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Gelatinolytic activity in dentin upon adhesive treatment

Xin Li[1,2], Jennifer Vandooren[3], Mariano Simón Pedano[1], Jan De Munck[1], Jorge Perdigão[4], Kirsten Van Landuyt[1] & Bart Van Meerbeek[1]✉

In this multi-parameter study, the effect of diverse factors related to adhesive application on the activation of host-derived gelatinases was investigated by gelatin zymography, *in-situ* zymography, fluorogenic DQ-gelatin assay and micro-tensile bond-strength (μTBS) testing. Gelatin zymography disclosed the presence of gelatinases in phosphoric acid-etched dentin powder, while two gold-standard adhesives generated no measurable MMP activation. *In-situ* zymography revealed that the interfacial gelatinolytic activity from specimens treated with the two adhesives appeared similar as that of the EDTA negative control, indicating no detectable gelatinases were activated upon adhesive treatment. In solution, MMP-2/9 activity significantly decreased upon interaction with both adhesives (two-way linear mixed effects model [LMEM]: $p < 0.05$); gelatinases were almost completely deactivated upon 1-week incubation at 37 °C (general linear model: $p < 0.05$); light-curing adhesives increased temperature up to 55 °C, which appeared sufficient to dramatically decrease MMP-2/9 activity (two-way ANOVA: $p < 0.05$). Finally, challenging adhesive-dentin interfaces with highly concentrated MMP-9 (at a much higher concentration than present in saliva) for 1 m did not significantly affect μTBS (two-way LMEM: $p > 0.05$). Taken together, the two adhesives did not activate but rather inhibited the release and activation of dentinal gelatinases.

Keywords Dentin-bonding agents, Matrix metalloproteinase 2, Matrix metalloproteinase 9, Gelatinases, Dental bonding, Biodegradation

Matrix metalloproteinases (MMPs) are calcium-dependent endopeptidases that can cleave almost all components of extracellular matrix, growth-factor binding proteins, receptor tyrosine kinases, cell-surface receptors, and other proteinases[1,2]. On basis of their substrate specificity, sequence similarity and domain organization, MMPs are classified as collagenases, gelatinases, stromelysins, matrilysins, membrane-type and other MMPs[3]. MMP-2 (gelatinase A, 72 kDa) and MMP-9 (gelatinase B, 92 kDa) are gelatinases which can cleave gelatin (denatured type I collagen), type-IV collagen and laminin[4,5]. In addition, MMP-2 and MMP-9 demonstrate a collagenolytic activity, which can degrade type-I and -III collagens in their native forms[6,7].

Gelatinases MMP-2 and MMP-9 are responsible for the remodelling and degradation of the organic dentin matrix, and are therefore actively involved in enamel and dentin development[8], caries progression[9,10], pulpal and periapical inflammation[11], as well as periodontal diseases[12,13]. More recent findings indicate that gelatinases may also be responsible for enzymatic biodegradation of adhesive-dentin interfaces. Such MMP-induced biodegradation may challenge the clinical longevity of adhesive tooth restorations[14–16], because of which various MMP inhibitors have been suggested to counteract enzymatic biodegradation. However, the proportional contribution of enzymatic activity to bond degradation remains unclear[17]. Data regarding bond degradation-retarding/arresting effects upon the use of MMP inhibitors, like for instance chlorhexidine digluconate (CHX) and proanthocyanidins (PA) are not always consistent. A systematic review and meta-analysis reported significant higher bond strength when 2% CHX was applied versus not applied (control) for specimens aged for 12 m, while no significant differences were observed for longer aging periods (> 12 m)[18]. A representative study by Sadek et al. showed that CHX could not prevent bond-strength to decrease, while still not being significant upon 9-m aging. However, a significant reduction in bond strength was detected upon 18-m aging that was no longer significantly different from the bond strength recorded for the experimental group that did not receive CHX treatment[19]. Clinical trials with a follow-up of 18, 24 or 36 m showed that the application of CHX or PA did not improve the clinical performance of adhesive restorations[20–24]. A meta- and trial sequential analysis of randomized clinical

[1]Department of Oral Health Sciences, BIOMAT & UZ Leuven, Dentistry, KU Leuven, Kapucijnenvoer 7, 3000 Leuven, Belgium. [2]Division of Restorative Dental Sciences, Faculty of Dentistry, The University of Hong Kong, Hong Kong, SAR, China. [3]Laboratory of Immunobiology, Department of Microbiology, Immunology and Transplantation, Rega Institute for Medical Research, KU Leuven, Leuven, Belgium. [4]Department of Restorative Sciences, University of Minnesota, Minneapolis, MN, USA. ✉email: bart.vanmeerbeek@kuleuven.be

trials showed that the risk of retention loss or restoration failure was not significantly decreased after CHX pretreatment, by which the authors concluded that "*Dentists can perform cavity pretreatments for inhibition of hybrid layer degradation, but a beneficial effect is not supported by sufficient evidence*" [25].

In our previous study, the effect of the three-step etch-and-rinse (E&R) adhesive Optibond FL ('OFL'; Kerr, Orange, CA, USA) and the two-step self-etch (SE) adhesive Clearfil SE Bond 2 ('CSE2'; Kuraray Noritake, Tokyo, Japan), both well-established adhesives having presented excellent long-term clinical bonding effectiveness within their respective adhesive category, was evaluated[26]. Gelatin zymography identified the presence of MMP-2 in untreated dentin powder, which was more intensively detected upon phosphoric-acid (PA) etching, while CSE2 revealed no gelatinolytic activity[26]. This result seems to contradict a previous study which reported that PA etching of dentin powder reduced gelatinolytic activity to 23%, while several simplified SE adhesives increased gelatinolytic activity[27].

In this study, we aimed to check if any cause-effect relationship between dental adhesive application and activation of dentinal gelatinases can be demonstrated. Co-determining factors that may influence gelatinolytic activity, among which the interaction with adhesives, 37 °C incubation and temperature increase during light-curing of adhesives were evaluated. Finally, the actual degree of involvement of enzymatic activity in bond degradation was investigated.

Methods

Materials

The considered gold-standard three-step E&R adhesive OFL (Kerr) and two-step SE adhesive CSE2 (Kuraray Noritake) were investigated, so the gathered data concerned well-performing adhesives. The composition of the adhesives is listed in the Supplementary Table S1.

Identification of gelatinases in human sound dentin

Healthy human third molars from patients under 25 yrs of age, extracted for orthodontic reasons, were gathered as approved by the Commission for Medical Ethics of KU Leuven under the file number S64350. All experiments were carried out in accordance with the Declaration of Helsinki and informed consent was obtained from all subjects. The extracted teeth were stored at 4°C and used within 1 m after extraction.

Gelatin zymography

Coronal dentin blocks free of enamel, pulp tissue and cementum were prepared using a slow-speed diamond saw (Micracut 151, Metkon, Bursa, Turkey) and a high-speed medium-grit (107 µm) diamond bur (882, Komet, Lemgo, Germany). Dentin blocks obtained from different patients were subsequently pulverized together using a water-cooled analytical grinder (A10, IKA, Staufen, Germany). After grinding, dentin powder was evenly divided into 9 portions and directly processed according to different treatment procedures (Table 1).

In the experimental Group NT, non-treated (NT) dentin powder served as blank control. In Group PA, dentin powder was demineralized/etched with 37.5% PA (Gel Etchant, Kerr) for 15 s, upon which etching was stopped with Tris–HCl. In Group PAA, dentin powder was treated the same as described in Group PA, followed by an additional acetone wash (3 times) to check if acetone itself would influence the outcome. In Group OFL, dentin powder was first treated as described for Group PA, upon which the re-collected dentin powder was mixed manually first with OFL-P for 15 s and then with OFL-A for 15 s (Table 1), followed by an acetone wash (3 times). In Group CSE2, dentin powder was mixed first with CSE2-P for 20 s and then with CSE2-B for 20 s, followed by an acetone wash (3 times), as described above. For Groups NT-1w-a, PA-1w-a, OFL-1w-a and CSE2-1w-a, dentin powder was treated following the same procedures as Groups NT, PA, OFL and CSE2, respectively, this followed by 1-w incubation at 37°C in PBS containing antibiotics ('a'; 1% penicillin–streptomycin, Gibco,

Group	Treatment procedure
NT	150 mg non-treated dentin powder
NT-1w-a	150 mg non-treated dentin powder was incubated in 1 mL PBS containing 1% antibiotics (penicillin–streptomycin, Gibco) at 37 °C for 1 w
PA	150 mg dentin powder was demineralized with 0.126 g 37.5% phosphoric acid for 15 s; etching was stopped with 1 mL 100 mM Tris–HCl and centrifugation (12,000 rpm for 2 min, 3 times)
PAA	150 mg dentin powder was demineralized with 0.126 g 37.5% phosphoric acid for 15 s; etching was stopped with 1 mL 100 mM Tris–HCl and centrifugation (12,000 rpm for 2 min, 3 times), followed by 1 mL acetone wash (centrifugation at 12,000 rpm for 2 min, 3 times)
PA-1w-a	150 mg dentin powder was demineralized with 0.126 g 37.5% phosphoric acid for 15 s; etching was stopped with 1 mL 100 mM Tris–HCl and centrifugation (12,000 rpm for 2 min, 3 times), followed by 1-w incubation in 1 mL PBS containing 1% antibiotics (penicillin–streptomycin, Gibco) at 37 °C
OFL	150 mg dentin powder was demineralized with 0.126 g 37.5% phosphoric acid for 15 s; etching was stopped with 1 mL 100 mM Tris–HCl and centrifugation (12,000 rpm for 2 min, 3 times), upon which the re-collected dentin powder was mixed manually with 105 µL OFL-P for 15 s and 105 µL OFL-A for 15 s, followed by 1 mL acetone wash (centrifugation at 12,000 rpm for 2 min, 3 times)
OFL-1w-a	150 mg dentin powder was demineralized with 0.126 g 37.5% phosphoric acid for 15 s; etching was stopped with 1 mL 100 mM Tris–HCl and centrifugation (12,000 rpm for 2 min, 3 times), upon which the re-collected dentin powder was mixed manually with 105 µL OFL-P for 15 s and 105 µL OFL-A for 15 s, followed by 1 mL acetone wash (centrifugation at 12,000 rpm for 2 min, 3 times) and 1-w incubation in 1 mL PBS containing 1% antibiotics (penicillin–streptomycin, Gibco) at 37 °C
CSE2	150 mg dentin powder was mixed manually with 105 µL CSE2-P for 20 s and 105 µL CSE2-B for 20 s, followed by 1 mL acetone wash (centrifugation at 12,000 rpm for 2 min, 3 times)
CSE2-1w-a	150 mg dentin powder was mixed manually with 105 µL CSE2-P for 20 s and 105 µL CSE2-B for 20 s, followed by 1 mL acetone wash (centrifugation at 12,000 rpm for 2 min, 3 times) and 1-w incubation in 1 mL PBS containing 1% antibiotics (penicillin–streptomycin, Gibco) at 37 °C

Table 1. Dentin-powder treatment procedures in different experimental groups.

Merelbeke, Belgium). Our pilot study showed that 1% penicillin–streptomycin generated no significant effect on MMP-9 activity.

After re-collection, the dentin powder was resuspended in 120 µL extraction buffer (100 mM Tris–HCl [pH = 6.8], 4% sodium dodecyl sulfate [SDS], 20% glycerol, and 200 µg/mL of Bromophenol Blue as tracking dye)[26,28] at 4 °C for 48 h under constant stirring. The samples were centrifuged at 12,000 rpm for 10 min at 4 °C, upon which the supernatants were collected and loaded into the gelatin zymography gels with a 7.5% separating gel, as previously described[29].

After electrophoresis, the gels were washed twice for 20 min with 2.5% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) to remove SDS, upon which they were incubated in the mixture of 50 mM Tris–HCl and 10 mM $CaCl_2$ (pH = 7.5) at 37 °C for 48 h to develop potential gelatinolytic bands. Finally, the gels were stained with 0.1% Coomassie Brilliant Blue R-350 (GE Healthcare, Piscataway, NJ, USA) for 20 min, and next de-stained in methanol/acetic acid. Along with each test, recombinant human MMP-2 (rhMMP-2, cat. No. 902-MP-010, R&D Systems, Lille, France) and recombinant MMP-9 (produced in house as previously described[30]) were loaded as standards to identify the molecular weight (MW) of the test samples. Gelatinases were visible as unstained bands on a blue background. This experiment was repeated with dentin powder obtained from another set of teeth originating from different patients.

Images of the gels were obtained using a digital camera (Canon 1200D, Canon, Tokyo, Japan) and further converted from RGB into 8-bit grayscale using an image-processing program (ImageJ 1.52a, Wayne Rasband, National Institutes of Health [NIH], Bethesda, MD, USA). For each lane, a line parallel to the long axis of the lane was drawn and a plot of pixel gray values along the line was obtained (ImageJ 1.52a).

In-situ zymography

Four extracted healthy human third molars from patients under 25 yrs of age were used. Each tooth was mounted in a gypsum block to facilitate manipulation. The occlusal third of the crown was removed using the slow-speed diamond saw (Micracut 151, Metkon). The tooth was sectioned parallel to the occlusal surface to obtain a 1-mm thick mid-coronal dentin disk. The lower occlusal surface of the dentin disk was ground manually with wet 320-grit silicon-carbide (SiC) paper (Hermes, Hamburg, Germany) for 30 s to produce a standard smear layer simulating clinical bur cutting. Each dentin disk was sectioned vertically into 4 subsections, which were randomly distributed to different treatment groups, including the treatment with the E&R adhesive OFL (Kerr) and the SE adhesive CSE2 (Kuraray Noritake), or without adhesive application (2 subsections for each tooth). The adhesives were applied strictly following the respective manufacturer's instructions, as detailed in Supplementary Table S2. A 2-mm thick layer of flowable composite SDR (Dentsply Sirona, Konstanz, Germany) was applied onto the bonded dentin sections and light-cured for 20 s using a light-emitting diode (LED) light-curing unit (Bluephase 20i, Ivoclar, Schaan, Liechtenstein) in 'high' power mode and a light output of 1200 mW/cm^2, as confirmed regularly during the experiment using a Marc Resin Calibrator (BlueLight Analytics, Halifax, Canada).

Upon preparation, the specimens were stored in distilled water at 4 °C overnight and then cross-sectioned to obtain 1-mm wide slabs using the water-cooled diamond saw (Micracut 151, Metkon). Each slab was glued to a plexiglass slide and subsequently polished using P1200 and P4000 SiC paper (Struers, Ballerup, Denmark) and polishing cloth paper (MicroCloth, PSA, Buehler, Lake Bluff, IL, USA) with 6- and 3-µm diamond suspensions (Kemet, Maidstone, UK) using a grinding/polishing machine (Beta Grinder-Polisher, Buehler). The polishing process was complete when dentin tubules appeared transparent under a light microscope at 40× -magnification (Axio Imager M2, Carl Zeiss Microscopy, Jena, Germany).

Dye-quenched (DQ) gelatin (Invitrogen, Carlsbad, CA, USA), which consists of quenched fluorescein isothiocyanate-labeled gelatin, was used as MMP substrate, and dissolved in water at 1 mg/mL. The gelatin stock solution was diluted with dilution buffer (50 mM Tris–HCl, 50 mM NaCl, 5 mM $CaCl_2$, and pH of 7.4) at a ratio of 1:8. The adhesive-dentin interface was next demineralized with 1% phosphoric acid (ortho-phosphoric acid 85%, AnalaR NORMAPUR, VWR International, Leuven, Belgium) for 10 s and rinsed with distilled water for 1 min. The adhesive-dentin surface was blot-dried with absorbent paper. For each tooth, 50 µL of the gelatin mixture was added individually on top of three specimens treated with the E&R adhesive OFL (Kerr) and the SE adhesive CSE2 (Kuraray Noritake), or without adhesive application. For a remaining specimen without adhesive application, 50 µL of the gelatin mixture including 250 mM ethylenediaminetetraacetic acid (EDTA) was applied, this having served as negative control. The interface was covered with a glass coverslip and the edges of the coverslip were sealed with nail varnish, upon which the specimens were kept overnight in a dark humid chamber at 37 °C.

Hydrolysis of quenched fluorescein-conjugated gelatin, indicative of endogenous gelatinolytic enzyme activity, was examined using a confocal laser scanning microscope ('CLSM') with a magnification of 63× (Leica TCS SP8X, Leica Microsystems, Wetzlar, Germany) at an excitation wavelength of 488 nm and emission wavelength of 530 nm. To compare the fluorescence intensity among different treatment groups, all confocal images were taken at a fixed gain value. To determine this value, confocal images of the negative control group were taken at different gain values (Supplementary Fig. S1). As EDTA inhibits MMPs, there should not be any green fluorescence indicative of gelatinolytic enzymes at the interface. A gain value of 700 V appeared appropriate as hardly any green fluorescence could be observed (Supplementary Fig. S1a and c), while at higher gain values of 800 and 900 V, the green fluorescence observed (Supplementary Fig. S1d, f, g and i) should be indicative of background noise due to the fluorescence property of human dentin, as has previously been reported[31]. Practically, the lack of green fluorescence made it however challenging to ensure that the surface of the specimen stayed in focus while the image was captured. Therefore, we selected 800 V instead of 700 V as the fixed gain value.

Along the adhesive-dentin interface of each specimen, two points that divide the interface into three equal segments were marked and selected as the two central spots where the confocal images were captured. Two-dimensional images captured 1 µm below the specimen-surface focus (where green fluorescence first appeared at the composite-dentin interface) were selected and processed using Fiji ImageJ (NIH).

Influence of adhesives on MMP-2/9 activity

To investigate the activity of MMP-2 and MMP-9 in contact with the adhesives, a sensitive and high-throughput fluorogenic DQ-gelatin assay was used[32]. Recombinant human MMP-2 (rhMMP-2, cat. No. 902-MP-010, R&D Systems) and recombinant MMP-9 (produced in house) were activated by incubation with the catalytic domain of stromelysin-1/MMP-3, as previously described[30]. The two adhesives investigated differ in pH, and in monomer and solvent composition. To differentiate between the influence of pH versus composition, the activated MMP-2/9 was diluted in Milli-Q water with a low buffering capacity and in 1 M Tris–HCl (pH = 8) with an alkaline buffering capacity. Therefore, the results for Milli-Q water may be induced by pH, monomer and solvent components, while the outcomes of Tris–HCl may represent mainly the influence of monomer and solvent components. Subsequently, OFL-P, OFL-A, CSE2-P or CSE2-B was added to the activated MMP-2 or MMP-9 at a volume ratio of 1:3. The mixture was centrifuged at 1000 rpm for 30 s, upon which the supernatant accounting for 3/4 of the volume was collected and the pH was measured a first time using pH-indicator paper (1 pH unit, pH-range 1–14, Merck Millipore, Burlington, MA, USA). The collected supernatant was buffered with 1 M Tris–HCl (pH = 8) at a volume ratio of 1:4, upon which the pH of the buffered solution was measured a second time. DQ-gelatin (Invitrogen) was added to the buffered solution at a concentration of 2.5 µg/mL, the mixture loaded to a 96-well plate (chimney, black, clear bottom, Greiner Bio-one, Frickenhausen, Germany) (n = 3, 100 µL for each well), and fluorescence intensity measured every 30 s for 10 cycles at 37°C (ex. 485 nm/em. 530 nm) using a microplate reader (FL600, Biotek, Highland Park, IL, USA). The slope of the fluorescence values of activated MMP-2 and MMP-9 without adhesive exposure was adjusted to 100%, so to calculate the relative activity of MMP-2 or MMP-9 in contact with adhesives. The test was repeated four times.

Two separate two-way linear mixed effects models (LMEMs) were prepared for Milli-Q water and 1 M Tris–HCl (pH = 8) medium, this with an additional random effect factor to account for each individual test and the significance level set at $\alpha = 0.05$.

Influence of 37 °C storage on MMP-2/9 activity

The activity of MMP-2 and MMP-9 upon 37 °C incubation was assessed using a DQ-gelatin assay[32]. The activated MMP-2 or MMP-9 was used at a concentration of 0.6 nM, upon which 900 µL activated MMP-2 or MMP-9 was prepared for each test. DQ-gelatin (Invitrogen) at a final concentration of 2.5 µg/mL was added to 300 µL enzyme. The mixture was loaded to a 96-well plate (Greiner Bio-one) (n = 3, 100 µL for each well), upon which fluorescence intensity was measured every 30 s for 10 cycles at 37°C (ex. 485 nm/em. 530 nm) using a microplate reader (FL600, Biotek). The remaining 600 µL activated MMP-2 or MMP-9 was incubated at 37°C for 24 h or 1 w (300 µL for each); at the end of incubation, DQ-gelatin was added, upon which the corresponding fluorescence intensity was measured. The slope of the fluorescence values of freshly activated MMP-2 or MMP-9 was adjusted to 100%, upon which the relative activity of MMP-2 or MMP-9 after 24-h or 1-w incubation was calculated. The test was conducted in triplicate.

A general linear model was constructed to model the relative activity in function of the two MMPs and the incubation time subjected to square root transformation, with the significance level set at $\alpha = 0.05$.

Influence of temperature change on MMP-2/9 activity

The temperature change during light-curing of the two adhesives (OFL, CSE2) was measured on the surface of a slide dryer (Klinipath, VWR), which was set at 37 °C to mimic the oral temperature (Supplementary Fig. S2a). A clean piece of parafilm (Bemis, St. Louis, MO, USA) was placed on the surface of the slide dryer and the curing light was mounted into a work-holding device (PanaVise, Reno, NV, USA), this to ensure that the distance between the tip of the curing light and the surface of the parafilm was 2, 4 or 8 mm (Supplementary Fig. S2b). The temperature was continuously recorded using a digital thermometer (Fluke 52II Dual Input Digital Thermometer, Fluke, Everett, WA, USA) during light-curing, after coating the tip of the thermometer with a thin layer of Vaseline (Pannoc, Olen, Belgium) and placing it on top of the parafilm (Bemis). Thereafter, 20 µL OFL-A (Kerr) or CSE2-B (Kuraray Noritake) was transferred onto the parafilm (Bemis) (Supplementary Fig. S2c). The adhesive was light-cured using an LED light-curing unit (Bluephase 20i, Ivoclar) in 'turbo' or 'high' power mode or another light-curing unit (SmartLite Pro, Dentsply Sirona) for 10 s (Supplementary Fig. S2d). The intensity of the light was repeatedly and regularly controlled using the Marc Resin Calibrator (BlueLight Analytics). The temperature change induced solely by the curing light without the application of adhesive was recorded as baseline reference. The test was repeated three times.

After having obtained the temperature-change trend during light-curing, 300 µL activated MMP-2 or MMP-9 was transferred to a 1.5 mL Eppendorf (Aarschot, Belgium) tube to be heated with a thermomixer (Eppendorf ThermoMixer C, Merck) with the temperature set at 37, 40, 50, 60 or 70 °C was used. As it took approximately 60 s to heat up the liquid inside the Eppendorf tube to the same temperature as that of the device, Eppendorf containing activated MMP-2 or MMP-9 was incubated in the thermomixer (Eppendorf ThermoMixer C, Merck) for 60 s, upon which DQ-gelatin (Invitrogen) was added at a concentration of 2.5 µg/mL. The mixture was loaded to a 96-well plate Macro-assay plate (chimney, black, clear bottom, Greiner Bio-one) (n = 3, 100 µL for each well), upon which fluorescence intensity was measured using a microplate reader (FL600, Biotek) every 30 s for 10 cycles at 37°C (ex. 485 nm/em. 530 nm). The slope of the fluorescence values for MMP-2 or MMP-9 incubated at 37°C was adjusted to 100% (reference), upon which the relative activity of the enzymes incubated at 40, 50, 60 and 70°C was calculated. The test was conducted in triplicate.

As the relative activity steeply decreased at a given temperature, the temperature was not transformed, nor included in the model as a linear predictor, but rather as a factor. Therefore, a two-way ANOVA model was constructed to predict gelatinolytic activity in function of the two MMPs and temperature, with the significance level set at $\alpha = 0.05$.

μTBS to dentin

Sixteen non-carious extracted human molars were cleaned using a sterile #15 blade (Swann Morton, Sheffield, UK) and stored in distilled water at 4°C for a period no longer than 1 m after extraction. The teeth were mounted in gypsum blocks to facilitate specimen manipulation. The occlusal third of the tooth crowns was removed at the level of mid-coronal dentin using the slow-cutting machine (Micracut 151, Metkon). A standardized bur-cut smear-layer was produced using the custom-adapted computer-controlled Micro-specimen Former (University of Iowa, Iowa city, IA, USA), equipped with a high-speed medium-grit diamond bur (107 μm; Komet). All dentin surfaces were carefully verified for absence of enamel and/or pulp tissue using a stereomicroscope (Stemi 2000-CS, Carl Zeiss Microscopy). Any residual enamel was removed by extra grinding the surface with a diamond bur.

The two adhesives were applied onto the bur-cut dentin surface strictly following the respective manufacturer's instructions (Supplementary Table S2). The adhesives were light-cured using the polywave LED light-curing unit (Bluephase 20i, Ivoclar) in 'high mode' at an output of approximately 1200 mW/cm^2, as measured and regularly confirmed using the MARC Resin Calibrator (BlueLight Analytics). After adhesive procedures, the surfaces were built up with the micro-hybrid composite Clearfil AP-X (Kuraray Noritake) in 3 layers up to a height of 5 mm. Each increment of max. 2 mm was light-cured for 20 s. Upon completion, the composite block was additionally light-cured for 20 s from each side, so to ensure optimum curing. Thereafter, the bonded specimens were immediately stored for 1 h at 100% humidity (37 °C) and subsequently for 23 h in water (37 °C). Upon 1-d water storage, the roots were removed about 5 mm below the adhesive-dentin interface using the slow-speed diamond saw (Micracut 151, Metkon), upon which a composite build-up was made at the root side using the two-step SE adhesive CSE2 (Kuraray Noritake) and Clearfil AP-X (Kuraray Noritake). These teeth were then glued to a square polymethylmethacrylate plate to section all specimens perpendicular to the interface using a water-cooled semi-automatic diamond saw (Accutom-50, Struers) to obtain rectangular sticks (approximately 12 specimens per tooth: 1 × 1 mm wide, 8–9 mm long).

Half of the specimens were stored in the MMP-assay buffer (50 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$, 0.01% Tween 20), with the other half stored in activated MMP-9 at a concentration of 0.01 μM. The storage solution was refreshed daily. After 1-m storage, each specimen was fixed to a BIOMAT jig using a cyanoacrylate-based glue (Model Repair II Blue, Dentsply-Sankin, Tochigiken, Japan) and stressed at a crosshead speed of 1 mm/min until failure in a universal testing machine (LRX, Lloyd, Hampshire, UK) using a load cell of 100N. The number of pre-testing failures (ptf's) per tooth (interface de-bonding during specimen preparation, but not because of manipulation errors) would be recorded as 0 MPa (no ptf's were recorded). Statistical analysis was performed with two-way LMEM, with the potential variance among individual teeth added to the model as an additional random effect factor and the significance level set at $\alpha = 0.05$.

The mode of failure was determined under a 50× magnification using the stereomicroscope (Stemi 2000-CS, Carl Zeiss Microscopy) and classified as either 'cohesive failure in dentin', 'cohesive failure in composite', 'adhesive failure at the interface', 'cohesive failure in the adhesive resin' or 'mixed failure'. The fractured surfaces (pairs of fractured dentin and composite side) were examined using scanning electron microscopy ('SEM'; JSM-6610LV, JEOL, Tokyo, Japan). Therefore, the specimens were processed using common SEM specimen processing, including fixation, gradual ethanol dehydration, and hexamethyldisilazane (HMDS) drying prior to thin gold-sputter coating (JFC-1300, JEOL).

Results

Gelatin zymography

Similar zymograms were observed in dentin powder originating from two different sets of teeth while having been treated the same way. The result from one representative set of teeth is presented in Fig. 1a. Non-treated dentin powder showed no detectable gelatinases; no obvious difference was detected after 1 w (Fig. 1a: NT, NT-1w-a). In dentin powder etched with PA, MMP-2 in latent and activated forms was identified (Fig. 1b: PA); less distinct gelatinolytic bands corresponding to human pro-MMP-9 and a higher MW MMP-9 (HMW MMP-9) were observed (Fig. 1b: PA, indicated as * and #, respectively). After 1-w incubation, faint bands of activated MMP-2, pro-MMP-2 and human pro-MMP-9 could be detected (Fig. 1a,b: PA-1w-a). In dentin powder processed with two adhesives immediately or upon 1-w storage, no gelatinases were detected (Fig. 1a: OFL, CSE2, OFL-1w-a and CSE2-1w-a).

In-situ zymography

To eliminate the influence of variation in the intensity of background noise among different teeth, we compared the results of dentin disks obtained from the same tooth while following different treatments. Representative confocal and light-microscopy photomicrographs of composite-dentin interfaces produced without adhesive (Fig. 2a-c), with OFL (Fig. 2d-f) or CSE2 (Fig. 2g-i) treatment appeared similar as those for the EDTA negative control (Fig. 2j-l), indicating no detectable gelatinases were activated upon adhesive treatment.

Influence of adhesives on MMP-2/9 activity

The two adhesives differ in pH, monomer and solvent composition. To differentiate the influence of these two factors, MMP-2/9 was diluted in Milli-Q water with a low buffering capacity or 1 M Tris–HCl with an alkaline buffering capacity. Therefore, the results for Milli-Q water may be induced by pH, monomer and solvent components, while the outcomes of Tris–HCl may represent mainly the influence of monomer and

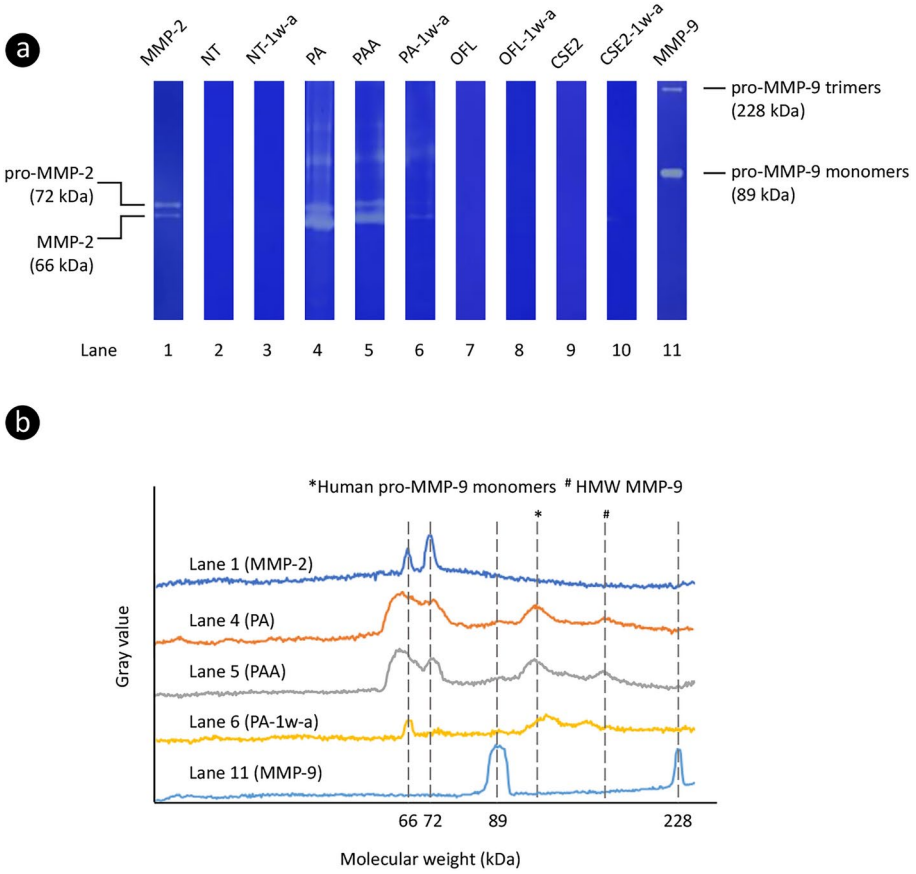

Fig. 1. Gelatin zymography analyses of MMP-2 and MMP-9 in untreated dentin powder or dentin powder treated with the different procedures listed in Table 1, immediately or after 1-w incubation in PBS containing antibiotics (**a**). The images were converted from RGB into 8-bit grayscale and the corresponding gray-values plotted in function of molecular weight (MW) (**b**). The recombinant human pro-MMP-2 and pro-MMP-9 were loaded as standards/references, respectively.

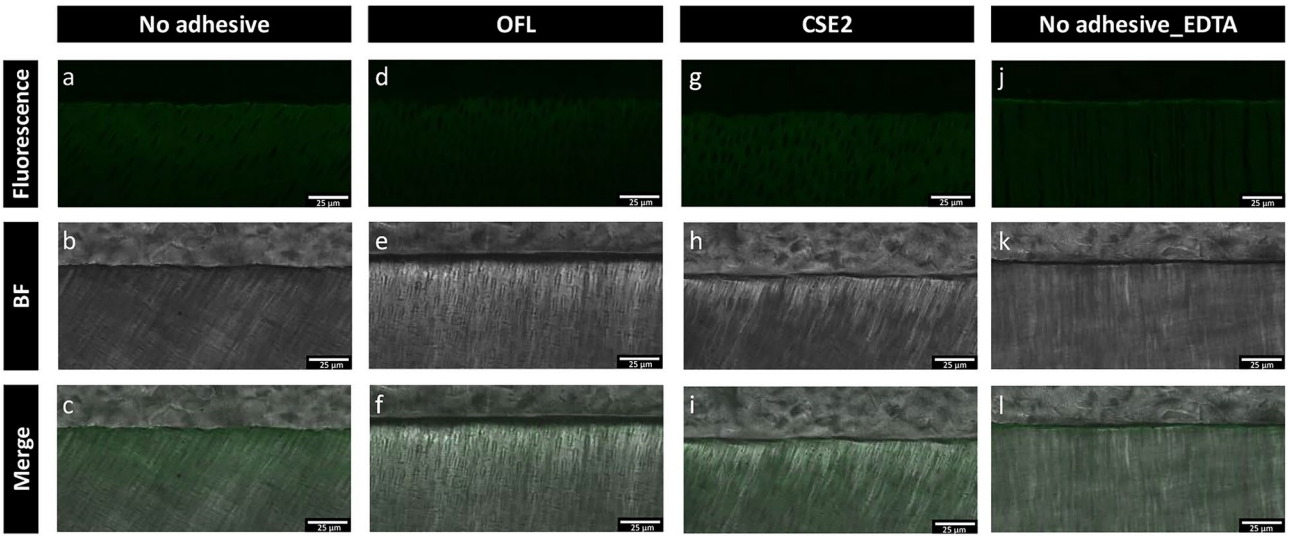

Fig. 2. Representative confocal and light-microscopy photomicrographs of composite-dentin interfaces produced without adhesive (**a**–**c**), with the E&R adhesive OFL (**d**–**f**), the SE adhesive CSE2 (**g**–**i**) or without adhesive and incubated in gelatin mixture containing EDTA as the negative control (**j**–**l**). Images acquired in green channel mode and bright field mode were presented as 'Fluorescence' and 'BF', respectively, with their merged images indicated as 'Merge'.

solvent components. The pH of the Tris–HCl-buffered OFL-P groups was in the range of 4–5, indicating that the buffering capacity of Tris–HCl was not sufficient to change the acidic nature of this mixture. Hence, the corresponding outcome was still generated by a combined effect of low pH, monomer and solvent components.

As the mixture of CSE2-P with MMP-2/9 was turbid and interfered with the measurement, the correlated data were discarded. Upon exposure to OFL-P, OFL-A and CSE2-B, the activity of MMP-2/9 diluted with Milli-Q water was significantly reduced ($p < 0.05$, Fig. 3a). When diluted in Tris–HCl, MMP-2/9 activity upon contact with OFL-P or CSE2-B was significantly reduced ($p < 0.05$, Fig. 3b). With regard to OFL-A, MMP-2/9 activity remained stable ($p > 0.05$, Fig. 3b).

Influence of 37 °C storage on MMP-2/9 activity

Upon 37 °C storage, MMP-2/9 activity decreased significantly over time ($p < 0.05$); complete deactivation was observed after 1 w (Fig. 3c).

Influence of temperature change on MMP-2/9 activity

Light-curing of adhesives at a distance of 2 mm increased the temperature up to 70°C when using Bluephase 20i (Ivoclar) in 'turbo' power mode or SmartLite Pro (Dentsply Sirona) (B_TURBO_OFL, B_TURBO_CSE2 and S_CSE2 in Fig. 3d). For Bluephase 20i (Ivoclar) in 'high' power mode, the highest temperature achieved was slightly lower, but still exceeded 60°C for both adhesives (B_HIGH_OFL and B_HIGH_CSE2 in Fig. 3d). At a distance of 4 or 8 mm, the temperature reached up to 55°C for both OFL (Kerr) and CSE2 (Kuraray Noritake) when using either Bluephase 20i (Ivoclar) in 'turbo' power mode or SmartLite Pro (Dentsply Sirona) (B_TURBO_OFL, B_TURBO_CSE2, S_OFL and S_CSE2 in Fig. 3e,f). At a distance of 8 mm, the highest temperature achieved with Bluephase 20i (Ivoclar) in 'high' power mode was below 55°C for both OFL (Kerr) and CSE2 (Kuraray Noritake) (B_HIGH_OFL and B_HIGH_CSE2, in Fig. 3f). MMP-2/9 activity was significantly affected by temperature; at 60 °C, the activity of MMP-2 and MMP-9 reduced to 32.2% and 15.3%, respectively ($p < 0.05$, Fig. 3g); at 70 °C, the activities further reduced to 11.7% and 9.9%, respectively ($p < 0.05$, Fig. 3g).

μTBS to dentin

1-m MMP-9 challenging generated no significant difference/decrease in μTBS for both adhesives ($p > 0.05$, Fig. 4a). No clear difference in failure pattern was observed; most failures were categorized as 'mixed failure' (Fig. 4b).

Discussion

To identify MMP-2 in human sound mineralized dentin, a previous study extracted dentin proteins from 24 individual permanent molars from patients at the age of 15–73 yrs[33]. Gelatin zymography showed the occasional presence of MMP-2 in latent and activated forms, but this MMP-2 expression was inconsistent and age-related[33]. It was more frequently observed in patients younger than 40 yrs, whereas for patients older than 41 yrs MMP-2 was barely detected[33]. Therefore, healthy human third molars from young patients under 25 yrs of age were used in this study to increase the chance of MMP-2 identification and potential activation. Moreover, to be consistent with the methodology of our previous study[26,28], we used teeth from patients under 25 yrs of age. In addition, we mixed the dentin powder extracted from teeth originating from different patients, this to avoid/limit the influence of variation in MMP-2 expression among individuals.

Although gelatin zymography has widely been used in previous studies, the methodology limitations should be addressed and the results obtained should be interpreted with care. First, the difference in hydrophilicity of dentin powder versus solid dentin may result in different infiltration extents of adhesives. Furthermore, the adhesive-treated dentin powder was tested uncured while coating dentin with resin that is subsequently polymerized, may protect dentin against degradation, as evidenced by the reported decrease in enzymatic activity in cured adhesive-coated dentin powder compared with uncured adhesive-treated dentin powder[27]. Hence, exposure of dentin powder may over/underestimate potential enzymatic activation effects during clinical procedures.

Gelatin zymography showed no detectable gelatinolytic band in untreated dentin powder, while upon 37.5% PA etching, MMP-2 in latent and activated forms, pro-MMP-9 and HMW MMP-9 were observed. This is in part consistent with our previous study that reported the appearance of MMP-2 in untreated and 35% PA-etched dentin powder, the latter at a higher expression level[28]. A possible explanation is that in our study the expression of gelatinases from non-treated dentin powder was very low (under picogram detection limit). PA etching exposed and/or activated gelatinases that resided within the dentinal collagen-fibril network, resulting in a higher detectable gelatinolytic activity. Compared with MMP-2, the gelatinolytic bands for human pro-MMP-9 monomers and HMW MMP-9 were less distinct, which is consistent with the results from another study that showed that the activity of MMP-2 was more evident than that of MMP-9 in demineralized coronal and radicular dentin[34]. Upon contact with OFL (Kerr), gelatinolytic activity of dentin powder disappeared completely or was reduced to a level that was below the detection limit. This should be attributed to enzymatic deactivation/denaturation or inhibition by both the acidic micro-environment induced by OFL-P (pH = 1.46), as well as the monomers and solvents present in the adhesive. This assumption was confirmed by the reduction in gelatinolytic activity when activated MMP-2 or MMP-9 was mixed with OFL-P and OFL-A (Fig. 3a,b). For CSE2, as applied without PA etching, no gelatinolytic activity was measured. Similar results were before observed in dentin powder treated with other SE adhesives, namely Clearfil Protect Bond (Kuraray Noritake) and G-Bond (GC)[28]. Due to the lack of data regarding MMP-2/9 activity upon contact with CSE2-P, the absence of gelatinolytic activity recorded upon application of CSE2 should be related to the combination of low pH and exposure to monomer and solvent components of CSE2-B, while any potential effect induced by CSE2-P cannot be excluded (Fig. 3a,b).

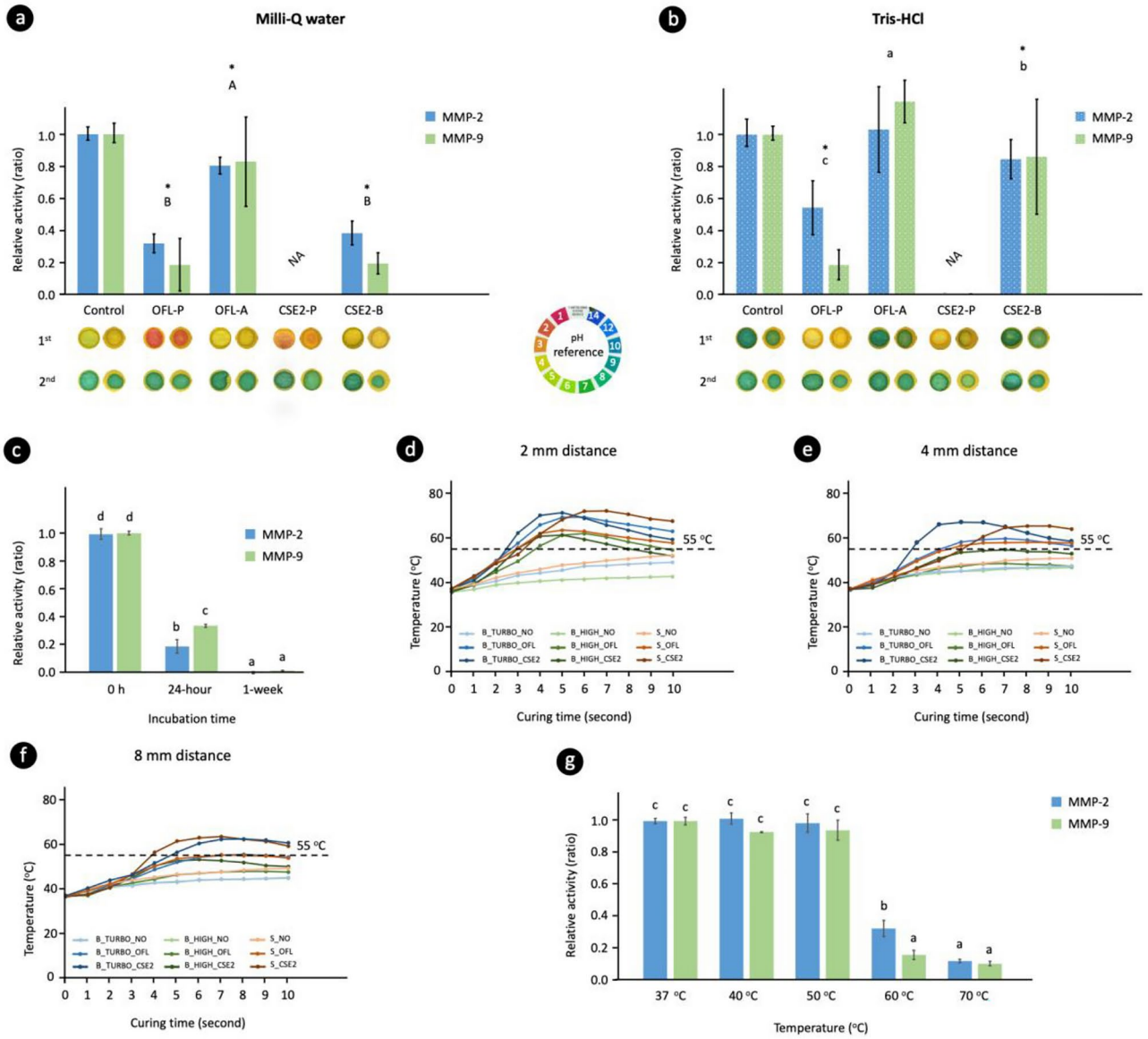

Fig. 3. Relative activity of activated MMP-2/9 diluted in Milli-Q water (**a**) or 1 M Tris–HCl (**b**) upon contact with Optibond FL Prime (OFL-P), Optibond FL Adhesive (OFL-A), Clearfil SE Bond 2 Primer (CSE2-P) or Clearfil SE Bond 2 Bond (CSE2-B) was measured using a fluorogenic DQ-gelatin assay; the slope of fluorescence values for activated MMP-2/9 without exposure to adhesives served as baseline, with the corresponding activities set as 100%. The corresponding pH values of the original and 1 M Tris–HCl buffered mixture were measured a 1st and 2nd time using pH-indicator paper (**a**,**b**). NA: the result of CSE2-P is not available because the mixture of CSE2-P and activated MMP-2/9 was turbid after centrifugation and may interfere with the measurement of the microplate reader. Relative activity of activated MMP-2/9 upon 24-h and 1-w incubation at 37 °C was measured using a fluorogenic DQ-gelatin assay (**c**); the slope of fluorescence values for freshly activated MMP-2/9 served as baseline, with the corresponding activities set as 100%. The real-time temperature change induced by light-curing of the adhesive resins (OFL-A and CSE2-B) at a distance of 2 (**d**), 4 (**e**) and 8 mm (**f**) was monitored using a digital thermometer; the LED light-curing unit (Bluephase 20i, Ivoclar: 'B') in 'turbo' or 'high' power mode, or the LED light-curing unit (SmartLite Pro, Dentsply Sirona: 'S') was used. The change of MMP-2/9 activity in response to temperature increase was measured using a fluorogenic DQ-gelatin assay (**g**); the slope of fluorescence values for activated MMP-2/9 at 37 °C served as baseline, with the corresponding activities set as 100%. Groups labeled with the same letter are not significantly different ($p > 0.05$). Groups denoted with an asterisk (* in **a**,**b**) are significantly different from their respective controls ($p < 0.05$).

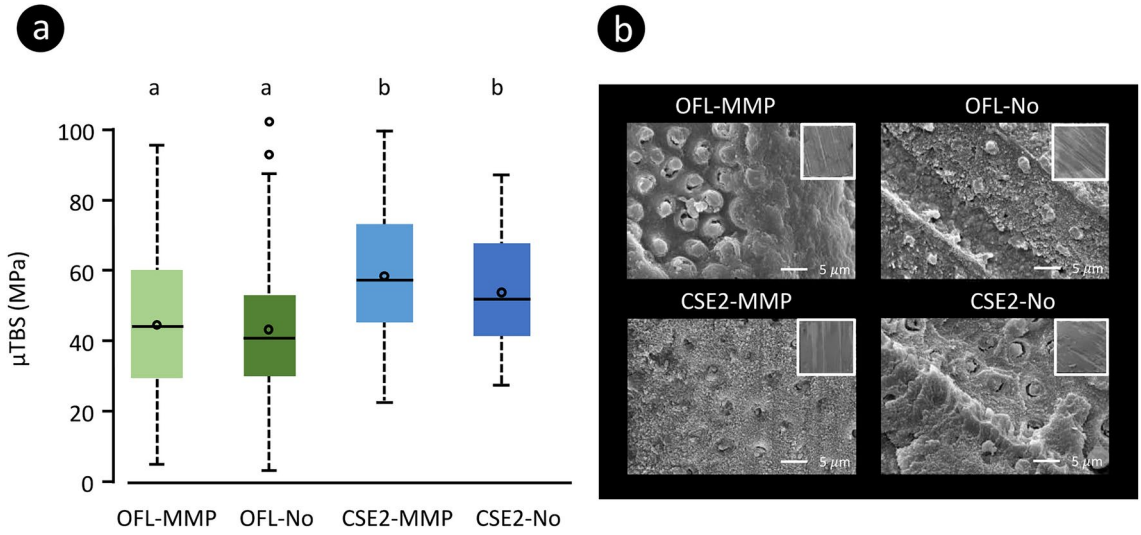

Fig. 4. Box-plot graph presenting the micro-tensile bond strength (μTBS) to dentin (**a**) and representative SEM photomicrographs of fractured surfaces that demonstrated a μTBS close to the mean of the respective experimental group (**b**) for 1-m stored specimens with (OFL-MMP, CSE2-MMP) and without MMP-9 challenging (OFL-No, CSE2-No). The box covers the middle 50% μTBS measured; the horizontal line and the dot in the box represent the median and mean values, respectively (**a**), along with the whiskers presenting the minimum and maximum values (excluding the outliers). Different letters indicate significant difference ($p < 0.05$).

Our gelatin zymography result contrasts with findings from a previous study, which showed that 10% PA etching (pH = 0.4) almost completely deactivated the inherent proteolytic activity of dentin powder[14]. Conversely, treating 10% PA-etched dentin powder with simplified E&R adhesives with a pH range from 2.7 to 3.6 significantly increased proteolytic activity, with a significant negative linear correlation between proteolytic activity and pH of the E&R adhesives[14]. Noteworthy is that the study utilized a collagen-conversion assay by which apart from gelatinases other types of MMPs may have been involved. Simplified SE adhesives with a pH range from 0.9 to 2.4 also significantly increased the gelatinolytic activity of dentin powder, while a positive correlation between gelatinolytic activity and pH of these SE adhesives was observed[27]. Irrespective of a positive or negative correlation, all data suggest that proteolytic/gelatinolytic activity appears pH dependent. However, it should be noticed that pH is not an independent parameter. The nature of the acid, being organic or inorganic, and the differences in chemical components, as present in the etchant and/or adhesive, will provoke different interactions with gelatinases and will all contribute to the overall effect adhesives exert upon contact with dentin substrate. With the intention to simulate clinical procedures, both adhesives investigated were in this study applied strictly following the application instructions recommended by the respective manufacturers. The composition of the etchant and adhesives used, as released by the respective manufacturers, is unfortunately incomplete. Compositional differences should nevertheless be considered when comparing the results of this study with future and previous studies, as besides inorganic phosphoric acid[14] for instance also carbon-based organic acetic acid and citric acid[35] have been used in other enzymatic biodegradation studies.

Overall, the activity of endogenous gelatinases is more complex than previously thought. Apart from acidity, factors such as the nature and composition of the etchant, the monomers and solvents contained in the adhesive may all influence gelatinolytic activity.

One-week (1-w) incubation in PBS containing antibiotics did not change the pattern of gelatinase expression in dentin powder, only the band intensities were much lower, which was probably caused by automatic degradation. This is proven by the reduction in MMP-2/9 activity over time (Fig. 3c). Within the limitations of this *in-vitro* study, the gelatin-degradation assay may not completely reflect the *in-vivo* gelatinase-degradation rate, especially for SE adhesives that partially demineralize dentin, by which collagen fibrils are still surrounded by residual hydroxyapatite (HAp)[36]. Although not much data is available, one study however revealed that *in-vitro* incubation of HAp crystals with MMP-1 and MMP-3 induced autolytic degradation[37]; this degradation was regulated by many factors such as the incubation time, free calcium concentration as well as the crystal concentration. We expect that HAp may also influence the activity of gelatinases, which will be the subject of further investigations. Irrespective of the mechanism involved in this degradation, by using both gelatin zymography and a gelatin-degradation assay we confirmed that the activated gelatinases undergo an automatic degradation over time.

Temperature may also participate in the regulation of gelatinolytic activity, as physiologically relevant temperature cycles (33–37°C) could drive fluctuations in MMP-2/9 activity[38]. Our study documented that light-curing of adhesives at a distance of 2 mm could increase the micro-environment temperature up to 60–70°C, which resulted in a dramatic decline in MMP-2/9 activity (Fig. 3g). A possible explanation is that this high temperature exceeded the thermal transition points of MMP-2/9[39], and therefore, induced denaturation of

gelatinases. At a longer distance of 4 or 8 mm, representing the clinical application in posterior cavities with deep boxes, the highest temperature achieved was slightly lower, but still reached up to 55°C when using either Bluephase 20i (Ivoclar) in 'turbo' power mode or SmartLite Pro (Dentsply Sirona), this is expected to generate a decrease in MMP-2/9 activity.

Regarding *in-situ* zymography, findings should be interpreted carefully, as differences in green fluorescence were detected for the EDTA negative control that included four different teeth (Supplementary Fig. S3). As EDTA inhibits MMPs, there should not be any green fluorescence indicative of gelatinolytic enzymes at the interface. These differences were caused by variation in the intensity of background noise among specimens rather than differences in gelatinolytic activity. Hence, the fluorescence intensity among different groups obtained from different teeth cannot directly be compared. Moreover, the gain value as a relative measure of amplification applied to the detection system influences not only the brightness of the images but also the amount of background noise. It is surprising that in the majority of previous studies, crucial metadata is missing. The gain value selected to capture the images is mostly not mentioned, and it is unclear if different experimental conditions were applied to different teeth, which makes direct comparison of fluorescence intensity not possible, as shown in this study. Additionally, previous research revealed that along the adhesive-dentin interface produced by the E&R adhesive Adper Scotchbond 1 XT (3M Oral Care, Seefeld, Germany), intense green fluorescence was present within the hybrid layer at the left side of presented photomicrograph, while negligible fluorescence was detected at the right side[40]. This discrepant observation could be attributed to variation in background noise, as presented in this study, or to the differences in MMP-2/9 immunoreactivity within the collagen fibrillar meshwork of dentin[35,41]. Apart from this, hardly any green fluorescence was previously observed along adhesive-dentin interfaces produced by OFL (Kerr); fluorescence was concentrated at the top of hybrid layer and even within resin composite[40], while we expect gelatinases, if present, to be active at the bottom of hybrid layer where resin less efficiently envelopes exposed collagen. This finding challenges the reliability and validity of *in-situ* zymography when used to qualitatively examine interfacial gelatinolytic activity.

Apart from endogenous MMPs, exogenous salivary gelatinases may play a role in enzymatic biodegradation of adhesive-dentin interfaces. In healthy participants and patients with periodontal diseases, the concentration of salivary MMP-9 was in the range of 83–131 and 75–385 ng/mL, respectively[42–45]. To maximize the potential effect, we challenged the interfaces with 0.01 µM MMP-9 (equals to 920 ng/mL), which was much higher than the clinically relevant concentrations. As activated gelatinases may undergo autolytic inactivation, we refreshed the medium daily. The exposure period was limited to 1 m due to the high cost of MMP-9 solution (commercial value of 8500 euro). On the other hand, a previous study showed that 1-m saliva microbial aging resulted in approximately 20% bond-strength loss for adhesive-dentin interfaces prepared using Clearfil SE Bond (Kuraray Noritake)[46]. The exposure of adhesive interfaces to neutrophils during a 30-day period, being the same period as we exposed adhesive-dentin interfaces (directly) to MMP-9 (and even refreshed daily) in this study, also resulted in a bond-degradation effect[47]. In another recent study, 0.1 mg/mL clostridium histolyticum collagenase solution was used to challenge adhesive-dentin interfaces prepared with experimental adhesives, and a significant decrease in micro-tensile bond strength was detected also upon 1-m challenging, again involving the same exposure period as in our study[48]. In addition, the specimens were sectioned to obtain rectangular sticks (1 × 1 × 10 mm) to ensure a larger surface area-to-volume ratio and thus to promote direct interaction of daily refreshed MMP-9 with the adhesive-dentin interfaces. Therefore, enzymatic biodegradation, if sufficiently intense, should have been detectable upon 1-m storage. The fact that this direct MMP-9 challenge generated no significant effect on µTBS, should, at least in part, be attributed to the use of (considered gold-standard) adhesives that employ a separate primer to be followed by the application of hydrophobic adhesive resin to stabilize/protect the adhesive interface.

It is important to point out that this study focused mainly on host-derived gelatinases that are bound to the collagen fibril network within dentin, while the effect of other intraoral free proteinases in saliva was only addressed in the µTBS study with daily refreshed MMP-9 exposure for 1 m. As another source of gelatinases, the dentin/pulp complex should also be taken into consideration in future studies. Recent findings indicated that human odontoblasts within the pulp are capable of synthesizing and secreting a wide scale of MMPs[49], but their reactions in response to adhesive treatments are poorly understood and insufficiently investigated. A previous *in-vitro* study indicated that the simplified SE adhesive Xeno III (Dentsply Sirona) generated no apparent effect on MMP-2 activity within dentinal tissue, while more intense MMP-2 activity was observed in the odontoblast layer and predentin[50]. An increase in MMP-2 expression was also observed in human pulp fibroblasts that were exposed to extracts from the SE adhesive TechBond (Isasan, Rovello Porro, Italy) and the E&R adhesive Optibond Solo (Kerr) for 96 hrs[51]. Exposing pulp cells to triethylene-glycol dimethacrylate (TEGDMA), a monomer frequently included in adhesives and resin composites, resulted in a significant increase in the expression of MMP-2 at concentrations of 0.1, 0.2 and 0.75 mM, while the expression of MMP-9 was also significantly upregulated at a concentration of 0.2 mM[52]. Another widely used monomer 2-hydroxyethyl methacrylate (HEMA), however, demonstrated a dose-dependent inhibitory effect on the activity of MMP-2 and MMP-9[53,54].

Noteworthy is that the expression of tissue inhibitors of metalloproteinases (TIMPs), the primary endogenous inhibitors of MMPs, was not assessed in this study. Also, the effect of other relevant MMPs, such as collagenases MMP-1, MMP-8 and MMP-13, remains to be determined.

In summary, it is questionable if the two tested adhesives generate an increase in dentinal gelatinolytic activity, for the following reasons: (1) application of the two gold-standard E&R and SE adhesives reduced MMP-2/9 activity (Figs. 1 and 3); (2) light-curing of adhesive raises the micro-environment temperature, hereby very likely denaturating gelatinases that potentially remained active after adhesive application; (3) expression of MMP-2 in dentin substrate is age-related[33]; we used extracted teeth from patients younger than 25 yrs, which may present with a higher MMP-2 expression; (4) challenging specimens bonded using two gold-standard adhesives with

highly concentrated recombinant MMP-9 for 1 m had no apparent effect on μTBS; and (5) enzymatic activation/stimulation by adhesives, even when existing, would be a one-time event and it is unlikely that these adhesive-activated gelatinases could stay functional over time.

This multi-parameter study puts a different light on the widely documented concept that host-derived gelatinases are released and activated by adhesives, and subsequently initiate biodegradation of hybrid layer and so compromise bond durability. The rationale behind the inhibited dentinal gelatinolytic activity recorded for the considered gold-standard E&R and SE adhesives needs to be explored further, as this contrast with findings documented for other adhesives investigated in previous enzymatic biodegradation research. Undoubtedly, the effect of adhesives on endogenous MMPs is much more complex than previously thought, by which further insight in the multi-factorial interplay between adhesives and adhesion-threatening enzymes is desired.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Author contributions

X.L. contributed to conception, design, data acquisition, analysis and interpretation, drafted and critically revised the manuscript; J.V. contributed to conception, design and data interpretation, and critically revised the manuscript; M.S.P. contributed to data acquisition and interpretation, and critically revised the manuscript; J.D.M. contributed to conception and design, performed all statistical analyses, and critically revised the manuscript; J.P. contributed to conception and design, and critically revised the manuscript; K.V.L. contributed to conception and design, and critically revised the manuscript; B.V.M. contributed to conception, design and data interpretation, and critically revised the manuscript. All authors gave their final approval and agree to be accountable for all aspects of the work.

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Declarations

Competing interests
The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to B.V.M.

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