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Original Article

Development of a zinc chloride-based chemo-mechanical system for potential minimally invasive dental caries removal system

Yu-Tzu Wu [a†], Po-Wen Lu [b,c†], Chih-An Lin [a], Liang-Yu Chang [a], Chonlachat Jaihao [b], Tzu-Yu Peng [d], Wei-Fang Lee [e], Nai-Chia Teng [d], Sheng-Yang Lee [d], Ram Prakash Dwivedi [f], Poonam Negi [g], Jen-Chang Yang [a,h,i,j*]

[a] *Graduate Institute of Nanomedicine and Medical Engineering, Taipei Medical University, Taipei, Taiwan*
[b] *Graduate Institute of Biomedical Materials and Tissue Engineering, Taipei Medical University, Taipei, Taiwan*
[c] *Division of Gastroenterology and Hepatology, Department of Internal Medicine, Shuang Ho Hospital, New Taipei, Taiwan*
[d] *School of Dentistry, Taipei Medical University, Taipei, Taiwan*
[e] *School of Dental Technology, Taipei Medical University, Taipei, Taiwan*
[f] *School of Electrical and Computer Science Engineering, Shoolini University, Himachal Pradesh, India*
[g] *School of Pharmaceutical Sciences, Biotechnology and Management Sciences, Shoolini University, Himachal Pradesh, India*
[h] *International Ph.D. Program in Biomedical Engineering, Taipei Medical University, Taipei, Taiwan*
[i] *Research Center of Biomedical Device, Taipei Medical University, Taipei, Taiwan*
[j] *Research Center of Digital Oral Science and Technology, Taipei Medical University, Taipei, Taiwan*

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KEYWORDS
Chemo-mechanical 2;
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Abstract *Background/purpose:* The chemo-mechanical caries-removal technique is known to offer advantages of selective dentin caries treatment while leaving healthy dental tissues intact. However, current sodium hypochlorite based reagents usually excessively damage

* Corresponding author. Graduate Institute of Nanomedicine and Medical Engineering, College of Biomedical Engineering, Taipei Medical University, 250, Wu-Xing Street, Taipei 11031, Taiwan.
E-mail address: yang820065@tmu.edu.tw (J.-C. Yang).
† These authors contributed equally to this work.

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Sodium hypochlorite 4;
Collagen

dentin collagen. Therefore, the purpose of this study was to develop a novel chemo-mechanical caries-removal system to preserve the collagen network for subsequent prosthetic restorations.
Materials and methods: The calfskin-derived collagen was chosen as a model system to investigate the dissolution behavior of collagen under different operating conditions of chemical-ultrasonic treatment systems. The molecular weight, triple-helix structure, the morphology, and functional group of collagen after treatment were investigated.
Results: Various concentrations of sodium hypochlorite or zinc chloride together with ultrasonic machinery were chosen to investigate. The outcomes of circular dichroism (CD) spectra demonstrated stability of the triple-helix structure after treatment of a zinc chloride solution. In addition, two apparent bands at molecular weights (MWs) of 130 and 121 kDa evidenced the stability of collagen network. The positive 222 nm and 195 nm negative CD absorption band indicated the existence of a triple-helix structure for type I collagen. The preservation of the morphology and functional group of the collagen network on the etched dentin surface were investigated by in vitro dentin decalcification model.
Conclusion: Unlike NaOCl, the 5 wt% zinc chloride solution combined with ultra-sonication showed dissolution rather than denature as well as degradation of the dentin collagen network. Additional in vivo evaluations are needed to verify its usefulness in clinical applications.

Introduction

Dental caries, a chronic infectious disease that demineralizes the tooth structure over time due to a metabolizing acid primarily from the cariogenic Streptococcus mutans, affects close to 2 billion people worldwide.[1] Dental caries may affect the quality of life even after treatment.[2] A deep carious lesion is commonly recognized as a caries when it reaches the inner third or inner quarter of the dentin thickness based on radiographic evidence.[3] Traditionally, deep carious lesions were treated by aggressively nonselective removal of all carious tissues to ensure the longevity of the subsequent prosthetic restoration,[4] which might lead to pulp exposure. Typical therapeutic management strategies for dental carious lesions include mechanical/rotary burs, air abrasion, lasers, atraumatic restorative therapy, and chemo-mechanical caries removal (CMCR).[5] Perhaps other antimicrobial agents such as quaternary ammonium compounds, electrolyzed water, silver-containing mesoporous bioglass etc. can be also tested for their possible use in CMCR.[6–8] Unlike conventional caries-removal techniques, CMCR methods offer advantages of minimal intervention and consideration of patient comfort by selectively eliminating infected dental tissue without inherent drawbacks like local anesthesia and possible adverse effects owing to heat and pressure.

Dentin consists of 70 wt% hydroxyapatite (HAp), 10 wt% water, and 20 wt% collagen as well as non-collagenous proteins.[9] Collagen, which contains proline and hydroxyproline chains, is energetically favorably folded into a triple-helical conformation of tropocollagen and is stabilized by inter-chain hydrogen bonds.[10] Tropocollagen aggregates to form a stable fibril network by covalent bond crosslinking.[11] In dentin, these fibrils are further hierarchically organized into a dense three-dimensional organic network reinforced by apatite mineral crystallites.[12] When dentin caries occur, demineralization tends to weaken the interface between the collagen network and HAp in nanocomposites and provide access for penetrating acids produced by oral microorganisms, thus leading to dentin collagen matrix degradation by proteolytic enzymes such as matrix metalloproteinases (MMPs).[13]

CMCR is an effective method using non-cutting hand instruments together with chemical agents for minimally invasive caries excavation of deep caries and root caries.[14] Removal agents are either sodium hypochlorite (NaOCl)-based agents such as Caridex[15] and Carisolv,[16] or papain enzyme-based agents such as Papacarie[17] and Biosolv.[18] Modified Carisolv gels were reported to have a formulation of 0.1 M amino acids (glutamic acid, leucine, and lysine), sodium chloride, erythrosin, carboxymethyl cellulose (CMC), and 0.475% NaOCl.[19] Possible mechanisms of NaOCl-based CMCR reagents on weakening the dentin collagen network were attributed to hypohalite radicals (•ClO) which interact with tropocollagen at sites of glycine or hydroxyproline to weaken inter-strand hydrogen bonds by a chlorination reaction[20] and form nitrogen-centered radical species by oxidization reactions inducing further protein fragmentation.[21]

The essential objective of adhesive restoration is to secure a long-lasting adaptation of the restorative material to a tooth without microleakage or recurrent caries.[22] The key challenge of dentin adhesion is to prevent a collapse of the dentin collagen network and blockage of the primer and bond infiltration.[23] The formation of a hybrid layer (HL) between the adhesive and dentin interface is of great importance to ensure micromechanical retention in adhesive procedures.[24,25] However, there is no collagen network available after any of the current caries removal treatments either by mechanical or CMCR techniques. Prior to

filling a cavity with resin composites, removal of the mineral by acid-etching becomes a necessary step to condition the collagen network for resin monomer infiltration into the demineralized dentin matrix.

Metal protein interactions play important roles in structural stability, signaling, regulation, transport, and many other biological functions.[26] Zinc ions are commonly observed in many biological systems. Zinc fingers are protein structural motifs that coordinate with zinc ions through cysteine and histidine side-chains to stabilize their folding.[27] Zinc chloride ($ZnCl_2$) is a white, odorless, crystalline solid with high water solubility at ambient temperature. Zinc revealed a protective effect on MMPs in reducing collagen degradation at the resin-dentin hybrid layer.[28] In addition, a $ZnCl_2$ solution was reported to be a good solvent system to completely solubilize the degummed silk fibroin at a temperature of as low as 45 °C within 1 h.[29]

Based on the good solubility and the collagen-protective effect of the $ZnCl_2$ solution, we hypothesized the possibility of developing a $ZnCl_2$-based CMCR system to dissolve rather than denature or degrade the collagen network. After removing dentin caries, if the dentin collagen network can still be preserved, then adhesive restoration might can directly proceed without the acid-etching step. Therefore, this study hoped to improve the CMCR formula for preserving the collagen structure and provide caries patients with a milder, less-invasive caries treatment for clinical use.

Materials and methods

Materials

Hydrogen chloride (HCl, 12 N), methanol (CH_4OH), perchloric acid ($HClO_4$), potassium hydroxide (KOH), and sodium chloride (NaCl), were purchased from J.T. Baker (Phillipsburg, NJ, USA). Citric acid ($C_6H_8O_7$), L-glutamic acid ($C_5H_9NO_4$), L-leucine ($C_6H_{13}NO_2$), type I collagen (purified from calfskin), methyl red ($C_{15}H_{15}N_3O_2$), *para*-dimethylaminobenzalaldehyde ($C_9H_{11}NO$), L-lysine ($C_6H_{14}N_2O_2$, 97%), chloramine-T ($CH_3C_6H_4SO_2N(Cl)Na\bullet H_2O$, 98%), isopropyl alcohol (C_3H_8O), zinc chloride, ($ZnCl_2$ ≥98% purity), and hydroxyethyl cellulose (of medium viscosity) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Acetic acid ($C_2H_4O_2$), calcium chloride ($CaCl_2$), sodium thiosulfate pentahydrate ($Na_2O_3S_2\bullet H_2O$), thymol ($C_{10}H_{14}O$), tetramethylenediamine (TEMED), ethylenediaminetetraacetic acid (EDTA), and sodium dodecylsulfate (SDS) were purchased from Acros Organics BV (Geel, Belgium), and anti-acid varnish was ordered from Max Factor (Tokyo, Japan). Sodium hypochlorite (NaOCl, 12%) was from Showa (Nagoya, Japan). All chemicals were used as received without further purification.

Dentin collagen extraction

Fifteen extracted caries-free and unrestored human teeth were collected and stored in a 1% thymol-containing ethanol solution at 4 °C according to a protocol approved by the Joint Institutional Review Board of Taipei Medical University (N201805095). Each tooth was embedded in acrylic resin (Ortho-Jet; Lang Dental Manufacturing, Wheeling, IL, USA). The crown and occlusal enamel were removed, and the remainder was cut into 10 dentin disks with a thickness of around 600–800 μm with a low-speed 0.15-mm diamond saw (TechCut 4™, Allied High Tech Products, Rancho Dominguez, CA, USA) parallel to the occlusal surface. The first group of specimens ($n = 10$) was used to prepare artificial caries dentin disks to test the efficacy of various CMCR systems, while the second group ($n = 5$) was used for dentin collagen extraction to establish the feasibility of using reagent-grade type I collagen (Sigma-Aldrich) as a substitute for human dentin collagen. The dentin discs were demineralized by soaking them for 2 days in 10 wt% EDTA at pH 7.4 adjusted using 10 N KOH. Specimens were rinsed with deionized (DI) water three times, then transferred into a 0.5 M acetic acid and 0.5 mg/ml pepsin bath at 4 °C for 24 h. Then the supernatant was collected, NaCl was added until the concentration reached 0.9 M, it was allowed to sit for 24 h, it was centrifuged at 1000 rpm for 1 h, and the precipitant was freeze-dried. The harvested dentin collagen was stored for further characterization.

Collagen dissolution test

After confirming the feasibility of using commercial calfskin-derived collagen (Sigma-Aldrich) as a model system, a collagen-dissolution test was carried out to screen each CMCR system. Concentrations of 0.1%, 0.5%, 1%, 2%, 3%, 4%, and 5% of a sodium hypochlorite (NaOCl) and zinc chloride ($ZnCl_2$) solution were prepared. Collagen fibers were collected under a bath ratio of 1 mg/1 ml of various CMCR reagents. For the static dissolution test, each designed specimen was hand-shaken for mixing for 10 s and then allowed to sit for 3 min. The status of full dissolution, partial dissolution, and non-dissolution by visual observation was recorded. For the ultra-sonicating dissolution test, each designed specimen was hand-shaken for mixing for 10 s and treated by ultrasonic Newtron® P5 XS B.LED Booster (ACTEON North America, Mount Laurel, NJ, USA) at a frequency of 32 kHz for 3 min, and then the dissolution states were recorded. After the dissolution test, the supernatant of each specimen was collected to investigate variations in molecular weights (MWs) by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and the secondary structure by circular dichroism (CD).

Artificial caries dentin disks

Artificial caries dentin disks were prepared according to procedures reported by Sattabanasuk et al.[30] Flat dentin disks obtained from the superficial occlusal dentin were polished with 1000-grit wet silicon carbide paper and 1200-grit water sandpaper at 500 rpm to create a smooth surface. The bottom surface of each disk was coated with two layers of nail varnish. Dentin disks were immersed in an acid buffer solution of 0.1 M lactic acid at pH 4.5 adjusted with 10 N KOH for 4 days at 4 °C to create the demineralized dentin layer. After demineralization, each disk was rinsed with DI water for 15 min, and the varnish coating was removed. Specimens were randomly divided into three

equal groups ($n = 10$) and treated with the following CMCR reagents for 30 s.

(1) Untreated (blank control),
(2) 0.5% NaOCl + 0.2 M amino acids (CarisolvTM-like CMCR), and
(3) 5% $ZnCl_2$ (experimental group).

After treatment, all specimens were immediately immersed in 10% neutral buffered formalin (Electron Microscope Sciences, Fort Washington, PA, USA) for 24 h to fix the dentin collagen. After fixation, disks were rinsed with DI water for 15 min. They were then dehydrated in ascending grades of ethanol (10%, 20%, 30%, 50%, 70%, and 90% each for 20 min). In the final run, specimens were soaked in 100% ethanol for 20 min, with three changes, and then air dried at ambient temperature. Field emission scanning electronic microscopy (FE-SEM) was used to investigate morphological changes in the dentin collagen network due to treatment with the CMCR systems.

Poly-acrylamide-gel-electrophoresis

For a qualitative analysis of the stability of collagen, we used SDS-PAGE with the Hoefer SE250/SE260 small-format vertical electrophoresis system (Hoefer, Holliston, MA, USA). SDS-PAGE was carried out using a discontinuous Tris-HCl/glycine buffer system, with 5% (w/v) polyacrylamide (Bio-Rad, Hercules, CA, USA) as the separating gel, and 3% (w/v) polyacrylamide as the stacking gel. Various designed CMCR reagents and a standard (1 mg of bovine type I collagen in 1 ml of 0.05 M acetic acid) were prepared in 0.5 M Tris-HCl buffer (pH 6.8) containing 10% SDS, 30% glycerol, 1% 2-mercaptoethanol, and 0.02% bromophenol blue, and then the solution was heated to 65 °C for 30 min. Next, 10 μL of protein markers (BioTools, New Taipei City, Taiwan), 10 μL of the standard, and specimens were loaded and electrophoresed at 110 V on vertical slab gels until the bromophenol blue had moved out of the gel. Then 10 μl of 1 mg/ml actin (MW 43 kDa) was added to each sample well for referencing. Polyacrylamide gels were stained for 30 min with 0.3% Coomassie blue R-250 in acetic acid/methanol/water 1:5:4 (v/v/v) and were de-stained in 10.0% acetic acid/15% methanol for 1 h.

Circular dichroism

Secondary structure conformation changes of the collagen solution after being treated with various formulations were characterized by CD (circular dichroism). Each treated collagen solution was diluted to 0.3 mg/ml, and a 220-μl specimen was loaded into a rectangular quartz cuvette with a 1-mm path length, and measured using a Jasco spectropolarimeter (J-810, Jasco, Tokyo, Japan). The CD spectra were recorded at 180—240 nm under a scan rate of 50 nm/min, and eight scans were averaged. CDNN 2.1 software (Applied Photophysics Ltd., Surrey, United Kingdom) was then used to calculate secondary structure proportions from the obtained CD spectrum.

Attenuated total reflectance fourier-transformed infrared spectroscopy

ATR-FTIR spectroscopy was performed to investigate the treatment effects on the functional groups of decalcified dentin collagen after being treated with various CMCR reagents and formalin fixation. In this experiment, the autoimage ATR mode of the FTIR system (Spectrum 2000, PerkinElmer, Llantrisant, UK) was used. ATR-FTIR spectra were recorded from 400 to 4000 cm^{-1} (2.5—25 μm), and 128 scans were averaged at a resolution of 4 cm^{-1}.

Field emission scanning electronic microscopy

Samples of decalcified dentin collagen after being treated with various CMCR reagents and formalin fixation were sputtered with a thin layer of gold palladium by an ion sputter (E—1010, Hitachi, Minato-ku Tokyo, Japan), and the morphologies and microstructures were observed using FE-SEM (JSM-6700F, JEOL, Akishima, Tokyo, Japan).

Results

Fig. 1 shows a typical SDS-PAGE pattern for type I collagen with two apparent bands at MWs of 130 and 121 kDa, which were calculated from a graph of log MW vs. Rf (the relative migration distance). NaOCl solutions were basic in the pH range of 10.78—12.44, while $ZnCl_2$ solutions were acidic in the pH range of 6.57—5.42 depending on their concentrations. Dissolution behaviors of type I collagen fibers under various CMCR systems are summarized in Table 1. Under the static dissolution mode, type I collagen fibers were partially soluble under NaOCl concentrations of 0.1—0.5 wt%, and fully soluble under NaOCl concentrations of 1.0—5.0 wt%, while they were insoluble under $ZnCl_2$ concentrations of 0.1—5.0 wt%. The ultra-sonicating dissolution test showed the fibers to be fully soluble in NaOCl and in $ZnCl_2$ under a concentration range of 0.1—5.0 wt%.

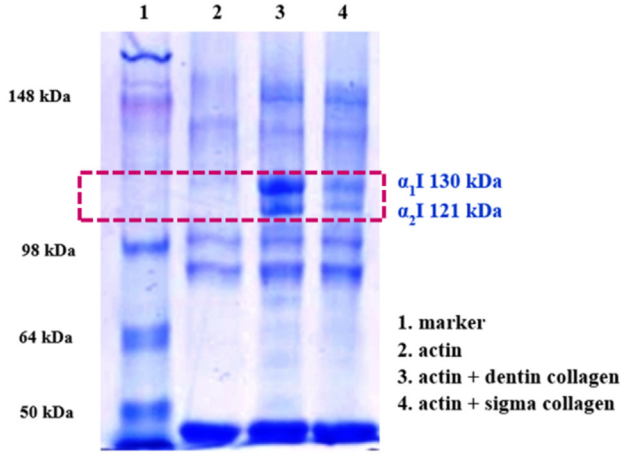

Figure 1 SDS-PAGE analysis of collagen extracted from the dentin collagen matrix. Lane 1: molecular weight (MW) marker; lane 2: actin (43 kDa); lane 3: actin + dentin collagen; lane 4: actin + collagen purchased from Sigma-Aldrich.

Table 1 Dissolution test results for type I collagen fibers under various CMCR systems.

Conc. (%)	NaOCl solution			$ZnCl_2$ solution		
	pH	Static 3 min	Ultrasonic 3 min	pH	Static 3 min	Ultrasonic 3 min
0.1	10.78	▲	●	6.57	×	●
0.5	11.54	▲	●	6.32	×	●
1.0	11.82	●	●	6.13	▲	●
2.0	12.08	●	●	5.88	▲	●
3.0	12.25	●	●	5.76	▲	●
4.0	12.36	●	●	5.61	▲	●
5.0	12.44	●	●	5.42	▲	●

Notes: ●, soluble; ▲, partially soluble; ×, insoluble.
CMCR, chemo-mechanical caries removal.

After the dissolution test, the supernatant of each specimen was collected, and MW changes were examined by SDS-PAGE and secondary structure changes were examined by CD (circular dichroism). Experiments monitored CD spectral changes after type I collagen was treated with various concentrations of NaOCl or $ZnCl_2$ solutions (Fig. 2). With the exception of the 0.1 wt% NaOCl solution treatment group which showed a negative band at 200 nm, all other concentration groups showed only noise in the CD spectra. On the contrary, the spectra revealed a positive band at 222 nm and had a pronounced negative band at around 198–202 nm depending on the concentration of the $ZnCl_2$ solutions.

The influence on the collagen MW by SDS-PAGE (SDS poly-acrylamide-gel-electrophoresis) was investigated in Fig. 3 indicating the existence of fingerprint MWs of type I collagen treated with various concentrations of NaOCl and $ZnCl_2$. solutions. Unlike dissolution in NaOCl solutions, calfskin-derived collagen dissolved in 0.1–5.0 wt% $ZnCl_2$ solutions revealed the typical SDS-PAGE pattern for type I collagen with MW bands at 121 and 130 kDa. In addition, the band intensity at 130 kDa appeared roughly twice that of the 121-kDa band, suggesting a 2: 1 ratio of α1(I) to α2(I) chains of the triple helix structure. For groups treated with NaOCl solutions, the lack of characteristic MW bands for α1(I) to α2(I) might be attributed to the severe oxidization and degradation of the collagen molecular chains.

Fig. 4 presents FE-SEM (field emission scanning electron microscope) micrographs of the morphology of acid-etched dentin collagen matrix after being treated with various reagents. Fig. 4a is the control group showing typical dentin tubules on the surface of untreated dentin and the collagen fibril network on acid-etched dentin (4000 ×). The rough appearance of the dentin surface was attributed to exposure of the collagen fibril chain-ends at the top of the untreated dentin under 20,000 × magnification. On the contrary, a smooth dentin surface and no observable collagen fibril residues inside the dentin tubules were evident after treatment with 0.5 wt% NaOCl as shown in Fig. 4b. A similar dentin surface morphology but with less roughness is shown in Fig. 4c and d with treatment with 0.5 wt% NaOCl +0.2 M amino acids and 5.0 wt% $ZnCl_2$, respectively. The modification by 0.5 wt% NaOCl with amino acids was adopted from the current commercial product of Carisolv® gels. Compared to the group treated with 0.5 wt% NaOCl (Fig. 4b), there are few collagen fibril residues on the dentin surface in Fig. 4c. As to the demineralized dentin treated with 0.5 wt% $ZnCl_2$, collagen fibrils were notable

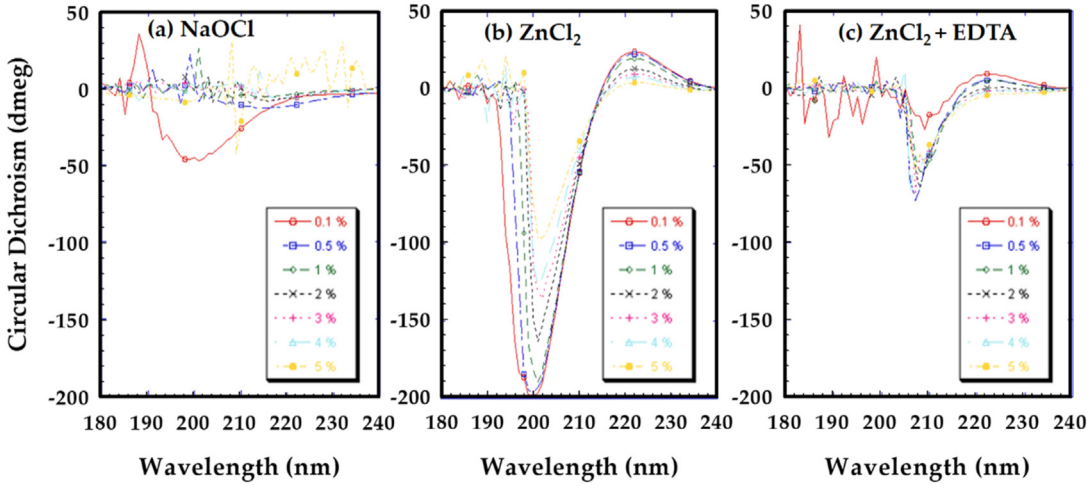

Figure 2 Circular dichroism spectra for type I collagen after being treated with various concentrations of (a) NaOCl solutions and (b) $ZnCl_2$ solutions (c) $ZnCl_2$ solutions + EDTA.

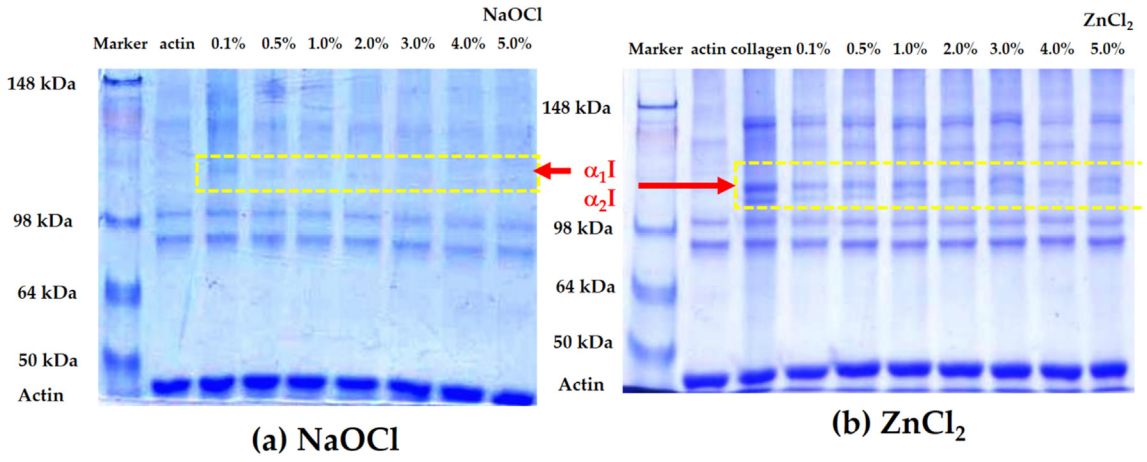

Figure 3 SDS-PAGE analysis of calfskin-derived collagen after treatment with various concentrations of (a) NaOCl and (b) $ZnCl_2$ solutions. Lane 1: molecular weight (MW) marker; lane 2: actin (43 kDa); lane 3: actin + collagen; Other lanes: solution concentrations as specified.

inside the dentin tubules and on the dentin surface (Fig. 4e). In contrast to the collagen fibril network of the control group, these collagen fibrils had collapsed and were deposited on the dentin surface due to their partial dissolution.

Fig. 5a and b are the respective representative FE-SEM images of untreated dentin and acid-etched dentin collagen matrix, while Fig. 5c and d shows their morphologies after being treated with 0.5 wt% NaOCl +0.2 M amino acids and 5.0 wt% $ZnCl_2$ combined with 30 s ultrasonication, respectively. FE-SEM images of demineralized dentin showed round, aligned and intact dentinal tubules. From an anatomical viewpoint, the dentin structure is a nanocomposite of nanocrystalline HAp and collagen fiber matrix with a network of dentin tubules. After acid-etching with 0.1 M lactic acid, the FE-SEM image of the demineralized dentin surface showed a dense dentinal collagen network (at 4000 ×). Compared to the untreated dentin surface, large diameters of dentin tubules in the acid-etched dentin collagen matrix were observed due to the acid removal of HAp. The broken fibril-ends of collagen were artifacts due to sample preparation and treatments. In Fig. 4c and d, there is no observable collagen matrix on the demineralized dentin surface after treatment with

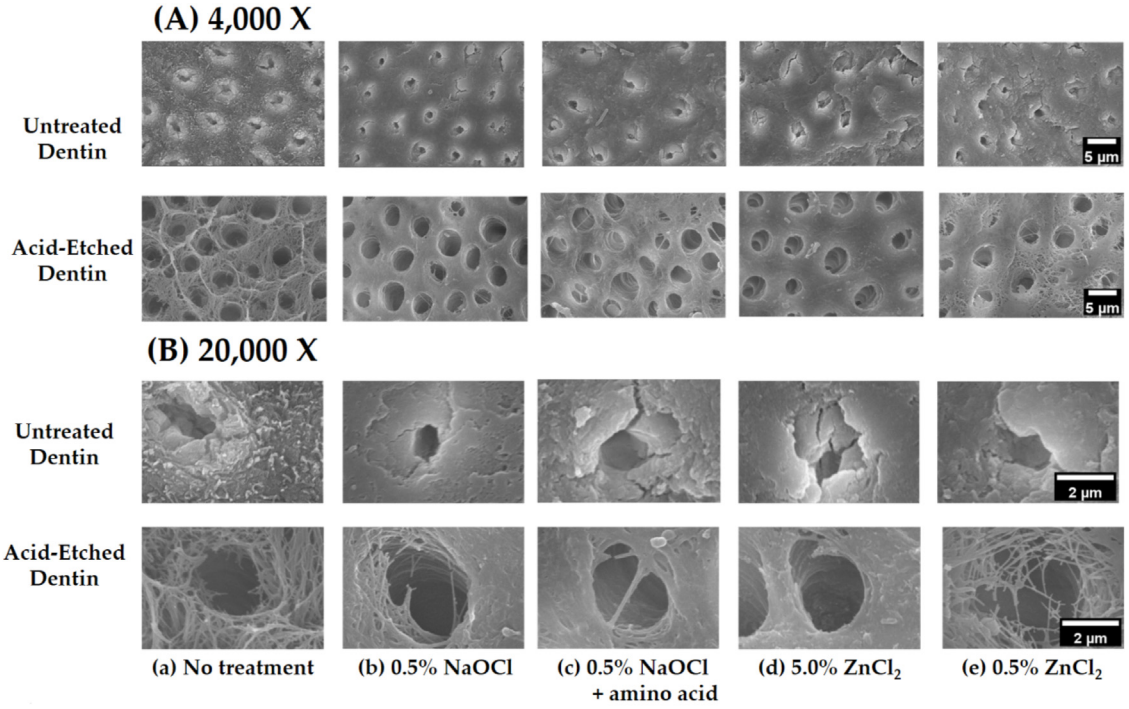

Figure 4 FE-SEM (field emission scanning electronic microscopy) micrographs at various magnifications of untreated dentin and acid-etched dentin collagen matrix morphologies with (a) no treatment or after being treated with (b) 0.5 wt% NaOCl, (c) 0.5 wt% NaOCl + 0.2 M amino acids, (d) 5.0 wt% $ZnCl_2$, or (e) 0.5 wt% $ZnCl_2$ combined with 30 s of ultrasonication.

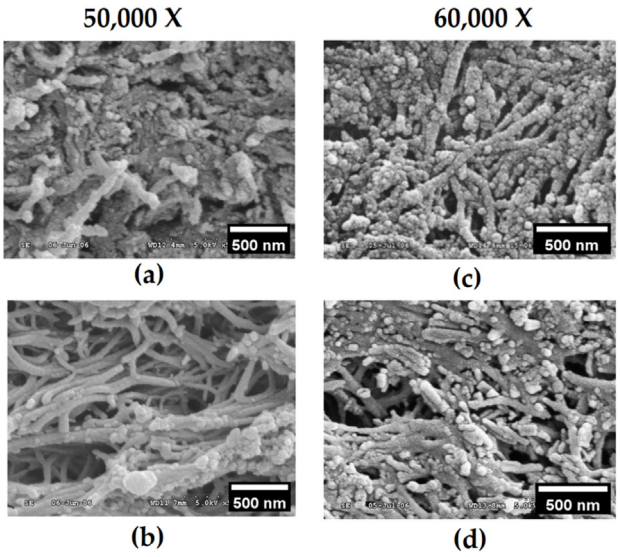

Figure 5 FE-SEM (field emission scanning electronic microscopy) micrographs of (a) untreated dentin, (b) acid-etched dentin collagen matrix, and their morphologies after being treated with (c) 0.5 wt% NaOCl + 0.2 M amino acids, (d) 5.0 wt% $ZnCl_2$ combined with 30 s of ultra-sonication under various magnifications.

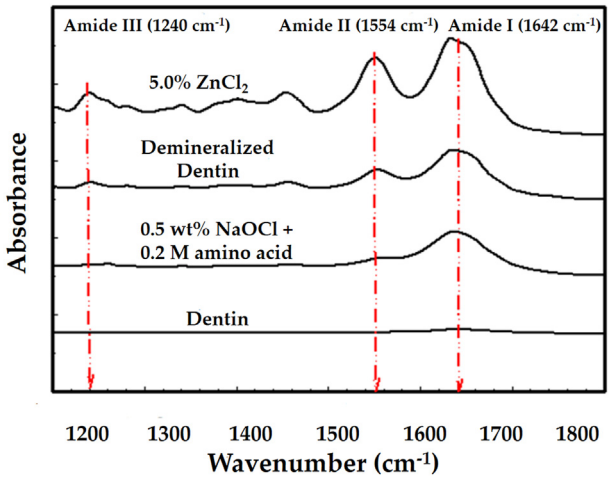

Figure 6 ATR-FTIR (attenuated total reflectance fourier-transformed infrared spectroscopy) spectra of untreated dentin, demineralized dentin, and the demineralized dentin matrix treated with NaOCl and $ZnCl_2$ solutions.

NaOCl and $ZnCl_2$ (at 10,000 ×). However, there were some residual fibers inside the dentin tubules, and the surface showed a deposition layer which left the surface with many overhangs and undercuts in the group treated with 5.0 wt% $ZnCl_2$. In addition, the texture of the collagen fiber matrix differed between treatments with NaOCl and $ZnCl_2$ (at 60,000 ×). More broken fibril-ends and shorter fiber lengths were collapsed on the demineralized dentin surface after being treated with the NaOCl solutions.

The ATR-FTIR (attenuated total reflectance fourier-transformed infrared spectroscopy) was carried out to investigate the characteristic absorption peaks for type I collagen were 1642 cm^{-1} (amide I), 1554 cm^{-1} (amide II), and 1240 cm^{-1} (amide III) (see Fig. 6). Unlike the untreated dentin surface, the ATR-FTIR spectra for the demineralized dentin surface had pronounced signature absorption peaks. The weak FTIR absorption peaks for the untreated dentin surface might be attributed to the collagen fibers being deeply buried in the HAp/collagen composite matrix.

Discussion

To establish a reagent evaluation platform, SDS-PAGE offered the fingerprint MW to confirm the purity of the collagens between extracting the dentin collagen and commercial calfskin-derived collagen (Sigma-Aldrich). Similar to the SDS-PAGE pattern of the dentin collagen matrix, the calfskin-derived collagen was lighter but had similar band locations at α1I (130 kDa) and α2I (121 kDa) for type I collagen. Bands of actin (43 kDa) appeared in lanes 2–4. Under the assumption that the SDS-PAGE band intensity was related to the amount of protein, the solubility of calfskin-derived collagen was less than that of the extracted dentin collagen. The commercial calfskin-derived collagen could thus serve as a model system for evaluating the designed CMCR systems. The type I collagen molecule was revealed to have a MW of about 283 kDa with two α1 (1056 amino acid) and one α2 (1029 amino acid) indicating a typical triple-helix structure.[31] Inanc et al. reported that type I collagen extracted from rat-tail tendon had bands at apparent MWs of 217 (β), 131 (α1), and 120 kDa (α2).[32] Similar results were also observed in this study.

During development of dentin caries, demineralization of HAp and the destruction of dentin organic matrix progressed. Potentially active MMPs are collagenases (MMP-1, MMP-8), gelatinases (MMP-2, MMP-9), stromelysin (MMP3), and enamelysin (MMP-20).[33] Among host-derived MMPs detected in dentin, MMP-8 was suggested to be a major dentinal collagenolytic MMP which hydrolyzes the collagen matrix.[34] In addition, dentinal collagen degraded by Streptococcus mutans proteases was evident on SDS-PAGE gel images, and two of the resultant fragments were further identified as α1 chains of human type I collagen based on a sequence homology analysis.[35]

Since all of the specimens were fully soluble in 0.1–5.0 wt% of NaOCl and $ZnCl_2$ solutions under the ultra-sonicating dissolution test, it was of great importance to distinguish their influence on the secondary structures of type I collagen. The type I collagen molecule, a 300-nm-long triple helix comprising two α1(I) and one α2(I) chains, has a distinctive triple-helical structure composed of three left-handed polyproline–II–type polypeptide chains.[36] CD, defined as the absorption difference between left-handed and right-handed circularly polarized light due to structural asymmetry, is a useful technique for analyzing secondary structures and folding properties of proteins.[37] For randomly coiled protein structures, a typical spectrum revealed a negative band of great magnitude at around 200 nm,[38] while the triple helix protein backbone exhibited a positive absorption band at 222 nm and a negative absorption band at 195 nm.[39] The CD spectral signature band with positive absorption at

222 nm indicated the existence of a triple-helix structure for type I collagen after being treated with $ZnCl_2$ solutions. For groups treated with NaOCl solutions, the lack of triple-helix structures might be attributed to serious oxidation and degradation. As to the negative band located around 198—202 nm but not 195 nm for a typical triple-helix in this study, one hypothesis is that partial type I collagen chains might reveal a phase transformation from a triple-helix to a randomly coiled structure during the dissolution step in $ZnCl_2$ solutions. These spectra might be attributed to a superposition of denatured random coils and triple-helical spectra. However, the CD spectral signature band for triple helix structures diminished after adding the chelation agent of EDTA to the $ZnCl_2$ solutions (Fig. 2c). On a larger scale, super-secondary structures are known to be combinations of basic secondary structural units, of either α-helix or β-sheets. In a classical zinc finger motif, two antiparallel β-sheets and an α-helix are brought together by zinc ions to stabilize the motif structure.[40] The possible protective effect of zinc ions on triple helix structures was proposed according to the zinc finger-like metal-protein complex super-secondary structure that contributes to the overall stability of type I collagen.[41] A consequence of the chelation of zinc ions by EDTA is that the triple-helix structure of type I collagen might collapse.

As evidenced by CD spectra, NaOCl effectively disrupted the triple-helix structure of collagen. It is well known that collagen fibrils, architecturally organized tropocollagen units, are stabilized by crosslinking between polypeptides and tropocollagen and aggregate to form a dense network in the dentin. Denaturation of collagen proteins by NaOCl will weaken the strength of collagen fibrils and the integrity of demineralized collagen network, thus suggesting the feasibility of using hand instruments in chemo-mechanical caries removal operations. In addition to dissolution and denaturation, NaOCl might play a role in collagen degradation. Collagen is a protein containing up to 57% of total amino acids (AAs) of glycine, proline, and hydroxyproline.[42] Possible cleavage sites were reported in glycine or hydroxyproline during degradation by NaOCl.[43] However, more-detailed studies revealed that chloramine formation at lysine side-chains and nitrogen-centered radicals are key elements in the induced protein fragmentation based on an electron spin resonance (ESR) technique.[21] NaOCl molecules can infiltrate into the apatite/collagen matrix, oxidize the organic matrix, denature the collagen, and change the chemical composition of dentin.[44]

Burke et al. reported the mean bond strength of glass polyalkenoate cement showed no significant difference between the Carisolv® gel-treated groups or a conditioner of 25% poly(acrylic acid)-treated groups.[45] Differences in the surface morphology between the dentin surface treated with $ZnCl_2$ solutions were evident and were believed to be the suitable for restorations with modern adhesive materials according to the collagen fibril residues.

This CMCR procedure avoids painful caries removal; however, there are some limitations when using this system. The efficiency and effectiveness among various carious dentine excavation techniques (bur, air-abrasion, sono-abrasion, and Carisolv™ gel) were evaluated and compared to conventional hand excavation.[46] In terms of the operative time to prepare the cavities, bur excavation was shortest and most efficient, while Carisolv gel was the longest among dentists in clinical practice.

Only exposed collagen fibrils could contribute to the FTIR spectra. Similar phenomenological estimations might be extendable to the ATR-FTIR spectra for the demineralized dentin matrix treated with NaOCl and $ZnCl_2$ solutions. The pronounced FTIR absorption peaks for the demineralized dentin surface treated with $ZnCl_2$ solutions indicated a

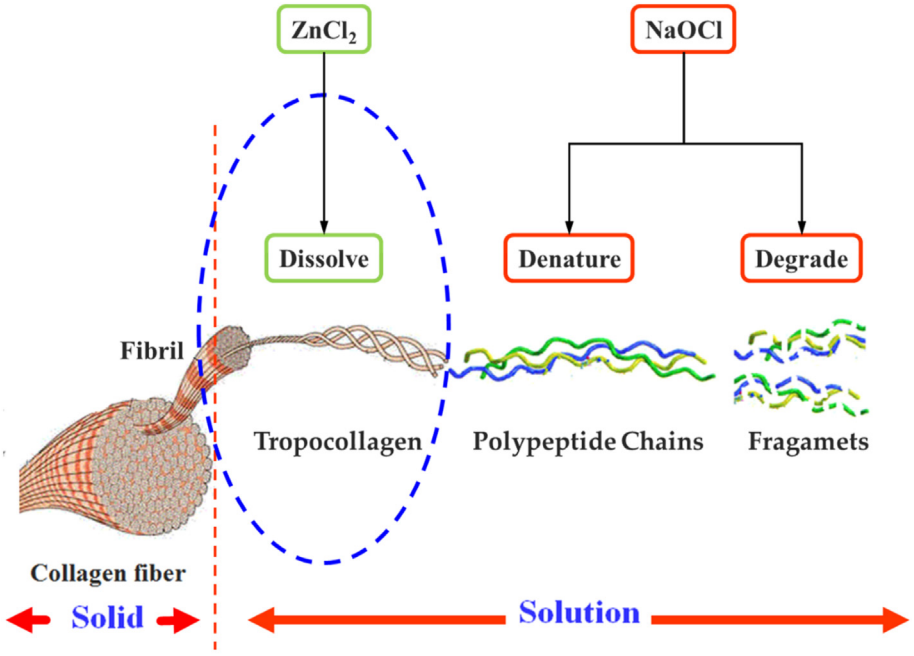

Figure 7 Proposed mechanisms of the caries dentin removal system based on $ZnCl_2$ and NaOCl solutions.

large quantity of residual collagen fiber deposition on the dentin surface.

The schematic comparison between the different caries dentin-removal systems based on $ZnCl_2$ and NaOCl solutions is summarized in Fig. 7. Calfskin-derived collagen was shown to be fully soluble in both the NaOCl and $ZnCl_2$ solutions. The strong oxidization potential of the NaOCl solutions tended to dissolve but not denature or degrade the calfskin-derived collagen molecules. Unlike in the NaOCl solutions, the $ZnCl_2$ solution offered an effective dissolution and protective effect of the triple helix structures (CD spectra), and preservation of the MWs of the α1(I) to α2(I) chains (SDS-PAGE) and the functional groups (ATR-FTIR spectra).

In fact, carious tissue removal is never the end of dental treatment, but purely to create conditions for further long-lasting restorations. The potential risk of pulp exposure during carious tissue removal in deep caries might be avoided by current NaOCl-based minimally invasive CMCR systems. However, the degradation and denaturation of collagen fibrils in the dentin network result in unavailability of the use of collagen fibrils for direct prosthetic restoration. Current self-etching or etch-and-rinse adhesives still need to etch and prime the tooth.[47] Furthermore, the reduction of the bond strength between adhesive systems and dentin walls by NaOCl might occur because of loss of collagen fibrils from the dentin surface and obstruction of the formation of a consistent hybrid layer.[48] To ensure the longevity of the subsequent restoration, maintaining the collagen network structure is still meaningful after treatment with CMCR systems.

Based on the results of good solubility, a protective effect of triple helix structures, and preservation of the MW of type I collagen, the $ZnCl_2$-based chemo-mechanical caries-removal system can dissolve rather than denature or degrade the collagen network. After removing the dentin caries, if the dentin collagen network can still be maintained, then the adhesive restoration might can directly proceed without the acid-etching step. Therefore, this study sheds some light on developing a novel CMCR system to offer caries patients a milder, less-invasive alternative for caries treatment. Additional in vivo evaluations are needed to verify its usefulness in clinical applications.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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References

1. *Global Oral Health Status Report: Towards Universal Health Coverage for Oral Health by 2030*. Geneva: World Health Organization, 2022.
2. Yeh DY, Kuo HC, Yang YH, Ho PS. The responsiveness of patients' quality of life to dental caries treatment-a prospective study. *PLoS One* 2016;11:e0164707.
3. Nagarathna C, Rahul C, Umapathy T. Characteristics of the deep carious lesions of primary and permanent molars using IOPA radiographs. *Eur J Dent Oral Health* 2021;2:1—3.
4. Ali AH, Thani FB, Foschi F, Banerjee A, Mannocci F. Self-limiting versus rotary subjective carious tissue removal: a randomized controlled clinical trial-2-year results. *J Clin Med* 2020;9:2738.
5. Abdelaziz E, Badran A, Allam G. Chemomechanical caries removal agents and their applications in pediatric dentistry. *Adv Dent J* 2022;4:11—8.
6. Qiu W, Zhou Y, Li Z, Huang T, Xiao Y, Cheng L. Application of antibiotics/antimicrobial agents on dental caries. *BioMed Res Int* 2020;2020:5658212.
7. Chen KK, Wu JH, Wei SI, Du JK. Influence of the acidity of electrolyzed water on the microhardness of inner layer dentin. *J Dent Sci* 2019;14:419—25.
8. Chiang YC, Wang YC, Kung JC, Shih CJ. Antibacterial silver-containing mesoporous bioglass as a dentin remineralization agent in a microorganism-challenged environment. *J Dent* 2021;106:103563.
9. Ten Cate AR. *Oral Histology : Development, Dtructure, and Function*, 5th ed. St. Louis: Mosby, 1998.
10. Bansal M, Ananthanarayanan VS. The role of hydroxyproline in collagen folding: conformational energy calculations on oligopeptides containing proline and hydroxyproline. *Biopolymers* 1988;27:299—312.
11. Bertassoni LE, Orgel JP, Antipova O, Swain MV. The dentin organic matrix - limitations of restorative dentistry hidden on the nanometer scale. *Acta Biomater* 2012;8:2419—33.
12. Marshall Jr GW, Marshall SJ, Kinney JH, Balooch M. The dentin substrate: structure and properties related to bonding. *J Dent* 1997;25:441—58.
13. Hedenbjörk-Lager A, Hamberg K, Pääkkönen V, Tjäderhane L, Ericson D. Collagen degradation and preservation of MMP-8 activity in human dentine matrix after demineralization. *Arch Oral Biol* 2016;68:66—72.
14. Hamama H, Yiu C, Burrow M. Current update of chemo-mechanical caries removal methods. *Aust Dent J* 2014;59:446—56.
15. Vougiouklakis G, Paroussis D. Chemomechanical means of removing caries–Caridex system. *Hell Stomatol Chron* 1988;32:97—102.
16. Kathuria V, Ankola AV, Hebbal M, Mocherla M. Carisolv- an innovative method of caries removal. *J Clin Diagn Res* 2013;7:3111—5.
17. Cardoso M, Coelho A, Lima R, Amaro I, Paula A. Efficacy and patient's acceptance of alternative methods for caries removal—a systematic review. *J Clin Med* 2020;9:3407.
18. Magalhães CSd, Moreira AN, Campos WRdC, Rossi FM, Castilho GAA, Ferreira RC. Effectiveness and efficiency of chemomechanical carious dentin removal. *Braz Dent J* 2006;17:63—7.
19. Nagaveni N, Radhika N, Satisha T, Ashwini K, Neni S, Gupta S. Efficacy of new chemomechanical caries removal agent compared with conventional method in primary teeth: an in vivo study. *J Int Oral Health Sci* 2016;6:52—8.
20. Ogushi K, Fusayama T. Electron microscopic structure of the two layers of carious dentin. *J Dent Res* 1975;54:1019—26.
21. Hawkins CL, Davies MJ. Hypochlorite-induced damage to proteins: formation of nitrogen-centred radicals from lysine residues and their role in protein fragmentation. *Biochem J* 1998;332:617—25.
22. Zhou W, Liu S, Zhou X, et al. Modifying adhesive materials to improve the longevity of resinous restorations. *Int J Mol Sci* 2019;20:723.
23. Perdigão J. Current perspectives on dental adhesion: (1) Dentin adhesion - not there yet. *Jpn Dent Sci Rev* 2020;56:190—207.
24. Nakabayashi N, Pashley DH. *Hybridization of Dental Hard Tissues*. Tokyo ; London: Quintessence Pub. Co, 1998.

25. Nakabayashi N, Kojima K, Masuhara E. The promotion of adhesion by the infiltration of monomers into tooth substrates. *J Biomed Mater Res* 1982;16:265—73.
26. Lin HH, Han LY, Zhang HL, et al. Prediction of the functional class of metal-binding proteins from sequence derived physicochemical properties by support vector machine approach. *BMC Bioinf* 2006;7:S13.
27. Matthews JM. Zinc finger folds and functions. In: Kretsinger RH, Uversky VN, Permyakov EA, eds. *Encyclopedia of Metalloproteins*. New York, NY: Springer New York, 2013:2420—8.
28. Osorio R, Yamauti M, Osorio E, et al. Zinc reduces collagen degradation in demineralized human dentin explants. *J Dent* 2011;39:148—53.
29. Wöltje M, Kölbel A, Aibibu D, Cherif C. A fast and reliable process to fabricate regenerated silk fibroin solution from degummed silk in 4 hours. *Int J Mol Sci* 2021;22:10565.
30. Sattabanasuk V, Shimada Y, Tagami J. Bonding of resin to artificially carious dentin. *J Adhesive Dent* 2005;7:183—92.
31. Miller EJ. Chemistry of the collagens and their distribution. In: Piex KA, Reddi AH, eds. *Extracellulose Matrix Biochemistry*. New York: Elsevier, 1984.
32. Inanc S, Keles D, Oktay G. An improved collagen zymography approach for evaluating the collagenases MMP-1, MMP-8, and MMP-13. *Biotechniques* 2017;63:174—80.
33. Mazzoni A, Tjäderhane L, Checchi V, et al. Role of dentin MMPs in caries progression and bond stability. *J Dent Res* 2015;94:241—51.
34. Chaussain-Miller C, Fioretti F, Goldberg M, Menashi S. The role of matrix metalloproteinases (MMPs) in human caries. *J Dent Res* 2006;85:22—32.
35. Huang B, Stewart CA, McCulloch CA, Santerre JP, Cvitkovitch DG, Finer Y. Streptococcus mutans proteases degrade dentinal collagen. *Dent J* 2022;10:223.
36. Makareeva E, Mertz EL, Kuznetsova NV, et al. Structural heterogeneity of type I collagen triple helix and its role in osteogenesis imperfecta. *J Biol Chem* 2008;283:4787—98.
37. Büyükköroğlu G, Dora DD, Özdemir F, Hızel C. Chapter 15 - techniques for protein analysis. In: Barh D, Azevedo V, eds. *Omics Technologies and Bio-Engineering*. Academic Press, 2018:317—51.
38. Corrêa D, Ramos C. The use of circular dichroism spectroscopy to study protein folding, form and function. *Afr J Biochem Res* 2009;3:164—73.
39. Greenfield NJ. Using circular dichroism spectra to estimate protein secondary structure. *Nat Protoc* 2006;1:2876—90.
40. Brodsky B, Shah NK. Protein motifs. 8. The triple-helix motif in proteins. *FASEB* 1995;9:1537—46.
41. Preciado LM, Pereañez JA. Low molecular mass natural and synthetic inhibitors of snake venom metalloproteinases. *Toxin Rev* 2018;37:19—26.
42. Li P, Wu G. Roles of dietary glycine, proline, and hydroxyproline in collagen synthesis and animal growth. *Amino Acids* 2018;50:29—38.
43. Dow J, Lindsay G, Morrison J. *Biochemistry: Molecules, Cells and the Body*. Addison-Wesley, 1996.
44. Sim TP, Knowles JC, Ng YL, Shelton J, Gulabivala K. Effect of sodium hypochlorite on mechanical properties of dentine and tooth surface strain. *Int Endod J* 2001;34:120—32.
45. Burke FM, Lynch E. Glass polyalkenoate bond strength to dentine after chemomechanical caries removal. *J Dent* 1994;22:283—91.
46. Banerjee A, Kidd EA, Watson TF. In vitro evaluation of five alternative methods of carious dentine excavation. *Caries Res* 2000;34:144—50.
47. Ozer F, Blatz MB. Self-etch and etch-and-rinse adhesive systems in clinical dentistry. *Comp Cont Educ Dent* 2013;34:12—4.
48. Tartari T, Bachmann L, Maliza AG, Andrade FB, Duarte MA, Bramante CM. Tissue dissolution and modifications in dentin composition by different sodium hypochlorite concentrations. *J Appl Oral Sci* 2016;24:291—8.