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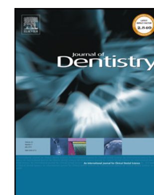

Effect of a novel quaternary ammonium silane on dentin protease activities

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ABSTRACT

Objectives: Demineralized dentin collagen release C-terminal cross-linked telopeptide (ICTP) and C-terminal peptide (CTX) during degradation. The present study evaluated the effects of dentin pre-treatment with K21, a quaternary ammonium silane (QAS), on matrix metalloproteinase (MMP) and cathepsin K-mediated collagen degradation.
Methods: Dentin beams were demineralized with 10% H_3PO_4 for 24 h. After baseline dry mass measurements, the beams were divided into 5 groups (N = 10) according to protease inhibitors. The beams were pre-treated for 2 min with 2% chlorhexidine (CHX), 2%, 5% or 10% QAS; no pre-treatment was performed for the control group. The beams were subsequently incubated in calcium- and zinc-containing medium for 3, 7 or 14 days, after which changes in dry mass were measured and incubation media were examined for ICTP and CTX release. The MMP-2 and cathepsin K activities in QAS-treated dentin powder were also quantified using ELISA.
Results: The two factors (disinfectants and time) had a significant effect on dry mass loss, ICTP and CTX release ($p < 0.001$). The percentage of dry mass loss increased with time and was significantly lower in all experimental groups when compared to the control at 14 days ($p < 0.001$). Conversely, the rate of ICTP and CTX release was significantly lower in the experimental groups, compared to the uninhibited control at 7 and 14 days ($p < 0.001$). Dentinal MMP-2 and cathepsin K activities were significantly reduced after demineralized dentin was pre-treated with QAS.
Conclusion: The experimental QAS is a good inhibitor of MMP and cathepsin K activities in demineralized dentin.
Clinical significance: The newly developed antibacterial quaternary ammonium silane increases the resistance of dentin collagen to degradation by inhibiting endogenous matrix metalloproteinases and cysteine cathepsins. The quaternary ammonium silane cavity disinfectant is promising for use as a protease inhibitor to improve durability of resin-dentin bonds.
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1. Introduction

Dentin contains type I collagen and non-collagenous proteins embedded in a matrix of carbonated apatite [1]. Matrix metalloproteinases (MMPs) and cysteine cathepsins are found extensively in sound [2,3], caries-infected [4] and caries-affected dentin [5,6]. These endogenous dentin enzymes are entrapped within the mineralized collagen matrix during dentinogenesis. Matrix metalloproteinases are responsible for degradation of extracellular matrix components, especially the highly cross-linked triple helical type I collagen [7,8]. Whereas cathepsin B and cathepsin L cleave only the non-helical telopeptide extensions of collagen, cathepsin K is the only cysteine cathepsin that cleaves collagen in the triple helical region [9,10].

The hybrid layer is produced by acid etching and infiltration of solvated methacrylate resin comonomers into the demineralized dentin collagen matrix. Treatment of dentin with 37% phosphoric acid does not denature the endogenous MMP and cathepsin activities of dentin matrices [11,12]. Subsequent application of acidic resin reactivates endogenous MMPs [13]. Similar to MMPs, cysteine cathepsins may be activated in mildly acidic environments; acid activation of dentin-bound cathepsins may further

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result in activation of the matrix-bound MMPs. A highly significant correlation between cysteine cathepsin and MMP activities in intact [2] and carious dentin [4] suggests that both proteases may be responsible for the breakdown of resin-sparse, unprotected collagen fibrils within the hybrid layer, eventually resulting in debonding of the overlying restorations.

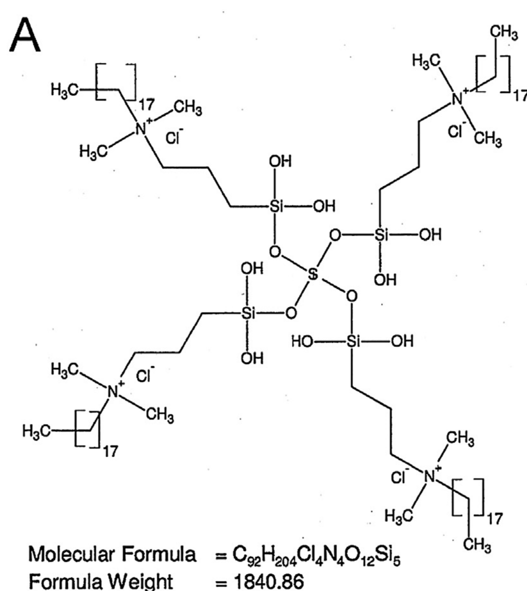
Incomplete removal of caries-infected dentin during cavity preparation results in entrapment of bacteria within the cavity [14]. Open restoration margins enable the oral microflora to invade the resin-dentin interface. These microorganisms continue to multiply and cause secondary caries formation, which is one of the major causes for failure of resin-based restorations [15]. Disinfection of the cavity using an antimicrobial prior to restorative procedures has been recommended to prevent secondary caries [16].

Chlorhexidine (CHX) is commonly used as a cavity disinfectant to eliminate residual bacteria in caries-affected dentin and dentinal tubules after mechanical caries removal [17,18]. This bisbiguanide is also a potent, non-specific inhibitor of MMP −2, −8 and −9 [19,20] and cysteine cathepsins [21]. Chlorhexidine is water-soluble and binds only electrostatically to the demineralized dentin matrix. Thus, it may be displaced by competing cations from the dentinal fluid and saliva, compromising its long-term protease inhibitory effect. In the presence of water at the bonded interface, CHX may debind from collagen and slowly leach out of the hybrid layer over time [22]. Recent studies have shown that CHX pretreatment of demineralized dentin has limited effect in preventing bond degradation after 9–12 months [23,24]. In addition, CHX has recently been reported to exert dose-dependent, mild trans-dentinal toxic effects on odontoblast-like cells [25], as well as inhibiting the mineralization potential of multipotent stem cells derived from human exfoliated deciduous teeth [26]. Therefore, there is a need to look for other antibacterial cavity cleansers of lower toxicity to inhibit oral biofilms and caries.

Quaternary ammonium compounds are commonly used as disinfectants of intact skin, non-critical surfaces and mucous membranes [27]. However, concerns of resistance development, potential toxicities and loss of antimicrobial activity over time as a result of leaching from the bound surfaces have limited their clinical use. The organosilicon quaternary ammonium chloride, 3-(trimethoxysilyl)-propyldimethy-octadecyl ammonium chloride (Si-QAC; $C_{26}H_{58}ClNO_3Si$; CAS number 27668-52-6), possesses potent antibacterial activities. Being an antimicrobial quaternary ammonium silane (QAS), Si-QAC has been used for antimicrobial coatings of fabrics [28,29] and medical devices [30–32]. The antimicrobial property of Si-QAC is attributed to its long, lipophilic C18 alkyl chain that penetrates bacterial cell membranes and causes cell death of bacteria by direct contact and leaching of intracellular components [33]. Being a trialkoxysilane, SiQAC possesses hydrolyzable and condensable methoxy groups which enable it to covalently attach to other alkoxysilanes or silanol-containing substrate surfaces via the formation of siloxane bridges [34,35].

Because methanol is produced during the hydrolysis and condensation reactions of SiQAC, the molecule is potentially toxic for intraoral use [36–38]. Hence, SiQAC has been substituted with 3-(triethoxysilyl)-propyldimethyloctadecyl ammonium chloride (i.e. the ethoxy version of SiQAC, abbreviated as Et-SiQAC) for coupling with tetraethoxysilane (TEOS) via sol-gel synthesis. This resulted in the generation of an ethanol- or acetone-soluble, fully-hydrolysed, partially-condensed QAS (1-octadecanaminium, N,N'-[[3,3-bis[[[3-(dimethyloctadecylammonio) propyl]dihydroxysilyl] oxy]-1,1,5,5,-tetrahydroxyl-1,5-trisiloxanediyl]di-3,1-propanediyl] bis[N, N-dimethyl] chloride (1:4); CAS number 1566577-36-3; codenamed K21). Because of the elimination of methanol, K21 may be used, without further purification, as an intraoral disinfectant. This ethoxylated QAS molecule has been shown to be effective against *Porphyromonas gingivalis* and *Enterococcus faecalis* when used as coatings for surgical sutures and dental flosses [39].

Because quaternary ammonium compounds possess MMP [40–42] and cysteine cathepsin [43] inhibitory effects, it is speculated that the ethoxylated QAS may also inhibit endogenous dentin proteases. Nevertheless, the inhibitory effect of ethoxylated QAS on MMPs and cysteine cathepsins in dentin has not been investigated. The combined antimicrobial and anti-collagenolytic effects of QAS may be beneficial in preventing degradation of resin-dentin bonds and development of secondary caries. Thus, the objective of the present study was to evaluate the effects of



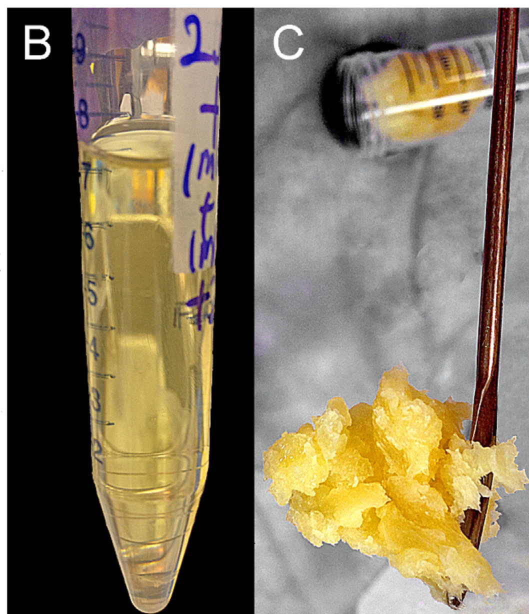


Fig. 1. A. Proposed chemical formula of the QAS (K21) molecule. B. A yellow solution was obtained after compete hydrolysis of the reaction mixture. C. The partially-condensed solid after heating of the completely hydrolyzed solution. (For interpretation of the references to colour in text, the reader is referred to the web version of this article.)

different concentrations of an experimental ethoxylated QAS, K21, in inactivating dentin proteases. The null hypotheses tested were that (i) pre-treatment of demineralized dentin matrices with QAS has no effect on their loss of dry mass by collagen degradation; and (ii) pre-treatment of demineralized dentin with QAS has no effect on inhibition of dentinal MMP or cathepsin K activities.

2. Materials and methods

2.1. Synthesis and characterization of K21

The experimental QAS (codenamed K21) was synthesized by sol-gel reaction between 1 mol of TEOS (Mw 208) and 4 mol of Et-SiQAC (Mw 538). The chemical structure of K21 and the processes of hydrolysis and condensation are shown in Fig. 1A. In a typical synthesis, 2.08 g of TEOS (Millipore Sigma, St. Louis, MO, USA) was blended with 29.89 g of Et-SiQAC (72 wt% of Et-SiQAC dissolved in ethanol; Gelest Inc., Morrisville, PA, USA) and 5 mL of ethanol (to render the blend more homogeneous). Hydrolysis was initiated by the addition of 10.08 g of 0.02 M HCl-acidified water (pH 1.66, representing 3.5 times the stoichiometric molar concentration of water required, to ensure complete hydrolysis). Hydrolysis and condensation of the two ethoxysilanes were monitored by attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR; Nicolet 6700, Thermo Fisher Scientific, Waltham, MA, USA) at a resolution of 4 cm^{-1} and an average of 32 scans per spectrum.

A yellow solution mixture was obtained after completion of the hydrolysis reaction (Fig. 1B). The yellow solution mixture was then maintained at 80 °C for 6 h to remove as much as possible the reaction by-products (ethanol and water), until a pale yellow rubbery solid material was produced (Fig. 1C). This yellow, partially-condensed solid was characterized using $^{1}H \rightarrow ^{29}Si$ cross polarization-magic angle spinning (CP-MAS) solid-state nuclear magnetic resonance spectroscopy (NMR). ^{29}Si solid-state NMR was performed at ambient temperature using a 270 MHz spectrometer (JEOL, Tokyo, Japan) equipped with a 7 mm MAS probe. Spectra were acquired in the $^{1}H \rightarrow ^{29}Si$ CP mode, using a MAS frequency of 4 kHz, with a 45° pulse angle of 5 s. The ^{1}H Larmor frequency for ^{29}Si was 53.76 MHz. Chemical shifts were referenced to external tetramethylsilane at 0 ppm. The yellow, partially-condensed K21 solid was immediately dissolved in absolute ethanol to produce solutions containing 2 wt%, 5 wt% or 10% of the QAS to be used as the experimental protease inhibitors in the present study.

2.2. Demineralized dentin beams

Fifty extracted non-carious human third molars were obtained from patients (18–21 year-old) with their informed consent under a protocol reviewed and approved by Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster (UW14-406). All the experiments were performed in accordance with the approved guidelines and regulations. The teeth were stored in 0.9% NaCl with 0.02% sodium azide at 4 °C to prevent bacterial growth and were used in within 1 month after extraction. Demineralized dentin beams were prepared by removing the occlusal enamel and superficial dentin of each tooth with an Isomet saw (Buehler Ltd., Lake Bluff, IL, USA) under water cooling. A 1-mm thick mid-coronal dentin disk was prepared from each tooth. One dentin beam with dimensions 6 mm × 2 mm × 1 mm was then sectioned from the center of each disk and a total of 50 beams were prepared. The dentin beams were completely demineralized in 10 wt% H_3PO_4 for 24 h at 25 °C. The demineralized beams were thoroughly rinsed in deionized water with constant stirring at 4 °C for 1 h. Absence of residual minerals in each beam was verified using digital radiography.

The demineralized beams were randomly allocated to each of the five groups (N = 10) so that there was no statistically significant difference in the mean dry mass among the groups. Group 1 was the negative control, which was not treated with any disinfectant. Groups 2, 3, 4 and 5 were treated respectively with 2% CHX (Millipore Sigma), 2% QAS, 5% QAS and 10% QAS, by dipping the beams in the respective medium for 2 min each. After treatment with the disinfectants, each beam was blot-dried using Kimwipes (Kimberly Clark Corp, Roswell, GA, USA) and placed separately in a labeled polypropylene tube containing 1 mL of complete storage medium (CM). The CM is a calcium- and zinc-containing storage medium containing 5 mM HEPES, 2.5 mM $CaCl_2 \cdot H_2O$, 0.05 mM $ZnCl_2$ and 0.3 mM NaN_3 (pH 7.4).

2.3. Loss of dry mass over time

Loss of dry mass was used as an indirect measurement of dissolution of the demineralized dentin matrix by endogenous matrix-bound proteases after each incubation period (3, 7 or 14 days) [44]. Prior to treatment with the respective disinfectant, the beams were transferred to individually labeled polypropylene tubes and placed in a vacuum desiccator chamber containing anhydrous calcium sulfate (Drierite, W.A. Hammond Drietite Co. Ltd, Xenia, OH, USA). With the vial cap off, each beam was desiccated to a constant mass for 72 h. The initial dry mass was measured to the nearest 0.001 mg using an analytical balance (XP6 Microbalance, Mettler Toledo, Hightstown, NJ, USA). After dry mass measurement, the beams were rehydrated in deionized water at 4 °C for 1 h before treatment with the designated disinfectants. The sealed tubes were placed in a shaking-water bath (Thermo Fisher Scientific) with a shaking speed of 60 cycles/min at 37 °C for 3, 7 or 14 days. The dry mass was re-measured using the same conditions after each incubation period. At the end of each incubation period, the storage medium was replaced with fresh incubation medium. The medium obtained after each incubation period was stored in a freezer (−80 °C) and subsequently analyzed for C-terminal cross-linked telopeptide (ICTP) and C-terminal peptide (CTX) fragments.

2.4. Solubilized collagen peptides

Degradation of the demineralized collagen matrix by MMPs was determined by measuring the amount of solubilized type I collagen ICTP fragments using an ICTP enzyme-linked immunosorbent assay (ELISA) kit (UniQ EIA, Orion Diagnostic, Finland). The rationale for this analysis was based on the finding that ICTP telopeptide fragments are solely derived from the telopeptidase activity of MMPs in a degrading dentin collagen matrix [45]. The collagen matrix degradation activity of cathepsin K was determined by measuring the amount of CTX fragments within the incubation medium, using a Serum Crosslaps ELISA kit (Immunodiagnostic System, Farmington, UK). The rationale for this analysis was based on the finding that CTX fragments are solely derived from the enzymatic activity of endogenous cathepsin K within a degrading collagen matrix [44]. At the end of each incubation period, the sealed tubes were retrieved from the shaker-water bath and the entire volume (i.e. 1 mL) of the medium was removed. Ten to twenty μL aliquots of the incubation medium were used to measure solubilized ICTP and CTX collagen fragments.

2.5. Protease detection from demineralized collagen

2.5.1. Specimen preparation

Thirty human third molars (obtained from 19 to 35 year-old subjects) were ultrasonically cleaned and stored in 1% thymol solution at 4 °C. The roots were removed and occlusal enamel was sectioned perpendicular to the longitudinal axis of each tooth with

the Isomet saw using water cooling, exposing a flat mid-coronal dentin surface (1 mm below the dentinoenamel junction). The tooth segments were meticulously rinsed with deionized water after the pulpal tissues were removed from each of the cut tooth section and rinsed with deionized water.

The cleaned tooth segments were pulverized in liquid nitrogen into a fine powder using a steel mortar/pestle (Reimiller, Reggio Emilia, Italy). Five one-gram aliquot of dentin powder were demineralized with 0.5 M EDTA (pH 7.0). The demineralized dentin powder was rinsed with deionized water for 5 times, dried and divided into five groups. Group 1 was left untreated and served as control. The remaining four groups of dentin powder were treated respectively with 2% CHX (Group 2), 2% QAS (Group 3), 5% QAS (Group 4) and 10% QAS (Group 5) for 2 min. The treated dentin powder was re-suspended in extraction buffer [50 mM Tris-HCl at pH 7.5 containing 0.2% Triton X-100, 5 mM $CaCl_2$, and 100 mM NaCl] for 24 h to extract the proteases. The vials were centrifuged at 20,000 rpm for 30 min at 4 °C. The supernatants were collected, dialyzed in bags with 30-kDa molecular cut-off overnight, lyophilized and frozen at −20 °C until they were analyzed.

2.5.2. MMP-2 and cysteine cathepsin ELISAs

The concentrations of endogenous MMP-2 and cathepsin K in the supernatants derived from different groups of dentin powder were quantified using ELISA (Human MMP2 ELISA Kit – Lot #5619 for MMP-2; Human CTSK/Cathepsin K ELISA Kit – Lot #5614 for cathepsin K, both from Lifespan Biosciences, Seattle, WA, USA). The kits were used according to the manufacturer's instructions. A calibration curve correlating protease concentration with absorbance intensity was prepared for each protease. The dialyzed supernatants were incubated in the respective assay buffers for MMP-2 and cathepsin K for 1.5 h at 37 °C. Detection reagents were added after several rinses and the absorbance was recorded with a spectrophotometer at 405 nm (Bio-Rad Laboratories, Inc. Hercules, CA, USA). All tests were conducted in a triplicate and the protease concentrations were expressed as ng/mL.

2.6. Statistical analyses

The percent loss of dry mass over incubation time and the rate of release of ICTP and CTX (ng telopeptide/mg dry dentin/unit time) from all groups were compared for normality (Shapiro-Wilk test) and equality of variance (modified Levine test). When the normality and equality variance assumptions of the original data were violated, the data were nonlinearly transformed to satisfy those assumptions prior to the use of parametric statistical methods. Two-factor analysis of variances (ANOVA) was employed to examine the effects of incubation time and disinfectants, and the interaction of those two factors on the respective parameters investigated. Post-hoc multiple comparisons were performed with the Tukey statistic. Dentinal MMP-2 and cathepsin K concentrations in the five groups were separately analyzed using one-way ANOVA and Tukey multiple comparison test. For all tests, statistical significance was preset at $\alpha = 0.05$.

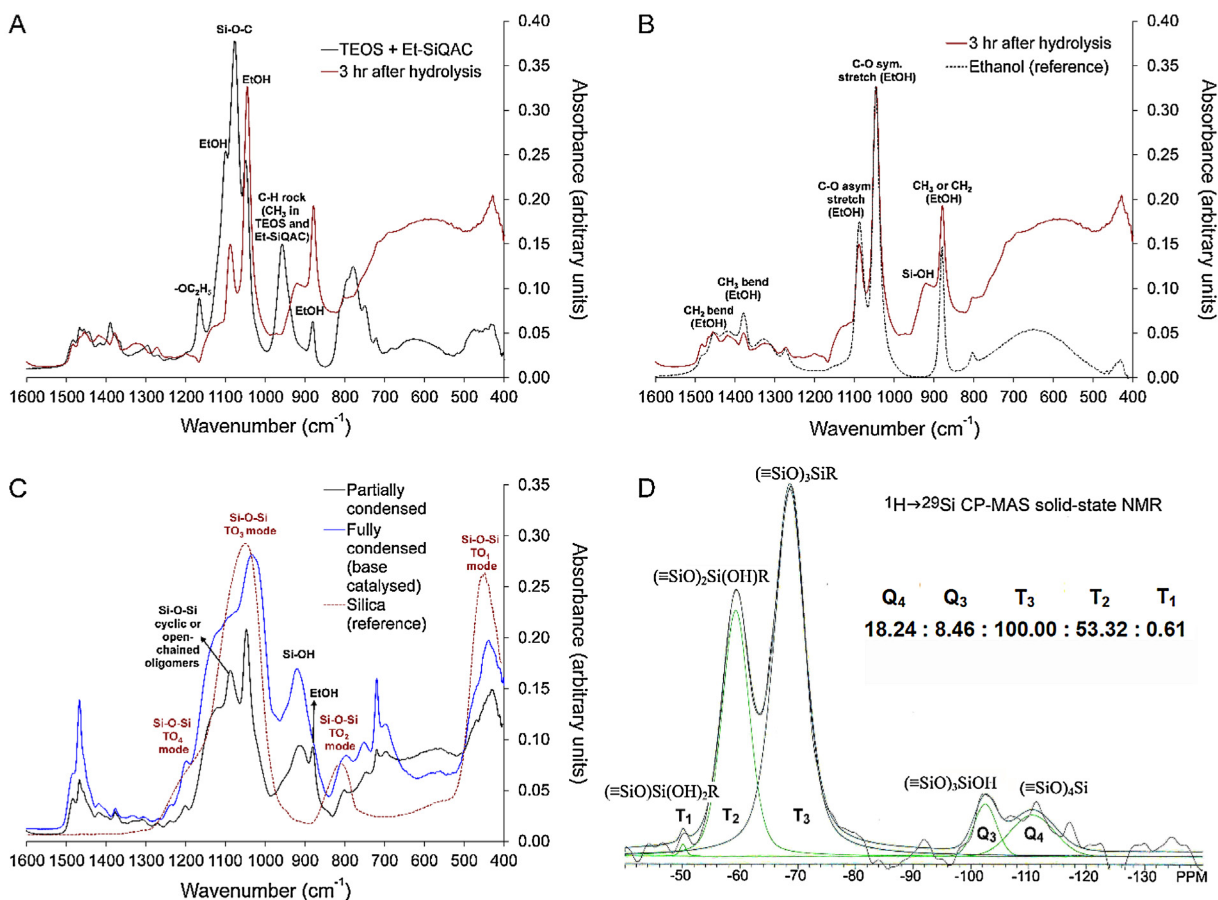


Fig. 2. A–C. Infrared spectra of A. the hydrolysis reaction between TEOS and Et-SiQAC; B. Comparison between the hydrolysis reaction products with spectrum of ethanol as the reference; C. Comparison between the partially and fully condensed K21 QAS. D. ^{29}Si CP-MAS solid-state NMR spectrum of the partially condensed K21 used for the solubilized protease inhibitor in the present study.

3. Results

3.1. Characterization of QAS

Completion of the hydrolysis reaction (approximately 3 h) was monitored by ATR-FTIR and was indicated by the disappearance of infrared absorbance peaks of the Si—O—C peak at 1078 cm^{-1} and the $—OC_2H_5$ peak at 1172 cm^{-1} (Fig. 2A). The IR spectrum of the fully hydrolyzed reaction between the tetraethoxysilane and triethoxysilane is shown in Fig. 2B, and was characterized by the presence of silanol groups, ethanol and water. The presence of ethanol, and not methanol as the product of hydrolysis was confirmed using the infrared spectrum of ethanol as reference. Infrared spectra of the partially-condensed and fully-condensed versions of the QAS are shown in Fig. 2C. Oligomers containing Si—O—Si cyclic or open-chained species could be identified as impurities from the partially-condensed version of QAS.

Solid-state ^{29}Si MAS NMR spectroscopy was used to identify the chemical environment of the ^{29}Si atoms. Fig. 2D shows the ^{29}Si solid-state CP-MAS NMR spectrum of the desiccated K21 QAS. The resonances of different species of the Si atoms in silica are located between −85 and −115 ppm, and may be denoted by the Q_n terminology [46]. The latter denotes Si with n Si—O—Si linkages with values $n = 1–4$. The characteristic tetrafunctional Q_4 peak was observed at −110 ppm, and the Q_3 peak at −102 ppm, with negligible presentation of the Q_2 peak at −93 ppm. The data suggests the bulk of the silanol groups were condensed during the sol-gel reaction, and indicates that the QAS possessed a networked structure. The same figure confirms the presence of alkyl silicon with n Si—O—Si linkages ($n = 1$-3), as denoted by the trifunctional T_n terminology. The peaks at −70 ppm, −60 ppm and −50 ppm were assigned to the T_3, T_2 and T_1 Si, respectively. The appearance of the T portions validates that Et-SiQAC was co-condensed with TEOS in the sol-gel reaction product. The predominance of the T_3 and T_2 peaks may be attributed to the use of Et-SiQAC and TEOS in the molar ratio of 4:1 in the sol-gel reaction.

3.2. Loss of dry mass

The percentage loss of dry mass after 3, 7 and 14 days of incubation are shown in Table 1 and Fig. 3. Both the factors incubation time and disinfectants had a significant effect on loss of dry mass ($p < 0.001$). The interaction between the two factors was also significant ($p < 0.001$). The percentage of dry mass loss in all groups increased with time and was significantly lower in the experimental groups (2% CHX, 2% QAS, 5% QAS, 10% QAS) compared to the uninhibited control ($p < 0.001$) at 7 and 14 days. The three QAS groups lost 14–16% of dry mass, compared to 27% in the control group after 14 days of incubation. No significant difference in dry mass loss was observed among the experimental groups at 3, 7 and 14 days. Increasing the QAS concentration did not further inhibit the dry mass loss.

Table 1
Mean percent loss of dry mass from various treatment groups for each incubation period.

	% Loss of dry mass		
Groups	3rd day	7th day	14th day
Control	7.46 ± 1.86 $^{A\alpha}$	16.47 ± 3.2 $^{b\beta}$	26.94 ± 3.69 $^{II\chi}$
2% CHX	6.69 ± 2.57 $^{A\alpha}$	9.57 ± 3.48 $^{a\alpha}$	16.54 ± 5.51 $^{I\beta}$
2% QAS	2.93 ± 2.57 $^{A\alpha}$	7.95 ± 3.86 $^{a\alpha}$	16.09 ± 6.51 $^{I\beta}$
5% QAS	4.29 ± 1.75 $^{A\alpha}$	10.44 ± 3.01 $^{a\alpha}$	15.09 ± 5.36 $^{I\beta}$
10% QAS	4.02 ± 1.92 $^{A\alpha}$	5.20 ± 2.22 $^{a\alpha}$	13.67 ± 7.61 $^{I\beta}$

N = 10.
Uppercase letters/lowercase letters/numerals represent differences in each column (3rd day/7th day/14th day) ($p < 0.05$). Symbols α, β and χ represent differences in each row ($p < 0.05$).

3.3. Inactivation of endogenous dentin proteases

3.3.1. ICTP release

The quantities of ICTP telopeptides released after incubation of the demineralized dentin beams for 3, 7 and 14-day are shown in Table 2 and Fig. 4. Both factors, disinfectants and time, had a significant effect on the ICTP release and the interaction of the two factors was also significant ($p < 0.001$). In the control group, the rate of ICTP release increased significantly after 7 days ($p < 0.05$) and then declined significantly after 14 days ($p < 0.05$). For the CHX group, the rate of release of ICTP was relatively high after 3 days and decreased significantly after 14 days ($p < 0.05$). The rate of release of ICTP was significantly lower in the QAS groups when compared to the untreated control and CHX group after 3 days, indicating that QAS was initially more effective than CHX in inactivation of MMPs. For the QAS groups, no significant difference in the rate of release of ICTP was observed across the three time periods. The percentage of ICTP release was significantly lower in the experimental groups, compared to the uninhibited control ($p < 0.001$) at 7 and 14 days. Increasing the QAS concentration did not further inhibit the rate of ICTP release after 3 and 14 days.

3.3.2. CTX release

The quantities of CTX released after incubation of the demineralized dentin beams for 3, 7 and 14-day are shown in Table 3 and Fig. 5. Both factors, disinfectants and time, had a significant effect on CTX release and the interaction of the two factors was also significant ($p < 0.001$). Similar to ICTP release, the rate of CTX release increased significantly after 7 days ($p < 0.05$) in the untreated control group and then decreased significantly after 14 days ($p < 0.05$). The percentage of CTX release was significantly lower in the experimental groups compared to the untreated control after 7 and 14 days ($p < 0.001$). No significant difference in the percentage of CTX release was observed among the experimental groups at 7 and 14 days ($p < 0.05$). Increasing the QAS concentration had no effect on the rate of CTX release at 7 and 14 days.

3.3.3. Concentrations of endogenous MMP-2 and cathepsin K

The concentrations of MMP-2 and cathepsin K (ng/mL) recorded in demineralized dentin powder (control) and treated-dentin powder are depicted in Table 4. The concentrations of active enzymes present in the CHX and QAS groups were significantly lower than the control group ($p < 0.05$). No significant difference in MMP activities was observed between the 2% CHX group and the 2% QAS group. Increasing QAS concentration significantly reduced the MMP-2 activities in demineralized dentin. The concentrations of active cathepsin K in the QAS groups were significantly lower than the CHX group ($p < 0.05$). Increasing the QAS concentration had no significant effect on cathepsin K activity in demineralized dentin.

4. Discussion

Using sol-gel chemistry, ethanol-soluble tetraethyl TEOS was used to react with Et-SiQAC (i.e. the ethoxy version of Si-QAC) to produce the raw materials for solubilization in ethanol to create the protease inhibitor. Heating of the fully hydrolyzed liquid alone did not result in optimal condensation of the two ethoxysilanes; full condensation may only be achieved by adding an alkali solution to raise the pH of the solution to 7.2 [47] to generate an ethanol-insoluble precipitate. The rationale for synthesizing a partially-condensed version of the QAS is that the product may be dissolved in solvents such as ethanol, acetone, 1,3-butanediol or 2-hydroxyethyl methacrylate, whereas the optimally-condensed version of the QAS is insoluble in these solvents.

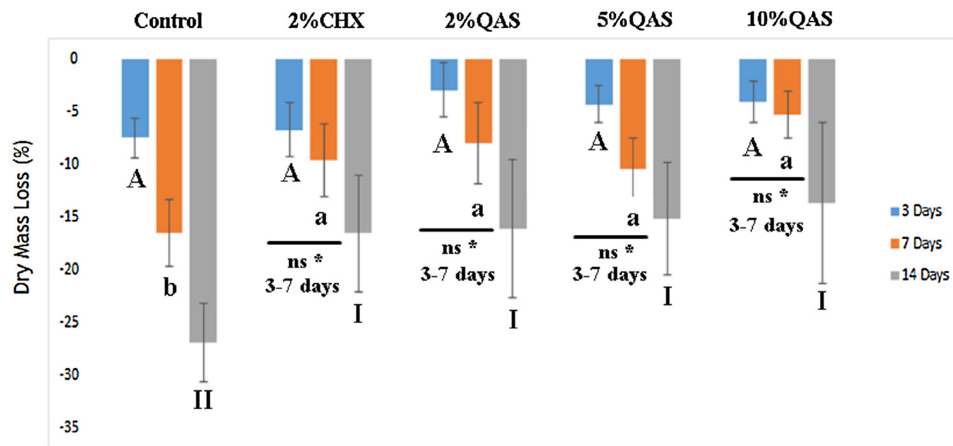


Fig. 3. Mean percent loss of dry mass of demineralized dentin beams from various treatment groups at 3, 7 and 14 days in the incubation medium. Values are means and standard deviations of the percentage dry mass loss (N = 10). The loss of dry mass from each beam was calculated as a percentage of the original dry mass of that beam at baseline. Uppercase letters/lowercase letters/numerals represent differences between each pre-treatment (3rd day/7th day/14th day) ($p < 0.05$); ns: no significant difference.

Table 2
Amount of ICTP released from various treatment groups for each incubation period.

	Release of ICTP telopeptides (ng/mg dentin)		
Groups	3rd day	7th day	14th day
Control	11.82 ± 1.69 $^{B\alpha}$	25.96 ± 5.55 $^{c\chi}$	20.73 ± 3.10 $^{II\beta}$
2% CHX	14.22 ± 1.86 $^{B\beta}$	9.94 ± 3.06 $^{b\alpha}$	6.15 ± 2.21 $^{I\alpha}$
2% QAS	7.21 ± 1.88 $^{A\beta}$	8.04 ± 1.59 $^{b\beta}$	3.36 ± 2.06 $^{I\alpha}$
5% QAS	4.06 ± 1.30 $^{A\alpha}$	7.18 ± 3.69 $^{b\alpha}$	3.40 ± 2.83 $^{I\alpha}$
10% QAS	3.34 ± 0.80 $^{A\alpha}$	2.30 ± 0.83 $^{a\alpha}$	2.69 ± 0.76 $^{I\alpha}$

N = 10.
Uppercase letters/lowercase letters/numerals represent differences in each column (3rd day/7th day/14th day) ($p < 0.05$). Symbols α, β and χ represent difference in each row ($p < 0.05$).

Because the hydrolysis by-product of the sol-gel reaction is ethanol, the QAS may be used, without further purification, as an intraoral cavity disinfectant and potential protease inhibitor. The inclusion of TEOS as the network forming agent enables a three-dimensional organically modified silicate to be produced by condensation of additional tetraethoxy and triethoxy molecules with remnant silanol groups within the K21 molecule. The K21 molecule has four quaternary ammonium arms that impart four positive charges to the molecule, thereby conferring its antimicrobial and antiviral properties [48]. The mechanism of the bactericidal action of quaternary ammonium compounds involves destructive electrostatic interactions with the bacterial cell wall and cytoplasmic membranes, thereby inhibiting bacterial survival [49].

The present study was designed to determine whether the QAS has any inhibitory effects on endogenous MMPs and cathepsin K retained in demineralized dentin matrices. Dry mass loss is an indirect measurement used for evaluating the dissolution of collagen from demineralized dentin matrices by collagen-bound proteases [44]. In a relatively short time (2 weeks), it was possible to identify significant changes in the solubility features of demineralized dentin matrices after pre-treatment with QAS at different concentrations. Because the losses of dry mass from the demineralized dentin beams pretreated with QAS were significantly lower when compared to control, the results were indicative of reduced endogenous protease activities in the QAS-pretreated demineralized dentin beams. Hence, the first null hypothesis that pre-treatment of demineralized dentin matrices with QAS has no

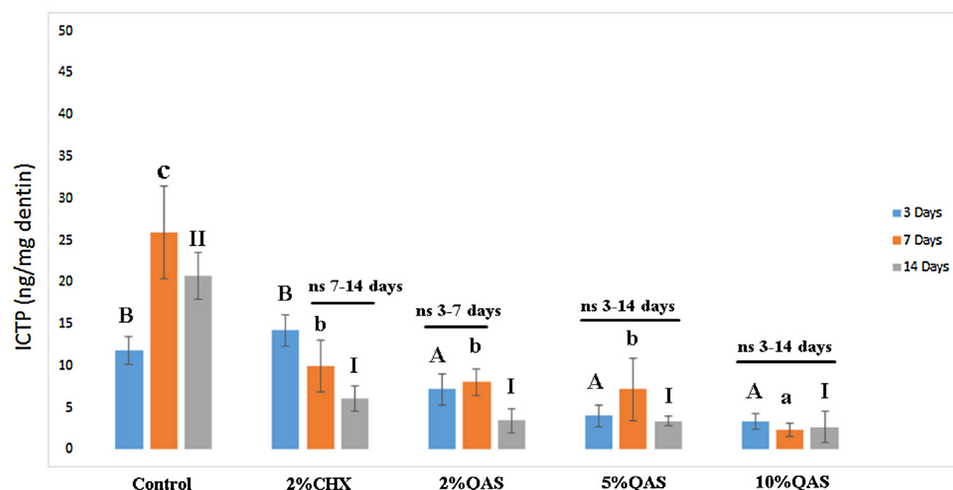


Fig. 4. The amount of ICTP telopeptides released per ng/mg dentin from various treatment groups at 3, 7 and 14 days in the incubation medium. Values are means and standard deviations (N = 10). Uppercase letters/lowercase letters/numerals represent differences between each pre-treatment (3rd day/7th day/14th day) ($p < 0.05$); ns: no significant difference.

Table 3
Amount of CTX released from various treatment groups for each incubation period.

	Release of CTX telopeptides (ng/mg dentin)		
Groups	3rd day	7th day	14th day
Control	$0.95 \pm 0.57^{A\alpha}$	$5.53 \pm 1.50^{b\chi}$	$2.57 \pm 1.40^{II\beta}$
2% CHX	$0.84 \pm 0.38^{A\alpha}$	$0.76 \pm 0.50^{a\alpha}$	$0.66 \pm 0.20^{I\alpha}$
2% QAS	$0.52 \pm 0.34^{A\alpha}$	$0.93 \pm 0.50^{a\alpha}$	$0.87 \pm 0.20^{I\alpha}$
5% QAS	$0.45 \pm 0.24^{A\alpha}$	$0.29 \pm 0.10^{a\alpha}$	$0.30 \pm 0.20^{I\alpha}$
10% QAS	$0.26 \pm 0.25^{A\alpha}$	$0.22 \pm 0.10^{a\alpha}$	$0.32 \pm 0.10^{I\alpha}$

N = 10.
Uppercase letters/lowercase letters/numerals represent differences in each column (3rd day/7th day/14th day) ($p < 0.05$). Symbols α, β and χ represent difference in each row ($p < 0.05$).

Table 4
Quantification of active MMP-2 and Cathepsin K in dentin powder derived from the control and experimental groups.

Groups	MMP-2	Cathepsin K
Control	17.00 ± 2.05^{A}	3.37 ± 1.99^{a}
2% CHX	8.38 ± 1.49^{B}	2.30 ± 1.17^{b}
2% QAS	5.62 ± 1.3^{C}	0.93 ± 0.22^{c}
5% QAS	$3.82 \pm 0.7^{C,D}$	0.70 ± 0.42^{c}
10% QAS	3.40 ± 1.62^{D}	0.62 ± 0.35^{c}

Values are means ± standard deviations, in ng/mL.
For MMP-2, groups designated with the same upper–case letter superscripts are not significantly different ($p > 0.05$).
For cathepsin K, groups designated with the same lower–case letter superscripts are not significantly different ($p > 0.05$).

effect on their loss of dry mass by collagen degradation has to be rejected.

Release of ICTP and CTX fragments from degrading collagen fibrils represents another indirect measurement of collagen matrix degradation. Analyses of the longer C-telopeptide of the collagen breakdown, ICTP, and the shorter eight amino acid CTX peptide provide more specific information on the contribution of endogenous MMPs and cysteine cathepsins to the breakdown of dentin collagen fibrils over time. Results of the present study showed that the amounts of ICTP and CTX release were significantly lower in the demineralized dentin beams treated with various concentrations of QAS compared to the uninhibited control. In addition, the concentrations of active MMP-2 and cathepsin K after the application of QAS were significantly lower than the control group. Results obtained from ELISAs used to detect these two proteases provide direct evidence of the inhibitory effect of QAS on dentinal MMP-2 and cathepsin K. Hence, the second null hypothesis that pre-treatment of demineralized dentin with QAS has no effect on inhibition of dentinal MMP or cathepsin K activities has to be rejected.

The decrease in dry mass loss in the present study is due to inhibition of ICTP and CTX release from the demineralized collagen matrices. It has been reported that demineralized dentin beams, when incubated in control medium, resulted in more dry mass loss over time than beams that were incubated in calcium-free or zinc-free media (calcium and zinc are required for MMP functioning) [50]. The same trend was observed in the present study; dentin beams incubated in control medium without any disinfectant pre-treatment lost 27% of the dry mass after 14 days. The dry mass loss for CHX (17%) and QAS groups (14%–16%) was significantly less than the control group. Results of the present study confirmed that QAS, similar to CHX, has potent inhibitory effect against matrix-bound proteases. Both ICTP and CTX telopeptide releases in the control group peaked at 7 days, but decreased after 14 days. The telopeptides released at day 7 were the soluble telopeptides at the matrix surfaces that were cleaved from the collagen telopeptides and diffused from the demineralized dentin into the storage medium [44]. The matrix-bound proteases have a limited degradation zone, which is tethered closely to the enzymes. Hence, the peptides are hydrolyzed by the proteases only within their immediate vicinity, causing a possible decrease of telopeptide degradation products at 14 days.

Dentin organic matrix contains endogenous proteolytic enzymes, MMPs and cysteine cathepsins, that can cause degradation of type I collagen. The MMPs belong to a family of endogenous zinc- and calcium-dependent endopeptidases, with 23 members classified into 6 groups based on their substrate specificity and homology [7]. Some of these MMPs, specifically, MMP-2, MMP-8 and MMP-9, are trapped within the calcified dentin matrix after dentinogenesis. These host-derived proteases contain at least a prodomain and a catalytic domain [7]. In non-activated MMPs, the unpaired cysteine in the prodomain forms a bridge with the catalytic zinc (referred to as the cysteine switch mechanism),

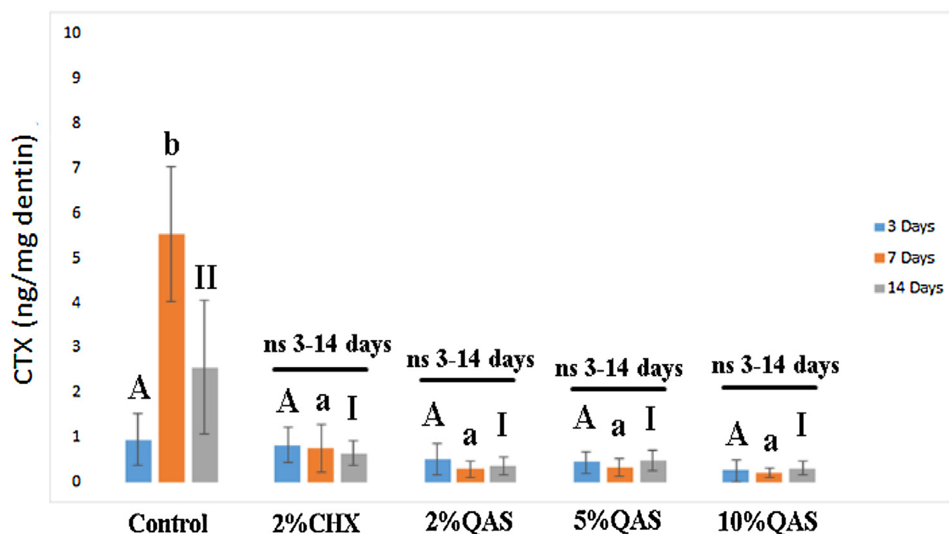


Fig. 5. The amount of CTX telopeptides released per ng/mg dentin from various treatment groups at 3, 7 and 14 days in the incubation medium. Values are means and standard deviations (N = 10). Uppercase letters/lowercase letters/numerals represent differences between each pre-treatment (3rd day/7th day/14th day) ($p < 0.05$); ns: no significant difference.

preventing enzymatic activity and acting as a ligand for the catalytic zinc atom [51]. Dissociation of the prodomain from the catalytic domain results in activation of the MMPs.

Matrix metalloproteinases are activated by the mildly acidic adhesive resins [13,52]. Activated MMP-2, −8 and −9 progressively degrade unprotected collagen fibrils in the hybrid layer, causing deterioration of resin-dentin bonds over time. The collagenase MMP-8 attacks collagen at specific peptide bonds and cleaves collagen molecules into a $^1/_4$ and $^3/_4$ segments [45]. These peptides lose their triple helical conformation, which can then be further degraded by the gelatinases MMP-2 and MMP-9 [6]. Conversely, MMP-2 and MMP-9 attack type I collagen telopeptides to release a long C-telopeptide segment referred to as the ICTP segment, which includes at least two cross-linked telopeptides and the first phenylalanine of the phenylalanine-rich region [12,45,51]. Release of ICTP has been used to examine the MMP inhibitory effect of several quaternary ammonium compounds [40–42].

Effective inhibition of endogenous MMPs by QAS occurs due to several reasons. Firstly, QAS is similar to CHX in that it is cationic with four positive charges. Demineralized dentin has fixed negative charges due to the presence of carboxylic groups in the collagen fibrils and noncollagenous proteins. This results in binding of QAS electrostatically to the insoluble collagen. Furthermore, QAS can hydrogen bond with the carboxyl groups and free amino groups of collagen fibrils. This non-specific binding of QAS may alter the configuration of the active site of MMPs, making them unable to accept the complementary peptide sequence for collagen. Secondly, the catalytic domains of MMPs are cysteine-rich sites, having a glutamic acid residue that contains negative charge [42]. The cationic QAS may bind to the negative charge of glutamic acid residues via electrostatic binding, thereby altering the configuration of the catalytic site of the MMPs, blocking and inhibiting their proteolytic activities. This QAS-MMP complex may make the MMP unable to hydrolyze the specific peptide bond of collagen, thus preserving the integrity of denuded collagen fibrils within the hybrid layer [42,53]. Thirdly, the MMP inhibitory effects of QAS may also be attributed to the binding of metallic ions such as Ca^{2+} and Zn^{2+} by the chloride ions in QAS, which are required for MMP activation.

Cysteine cathepsins, activated by low pH, are also responsible for degradation of collagen fibrils [2,51]. A highly significant correlation between the proteolytic activities of MMPs and cysteine cathepsins has been shown in intact and carious dentin [2,4]. That correlation indicates that the proteolytic activities of dentin are due to synergistic effects between MMPs and cysteine cathepsins. Among the different cathepsins, only cathepsin K has been shown to have both telopeptidase and collagenase activities [43,45]. Cathepsin K is the only mammalian collagenase capable of cleaving the triple helical collagens at multiple sites to completely hydrolyze collagen fibrils into small peptide fragments [9,54]. Cathepsin K is known to cleave non-helical C-terminal telopeptide extensions of fibrillar collagen to release CTX, which is the smaller eight amino acid C-terminal peptide [43].

Release of CTX from demineralized dentin is an indirect measure of cathepsin activity. The active sites of cysteine cathepsins contain cysteine, histidine and aspartane residues, whereas cysteine forms a catalytic thiolate-imidazolium ion pair. The cysteine thiolate acts as a nucleophile for attack on the carbonyl carbon atom of the peptide bond [55]. The cathepsin inhibitory effect of QAS is likely to be electrostatic binding between QAS and the cathepsin active site, providing a direct and potent enzyme inhibition mechanism. There was no difference in CTX telopeptide release of the treatment groups at 7 and 14 days, indicating that CHX and QAS pre-treatments may have completely inactivated the cathespin K activity at pH 7.4.

The ICTP telopeptides released from the treatment groups generally decrease over time. The difference in release profiles of ICTP and CTX may be explained by the difference in molecular mass between ICTP (10,249 Da) [56] and CTX (<3000 Da) [57]. According to the size exclusion characteristics of type I collagen fibrils, small molecules (<1000 Da) can diffuse in and out of the collagen fibrils; while molecules approximating 10,000 Da begin to be excluded from entering or leaving insoluble type I collagen and large molecules (66,000 Da) are completely excluded [58]. Hence, the diffusion of ICTP may be slower than CTX and takes a longer time to diffuse out of the demineralized dentin. The CTX release was around one-tenth that of ICTP, because cathepsin K was operating at pH 7.4, instead of its optimum pH of 5.5 and the results are in agreement with previous studies [43]. Further studies should evaluate the release of CTX by QAS at its optimal pH of 5.5. The 2% QAS also showed significantly less cytotoxicity to human dental pulp stem cells than 2% CHX (data not shown). The long-term stability of resin-dentin bonds in etched dentin specimens that have been treated with the QAS prior to bonding with different etch-and-rinse adhesives will require further investigations.

5. Conclusion

Within the limits of the present study, it may be concluded that the newly developed quaternary ammonium silane, K21, increases the resistance of dentin collagen to degradation by inhibiting endogenous matrix metalloproteinases and cysteine cathepsins. The quaternary ammonium silane is promising for use as a protease inhibitor to improve durability of resin-dentin bonds.

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