Mussel-inspired polymer with catechol and cationic Lys functionalities for dentin wet bonding

Zunhan Hu a,1, Wenzhen Wu b,1, Meizhe Yu c,1, Zhi Wang a, Zhenyu Yang d, Xiaodong Xing c, Xiaofang Chen e, Lina Niu d, Fan Yu d,*, Yuhong Xiao a,*, Jihua Chen f,*

a Department of Stomatology, 920 Hospital of Joint Logistics Support Force, PLA, Kunming, 650032, China
b The First Dongguan Affiliated Hospital, Guangdong Medical University, Dongguan, 523710, China
c College of Chemical Engineering, Nanjing University of Science and Technology, Nanjing, 210094, China
d National Clinical Research Center for Oral Diseases & Shaanxi Key Laboratory of Stomatology, Department of Prosthodontics, School of Stomatology, The Fourth Military Medical University, Xi’an, 710032, China
e Department of Stomatology, Chongqing Yubei District People’s Hospital, Chongqing, 401120, China
f Department of Stomatology, 927 Hospital of Joint Logistics Support Force, PLA, Pu’er, 665000, China

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ABSTRACT

Mussel-inspired polymers have been evaluated extensively in the field of underwater bonding, which remains a difficult task that was once considered impossible for a man-made adhesive [9-11].

At present, composite resin restoration based on dentin–resin bonding technology has become the primary method for repairing tooth defects because of its good immediate adhesion performance, excellent aesthetic effect, and minimally invasive nature [12,13]. Similar to mussel adhesion, dentin bonding occurs in wet conditions in which water from saliva, dentinal tubules, rinse fluids, and dental equipment is in contact.

1. Introduction

Mussel-inspired polymers have been evaluated extensively in the field of underwater bonding, which remains a difficult task that was once considered impossible for a man-made adhesive [9-11].

At present, composite resin restoration based on dentin–resin bonding technology has become the primary method for repairing tooth defects because of its good immediate adhesion performance, excellent aesthetic effect, and minimally invasive nature [12,13]. Similar to mussel adhesion, dentin bonding occurs in wet conditions in which water from saliva, dentinal tubules, rinse fluids, and dental equipment is in contact.
with the dentin substrate. However, unlike mussel bonding, long-lasting dentin bonding, to which water is considered the main barrier, is difficult to achieve [14–16]. In general, dentin bonding is a process in which exogenous resins penetrate the demineralized collagen network [12,13]. The presence of an adequate amount of water within the demineralized dentin substrate can help maintain the three-dimensional structure of the collagen matrix and facilitate adhesive infiltration; however, excess water may hinder close contact between the adhesive monomers, thereby impeding complete hybridization. Under clinical conditions, precisely controlling the water content in the dentin matrix is impossible. Moreover, after hybridization is complete, it is difficult to remove excess water from the bonding interface. This residual moisture in the cured bonding interface can induce a series of degradation processes and undermine the durability of the bonding interface [17]. Recently, chemical dehydration protocols and extrabrilharial demineralization techniques have been introduced to address water-associated issues during dentin bonding; however, limitations still exist [16–21].

Considering the similarities between dentin bonding and mussel adhesion, researchers have attempted to use mussel-inspired polymers to achieve reliable dentin bonding. The dynamic underwater bonding process by mussels is initiated with the deposition of the vanguard mussel foot catecholic proteins; i.e., protein-3 (Mfp-3) and Mfp-5, on the wet surface, which form an interfascial adhesion layer [3,22]. These catecholic proteins play a vital role in mussel bonding as the catechol moiety can form various chemical bonds with the substrate, including hydrogen bonds, Michael additions, cation–π interactions, and π–π coupling [23–28]. Accordingly, recombinant mussel adhesive protein and catechol-functionalized polymers have been used as primers or etchant additives in dentin wet bonding [29–34]. According to previous studies, these highly active monomers can improve the types of chemical bonding forces and reduce collagen degradation at the bonding interface, thereby improving the durability of dentin bonding [29–34]. However, these previous studies did not involve in-depth research on the dynamic bonding process involved in water contamination. Mussel adhesion is a process involving multiple components, especially in the eviction of the interfascial hydration layer [23]. A previous study demonstrated that although Mfp-1 and Mfp-3 exhibited different adhesion performances, their catechol contents were similar [35]. Thus, it is reasonable to speculate that using only catechol moiety-functionalized polymers fails to achieve a bonding performance equal to that of natural mussels [23].

Although catechol can provide abundant interaction force for the bonding interface, studies have indicated that interactions between other functional groups and a moist substance underlie the action of catechol in natural mussels. Recently, a study identified the synergistic effect of cationic lysine (Lys) and catechol on the evaporation of the hydration layer. Maier et al. found that catechol- and Lys-functionalized siderophores, together with their analogs, achieved robust adhesion on wet mica surfaces, wherein Lys repelled the hydration layer from the mineral surface and enabled catechol binding to the underlying substance [36]. Subsequent studies have shed additional light on the optimal synergy obtained when coupling cationic amine residues and catechol within the same molecule [37,38]. However, to date, no catechol- or cationic Lys-functionalized polymers have been used in the field of dentin bonding. Therefore, to achieve reliable and durable dentin adhesion under wet conditions, a catechol- and Lys-functionalized polymerizable polymer (catechol–Lys–methacrylate [CLM]) was designed as inspired by the synergistic process of wet adhesion in mussels (Fig. 1). In this study, we synthesized a CLM monomer and evaluated its dentin-bonding potential. We also investigated the potential effects of CLM on the physicochemical properties of dentin collagen.

2. Materials and methods

2.1. Materials

The materials used in this study are listed in Table 1.

![Fig. 1. Schematic diagram of the synthesis of catechol–Lys–methacrylate (CLM). HOBut, 1-Hydroxybenzotriazole; EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; DIPEA, N,N-diisopropylethylamine; DEA, diethylamine; TEA, triethylamine.](image)
2.2. Synthesis and characterization of CLM

CLM was synthesized step-by-step (Fig. 1) as follows:

Step 1. Briefly, 5 g of Fmoc-L-Lys (Boc, 10.67 mmol) was completely dissolved in 25 mL of dichloromethane (DCM) in a three-necked flask, followed by the addition of hydroxybenzotriazole (HOBt; 1.59 g, 11.74 mmol) and N-(3-dimethylaminopropyl)-N-ethylcarbodiimide (EDC; 2.25 g, 11.74 mmol) while stirring at room temperature. The resulting mixture was stirred at 0 °C for 30 min in a nitrogenous atmosphere. The mixture was washed with sodium bicarbonate and the collected DCM layer was washed with distilled water and saturated brine. The organic phases were dried over anhydrous sodium sulfate. After removing the DCM solvent, the raw product of 2-(methacryloyloxy)ethyl 2-[(1H-fluoren-9-yl)methoxy]carbonyl]amino]-6-[(tert-butoxycarboxy] amino) hexanoate (referred to as P1) was purified using silica gel column chromatography (ethyl acetate/petroleum ether, 1/2) to obtain the product (white solid, 4.2 g), with a yield of 67.79%.

Step 2. P1 was dissolved in 70 mL of DCM/diethylamine (v/v, 1/1) and stirred for 6 h at room temperature. DCM was added after the solvent evaporated. The crude product was purified using fast-gradient silica gel column chromatography (ethyl acetate/petroleum ether, 1/1; DCM/ methanol, 15/1). After removing the solvent initially, all traces of the solvent were subsequently eliminated in a vacuum and 2.4 g of 2-(methacryloyloxy)ethyl 2-amino-6-[(tert-butoxycarbonyl]amino] hexanoate (referred to as P2, 93% yield) was obtained.

Step 3. P2 (6.71 mmol, 2.4 g), 2,3-dihydroxybenzoic acid (3.36 mmol, 0.518 g), and HOBt (3.36 mmol, 0.454 g) were shaken in 30 mL of tetrahydrofuran at room temperature for 5 min in a nitrogenous atmosphere. Subsequently, triethylamine (10.08 mmol, 1.40 mL) and EDC-HCl (10.08 mmol, 1.93 g) were added. The reaction was allowed to continue for 16 h at 35 °C. The crude material was extracted with DCM and the solution was washed with 0.1 N HCl, distilled water, and saturated brine. The organic layer was dried over anhydrous sodium sulfate and the crude product of 2-(methacryloyloxy)ethyl 6-[(tert-butoxycarbonyl]amino]-2-(3,3-dihydroxybenzamid) hexanoate (P3) was purified using gradient column chromatography on a silica gel (DCM/ methanol, 20/1). The solution was concentrated and 1.15 g of P3 (light yellow oil, 69% yield) was obtained.

Step 4. P3 was stirred with 50 mL of HCl-dioxan (4 N) for 1–2 h at room temperature. After the reaction was complete, the mixture was concentrated under a high vacuum and brown-colored products were obtained (0.81 g, oily liquid, 89% yield).

During synthesis, the proton nuclear magnetic resonance (1H NMR) spectra of the intermediate products and CLM were acquired using the Bruker Avance III HD 500-MHz system (Bruker BioSpin, Switzerland). In addition, the cytotoxicity of CLM was compared with that of commercially available bisphenol-A glycol dimethacrylate (Bis-GMA), triethylene glycol dimethacrylate (TEGDMA), hydroxyethylmethacrylate (HEMA), and diurethane dimethacrylate (UDMA) using the Cell Counting Kit-8 reagent as previously described [59]. Briefly, the monomers were dissolved in dimethyl sulfoxide and mixed with a serum-free medium to prepare the test solution. The test solution was added to each well of the microplate, and the cells were then cultured for 6 h at room temperature. The resulting mixture was stirred at 0 °C for 3 min, over 10,000 cycles.

A total of 128 teeth were randomly divided into four groups of 32 each. The occlusal one-third of the crown was removed using a diamond-impregnated cutting machine (SJV-150A, Kejing, Shenyang, China). To create a standard smear layer, the obtained dentin surface was polished with 600-grit carbide paper for 60 s under running water. After etching with 37% phosphoric acid for 15 s and rinsing, the specimens in each group were treated with deionized water and 1, 5, and 10 mg/mL CLM primers for 60 s. The SB2 adhesive was then applied and cured per the manufacturer's instructions. A cured 6-mm-thick composite buildup was established with the Z250 resin (Filtreck Z250, 3 M ESPE) for each specimen. The prepared bonding specimens in each group were divided into two subgroups: the immediate group (immersed in distilled water for 24 h) and the aging group (thermocycling, 5 °C for 1 min, followed by 55 °C for 1 min, over 10,000 cycles).

Four 10 × 1 × 1-mm sticks perpendicular to the long axis of each tooth were used in the bond strength test as previously described [16]. The microtensile bond strength (μTBS) value of each specimen was measured using a microtensile testing machine (EZ-TEST 500 N, Shimazu Co.) at a crosshead speed of 1 mm/min. Four μTBS values for each tooth were averaged (n = 10). The debonded interface was observed under a stereomicroscope (MLC-150, Motic, Decatur, GA, USA) to determine the failure modes as described in a previous study: the failure modes were adhesive failure, cohesive failure within the dentin, cohesive failure within a resin composite, and mixed failure involving both interfacial and cohesive fractures [40].

The bonding interface was observed using field-emission scanning electron microscopy (FE-SEM, S-4800, Hitachi, Tokyo, Japan) as previously described [16]. Briefly, 1-mm-thick specimens perpendicular to the bonding interface were polished with silicon carbide paper, etched with 37% phosphoric acid, immersed in 5% sodium hypochlorite, ultrasonically irrigated, fixed, dehydrated, sputter-coated with gold, and examined using FE-SEM.

The nanoleakage level of bonding interface was evaluated using FE-SEM as previously described [16]. Four 4 × 1 × 1-mm slices along the long axis from each tooth were coated with nail varnish, leaving an uncoated region of approximately 1 mm around the bonding interface. All specimens were then immersed in 50 wt% ammoniacal silver nitrate solution for 24 h, ultrasonically cleaned, soaked in a photo-developing solution, and exposed to fluorescent light irradiation for 8 h. All
specimens were subsequently cleaned ultrasonically, dried with filter paper, sputter-coated with gold, and examined via FE-SEM in the back-scattered mode.

The collagenase activity within the bonding interface after aging was also evaluated using confocal laser scanning microscopy (Fluoview FV1000; Olympus, Japan). SB2 was premixed with tetramethylrhodamine B isothiocyanate (Miliporte Sigma, St. Louis, MI, USA) and three teeth from each group were bonded and aged as described above. Two-millimeter thick slabs that contained the bonding interface were obtained and subjected to an in situ zymography test using the EnzChek collagenase kit (E–12055; Molecular Probes, Eugene, OR, USA) as previously described [41].

2.4. Interaction between CLM and collagen

To evaluate the binding affinity of CLM to dentin collagen, molecular docking was performed using Molecular Operating Environment (Chemical Computing Group, Montreal, QC, Canada) [34,42]. Three crystal structures of type I collagen were selected from the Protein Data Bank database (IDs: 4OYS, 1CGD, and 1QSU). The two-dimensional structure of CLM was converted into a three-dimensional structure in Molecular Operating Environment according to the energy minimization process. The protonation state of the target and the orientation of hydrogen were optimized using LigX at a pH of 7 and temperature of 300 K. Docking was performed in the force field of AMBER10:EHT and the implicit solvent model of R-field. The binding site of collagen was identified using the Site Finder module in Molecular Operating Environment. Before the docking workflow, an “induced-fit” protocol was used to make the side chains of the collagen pocket mobilizable to ligand conformations, with a constraint on their positions. A weight of 10 was used for tethering sidechain atoms to their original positions. All docked conformations were ranked by London dG scoring, followed by force field refinement on the top 30 poses and rescoring based on the GBVI/WSA dG bonding free-energy scoring method [34].

To further characterize the interaction between CLM and collagen, we used time-resolved FTIR detection. A 1-mm-thick dentin slab was prepared perpendicular to the long axis of the tooth using a low-speed diamond saw and polished using 600-grit silicon carbide paper under running water. The slabs were etched with 10% phosphoric acid for complete demineralization, in which the endpoint of demineralization was determined by the dropwise addition of a 30% potassium oxalate solution [39]. The slabs were scanned using ATR-FTIR in the range of 4000–500 cm⁻¹ with a resolution of 4.0 cm⁻¹. The specimens were then immersed in CLM solution for 5 min. After ultrasonic cleaning, the specimens were scanned using ATR-FTIR.

2.5. Effects of CLM on the physicochemical properties and enzymatic stability of collagen

Tooth enamel was removed using a high-speed handpiece, and 1 × 1 × 6-mm dentin slabs parallel to the long axis of the tooth were prepared using a low-speed diamond saw. In total, 200 dentin specimens were etched with 10% phosphoric acid for 24 h and thoroughly rinsed with distilled water. All specimens were further treated with distilled water (blank control), 1 mg/mL CLM, 5 mg/mL CLM, 10 mg/mL CLM, or 2.5% glutaraldehyde (GJD; positive control) for 5 min. Subsequently, 20 specimens in each group were evaluated for ultimate tensile strength (UTS), while the other 20 specimens were treated with 0.1 mg/mL collagenase (type IV Clostridium histolyticum, 160 units/mg, Aladdin) for 24 h. The UTS test was performed using a universal testing machine (EZ-TEST 500 N; Shimadzu) at a crosshead speed of 1 mm/min.

Thermogravimetric analysis was performed to evaluate the effects of CLM on the thermostability of demineralized dentin collagen. Briefly, dentin specimens without enamel, cementum, and pulp tissue were processed in liquid nitrogen for 30 min, pulverized, etched with 10% phosphoric acid for 48 h, and lyophilized. A total of 150 mg demineralized dentin powder was divided into five groups and treated with distilled water (blank control), 1 mg/mL CLM, 5 mg/mL CLM, 10 mg/mL CLM, or 2.5% GD for 5 min (n = 5). The lyophilized powder for each group was processed using a thermogravimetric analyzer (TA Instruments, New Castle, DE, USA) from 20 °C to 200 °C at a heating rate of 10 °C/min.

One gram of the dried dentin powder was divided into five groups and treated with the same abovementioned solutions for 5 min. After being rinsed with distilled water, the powder for each group was placed in Eppendorf tubes containing 2 mL of 0.1 mg/mL type IV collagenase solution for 24 h at 37 °C. Subsequently, 500 µL of the supernatant was collected from each tube for hydroxyproline (HYP) estimation using an HYP detection kit (Solarbio, Beijing, China) per the manufacturer's instructions. The remaining powder was dried in a vacuum, and the dry mass loss of each group was calculated (n = 5 for each group).

Zymography of demineralized dentin treated with CLM was evaluated using the EnzChek collagenase kit. The reaction solution was prepared by mixing 1 mg/mL DQ gelatin, 1 × buffer, and an anti fluorescein quenching agent (DAPI H-1200) in a ratio of 1:8:1. Further, 2-mm-thick dentin slabs were etched with 37% phosphoric acid for 15 s and treated with distilled water, 1 mg/mL CLM, 5 mg/mL CLM, 10 mg/mL CLM, or 2.5% GD for 5 min (n = 5). Mineralized dentin slabs served as blank controls. All slabs were treated with the reaction solution for 24 h in the dark (37 °C, 100% humidity), rinsed thoroughly, and examined using confocal laser scanning microscopy (Fluoview FV1000; Olympus, Japan) as previously described [16].

The effects of collagenase inhibition of CLM were evaluated using the EnzChek collagenase kit. Briefly, 0.4 U/mL collagenase solution, 1 × buffer, and 100 µg/mL DQ gelatin solution were prepared and kept in the dark before use. Further, 20 µL of the DQ gelatin solution and 100 µL of the collagenase solution (type IV collagenase, 0.4 U/mL) were added to each well of a 96-well plate. Finally, 80 µL of deionized water, 1 mg/mL CLM, 5 mg/mL CLM, 10 mg/mL CLM, or 1,10-phenanthroline was added into each well (n = 5). The fluorescence intensities of each well before and after 24 h of incubation in the dark were measured using a GloMax® Discover Microplate Reader (Promega, Madison, WI, USA) at absorption and emission wavelengths of 495 and 515 nm, respectively.

2.6. Statistical analyses

All quantitative data are expressed as the mean ± standard deviation. All data analyses were performed using SPSS 20.0 and the threshold for statistical significance was set at α = 0.05. The data of failure mode distribution was analyzed using the chi-square test. A two-way analysis of variance was performed to characterize the effects of conditioning and aging on the dentin bond strength of the bonding interface and on the UTS of collagen. Least significant difference tests were used for pairwise comparisons. We also used one-way analysis of variance and post-hoc Fisher’s least significant difference test after ascertaining normality and homoscedasticity.

3. Results

3.1. Synthesis and characterization of CLM

Fig. 2 presents the 1H NMR image of P1, P2, and CLM. The signals at 5.5–6.25 ppm were attributed to the protons of C=C of HEMA (Fig. 2a), indicating the interaction between the –OH of HEMA and the –OH of Fmoc-L-Lys (Boc). The removal of Fmoc was confirmed by the disappearance of the signals at 7.25–8.00 ppm in the 1H NMR spectra of P2 (Fig. 2b) [43,44]. The signals at 6.42–7.04 ppm and 2.73 ppm confirmed the presence of catechol and Lys groups, respectively, in CLM (Fig. 2c) [43,44].

Fig. 3 shows the viability of human dental pulp stem cells exposed to different monomers after 24 h. Inhibited cell viabilities were observed for Bis-GMA, UDMA, and CLM at test concentrations. However, CLM showed

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Fig. 2. $^1$H NMR spectra of intermediate products and catechol-lys-methacrylate (CLM). (a) $^1$H NMR spectra of P1. $^1$H NMR (500 MHz, chloroform-d) = $\delta$ 7.77 (d, 2H); 7.61 (dd, 2H); 7.40 (t, 2H); 7.32 (t, 2H); 6.12 (s, 1H); 5.59 (t, 1H); 4.63–4.31 (m, 8H); 3.10 (t, 2H); 1.93 (s, 3H); 1.89–1.68 (m, 2H); 1.49 (q, 2H); 1.43 (s, 9H); 1.40–1.32 (m, 2H). (b) $^1$H NMR spectra of P2. $^1$H NMR (500 MHz, chloroform-d) = $\delta$ 6.10 (s, 1H); 5.58 (t, 1H); 4.44–4.22 (m, 4H); 3.43 (dd, 1H); 3.08 (q, 2H); 1.92 (s, 3H); 1.72–1.68 (m, 2H); 1.46 (t, 2H); 1.41 (s, 9H); 1.38–1.07 (m, 2H). (c) $^1$H NMR spectra of CLM. $^1$H NMR (500 MHz, deuterium oxide) = $\delta$ 7.04–6.96 (t, 1H); 6.80–6.71 (d, 1H); 6.56–6.42 (m, 1H); 5.83–5.56 (t, 1H); 5.39–5.15 (t, 1H); 4.44–3.93 (m, 5H); 2.73 (q, 2H); 1.04–1.49 (m, 3H); 1.44 (m, 2H); 1.30–1.12 (m, 2H).
a median lethal concentration (between 140 and 160 μg/mL) similar to that of UDMA, both of which were higher than that of Bis-GMA, thereby indicating the safety of CLM when used as a dental monomer.

3.2. Bonding performance of CLM

Fig. 4a shows the effects of CLM primers on the DC of SB2. At each time point, the DC values of the 10-mg/mL group were lower than those of the control group (all \( P < 0.05 \)). Compared with the control group, the DC decreased in the 1- and 5-mg/mL CLM groups; however, the differences were not statistically significant (\( P > 0.05 \)).

Fig. 4b presents the μTBS values of SB2 under different primer conditions. The bond strength was affected by the concentration of CLM and the aging mode (\( P < 0.05 \)). Before aging, treatment with 5 mg/mL CLM yielded the highest μTBS value among the groups (\( P < 0.05 \)). After aging, the μTBS values of all groups decreased significantly (\( P < 0.01 \)) and dentin conditioned with 5 mg/mL CLM showed the highest μTBS value (\( P < 0.01 \)). The μTBS value of the control group showed a drastic decrease of 45.03%, whereas that of the 5-mg/mL CLM group decreased by only 17.54%. Fig. 4c shows the distribution of the failure mode. The most common failure mode among the groups was mixed failure, and neither the aging mode nor the conditioning agent had any significant effect on the distribution of failure modes (\( P > 0.05 \)).

As shown in Fig. 5a, an intact hybrid layer with abundant underlying resin tags was found in all groups before aging. There was no discernible visual difference in terms of bonding interfaces created by the different conditioning modes. After the aging process, a rough-looking hybrid layer and fractured resin tags were observed in the control group, whereas a more integrated hybrid layer with intact resin tags was preserved in the 5-mg/mL group. Fig. 5b presents representative images of the nanoleakage level. Before aging, only a few white silver depositions could be identified in the hybrid layer in the three CLM-conditioned groups, whereas distinct linear silver depositions were noted in the control group. After aging, all groups exhibited silver deposition at the bottom of the hybrid layer, and the 5-mg/mL CLM group had the lowest silver deposition of all groups.

We performed an in situ zymography test to reveal the collagenase activity within the bonding interface (Fig. 5c). The adhesive mixed with tetramethylrhodamine B isothiocyanate appeared as a red fluorescent region, while the enzymatic degradation product of exogenous gelatin substrates formed by the action of endogenous collagenase appeared as green fluorescence. In the control group, the bottom of the bonding interface showed marked green fluorescence; however, this phenomenon was rare in other CLM-treated groups.

3.3. Interactions between CLM and collagen

Fig. 6a illustrates the docking patterns for targeted type I collagen variants. As shown by molecular docking, CLM can form a suitable steric complementarity with the binding site of 1QSU, 1CGD, and 4OY5, separately. The binding free-energy between CLM and type I collagen 1QSU was \(-3.83\) kcal/mol. Both hydrogen bonds and covalent interactions were formed between CLM and 1QSU. The nitrogen atom in CLM, which is regarded as a hydrogen acceptor, formed a hydrogen bond with the backbone nitrogen atom of Gly12. The sidechain nitrogen atom of Lys14 formed a covalent bond with CLM. Van der Waals (VDW) interactions were formed between CLM and the surrounding residues. The binding affinity between 1CGD and CLM was \(-4.71\) kcal/mol. A hydrogen bond was formed between CLM and 1CGD. The oxygen atom in CLM, which is considered the hydrogen acceptor, formed a hydrogen bond with the backbone nitrogen atom of Gly24. The other oxygen atom in CLM formed a hydrogen bond with the sidechain oxygen atom of Hyp26. VDW interactions were formed between CLM and the surrounding residues. The binding affinity between 4OY5 and cpd2 was \(-4.60\) kcal/mol. Both hydrogen bonds and cation–π interactions were formed between CLM and the surrounding residues.
interactions were formed between CLM and 4OY5. The oxygen atom in CLM, considered a hydrogen acceptor, formed a hydrogen bond with the backbone nitrogen atom of Gly4. The benzene ring in CLM formed a cation–π interaction with the nitrogen atom of Gly1. VDW interactions were formed between CLM and the surrounding residues.

As shown in Fig. 6b, the inherent Amide A (3297 cm$^{-1}$), Amide I (1632 cm$^{-1}$), Amide II (1551 cm$^{-1}$), and Amide III (1239 cm$^{-1}$) of collagen was found in untreated as well as CLM-treated collagen. After CLM treatment, the Amide I peak shifted to a lower wave number. After the reaction, the appearance of peaks at 1638 cm$^{-1}$, 1722 cm$^{-1}$, and 669 cm$^{-1}$ originating from the –C=C, –C=O, and –OH groups in the phenol ring of CLM indicated the grafting of CLM onto collagen.

### 3.4. Effects of CLM on the physicochemical properties and enzymatic stability of collagen

![Fig. 4. Bonding performance of CLM-based adhesion schemes.](image)

**Fig. 4.** Bonding performance of CLM-based adhesion schemes. (a) Degree of conversion. Data are expressed as the mean ± standard deviation. *P* < 0.05 vs. control. (b) μTBS values of the different bonding schemes. Data are expressed as the mean ± standard deviation. For μTBS evaluated before aging, groups labeled with the same uppercase letter were not significantly different (*P* > 0.05). After aging, groups labeled with the same lowercase letter were not significantly different (*P* > 0.05). (c) Distribution of failure modes.

Collagenase-mediated aging, the UTS decreased significantly in all groups (*P* < 0.01). The $T_{95}$ value of demineralized dentin collagen increased significantly after CLM and GD treatment (*P* < 0.01), indicating improved thermal stability of collagen (Fig. 7b).

After collagenase-mediated aging, collagen in the control group exhibited the highest loss of dry mass among all the groups (*P* < 0.01; Fig. 7c). As evidenced by the lower mass loss values than those of controls, both CLM and GD treatment can prevent degradation by enzymolysis (all *P* < 0.01). To detect the degree of collagen degradation, we performed an HYP release test (Fig. 7d), and its results were consistent with those of the dry mass loss test in which a lower amount of HYP could be found in the CLM and GD groups than in the control group (*P* > 0.05).

After etching, the host-derived enzymes embedded in the dentin mineral were released and activated in an acidic environment, initiating a cascade of the proteolytic degradation of collagen [45]. As shown in Fig. 7e and f, green fluorescence represents the activity of the endogenous enzymes of dentin. A lower intensity of green fluorescence can be observed in the CLM and GD-treated groups, indicating inhibited collagenase activity. Our results also indicated that CLM can inhibit the activity of type IV collagenase in a concentration-dependent manner (Fig. 7g; *P* < 0.01).
4. Discussion

Water has long been considered a double-edged sword in dentin bonding. A certain amount of water during dentin demineralization is a prerequisite for the penetration of adhesive resin monomer to keep the collagen network from collapsing. However, the water that cannot be removed completely from the bonding interface negatively affects the interaction between collagen and resin monomers, adhesive polymerization, and other hazards of bonding durability. Inspired by the remarkable underwater bonding efficiency of mussels, we synthesized a water-expelling dentin adhesive monomer and confirmed its bonding-promoting potential in an in vitro wet dentin-bonding model characterized by improved bond strength and bonding durability. In addition, the potential benefits of CLM on the physicochemical properties of collagen were verified.

To address the disadvantages of the presence of water on dentin adhesion, researchers have proposed a few mussel-inspired catechol-containing polymers for use in dentin bonding that showed promising...

Fig. 5. Bonding interface observations. (a) SEM observation of the bonding interface before and after aging. Images on the right are magnified images of those on the left. Bar = 10 μm. (b) Nanoleakage levels of the bonding interface before and after aging. Bar = 10 μm. (c) In situ zymography of the bonding interface. Bar = 50 μm.
results \([30-32,34]\). However, these simplified polymers failed to capture the complex synergy between amino acids and catechol in natural Mfps. Inspired by the synergistic effects of catechols and cationic amino residues within vanguard Mfp-3 and Mfp-5 proteins on mussel underwater bonding, we synthesized a multifunctional CLM monomer containing a functional catechol group and cationic Lys to achieve dynamic water-expelling properties and promote robust adhesion during dentin bonding. To ensure the capacity of copolymerization with other resin monomers, we also grafted a methacrylate group onto CLM. The structure of CLM was confirmed via NMR spectroscopy (Fig. 2c). In addition, CLM exhibited comparable cytotoxicity to common commercially used dental monomers, including Bis-GMA, TEGDMA, and HEMA, indicating that it can be used in a dental adhesive formula.

To evaluate the bonding potential of CLM, we formulated an experimental primer containing CLM and established an \textit{in vitro} dentin-bonding model. The \(\mu\)TBS test revealed that pretreatment with 5 mg/mL CLM achieved the highest \(\mu\)TBS among all groups, regardless of aging (Fig. 4b). Compared with the control group, the value of immediate bond strength increased by 12.64% in the 5-mg/mL CLM group. After aging, the control group showed a drastic decrease of 45.03%, which was consistent with the results of previous studies \([29,31-34]\). However, the bond strength in the 5-mg/mL CLM group decreased by only 17.54%, indicating improved bonding durability. In addition, a more integrated bonding interface was observed in the 5-mg/mL CLM group via FE-SEM.
Fig. 7. Effects of CLM on the physicochemical properties and enzymatic stability of collagen. (a) Ultimate tensile strength. (b) 5% mass loss temperature. (c) Dry mass loss. (d) HYP release. (e) Zymography of the dentin matrix under different treatments. Bar = 50 μm. (f) Quantification of collagenase activity. Data are expressed as the mean ± standard deviation. Groups designated with different letters were significantly different (P > 0.05). (g) Inhibitory effect of CLM on type IV collagenase. All data are expressed as the mean ± standard deviation. Groups labeled with the same letter were not significantly different (P > 0.05).
and the nanoleakage assay, indicating adequate adhesive penetration into the demineralized collagen matrix. Our results were in accordance with those of previous mussel-inspired dentin-bonding studies in which catechol-functionalized adhesives exhibited improved bond strength and durability [31–34]. However, it is difficult to make exact comparisons among these works owing to different solvent types, adhesive types, treatment times, and test conditions.

There may be several reasons for the improved bonding performance of the experiment primer. The water-expelling effect of Lys and the high chemical affinity of catechol provide a ‘one–two punch,’ thereby remarkably increasing the strength of the bonding interface [36–38]. The cationic Lys of CLM acted as a vanguard moiety to break through the hydration layer on the surface of the substance. Additionally, the hydrophobic groups of CLM, such as ester and alkyl groups, may move spontaneously toward the hydrophobic amino residues in demineralized collagen via hydrophobic interactions, further facilitating the evicition of water [46]. Accordingly, CLM can achieve close contact with wet demineralized dentin and a myriad of interfacial interactions can be obtained by introducing the catechol group into the interface, including hydrogen bonding, metal-ion coordination bonds, Michael additions, π–π interactions, cation–π interactions, and electrostatic interactions [23–28,47]. In addition, to remove the hydration layer, Lys can amplify the cation–π interactions and enhance cohesion [48,49]. These above-mentioned interfacial interactions of CLM may play a vital role in the efficiency of dentin bonding, as additional chemical adhesion within the bonding interface was reported to contribute to the high quality of the hybrid layer against harmful biological and chemical activities [39,50,51].

In addition, the high bond strength and integrated hybrid layer in the 5-mg/mL group indicated an acceptable polymerization of the adhesive resin. This was evidenced by the results of DC. Our results showed that low concentrations of CLM (1 and 5 mg/mL) did not affect resin polymerization, whereas a high concentration of CLM (10 mg/mL) showed an inhibitory effect. This might be attributed to catechol's reducibility. Catechol acts as a free radical scavenger and eliminates oxygen free radicals that are generated during polymerization. Previous studies have shown that catechol-functionalized dopamine methacrylamide decreases the DC of Adper SB2 at high concentrations [31,33]. In addition, high concentrations of catechol-rich proanthocyanidin were also reported to interfere with polymerization [52,53]. These findings are in agreement with the results of our study in which 10 mg/mL showed decreased DC and minimal satisfactory bonding performance regarding the bond strength values and silver nanoleakage levels.

The biodegradation of collagen components within the bonding interface mediated by matrix metalloproteinases (MMPs) has been well acknowledged as one of the main causes of bonding failure. Strategies including the use of collagen cross-linkers and MMP inhibitors have been recommended to improve the stability of collagen within the bonding interface [34,55]. It has been well documented that catechol-containing agents, including proanthocyanidin and DMA, have the ability to cross-link collagen and inhibit MMP activity [31–34,56,57]. In this study, in situ zymography revealed that the activity of endogenous MMPs was inhibited significantly in the CLM group, indicating that CLM might have similar effects on the enzyme-mediated collagen degradation process to those of proanthocyanidin and DMA. Therefore, we further explored the interaction between CLM and collagen and its effects on collagen stability.

The reactivity between CLM and collagen was confirmed by molecular docking using FTIR spectroscopy. The results of molecular docking indicated that CLM could bind to type I collagen through covalent bonds, hydrogen bonds, VDW interactions, and cation–π interactions. The appearance of C=O (1724 cm⁻¹) and C=O (1638 cm⁻¹) bonds in CLM-treated collagen indicated the grafting of CLM (Fig. 5b). In addition, the shifts of Amide I, Amide II, and Amide III could be attributed to the formation of hydrogen bonds, Michael additions, electrostatic interactions, and cation–π interactions between catechol and collagen. The abovementioned results are consistent with the findings of previous studies [58,59]. Catechol on Mfps can form not only hydrogen bonds with the O, F, and N atoms on the surface of organic and inorganic materials but also cation–π interactions with cationic amino acid residues in collagen, such as Lys and arginine [34,60]. Furthermore, the catechol moiety of DMA has been confirmed to form hydrogen bonds, covalent bonds, and VDW interactions with collagen, which is in agreement with the results of our study [34]. Accordingly, the collagen–CLM complexes formed can chemically bond to other adhesive resin monomers via the polymerization of methacrylate bonds. Although mechanical inter-locking has always been regarded as the main bonding mechanism for dentin bonding, additional chemical bonding forces between collagen and adhesive resin may strengthen the bonding interface [39,40,50,51]. In this study, the high chemical affinity of binding of the catechol structure on CLM to collagen and other methacrylic resins may facilitate the formation of a more perfect bonding interface.

Our results also showed improvement in the mechanical strength, thermal stability, and enzymatic stability of CLM-treated collagen. These results are consistent with the findings of previous studies in which catechol-functionalized DMA improved the physicochemical stability of collagen due to its collagen-crosslinking effects [34,40]. Similar to proanthocyanidin and DMA, CLM also showed a collagenase inhibitory effect, as evidenced by the collagenase inhibition test. This was further confirmed by zymography of the demineralized dentin, in which endogenous collagenase activities were inhibited in the CLM-treated groups. This might be attributable to the metal-chelating ability of the catechol structure on CLM, which competitively ‘preys' on Zn²⁺ and Ca²⁺ (both of which are necessary for the activation of collagenase) in the surrounding environment.

5. Conclusion

We propose that CLM can act, similarly to the natural mussel protein Mfp-3, as a “molecular vanguard” in the bonding process, with catechol and Lys providing a synergistic ‘one–two punch’ to destroy the hydrated layer on its surface and create a water-free microlayer. This creates a local water-free microenvironment, facilitating the introduction of the bonding agent to form a more perfect bonding interface. The polymerizable C–C bond on CLM enables copolymerization between the CLM–collagen complex and other resin monomers, leading to the formation of a monoblock. Furthermore, the interactions between CLM and collagen can enhance the mechanical and chemical stability of collagen, increasing its enzymatic resistance and providing additional physical/chemical adhesion to the dentin bonds, which results in the formation of a tough and long-lasting bond, similar to that formed by natural mussels.

Author contributions

Z.H. Hu: Conceptualization, Methodology, Investigation Validation, Formal analysis, Writing - Original Draft. W.Z. Wu: Methodology, Investigation Validation, Formal analysis, Writing - Original Draft. M.Z. Yu: Methodology, Investigation Validation, Formal analysis, Writing - Original Draft. Z. Wang: Validation, Formal analysis, Resources, Visualization. Z.Y. Yang: Validation, Resources. X.D. Xing: Conceptualization, Validation, Resources. X.F. Chen: Formal analysis. L.N. Niu: Validation, Resources. F. Yu: Conceptualization, Methodology, Investigation Validation, Formal analysis, Writing - Original Draft, Writing - Review & Editing, Supervision. Y.H. Xiao: Conceptualization, Writing - Review & Editing, Supervision, Funding acquisition. J.H. Chen: Conceptualization, Writing - Review & Editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
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