Kalsoom et al 2016). Interest in printable, biologically 'smart' materials with extensive features such as bacterial killing and desirable mechanical strength is growing. The two strategies we presented in chapter 6-I demonstrated potent killing effect on bacteria associated with dental caries, without compromising the biological and mechanical properties of the materials required for their clinical performance, attributed to the incorporation of QAC in a resin matrix. For eventual clinical use, *in vivo* evidence about the prevention of caries or gingivitis due to the use of antibacterial composite is valuable, but not yet available (Pereira-Cenci et al. 2013). Future research into the long-term effect and *in vivo* effects of contact-killing materials is worth exploring.

A combination of different antibacterial measures decreases the risk for oral biofilm related infections in orthodontics and oral health in general. Oral biofilm control requires a well-maintained balance between efficacy and safety of the measure used. For optimal clinical application an anti-bacterial measure should be effective in killing pathogenic bacteria, disrupting biofilm matrix, and ideally aiming for a healthy equilibrium in the oral microbiome. In parallel, measures used should be save without detrimental effects in any part of the body.

Summarizing, in this thesis we explored pathways for modification of the composition of biofilm, mechanisms of antimicrobial resistance of oral bacteria and control of biofilm formation by 3D printable and contact-killing materials.

Based on the main findings from this thesis, a number of conclusions can be put forward:

1. Powered toothbrushing enhances the action of oral antimicrobials and results in significant reductions of oral biofilm and shifts in its compositions.

2. Different species of bacteria have different responses and repair mechanisms that may play a role in development of resistance toward chlorhexidine.

3. Among five commonly used methods, including an established ASTM, to evaluate the efficacy of contact killing, three are unsuitable. Methods found suitable (Petrifilm® and JIS Z 2801) should be used in combination with a zone-of-inhibition-assay to establish absence of antimicrobial leaching.

4. A biocompatible, 3D printable composite resin has been developed demonstrating potent bacterial killing upon contact with good clinical handling properties.

5. When considering societal impact of research, the opinion of end-users is valuable in selecting research topics and the role of (social) media cannot be denied in spreading scientific results.

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Summary

In this thesis different antibacterial measures for oral biofilm control were investigated. **Chapter 1** identifies problems related to oral biofilm infections with emphasis on orthodontics and describes different approaches for antibacterial measures.

In selecting research topics for (biomedical) research the involvement of end-users becomes increasingly important, particularly in a field as orthodontics with which a major part of the population has become in contact with. A case example of the participation of orthodontic end-users in selecting research topics is presented in **Chapter 2**, in which patients, parents and care providers are involved in the set-up and topic selection of a part of this thesis, using a structured questionnaire. The questionnaire addressed different aspect of oral biofilm control in orthodontic patients and asked what aspects and new developments would be valued most by them as end-users. All respondents, including patients, parents of patients, orthodontists and paramedics scored highest for 'non-compliance' bacterial-killing adhesives with lasting killing effect. The results demonstrate that end-users can make a valuable contribution for scientists in the selection for societally-relevant research topics, when the main purpose of the research work is to reach its potential end-users and provide benefit for their health and wellbeing. Moreover, public opinion can help scientists to better understand the needs of end-users.

In daily life, manual or powered brushing are still by far the most effective measure for oral hygiene maintenance in orthodontic patients. In **Chapter 3** orthodontic, multi-strand retention-wires are used as a generalized model for oral retention sites to investigate whether biofilm left-behind after powered toothbrushing *in-vivo* enabled better penetration of antibacterials as compared with manual brushing. 2-cm multi-strand, stainless-steel retention-wires were placed in brackets bonded bilaterally in the upper arches of 10-volunteers. Volunteers used a NaF-sodium-lauryl-sulphate-containing toothpaste and antibacterial, triclosan-containing toothpaste supplemented or not with an essential-oils containing mouthrinse. Opposite sides of the dentition including the retention-wires, were brushed manually or with a powered toothbrush. Health-care-regimens were maintained for 1-week, after which wires were removed and oral biofilm was collected. When powered toothbrushing was applied, slightly less bacteria were collected than after manual brushing, regardless whether an antibacterial-regimen was used or not. Powered-toothbrushing combined with antibacterial-regimens yielded lower biofilm viability

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than manual brushing, indicating better antibacterial penetration into biofilm left-behind after powered brushing. Major shifts in biofilm composition, with a decrease in prevalence of both cariogenic species and periodontopathogens, were induced after powered brushing using an antibacterial-regimen. Oral biofilm left-behind after powered brushing *in-vivo* enabled better penetration of antibacterials than after manual brushing.

Oral healthcare products with antibacterial components may be effective for oral biofilm control, but at the same time bear the threat of developing bacterial resistance. Clinically, strains such as *Staphylococcus aureus* have been found resistant to chlorhexidine, while in dental practice oral bacterial strains, including *Streptococcus mutans* have remained largely susceptible to chlorhexidine. The aim of **Chapter 4** is to speculate on the mechanisms through which *S. aureus* adapts resistance against chlorhexidine *versus S. mutans* remaining susceptible. Chlorhexidine exposure of adhering bacteria to (sub)-MIC concentrations of chlorhexidine yielded reversible, nanoscopic cell wall deformation in *S. mutans*, but not in *S. aureus*, indicative of loss of intracellular, cytoplasmic pressure in *S. aureus*. Although overall cell surface properties of both strains did not significantly change, propidium iodine staining demonstrated that the *S. aureus* cell membrane was indeed more easily damaged than the *S. mutans* cell membrane. Significantly, metabolic activity of *S. mutans* changed little upon exposure to chlorhexidine, while *S. aureus* metabolic activity became much higher. Concurrently, repeated culturing in presence of chlorhexidine demonstrated that chlorhexidine resistance was easy to induce in *S. aureus*, but not in *S. mutans*. Exact interpretation of these data is difficult. *S. aureus* may adapt a high metabolic activity to survive chlorhexidine attack, e.g. by activating efflux pumps or opening of membrane channels to decrease the intracellular chlorhexidine concentration. This may cause loss of intracellular pressure yielding cell wall deformation, and at the same time stimulate development of chlorhexidine resistance. In *S. mutans*, cell wall deformation was reversible within 15 min after exposure to chlorhexidine, suggesting spontaneous, strong cell wall self-repair. Due to cell wall self-repair, *S. mutans* may be unable to effectively reduce the chlorhexidine concentration in its interior, preventing its survival and development of a resistant progeny.

Like resistance to antimicrobials can develop differently in different strains, antibiofilm activity of an antimicrobial may be achieved by different mechanisms of

action: preventing bacterial adhesion, limiting bacterial growth, disrupting an already established biofilm or altering the composition and/or pathogenicity of the biofilm. One strategy of particular interest in dentistry is modifying dental materials to equip them with antimicrobial properties. Cationic surfaces with alkylated quaternary-ammonium groups kill adhering bacteria upon contact by membrane disruption and are considered increasingly promising as a non-antibiotic based way to eradicate bacteria adhering to surfaces. However, reliable *in-vitro* evaluation methods for bacterial contact-killing surfaces do not yet exist. More importantly, results of different evaluation methods are often conflicting. Therefore, we compared in **Chapter 5** five methods to evaluate contact-killing surfaces. To this end, we have copolymerized quaternary-ammonium groups into diurethane dimethacrylate/glycerol dimethacrylate (UDMA/GDMA) and determined contact-killing efficacies against five different Gram-positive and Gram-negative bacterial strains. Spray-coating bacteria from an aerosol onto contact-killing surfaces followed by air-drying as well as ASTM E2149-13a (American Society for Testing and Materials) were found unsuitable, while the Petrifilm® system and JIS Z 2801 (Japanese Industrial Standards) were found to be excellent methods to evaluate bacterial contact-killing surfaces. It is recommended however, that these methods be used in combination with a zone of inhibition on agar assay to exclude that leakage of antimicrobials from the material interferes with the contact-killing ability of the surface.

For clinical applications, it would be advantageous to incorporate contact-killing properties in a material with other unique features, e.g. 3D printability and mechanical versatility. 3D printing is seen as a game-changing manufacturing process in many domains, including general medicine and dentistry, but the integration of more complex functions into 3D-printed materials remains lacking. In **Chapter 6-I**, we demonstrated the development of a 3D-printable antibacterial material. Monomers containing antimicrobial, positively charged quaternary ammonium groups with an appended alkyl chain are either directly copolymerized with conventional diurethanedimethacrylate/glycerol dimethacrylate (UDMA/GDMA) resin components by photocuring or prepolymerized as a linear chain for incorporation into a semi-interpenetrating polymer network by light-induced polymerization. For both strategies, dental 3D-printed objects fabricated by a stereolithography process kill bacteria on contact when positively charged quaternary

ammonium groups are incorporated into the photocurable UDMA/GDMA resins. Leaching of quaternary ammonium monomers copolymerized with UDMA/GDMA resins is limited and without biological consequences within 4–6 d, while biological consequences could be confined to 1 d when prepolymerized quaternary ammonium group containing chains are incorporated in a semi-interpenetrating polymer network. Routine clinical handling and mechanical properties of the pristine polymer matrix are maintained upon incorporation of quaternary ammonium groups, qualifying the antimicrobially functionalized, 3D-printable composite resins for clinical use.

Our manuscript on the development of a 3D printable antimicrobial composite resin, within the first weeks after being published in Advanced Functional Materials, immediately received tremendous attention from both national and international mainstream media. Even though our research project received enormous attention and positive recognition at the social media platforms, the impact of these reports is a double-edged sword, as discussed in **Chapter 6-II**. The public attention of this research project could be largely attributed to recognition by the general public of its potential societal impact that the research outcome might generate but was not based on its true scientifically proven content. Therefore, it remains a challenge for researchers to reach the public with their research project, and properly guide this process so that the right information and interpretation are spread.

In the general discussion in **Chapter 7** the results of the studies are discussed from a clinical perspective and suggestions for future research opportunities are made.

Samenvatting

In dit proefschrift werden verschillende antibacteriële maatregelen tegen de orale biofilm onderzocht. **Hoofdstuk 1** beschrijft de problemen, met name in de orthodontie, die veroorzaakt worden door orale biofilm en beschrijft verschillende benaderingen voor antibacteriële maatregelen.

Bij het selecteren van onderzoeksthema's voor (biomedisch) onderzoek wordt de betrokkenheid van eindgebruikers steeds belangrijker, vooral in het vakgebied orthodontie, waar een groot deel van de bevolking mee in contact komt. Een voorbeeld van de participatie van eindgebruikers in de orthodontie wordt gepresenteerd in **Hoofdstuk 2**. Patiënten, ouders en zorgverleners zijn betrokken geweest bij de opzet en onderwerpselectie van een deel van dit proefschrift door middel van gestructureerde vragenlijsten. De vragenlijst behandelt verschillende aspecten van antibacteriële maatregelen bij patiënten met orthodontische apparatuur en vroeg welke aspecten en nieuwe ontwikkelingen door hen als eindgebruikers het meest zouden worden gewaardeerd. Alle respondenten, inclusief patiënten, ouders van patiënten, orthodontisten en paramedici scoorden het hoogst voor 'non-compliance' bacteriedodende composiet met een langdurig bacteriedodend effect. De resultaten tonen aan dat eindgebruikers een waardevolle bijdrage kunnen leveren aan wetenschappers bij de selectie van maatschappelijk relevante onderzoeksthema's, wanneer het hoofddoel van het onderzoek is om de potentiële eindgebruikers te bereiken en voordelen te bieden voor hun gezondheid en welzijn. Bovendien kan de publieke opinie wetenschappers helpen om de behoeften van eindgebruikers beter te begrijpen.

In het dagelijks leven zijn handmatig of elektrisch poetsen nog steeds verreweg de meest effectieve maatregel voor het onderhouden van de mondhygiëne bij patiënten met orthodontische apparatuur. In **Hoofdstuk 3** worden orthodontische, multi-streng retentiedraden gebruikt als een gegeneraliseerd model voor orale retentieplaatsen om te onderzoeken of biofilm die achter blijft na elektrisch tandenpoetsen *in- vivo* betere penetratie van antibacteriële middelen mogelijk maakt in vergelijking met handmatig poetsen. 2-cm meerstrengs roestvrijstalen retentiedraden werden bilateraal van de boventandbogen, tussen brackets geplaatst bij 10 vrijwilligers. Vrijwilligers gebruikten een NaF-natrium-lauryl-sulfaat-bevattende tandpasta en antibacteriële, triclosan-bevattende tandpasta aangevuld of niet met een mondspoeling die etherische oliën bevat. De beide zijden van het gebit met inbegrip van de retentiedraden werden handmatig of met een elektrische

tandenborstel gepoetst. De mondhygiëne regimes werden gedurende 1 week gehandhaafd, waarna draden werden verwijderd en orale biofilm werd verzameld en geëvalueerd. Wanneer elektrische tandenborstels werden toegepast, werden er iets minder bacteriën verzameld dan na handmatig poetsen, ongeacht of er een antibacterieel regime was toegepast of niet. Opvallend is dat elektrisch poetsen gecombineerd met een antibacterieel regime leidde tot lagere levensvatbaarheid van de biofilm dan na handmatig poetsen, wat aan geeft dat er een betere penetratie is van antibacteriële middelen in de biofilm die achterblijft na elektrisch poetsen. Ook werden na elektrisch poetsen met een antibacterieel regime grote verschuivingen in de samenstelling van biofilm geïnduceerd, met een daling van de prevalentie van zowel cariogene als paropathogene soorten. Hoofdstuk 3 laat hiermee voor het eerst zien dat er een synergie bestaat tussen de manier van poetsen en het gebruik van antibacteriële middelen met klinisch aantoonbare effecten.

Mondhygiëne producten met antibacteriële componenten kunnen effectief zijn tegen orale biofilm, maar dragen tegelijkertijd bij aan de dreiging van ontwikkeling van bacteriële resistentie. Klinische stammen zoals *Staphylococcus aureus* zijn resistent gebleken tegen chloorhexidine, terwijl orale bacteriële stammen, waaronder *Streptococcus mutans*, in de tandheelkundige praktijk grotendeels gevoelig blijven voor chloorhexidine. Het doel van **Hoofdstuk 4** is het speculeren over de mechanismen die een rol spelen bij het resistent worden tegen chloorhexidine van *S. aureus* versus *S. mutans*, die vatbaar blijft. Chloorhexidine blootstelling van aangehechte bacteriën aan (sub) -MIC concentraties van chloorhexidine leverde reversibele, nanoscopische celwandvervorming op in *S. mutans*, maar niet in *S. aureus*, een aanwijzing voor verlies van intracellulaire, cytoplasmatische druk in *S. aureus*. Hoewel de algehele celoppervlakte-eigenschappen van beide stammen niet significant veranderden, toonde propidium-jodiumkleuring aan dat de celmembraan van *S. aureus* inderdaad meer was beschadigd dan het celmembraan van *S. mutans*. De metabolische activiteit van *S. mutans* veranderde opvallend weinig bij blootstelling aan chloorhexidine, terwijl de metabolische activiteit van *S. aureus* veel hoger werd. Tegelijkertijd toonde herhaald kweken in aanwezigheid van chloorhexidine aan dat chloorhexidineresistentie gemakkelijk te induceren was in *S. aureus,* maar niet in *S. mutans*. Exacte interpretatie van deze gegevens is moeilijk. *S. aureus* kan met een hoge metabole activiteit zich aanpassen om de chloorhexidine-aanval te overleven, b.v. door efflux pompen of opening van membraankanalen te

activeren om de intracellulaire chloorhexidineconcentratie te verlagen. Dit kan verlies van intracellulaire druk veroorzaken waardoor celwandvervorming ontstaat, en tegelijkertijd de ontwikkeling van chloorhexidineresistentie stimuleren. In *S. mutans* was de vervorming van de celwand omkeerbaar binnen 15 minuten na blootstelling aan chloorhexidine, wat duidt op spontane, sterk zelf herstellend vermogen van de celwand. Als gevolg van celwandzelfreparatie, is *S. mutans* mogelijk niet in staat de chloorhexidine-concentratie in het inwendige effectief te verminderen, waardoor het overleven en de ontwikkeling van een resistent nageslacht wordt voorkomen.

Net zoals resistentie tegen antimicrobiële middelen zich in verschillende stammen verschillend kan ontwikkelen, kan anti-biofilm activiteit van een antimicrobieel middel worden bereikt door verschillende werkingsmechanismen: het voorkomen van bacteriële adhesie, het beperken van bacteriegroei, het verstoren van een reeds gevestigde biofilm of het veranderen van de samenstelling en / of pathogeniteit van de biofilm. Een belangrijke strategie in de tandheelkunde is het modificeren van materialen met antimicrobiële eigenschappen. Cationische oppervlakken met gealkyleerde quaternaire ammoniumgroepen doden gehechte bacteriën bij het contact maken op het oppervlak door het beschadigen van de celmembraan. Deze 'contact-dodende' oppervlakken zijn veelbelovend als een niet-antibioticum-gebaseerde manier om bacteriën te verwijderen die zich aan oppervlakken hechten. Er bestaan echter nog geen betrouwbare *in-vitro*-evaluatiemethoden voor oppervlakken die bacteriën doden door contact met het oppervlak. Opvallend is dat de resultaten van verschillende evaluatiemethoden vaak tegenstrijdig zijn. Daarom hebben we in **Hoofdstuk 5** vijf methoden vergeleken om contact-dodende oppervlakken te evalueren. Hiertoe hebben we quaternaire ammoniumgroepen in diurethaandimethacrylaat / glyceroldimethacrylaat (UDMA / GDMA) gecopolymeriseerd en de contact-dodende werkzaamheid tegen vijf verschillende Gram-positieve en Gram-negatieve bacteriestammen geëvalueerd. Sprayen van bacteriën op contact-dodende oppervlakken gevolgd door luchtdroging en ASTM E2149-13a (American Society for Testing and Materials) bleken niet geschikt, terwijl het Petrifilm®-systeem en JIS Z 2801 (Japanese Industrial Standards) uitstekende methoden bleken te zijn voor het evalueren van oppervlakken die contact met bacteriën doden. Het wordt echter aanbevolen om deze methoden te gebruiken in combinatie met een inhibitiezone-test op een agarplaat om lekkage van antimicrobiële stoffen uit het materiaal uit te sluiten.

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Voor klinische toepassingen zou het gunstig zijn om contact-dodende eigenschappen op te nemen in een materiaal met andere unieke kenmerken, b.v. 3D-printbaar en mechanisch veelzijdig. 3D-printen wordt gezien als een veranderende technologie in productieprocessen in vele domeinen, waaronder algemene geneeskunde en tandheelkunde, maar de integratie van meer complexe functies in 3D-geprinte materialen ontbreekt nog steeds. In **Hoofdstuk 6-I** demonstreerden we de ontwikkeling van een antibacterieel materiaal dat 3D printbaar is. Monomeren die antimicrobiële, positief geladen quaternaire ammoniumgroepen met een aangehechte alkylketen bevatten, werden ofwel direct gecopolymeriseerd met conventionele diurethaandimethacrylaat / glyceroldimethacrylaat (UDMA / GDMA) composiet of voor-gepolymeriseerd als een lineaire keten voor integratie in een semi-interpenetrerend polymeernetwerk. Voor beide strategieën werden dentale 3D-geprinte objecten vervaardigd door een printer middels een stereolithografieproces, waarna de bacterie contact-dodende eigenschappen zijn aangetoond. Het lekken van quaternaire ammoniummonomeren gecopolymeriseerd met UDMA / GDMA-composiet is beperkt en zonder biologische consequenties binnen 4-6 d, terwijl biologische consequenties nog verder beperkt zouden kunnen zijn tot 1 d wanneer geprepolymeriseerde quaternaire ammoniumgroep bevattende ketens worden opgenomen in een semi-interpenetrerend polymeernetwerk. De klinische handteerbaarheid en mechanische eigenschappen van de polymeermatrix blijven gehandhaafd na opname van quaternaire ammoniumgroepen. Hierbij zijn de antimicrobieel 3D-printbare composieten gekwalificeerd voor klinisch gebruik.

Ons manuscript over de ontwikkeling van een 3D-printbaar antimicrobieel composiet, kreeg binnen de eerste weken na publicatie in *Advanced Functional Materials* onmiddellijk enorme aandacht van zowel nationale als internationale reguliere media. Hoewel ons onderzoeksproject op de platforms voor sociale media enorme aandacht en positieve erkenning kreeg, is de impact van deze rapportages een tweesnijdend zwaard, zoals besproken in **Hoofdstuk 6-II**. De publieke aandacht van dit onderzoeksproject kan grotendeels worden toegeschreven aan de erkenning door het grote publiek en de potentiële maatschappelijke impact die de onderzoeksresultaten zouden kunnen genereren. Het was echter niet gebaseerd op de ware wetenschappelijk bewezen inhoud. Daarom blijft het een uitdaging voor onderzoekers om het publiek op een goede manier te bereiken met hun

onderzoeksproject en dit proces te begeleiden, zodat de juiste informatie en interpretatie worden verspreid.

In de algemene discussie in **Hoofdstuk 7** worden de resultaten van de onderzoeken vanuit een klinisch perspectief besproken en worden suggesties voor toekomstige onderzoeksmogelijkheden gedaan.

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Curriculum Vitae

Marieke van de Lagemaat (1987) was born in Enschede, The Netherlands. From 2006 she studied Dentistry at the University of Groningen (RUG). She did her masterthesis on research of biofilm on orthodontic retention wires. After graduating as a dentist in 2012, she worked for one year as a general dentist in two different practices in Enschede. In 2013, she started the postgraduate program in orthodontics at the Department of Orthodontics at the University Medical Center Groningen (UMCG) and was given an opportunity to start a PhD project in line with her masterthesis. This PhD project was a collaboration of the Department of Orthodontics at the UMCG and the Department of Biomedical Engineering, part of the W.J. Kolff Institute.

During the postgraduate program in orthodontics she was board member of the VOIO (Verening van Orthodontisten In Opleiding) and editor of the W.J. Kolff Newsletter. She gave several presentations about her research project during clinical and biomedical congresses and won the award for best societal impact of the year during the Kolff days in 2016.

Marieke finished her postgraduate program in orthodontics in 2017 and started working as an orthodontist in different private practices in Maarssen and Katwijk. Currently, she works in one orthodontic practice and participates in the Young Leadership Program of the Koninklijke Nederlands Maatschappij voor Tandheelkunde (KNMT).