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Effects of water aging on the mechanical and anti-biofilm properties of glass-ionomer cement containing dimethylaminododecyl methacrylate

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ABSTRACT

Objectives. The aims of this study were to investigate the effects of water aging for up to 6 months on the mechanical and anti-biofilm properties of a novel antibacterial glass ionomer cement (GIC) containing dimethylaminododecyl methacrylate (DMADDM).

Methods. GIC specimens (n = 180) which contained DMADDM (0 wt.%, 1.1 wt.% or 2.2 wt.%) were prepared. The mechanical properties surface roughness, microhardness and the surface charge density of ammonium groups were measured before and after water aging for 3 and 6 months at 37 °C. Further six months aged specimens (n = 216) were worn by 6 volunteers in their oral cavities for 24 h and 72 h. Biofilm formation was analyzed and rated by fluorescence microscopy (FM) and by scanning electron microscopy (SEM). Biofilm viability was analyzed by FM.

Results. Water aging did not show any adverse effects on the surface roughness and hardness of the material. The surface charge density of the GIC samples containing DMADDM decreased due to the aging procedure, however, was still higher than that of the GIC without DMADDM. *In situ* biofilm formation was significantly reduced after 24 h on DMADDM containing GIC ($p < 0.05$). FM results showed a higher ratio of red/green fluorescence on GIC-DMADDM samples.

Significance. Incorporating DMADDM into GIC affected the material properties in a tolerable manner even after 6 months of storage in water. The new GIC is a promising material to affect the biofilm formation on the surface of restorations.

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1. Introduction

Glass ionomer cement (GIC) is the mainstream bioactive restorative material and is the first choice for the atraumatic restorative therapy (ART), as it adheres chemically to the tooth structure and releases fluoride. Hence it not only contributes to the reduction in the amount of residual bacteria underneath the restoration, but also favors remineralization of the affected dentin [1,2]. Unlike light activation, GIC is chemically activated and requires no special appliances. Meanwhile, it also provides biocompatibility, less volumetric contraction and a coefficient of thermal expansion similar to that of tooth structure [3]. The advantages of GICs over other direct restoratives ensure their versatility and hence they remain an important material type available to dentists.

Clinical surveys showed that, similar to composites, the most common reason for failure of GIC was secondary caries [4,5], indicating that fluoride release is not sufficient enough to inhibit growth of bacteria and to combat bacterial destruction of tooth substances [6,7]. Therefore, a GIC which offers a genuinely antimicrobial and antibiofilm efficacy would be of considerable clinical benefit [8]. Such a material could reduce recurrent decay in the vicinity of a restoration and could provide an antibacterial seal under other materials, protecting the pulp from bacterial ingress.

Several efforts have been undertaken to develop antibacterial GICs that could kill bacteria and reduce or avoid the formation of biofilms [9–12]. In a former study [13], a novel antimicrobial quaternary ammonium monomer dimethylaminododecyl methacrylate (DMADDM) was synthesized and incorporated into a GIC, which showed strong antibacterial and antibiofilm effects *in situ*. However, the long-term antibacterial effects of GIC containing DMADDM have not been investigated, and the mechanical properties of GIC containing DMADDM after long-term water aging need to be determined. Hence, the objectives of the present study were to determine if the addition of DMADDM would influence the mechanical properties of GIC and to investigate the effects of water aging on the mechanical and anti-biofilm properties of GIC containing DMADDM. It was hypothesized that: (1) the GICs containing DMADDM have similar mechanical properties compared with the parent material, (2) the long-term water aging procedures had no adverse effects on the mechanical properties of GIC containing DMADDM, (3) the new materials would affect biofilm formation after 6 months of water aging.

2. Materials and methods

2.1. Specimen preparation

Dimethylaminododecyl methacrylate (DMADDM) was synthesized via a modified Menschutkin reaction method. Briefly, 10 mmol of 1-(dimethylamino)docecane (DMAD) (Tokyo Chemical Industry, Tokyo, Japan) and 10 mmol of 2-bromoethyl methacrylate (BEMA) (Monomer-Polymer and Dajac Labs, Trevose, PA) were added in a 20 mL vial with a magnetic stir bar. The vial was capped and stirred at 70 °C for 24 h. After the reaction was complete, the ethanol solvent was removed via evaporation, yielding DMADDM as a clear, colorless, and viscous liquid. The reaction and product of DMADDM were verified via Fourier transform infrared spectroscopy (FTIR, Nicolet 6700, Thermo Scientific, Waltham, MA) as reported in a previous study [14].

The glass ionomer cement chosen for the current study was a conventional GIC (Fuji IX GP, GC Corporation, Tokyo, Japan). The parent material was modified by adding 5% or 10% DMADDM (w/w) to the liquid of the GIC while keeping the original powder/liquid ratio of 3.6 g: 1.0 g, thus achieving finial mass fractions of 1.1 wt.% and 2.2 wt.% DMADDM in GIC. GIC without DMADDM (0 wt.%) served as control. Specimens with nominal dimensions of 5 mm in diameter and 1 mm thickness were produced by mixing the GIC according to the manufacturers' instructions and packing into silicon molds covered by a mylar strip and glass plate under hand pressure. The mixing was carried out by one individual with extensive experience in GIC handling. Specimens were removed from the molds and coated with a thin layer of adhesive. They were placed in a chamber at 37 °C for 1 day that contained wet tissue paper not in direct contact with the specimens, to achieve an atmosphere of 100% humidity but prevent the specimens being in contact with water which could result in dissolution during the critical early phases of setting [15,16]. After this, the specimens were polished by wet SiC paper (grit size 2500) at 300 rpm (Phoenix 3000, Buchler, Braunschweig, Germany) and disinfected in ethanol (70%) for 30 min and subsequently washed several times in distilled water.

2.2. Water-aging procedures

Specimens were stored in sterile deionized (DI) water at 37 °C for 3 months and 6 months. For the immersion specimens, each group of six specimens of the same material was immersed in 100 mL of water in a sealed glass reagent bottle. The water was changed once every week.

2.3. Surface characterization

2.3.1. Surface roughness

Quantitative three-dimensional topographical analysis was performed on polished specimens using a white light interferometer (MicroProf WLI, FRT, Germany). Each specimen was individually fixed in a clamping apparatus and characterized by the roughness parameter Ra which describes the average surface roughness. The results were obtained by employing a scanning interferometry technique, a scan length of 20 μm at working distance of 5 mm. Each measurement was performed within a field-of-view of 90 μm × 90 μm. Five equidistant locations were measured on each disc, starting from its center and moving towards its periphery. Each experimental group comprised of six samples (non-aged, three or six months aged, 0 wt.%, 1.1 wt.% or 2.2 wt.% DMADDM: n = 54).

2.3.2. Surface microhardness

The microhardness test was performed with a Vickers hardness testing machine (Duramin, Struers, Denmark) with a 0.98 N load applied through the indenter and a loading time of 10 s. Five indentations were made on each sample disc; in its center and in four other locations, so that uneven cur-

ing deviations could be detected and taken into account. Each experimental group comprised six samples (non-aged, three or six months aged, 0 wt.%, 1.1 wt.% or 2.2 wt.% DMADDM: n = 54). A mean value was recorded as Vickers hardness number (VHN).

2.3.3. *Surface charge density*

The charge density of quaternary ammonium groups present on the GIC specimen's surfaces was quantified using a fluorescein dye method [17–19]. Samples were placed in a 48-well plate. Fluorescein sodium salt (200 μL of 10 mg/mL) in DI water was added into each well, and specimens were left for 10 min at room temperature in the dark. After removing the fluorescein solution and rinsing extensively with DI water, each sample was placed in a new well, and 200 μL of 0.1% (by mass) of cetyltrimethylammonium chloride (CTMAC) in DI water was added. Samples were shaken for 20 min at room temperature in the dark to desorb the unbound dye. The CTMAC solution was supplemented with 10% (by volume) of 100 mM phosphate buffer at pH 8.0. Samples were taken out and absorbance was read at 501 nm using a plate reader (Infinite® M200, Tecan, Switzerland). The fluorescein concentration was calculated using Beers Law and the molar extinction coefficient of 7.7×10^4 L mol^{-1} cm^{-1}. Using a ratio of 1:1 for fluorescein molecules to the accessible quaternary ammonium groups, the surface charge density was calculated as the total molecules of charge per exposed surface area (sum of top, bottom and side edge area, measured independently for each GIC disk due to slight variations in disk diameters). Six replicates were tested for each group (non-aged, three or six months aged, six months aged and polished, 0 wt.%, 1.1 wt.% or 2.2 wt.% DMADDM: n = 72).

2.4. *In situ antibiofilm model*

2.4.1. *Study design and subjects*

Biofilms were formed intra-orally on a total of 216 GIC specimens that were water aged for 6 months in a prospective, volunteer blinded *in situ* study. The study protocol was approved by the ethical committee of the Saarland Medical Association (vote number: 193/08). Six healthy volunteers were involved after signing an informed consent form. Inclusion criteria were: full dentition, sufficient compliance, no periodontal or restorative treatment needs, no local or systemic hypersensitivity to the materials used (splints, silicone impression material, resin composite, antimicrobial agent), no systemic disease(s), no pregnancy, no smokers and, no antibiotic treatment in the last six months. The volunteers received detailed information on the handling of the intraoral splints containing the specimens (see below).

2.4.2. *In situ formation of oral biofilms*

Alginate impressions (Blueprint cremix®, Dentsply DeTrey, Konstanz, Germany) were taken from the upper jaw of the six volunteers. Transparent custom made acrylic splints (Thermoforming foils®, Erkodent, Pfalzgrafenweiler, Germany) were fabricated as carriers of the GIC specimens. Six samples were fixed in the left and right buccal position in the molar and premolar regions with silicon impression material (President light body®, Coltène, Altstaetten, Switzerland) onto the splints [20]. The splints were exposed intraorally for 24 h and 72 h, respectively. During meals or for tooth brushing, splints were removed and stored in a wet chamber. Tooth brushing was performed twice daily just using tap water without tooth pastes. Neither additional cleaning procedures nor agents for chemical plaque control were applied. Splints with fixed specimens were not subjected to any cleaning measures. Volunteers were advised to maintain their normal eating habits.

After intraoral exposure, specimens were rinsed for 10 s with sterile NaCl-solution (0.9%) and processed immediately for fluorescence microscopy or scanning electron microscopy analysis.

2.5. *Vital fluorescence microscopy (FM)*

Biofilms coverage as well as the viability of the biofilms on 6 months aged specimens was assessed by fluorescence microscopy in triplicates (24 h or 72 h oral exposure, 0 wt.%, 1.1 wt.% or 2.2 wt.% DMADDM, six volunteers: n = 108). The biofilms on the GIC specimens were stained using a live/dead staining kit (BacLight® Bacterial Viability Kit L7012, Molecular Probes, Carlsbad, USA). The live/dead stain was prepared by diluting 1 μL of SYTO 9 (green; living bacteria) and 1 μL of propidium iodide (red, dead bacteria) in 1 mL of distilled water. Specimens were placed in 24-well plates and 100 μL of the reagent mixture were added to each well followed by incubation at room temperature in the dark for 15 min.

Each specimen was carefully positioned on a glass slide covered with mounting oil. Samples were evaluated under a reverse light fluorescence microscope (Axio Scope, Carl Zeiss AG, Oberkochen, Germany) in combination with the image processing software AxioVision 4.8 (Carl Zeiss Microimaging GmbH, Goettingen, Germany). One reading of biofilms on each quadrant and center area per specimen (magnification 1000 ×, oil immersion) were carried out (=5 FM-micrographs per specimen). Green and red FM-micrographs of the same section of the specimen were recorded separately and assembled hereafter using the AxioVision software.

ImageJ 1.48 [National Institutes of Health (NIH), Bethesda, MD, USA; freeware from http://rsb.info.nih.gov/ij/] was used to quantify the coverage area and the viability of the biofilms. The images for each color channel were assembled into image stacks. Total fluorescence area of each section was calculated as biofilm coverage. The images of green/red channel were calculated separately.

For each specimen, the biofilm coverage was assessed using the biofilim formation scores. For the assessment of biofilm vitality a 5-step scoring system was used regarding ratios between red and green fluorescences. These scoring systems were developed based on the experience of a previous study [21].

2.6. *SEM-evaluation*

The 6 months aged specimens were fixed in a solution containing 2% glutaraldehyde and 0.1 M cacodylate buffer for 2 h at 4 °C. This was followed by washing in 0.1 M cacodylate buffer, and dehydration in an ascending series of 50–100% ethanol. After drying in 1, 1, 1, 3, 3, 3-hexamethyldisilazan, the samples were sputtered with carbon. SEM analysis was carried

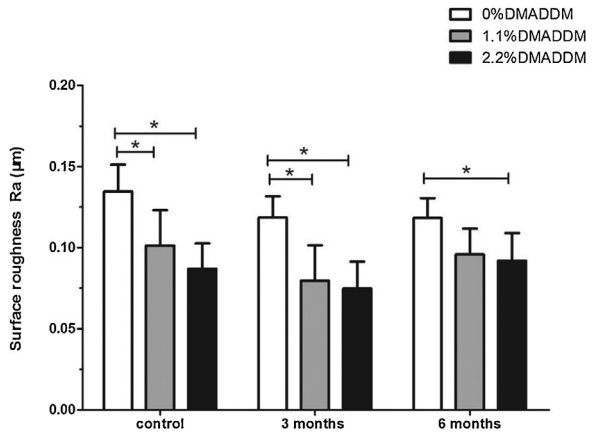

Fig. 1 – Surface roughness of GIC containing different mass fractions of DMADDM after different aging time. Each values is mean ± sd (n = 6) (*p < 0.05).

out using a FEI XL30 ESEM FEG (FEI Company, Eindhoven, NL). Analogous to FM, the biofilm coverage and its structure was also assessed using the biofilim formation scores [21]. Experiments were conducted 3-fold (24 h or 72 h oral exposure, 0 wt.%, 1.1 wt.% or 2.2 wt.% DMADDM, six volunteers: n = 108).

2.7. *Statistical analysis*

The collected data were statistically analyzed using the software SPSS (release 19, SPSS Inc., Chicago, IL, USA). In order to find performance differences in materials before and after aging, one way and two way analysis of variance (ANOVA) followed by Tukey's multiple comparison test were performed. The Kruskal-Wallis test and the Dunn's Multiple comparison test were used to test the influence of the DMADDM concentration on biofilm scoring. Median values and interquartile ranges (25–75th percentiles) of biofilm formation and viability were calculated. All statistical analyes were carried out at a significance level of 0.05.

3. Results

3.1. *Surface roughness*

Fig. 1 reports the quantitative topographical analysis of the experimental cements. The surface roughness of GIC containing DMADDM decreased compared to GIC without DMADDM ($p < 0.05$). There were no significant differences between groups of 1.1 wt.% and 2.2 wt.% DMADDM samples ($p > 0.05$). Even after 6 months of water aging, 2.2 wt.% DMADDM surface roughness decreased significantly comparing to 0 wt.% DMADDM ($p < 0.05$). Notably, it was found that there was no significant difference of Ra values between non-aged controls and aged specimens in each concentration group ($p > 0.05$).

3.2. *Surface microhardness*

Fig. 2 shows typical indentations on GIC and GIC containing DMADDM following the Vickers micro-hardness test. The micrographs indicate an increase in diagonal length in accordance with the increase of DMADDM concentrations. Moreover, no cracks from the diagonals of the indentation were observed. 6 months aged samples showed no obvious differences from control groups.

The mean microhardness values were found to decrease accordingly to the increase of DMADDM concentrations (Fig. 3). GIC without DMADDM showed the highest hardness values. There were no significant differences between 1.1 wt.% and 2.2 wt.% DMADDM groups ($p > 0.05$). Compared with non-aged controls, 3 and 6 months aged samples showed no significant differences of hardness values for each DMADDM concentration ($p > 0.05$).

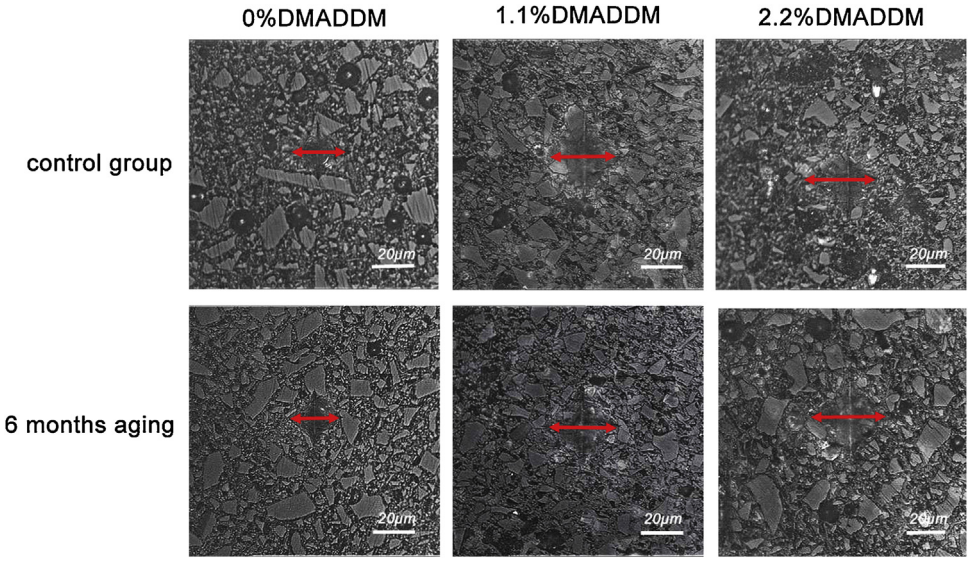

Fig. 2 – Micrograph of indentations on GIC and GIC containing DMADDM showing impressions of diamond indenter. The red arrow indicates the length of the indentation. Bar scale = 20 µm.

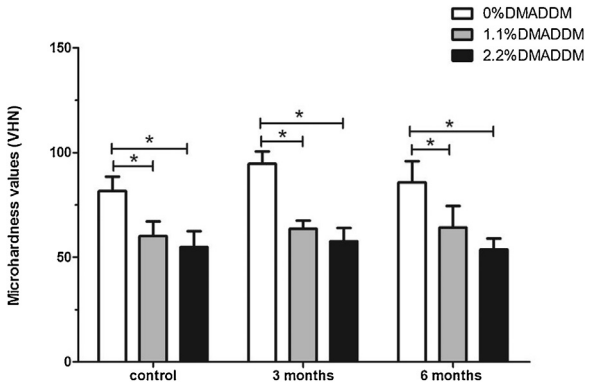

Fig. 3 – Microhardness values of GIC containing different mass fractions of DMADDM after different aging time. Each values is mean ± sd (n = 6) (*p < 0.05).

3.3. *Surface charge density*

The surface charge density values of GIC containing DMADDM are plotted in Fig. 4. (1) In general, fluorescein binding to the cationic quaternary groups revealed statistically significant increases in the quaternary ammonium sites present on the surfaces of the specimens with increasing DMADDM concentrations ($p < 0.05$). Specimens with 2.2 wt.% DMADDM had approximately 3 times more quaternary ammonium sites present on the surfaces compared with the 0 wt.% DMADDM samples for non-aged control group. (2) After 3 and 6 months of water aging, the samples surface charge density showed a trend that increased values are in accordance with increasing DMADDM concentrations. Specimens with 2.2 wt.% DMADDM in aged groups showed statistically significant higher values compared with samples of the 0 wt.% DMADDM group ($p < 0.05$). (3) For samples with 0 wt.% DMADDM no significant differences between non-aged controls and aged samples were measured. (4) Compared with non-aged control samples, aged DMADDM-GIC samples showed lower surface charge densities ($p < 0.05$). Six months aged specimens were polished again and about 0.1 mm surface were removed. It was found that the charge densities of polished DMADDM containing specimens increased when compared with non-polished samples. Compared with non-aged controls, polished specimens showed no significant differences ($p > 0.05$, Fig. 4).

3.4. *In situ formation of oral biofilms*

The FM images showed in general higher ratios of dead (red) to living (green) bacteria on samples containing 1.1 wt.% or 2.2 wt.% DMADDM in comparison with control samples without DMADDM (Fig. 5). The Kruskal–Wallis test revealed a significant influence of DMADDM added to the GIC on biofilm viability for intraoral exposure (24 h and 72 h) on 6 months aged samples ($p < 0.05$, Fig. 6). The scores for biofilm viability (Fig. 6a and c) were significantly lower in the groups of DMADDM containing samples compared to the controls (Dunn's test: $p < 0.05$). The biofilm formation scores were significantly lower in DMADDM containing GIC samples for 24 h aged specimens (Dunn's test: $p < 0.05$, Fig. 6b), while there were no differences in scores between DMADDM containing GIC specimens and controls for 72 h aged samples (Dunn's test: $p > 0.05$, Fig. 6d).

The results obtained from SEM confirmed the FM results. The SEM images of 6 months aged samples after intraoral exposure are shown in Fig. 7(A–F). After 24 h intraoral exposure, the group with 1.1 wt.% DMADDM had less microbial colonization and more damaged bacteria with dispersed extracellular matrix structure. On 2.2 wt.% DMADDM specimens, less microbial colonization was apparent and the GIC surface pattern was visible (Fig. 7B and C). After 72 h, multiple microbial aggregations were detectable on the GIC-DMADDM samples, however, more damaged bacteria were seen compared to controls (Fig. 7E and F). The Kruskal-Wallis test revealed that after 24 h intraoral exposure, the scores for biofilm coverage were significantly lower (Dunn's test: $p < 0.05$) in the groups of GIC-DMADDM samples compared to the controls (Fig. 7a). After 72 h, the scores for samples containing

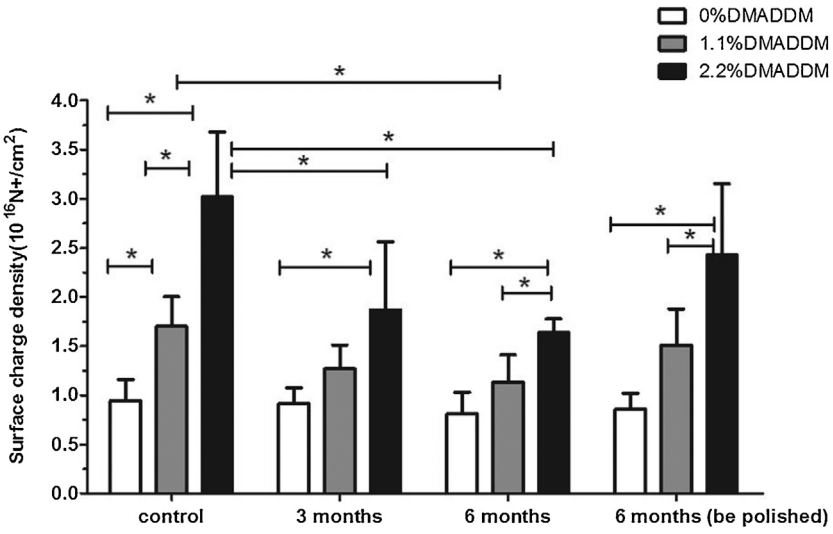

Fig. 4 – Surface charge density of GIC containing different mass fractions of DMADDM after different aging time. Each values is mean ± sd (n = 6) (*p < 0.05).

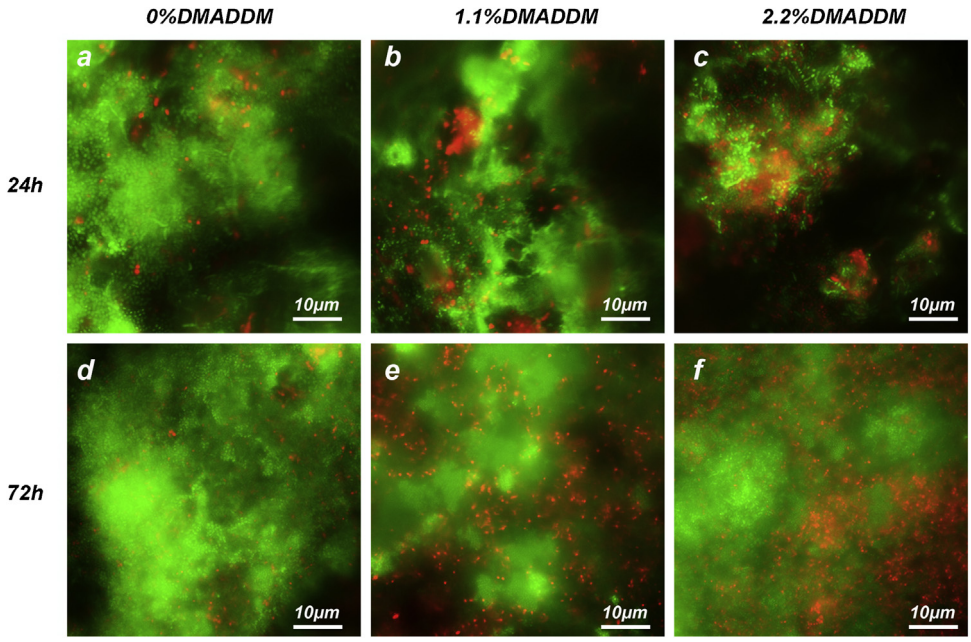

Fig. 5 – Representative FM images of 24 h and 72 h biofilms formed on 6 months aged GIC specimens without (a and d), with 1.1 wt.% (b and e) and 2.2% (c and f) DMADDM. Bar scale = 10 μm.

2.2 wt.% DMADDM were significantly lower compared to the GIC without DMADDM, however, there were no statistically significant differences between the 1.1 wt.% group and the 0 wt.% control (Fig. 7b).

4. Discussion

Recurrent caries and fractures are two major reasons for restoration failure after long-term clinical use of GICs [5,22]. When GIC restorations are placed, residual bacteria may remain viable over years, and the resulting infected dentin may cause restoration failure [23,24]. The complex oral environment provides constant challenges to any restorative material due to the presence of ions, enzymes, bacteria, pH changes and temperature fluctuations [25,26]. This may result in degradation of the filling materials, progressively yielding increase of wear and roughness, and decrease of the mechanical properties. For dentists and patients alike, longevity is thought as the most important considerations of filling materials. Therefore, dental restorative materials must be evaluated to determine if they are susceptible to degradation during long-term use. Water aging is one of the most widely used procedures in experimental studies to evaluate the materials' performance and to simulate the physiological aging of biomaterials [27].

The present study evaluated the effects of DMADDM addition to GIC and long-term water aging on the mechanical properties *in vitro* as well as the intra-oral antibiofilm activity of GIC containing DMADDM for the first time. It was demonstrated that the DMADDM-GIC could achieve a long-term biofilm growth-reducing capability and significantly biofilm viability reduction after 6 months of water aging, while possessing a much higher surface charge density and lower surface roughness than that of the commercial control containing no DMADDM.

A physical factor that influences bacterial adhesion is the average surface roughness of the material. Surface roughness of restorative materials has several clinical implications, and alterations on the surface topography and roughness are often used to determine the wear of a material. Increased roughness might be a predisposing factor to microbial colonization, which could potentially increase the risk of oral diseases [28]. In addition, an increase in surface roughness might indicate material deterioration [29]. Bollen et al. considered the critical surface roughness (Ra) mean for bacterial colonization of several dental materials to be 0.2 μm [30]. Ra values higher than 0.2 μm are likely to increase significantly bacterial adhesion, dental plaque maturation and acidity, which act on the material surface, thereby increasing the caries risk [31]. In this study, the surfaces roughness of the GIC specimens decreased with the increase of the DMADDM concentration. All samples presented Ra values below 0.15 μm. After 6 months of water aging, all experimental groups revealed no significant changes of the surface roughness, which is in agreement with the findings of Zhou et al. [27], indicating that the surface morphology and average roughness of GIC displayed no changes after water aging. Therefore, the results of this study showed that the GIC containing DMADDM presented the required surface roughness after the setting reaction and aging treatment.

Surface hardness is an important factor in controlling wear resistance and thus can be used as a predictor of the long-term durability of materials [32]. Recently, it has been shown that microhardness is a valid indicator of the surface properties of GIC [24]. In this study, it was found that the microhardness values of DMADDM containing specimens are reduced as compared with the control group. One possible reason might be the changes in the powder/liquid ratio. The increasing con-

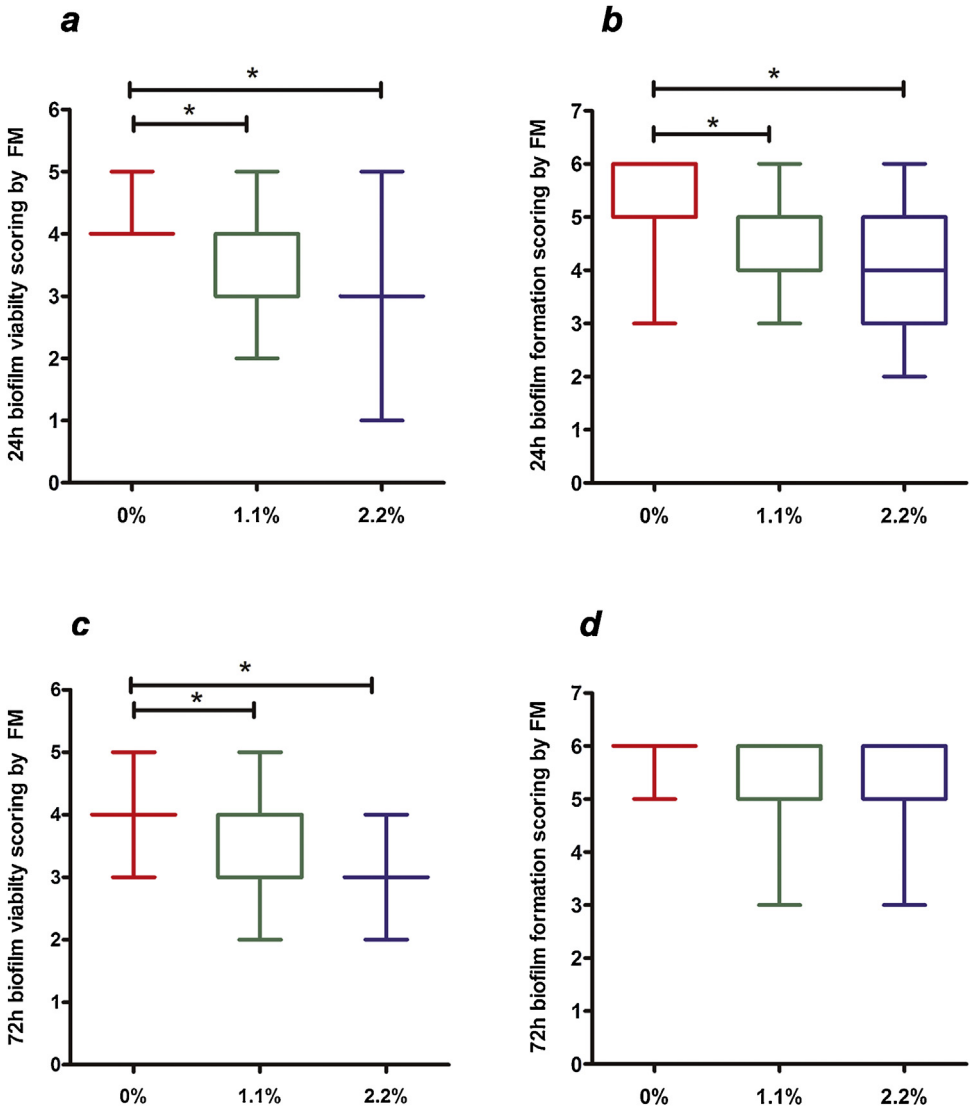

Fig. 6 – Results of biofilm viability (a and c) and biofilm coverage (b and d) scores by FM on 6 months aged samples. The scores for biofiom viability were significantly decreased on DMADDM containing specimens compared to controls without DMADDM. The biofilm formation scores were significantly lower in GIC-DMADDM specimens for 24 h, while there were no differences for 72 h (*$p < 0.05$).

centration of DMADDM monomer may decrease the reaction between the glass particles and the liquid of the cement, thereby increasing the number of unreacted particles in the structure. GICs have a major role in the ART, which is widely used in developing countries in rural areas. In addition, the use of GIC has been increasing in both pediatric and geriatric dentistry. The primary demand of the fillings that are used for extensive carious processes on dentine and root surfaces under wet conditions is not resistance to chewing forces, but rather the sealing and preservation of the remaining tooth substance. Fuji IX, as a high viscosity GIC, was selected as the base GIC material in our study because it has higher mean wear resistance and material surface hardness values when compared with the conventional GIC and some of the resin-modified GICs [33]. According to the results of this study, even the lowest microhardness values of 2.2 wt.% DMADDM containing GIC specimens are higher than that of conventional GIC depicted by Shintome et al. [32], which means the DMADDM-GIC processing acceptable hardness properties for clinical use. Moreover, water-aging had no significant adverse effect on the microhardness values of the specimens, indicating that the novel material maintained stable and durable surface properties.

Even though the precise antimicrobial mechanism of quaternary ammonium remains to be fully understood, the positive quaternary amine surface charge of the quaternary ammonium material plays an important role in disruption of the bacterial membrane [34,35]. The commonly accepted hypothesis is that quaternary ammonium methacrylates (QAMs) have bacteriolytic effects, because their positively charged quaternary amine N+ can attract the negatively charged cell membrane of bacteria, which could disrupt the cell membrane and cause cytoplasmic leakage [36–38]. In the present study, the surface charge density of GIC containing

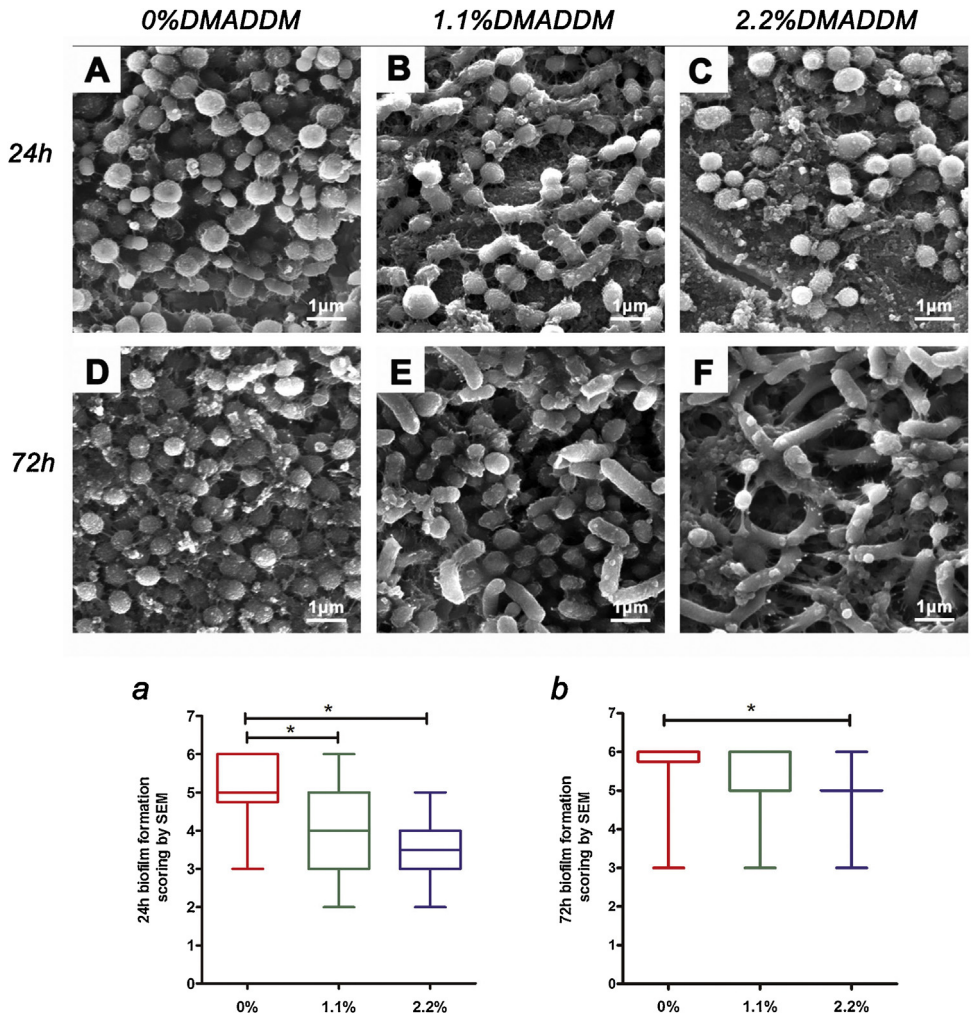

Fig. 7 – Representative SEM micrographs of 24 h (A–C) and 72 h (D–F) biofilms on 6 months aged samples. Results of scoring of biofilm formation (a and b) showed lower scores on DMADDM containing specimens compared to controls without DMADDM (*$p < 0.05$). Magnification: 10,000×, bar scale = 1 μm.

DMADDM significantly increased with increasing DMADDM concentration. After 3 months and 6 months of water aging, specimens containing DMADDM showed decreased surface charge densities compared with non-aged specimens, but still higher values than controls without DMADDM. These findings indicate that the DMADDM modified Fuji IX maintains a long term and durable charge density on the material surface. An interesting observation in this experiment is that removing about 0.1 mm of the surface could recover the high charge density of the specimens, which indicates that daily tooth brushing might re-establish the original surface charge density value, thereby providing a clinical benefit.

In our previous study, the GIC containing DMADDM strongly affected quality as well as quantity of biofilm formation under *in vivo* conditions over a period of 72 h [13]. However, long-term experiments are still needed to investigate the antibacterial durability of this material. For the first time in the present experiments, GIC-DMADDM samples were shown to reduce biofilm formation even after 6 months of water aging. DMADDM is an antibacterial quaternary ammonium monomer with an alkyl chain length of 12 which possessed a strong antibacterial potency [14,18,39,40]. Regarding the mechanism of antibiofilm activity of the GIC containing DMADDM, it was hypothesized in our previous study that the direct contact killing effect on bacteria could be the main reason for the early stage biofilm inhibition [13]. This effect might kill early colonizing bacteria directly and influence the development of biofilm indirectly. While for the mature biofilm, it was likely that metabolic communication and quorum-sensing were pivotal regulatory factors. The results of this *in situ* study confirm our previous hypothesis. The current results suggest that the density of positive surface charges increased with the concentration of DMADDM in the GIC samples, even after 6 months of water aging. Consistent with the surface charge density, a higher ratio of dead/live bacteria was observed on the DMADDM containing materials. Biofilm viability and biofilm formation after 24 h in the water aging group were significantly inhibited on DMADDM containing samples as compared to controls. After 72 h of oral *in situ* biofilm formation on water aged specimens, the biofilm viability, and to a lesser extent the biofilm formation scores, were also inhibited on the DMADDM con-

taining material compared to controls. DMADDM containing GIC showed decreased surface charge density after water aging, which might be one reason for more microbial colonization observed on the aged specimens in comparison to previously published results for DMADDM containing GICs. Regarding the antibacterial features, previous studies indicated that adding QAMs to composites and adhesives yielded a long-term antibiofilm property because the QAM was not released over time [35,41,42]. These results are also consistent with our previous study in which it had been found that the concentrations of the released DMADDM decreased to zero after 12 h for both 1.1 wt.% and 2.2 wt.% DMADDM containing GIC specimens, and support our assumption that the biofilm inhibiting properties are a surface contact effect.

Summarizing the results suggests beneficial long-term effects of DMADDM incorporated in GIC on biofilm formation. Although incorporating DMADDM into GIC yielded changes of mechanical properties, they are acceptable and stable. The DMADDM containing GIC might be promising for clinical applications as dental biomaterial which prevents or reduces the incidence of secondary caries and protects the tooth and soft tissues from bacterial infection. The present study focused on the effects of 6 months of water aging treatment on the mechanical and biofilm inhibiting properties of the DMADDM containing GIC, without investigating the remineralization of tooth lesions. Further studies are necessary to address this issue. The complex oral environment provides constant challenges to any restorative materials; hence, further studies are also needed to investigate whether the antibacterial potency decreases after longer aging time.

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