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MARI MIURA SUGII

QUATERNARY AMMONIUM COMPOUNDS TO PREVENT ORAL BIOFILM FORMATION

COMPOSTOS CONTENDO AMÔNIO QUATERNÁRIO PARA PREVENÇÃO DA FORMAÇÃO DE BIOFILME ORAL

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ABSTRACT

Background and objectives: Orthodontic appliances in the oral cavity favor biofilm accumulation. To minimize the negative impact of biofilm formation, coatings with antimicrobial effect have been developed. This thesis aims to present: 1) A literature review about quaternary ammonium compounds (QAC), its diverse applications and the variables influencing the antimicrobial activity of these compounds; 2.1) An in vitro study with development, synthesis and characterization of a quaternary ammonium salt (QAS) coupled to different polyhydroxyurethanes (PHU and PHU*) for stainless steel coating (PHU-QAS and PHU*QAS); 2.2) evaluate the antimicrobial activity of the coatings' surface and leachate against *Streptococcus mutans* (*S. mutans*); 2.3) evaluate the cytotoxicity of coatings' leachate over fibroblasts L929 and 2.4) evaluate the effect of washing in demineralized water or ion-exchange in sodium phosphate solution on cytotoxicity. Both coatings showed contact-killing and planktonic antimicrobial activity against cariogenic *S. mutans* up to 7 days tested. Both ion exchange and washing in demineralized water changed the coating nanostructure without diminishing antimicrobial efficiency. Detached QAS from the PHU and PHU* backbone leached out of the coating to give rise to planktonic antimicrobial activity and cytotoxicity. PHU-QAS coating when washed in demineralized water is suitable for orthodontic application aiming biofilm prevention. The literature review included 107 citations about the use of QAC in different fields and was divided in 9 topics: Background; biofilm treatment and prevention; quaternary ammonium compounds and their chemistry; cationic acrylates and cationic silanes; QAC disinfectants and preservatives; *in situ* quaternization of tertiary amines to form QAC and nanoparticles functionalization; variables influencing antimicrobial properties of QAC; cytotoxicity and antimicrobial resistance. In the in vitro study, a QAS with an alkyl chain of 12 carbons was coupled to PHU or PHU* originating PHU-QAS and PHU*-QAS compounds that were used to coat stainless steel. Compounds were characterized by infrared spectroscopy, nuclear magnetic resonance, X-ray photoelectron spectroscopy and grazing incidence scatteing X-ray. The antimicrobial effect was evaluated in two time points with planktonic *S. mutans* for the coatings' surface and leachate. The cytotoxicity of coatings' leachate was evaluated with fibroblasts L929. Two surface treatments were tested to diminish the cytotoxicity: washing in demineralized water for 3 days or ion-

exchange in sodium phosphate solution for 2 h. All coatings presented antimicrobial activity for *S. mutans* on both time points tested. Washing in demineralized water was able to diminish the cytotoxicity of PHU-QAS on the second time point tested. Ion-exchange though promoted chemical and nanostructural changes but was not able to diminish cytotoxicity. It was concluded that PHU-QAS and PHU*QAS presented effective antimicrobial activity against *S. mutans.* Surface treatments did not diminish the antimicrobial activity of the coatings. PHU-QAS coatings after washing can be considered suitable for biofilm prevention over stainless steel devices.

Keywords: Anti-Infective Agents; Dental Plaque; *Streptococcus mutans*.

RESUMO

A fixação de acessórios ortodônticos na cavidade bucal favorece acúmulo de biofilme dental. Para evitar os efeitos adversos do acúmulo de biofilme, materiais de revestimento com potencial antimicrobiano têm sido desenvolvidos. Dentre os objetivos desta tese estão: 1) Revisão narrativa da literatura sobre compostos de amônio quaternário (QAC), sua diversidade de aplicação e as variáveis que influenciam a ação antimicrobiana destes compostos; 2.1) Através de estudo *in vitro* desenvolver, sintetizar e caracterizar um sal de amônio quaternário (QAS) acoplado a diferentes polihidroxiuretanas (PHU ou PHU*) para aplicação como filmes de revestimento (PHU-QAS ou PHU*QAS) sobre superfície de aço inoxidável; 2.2) Avaliar o efeito antimicrobiano da superfície dos filmes de revestimento e de seus compostos lixiviados contra *Streptococcus mutans* (*S. mutans*); 2.3) Avaliar o efeito citotóxico sobre fibroblastos L929 dos compostos lixiviados dos filmes de revestimento e 2.4) Avaliar o efeito da lavagem dos filmes de revestimento em água demineralizada ou da troca iônica em solução de fosfato de sódio sobre a citotoxicidade dos compostos lixiviados. A revisão de literatura abrangeu 107 citações com uso de QAS em diferentes setores e foi subdividida em 9 tópicos: Contextualização; tratamento e prevenção de biofilmes; química dos QAS; acrilatos e silanos catiônicos; desinfetantes e conservantes à base de QAS; quaternização de aminas terciárias *in situ* e funcionalização de nanopartículas; variáveis que influenciam as propriedades antimicrobianas dos QAS; citotoxicidade e resistência bacteriana aos QAS. No estudo *in vitro* um QAS contendo uma cadeia alquílica de 12 carbonos foi acoplado à PHU ou PHU* derivando compostos PHU-QAS e PHU*QAS que foram aplicados sobre superfície de aço inox. Os compostos foram caracterizados por espectroscopia de infravermelho, espectroscopia de ressonância magnética nuclear, espectroscopia de fotoelétrons excitados por raios-X e dispersão de raios-X de incidência rasante. O efeito antimicrobiano foi avaliado em dois tempos com *S. mutans* UA159 em estado planctônico em contato com os compostos lixiviados ou sobre a superfície dos revestimentos. A citotoxicidade dos compostos lixiviados dos filmes em dois tempos foi avaliada para fibroblastos L929. Dois tratamentos de superfície foram testados para averiguar sua influência na citotoxicidade: Lavagem em água demineralizada por 3 dias ou troca iônica em solução de

fosfato de sódio por 2 h. Todos os revestimentos apresentaram atividade antimicrobiana para *S. mutans* nos dois tempos avaliados. O tratamento de lavagem em água foi capaz de diminuir a citotoxicidade do filme de revestimento PHU-QAS no segundo tempo testado. O tratamento de troca iônica porém, promoveu alterações químicas e nanoestruturais não sendo capaz de diminuir a citotoxicidade. Concluiu-se que os filmes de revestimento PHU-QAS e PHU*QAS apresentaram atividade antimicrobiana eficaz contra *S. mutans*. Os tratamentos de superfície não diminuíram o potencial antimicrobiano dos filmes. Filmes de PHU-QAS após lavagem em água demineralizada podem ser considerados adequados para prevenção da formação de biofilme em aparatos de aço inoxidável.

Palavras-chave: Agentes Antimicrobianos; Biofilme Dentário; *Streptococcus mutans.*

SUMÁRIO

1 INTRODUÇÃO

A movimentação dentária envolvida no tratamento ortodôntico depende da colagem de aparatos (bráquetes, botões, tubos, bandas) sobre a superfície dental. Bráquetes metálicos têm se mostrado mais resistentes à fratura (20 vezes menos friáveis) (Lindauer et al. 1994; Scott 1988) do que bráquetes cerâmicos, suportando deformações estruturais de até 20% sem que ocorram danos permanentes (Elekdag-Türk and Yilmaz 2019; Scott 1988; Viazis et al. 1993) por isso, seu manuseio durante o tratamento ortodôntico e sua remoção ao término são facilitadas.

Todos os acessórios dificultam a higienização, devido ao detalhamento de sua geometria além de impedir a ação da musculatura e da saliva na superfície às quais estão cimentados ocasionando acúmulo de biofilme bacteriano (Gorelick et al. 1982; Karadas et al. 2011; Mattousch et al. 2007; Øgaard et al. 1988; Rosenbloom and Tinanoff 1991).

Na literatura encontram-se diferentes descrições do processo contínuo e dinâmico de formação de biofilme (Mancl et al. 2013; Riga et al. 2017; Wilkins et al. 2014) em sua maioria caracterizada por 5 fases: 1) Adesão bacteriana reversível; 2) Adesão irreversível; 3) Início do processo de secreção de polissacarídeos extracelulares (PEC) da matriz, do mecanismo de *quorum sensing* (QS) e da conformação tridimensional do biofilme; 4) Biofilme maduro com sistema de canais para água e nutrientes e QS; 5) Dispersão através do desprendimento de segmentos do biofilme e retorno dos microrganismos liberados ao estado planctônico. Uma vez que o bioflme atinge a maturação, os microrganismos contidos na matriz de PEC tornam-se menos suscetíveis a respostas imunológicas do hospedeiro, substâncias antimicrobianas e tensões mecânicas (Bowen et al. 2018; Bowen and Koo 2011; Wilkins et al. 2014).

A placa dental bacteriana é um biofilme formado na cavidade bucal sobre tecidos duros e moles. Microorganismos presentes no biofilme como *Streptococcus mutans* (*S. mutans*), são responsáveis pela fermentação de carboidratos alimentares (Conrads et al. 2014; Hamada and Slade 1980; de Soet et al. 2000) podendo levar à doença cárie (Sundararaj et al. 2015), prejuízos à polpa e em última instância perda precoce do elemento dental (Fontana et al. 2010). Sabe-se que, após a cimentação

do aparato ortodôntico na cavidade bucal, há uma mudança na microbiota com aumento do número de *S. mutans* (Komori et al. 2012).

O processo de desenvolvimento de lesões iniciais de cárie ocorre mais rapidamente em pacientes ortodônticos devido à dificuldade de higienização. Lesões iniciais de cárie ao redor de bráquetes podem ocorrer em até 1 mês após a cimentação dos acessórios (Höchli et al. 2016). Há evidências clínicas de que cerca 50% dos pacientes sob tratamento ortodôntico apresentam lesões de mancha branca enquanto do uso de aparelhos fixos (Jiang et al. 2015).

A reeducação quanto aos hábitos de higiene oral depende grandemente da colaboração e comprometimento do paciente além de exigir tempo (Cozzani et al. 2016; Gao et al. 2014; Jönsson et al. 2006; Novaes-Júnior and Novaes 1999). Materiais de revestimento de superfície com potencial antimicrobiano têm sido utilizados para prevenção do acúmulo de biofilme em aparatos (Cloutier et al. 2015). Por isso, o uso de materiais de revestimento com atividade antimicrobiana na odontologia poderia representar uma alternativa para prevenção da cárie durante o tratamento ortodôntico.

Compostos de amônio quaternário (QAS) são conhecidos como substâncias antimicrobianas utilizadas desde a década de 1930 em desinfetantes (Domagk 1935). Seu uso em produtos de higiene bucal datam de 1970 (Ge et al. 2015). Atualmente têm sido incluído em revestimentos de aparatos médicos e da indústria alimentícia para prevenir a contaminação por microorganismos (Kenawy et al. 2007). Outras aplicações incluem soluções de limpeza para lentes de contato, sabonetes, xampus, cosméticos e soluções antisépticas. As moléculas de QAS mais conhecidas são: cloreto de benzalcônio, brometo/cloreto de cetiltrimetilamônio, cloreto de cetilpiridínio, metacrilatos de amônio quaternário. Há extensa literatura sobre a eficácia destes compostos contra uma ampla variedade de microrganismos, incluindo bactérias orais e espécies fúngicas. O mecanismo de ação antimicrobiana destes compostos é baseado em interações eletrostáticas entre membrana bacteriana, carregada negativamente, e a carga parcialmente positiva dos QAS. Tal interação poderia levar à lise da membrana celular resultando em extravazamento do conteúdo citoplasmático e morte celular (Jennings et al. 2016; Jiao et al. 2017).

Materiais como poliuretanas já são utilizadas em catéteres, válvulas cardíacas, balões intra-aórticos curativos e materiais de revestimento por

apresentarem estabilidade térmica e oxidativa, baixa toxicidade e baixo módulo de elasticidade (Bergmann et al. 2014; Lamba et al. 2012). A produção deste tipo de material porém, envolve reagentes como fosgênio e isocianato, conhecidos por sua toxicidade e carcinogenicidade.

Dentro deste contexto, estudos comprovaram que uretanos sintetizados a partir de ciclocarbonatos (polihidroxiuretanas - PHU) além de apresentarem resistência à hidrólise e boa força de adesão a substratos metálicos (Aguiar 2015) reúnem boa estabilidade térmica e oxidativa (cadeias poliméricas mais resistentes através ligações cruzadas), baixa energia de superfície e boa flexibilidade das ligações uretânicas (Aguiar 2015). Além das propriedades físicas vantajosas, as PHU são sintetizadas sem a utilização dos isocianatos ou fosgênio.

Visto que o acúmulo de biofilme na superfície e ao redor de acessórios ortodônticos é um problema frequente devido à dificuldade de higienização, o uso de compostos PHU contendo sais de amônio quaternário como revestimento poderiam auxiliar na prevenção da formação de biofilme sobre estes aparatos. Desta forma o objetivo desta tese foi sintetizar PHUs contento QAS, utilizando os materiais finais como revestimentos de superfície de aço inox com a finalidade de diminuir formação de biofilme de *S. mutans*.

2 ARTIGOS

2.1 Artigo: Quaternary ammonium compound derivatives for biomedical applications

Capítulo de livro publicado em *Materials for Biomedical Engineering, Inorganic micro and nanostructures*, Chapter 5; 153-169 (Anexo 1)

Mari Miura Sugii; Fábio Augusto de Souza Ferreira; Karina Cogo Müller; Ubirajara Pereira Rodrigues Filho; Flavio Henrique Baggio Aguiar

Background

Although medical devices, implants, prostheses and equipment are sterilized by autoclave or radiation, exposure to air can infect these surfaces [1]. Given acceptable growth conditions, they can multiply from one organism to more than one billion in just 18 hours. Contamination and colonization by microorganisms on surfaces can result in problems as insignificant as bad odor up to serious human infections [2].

Bacteria can settle and build biofilms. Biofilms are cohesive and protective communities sheltering microorganisms in a three dimensional extracellular polysaccharide matrix. Bacteria embedded in biofilms are much more resistant to antibiotics and host immune system than planktonic ones making its eradication more difficult. Thus, biofilms are usually related to chronic and persistent infections. Chronic sinusitis and otitis, dental caries, periodontal diseases, pulmonary or urinary infections, chronic wounds are among the complications that biofilms unleash [3,4]. Not surprisingly, bacterial contamination is still the most common cause of prosthesis losses [2].

We are facing what some call the “post antibiotic era” in which infections that were easily treatable are now a threaten to life, therefore alternative strategies must be found (WHO). Materials with antimicrobial properties for biomedical purposes are a promising field to be explored [3,5–7]. The term “antimicrobial agent” refers to a broad range of substances able to kill pathogenic microorganisms, providing varying degrees of protection [8]. In this context, a group of antimicrobial agents called quaternary ammonium compounds (QAC) have met dental and medical requirements for fighting bacteria.

The aim of this chapter is to cover biofilm-arising problems, current antimicrobial materials containing QAC for dental and biomedical purposes, means of obtaining these materials, proposed mechanisms of action and variables influencing the antimicrobial activity.

Biofilm treatment and prevention

The first step of biofilm formation is bacterial adherence to a conditioning layer comprised of proteins, at this stage bacterial adhesion is still reversible. Bacteria will start dividing and others will adhere initiating the irreversible attachment. Extracellular polymeric substances (EPS) secretion and quorum sensing mechanism will start and three dimensional structuring will begin. After this stage, biofilm is considered mature. With a mature biofilm, dispersal step will take place in which small segments of the biofilm will be detached releasing bacteria to colonize other surfaces [3,7]. Figure 1 depicts biofilm formation steps.

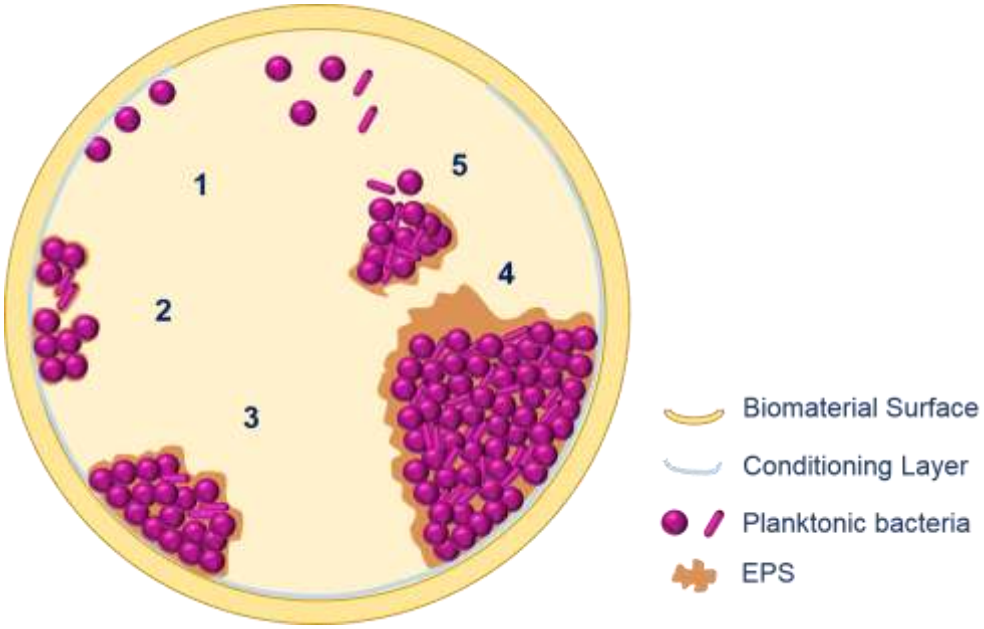

Figure 1. Biofilm formation steps: 1) Attachment; 2) Irreversible attachment; 3) EPS and quorum sense starts triggering three dimensional structuring; 4) Mature Biofilm with water channels and 5) Detachment of biofilm segments releasing planktonic bacteria.

Antibiotic therapy is the main approach against infections nowadays. Yet, diffusion and penetration of antimicrobials is hindered due to the EPS surrounding bacteria. Bacteria sheltered in biofilms can be 1000 times more resistant to antibiotics than in planktonic form. Additionally, the widespread production and extensive use of antibiotics have contributed to the emergence of

multiple drug-resistant infectious organisms, the so-called superbugs (e.g., methicillin-resistant *Staphylococcus aureus*) [9].

Materials with antimicrobial capability came across as a reasonable alternative to antibiotics. Antimicrobial materials can be classified in two main types: leaching materials and contact-active materials. Leaching materials are biocide carriers and their mechanism of action relies on release of the biocide, usually low-molecular-weight compounds, in the environment and microorganisms' chemical eradication. Contact-active materials have a modified surface that will prevent bacterial adhesion or kill bacteria upon contact [7].

Leaching materials are mostly preferable in cases in which high initial doses are advantageous, for instance for preventing contamination over newly placed implants. Some biocides have been cited for leaching purposes such as silver ions, antimicrobial peptides and even some low-molecular weight QAC [7]. When the biocide is released in the body, it does not exert a specific and localized action [2,5,10,11]. In this regard, some thermo or pH-responsive leaching material could amend this issue [12]. Concerns over leaching materials are related to the toxicity of released doses and to loss of the antimicrobial potential over time [10,13].

In contact-active materials, biocides are not leached but presented in the bulk of the material or as a coating on the surface thus diminishing the possibility of toxicity, enhancing selectivity and effectiveness, and preventing loss of antimicrobial activity in the long term [5]. Contact-active materials can perform antimicrobial activity by preventing protein and bacterial adhesion or by damaging bacterial membrane. With respect to the former mechanism, proteins or organism-specific interactions with the surface are minimized and therefore the adhesion is inexistent or easily reverted. Examples are poly(ethylene glycol) (PEG), Teflon or poly(dimethylsiloxane) (PDMS)-based materials [7]. Regarding the second mechanism a cationic charged surface will interact with bacterial membrane to kill bacteria, as it is the case of QAC mechanism of action.

Quaternary ammonium compounds (QAC) and their chemistry

Antimicrobial agents, including antibiotics, disinfectants, and antiseptics have been substantially developed [14–16]. Antimicrobials can vary in their

chemical nature, mechanism of action, impact on the human body and environment, half-life characteristics, endurance on various substrates, synthesis and costs. The ideal antimicrobial polymer would exhibit: antimicrobial activity against a broad range of microorganisms; long-term properties; chemical stability (should not leach out toxic subproducts); environment friendly synthesis; low-cost and insolubility in body fluids [17]. Quaternary ammonium polymers have matched great part of the requirements as antimicrobial materials.

Quaternary ammonium compounds are conceived as antimicrobial agents extensively studied since Domagk discovered the antimicrobial property of benzalkonium chlorides in 1935. QAC constitute a group of cationic antimicrobial agents that contain functional groups covalently bonded to a central nitrogen atom (R_4N^+), with at least one of the R groups consisting of an alkyl group [18].

The classical Menschutkin reaction is one of the most common routs to obtain quaternary ammonium cations. The reaction is based on the addition reaction between tertiary amines and organo-halides thus representing a facile approach to produce a wide variety of potentially antibacterial monomers, oligomers, and polymers. This technique was adapted to synthesize free radical, photocurable, dimethacrylate monomers containing quaternary ammonium functionalities, miscible with common dental resinous composite [19–21]. When QAC is obtained it could be included or attached to different sorts of material as this chapter will explore.

QAC mechanism of action relies on strong electrostatic interactions between the positively charged nitrogen and the negatively charged bacterial membrane resulting in its disruption and loss of cytoplasmic content. Generations of QAC with various structures have been explored as disinfectants [22] in many fields, such as water treatment, agriculture, medicine and healthcare products, food, and the textile industry [23,24].

The use of a quaternary ammonium biocide can provide durable antimicrobial protection against a wide variety of microorganisms without the side effect of leaching heavy metals, phenolic compounds, or other toxic compounds. The well-established fungicidal and bactericidal properties make QAC a promising candidate for modifying a great variety of surfaces [25]. Stable binding and immobilizing of quaternary ammonium moieties into biomaterials are

commonly achieved by means of covalent bonds. Routes for obtaining a polymeric material containing stable quaternary ammonium biocides attached are: Polymerization of quaternary ammonium-bearing monomers; hydrolysis and condensation of silanized quaternary ammonium groups; binding to a previously prepared material [26] and functionalizing nanoparticles [27].

Cationic acrylates and cationic silanes

Recent scientific reports evidence the need for materials with long-lasting antimicrobial properties to overcome biofilm in dentistry. The most common and costly biofilm-dependent oral disease worldwide is dental caries [28,29]. Biofilm plays important role on dental caries development. Oral biofilms also follow the steps of biofilm formation formerly described. The initial colonization occurs over acquired pellicle (glycoproteins, mucins, statherins, α-amylase, agglutinins) microorganisms like *Streptococcus sanguinis* (*S. sanguinis*), *Streptococcus gordonii* (*S. gordonii*), *Streptococcus oralis* (*S. oralis*), *Streptococcus mitis* [30–32], pathogenic *Streptococcus mutans* (*S. mutans*) and *Actinomyces spp*. will interact with each other [33]. If the individual diet is rich in fermentable sugars, acidogenic species will prevail and fermentation of these dietary carbohydrates will bring the environment pH down [34–36]. A pH lower than 5.5 initiates demineralization of tooth structure which will lead to white spot lesions (initial dental caries lesions) [37].

Once a cavitation is originated and bacteria infiltrate in dentin tubules it is hard to obtain aseptic dentin for restoration placement [38,39]. In this setting, studies about bonding systems containing antimicrobial properties launched. A series of studies were dedicated to evaluate antimicrobial activity of quaternary ammonium monomers inserted into primers or bonding agents. Methacrylate monomers containing quaternary ammonium groups were developed to this end: methacryloyloxydodecyl pyridinium bromide (MDPB), dimethylaminododecyl methacrylates (DMADDM), methacryloxyethyl cetyl dimethyl ammonium chloride (DMAE-CB) are the most common [40].

MDPB monomers were the first successfully included in commercial bonding systems which exhibited antimicrobial activity against seven oral streptococci, some lactobacilli, anaerobic and endodontic pathogens like *Enterococcus faecalis* (*E. faecalis*), *Fusobacterium nucleatum*, and *Prevotella*

nigrescens. Due to this large spectrum of action some have tried incorporation of MDPB into restorative composite resins but reported diminished inhibitory effects after polymerization of the monomers [39,41]. DMAE-CB is another effective monomer against cariogenic *S.mutans* [21], *S. sanguinis* and *Streptococcus sobrinus* (*S. sobrinus*) when incorporated in commercialized bonding systems [40]. DMADDM exhibited antimicrobial properties against *S.mutans* when inserted in primers and [42–44] adhesives from bonding systems, in nanocomposites for tooth restoration [45] and in composite resins for orthodontic cementation [46].

Biofilm accumulation around orthodontic devices is a common situation in dental practice. All cemented appliances hinder hygiene because of their complex geometry and also block muscle and saliva clearance activity, leading to biofilm accumulation [44,46–50]. The development of oral biofilms in orthodontic composite resins, as well as on other accessories, such as brackets, metal ligature, wires, and elastomeric rings, may compromise patients' oral health, jeopardizing the efficiency of orthodontic treatment [26]. White spot lesions take 6 months to develop in normal conditions and in patients with fixed orthodontic appliances they can occur much faster, in up to 1 month after cementation [51,52].

To address this problem, this group has developed a new silane based material containing QAC (iodide quaternary ammonium methacryloxy silicate (IQAMS)). IQAMS was inserted in a commercial composite resin for bracket cementation (Transbond XT Light Cure Adhesive) or applied as a coating over this composite surface. IQAMS exhibited *S. mutans* biofilm inhibition effect when applied as coating but not when inserted into the composite resin. Like others hypothesized, the diminished antimicrobial activity of IQAMS inserted into the composite could be due to unavailability of the compound on the surface after photoactivation. Therefore application as a coating was advocated for enhanced antimicrobial properties [53].

Considerable interest emerged over application of functional quaternary ammonium-containing silanes and polysiloxanes. These materials differ from the methacrylate based ones because the anchoring unit is an organofunctional trialkoxy or tetralkoxysilane and quaternary ammonium groups are linked by

siloxane bonds (Figure 2). These materials are commonly synthesized via the sol–gel process and present the possibility of adjusting the properties of the final product at a molecular level. Different end-functional macromonomers may be synthesized with non-leaching QAC distributed into the bulk of the material [54].

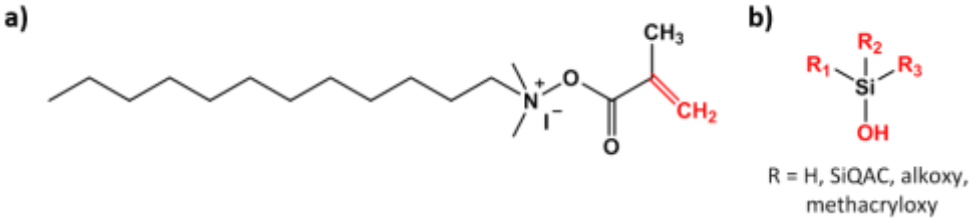

Figure 2. a) Quaternary ammonium methacrylate based monomer with reactive double bond ready for free radical polymerization, b) Anchoring units of silane after hydrolysis ready for further condensation reaction.

Such materials are extremely versatile in terms of application and kinetically and thermodynamically stable in both strongly acidic and slightly basic media [55]. Organosilanes matrices offer tailored hydrophilic, hydrophobic, ionic, and hydrogen bonding capacities, as well as electrochemical properties and adjustable porosity [56]. They assemble inert, non-biodegradable materials and promising antimicrobial results have been reported considering functionalization with quaternary ammonium groups. In the mid-1960s, researchers discovered that antimicrobial functionalized silanes could be strongly bonded to reactive substrates by siloxane (Si–O) linkages [57]. Hydrolyzable groups (halogens or alkoxy groups) on the silicon atom enable silanes carrying specific functions to be bonded to the substrate. Thereafter, the antimicrobial activity of the [3-(trimethoxysilyl)propyldimethyloctadecyl] ammonium chloride (SiQAC) has been studied extensively on a variety of treated surfaces. Because of the presence of reactive silanol groups generated during hydrolysis, quaternary ammonium silanes can attach covalently to substrate surfaces via Si–O linkages to exert non-leachable antimicrobial functions [57]. The surfaces on which they can be used include metal, plastic, glass, rubber, ceramic, porcelain, marble, cement, granite, tile, silica, sand, appliances that are melamine or phenolic, siliceous, polycarbonate, and wood. The bridge-building of organofunctional silanes is particularly important in three fields of application: adhesion promotion, surface modification, and polymer cross-linking [58].

The sol–gel process is based on a sol, generated from alkoxy metal or metalloid. Such compounds readily react with water via hydrolysis, generating products with hydroxyl groups bonded to the metal or metalloid atom. The hydrolyzed molecules will bond via condensation reactions, from which smaller molecules, such as water and ethanol, are formed and the result is a colloidal suspension of solid particles or polymers in a liquid. The process continues forming bigger molecules until the sol turns into a gel, colloidal, or polymeric network non-fluidic containing cross-linked covalent bonds. The solvent evaporation from the gel can lead to a xerogel or an aerogel, if the solvent is removed under supercritical conditions [59]. Figure 3 is a schematic representation of the reactions involved in the sol–gel process.

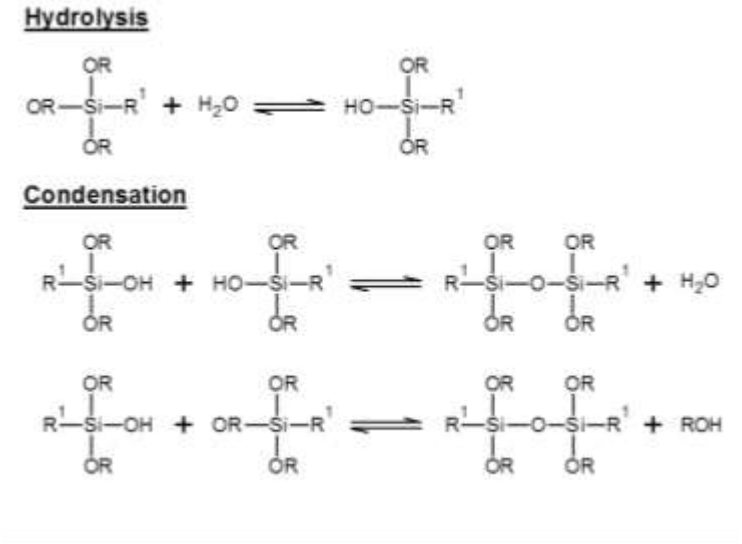

Figure 3. Representation of the reactions that take place during the sol–gel process.

The sol–gel process is influenced by temperature, synthesis duration, presence of catalysts, concentration of reagents, etc. All these factors will determine the characteristics of the final material. Regarding the catalysts, an acid environment is usually applied when the aim is film development, while bases are mainly used for synthesis of particles that can be incorporation into different matrices [59].

The disadvantage of the sol–gel process versus controlled polymerization techniques is the lack of control over polymer polydispersity and architecture. Nevertheless, polymers prepared from quaternized ammonium silane macromonomers may have improved toughness and damping properties, due to the flexibility of the siloxane backbone as compared with rigid C–C bonds.

Additionally, the incorporation of more flexible siloxane linkages can demonstrate enhanced polymerization characteristics and the ability to self-repair damage caused by water sorption and mechanical stress relief over time [60].

Important studies to dentistry brought together a quaternary ammonium silane-functionalized methacrylate (QAMS) groups to be inserted in an experimental photocurable composite resin [60] and in a commercial auto-polymerizing acrylic resin for removable orthodontic devices [61]. In both cases, materials containing QAMS demonstrated contact-killing effect for *S. mutans*, *A naeslundii* and *C. albicans*. Authors hypothesized that the presence of siloxane cross-linking could delay cracking propagation in the restorative composite resin since the silicate network could deflect and dissipate energy [60]. For the orthodontic acrylic resin QAMS insertion improved toughness without affecting flexural strength and modulus [61].

In biomedical devices, quaternary ammonium silanes (QAS) were used to coat silicon rubber tracheoesophageal shunt prostheses. Biofilms readily settle and impair the functioning of these prostheses leading to a short useful lifetime, ranging from 3 to 6 months [62]. The silane containing quaternary ammonium groups formed a positively charged surface with great efficacy in preventing mixed biofilms (*Candida tropicalis*, *C. albicans*, *S. aureus*, *Staphylococcus epidermidis* (*S. epidermidis*) and *Streptococcus salivarius*). Besides the inhibitory effect, the QAS was non-cytotoxic to mammalian cells and stable even in a moist environment. QAS coating could increase tracheoesophageal prostheses' lifetimes and could also be useful in other biomedical devices [63].

Infections starting in catheters are also a concern. Hospitalized patients can remain with catheters for extended periods of time and bacterial biofilms can easily form leading to infections. Zanini et al. modified the surface of a commercialized catheter with a silane based QAC and found that the polyurethane catheters could display antimicrobial activity against *E. coli* for 4 h up to 24 h [64].

Surgical sutures and wound dressings are also a concern in biomedical field once surgical sites are easily infected by bacteria, regardless of the spot in the human body and represent a risk for bacteremia [65], increased morbidity and hospital stay [66]. Preliminary studies with chromic gut, nylon and polyester

sutures impregnated with silane based QAC - QACK21 – demonstrated antimicrobial action against *Porphyromonas gingivalis* and *Enterococcus faecalis* [65]. Wound dressings and textile fibers impregnated with silane based QAC can also help preventing wound contamination and hospital cross-infections. Silane based QAC can be covalently bound to these fibers and therefore resistant to laundering. Functionalized coatings were effective against *E. coli*, *Pseudomonas aeruginosa* (*P. aeruginosa*), *S. aureus*, *S. epidermidis* and fungi *Saccharomyces cerevisiae* and *C. albicans* [67–69].

Bone cements used to immobilize prosthesis or fragments of fractured bone, as other medical devices are prone to biomaterial associated infections. Quaternized chitosan derivatives inserted into bone cement prevented biofilm formation over this surface better than gentamicin-loaded bone cement. This effect was observed for *S. epidermidis*, *S aureus* and methicillin-resistant strains. Besides the robust antimicrobial property, quaternized chitosan-loaded bone cements were also biocompatible with osteogenic cells [70].

From industrial point of view bacteria, fungi, algae, and other organisms can consume and degrade surfaces during shipment, storage, and use, causing loss of product as well as exposing the consumer to contamination. Once the material is anchored on the substrate it can protect it from microbial contamination and guarantee product quality [71].

QAC disinfectants and preservatives

Chlorhexidine digluconate is one of the most notorious quaternary ammonium based disinfectants and undoubtedly efficient for a wide spectrum of pathogenic bacteria. In dentistry is the gold standard chemotherapist being recommended for amending gingivitis, periodontal therapy, adjuvant treatment for patients with high-risk of caries, disinfecting prostheses, pre and post-operative rinsing and root canal irrigation [72]. The prescription should be precautious though once extensive use of chlorhexidine can induce tooth staining, calculus formation and changes in taste perception. Other applications of chlorhexidine include antimicrobial soaps, hand antisepsis and skin disinfection in hospitalized patients [73,74].

Alkyl trimethyl ammonium bromide and chloride, cetyltrimethyl ammonium bromide and chloride, lauryltrimethyl ammonium bromide, behentrimonium chloride, Stearyltrimethylammonium chloride have been added in hair products and face cosmetics [75]. Chitosan and its derivatives and quaternary ammonium cellulose derivatives have been reportedly used in hair conditioners, shampoos and skin products [5].

QAC is also present in food processing industry [5]. Concerns over food-born poisoning and demand over increased shelf-life for food products lead to the use of QAC in food packaging and processing. QAC could be used as disinfectants or edible films against food spoilers such as *E. coli, S. aureus, P. aeruginosa, Campylobacter jejuni* [76], *Listeria monocytogenes* and *Salmonella* [77]. Benzalkonium chloride, benzethonium chloride, cetyl pyridinium chloride didecyldimethylammonium chloride are some of the already commercialized disinfectants [76] and quaternized chitosan derivatives have been studied for edible films [17].

As QAC can bind to surfaces in a stable manner it also caught attention for application in water treatment filters because of diminished risk of toxicity for consumer. Quaternized chitosan derivatives in combination with graphene oxide was able to reduce 99,99% of *E. coli* from contaminated water without leaching of QAC [78]. Other chitosan quaternary ammonium salts [79] and quaternary ammonium cationic polymers such as poly(diallyldimethylammonium chloride) and epichlorohydrin-dimethylamine have been used as flocculating agentes [80].

In situ quaternization of tertiary amines to form QACs and Nanoparticles functionalization

Cationic Poly(ethylene imines) (PEI) are an example of QAC obtained by means of *In situ* quaternization of tertiary amines. For this kind of approach the surface or polymeric structure should present reactive alkyl groups. If the substrate originally do not present these groups it is possible to functionalize via plasma and akylation treatments prior to the quaternization. Once the substrate or polymer has alkyl groups it is possible to react them with tertiaty amines existing in PEI [81]. In situ quaternization have a drawback of limited functionalization due to steric hindrance [82].

Minimal Inhibitory Concentration (MIC) test was performed for a series of cationic PEI solutions with different molecular structures for: *S. aureus*, *E. coli* and *Bacillus subtilis* (*B. Subtilis*). It was concluded that polymeric solutions of cationic PEI are effective against the three bacterial strains and the best molecular arrangement for enhanced antimicrobial activity was a cationic group directly connected to an alkyl chain resulting in an amphiphilic molecule which then could be linked to PEI without spacers or intermediate molecules [83].

For application in dentistry purposes quaternary ammonium functionalized PEI nanoparticles were synthesized and inserted in 3 different commercial products: a restorative composite resin (Filtek Z 250, 3M ESPE); a low viscosity composite resin (Filtek Flow, 3M ESPE) and a bonding agent (Adper Single Bond, 3M ESPE). Materials incorporated with 1% (w/w) quaternary ammonium functionalized PEI nanoparticles exhibited antimicrobial properties against *S. mutans*. Flexural strength though decreased for the low viscosity composite resin after insertion of quaternary ammonium functionalized PEI nanoparticles [84]. Quaternary ammonium functionalized PEI nanoparticles were also tested in root canal sealers with broader spectrum of action: besides *S. mutans* also *A. naeslundii*, *E. faecalis*, *C. albicans* [85].

Silica nanoparticles (NP) can deliver biocides entrapped in their structure or immobilized in their surface. Because of high surface area NP are capable of carrying and releasing great amounts of biocides (Figure 4). Incorporating antimicrobial compounds by modifying nanoparticles surface and entangling these particles into a polymeric matrix can also enhance mechanical features. Dental materials profited from inclusion of QAC-functionalized nanoparticles [85].

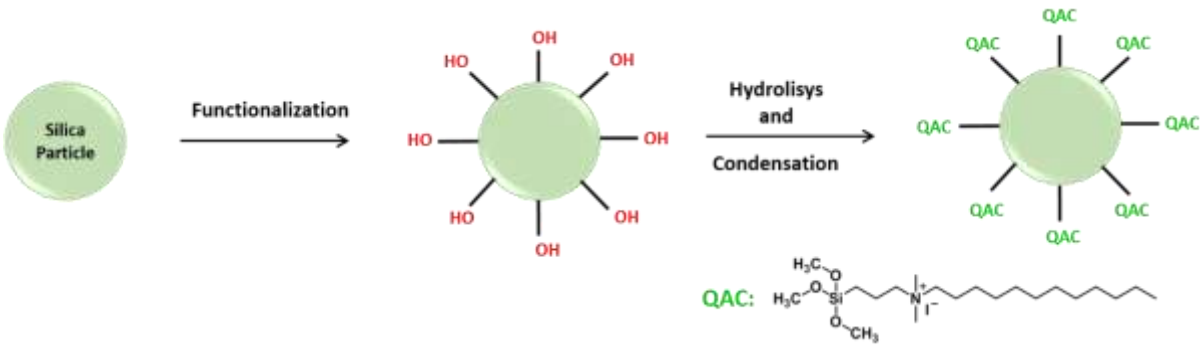

Figure 4. Schematic representation of nanoparticles quaternization.

Quaternary ammonium compounds were attached to the surface of nanosilica particles and the results revealed that composite filled with these quaternary ammonium methacrylate-modified nanosilica (QMSNs) have inhibited growth of gram-positive *S. mutans*, *S. aureus*, *B. Subtilis*, gram-negative *E. coli*, *P. aeruginosa*, and fungi *C. albicans*. These modified nanoparticles functioned as reinforcement particles and composites showed improved mechanical properties with higher values of flexural strength [50].

There are disadvantageous aspects of functionalizing silica nanoparticles in the sense that the particle could be lost by wear if they are not strongly chemically bound to the matrix or if they have to transpose thick mature biofilms when used as drug carriers. NP can bind to EPS but their variability in size, charge and shape can also change this interaction. Therefore, researchers have been trying to understand how NP's characteristics play a role on the attachment and diffusion through the biofilm. Evidence point out that for some *Pseudomonas* and *Escherichia coli* biofilms, positive charged NP could attach and diffuse more easily into the biofilm if compared to negatively charged or neutral NP.

Variables influencing antimicrobial properties of QAC

There are some factors to be considered when analyzing the antimicrobial properties of polymeric materials containing QAC including surface charge density [86]; effect of molecular weight [2]; counterion effect [6]; effect of alkyl chain length [5]; and bacterial singularities [8].

The influence of the surface charge was tested between different compounds containing QAC. It was noticed that positively charged surfaces, even when attracting more bacteria at first, prevented biofilm growth for gram-negative bacteria. A threshold of 10^{14} charges per cm^2 have been reported for a surface to exert antimicrobial activity [87]. The authors inferred that a strong attachment between positive surface charge and negative bacterial membrane impeded the elongation necessary for cell division. Gram-positive bacteria were not affected as much because of their thicker and more rigid peptidoglycan layer. Negatively charged surfaces, in contrast, promoted exponential gram-positive and gram-negative growth, even though the initial adhesion was lower [88].

The molecular weight was also correlated with the antibacterial potential. It was demonstrated through synthesis of polymeric biocides with different molecular weights that antibacterial activity increases as the molecular weight rises, as does the cationic charge density. As the bacterial cell surface is negatively charged, the higher the cationic charge density of a surface, the easier is the adsorption of the polymeric biocide to the cell membrane and the consequent process of membrane lyses, cytoplasmic material leakage and cell death [89].

It has been discussed that the counterion also plays a role in the antimicrobial activity. Some have stated that there was an increase or decrease in the antimicrobial efficacy against *E. coli* and *S. epidermidis* depending on the counterion. The exact mechanism for these changes, however, could not be elucidated [90]. Counterions with weak ionic bonds, that could easily dissociate into free ions, exhibited higher antimicrobial activity than tight ion pairs [91]. Concerning quaternary ammonium groups, it was found that bromide counterions were more efficient than chloride. On the other hand, some authors found no difference between chloride, bromide, and iodide counterions [92]. Some have found that chloride-containing quaternized amine polyurethanes were less bactericidal than iodide-containing quaternized amine polyurethanes. Although the antimicrobial activity of iodine has already been established, the influence of iodide counterions was not conclusive in this study [93].

Polymer final conformation and charge density vary according to spacer length. This characteristic could also play a role on the way that the polymer interacts with the cytoplasmic membrane [83,89]. When considering quaternary ammonium chlorides, the hydrophilic–lipophilic balance affected its antimicrobial potential. It was found that even low concentrations of quaternary ammonium compounds were capable of hindering osmoregulatory activity and leakage of K+ and H+ [94] when a long carbon chain is attached to the N+. This additional antimicrobial mechanism was explained by chain intake into the bacteria cell enhancing the process of membrane physical disruption [95].

At this point, it is important to mention that the carbon chain length has also been reported as an important factor influencing the antimicrobial activity. Longer

chains seem to demonstrate greater antibacterial activity [45,95,96]. An optimum chain length has been cited as between 16 and 18 carbon atoms [97].

To prove the influence of the carbon chain in the antimicrobial activity of compounds a series of polymeric iodine QAC with different alkyl chain lengths obtained by reacting dimethylaminoethyl with different alkyl iodides were synthesized. MIC determination showed that all chain lengths between C10 up to C18 showed significant antibacterial activity. The antibacterial activity increased with increasing alkyl chain length of from 5 to 16. Increasing the chain length to more than 16 carbons did not reflect on effectiveness [23].

Another comparative analysis was made between methacrylate containing quaternary ammonium groups with different carbon chain lengths inserted on a dental bonding agent. Bacterial early attachment and biofilm CFU decreased by 4 log when increasing the chain size up to 16 carbons. Also, with longer chains the MIC and minimum bactericidal concentration decreased by five orders of magnitude. However, when the chain size was extended to 18, the antibacterial efficacy decreased. This was attributed to chain bending and consequent prevention of electrostatic interactions between the quaternary ammonium group and bacterial membrane. The improvements in antimicrobial activity did not compromise cytotoxicity or the bond strength to dental structure of the bonding agent [96].

It has been stated that a maximum antimicrobial efficiency is granted for gram-positive bacteria when the chain length is between 12 and 14 carbons. As for gram-negative bacteria, the ideal chain length would be between 14 and 16 [98,99].

It is important to emphasize that differences in bacterial structure also influence antimicrobial activity. Gram-negative bacteria present besides the cell wall an outer membrane that enhances the barrier against antimicrobial substances. Studies with *S. aureus* (gram-positive) showed that molecules weighing in a range of 5×10^4 to 9×10^4 Da could diffuse across the cell wall without difficulty [5]. On the other hand, it is worth remembering that gram-positive strains have a thicker and more rigid peptidoglycan layer [88] that can work as a barrier against molecules with high molecular weight [100].

Differences between bacteria and fungi must be cited. Both, gram-positive and gram-negative bacteria are prokaryotic cells, whereas fungi are eukaryotic cells. Because of the more complex structure of the eukaryotic cells, higher resistance to antimicrobial activity for these is expected [101].

The most common methods described in microbiology to evaluate the antimicrobial activity of materials are the agar or disk diffusion test, and the quantitative methods that include MIC and broth macro/microdilution due to their simplified methodology and cost–benefit. Unfortunately, as a lot of adaptations in methodologies have been done to test the antibacterial activity of antimicrobial materials (different bacterial strains or growth media) it may be difficult to compare the results of different studies, suggesting a need for a standardized method as reported by other researchers. Another issue is the duration of the antibacterial effect. Most studies for dental material science only document the short-term effects on antibacterial activity of the material. With a maximum aging period of 6 months in *in vitro* studies, while the maximum aging in *in vivo* studies was 12 months, suggesting the need for further long-term randomized controlled trials for assessing dental materials with antimicrobial agents [27].

Based on the data survey, there is a multitude of factors to be considered when developing polymeric materials containing antimicrobial moieties. It is necessary to take into count the characteristics of the polymer to which the immobilization will be carried out, such as thermal and chemical stability, mechanical properties, and affinity to the antimicrobial moiety chosen. Also, the antimicrobial moiety peculiarities concerning potential toxicity (depending on the molecular weight), the hydrophobicity promoted by the carbon chain, which in turn will directly impact the antimicrobial activity, and the immobilization of the group on the surface of the substrate should be pondered. The characteristics of the microorganisms to which the antimicrobial effect is aimed will also guide the features of the molecule.

Cytotoxicity

In general, low molecular weight antimicrobial agents tend to be more toxic than the high molecular weight ones but given the mechanism of bacterial membrane-targeting of QAC, cytotoxicity is of obvious concern and still under debate.

Important studies compared the toxicity of MDPB monomer to others already in use in dental materials like triethylene glycol dimethacrylate (TEGDMA), bisphenol A-glycidyl methacrylate (Bis-GMA) and Methacryloyloxydecyl phosphate (MDP) over mouse fibroblasts L929, odontoblast-like cells and human pulpal cells. Toxic concentrations of MDPB for fibroblasts L929 were in the same range as for TEGDMA [102]. Bis-GMA and MDP caused higher mineralization inhibition from odontoblast-like cells compared to MDPB [103].

After these evidences the first antibacterial adhesive system containing MDPB started to be commercialized by Kuraray Medical (Clearfil SE Protect in USA and Clearfil Mega Bond FA in Japan). Subsequently studies followed comparing Clearfil SE Protect to other commercial dental adhesives. Clearfil SE Protect was less cytotoxic than Adper Scotchbond 1, Excite, Tyrian SPE, and One Step plus [104]. Also, Clearfil SE Protect was as cytotoxic as Clearfil SE Bond which do not contain antimicrobial monomer [105].

Gong and collaborators also tested cytotoxicity by means of MTT assay and flow cytometry. Quaternary ammonium-containing acrylic resins were compared to quaternary ammonium-free acrylic resins on murine dental papilla-derived odontoblast-like cells (MPDC-23). Authors concluded that acrylic resins containing QAC were as biocompatible as the ones free of QAC and that cytotoxicity observed could be related to leaching of residual methacrylate monomers from the acrylic resin [26].

Composite resins containing QPEI showed similar effect on cell viability of MBT cell to composites that did not contain QPEI. *In vivo* studies in rats were also carried out and revealed no inflammatory signals after implantation of the restorative composite resin containing QPEI 2% (w/w). DMADDM also had much lower cytotoxicity than Bis-DMA on human gingival fibroblasts [106]. Rat tooth models evidenced that DMADDM promoted little pulpar inflammation when compared to a commercial adhesive and a glass-filled composite resin [96].

Antimicrobial resistance

Antimicrobial resistance is an alarming issue worldwide being target of discussions not only in academic sphere but among public health leaderships. QAC emerged in this scenario as a good approach to counteract antimicrobial resistance.

Antimicrobial resistance to QAC has already been reported related to the presence of sub-inhibitory concentrations. Environments such as sewage, sediments and water treatment stations can contribute to the selection of QAC-resistant bacteria [85]. Therefore, special care should be taken concerning discard of QAC. Soil and sediments are naturally negatively charged which makes contamination by QAC much easier [18].

Resistance mechanism arose from both kinds: intrinsic and acquired means. Intrinsic resistance are related to components naturally found in bacteria (structural, physiological or biochemical) that contribute to decreased susceptibility to biocides, for instance the outer membrane of Gram-negative bacteria. Acquired mechanisms are related to modifications on the hyper expression of efflux pump-gene due to oxidative stresses, stress-induced mutagenesis and acquisition of efflux pump genes through integrons, plasmids or transposon [18,85,107]. Genes associated to acquired resistance to QAC are *qacA*, *qacB*, *qacC-H*, *qacJ* and *qacZ*, which are responsible for efflux pump expression [107].

Up-to-date list of QAC-resistant bacteria point to: *Salmonella typhimurium, Acinetobacter baumannii, L. monocytogenes, E. coli*, *S. aureus*, *E faecalis*, *K. pneumoniae* and *P aeruginosa*. Further developments have been directed to inactivation of efflux pumps as a measure to fight resistant bacteria [85].

Remarks

Bacterial contamination is an everlasting mankind problem. Combating diseases caused by bacterial infection is not so simple in this post-antibiotic era. Therefore, there is urgency for alternative strategies to tackle bacterial associated infections. In view of this worrying ambience, grafting antimicrobial activity to materials used for dentistry and biomedical purposes is meaningful.

Quaternary ammonium compounds have been for decades included in daily-life products and more recently also in biomaterials with undeniable efficiency against pathogens. Given the variety of quaternary ammonium compounds and means for achieving a QAC-containing material it is of major importance for investigators to know variables affecting the antimicrobial activity, how to better benefit from the mechanism of action and what is the aimed final application. Queries about

quaternary ammonium compounds still have to be addressed such as cytotoxicity to human body and the QAC-resistant bacteria.

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2.2 Artigo: Quaternary Ammonium-Containing Isocynate Free Polyhydroxyurethane Coatings to Prevent *Streptococcus mutans* Colonization on Orthodontic Devices

Artigo submetido ao periódico *Chemistry of Materials* (Anexo 2)

Mari Miura Sugii; Carlos Alexandre Holanda; Apostolos Vagias; Giuseppe Portale; Henny C. van der Mei; Hidetake Imasato; Ubirajara Pereira Rodrigues Filho; Flavio Henrique Baggio Aguiar; Prashant Kumar Sharma

ABSTRACT

Oral biofilm growth is a common and detrimental obstacle to dentistry, especially for orthodontic patients for which adequate brushing is constrained. Patient collaboration to maintain good oral hygiene is often difficult, thus the use of materials with antimicrobial activity would diminish the risk of caries during the treatment. Long-lasting antimicrobial activity associated to low cytotoxicity is not yet a reality for metallic surfaces though. Aiming a material to address these problems, isocyanate-free polyhydroxyurethanes (PHU) containing quaternary ammonium salt (QAS) is developed to coat stainless steel. Two hybrid materials are designed and coatings are produced on stainless steel slabs. Two treatments are applied aiming reduced cytotoxic effect, a short-duration ion exchange with sodium phosphate and an extensive washing with demineralized water. Antimicrobial activity of coating leachate is assessed using Streptococcus mutans. Syntheses and coating preparation were successfully achieved as confirmed by characterization methods. Coatings exhibit strong killing by leaching of antimicrobial QAS. None of the treatments affects the antimicrobial properties of the coatings. Unlike ion exchange, washing for 3 days previously to inserting the coating in culture media does decrease the cytotoxicity. In conclusion, PHU-QAS washed coating is a reliable approach to render stainless steel antimicrobial properties.

1 INTRODUCTION

Although orthodontic treatment amends oral health and function,[1] during its course it is difficult to establish an optimal oral hygiene routine. Orthodontic appliances present complex geometries, which demands dexterity and diligence to properly clean. Since most of the patients are young, cooperation to dentist instructions remains low [2] which will lead to a poor oral hygiene and biofilm accumulation on orthodontic appliances and surrounding areas.[3–5] Oral biofilms can trigger problems such as caries, pulp injuries, periodontal diseases, implant losses and endocarditis.[6] The use of antimicrobial materials can help to maintain oral health during orthodontic treatment, by preventing bacterial adhesion, proliferation and biofilm formation. Thus, development of antimicrobial coatings on stainless steel, a prevalent orthodontic material, is appealing.

Antimicrobial activity from a coating or a material can be achieved either through slow leaching of biocides [7–10] or by immobilizing antimicrobials to the surface. Leaching materials will release the biocide in the environment and kill both sessile and planktonic bacteria by chemical means. Antimicrobial activity can also be obtained through immobilizing antifouling or biocides on the surface, usually called contact-killing surfaces, which would promote bacterial killing upon contact.[8,11–15]

Quaternary ammonium salts (QAS) are widely used for contact-killing coatings.[16] They are known for their antimicrobial activity against a great variety of fungi and bacteria and have been successfully used for preventing biomaterial-associated infections. QAS are cationic molecules acting by electrostatic interaction with the negatively charged microorganism membrane causing leakage of cell content and cell death. Besides, the long alkyl chain can interact with the lipoprotein layer on the microbial cell membrane and contribute to QAS antimicrobial activity.

Grafting QAS molecules directly on the stainless steel is a challenge.[17,18] A carrier layer is necessary to keep the QAS molecules available at the orthodontic device surface. The use of polymeric coatings can facilitate grafting bactericidal agents on the surface.[19] Long-established polyurethanes are already in use in biomedical field as catheters, tissue sealant and cardiac valves due to their excellent biocompatibility, thermal stability, and good malleability.[19] However, synthesis of polyurethanes often involves the use of isocyanate reagents. Isocyanates are highly reactive and toxic agents therefore the chemical process and disposal of the product

remains environmentally-unfriendly.[20–22] Furthermore, it carries a risk of isocyanate release in the oral cavity due to either mechanical wear.

In this study we use isocyanate-free polyhydroxyurethanes (PHU) derived from cyclic carbonates containing polydimethylsiloxane segments.[23] Just like classic polyurethanes, PHU have proven to function as coatings on metallic surfaces with excellent adhesion properties.[20,24] Therefore, the aim of this study was to develop hybrid coatings of isocyanate-free PHU containing QAS to be applied on stainless steel substrates as an antimicrobial coating. For coupling QAS to PHU, silanes with two different hydrolysable ends were used, giving rise to two hybrids i.e. PHU-QAS (ethoxy end) and PHU*QAS (methoxy end). PHU, PHU* and QAS were synthesized separately and coupled by a sol-gel process. PHU-QAS and PHU*QAS compounds were characterized by fourier-transform infrared spectroscopy (FTIR), nuclear magnetic resonance (NMR), grazing-incidence X-ray scattering (GIXS) and X-ray photoelectron spectroscopy (XPS). Coatings were applied by drop-casting on stainless steel slabs. For planktonic killing and contact-killing the S. mutans UA159 was used. The cytotoxicity of the coating's leachate was tested using the XTT assay with mice fibroblasts L929. Two treatments were performed to the coating in order to reduce the cytotoxicity i.e. 2 h ion exchange with sodium phosphate and 3 days of washing in demineralized water.

2 MATERIALS AND METHODS

2.1 Synthesis of polyhydroxyurethane (PHU) and modified polyhydroxyurethane (PHU*) containing QAS

PHU backbone was synthesized as previously described.[23] Briefly, cyclic carbonates (CCPDMS) were obtained from the reaction of 24.5 mmol polydimethylsiloxane diglycidylether (Sigma-Aldrich, USA) and 1.6 mmol hexadecyltrimethylammonium bromide (Fluka, USA) in 10 mL ethoxyethanol (Sigma-Aldrich, USA) for 8 h, 1 MPa, 100°C and 300 rpm. One and a half mmol of CCPDMS was then reacted with 0.75 mmol 5-amino-1,3,3-trimethylcyclohexanemethylamine (IPDA) (mixture of cis-trans, Sigma-Aldrich, Germany) at 50°C for 3 h (Figure 1a). After this step, the compound was silylated for 3 h either with 3 mmol (3-(aminopropyl)triethoxysilane (APTES) (Sigma-Aldrich, China) leading to hybrid PHU compound (Figure 1b) or 3 mmol of N-[3-(trimethoxysilyl)propyl]ethylenediamine (TMSPE) (Sigma-Aldrich, USA) leading to hybrid PHU* compound (Figure 1c).

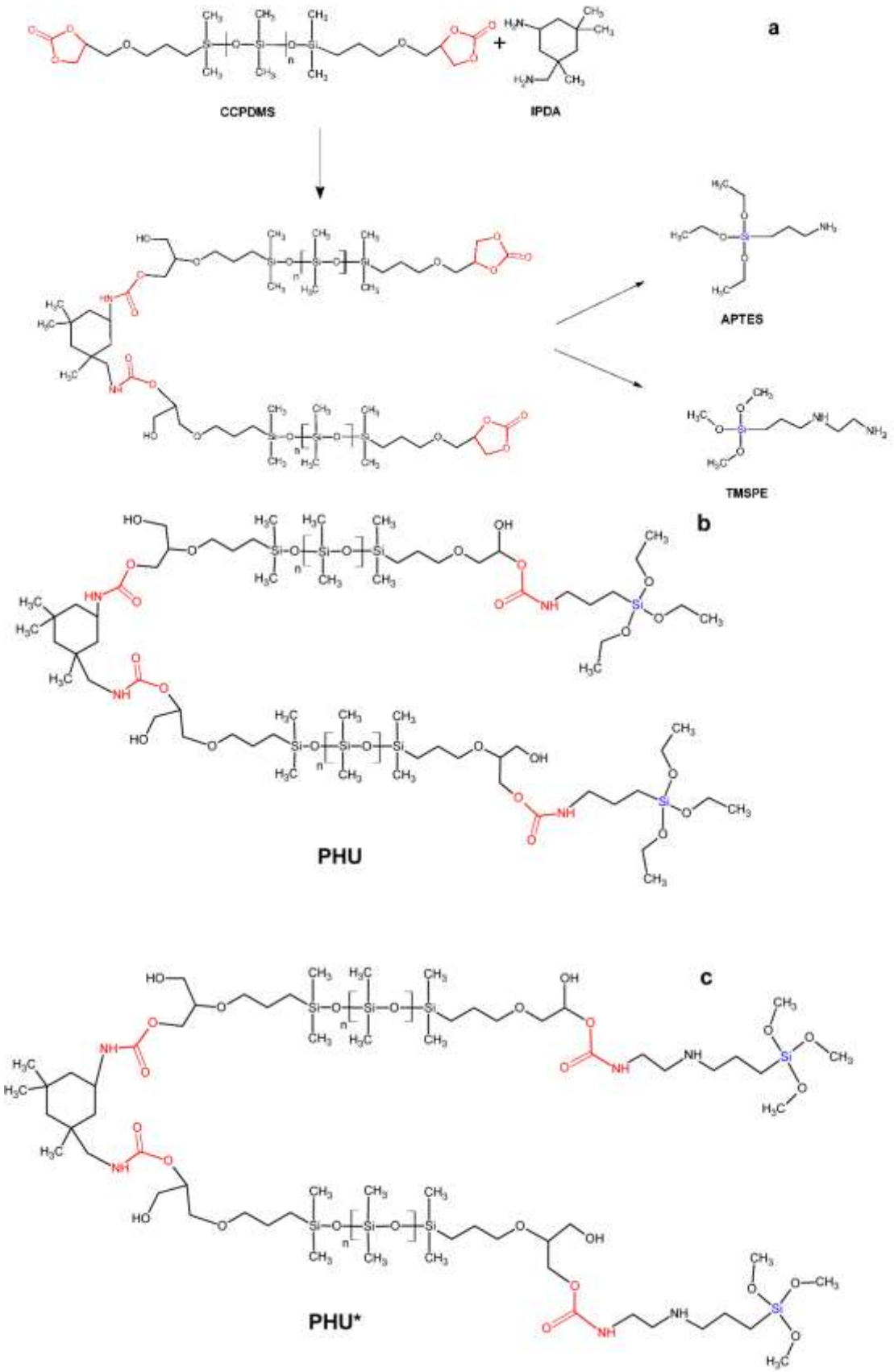


Figure 1a) Schematic representation of reaction between CCPDMS and IPDA and silylation of resultant molecule with APTES or TMSPE. Final structure of **(b)** PHU and **(c)** PHU* molecules**.**

QAS was obtained from the reaction between 6 mmol of tertiary amine N,N-dimethyldodecylamine (DMDA) (Sigma-Aldrich, Switzerland) and 6 mmol (3-iodopropyl)trimethoxysilane (IPTMS, Sigma-Aldrich, USA) at 80˚C for 24 h in 25 mL N,N dimethylformamide (DMF) (Sigma-Aldrich, Germany). The DMF was later removed by rotaevaporation (Ika RV 10 Digital, Ika, Germany).[25]

PHU/PHU* and QAS products were coupled to form two hybrid compounds, PHU-QAS (Figure 2a) and PHU*QAS (Figure 2b), by a sol-gel process in ethanol. Hydrolysis and condensation were performed at 50°C with HCl 0.3 M solution and 3 mmol tetraethyl orthosilicate as a coupling agent (TEOS) (Sigma-Aldrich, China).

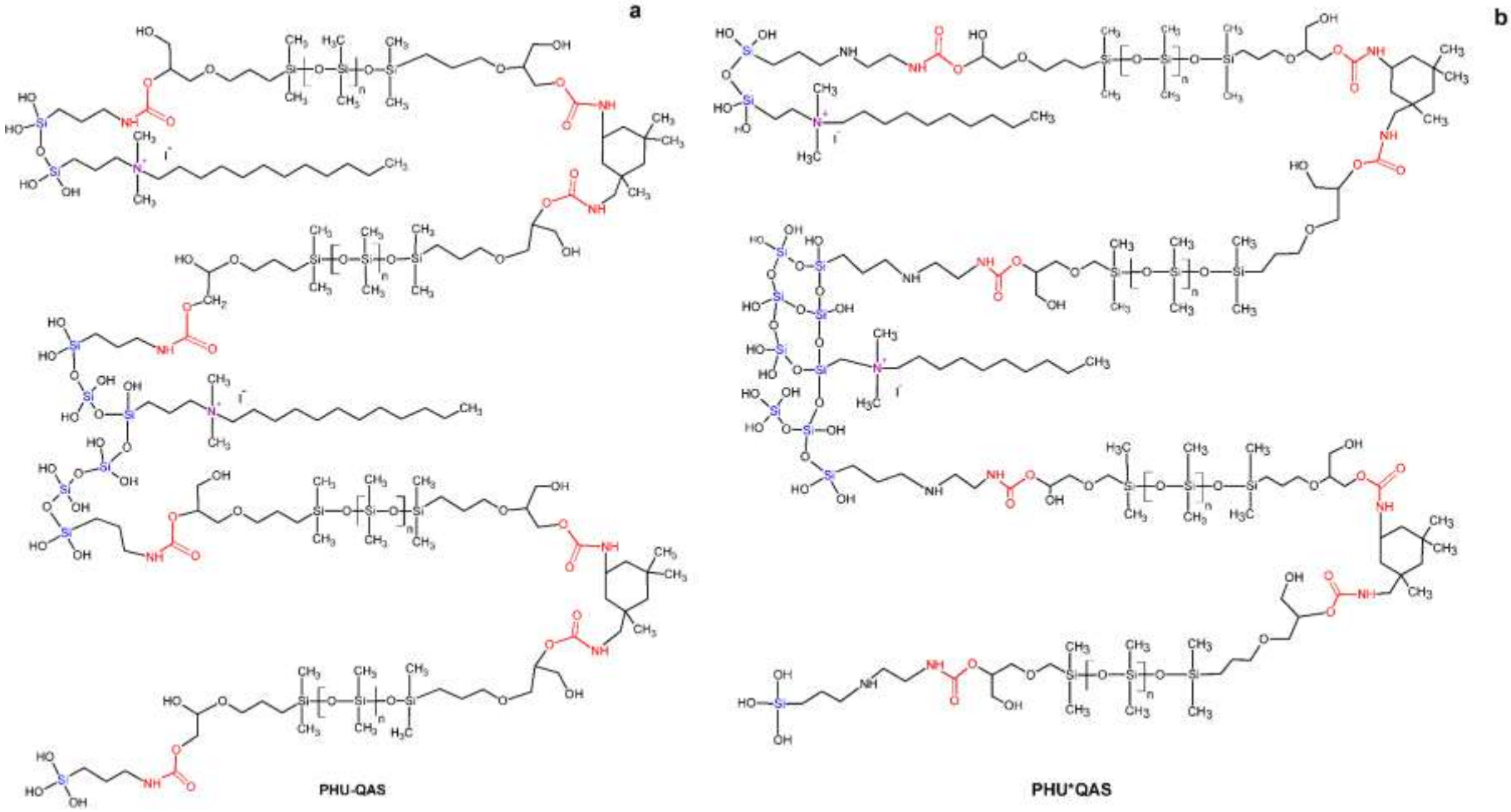


Figure 2. Schematic representation of (a) PHU-QAS and (b) PHU*QAS molecules. The sol-gel process can lead to different structural configurations. The siloxane network can be more branched and some molecules could adopt a circular configuration.

2.2 Synthesis and coating characterization

2.2.1 Attenuated Total Reflectance Fourier-transform infrared spectroscopy (FTIR): After synthesis, ATR-FTIR spectra from PHU and PHU* in ethanol were performed, to detect the conversion of cyclic carbonates into urethane bonds using an IRAffinity-1S (Shimadzu, Japan) spectrometer equipped with ZnSe ATR crystal using the wave number range of 650–4000 cm^{-1}, 32 scans and resolution of 4 cm^{-1}.

2.2.2 Nuclear Magnetic Resonance (NMR): The chemical composition of each step of synthesis dissolved in acetone-d6 (Sigma-Aldrich, Germany) was determined using NMR. 13C NMR spectra of CCPDMS (600 µg/mL), PHU (600 µg/mL), PHU* (600 µg/mL) and QAS (150 µg/mL) were recorded using a BioSpin GmbH 500 MHz (Bruker, Germany) with acquisition parameters of 4000 scans, acquisition time 0.5 s, relaxation delay of 1 s, and pulse width of 12 s.

2.2.3 X-Ray Photoelectron Spectroscopy (XPS): XPS was performed on PHU-QAS and PHU*QAS coatings and extracted leachate solutions (see section 2.3) casted on gold wafers at room temperature. Coating and leachate solutions were assessed by a S-probe spectrometer (Surface Science Instruments, Mountain View, CA), equipped with a monochromatic X-ray source (Al Kα anode, 1486.8 eV X rays), operated at 10 kV accelerating voltage and 22 mA current. A wide scan range from 0 eV to 1200 eV was taken with pass energy of 150 eV in a spot size of 250 x 1000 µm. High resolution scans of C1s and N1s peaks were made using pass energy 50 eV. Charge correction was performed with a flood gun set to 10 eV. Peak fitting procedure was performed by software Hawk Data Analyisis 7 (version V7.03.04, Service Physics Inc., Bend, USA). The binding energy of aliphatic and silicon carbons was set at 284.6 eV, the baseline function was Shirley background and a binding energy precision of 0.1 eV was adopted. The C1s peak was then decomposed into 289.1, 287, 286.1 and 284.6 eV peaks. The N1s peak was decomposed in 399.5 and 402.8 eV peaks. Quaternary and amine nitrogens percentages were calculated based on the area under the peaks with respect to the total nitrogen peak.[26]

2.2.4 Grazing-incidence X-Ray scattering (GIXS): Sodalime glass slides (76 mm x 25 mm, Thermo Fisher, Germany) were cleaned with the same protocol described for stainless steel (section 2.3). Coatings were prepared on only one side

of the glass slide at room temperature with 30 µL of PHU-QAS or 100 µL of PHU*QAS suspensions. An aluminum slot-die with gap clearance of 120 µm was used to produce uniform film thickness along the spreading direction.

GIXS experiments were conducted using a home-built X-ray scattering instrument comprised of a Cu rotating anode source using 8 keV irradiation energy and a wavelength (*ʎ*) of 1.541 Å at 23°C, the so-called Multipurpose Instrument for Nanostructure Analysis (MINA). The X-Ray beam was 500 µm (perpendicular to the film surface) x 500 µm (along the film surface). 2D GIXS patterns were collected using a Vantec500 detector (array of 1024 × 1024 pixels, with pixel size 136 µm × 136 µm) located 275.6 mm away from the sample. The sample-to-detector distance allowed measurements with in-plane range qy = 0.33–9.7 nm−1 (i.e., 0.64 - 19 nm, range of spatial resolution) and out-of-plane range of qz = 0.61 to 20 nm-1. The qy is the modulus of the in-plane scattering wave vector:

$$q_y = \left(\frac{2\cdot\pi}{\lambda}\right)\cdot\cos(\alpha_f)\cdot\sin(\psi) \qquad (1)$$

With ψ being the in-plane scattering angle in parallel direction and α_f being the exit scattering angle in the vertical direction. Similarly, q_z is the modulus of the out-of-plane scattering vector:

$$q_z = \left(\frac{2\cdot\pi}{\lambda}\right)\cdot[\sin(\alpha_f) + \sin(\alpha_i)] \qquad (2)$$

With α_i the incident angle of the X-Ray beam with respect to the sample surface and α_f being the exit scattering angle in the vertical direction. The nominal critical angle α_c of the polymer films and of the sodalime glass substrate was 0.16° and 0.24°, respectively. The measurements were conducted in reflection geometry at incident angles α_i~ 0.3± 0.05° with respect to the direct beam using a high-resolution Huber goniometer.[27,28] This incident angle α_i (α_i > $\alpha_{c,polymer}$) allowed to probe the nanostructure in the films close to the air-film interface and in the bulk of the film e. An exposure time between 5 min and 3 h per pattern was used. The direct beam center position on the detector and the sample-to-detector distance were calibrated using the diffraction rings from a standard silver behenate powder. Azimuthal integrations of the scattered intensity over all pixels were performed from 0 to 180 degrees in order to obtain the 1D intensity profiles I(q) vs q.

2.3 Coating preparation, washing, ion exchange and leachate extraction

After polishing, samples were sonicated for 15 min in 2% RBS 35 detergent (Chemical Products R. Borghgraef S.A., Belgium) aqueous solution. Subsequently, thorough washing with demineralized water followed by 2 h immersion in methanol. Stainless steel slabs were dried with nitrogen air jet and 30 µL of PHU-QAS (n=36) or PHU*QAS (n=36) were applied on the slabs. Coatings were applied on both sides with an interval of 10 days for PHU-QAS and 2 days for PHU*QAS. PHU*QAS needed a shorter drying time than PHU-QAS due to the faster molecular rearrangement when forming a gel.

Twelve dry coatings of each group PHU-QAS and PHU*QAS were washed with demineralized water for 72 h (265 mL refreshed every 24 h) leading to groups *PHU-QAS washed* and *PHU*QAS washed*. Another 12 samples of each group were immersed for 2 h in 0.2 M sodium phosphate (Sigma-Aldrich, Germany) at room temperature to promote $(PO_4)^{3-}$ ion exchange with I^- leading to groups *PHU-QAS ion exchanged* and *PHU*QAS ion exchanged*. Pristine coatings, which did not undergo any further steps besides drying at room temperature, were called *PHU-QAS* or *PHU*QAS*. Coatings from all the above groups were stored in 3 mL growth media – Brain Heart Infusion (BHI) broth (Oxoid, UK) for planktonic bacterial killing test and in Gibco™ Minimum Essential Media (MEM) (Gibco-Life Technologies, USA) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 2% Glutamax for cytotoxicity test at 37°C. The 3 mL of growth media in contact with the coatings and containing leachates were collected and refreshed every 24 h. Growth media collected on day 1 and day 7 were tested for their antimicrobial and cytotoxicity.

2.4 Cationic charge density measurement

Cationic charge density of the QAS coatings was determined by fluorescein staining.[26] Coatings were immersed in 4 mL 1 wt% fluorescein sodium salt (Sigma Aldrich, USA) aqueous solution for 10 min. To remove the dye not bound to the cationic charges, the coatings were washed and sonicated in 5 mL of demineralized water. Subsequently, samples were immersed in 4 mL 0.1 wt% cetyltrimethylammonium chloride aqueous solution and sonicated for 10 min, followed by addition of 10 v/v% of 100 mM phosphate buffer, pH 8. UV/VIS measurements were then carried out in the Spectronic®20 Genesys™ (Spectronic

Instruments, USA) at 501 nm to calculate the molar concentration of extracted fluorescein ([Dye]) from the QAS coatings according to:

$$[Dye] = Abs_{501}/(\varepsilon_{501} \times L) \quad (3)$$

In which Abs_{501} is the UV absorption at 501 nm, ε_{501} is the extinction coefficient (77 $mM^{-1}cm^{-1}$ for fluorescein) and L is the transversal width of a polystyrene cuvette (1 cm) crossed by the UV-light beam. Next, the cationic charge density per cm^2 coated surface was calculated based on:

$$Charge\ Density = [Dye] \times V \times (N/A) \quad (4)$$

In which V is the volume of the extraction solution (4 mL), N is Avogadro's number (6.023×10^{23}) and A is the surface area of the QAS coated surface (2.5 cm^2).

PHU-QAS was applied on stainless steel slabs (SS316L, 10x10x1 mm; 0,03 %m C; 2 %m Mn; 0,75 %m Si; 16-18 %m Cr; 10-14 %m Ni; 2-3%m Mo; 0,045 %m P; 0,030 %m S; 0,10%m N) (Rocholl GmbH) of 1 cm width and 2.5 cm length previously polished and cleaned.

Films were formed on the slabs by casting, where 12 μL of the PHU-QAS were deposited on the surface and scattered with a micropipette.

2.5 Release and contact-killing antimicrobial activity of the coatings and leachate cytotoxicity

2.5.1 Antimicrobial activity of the coating leachate against planktonic *S. mutans* UA159

S. mutans UA159 was taken from -80°C stock in 7 v/v% dimethyl sulfoxide/BHI, streaked on blood agar plates and grown for 24 h at 37˚C with 5% CO_2. Colonies were inoculated in BHI overnight, centrifuged for 5 min at 5000 g and 10˚C and subsequently washed two times with 10 mM potassium-phosphate buffer, pH 7. The collected coating leachates in growth media on day 1 and day 7 were inoculated with *S. mutans* (2×10^6 bacteria/mL) and incubated for 24 h at 37°C and 5% CO_2. After incubation and serial dilution, the bacterial suspension was plated on BHI agar and incubated for 48 h after which colony-forming units per mL (CFU/mL) were determined.

2.5.2 Contact-killing of QAS containing coatings

To detect the contact-killing potential of the coatings after washing, ion exchange and leachate extraction in growth media, the Petrifilm® Aerobic Count plate system (3M Microbiology, USA) was used. Petrifilm® plates consist of two foils containing nutrients, cold-water soluble gel, and tetrazolium indicator for colony counting. These plates were prepared by swelling the gel with 1 mL of sterile demineralized water. Next, the sample was enclosed between the two foils in contact with 15 µL of the overnight inoculum (3 x 10^5 bacteria/mL) , ensuring bacterial suspension to be evenly spread over the surface area of the sample. The Petrifilm® was incubated at 37˚C with 5% CO_2 for 48 h. The absence of colonies in gel contacting the coating was interpreted as positive for contact-killing and the presence of colonies interpreted as negative for contact-killing.

2.5.3 Cytotoxicity of the coating leachate

Mice fibroblasts L929 (Bioscan, USA) were used according to ISO Standard 10993-5 for cytotoxicity assessment. The leachate test, evaluates the cytotoxicity of any leachable molecules from the QAS coatings. Cells were cultured in MEM supplemented with 10 v/v% fetal calf serum, 1 v/v% penicillin (100 units/mL) with streptomycin (100 µg/mL) and 2 v/v% GlutaMAX, at 37˚C in 5% CO_2. All products for culturing were manufactured by Gibco-Life Technologies. Confluent cells were detached from the culture flask with 0.05% trypsin-EDTA, centrifuged and resuspended. Cells were then cultured in a 96 wells plate at a concentration of 10,000 cells/well for 24 h at 37˚C in 5% CO_2 incubator prior to exposure to growth medium with leachates. After 24 h the growth medium was replaced by the growth medium with the leachates collected on days 1 and 7. Cells were incubated for another 24 h under the same conditions.

XTT (Roche, Germany) was used following manufacturer's instructions to quantitatively determine cell metabolic activity. Cells cultured under normal conditions in MEM were used as a control and growth media containing leachates without cells was used as a blank. The XTT solution was prepared in a 1:50 activator to reagent ratio. Then 50 µL of XTT solution was added to each well and the plate was incubated for 3 h at 37˚C in 5% CO_2 incubator. Absorbance was measured with FLUOstar OPTIMA (BMG Labtech, Germany) at wavelengths 485 nm and at 690 nm. Cell viability was calculated according to the formula:

$$\frac{(OD_{485}-OD_{690})-(ODb_{485}-ODb_{690})}{(ODc_{485}-ODc_{690})} \times 100 \qquad (5)$$

in which OD_{485} and OD_{690} are absorbances from tested growth media containing leachates at the two wavelengths measured, ODb_{485} and ODb_{690} are absorbance from the blank and ODc_{485} and ODc_{690} from the control.

3 RESULTS

3.1 Coating design and characterization: PHU-QAS and PHU*QAS coatings were successfully fabricated with outstanding differences in drying time: 10 days for PHU-QAS and 2 days for PHU*QAS.

3.1.1 FTIR: Figure 3 (a-c) shows spectra for CCPDMS, PHU and PHU* molecules. CCPDMS cyclic carbonates are represented by υ(C=O) stretching vibrations occurring at 1789 cm^{-1} on CCPDMS spectrum (Figure 3a). On PHU and PHU* spectra, peak 1789 cm^{-1} is shifted to 1713 cm^{-1} related to υ(C=O) stretching vibration followed by 1537 cm^{-1} related to δ(NH) angular vibration both distinctive of urethane groups.

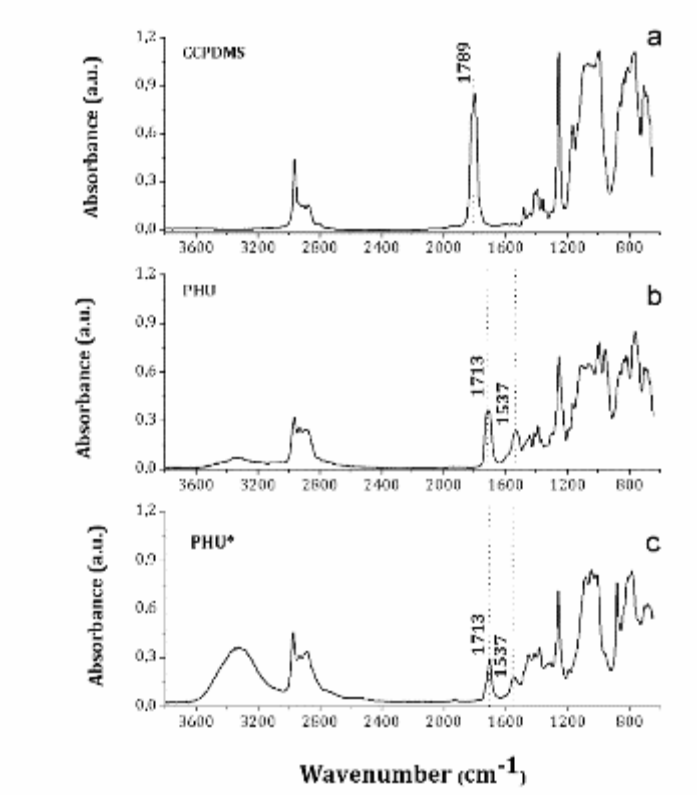


Figure 3. FTIR-ATR spectra: a) CCPDMS; **b)** PHU and **c)** PHU* polymer solutions.

3.1.2 NMR: Figure 4a shows ^{13}C NMR spectrum of CCPDMS. Cyclic carbonate signals were detected at δ155.69 ppm attributed to carbon **1** (C=O), δ66.84 ppm attributed to carbon **2** (–CH_2–O–) and δ76.33 ppm to carbon **3** (–CH–). Carbons **4** and **5** from ether linkage were detected at δ74.91 ppm and at δ70.66 ppm respectively, carbon **6** and **7**, from propyl segments linked to terminal silicon of poly(dimethylsiloxane) segment, at δ24.09 ppm and δ14.6 ppm respectively. Methyl carbons **8** and **9** from the poly(dimethylsiloxane) chain were assigned to peaks δ0.31 ppm and δ1.4 ppm, respectively.

Figure 4b presents the ^{13}C NMR spectrum of PHU and Figure 4c of PHU*. Urethane bonds (carbons **1** and **1'**) were detected in the range between δ156.5 ppm and δ158.5 ppm for both PHU and PHU*. Urethane bonds could be bounded to a primary or secondary alcohol. In primary alcohol, the hydroxyl group is linked to a primary carbon (–CH_2OH) and therefore signals will differ from secondary alcohol (hydroxyl group attached to a carbon linked to two other carbons). For the urethane bond attached to a primary alcohol, carbons in sequence were numbered as **1**, **2**, **3** and **4**. For the urethane bond attached to a secondary alcohol, carbons in sequence were named **1'**, **2'**, **3'** and **4'**.

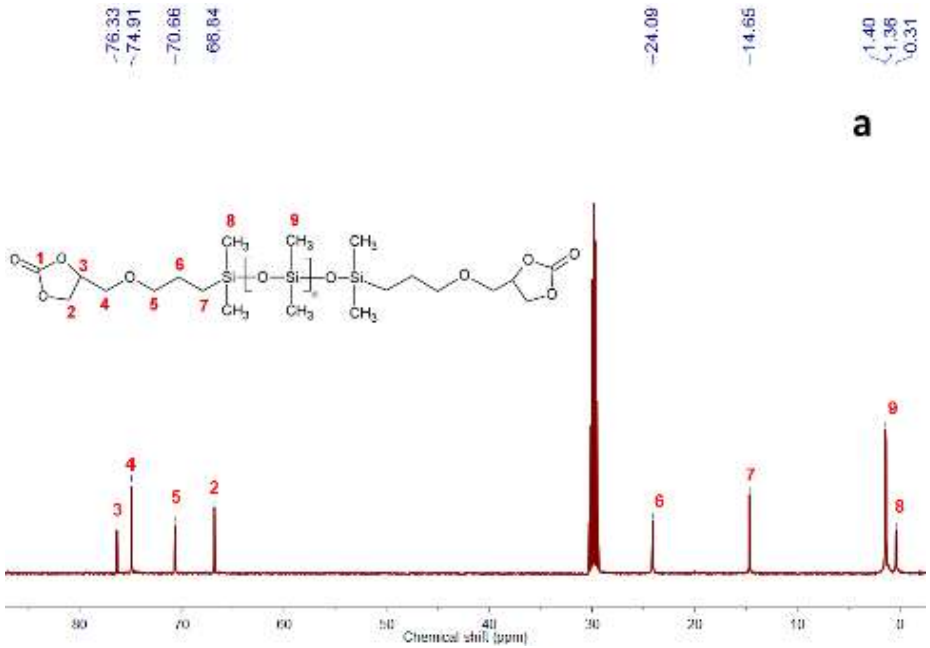
For the PHU molecule: the peak at δ66.8 ppm was assigned to carbon **2**, δ69.6 ppm to carbon **3** and δ74.68 ppm to carbon **4**. The second terminal end in the PHU molecule showed peaks at δ62.1 ppm, δ74.6 ppm and δ70.31 ppm assigned to carbons **2'**, **3'** and **4'** accordingly. Carbon **5** was detected at δ72.81 ppm, carbons **6** and **13** superimposed at δ24.22 ppm and carbon **7** at δ14.8 ppm. Carbons **8** and **9** from the poly(dimethylsiloxane) chain were assigned to δ0.37 ppm and at δ1.38 ppm respectively. For the PHU molecule, terminal ends will present ethoxy groups (CH_3CH_2O–) characteristic of APTES silane thus at the end of the molecule: carbons **10** (–CH_3), **11** (–O–CH_2–) and **12** (–Si-CH_2–) were attributed to peaks δ18.7 ppm, δ58.76 ppm and the duplet δ8.27 and δ 8.84 ppm, respectively.

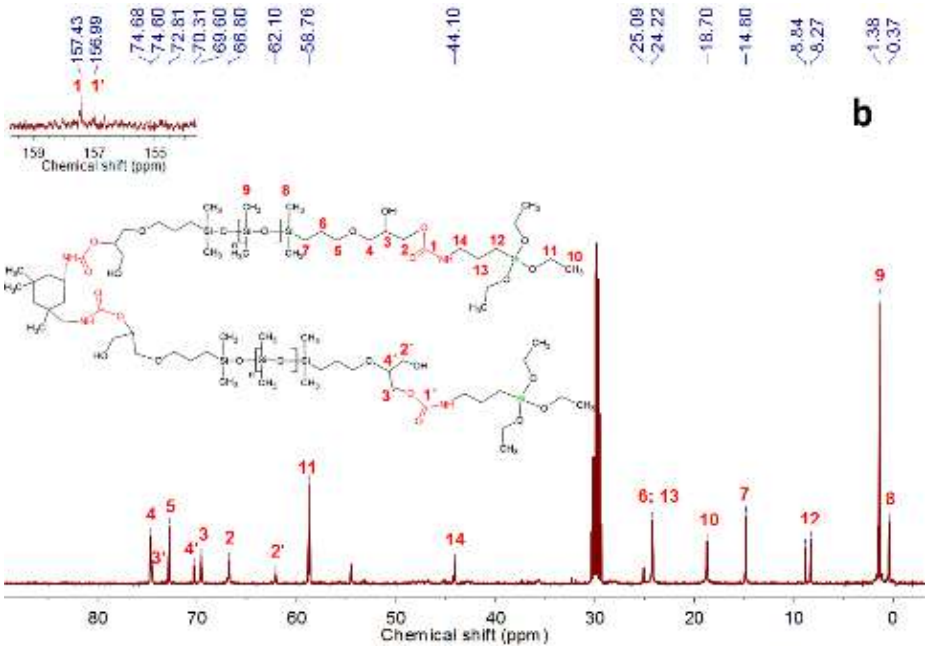
Peaks assigned for PHU* are similar to the ones described for PHU: carbon **2** at δ66.77 ppm, carbon **3** at δ69.49 ppm, carbon **4** at δ74.85 ppm. The second terminal end in PHU* was identified through peaks δ61.97 ppm accredited to carbon **2'**, δ74.57 ppm to carbon **3'** and δ70.22 ppm to carbon **4'**. Carbon **5** at δ72.76 ppm,

carbons **6** and **13** superimposed at δ24.17 ppm and carbon **7** at δ 14.76 ppm. Carbons **8** and **9** from the poly(dimethylsiloxane) chain were assigned to δ0.37 ppm and to δ1.35 ppm respectively. For PHU* molecule, terminal ends are methoxy groups (CH_3O–) so carbon **10** is absent and two other carbons were identified: **15** and **16** arising from TMPSE silane, which were not present on APTES: carbons **11** (–O–CH_2–) and **12** (–Si-CH_2–) were attributed to peaks δ49.57 ppm and the duplet δ7.20 and δ7.25 ppm respectively. Carbons **14**, **15** and **16** were identified at: δ43.9 ppm, δ47.28 ppm and δ44.99 ppm respectively.[29] All high intensity signals around δ29.9 ppm in ^{13}C NMR spectra were attributed to acetone d_6 solvent.

Some of the peaks in PHU and PHU* spectra were not classified as they are related to the side chains of the molecule where two CCPDMS were linked to each other. The ^{13}C NMR characterization was meant to identify the differences between silylation with APTES and TMSPE which generated PHU and PHU*, respectively.

^{13}C NMR of pure QAS is shown in Figure 4d. Methyl (from the alkoxysilane) and ammonium carbons, **a** and **e**, were superimposed at δ51.32 ppm. Carbon **b** was assigned to peak δ11.09 ppm, **c** and **i**-**n** superimposed on acetone d_6 peaks, **d** to δ65.75 ppm, **f** to δ67.22 ppm and **g** to δ27.19 ppm. At the end of the chain, carbons **o** at δ32.69 ppm, **h** and **p** superimposed on δ23.35 ppm and **q** at δ14.42 ppm.


a
Chemical shift (ppm)


b
Chemical shift (ppm)

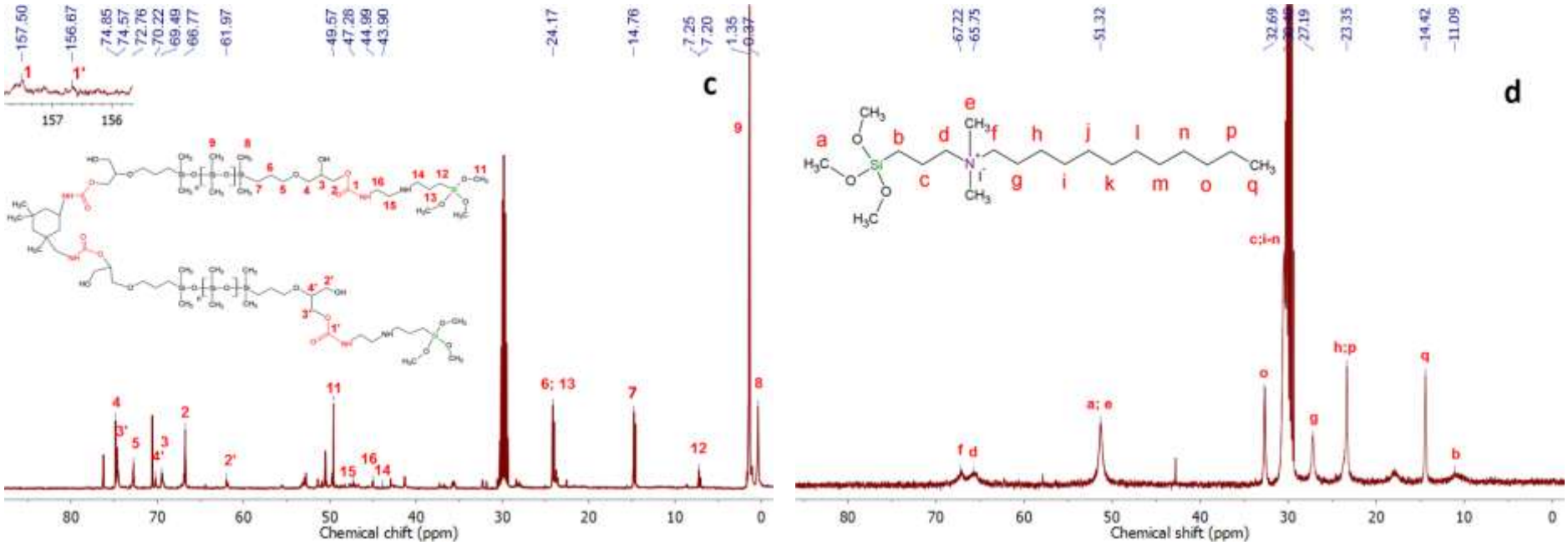


Figure 4. ^{13}C NMR spectra: **a)** CCPDMS; **b)** PHU; **c)** PHU* and **d)** QAS.

3.1.3 XPS: From both materials, PHU-QAS and PHU*QAS, focus was given to carbon (C_{1s}), nitrogen (N_{1s}), iodide (I_{3d}), oxygen (O_{1s}) and phosphorus (P_{2p}) from the wide scan. Figures 5a,b and 6a,b show C_{1s} peaks of PHU-QAS and PHU*QAS, respectively. Aliphatic and silicon linked carbons (***C***–C /***C***H_3–Si) were assigned to a binding energy of 284.6 eV. Carbon linked to quaternized nitrogen ≡N^+–***C*** had a binding energy of 287.0 eV and 289.1 eV belongs to carbon in urethane O–(***C***=O)–NH. Carbon linked to urethane nitrogen (OC=O)NH–***C***, ethanol –***C***–OH and ether –***C***–O–C had a binding energy at 286.1 eV. Amine -NH–***C***, only present in PHU*QAS also had a binding energy of 286.1 eV.[23,30]

Figures 5c-f and 6c-f show the N_{1s} peaks for PHU-QAS and PHU*QAS, respectively. For both coatings: Quaternary ammonium group was identified at a binding energy of 402.8 eV and nitrogen species related to urethane group (C=O)–***N***H–C provided signals at 400.2 eV for both coatings PHU-QAS and PHU*QAS. The peak at binding energy 399.5 eV was attributed to primary and secondary amines. The difference between PHU-QAS and PHU*QAS can be denoted with an extra peak at 399.5 eV in PHU*QAS (Figure 6c-e, red line), representative of secondary amine C–***N***H–C derived from TMSPE silane and not present in PHU-QAS (Figure 2).[30,31]

In the PHU-QAS and PHU*QAS pristine coating (Figure 5c and 6c) the quaternary ammonium peak is more intense than the urethane nitrogen peak. This pattern is the same for ion exchanged coating (Figure 5e and 6e) but not for the washed (Figure 5d and 6d), in which the intensity of quaternary ammonium group decreased compared to the urethane peak.

In the leachate solutions the excess of APTES and TMSPE were collected therefore the primary and secondary amine peak at 399.5 eV is present. Also, in the leachate solutions there is a prominent peak of quaternary ammonium at 402.8 eV (Figure 5f and 6f). The absence of the 289.1 eV peak from urethane carbons and of peak 400.2 eV from urethane nitrogens in both leachates denote that PHU/PHU* segments were not detached from the coating and leached out (Figure 5b,f and 6b,f).

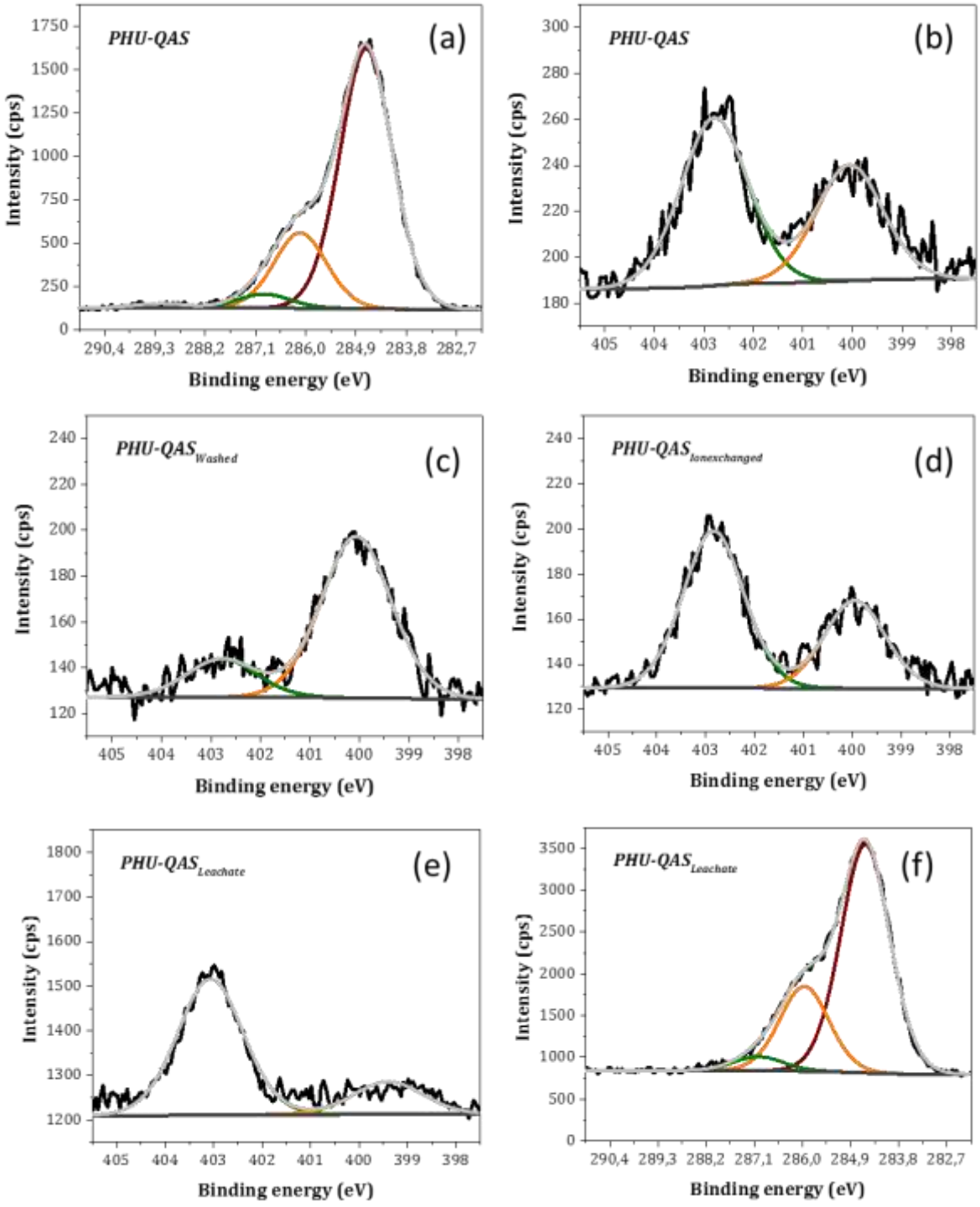


Figure 5. XPS narrow scans for: **a)** C_{1s} for PHU-QAS coating; **b)** C_{1s} of PHU-QAS leachate solution; **c)** N_{1s} for PHU-QAS coating; **d)** N_{1s} for PHU-QAS washed coating; **e)** N_{1s} for PHU-QAS ion exchanged coating and **f)** N_{1s} of PHU-QAS leachate solution.

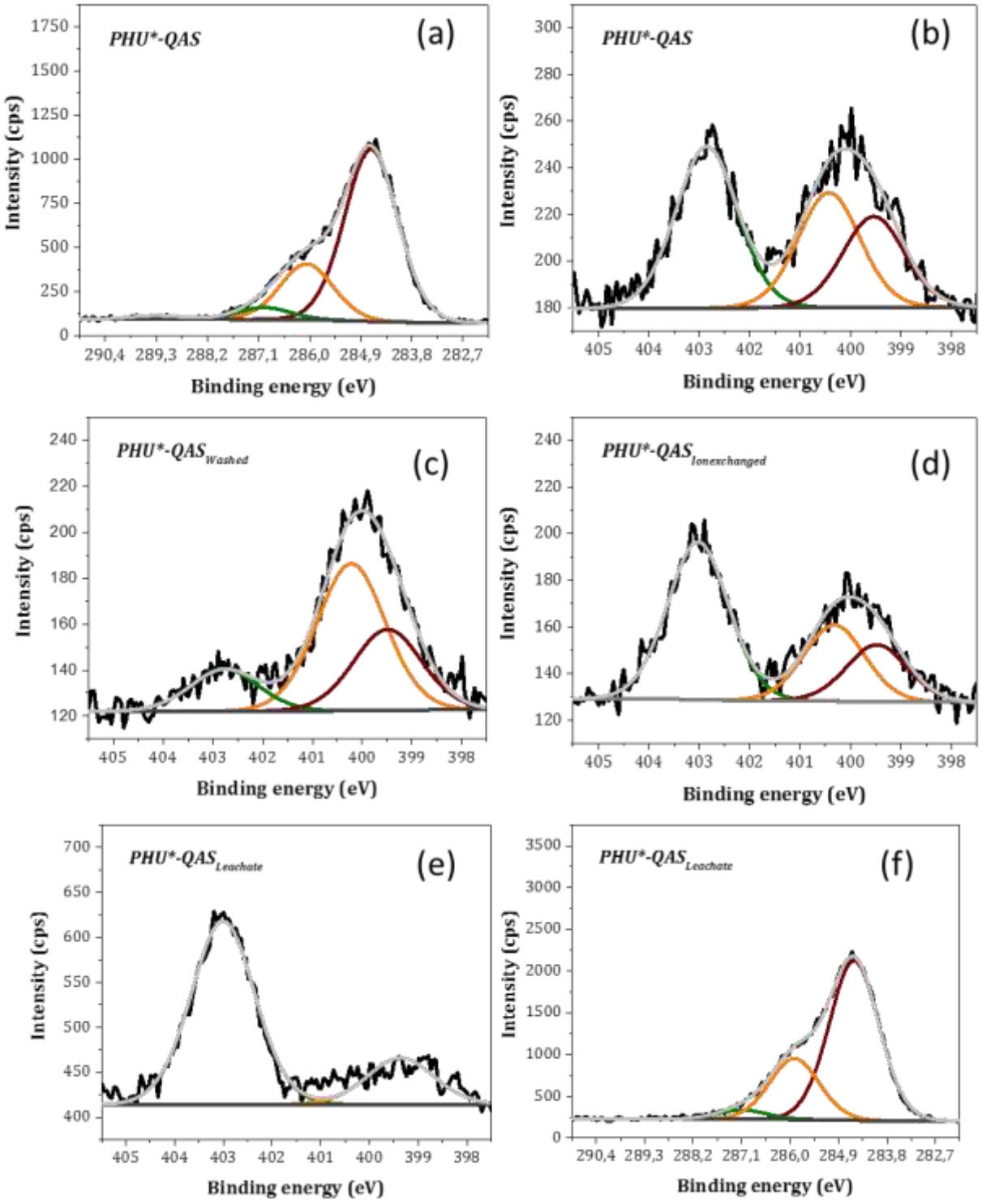


Figure 6. XPS narrow scans obtained for: **a)** C_{1s} for PHU*QAS coating; **b)** C_{1s} of PHU*QAS leachate solution; **c)** N_{1s} for PHU*QAS coating; **d)** N_{1s} for PHU*QAS washed coating; **e)** N_{1s} for PHU*QAS ion exchanged coating and **f)** N_{1s} of PHU*QAS leachate solution.

Table 1 displays chemical composition in each coating group in atomic percentages. When coatings were washed, the percentage of quaternary ammonium and the counter-ion (I^-) decreased on the surface of PHU-QAS and PHU*QAS. After

ion exchange (PHU-QAS ion exchange and PHU*QAS ion exchange) the percentage of quaternary ammonium remained similar to pristine samples and the counter-ion concentration decreased, indicating exchange of I^- for $(PO_4)^{3-}$. Leachate solution showed the highest percentages of quaternary ammonium and N^+/N ratio. The higher percentage of I^- in PHU*QAS leachate indicates a higher release of quaternary ammonium from PHU*QAS.

Table 1. Means and standard deviations (n=2, read in two different spots) of coatings surface atomic percentages of quaternary ammonium (N^+); amine nitrogens (N), ratio between quaternary ammonium and amine nitrogens (N^+/N), iodide (I), carbon (C), oxygen (O) and phosphorus (P) determined by XPS.

	N^+ (402.8 eV)	N (400.2 eV)	N^+/N	I	C	O	P
PHU-QAS	2.3±0.6	1.6±0.4	1.5±0.0	1.2±0.1	61.4±2.2	12.7±1.3	-
PHU*QAS	2.3±0.1	2.8±0.2	0.8±0.0	1.6±0.5	65.6±3.6	14.6±0.5	-
PHU-QAS washed	0.8±0.1	3.8±0.2	0.2±0.0	0.6±0.1	70.8±0.1	18.2±0.0	-
PHU*QAS washed	0.7±0.1	3.6±0.2	0.2±0.0	0.3±0.0	59.4±0.0	17.6±0.6	-
PHU-QAS ionexchange	2.8±0.1	1.6±0.1	1.8±0.1	0.3±0.0	68.6±0.8	19.1±0.5	0.0±0.0
PHU*QAS ionexchange	2.1±0.1	1.7±0.1	1.3±0.1	0.2±0.0	58.1±0.7	16.8±0.1	0.0±0.0
PHU-QAS Leachate	5.1±2.5	2.9±1.4	1.8±0.2	1.1±0.8	68.8±2.1	11.5±1.4	-
PHU*QAS Leachate	4.6±0.7	1.5±0.1	3.0±0.4	2.6±0.3	70.5±0.8	11.2±1.1	-

3.1.4 GIXS: PHU*QAS coatings (Figure 7 a-c) demonstrated a nanostructural order with their main diffraction planes preferentially aligned parallel to the coating surface. The 2D scattering patterns showed arc-shaped diffraction features with different degree of ordering for the pristine, washed in demineralized water or ion exchanged coatings. Pristine coatings had the strongest lamellar arrangement along the surface plane (Figure 7a) showing a strong 1st order signal along the q_z direction, together with a clear 2nd order diffraction spot indicating high ordering. The ratio between the 1st diffraction spot (q^*= 1.37 nm^{-1} - black line, Figure 7d) and the 2nd diffraction spot is 1:2 implying a layered arrangement of the sample nanostructure.[32]

After washing of PHU*QAS coating with demineralized water (Figure 7b), the scattered intensity was partially lost especially along q_z and the 2nd order diffraction signal disappeared. Washing with demineralized water led to a significantly more open structure (large shift of the q^* peak to smaller values). This is particularly more

evident for the PHU-QAS film where the ratio between the 1st order peak position in the pristine sample and washed is 1.56, versus only 1.06 for the PHU*QAS. This suggests that water diffusion into the coating result on loss of ordering.

The *q*-spacing for the 1st order diffraction spot of the washed sample was smaller (q^*= 1.29 nm^{-1} – blue line, Figure 7d) as compared to the pristine sample (q^*= 1.37 nm^{-1} - black line, Figure 7d) suggesting an increase in the average lamellar spacing ($d^*=2\cdot\pi/q^*$, Table 2). Despite the increased spacing, the structure remained layered-like as a weak 2nd order, which is visible in Figure 7b. Finally, the PHU*QAS ion exchanged coating (Figure 7c) exhibited intermediate order between the pristine and the washed sample: its scattering pattern demonstrated a strong ring-shaped signal that is more uniformly spread radially and also less pronounced along q_z compared to the pristine one (Figure 7a).

The ion exchange exerted weaker alteration of the q^* and its impact seems to be different for each polymeric structure once for PHU*QAS, the q^* value increased compared to pristine coating while for PHU-QAS it decreased. Moreover, the ion exchange seems to affect the order at q > 2 nm^{-1} with a weaker decay of I(q) vs. q profile as q increased. The ion exchanged sample exhibited intermediate value of the average global intensity between the pristine and the washed sample. Especially at q > 2 nm^{-1}, the ion exchanged sample showed the highest average global intensity, associated to an increase in contrast due to higher electron density of phosphate and higher trend to electrostatic cross-linking, i.e. self-assembling.

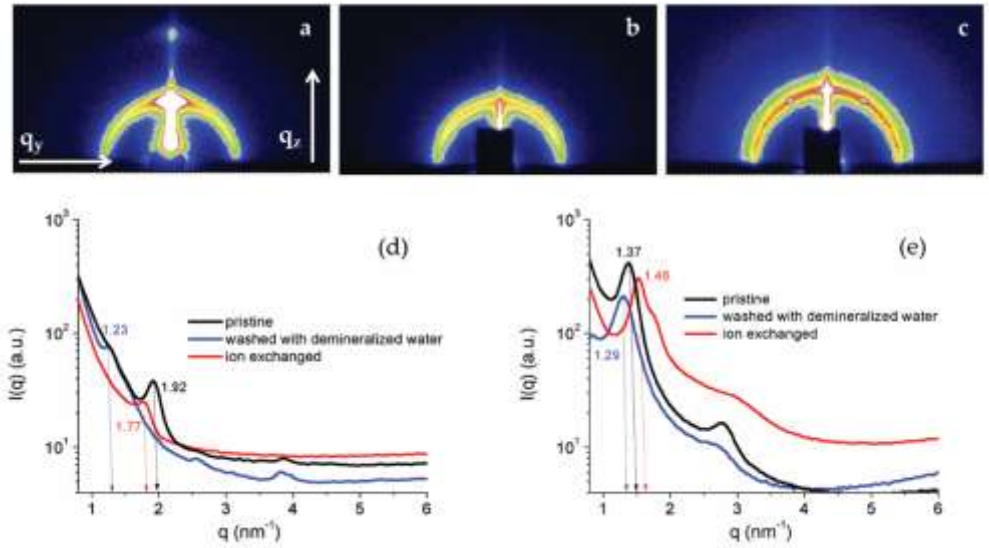


Figure 7. Two-dimensional GIXS patterns acquired utilizing an incident angle of α_i =0.3° for PHU*QAS films slot-die coated on Sodalime glass substrates. **(a)** PHU*QAS pristine coating; **(b)** PHU*QAS after washing with demineralized water and **(c)** PHU*QAS after ion exchange. The intensity scale was the same for all designated scattering patterns, spanning from 0 to 400 (a.u.). Panels **(d)** and **(e)** depict the 1D SAXS intensity curves, *I*(*q*), (a.u.) vs. wavevector *q,* in nm^{-1}, for the PHU*QAS and PHU-QAS coatings, respectively. The vertical arrows point to the peak positions *q** (values shown in each panel with color-matching numbers) related to the Bragg spacings *d** in the color-matching *I*(*q*) vs. *q* datasets.

Table 2. GIXS-resolved average lamellar spacings, d*(=2·π/q*), for the PHU-QAS and PHU*QAS coating nanostructure according to exposure to washing or ion exchange.

	Pristine	Washed	Ion exchanged
d* PHU-QAS (nm)	3.3	5.1	3.6
d* PHU*QAS (nm)	4.6	4.9	4.3

Similar behavior but with a weaker scattering intensity have been recorded for the PHU-QAS coatings (Figure 7e). Consequently, the azimuthal integrations of the scattered intensity expressed by 1-dimensional scattered intensity plots, I(q) vs. q, also show lower scattering intensity (weaker contrast) for the PHU-QAS than the PHU*QAS system. The qualitative effects of film perturbation by washing with respect to a pristine coating in the nanostructure are assured, regardless of the chemical structure of the polyhydroxyurethane used (PHU-QAS or PHU*QAS).

3.2 Antimicrobial activity of the coating

3.2.1 Antimicrobial activity of the leachate: The BHI medium in which the coatings were immersed was collected on day 1 and day 7 and used to determine

the antimicrobial activity against planktonic *S. mutans*. Figure 8 shows that THB medium containing the leachate from all the coatings was able to kill the planktonic *S. mutans* after 24 h. This led to a 8 log reduction in colony forming units as compared to the control. This killing ability remained the same for day 1 and day 7, even with daily THB renewal. Washing or ion exchange treatment did not exert changes on the antimicrobial activity of the coatings for both time points tested.

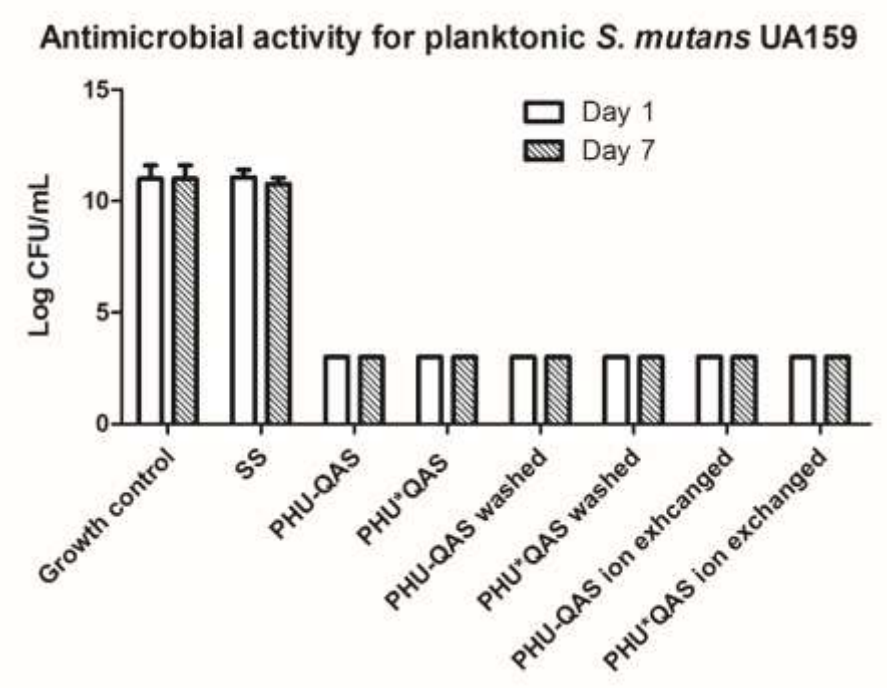


Figure 8. Antimicrobial activity against *S. mutans* UA159 of PHU-QAS and PHU*QAS leachate from pristine, washed and ion exchanged coatings collected on day 1 and day 7 compared to growth control in media and stainless steel leachate. Means and standard deviation of three different samples tested with three different inoculums of *S. mutans*. Planktonic growth presented in log CFU mL^{-1}.

3.2.2 Antimicrobial effect in contact-killing mode: Petrifilm® system was used to assess the contact-killing ability of all coatings and surface cationic charge density was measured for determining if the charge density was high enough to kill bacteria (Table 3). All coatings possessed a cationic charge density above $10^{14}/cm^2$, which led to complete killing of all *S. mutans* bacteria in contact with the coatings. The cationic charge density for stainless steel ($10^{12}/cm^2$) was two orders of magnitude lower and no contact-killing was observed. Despite the thorough washing in demineralized water, PHU-QAS washed and PHU*QAS washed coatings maintained their cationic charge density and contact-killing capability. Ion exchange increased the cationic charge density one log and did not affect the contact-killing

performance. For the samples stored in growth medium for 7 days their contact-killing capability was kept. The cationic charge density increased for PHU-QAS, PHU*QAS, PHU-QAS washed and PHU*QAS washed (Table 3).

Table 3. Means and standard deviation (n=3) of coatings cationic charge density/cm^2 by means of fluorescein staining and contact-killing capability using the Petrifilm® system before and after storage in media for 7 days.

Coating	Log cationic charge density/ cm^2	Contact-killing	Log cationic charge density/ cm^2 after 7 days in growth media	Contact-killing after 7 days in growth media
SS	12.3 ± 0.6	NO	11.1 ± 0.1	NO
PHU-QAS	14.6 ± 0.2	YES	15.4 ± 0.2	YES
PHU*QAS	14.7 ± 0.1	YES	15.8 ± 0.0	YES
PHU-QAS washed	14.0 ± 0.3	YES	15.3 ± 0.3	YES
PHU*QAS washed	14.7 ± 0.2	YES	15.7 ± 0.0	YES
PHU-QAS ion exchanged	15.7 ± 0.3	YES	15.9 ± 0.1	YES
PHU*QAS ion exchanged	16.0 ± 0.1	YES	15.9 ± 0.1	YES

3.3 Coating leachate biocompatibility

The Cell Proliferation Kit II (XTT) was used to assess the cytotoxicity of the coating leachate. The MEM medium in which the coatings remained for 1 and 7 days was used to measure the metabolic activity of fibroblasts L929 cells compared to fresh MEM medium and MEM which stored stainless steel slabs. Media extracted from stainless steel group on day 1 and day 7 presented 98.9% and 97.4% of cell viability, respectively (Table 4). Cells exposed to QAS coatings leachate from the 1st day exhibited a decreased metabolic activity of 3.0% and 5.1%. After 7 days of constant media renewal, the metabolic activity was 35.1% for PHU-QAS and 79.3% for PHU-QAS washed coatings, but did not show any increase for the PHU*QAS. Ion exchange though was not able to improve metabolic activity of L929 fibroblasts that remained low ranging from 5.6% to 6.4% even after 7 days of constant media refreshing for PHU-QAS ion exchanged and PHU*QAS ion exchanged coatings leachate.

Table 4. Mean metabolic activity and standard deviation (n=3) represented in percentage for fibroblasts L929 exposed to PHU-QAS and PHU*QAS leachates from pristine, washed or ion exchanged coatings collected on day 1 and day 7. The data was normalized to the control.

Group	Day 1	Day 7
CONTROL	100	100
Stainless steel	98.9±6.4	97.4 ± 7
PHU-QAS	4.1±0.1	35.1±0.7
PHU*QAS	4.4±0.1	3.4±0.1
PHU-QAS washed	3.0±0.1	79.3±1.7
PHU*QAS washed	3.8±0.1	3.9±0.1
PHU-QAS ion exchanged	5.1±0.1	5.6±0.1
PHU*QAS ion exchanged	3.8±0.1	6.4±0.1

4 DISCUSSION

Orthodontic patients have difficulties to follow the prescribed oral hygiene strategy which makes it difficult for the clinician to control risk for white spots and eventually caries. Therefore, devices that present antimicrobial features would benefit the orthodontic treatment and patient's oral health. Polyhydroxyurethanes synthesized from cyclocarbonates have proven to be suitable polymers for coating metallic substrates. They were produced without isocyanates or phosgene and are therewith environment-friendly materials. In this study it was possible to tether a quaternary ammonium compound, a prevailing antimicrobial substance, to a polyhyroxyurethane backbone and attest the antimicrobial activity of this material applied as a coating on stainless steel surfaces.

Nuclear magnetic resonance for ^{13}C confirmed cyclic carbonate conversion into PHU or PHU* and also pointed out the structural differences between PHU and PHU* molecules residing at the terminal ends. Both materials, PHU-QAS and PHU*QAS, applied as coatings had a self-assembling lamellar or layered-like ordering, confirmed by GIXS. Several works reported that this nanostructural arrangement can be obtained by acid catalyzed hydrolysis.[32,33] Both PHU-QAC and PHU*QAC demonstrated strong antimicrobial activity (Figure 8 and Table 3), but also high levels of cytotoxicity. Two treatments were used aiming to reduce this cytotoxic effect,

namely short-duration (2 h) ion exchange with sodium phosphate solution and long-duration (3 days) washing with demineralized water.

Ion exchange was expected to prevent or at least decrease release of QAS. The process did stabilize quaternary ammonium or smaller segments of polymer-QAS on the surface of the coatings as XPS analysis and GIXS confirmed probably by means of increasing ionic cross-links due to exchange of I^- ions for multivalent $(PO_4)^{3-}$ ions. XPS showed a very low or no P signal. It turns out that the X-ray photoionization cross section for the I 3d3/2 and I 3d5/2 peaks are 13.77 and 19.87 barns, respectively, while the P 2p3/2 and P 2p1/2 are 0.403 and 0.789 so the sensitivity is much higher for the I^- than for $(PO_4)^{3-}$. [34] Also, we hypothesize that the QAS present in the bulk of the coating forms a double electric layer when in contact with the surrounding fluid, due to concentration gradients, forming an ionic pair with $(PO_4)^{3-}$ and stabilizing the system. With this, the rate of $(PO_4)^{3-}$ uptake to the coating already decreases. Also, as 3 I^- are replaced for 1 $(PO_4)^{3-}$, the concentration of $(PO_4)^{3-}$ is expected to be even lower than that of I^-. Possibly a longer period of ion exchange, a higher phosphate concentration or a different anion could better stabilize the QAS inside the coating and could decrease the cytotoxicity much further without affecting the antimicrobial activity. The disruption of lamellar arrangement for ion exchanged coatings, observed with GIXS (Figure 7 and Table 2) suggests though an increase in ionic cross-links. Leaching was not completely prevented, because the leachates were still cytotoxic, according to XTT results (Table 4).

Washing in demineralized water was expected to leach out all loosely bound quaternary ammonium and lower the cytotoxicity.[8] The procedure did wash out quaternary ammonium groups, from both coatings PHU-QAS and PHU*QAS, as XPS analysis showed (Table 1). It has been hypothesized that siloxane bonds could be hydrolyzed when in presence of water which could explain the quaternary ammonium detachment from the pohyhydroxyurethane when coatings are washed.[35] Washing in demineralized water decreased cytotoxicity for fibroblast L929 of PHU-QAS washed coating on the 7th day, shown by a metabolic activity of 80% without loss of antimicrobial activity. After washing in demineralized water, QAS coatings continued to release quaternary ammonium and exert antimicrobial activity for 7 days. Planktonic killing was shown for *S. mutans* on the 1st and 7th day which was also observed in other studies.[16,25,36] For the PHU*QAS coating after washing still a small

inhibition zone was observed and therewith only contact-killing could not be confirmed (data not shown). The PHU-QAS washed did not show any inhibition zone and therewith the contact-killing is confirmed.[26]

Differences in cytotoxicity between the PHU-QAS and PHU*QAS could reside on the higher affinity of PHU* for water molecules. PHU* contains an extra nitrogen atom originating from TMSPE silane (see Figure 1a) which could explain the affinity for water molecules. The ratio between dispersive component and the polar component of PHU* is almost 2 times smaller than that of PHU making the former more prone to water interactions than the later.[29] The greater amount of hydrolysis of siloxane bonds,[35,37] detaching quaternary ammonium from PHU* could cause higher cytotoxicity still after washing and 7 days release in growth media.

Many commercialized dental materials are cytotoxic,[38] due to incomplete polymerization and release of monomer. Methacryloyloxydodecylpyridinium bromide (MDPB), an antimicrobial monomer was extensively investigated for insertion in dental adhesive systems. MDPB was pointed out to be as toxic as triethyleneglycol dimethacrylate (TEGDMA) and less toxic than bisphenol A-glycidyl methacrylate (Bis-GMA) monomers found in many formulations of restorative composite resin for dental filling. MDPB and TEGDMA at concentrations up to 50 µg/mL did not inhibit mitochondrial dehydrogenase activity on MTT tests with MDPC-23 cell type whilst Bis-GMA inhibited already at 10 µg/mL.[38–40] Minimum bactericidal concentration though for MDPB against a wide variety of oral bacteria ranged from 8.25 µg/mL to 62.5 µg/mL.[38]

The cytotoxicity acceptance level is dependent on the application of the material and the ratio between surface area and elution fluid.[41] Since the coatings developed in this paper aim metallic brackets and wires coverage, considerable elution in saliva is expected. Secretion of at least 1000 mL of saliva on a daily basis will elute the toxic compounds to a reasonable level in the oral cavity of healthy individuals.[42] Experiments in this study were conducted with 5 cm^2 coated surface area in a small volume of growth media (3 mL) refreshed every 24 h. A previous report [41] used 2 cm^2 sample eluted in 100 mL volume of growth media, refreshed every 24 h, meaning 80 times more diluted than in our study. These authors observed that the 1st day of eluted growth medium showed toxic effects on fibroblasts. Only eluted medium from days 4 and 6 were considered comparable to control growth media

which corroborates for the 7th day findings of the present study. Since we used a 80 times higher area/volume ratio we expect that adjusting elution could make PHU-QAS washed suitable to use as a coating on orthodontic appliances.

Conclusions

The stainless-steel surface was successfully coated with two different types of polyhydroxyurethanes (PHU and PHU*) coupled with quaternary ammonium salt (QAS). Both coatings showed contact-killing and planktonic antimicrobial activity against cariogenic *S. mutans* up to 7 days tested. Both ion exchange and washing in demineralized water changed the coating nanostructure without diminishing antimicrobial efficiency. Detached QAS from the PHU and PHU* backbone leached out of the coating to give rise to planktonic antimicrobial activity and cytotoxicity. PHU-QAS coating when washed in demineralized water is suitable for orthodontic application aiming biofilm prevention.

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3 DISCUSSÃO

O acúmulo de biofilme ao redor de aparelhos ortodônticos é uma situação bastante prevalente dada à dificuldade de higienização dos aparatos e à falta de colaboração por parte dos pacientes (Cozzani et al. 2016; Gao et al. 2014; Jönsson et al. 2006; Novaes-Júnior and Novaes 1999). Neste contexto, o desenvolvimento de materiais com potencial antimicrobiano que possam auxiliar na prevenção da formação de biofilme durante o tratamento ortodôntico é oportuno.

QAC são substâncias antimicrobianas consideradas efetivas contra a espécie cariogênica *S. mutans* (Antonucci et al. 2012; Gong et al. 2012; Gong et al. 2013; Sugii et al. 2017). A revisão de literatura apresentada sobre o uso de QAC em aparatos médicos e materiais odontológicos disserta sobre as variáveis que influenciam o potencial antimicrobiano destes compostos como: a presença de cadeias alquílicas, o tipo de polímero ao qual estão associados, os métodos obtenção e a controversa função do contra-íon. Esta revisão elucida fatores determinantes para a escolha da molécula de QAC com uma cadeia alquílica de 12 carbonos a ser aplicada em um material de revestimento de polihidroxiuretana no estudo *in vitro* apresentado no capítulo subsequente.

No estudo *in vitro*, duas moléculas de polihidroxiuretanas distintas foram sintetizadas – PHU e PHU* - e modificadas com QAS para fins de revestimento de superfície de aço inoxidável em forma de filmes. A molécula de QAS foi acoplada à PHU ou PHU* por meio de dois diferentes silanos resultando em materiais de revestimento com diferentes características. Para ambos os filmes de revestimento testados observou-se atividade antimicrobiana contra *S. mutans* e efeito citotóxico para fibroblastos devido à liberação de QAS nos dois tempos avaliados (dia 1 e dia 7).

Dois processos de tratamento pós-confecção dos filmes de revestimento foram testados: lavagem em água demineralizada por 3 dias ou troca iônica em solução de fosfato de sódio por 2 h. O processo de lavagem do revestimento PHU-QAS em água demineralizada resultou em diminuição da citotoxicidade do material sem prejuízos ao potencial antimicrobiano. O processo de troca iônica porém, apresentou apenas modificações químicas e nanoestruturais nos filmes de revestimento, não sendo capaz de diminuir o efeito citotóxico. Neste sentido, acredita-se que o aumento da concentração da solução de fosfato de sódio ou do tempo de imersão

na solução poderia aumentar a difusão de PO_4^{3-} aumentando a fixação de QAS no material de revestimento.

Assegurar mais ligações covalentes entre QAS e PHU/PHU* através de condições de síntese totalmente livres de umidade ou ainda a ligação de QAS a polihidroxiuretanas através das ligações uretânicas (mais estáveis que ligações siloxânicas) poderiam diminuir as taxas de liberação de QAS e por conseguinte diminuir a citotoxicidade do material de revestimento.

Outro aspecto importante para se avaliar o fator citotoxicidade *in vitro* é a relação área superficial de amostra/volume do meio de diluição. Em situações *in vivo*, na cavidade bucal onde existe fluxo salivar contínuo, a quantidade de QAS lixiviada poderia ser constantemente diluída podendo-se esperar uma diminuição da resposta de citotoxicidade local (Yue et al. 2015). Para isso, estudos de espectroscopia de massa para determinação da quantidade de QAS liberada devem ser realizados. Uma vez determinada a quantidade de QAS liberada pode-se proceder para um estudo de citotoxicidade considerando uma série de concentrações.

Uma vez que pacientes sob tratamento ortodôntico sofrem alterações da microbiota bucal, com aumento das espécies estreptocócicas (Rosenbloom and Tinanoff 1991) o estudo apresentado avaliou o potencial antimicrobiano apenas para a espécie cariogênica *S. mutans*. Para que o composto desenvolvido possa ser usado em outras aplicações odontológicas é importante que se ateste a eficácia contra outras espécies bacterianas e/ou fúngicas.

Superfícies inseriadas na cavidade bucal são prontamente embebidas em saliva, fluido composto por proteínas, enzimas, imunoglobulinas, mucinas e eletrólitos (Humphrey and Williamson 2001). Dado este fato, alguns estudos investigaram a influência da película salivar e constataram que esta não inibiu o potencial antimicrobiano do QAS (Gong et al. 2012; Gong et al. 2013; van de Lagemaat et al. 2017; Yue et al. 2015). Dado o caráter inovador do estudo experimental apresentado, em que novos materiais foram desenvolvidos, optou-se pela avaliação do potencial antimicrobiano diretamente sobre a superfície filme de revestimento ou do QAS lixiviado em meio de cultura.

Observou-se que a adição de QAS em materiais de uso odontológico é viável e pode trazer melhorias na manutenção da saúde bucal. Futuramente diferentes áreas da odontologia poderiam se beneficiar do uso de QAS. Porém, aspectos como a citotoxicidade e ação antimicrobiana contra outros microrganismos e influências da presença de saliva devem ser abordados.

4 CONCLUSÃO

Os filmes de revestimento sintetizados PHU-QAS e PHU*QAS demonstraram efeito antimicrobiano eficaz contra *S. mutans* de até 7 dias. O efeito citotóxico de PHU-QAS pode ser minimizado através do simples processo de lavagem em água demineralizada.

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APÊNDICE 1 – DETALHAMENTO DO PROCESSO DE SÍNTESE E REAGENTES UTILIZADOS

Síntese de Polihidroxiuretanas com sal de amônio quaternário (PHU-QAS)

Quadro 1 - Reagentes utilizados, pureza e número de registro do banco de dados do “Chemical Abstract Service” (CAS) correspondentes.

Reagente	Sigla	Pureza	CAS
N,N-Dimetildodecilamina	DMDA	97%	112-18-5
(3-Iodopropil)Trimetoxissilano	IPTMS	≥95%	14867
N,N-Dimethylformamida	DMF	99,8%	68-12-2
Tetraetilortossilicato	TEOS	98%	78-10-4
5 - Amino - 1,3,3 - trimetilciclohexanometilamina	IPDA	99%	2855-13-2
(3- Aminopropil)triethoxissilano	APTES	99%	919-30-2
Poly(dimethylsiloxane)	EPDMS	-	130167-23-6
2 Ethoxyethanol	EE	99%	110-80-5

1) Síntese de bis(ciclocarbonato) derivado de Poli(dimetilsiloxano) - CCPDMS

Foram utilizados para esta etapa: poli(dimetilsiloxano)diglicidil éter (PDMS, 0,99 g/mL a 25 °C), hexadeciltrimetilamônio (HTMA, 96%, Fluka) e 2-etóxietanol (EE, 99%). Todos os reagentes foram utilizados da forma como são comercializados por Sigma Aldrich, sem tratamento prévio.

Em um reator foram misturados 19,6 g de PDMS, 0,588 g de HTMA e 10 mL de EE. A reação ocorrerá em atmosfera de dióxido de carbono (CO^2) 99,99% em reator Parr tipo autoclave modelo 2192HC4 (Parr, Illinois, EUA) (Figura 1) com as seguintes condições de operação: pressão de 1 MPa, temperatura de 100 °C e agitação de 300 rpm.

Figura 1 – Reator Parr para síntese com cicloadição de CO_2.

2) Síntese PHU

Para a síntese de PHU, foram utilizados: CCPDMS sintetizado como descrito anteriormente; 5-amino-1,3,3-trimetilciclohexano metilamina (IPDA, nome comercial isoforeno diamina, >99%, mistura de cis/trans, Sigma Aldrich) e 3-(aminopropil)trietóxisilano (APTES, >98%, Sigma Aldrich).

Em uma primeira etapa reacional, foram misturados 0,375 mmol de CCPDMS com 0,19 mmol de IPDA sob agitação a 50 °C. Na segunda etapa (finalização da reação), foram adicionados 0,75 mmol de APTES ao sistema.

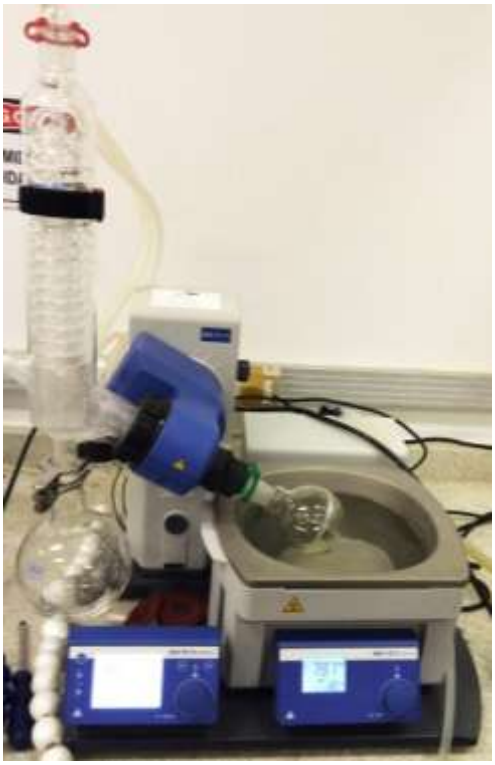
3) Síntese do sal de amônio quaternário (QAS)

A síntese sal de amônio quaternário (QAS) foi realizada em uma proporção molar de 1,5 mmol IPTMS : 1,5 mmol DMDA. O IPTMS e DMDA foram reagidos em balão de fundo redondo em solvente DMF, por 24 horas, protegidos da luz e sob refluxo constante (Figura 2).

Figura 2 - Balão reacional conectado ao condensador compondo sistema de refluxo.

Ao término da síntese, o solvente foi removido em Rota Evaporador Ika modelo RV 10 Digital e em estufa a 80 °C por 12 horas (Figura 3). O produto final foi diluído em etanol.

Figura 3 – Rota Evaporador Ika



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Title: "Quaternary Ammonium Containing-Isocynate Free Polyhydroxyurethane Coatings to Prevent Bacterial Colonization on Orthodontic Devices"
Author(s): Sugii, Mari; Holanda, Carlos; Vagias, Apostolos; Portale, Giuseppe; Imasato, Hidetake; Van der Mei, Henny; Rodrigues-Filho, Ubirajara; Aguiar, Flavio; Sharma, Prashant
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Dear Dr. Sharma:

Your manuscript cm-2019-01421z entitled "Quaternary Ammonium Containing-Isocynate Free Polyhydroxyurethane Coatings to Prevent Bacterial Colonization on Orthodontic Devices" has been assigned to the following editor:

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