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A novel dentin bonding scheme based on extrafibrillar demineralization combined with covalent adhesion using a dry-bonding technique

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ABSTRACT

Dentin bonding is a dynamic process that involves the penetration of adhesive resin monomers into the extrafibrillar and intrafibrillar demineralized collagen matrix using a wet-bonding technique. However, adhesive resin monomers lack the capacity to infiltrate the intrafibrillar space, and the excess water that is introduced by the wet-bonding technique remains at the bonding interface. This imperfectly bonded interface is inclined to hydrolytic degradation, severely jeopardizing the longevity of bonded clinical restorations. The present study introduces a dentin bonding scheme based on a dry-bonding technique, combined with the use of extrafibrillar demineralization and a collagen-reactive monomer (CRM)-based adhesive (CBA). Selective extrafibrillar demineralization was achieved using 1-wt% high-molecular weight (MW) carboxymethyl chitosan (CMCS) within a clinically acceptable timeframe to create a less aggressive bonding substance for dentin bonding due to its selectively extrafibrillar demineralization capacity. CMCS demineralization decreased the activation of in situ collagenase, improved the shrinking resistance of demineralized collagen, and thus provided stronger and more durable bonding than traditional phosphoric acid etching. The new dentin bonding scheme that contained CMCS and CBA and used a dry-bonding technique achieved an encouraging dentin bonding strength and durability with low technical sensitivity. This bonding scheme can be used to improve the stability of the resin-dentin interface and foster the longevity of bonded clinical restorations.

1. Introduction

Progress in adhesive dentistry plays a vital role in the practice of operative and conservative dentistry, including pit-and-fissure sealant therapy, orthodontic treatments, intra radicular posts, dentin hypersensitivity, direct aesthetic composite restorations, and other indirect prostheses [1–4]. Although successful bonding to enamel has been achieved, bonding to dentin remains unsatisfactory due to its limited longevity [5–7]. Unlike enamel, dentin contains both inorganic apatite and collagen fibrils with associated non-collagenous proteins. The main component of natural dentin, type I collagen, is reinforced both by the external mineral separating the fibrils and the intrafibrillar mineral within the fibril gaps. During dentin bonding, the minerals are removed by phosphoric acid or acidic monomers and resin monomers are conceived to occupy all the created spaces. In this manner, dentin bonding is a unique form of tissue engineering, in which demineralized dentin collagen serves as a scaffold for the infiltration of adhesive resins [8]. The hybrid, collagen/polymer, three-dimensional network then

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provides a tough and stable link between native dentin tissues and the resin composite [9]. However, this in situ process must be performed within the shortest possible time, making subtle control challenging. Numerous studies have proved that the collagen scaffold was not effectively protected by the infiltrated resin or the original hydroxyapatite [10–12]. Unimpregnated collagen was observed within the bonding interface and was inclined to degradation [13,14], leading to restorative failure and increasing the economic burden.

An ideal bonding interface must not have any structural defects to withstand the challenges inherent to the oral environment, including cyclic loading during mastication, water absorption, and the invasion of other biological destructive factors [15]. To achieve a completely hybrid state, all the demineralization space must be completely replaced by the resin. While the intrafibrillar mineral was removed by acid, without exceptions, complete infiltration into the interfibrillar spaces was impossible [16]. This was because the intermolecular space between microfibrils in a collagen fibril is 1.26–1.33 nm [17], which was too small for large resin monomers to access [18]. In addition, complete impregnation of the extrafibrillar space is also difficult to implement. The collagen scaffold is prone to collapse without the support of intrafibrillar minerals, hampering adhesive resin impregnation when using the simple dry-bonding technique. Water-wet bonding techniques were introduced to maintain the three-dimensional structure of the collagen scaffold for resin infiltration [19]. However, the trapped water molecules could not evaporate completely in a clinical setting, raising the concern of hydrolytic degradation of the bonding interface [20]. Thus, the wettability of wet dentin in water-wet bonding has been debated in clinical settings [21,22]. Conceptually, ethanol wet bonding facilitates infiltration of hydrophobic resins into the interfibrillar spaces, which improves the in vitro stability of the resin-dentin interfaces [23]. While optimized chemical dehydration using an ethanol wet bonding technique can be used to maintain the interfibrillar space for resin encapsulation, it is time-consuming and is too complicated to be performed satisfactorily in a clinical setting [4].

Emerging biomimetic strategies of biomineralization within the resin-sparse intrafibrillar spaces have been suggested to repair the faulty bonding interface [24,25], but these are not yet applicable in everyday clinical practice. Since the intrafibrillar minerals are protected by collagen molecules, partial dentin demineralization has been suggested as a method of increasing the bonding durability. This could be achieved by either reducing the acidity of the phosphoric acid solution or by using a mild demineralization agent, such as 5-wt% phosphoric acid [26] or ethylenediaminetetraacetic acid (EDTA) [27,28]. However, studies have confirmed that type I collagen has a size exclusion effect where molecules with a molecular weight (MW) < 6 kDa are free to penetrate the intrafibrillar space and those with a MW > 40 kDa are excluded [29,30]. EDTA and phosphoric acid have small MWs, such that they can access the intrafibrillar minerals and it is impossible to restrict demineralization to the extrafibrillar minerals during clinical procedures [31]. Recently, an extrafibrillar demineralization protocol involving chelating agents with MWs >40 kDa, including chitosan [32] and glycol chitosan-EDTA [33], was suggested for creating a unique collagen scaffold for adhesive hybridization. The collagen scaffold was then reinforced by the remaining intrafibrillar minerals to resist shrinkage stress, leaving stable, mineral-free, interfibrillar spaces. A strong and stable link between the dentin substrate and prosthesis was produced when combined with a simple dry-bonding strategy, indicating that extrafibrillar demineralization was a promising method for adhesive applications. The improvement in dentin bonding as a result of chitosan-based, extrafibrillar demineralization suggested that its derivatives had the potential to extend the longevity of resin-dentin bonds with short operational times. Compared with chitosan, commercially-available carboxymethyl chitosan (CMCS) is more water-soluble and has a higher affinity for alkali and alkaline earth metal ions such as those of Ca [34,35]. It would be valuable to test the bonding potential of high-MW CMCS within a clinically acceptable timeframe.

Another fundamental factor contributing to the success of in situ engineering is the interaction, both physical and chemical, between the resin monomer and collagen scaffold. Mechanical interlocking between the collagen scaffold and infiltrated resin components was recognized as the main source of adhesion, while additional interactions between these substrates are highly desirable [36,37]. One inspiring example is that functional polymers, such as 10-methacryloyloxydecyl dihydrogen phosphate, can bond ionically to the calcium of hydroxyapatite within the bonding interface. However, only reversible, secondary bonds, such as hydrophobic interactions and hydrogen bonds, were found between the collagen scaffold and adhesive resin monomer [38,39]. In our recent study, we synthesized a dual-function, collagen-reactive monomer (CRM), isocyanate-terminated urethane methacrylate, to target the amino groups on the collagen scaffold to form irreversible covalent bonds [40,41]. The CRM-based adhesive (CBA) achieved high-quality hybridization with phosphoric acid-demineralized collagen, improving the mechanical properties and integrity of the bonding interface [40,42]. However, the hybridization performance of CBA on the extrafibrillar, demineralized dentin remained unclear.

Based on the extrafibrillar demineralization concept and the combined covalent adhesion provided by CBA, a more compact and stronger bonding interface with higher resistance to degradation is expected to be produced using a dry-bonding technique. Thus, in the present study, a novel dentin bonding scheme containing CMCS and CBA was constructed by harnessing the benefits of these two novel principles. The bonding strength of this new scheme was evaluated, and the bonding interface was identified by atomic force microscopy (AFM). Specially, the degradation resistance of the bond was tested. Accordingly, the following null hypotheses were tested: 1) it is not possible to achieve extrafibrillar demineralization with retained intrafibrillar minerals by high-MW CMCS; 2) conditioning dentin with CMCS does not achieve dentin bond strength equivalent to what is achieved using 37% phosphoric acid and the same etch-and-rinse adhesive; 3) there are no differences in the bond strengths generated by the new bonding scheme (CMCS + Dry bonding + CBA) or the traditional bonding scheme (37% phosphoric acid etching + wet bonding + two-step etch-and-rinse adhesive).

2. Materials and methods

2.1. CMCS-based extrafibrillar demineralization

2.1.1. Preparation of CMCS

A solution of 37-wt% phosphoric acid was prepared using 85-wt% o-phosphoric acid solution (Millipore Sigma, USA). CMCS (MW: 100–1000 kDa, Yingxin Lab, Shanghai, China) dissolved in deionized water was processed with Zeba spin desalting columns (40 kDa MW cutoff, Thermo Fisher Scientific, USA) to exclude CMCS with MW < 40 kDa. Then, 100 mg of lyophilized CMCS powder was dissolved in 10 mL of deionized water to achieve a concentration of 1 wt% (pH = 6.7).

2.1.2. Inductively coupled plasma mass spectrometry

Non-carious human teeth were collected based on a protocol approved by the Committee of the Fourth Military Medical University, China. The teeth were stored in 0.9% (w/v) NaCl containing 0.02% sodium azide at 4 °C and used within one month.

The chelation efficiency of CMCS on Ca^{2+} from human dentin was evaluated using inductively coupled plasma mass spectrometry (ICP-MS) (NexIONTM 350D, PerkinElmer, USA). Dentin blocks (2×2×2 mm) were placed on a 96-well plate and supplied with either 200 μL CMCS or phosphoric acid. The dentin specimens were conditioned for 15, 30, and 60 s; then, the Ca^{2+} concentration was detected using ICP-MS in standard cell mode (radio frequency generator power of 1125 W, nebulizer gas flow of 0.945 L min^{-1}, auxiliary gas flow of 1.275 L min^{-1}, plasma gas flow of 16.0 L min^{-1}, sample uptake rate of 0.8 mL min^{-1}, and

integration time of 5 s). The experiment was repeated six times (n = 6).

2.1.3. Transmission electron microscopy

The dentin specimens were cut into 1×1×2 mm blocks and then conditioned with either 37-wt% phosphoric acid for 15 s or 1-wt% CMCS for 30 s. After demineralization, the blocks were rinsed with deionized water three times. For the transmission electron microscopy (TEM) observations, specimens from each group were fixed with Karnovsky's fixative for 12 h, rinsed with 1-wt% phosphate buffer, and postfixed with 1-wt% osmic acid for 2 h. The specimens were then rinsed with phosphate buffer for 20 min, gradually dehydrated by an ascending acetone series (50 wt%, 70 wt%, 80 wt%, 85 wt%, 90 wt%, 95 wt%, and 100 wt %, each for 1 h), and embedded in EMbed-812 resin (Electron Microscopy Sciences, Fort Washington, PA, USA). Unstained ultrathin sections (100-nm-thick) were observed using TEM (T12 Tecnai G2 Spirit Biotwin, FEI COMPANY, USA) at 110 keV.

2.1.4. Field emission scanning electron microscopy

To further characterize the demineralized bonding substrate, four conditioned-dentin specimens from each group were observed using two dehydration protocols to simulate wet and dry bonding, respectively. To create a standard smear layer, 2-mm-thick dentin specimens were polished with 600-grit wet silicon carbide paper for 1 min and then conditioned with either CMCS for 30 s or phosphoric acid for 15 s. Two specimens from each group were fixed with 2.5-wt% glutaraldehyde solution for 4 h, dehydrated by an ascending ethanol series (70 wt%, 80 wt%, 95 wt%, and 100 wt% ethanol, each for 30 min), and immersed in ascending polar hexamethyldisilazane (HMDS)-ethanol solutions (50 wt %, 75 wt%, and 100 wt%, each for 45 min). The other two specimens from each group were blow-dried for 5 s with oil- or water-free compressed gas and then placed in a desiccator for another 24 h. All the dehydrated specimens were sputter-coated with gold (E−1045, Hitachi, Tokyo, Japan) and examined using field emission scanning electron microscopy (FE-SEM) (S-4800, Hitachi, Tokyo, Japan).

2.1.5. Atomic force microscopy

To obtain a flat surface topography, 1-mm-thick dentin specimens were serially polished with #320, #600, #800, #1200, #4000, and #8000 grit wet silicon carbide paper and then processed with W1, W0.5, and W0.25 diamond paste. They were then conditioned with CMCS or phosphoric acid, as described above, dried with filter paper to remove surface moisture, and completely dehydrated in a vacuum desiccator. They were examined using AFM (Keysight 5500, Keysight Technologies, Inc., Santa Rose, CA, USA) in contact mode with a silicon probe (PPP-NCL-20, Nanosensors, Neuchâtel Switzerland), under a resonance frequency of 20 kHz, a spring-constant of 48 N m^{-1}, and a scan rate of 2 Hz. The surface image and roughness of each specimen were acquired.

2.1.6. Zymography of demineralized dentin

The endogenous collagenase activity within demineralized dentin specimens (3×3×1 mm), treated with either 37-wt% phosphoric acid for 15 s or 1-wt% CMCS for 30 s, was investigated. Dentin treated with deionized water served as a blank control. After conditioning, 100 μL of 1 mg mL^{-1} fluorescein-conjugated gelatin (E−12055; Molecular Probes) with an anti-fluorescence degeneration agent (Dapi H-1200, Vectashield, Vector Laboratories LTD) was dropped onto each specimen and covered by a glass slide. After incubation in the dark at 37 °C and 100% humidity for 48 h, excessive fluorescent dye was rinsed with deionized water. The samples were examined with a confocal laser scanning microscope (Fluoview FV1000, Olympus, Japan). The fluorescence intensity was quantified using ImageJ software (Version 1.52a, National Institute of Health, Bethesda, MD, USA).

2.2. Dentin bonding performance of CMCS-mediated extrafibrillar demineralization

2.2.1. Bonding procedure

Dentin bonding was performed under a stimulated physiological intrapulpal pressure (i.e., 10 cm water pressure) [42]. A commercial, two-step etch-and-rinse adhesive, Single Bond 2 (SB2; 3 M ESPE), was used for wet- and dry-bonding. Eighty dentin specimens with a standard smear layer were conditioned with CMCS (30 s) or phosphoric acid (15 s) and then rinsed with deionized water for 15 s. For wet bonding, the rinsed dentin surface was blotted dry with a cotton pellet, leaving a moist surface according to the manufacturer's directions. For dry bonding, the rinsed specimens were blow-dried for 5 s with either oil- or water-free compressed gas before the adhesive coating was applied. Two consecutive coats of SB2 were applied to the dentin surface for 15 s with a fully saturated applicator and then gently air-thinned for 5 s to evaporate the solvent. Finally, they were light-cured using a light emitting diode curing unit (Elipar S10, 3MESPE, St. Paul, MN, USA) for 10 s with an output intensity of 600 mW cm^{-2}. A 2-mm composite build-up was performed using Z250 resin (Filtek Z250, 3 MESPE, St Paul, MN, USA), light-cured according to the manufacturer's instructions. Twenty bonded specimens from each group were randomly assigned to one of two aging processes: storage in deionized water for 24 h or a thermocycling stimulation (5 °C followed by 55 °C for 1 min each, for 30000 cycles).

2.2.2. Microtensile bond strength and FE-SEM observation of bonding interface

The bonded specimen was cut perpendicular to the bonding interface to obtain four 8×1×1 mm sticks adjacent to the center of each tooth. A microtensile bond strength (μTBS) test was performed using a micro tensile testing machine (EZ-TEST 500 N, Shimadzu Co., Kyoto, Japan) [40]. The four μTBS values for the same tooth were averaged (n = 10 teeth for each group).

For bonding interface observations, 1-mm-thick specimens across the bonding interface were treated with 37% phosphoric acid, 5% sodium hypochlorite, fixed, dehydrated, and then scanned with FE-SEM.

2.3. Bonding performance of new adhesive scheme

2.3.1. Bonding procedure

The synthesis of the CRM and CBA formulation was described in our previous study [40,42]. Briefly, CBA contained 45% bisphenol A ethoxylated dimethacrylate (Bis-EMA), 40% CRM, 19% acetone, and 1% photoinitiator. The light-curing ability, cytotoxicity, wetting ability, and hydrophobic property were studied in our previous study. An acetone-based commercial adhesive, Solobond M (SM, VOCO), was used as the control.

The enamel crown and the root of a tooth were removed parallel to the cementoenamel junction, leaving the mid-coronal dentin disk. The disks were further polished with 320-grid SiC paper for 1 min to create a standard smear layer. The teeth were etched with either 37% phosphoric acid for 15 s or 1% CMCS for 30 s, then bonded with a CBA or SM adhesive using either a wet-bonding strategy or dry-bonding strategy. All 160 teeth were randomly divided into eight groups: 1) CMCS+ Wet bonding + SM; 2) CMCS+ Dry bonding + SM; 3) CMCS+ Wet bonding+ CBA; 4) CMCS+ Dry bonding + CBA; 5) Phosphoric acid + Wet bonding+ SM; 6) Phosphoric acid + Dry bonding +SM; 7) Phosphoric acid + Wet bonding + CBA; and 8) Phosphoric acid + Dry bonding + CBA. All the teeth were bonded under water pressure of 10 cm to stimulate pulp pressure, using a device as previously reported [43]. After adhesive application, a 4-mm-thick composite resin (Z250, 3 M ESPE) coating was applied incrementally and light-cured. Ten bonded specimens that formed each group were then removed from the device, stored in water for 24 h, and evaluated for immediate bonding strength. Another 10 bonded teeth from each group were then treated with a

thermocycling aging process (5 °C for 1 min and 55 °C for 1 min, for 30000 cycles). The μTBS values of each group were examined as described in Section 2.2.2.

2.3.2. AFM observations and elastic modulus

Four bonded teeth of each group were sectioned into 4×1×1 mm slices along the long axis of the tooth. Four slices adjacent to the center of each tooth were observed under an atomic force microscope (Keysight 5500, Keysight Technologies, Inc., Santa Rose, CA, USA). The slices were gradient polished with 320, 600, 1200, 2000, and 4000-grit wet SiC papers (1 min for each grid), dried under vacuum, and examined with a silicon probe (PPP-NCL-20, Nanosensors, Neuchâtel Switzerland) in contact mode (resonance frequency of 20 kHz, spring-constant of 48 N/m, and scan rate of 2 Hz). The hybrid layer was identified in the real-time topography image and the modulus of elasticity of each position at an indentation depth of 50 nm was quantified according to a previous study [44].

2.3.3. Nanoleakage test

Three bonded teeth from each group were sectioned into 4×1×1 mm slices along the long axis of the tooth. Nail varnish was applied on the slice surface, except for the 1-mm region surrounding the bonding interface. Then, the coated slices were immersed in 50 wt% ammoniacal silver nitrate solution and placed in the dark for 24 h, immersed into photo-developing solution (8 h), and then processed with fixing solution (8 h). The specimens were sputter-coated with gold and examined using FE-SEM at 10 kV.

2.4. Statistical analysis

All data were analyzed using SPSS 20.0 software with $\alpha = 0.05$. The data were expressed as mean ± standard deviation. For ICP-MS, surface roughness and collagenase activity quantification, one-way ANOVA and Tukey's multiple comparison tests were performed to determine differences among groups after ascertaining the normality and homoscedasticity of the data sets. Three-factor ANOVA and least significant difference (LSD) tests were used to examine the demineralization agent, bonding technique, and aging using the SB2 μTBS values. For each conditioner, two-way ANOVA and was performed to characterize the effects of "bonding mode" and "aging" on bond strength of SB2; we used Tukey's multiple comparisons test for pairwise comparisons.

To examine the effects of the adhesive, bonding mode, conditioner, aging, and their interactions on the dentin bond strength, the μTBS data obtained from section 2.3.1 were analyzed via multi-factor analysis of variance and post hoc Tukey's multiple comparisons test. These parameters were chosen as dependent variables. Shapiro-Wilk test and Bartlett test were performed sequentially and confirmed the normal distribution and the variance homogeneity of the obtained data. Then the μTBS data were analyzed via multi-factor analysis of variance and post hoc Tukey's multiple comparisons test. Two-way ANOVA was performed to evaluate the effects of "adhesive" and "bonding mode" on the elasticity modulus of the bonding interface for each conditioner; we used Tukey's multiple comparisons test for pairwise comparisons.

3. Results

3.1. CMCS-based extrafibrillar demineralization

CMCS extracted Ca^{2+} from dentin in a time-dependent manner (Fig. 1A). Phosphoric acid showed greater demineralization capacity than CMCS in less time ($p < 0.05$). As shown in Fig. 1B, CMCS conditioning for 30 s produced a 1-μm, partly demineralized zone with

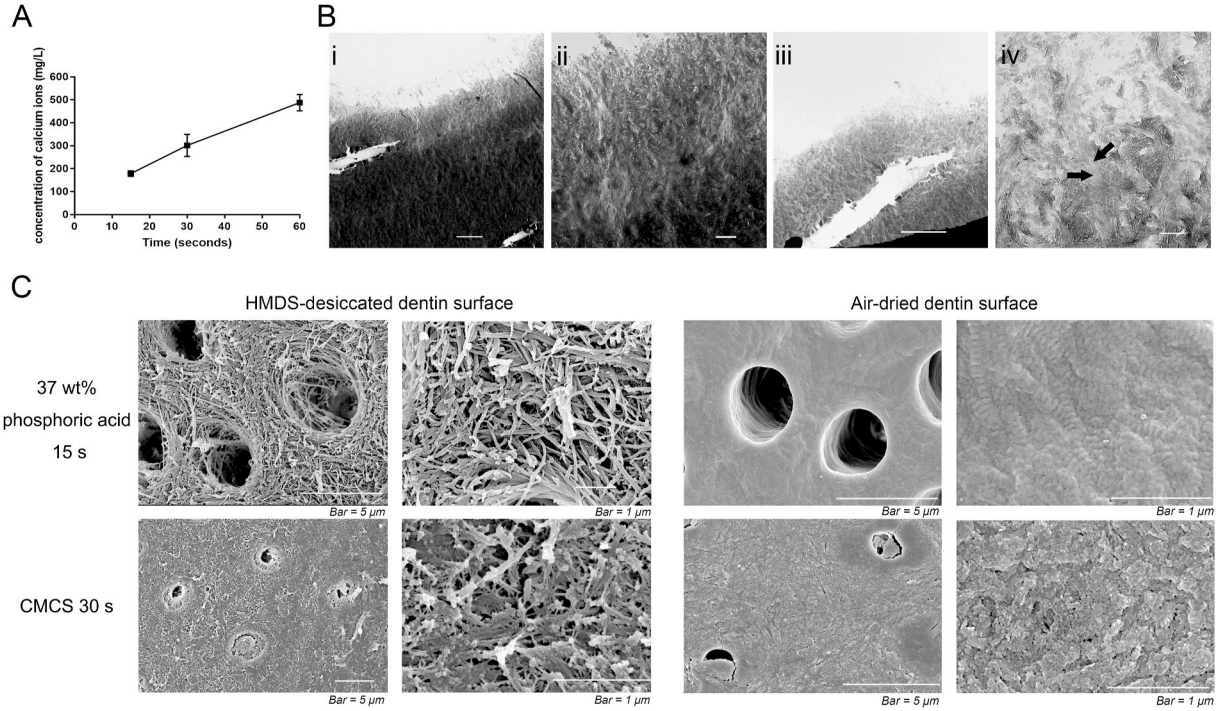

Fig. 1. Calcium-chelating ability of CMCS and characterization of CMCS-conditioned dentin. A. Concentration of Ca^{2+} chelated by 1-wt% CMCS from mineralized dentin within 15, 30, and 60 s. The Ca^{2+} concentration extracted by 37-wt% phosphoric acid within 15 s was 1592 ± 179 g L^{-1} (data not shown). Data are expressed as mean ± standard deviation. B. Representative TEM images of the dentin surfaces after different treatments. (i) Phosphoric acid etching for 15 s created a 5-μm, top-down demineralized layer; bar = 5 μm. (ii) Higher magnification of (i); bar = 1 μm. (iii) Conditioning with 1-wt% CMCS for 30 s; bar = 1 μm. (iv) Higher magnification of (iii); collagen fibrils with remaining intrafibrillar minerals can be seen (arrowheads). C. Representative SEM images of dentin surfaces after different treatments. Left, hexamethyldisilazane (HMDS)-desiccated dentin conditioned with 37-wt% phosphoric acid for 15 s (first row) and 1-wt% CMCS for 30 s (second row); the images on the right are higher magnification images of those on the left. Right, air-dried dentin surfaces conditioned with 37-wt% phosphoric acid for 15 s (first row) and 1-wt% CMCS for 30 s (second row); the images on the right are higher magnification images of those on the left.

interfibrillar gaps and some remaining intrafibrillar minerals. FE-SEM was used to assess whether the bonding interface created by CMCS was suitable for subsequent dry- or wet-bonding. As shown in Fig. 1C, CMCS-conditioned dentin had a less demineralized matrix than phosphoric acid-conditioned dentin, under HMDS-assisted FE-SEM observations. Detectable spaces were evident following CMCS conditioning in both the HMDS-assisted and air-drying modes (Fig. 1C). The smear plugs trapped in the dentinal tubules were partially removed by CMCS. However, phosphoric acid conditioning combined with air drying produced a constringent and smooth interface, which may be ill-suited to adhesive infiltration.

The AFM observations produced similar results for the air-dried CMCS- and phosphoric acid-conditioned specimens (Fig. 2A). The surface roughness of CMCS-conditioned dentin was significantly higher than that of phosphoric acid-conditioned dentin (Fig. 2B). As shown in Fig. 2C and D, the demineralized dentin produced by CMCS conditioning showed less green fluorescence than that produced by phosphoric acid conditioning; this indicated that there was less collagenase activity.

3.2. Dentin bonding performance of CMCS-mediated extrafibrillar demineralization

The μTBS of SB2 under different adhesion modes and aging processes is shown in Fig. 3A. The μTBS values were affected by the demineralization agent, bonding technique, and aging ($p < 0.001$ for all). Statistically significant interactions were identified between the demineralization agent and bonding technique and between the demineralization agent and aging. LSD analyses showed that, after water storage for 24 h, CMCS conditioning achieved μTBS values comparable to phosphoric acid conditioning when the wet-bonding technique was used ($p > 0.05$). There was no significant difference in the μTBS values when CMCS-conditioned dentin was bonded with the wet- or dry-bonding technique ($p > 0.05$). The CMCS dry-bonding group had the highest bonding strength after thermocycling ($p < 0.05$). There were no significant differences between the CMCS dry-bonding groups before and after aging ($p > 0.05$). However, the μTBS value for the phosphoric acid-conditioned dentin decreased dramatically after thermocycling, regardless of the bonding technique (both $p < 0.001$).

As shown in Fig. 3B, there was a clear difference between the CMCS and phosphoric acid groups. Although the bonding interfaces of the CMCS groups were continuous, no obvious resin tag was observed.

3.3. Bonding performance of the novel adhesive scheme

The μTBS values for each group are shown in Fig. 4A. The dentin bond strength was affected by all the test factors, including the adhesive, conditioner, bonding mode, and aging (all $p < 0.001$). Statistically significant interactions were identified for adhesive * aging ($p = 0.042$),

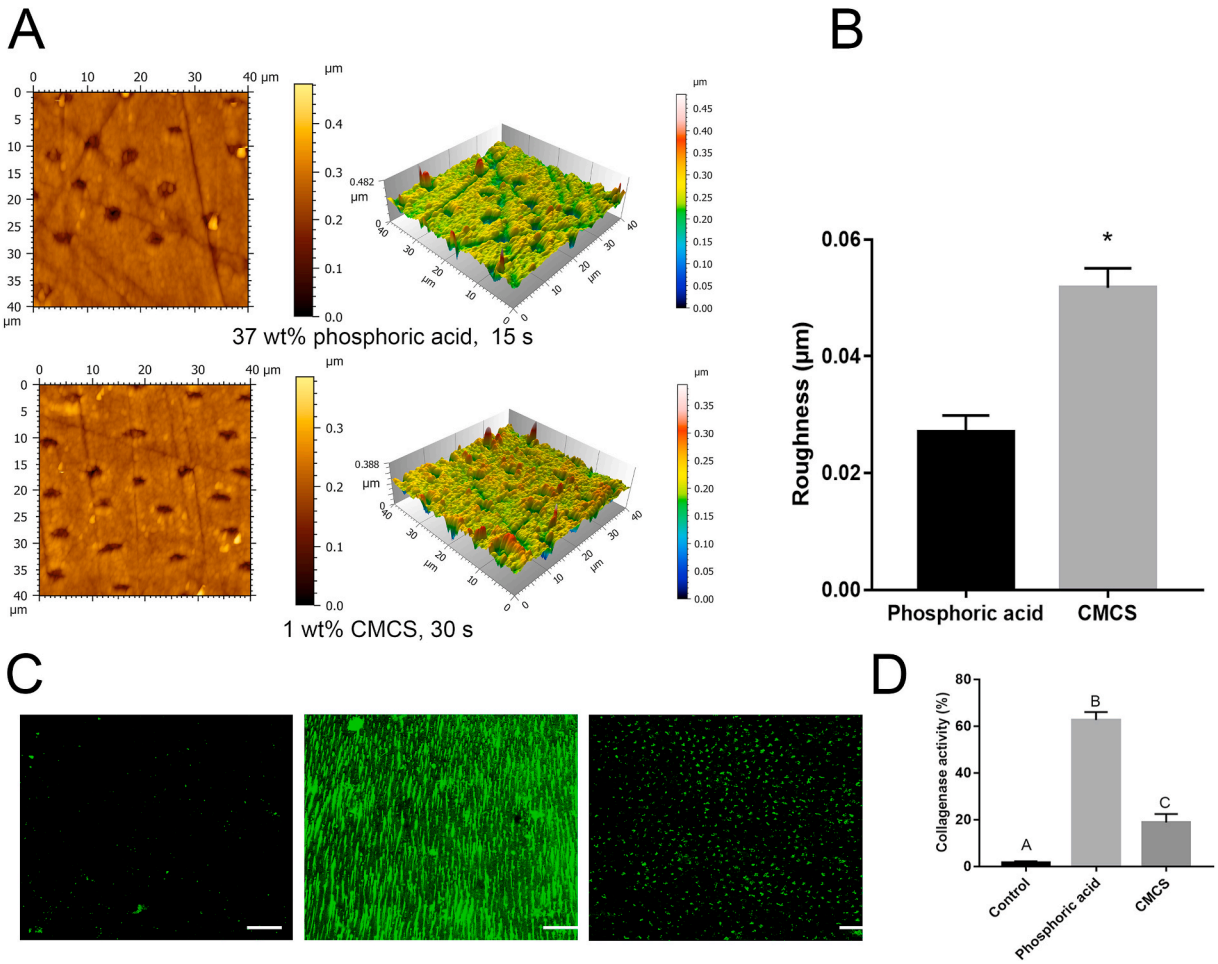

Fig. 2. AFM observations, surface roughness quantification, zymography, and collagenase activity for dentin surfaces after different treatments. A. Representative AFM images and images of dentin conditioned with CMCS for 30 s or phosphoric acid for 15 s; left, surface topography and right, 3D vitalization. B. Surface roughness quantification. Data are expressed as mean ± standard deviation. Asterisk above the column indicates statistically significant differences between the two groups. C. Representative images showing the zymography of dentin conditioned with 37-wt% phosphoric acid (15 s) or 1-wt% CMCS (30 s). Mineralized dentin served as control; bar = 50 μm. D. Quantification of collagenase activity. Data are expressed as mean ± standard deviation. Groups designated with different letters are statistically different ($p > 0.05$).

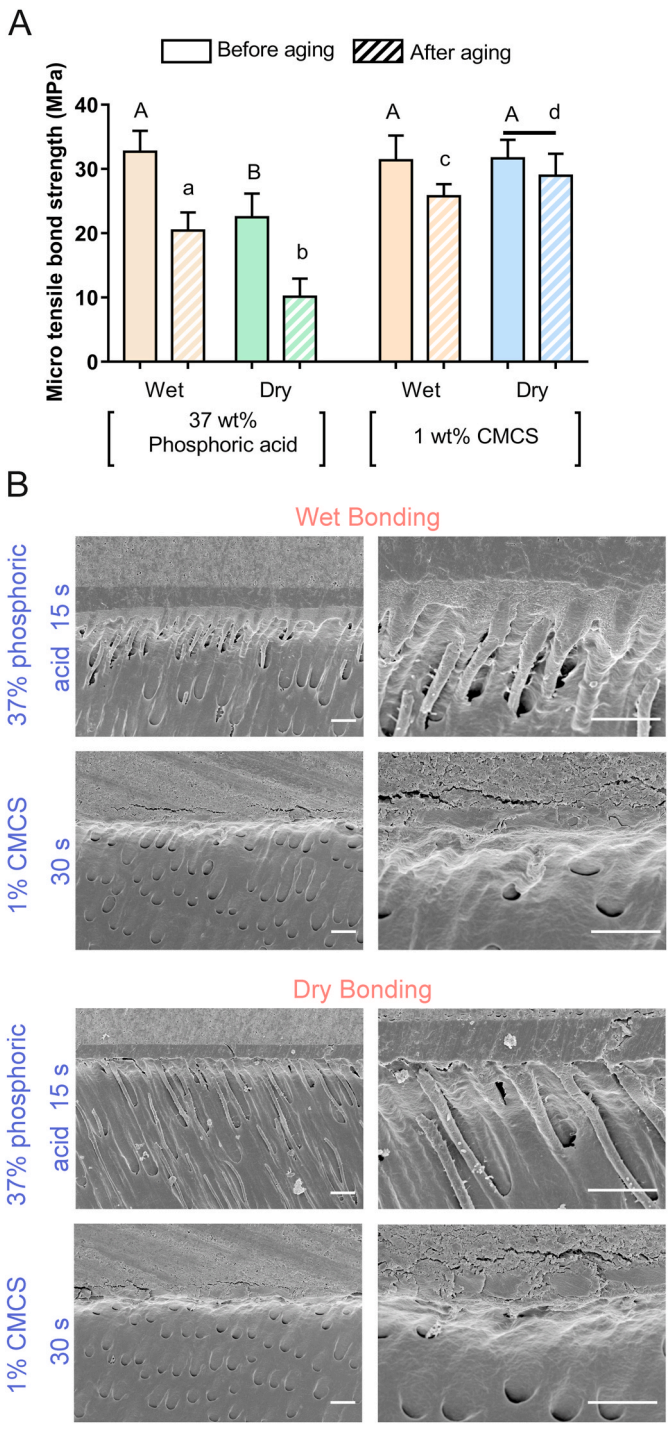

Fig. 3. Bond strength and bonding interface observation of SB2 under different demineralization modes with wet- and dry-bonding techniques. A. μTBS values of SB2 using different bonding modes. Data are expressed as mean ± standard deviation. For μTBS evaluated before aging, groups that are labeled with the same uppercase letter are not significantly different ($p > 0.05$). After aging, groups that are labeled with the same lowercase letter are not significantly different ($p > 0.05$). A horizontal bar indicates that there was no significant difference between the two columns ($p > 0.05$). B. Bonding interface observations. Images on the right are higher magnification images of those on the left. Bar = 10 μm.

conditioner * bonding mode ($p < 0.001$), demineralization agent *aging ($p < 0.001$), bonding mode *aging ($p < 0.001$), adhesive * conditioner * bonding mode ($p = 0.028$), and conditioner * bonding mode * aging ($p = 0.016$). When 37% phosphoric acid was used, CBA showed higher immediate μTBS values than SM for the wet-bonding technique ($p = 0.01$). Dry bonding achieved lower μTBS values than wet bonding for each adhesive when using phosphoric acid etching (both $p < 0.001$). However, different trends were found when phosphoric acid was replaced by CMCS. Regardless of the adhesive used, there was no significant difference between the μTBS values created by wet bonding and dry bonding (both $p > 0.05$). The most favorable μTBS value was achieved by CMCS+CBA, regardless of wet-bonding or dry-bonding techniques. After aging, the highest μTBS value was achieved by CMCS+CBA+dry bonding ($p < 0.05$), and its mean bonding strength decreased by only 11%.

As shown in Fig. 4B, regardless of the type of adhesive, the bonding interface achieved by the CMCS-extrafibrillar demineralization technique did not show any resin tags.

The representative AFM images of the bonding interface are shown in Fig. 5. CMCS combined with CBA created a constant bonding interface. During sample preparation, the area within the bonding interface with different mechanical strengths experienced different degrees of deformation; thus, an abruptness between the adhesive and the underlying dentin was observed in other groups. When phosphoric acid was used as the etching agent, an obvious linear depression occurred within the bonding interface when the dry-bonding protocol was used. However, a more gentle sink originated from the bottom of the adhesive layer and extended to the underlying dentin. The results regarding the elastic modulus of the hybrid layer further confirmed this observation (Fig. 5), in which the highest elastic modulus of the hybrid layer was found in the CMCS-CBA group ($p < 0.05$).

As shown in Fig. 6, the bonding interface of the CMCS groups showed better sealing capability than that of phosphoric acid, as indicated by the low silver deposition. When comparing SM and CBA, CBA showed less silver deposition within the bonding interface than SM when the wet-bonding strategy was used. However, when phosphoric acid-conditioned dentin was bonded with CBA by dry bonding, interface defects were observed for CBA.

4. Discussion

Dentin bonding is a dynamic process involving dentin demineralization and adhesive resin hybridization [8,36]. Currently, dentin bonding is achieved via a micromechanical interlocking mechanism involving the penetration of adhesive resin monomers into the extrafibrillar and intrafibrillar demineralized collagen matrix. However, it is generally recognized that the bonding interface is inclined to degradation and is the weakest link of resin restoration [3,5]. In the present study, we proposed a novel bonding scheme involving CMCS-based extrafibrillar demineralization, additional covalent adhesion by CBA, and a dry-bonding strategy. Our results showed that this combined adhesion scheme achieved the highest immediate dentin bonding strength and bonding durability with a strengthened bonding interface against degradation.

In the present study, CMCS with a MW > 40 kDa was used as a substitute for phosphoric acid to achieve extrafibrillar demineralization. The Ca^{2+} capture capacity of 1-wt% CMCS was confirmed by ICP-MS. The demineralization rate of CMCS was one fifth that of phosphoric acid when the application time was 15 s. As evidenced by TEM, CMCS removed the extrafibrillar minerals but left the intrafibrillar minerals in situ. These results were in line with those of a recent study where CMCS with a high MW was excluded from the fibrils [45]. This necessitates rejection of the first null hypothesis. A 1-wt% chitosan solution prepared with 0.2-wt% acetic acid has previously been used for extrafibrillar demineralization with an application time of 60 s [32]. This study investigated the ability of CMCS to remove extrafibrillar minerals from

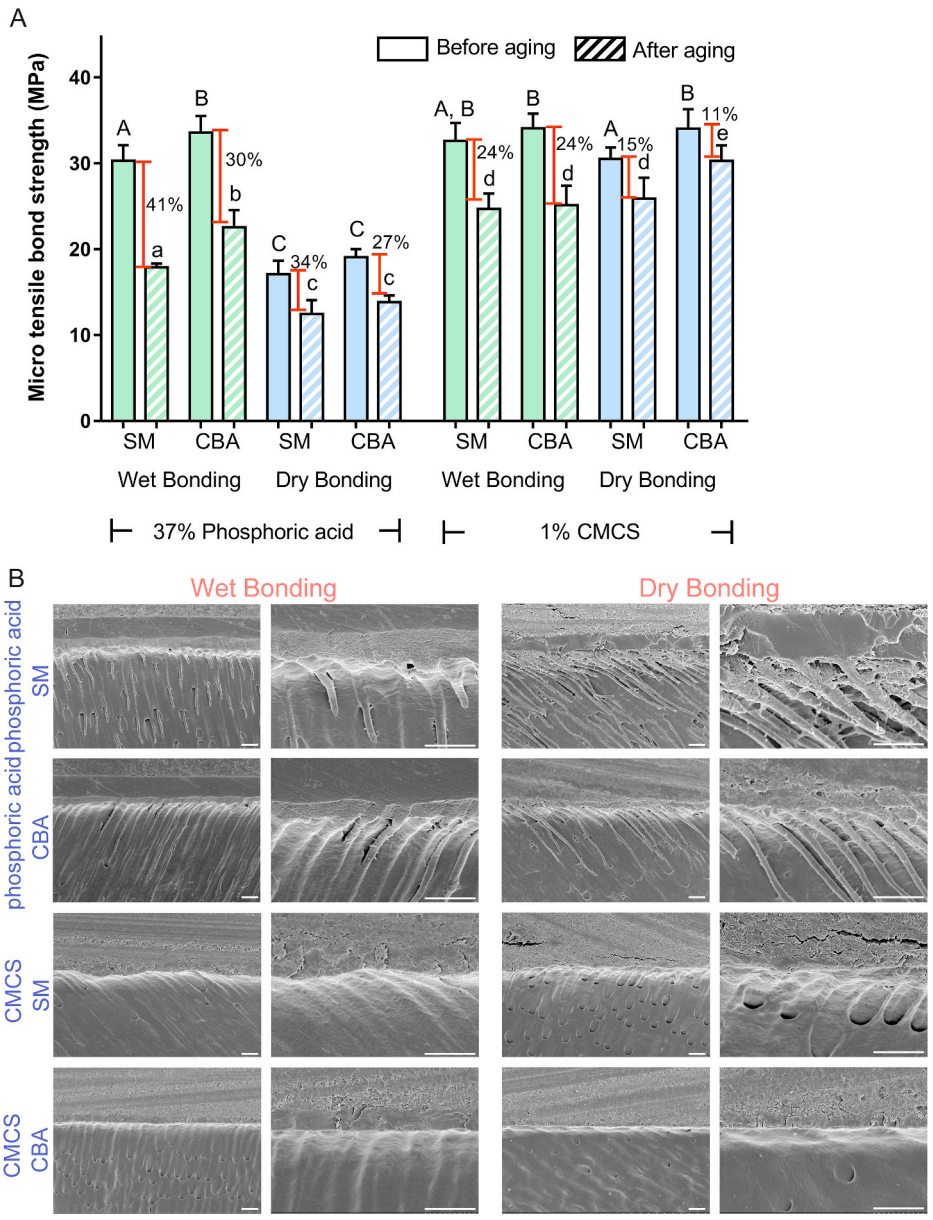

Fig. 4. Bond strength and bonding interface observation. A. μTBS values of different schemes. Data are expressed as mean ± standard deviation. For μTBS evaluated before aging, groups that are labeled with the same uppercase letter are not significantly different ($p > 0.05$). After aging, groups that are labeled with the same lowercase letter are not significantly different ($p > 0.05$). B. Bonding interface observation. Images on the right are higher magnification images of those on the left. Bar = 10 μm.

dentin with a shorter application time, as it had a greater Ca^{2+} capacity than chitosan. This reduced application time is much more user-friendly and will reduce the chance of bonding interface contamination by blood or saliva. Further, CMCS can be dissolved in water at near-neutral pH, which is more environmentally friendly when used in vivo [46,47].

The completely demineralized dentin collagen created by phosphoric acid conditioning lacked the reinforcement provided by intrafibrillar minerals and was inclined to collapse due to the inter/intramolecular hydrogen bonds. After air drying, phosphoric acid-etched dentin showed a smooth surface, which hampered the subsequent infiltration of adhesive resin [48,49]. As shown in Fig. 1, the water-wet bonding technique maintained the integrity of the interfibrillar spaces for inward diffusion of resin monomers. However, it was extremely difficult to completely remove residual water, which hampered the resin encapsulation of denuded collagen fibrils and limited the durability of the bonding interface.

As for the extrafibrillar demineralization technique, CMCS-conditioned dentin appeared to have a significantly rougher surface with visible crevices after severe drying. This indicated that the intrafibrillar minerals that remained within the collagen substrate created by CMCS resisted complete shrinking of the collagen fibrils during drying. As evidenced by the AFM observations, the surface roughness created by CMCS conditioning was considerably greater than that created by phosphoric acid conditioning; this may have improved the dentin wettability and resin spreading due to the capillary force [50,51]. Accordingly, we speculated that the CMCS-extrafibrillar demineralization protocol may be suitable for the dry-bonding technique, which has low technique sensitivity and is more clinically friendly. This was partially confirmed by the dentin bonding test, in which strong and durable dentin bonding was achieved with CMCS-conditioned dentin and two types of commercial adhesives using a dry-bonding technique; this indicated that adequate adhesive infiltration and hybridization occurred. These results were in accordance with those of previous studies [32,33,52].

Matrix metalloproteinases (MMPs)-mediated enzymatic hydrolysis of the collagen scaffold within the bonding interface plays a significant role in dentin-resin bond failure [31]. These host-derived enzymes are embedded in the intrafibrillar mineral and remain inactive [53]. During the traditional etch-rinse and self-etch procedure, these enzymes are released and activated in the acidic environment, initiating a cascade of

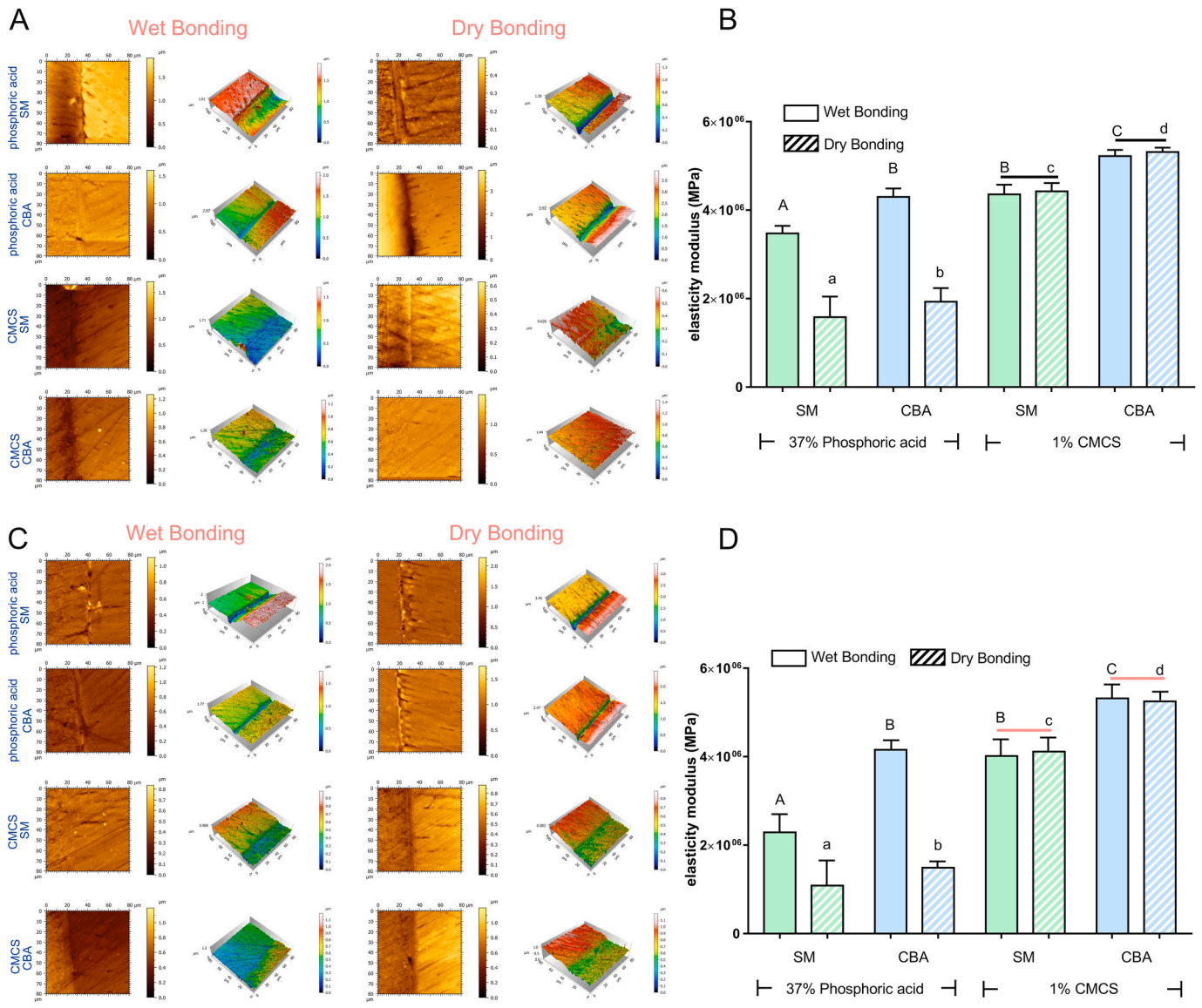

Fig. 5. AFM observation and elasticity modulus of the bonding interface. Representative AFM images of the bonding interface before (A) and after aging (C). Quantification of elasticity modulus of the hybrid layer before (B) and after aging (D). Data are expressed as mean ± standard deviation. Groups that are labeled with the same capital letter are not significantly different ($p > 0.05$). When the wet-bonding technique was used, groups that are labeled with the same capital letter are not significantly different ($p > 0.05$). When the dry-bonding technique was used, groups that are labeled with the same lowercase letter are not significantly different ($p > 0.05$). A horizontal bar indicates that there was no significant difference between the two columns ($p > 0.05$).

proteolytic degradation of the collagen scaffold [54–56]. Inspired by the organic component of natural dentin that maintains a steady level, a less destructive alteration on dentin should be adopted to maintain the organic collagen at steady state. This was achieved by the present extrafibrillar demineralization technique. The zymography of the CMCS-conditioned dentin indicated that the MMPs were fossilized by the remaining intrafibrillar minerals and remained inactivated in the CMCS-extrafibrillar demineralized dentin, leading to an insignificant amount of collagenase activity. This may prevent enzyme-mediated interfacial degradation from the source.

As shown in Fig. 3A, the bonding strength of CMCS-conditioned dentin was not lower than that of 37% phosphoric acid conditioned dentin. Thus, the second null hypothesis has to be rejected. The fact that the immediate bonding strength of the CMCS-conditioned dentin was acceptable when using both the wet- and dry-bonding techniques was also attributed to the reserved intrafibrillar mineral within the bonding interface, which allowed it to withstand shear stress. The relatively low content of intrafibrillar minerals (25–30 wt%) within dentin had a profound effect on its mechanical properties [57]. The intrafibrillar minerals that remained after CMCS conditioning may have prevented collagen shrinkage during resin infiltration and increased the stability of the collagen matrix [28]. The collagen fibrils with the preserved intrafibrillar minerals were assumed to improve the mechanical strength of the integrated structure. In contrast, the intrafibrillar, mineral-free, collagen components left by phosphoric acid conditioning suffered a significant deterioration in mechanical properties, which resulted in a weaker integrated structure. The bonding strength of the phosphoric acid-conditioned dentin showed a sharper decrease after thermocycling than that of CMCS-conditioned dentin, irrespective of the bonding technique; this indicated the importance of intrafibrillar mineral retention and MMP inactivation in collagen for bonding durability [52–54]. Improved dentin bonding durability and inhibition of MMP-mediated collagen degradation of the bonding interface were also observed in previous studies, in which intrafibrillar minerals were retained by EDTA [27,58].

Accordingly, improvement of dentin bonding with SB2 by CMCS-

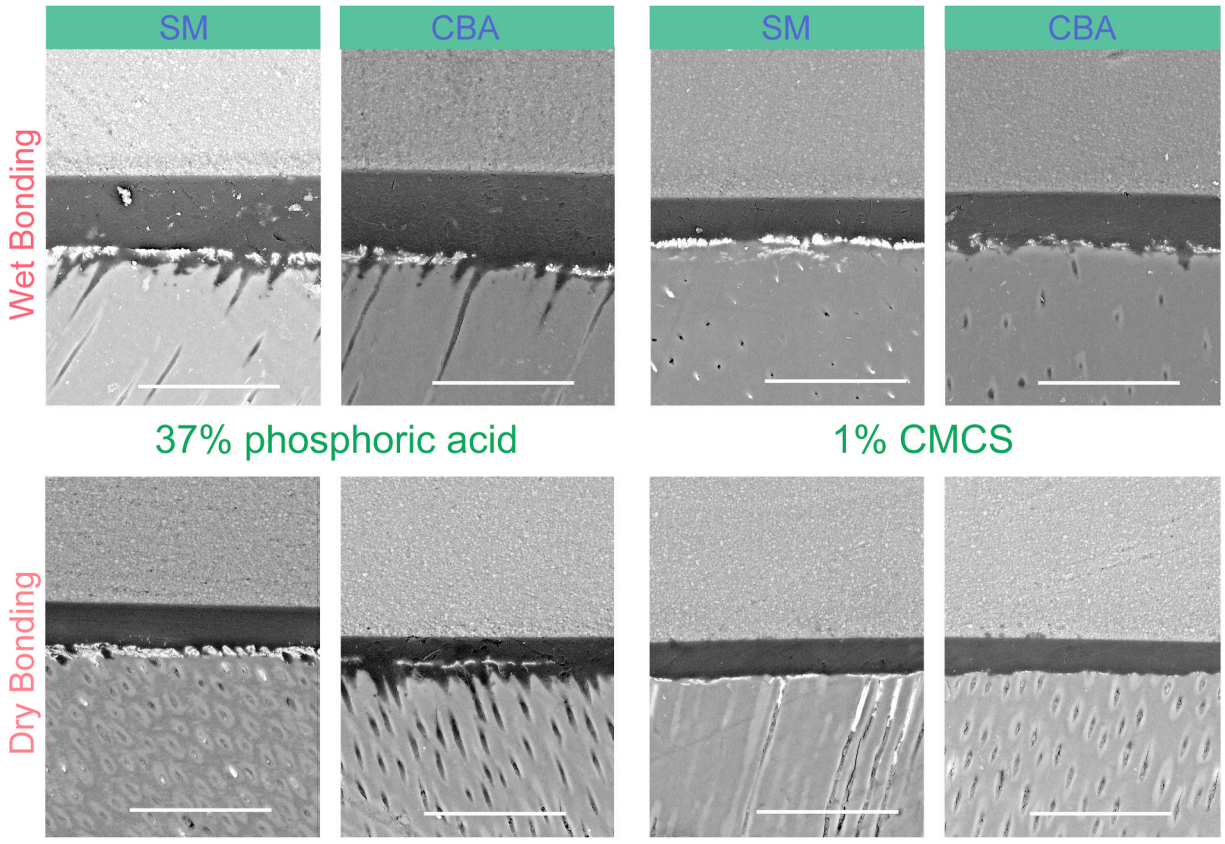

Fig. 6. Leakage level of bonding interface. Representative SEM images of bonding interfaces created by different bonding modes. Bar = 50 μm.

extrafibrillar demineralization using a dry-bonding technique justified evaluation of the potential of various adhesives for extending the longevity of resin-dentin bonds. Herein, we evaluated the effects of using two combined acetone-based adhesives: CBA with chemical bonding properties and a commercialized SM adhesive. Our results showed that both CBA and SM achieved higher immediate bonding strength values when using the CMCS-dry bonding technique, compared to those when using a traditional, phosphoric acid, dry-bonding technique. Acetone is the main solvent in these two adhesives, which is not able to re-expand shrunken demineralized collagen due to its low H-bonding capacity [59]. Thus, these results further verified that CMCS-conditioned collagen matrix remained expanded for adhesive infiltration and achieved a strengthened and stable bonding interface without the help of a wet-bonding technique. More encouragingly, while a sharp decrease was observed when using the traditional etch-rinse scheme for each adhesive, the bonding strength achieved by the new adhesion scheme remained stable after thermocycling, indicating less water-mediated hydrolytic degradation of the resin-dentin bond.

In the present study, SM achieved a similar immediate bonding strength to CBA when using the CMCS-dry bonding technique, while its strength was significantly lower than that of CBA after thermocycling. CBA combined with CMCS-dry bonding achieved the highest bonding strength and the lowest rate of decline. Based on these results, the third null hypothesis that "there are no differences in the bond strengths generated by the new bonding scheme (CMCS + dry bonding + CBA) or the traditional bonding scheme (37% phosphoric acid etching + wet bonding + two-step etch-and-rinse adhesive)" has to be rejected. Further, the elasticity modulus of the CBA-bonded, CMCS-conditioned dentin was considerably higher than that of the SM-CMCS group. When the two acetone-based adhesives, CBA and SM, were applied using the same bonding technique, CBA always achieved a higher bond strength and stability than SM, except when using phosphoric acid and a dry-bonding mode. These results partially confirmed the benefits of an additional covalent adhesion between the collagen scaffold and adhesive resin for dentin bonding. For CBA, terminal, light-curing, double bonds were introduced to collagen with the help of the isocyanate group of CRM, leading to the copolymerization of the collagen scaffold with methacrylate adhesive resin. An integrated, hybrid, collagen/polymer network was expected to be produced with no gaps between the collagen and resin components using this method [40,41]. These results were in line with those of our previous study, in which CBA achieved a more integrated and stable interface with a reinforced collagen scaffold by using CRM [42]. Accordingly, when the bonding interface was strengthened by the remaining intrafibrillar minerals and the additional covalent adhesion, derivable dentin bonding durability was achieved in this study.

An ideal dentin bonding interface was only formed if the demineralized space was completely replaced by resin components. However, trapped water within the formed hybrid resin/collagen composite, either the residual water caused by wet bonding or water originating from the adhesive solvent or the underlying natural dentin, has long been recognized as a major initial factor resulting in interface degradation [60]. Bond durability is unlikely to improve if this critical barrier to progress in dentin adhesion is not overcome [20]. In the present study, AFM was used to observe the surface topography of the dehydrated bonding interface [42]. During the sample preparation procedure, the hybrid layer collapsed to some extent due to the evaporation of residual water. As evidenced by the AFM observations, the immediate bonding interfaces created using the traditional etch-rinse strategy showed abrupt sags between the cured resin and underlying natural dentin. However, in this study, this issue was addressed by using the extrafibrillar demineralization technique combined with the dry-bonding technique; when adhesives were applied onto the CMCS-conditioned dentin surfaces, continuous and smooth bonding interfaces were observed. After thermocycling, the degraded components within the hybrid layer were replaced by water, as evidenced by

the collapsed morphology in the AFM observations and the silver nanoparticle infiltration in the leakage test. The CAB-CMCS-dry bonding scheme created a bonding interface with the highest elasticity modulus, lowest water content, and minimal leakage level. These results indicated that the novel bonding scheme reduced the residual water content of the adhesive interface and avoided the adverse effect of water on the adhesion durability.

Adhesive development has been moving toward simplified and less time-consuming bonding steps since the early 1950s; stable dentin-resin bonding has always been the pursuit of adhesive dentistry, based on the current state of the art [1]. In this study, the novel adhesion scheme using a 30 s CMCS-condition and 10 s blow-drying was user-friendly with no requirement for fine control of the humidity of the collagen scaffold. Otherwise, this promising result justified the amelioration of other chelating agents with higher MW to decrease the conditioning time or to achieve other appropriate biological properties, such as antibacterial, remineralization, enzymatic, inhibitory, and therapeutic capabilities. In addition, with the promising dentin bonding performance of CBA, a prolonged aging process should be performed to weight the contribution of combined covalent adhesion with collagen to bonding durability.

5. Conclusion

Within the scope of this study, extrafibrillar demineralization by 1-wt% CMCS achieved a promising dentin bonding performance within a clinically acceptable timeframe. Chelating demineralization with high-MW CMCS reduced the damage to natural collagen, decreased the activation of in situ MMPs, improved the shrinking resistance of demineralized collagen, and thus provided bonding that was stronger and more durable than that afforded by traditional phosphoric acid etching. The novel dentin bonding scheme containing 1% CMCS and CBA using a dry-bonding technique achieved an encouraging dentin bonding strength and durability. The extrafibrillar collagen demineralization technique and CBA are promising for the combined use of a less technical, sensitive dry-bonding strategy. The novel dentin bonding scheme can be used to improve the stability of the resin-dentin interface and foster the longevity of bonded clinical restorations.

CRediT authorship contribution statement

F. Yu: Conceptualization, Methodology, Investigation, Validation, Formal analysis, Writing – original draft. **M.L. Luo:** Methodology, Investigation, Validation, Formal analysis, Writing – original draft. **R.C. Xu:** Methodology, Investigation, Validation, Formal analysis, Writing – original draft. **L. Huang:** Validation, Formal analysis, Resources, Visualization. **H.H. Yu:** Validation, Resources. **M. Meng:** Validation, Resources. **J.Q. Jia:** Validation, Resources. **Z.H. Hu:** Validation, Resources. **W.Z. Wu:** Resources, Visualization. **F.R. Tay:** Conceptualization. **Y.H. Xiao:** Conceptualization, Writing – review & editing, Supervision. **L.N. Niu:** Conceptualization, Writing – review & editing, Supervision. **J.H. Chen:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare no competing interest.

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