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A Dynamic Mechanical Method to Assess Bulk Viscoelastic Behavior of The Dentin Extracellular Matrix

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Abstract

Objectives

To develop a protocol for assessment of the bulk viscoelastic behavior of dentin extracellular matrix (ECM), and to assess relationships between induced collagen cross-linking and viscoelasticity of the dentin ECM.

Methods

Dentin ECM was treated with agents to induce exogenous collagen cross-linking: proanthocyanidins (PACs) from *Vitis vinifera* – VVe, PACs from *Pinus massoniana* - PMe, glutaraldehyde – (GA), or kept untreated (control). A dynamic mechanical strain sweep method was carried out in a 3-point bending submersion clamp at treatment; after protein destabilization with 4 M urea and after 7-day, 6-month, and 12-month incubation in simulated body fluid. Tan δ, storage (E'), loss (E"), and complex moduli (E*) were calculated and data were statistically analyzed using two-way ANOVA and post-hoc tests ($\alpha = 0.05$). Chemical analysis of dentin ECM before and after protein destabilization was assessed with ATR-FTIR spectroscopy.

Results

Significant interactions between study factors (treatment *vs*. time points, $p < 0.001$) were found for all viscoelastic parameters. Despite a significant decrease in all moduli after destabilization, PAC-treated dentin remained statistically higher than control ($p < 0.001$), indicating permanent mechanical enhancement after biomodification. Covalently crosslinked, GA-treated dentin was unaffected by destabilization ($p = 0.873$) and showed the lowest damping capacity (tan δ) at all time points ($p < 0.001$). After 12 months, the damping capacity of PMe and VVe groups decreased significantly. Changes in all amide IR resonances revealed a partial chemical reversal of PAC-mediated biomodification.

Significance

Viscoelastic measurements and IR spectroscopy aid in elucidating the role of inter-molecular collagen cross-linking in the mechanical behavior of dentin ECM.

Keywords

Proanthocyanidins, Dynamic mechanical analysis, Dentin, Urea, Glutaraldehyde

Abbreviations

PAC proanthocyanidin
DMA dynamic mechanical analysis
GA glutaraldehyde
ECM extracellular matrix
SBF simulated body fluid
AFM atomic force microscopy
VV *Vitis vinifera*
PM *Pinus massoniana*
E* complex modulus
E' storage modulus
E" loss modulus
ATR-FTIR attenuated total reflectance-Fourier transform infrared spectroscopy

1. Introduction

The viscoelasticity of dentin is attributed to the extracellular matrix (ECM) enabling the combined behavior of an elastic solid and a viscous liquid [[1], [2], [3]]. Under uniaxial static forces, viscoelastic tissues commonly exhibit lack of linearity in the stress-strain curve [1,4]. In contrast to static methods, dynamic mechanical analysis (DMA) uses harmonic vibrations simulating the cyclic/dynamic loading of the dentin and, thus, enhancing mechanical characterization of viscoelastic materials.

Previous efforts showed that, at the nano-length scale, the viscoelasticity of the dentin (nanoDMA, small angle X-ray scattering, AFM) is attributed to the sliding and unfolding of collagen molecules and fibrils [5], while at a higher hierarchical level, the viscoelasticity is associated with the crosslinking and density of the collagen fibrils [6]. Consequently, dentin's mechanical behavior results from its complex hierarchical composition, and the analysis of its bulk viscoelastic properties leads to a better mechanical understanding. The storage modulus (E') represents the elastic properties of a material (capacity to store energy, solid-like response) while the loss modulus (E") refers to the viscous properties of a material (capacity to lose energy, fluid-like response). The ratio between both moduli (tan δ), also called damping capacity, describes the ability of the material to dissipate energy and is considered an important functional feature [2,3].

The determination of collagen crosslinking effects on the bulk viscoelasticity of dentin is possible through the analysis of the viscoelastic properties. Collagen crosslinking is a post-translational modification of proteins that directly impacts the viscoelastic properties of the ECM [2,7,8]. Plant-derived proanthocyanidins (PACs) and synthetic glutaraldehyde (GA) can induce non-enzymatic crosslinking that biomodifies the dentin matrix and promote the enhancement of their mechanical and biochemical properties [7,[9], [10], [11], [12]]. Such dentin biomodification takes place at different hierarchical levels, and these chemical interactions are the basis for the biological stability, tensile strength, and viscoelasticity of the resulting dentin matrix [7,9,11,13].

A two-fold aim was pursued in this study: (1) to develop a method for the investigation of the bulk dynamic mechanical behavior of dentin matrices; and (2) to determine the mechanical and chemical

stability of dentin ECM treated with biomodification agents using an accelerated method of protein destabilization. The null hypothesis tested was that the chemical and viscoelastic properties of the bulk dentin matrix, induced by different collagen cross-linkers, would not be affected by an accelerated protein destabilization protocol.

2. Methods

2.1. Preparation of dentin extracellular matrix and biomodification strategies

Mid-coronal dentin specimens of 1.5 × 7 × 0.5 mm (width × length × thickness) were prepared from extracted human sound molars (IRB no. 2018-0346) and demineralized in 10% phosphoric acid (Ricca Chemical Company, Arlington, TX, USA) for 5 h. A dimple created with a diamond bur (835.31.014 F G, Brasseler USA Dental, Savannah, GA, USA) in one edge of the specimen allowed constant specimen positioning throughout the entire experiment.

Demineralized specimens were assigned into groups of synthetic and natural dentin biomodification agents (n = 5). Biomodification strategies included 0.65% w/v of enriched extract from *Vitis vinifera* (VVe) [7,14] or *Pinus massoniana* (PMe) [[15], [16], [17]], in 20 mM HEPES; and 5% v/v glutaraldehyde – GA (Lot 894368, Fisher Scientific, Fair Lawn, NJ, USA) in distilled water (DW) [18]. A control group was exposed to HEPES buffer only. Specimens were immersed in 100 µL of solution (pH 7) for 1 h at room temperature and rinsed with DW. To leach any loosely bound biomodification agent, specimens were kept for 24 h in DW prior to testing.

2.2. Chemical protein destabilization

The protein destabilization protocol consisted of the incubation of specimens in 4 M urea (Lot. R25482, MP Biomedicals, Solon, OH, USA) diluted in DW (pH 6.8) for 24 h at 37 °C [7,19]. Then, specimens were rinsed and stored in simulated body fluid (SBF: 50 mM HEPES, 5 mM $CaCl_2 \cdot 2\ H_2O$, 0.001 mM $ZnCl_2$, 150 mM NaCl, and 3 mM NaN_3, pH 7.4) [20] for 12 months. SBF was changed every two weeks.

2.3. Dynamic mechanical analysis

A strain sweep was conducted to assess viscoelastic properties of the dentin ECM using a dynamic mechanical analyzer (Q800 DMA, TA Instruments, New Castle, DE, USA). A 3-point bending submersion clamp with a drive shaft that oscillates vertically under controlled stress forces was used with a 5-mm span (Fig. 1). The measurements correspond to the response of a viscoelastic material as it is deformed over a range of strain (deformation amplitude) monitored at a constant frequency and temperature. The viscoelastic mechanical components were calculated (modulus precision: ± 1%) using instrument signals (force, displacement, and stiffness) and recorded specimen dimensions (thickness and width).

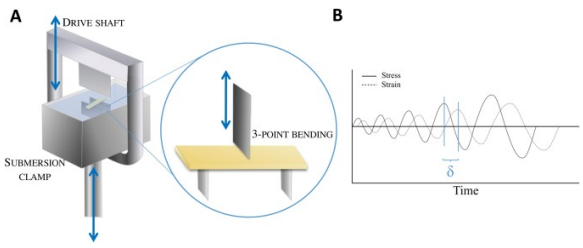

Fig. 1. Dynamic mechanical analysis. A. Illustration of the 3-point bending submersion clamp. A dynamic load is applied by the drive shaft in the middle of the specimen. B. Depiction of the phase angle δ or phase shift formed between the deformation and the response of a viscoelastic material *vs*.

Based on pilot studies, the adopted test parameters were as follows: preload force of 0.01 N, amplitude sweep gradually ascending from 1 to 100 µm (in 10 steps), frequency of 1 Hz at room temperature. All properties were calculated within the range of linear viscoelasticity, where the modulus is independent of the strain. The storage modulus or elastic component is calculated as $E' = (\tau_0/\varepsilon_0) \cos \delta$, the loss modulus or viscous component as $E'' = (\tau_0/\varepsilon_0) \sin \delta$, and the complex modulus as $E^* = (E'^2 + E''^2)^{0.5}$, where τ_0 is the peak stress, ε_0 is the peak strain, and δ is the phase lag between stress and strain (Fig. 1) [21]. The damping capacity (tan δ) was calculated as the ratio of the loss modulus to the storage modulus (E”/E’) and reflects a fluid-like (viscous, Newtonian) behavior when value is high and a solid-like (elastic, Hookean) behavior when tan δ is low. Thus, a purely elastic material has a tan δ = zero.

All demineralized dentin specimens were pre-screened to include specimens with complex modulus in the range of 6−10 MPa baseline values. These specimens were then distributed into the experimental groups. DMA measurements were carried out at the following time points: biomodification (T1), protein destabilization (T2); and at 7-days (T3), 6-months (T4), and 12-months (T5) incubation in SBF.

2.4. Physicochemical analysis of the dentin extracellular matrix

The structural and biochemical variation of the dentin collagen before and after protein destabilization was assessed using ATR-FTIR spectroscopy (Nicolet 6700 and Smart iTR, Thermo Fisher Scientific, Waltham, MA, USA). Dentin specimens (2 × 6 × 0.5 mm, width × length × thickness, n = 3) were demineralized and treated as described in sections 2.1 and 2.2. Absorbance spectra were collected in the range of 650 to 4000 cm^{-1}, using 128 acquired scans and a resolution of 4.0 cm^{-1}. The peak area of specific bands were determined following a two-point baseline correction, normalization, and band area integration using OMNIC Spectra Software (Thermo Fisher Scientific, USA). Variation in the secondary structure of the collagen triple helix was calculated by the ratio of the peak area of amide III (1240 cm^{-1}) to the CH_2 scissoring (1450 cm^{-1}) bands [22,23].

2.5. Statistical analysis

Levene’s intragroup variability test met the assumption of homogeneous distribution for the variable storage modulus ($p = 0.071$) but not for loss modulus ($p = 0.002$), complex modulus ($p = 0.04$) and tan δ ($p < 0.001$). Accordingly, data were analyzed using two-way ANOVA followed by Tukey or Games-Howell for multiple comparisons. Ratios showing structural variations of the dentin collagen were compared using one-way ANOVA and Tukey’s post hoc test. The level of significance for all tests was set at 5%.

3. Results

3.1. Dynamic mechanical analysis

Fig. 2 shows the range of linear viscoelasticity of the dentin ECM. Significant interactions between study factors (treatment groups *vs*. time points, $p < 0.001$) were found for E*, E’, E” and tan δ (Fig. 3). While all treatments (dentin biomodification) significantly increased E’ and E” moduli, dentin ECM treated with PMe and VVe showed the highest values after biomodification ($p < 0.001$). The damping

capacity (tan δ) among groups from highest to lowest was as follows: VVe > control = PMe > GA ($p < 0.001$).

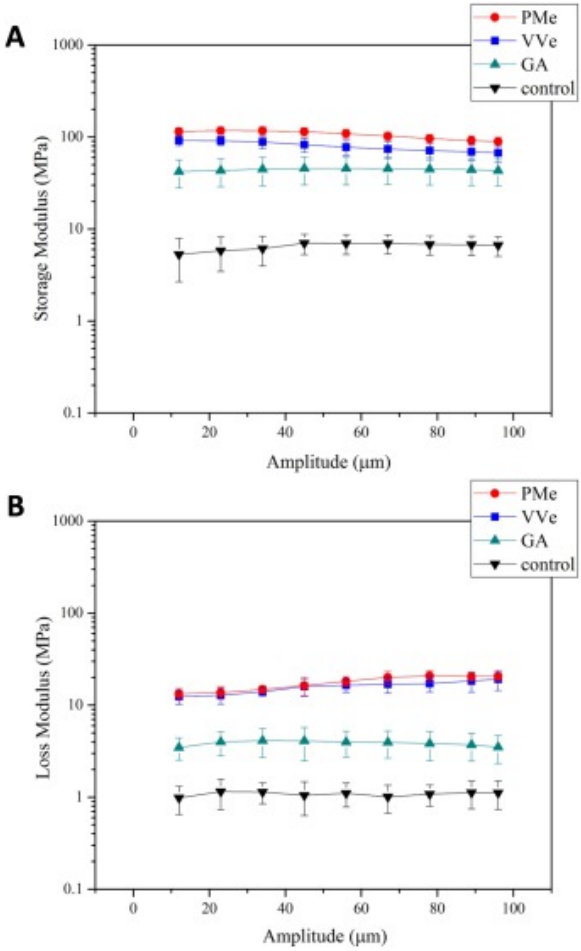

Fig. 2. Viscoelastic components. Mean and standard deviation of the storage (A) and loss moduli (B) of the biomodified dentin ECM as a function of strain amplitude. All moduli were calculated within the linear region of viscoelasticity.

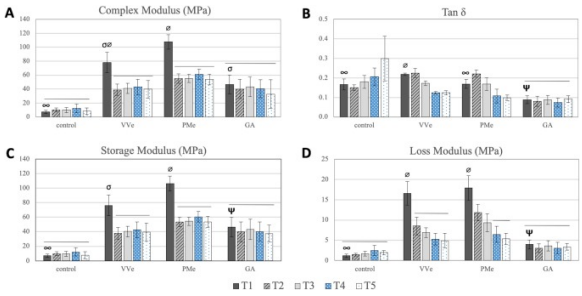

Fig. 3. Viscoelastic mechanical properties. Results (mean and standard deviation) of E* (A), tan δ (B), E' (C) and E" (D) of the dentin matrix according to treatment and time point. Different symbols depict statistical differences within the time point T1. Bars represent lack of statistical differences within treatment groups ($p > 0.05$). T1: biomodification, T2: protein destabilization, T3: 7-days, T4: 6-months and T5: 12-months.

The protein destabilization significantly affected PMe and VVe treated dentin ECM ($p < 0.001$), reducing all moduli up to 50% ($p < 0.001$) while sustaining statistically higher values than control ($p <$

0.001). The destabilization only decreased the damping capacity of PMe-treated ECM ($p = 0.009$). Neither GA-treated dentin matrices ($p = 0.873$) nor control group ($p = 0.385$) were affected by urea destabilization.

Long-term studies (up to 12 months) showed that E', E" and E* remained stable in all groups, except for a significant reduction in E", a total of 70%, of PMe-treated group ($p < 0.001$). The damping capacity of PMe- and VVe-treated dentin matrix decreased over time to values statistically similar to the GA group ($p > 0.07$); while the latter maintained stable at all timepoints ($p = 0.873$). Despite not showing statistical differences with time, tan δ of the untreated dentin ECM increased 1.8 times after 12-month storage.

3.2. Chemical analysis of the dentin matrix

Representative ATR-FTIR spectra from dentin ECM with typical type I collagen characteristic resonances are shown in Fig. 4. Spectra from PMe-treated and VVe-treated ECM exhibited a set of modifications not found in the GA-treated and untreated ECM spectra. Amide resonances exhibited decreased intensity and widening. The bands corresponding to amide I, II and CH_2 scissoring showed minor shifts, the shoulder at ~1402 cm^{-1} exhibited decreased intensity, and new band formation was detected at ~1116 cm^{-1}. Exclusively in PMe-treated dentin, the spectra displayed a rise of a shoulder at ~1522 cm^{-1}, a new band at ~1180 cm^{-1}, the loss of peak at ~1320 cm^{-1}, and additional band shifts at ~1160 and ~1060 cm^{-1}, which are possibly expressed at ~1145 and ~1068 cm^{-1}, respectively. There was no difference in the spectra from GA-treated dentin ECM. After biomodification, the ratio expressing structural variations of the collagen triple helix (A_{1240}/A_{1450}, Fig. 5A) exhibited significant decreased values ($p < 0.001$) in the PMe and VVe groups (0.30 and 0.58, respectively) when compared to the GA and control groups (1.24 and 1.30, respectively).

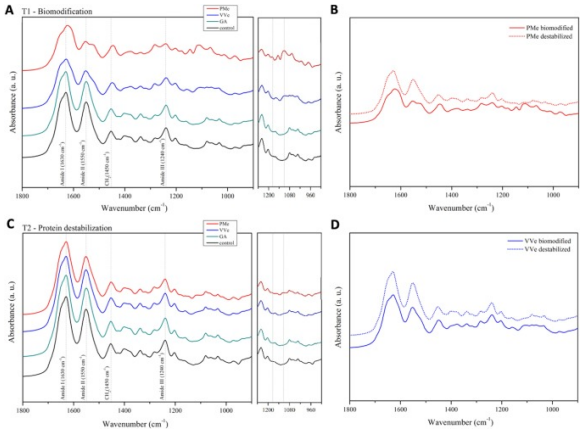

Fig. 4. FTIR spectroscopic analysis of the dentin matrix. Graphic shows representative ATR-FTIR spectra of the dentin ECM of all groups before (A) and after protein destabilization (C). Note exclusive peak in PAC-treated dentin ECM (~1116 cm^{-1}) sustained presence after protein destabilization. Graphics show differences among spectra from PMe (B) and VVe (D) before and after protein destabilization.

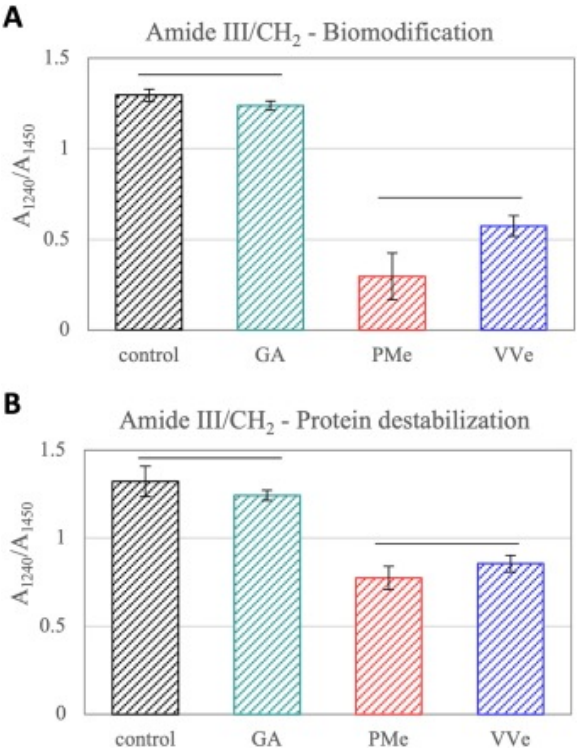

Fig. 5. Variations in collagen secondary structure. Results (mean and standard deviation) of ratio (A_{1240}/A_{1450}) depicting structural variations of the collagen triple helix after biomodification (A) and protein destabilization (B). Bars represent lack of statistical differences within groups ($p > 0.2$).

Protein destabilization resulted in a decrease in the CH_2 scissoring peak, increased intensity in all amide bands, and the emergence of a shoulder at 1402 cm^{-1} in PAC-treated ECM (VVe and PMe). Bands between 1160 cm^{-1} and 950 cm^{-1} became virtually the same as shown by the untreated dentin ECM spectrum. Collagen structural variations were not detected in the GA-treated and control group. Yet, the ratio significantly increased ($p < 0.005$) in both PMe and VVe groups (0.77 and 0.86, respectively, Fig. 5B), all changes revealing a reversal of the PAC-induced chemical biomodification. However, an interesting finding was the sustained stability of the ~1116 cm^{-1} peak, only in PAC-biomodified ECM spectra. The stability of this resonance, even after protein destabilization, indicates permanent chemical shift had occurred to the dentin-PAC complex.

4. Discussion

Pathological, chronological, and biomimetic modifications of dentin will likely manifest with different bulk mechanical functions. Elucidating the mechanisms by which the dentin withstands mechanical forces and environmental aggressions, requires understanding the complex hierarchical assembly it entails. The structural organization of dentin and the arrangement of collagen fibrils impact the viscoelastic behavior of bulk dentin tissue. The dynamic mechanical analysis enables the calculation of multiple components of the viscoelastic properties of the dentin, otherwise not possible under static methods. The complex modulus offers a snapshot of the main components, storage, and loss moduli. However, the relationship among these two moduli, expressed in tan δ, can gather deeper information on crosslinking, molecular structure, and dentin rearrangements [3,4]. Herein, we developed a method to assess the bulk dynamic mechanical behavior of dentin matrices that enables multiple

measurements for cumulative effects (treatment, accelerated, and chronological aging). The null hypothesis that the chemical and viscoelastic properties of the biomodified dentin ECM would not be affected by the protein destabilization method was rejected.

The selection of dentin biomodification agents, two PAC preparations from different plant sources and glutaraldehyde, was made to study different binding mechanisms and, thus, aid in the comparison of the chemical-physical modifications of the dentin ECM. All agents induced robust changes to the dynamic mechanical properties of dentin with pronounced differences among biomodification agents. Glutaraldehyde-induced biomodification yielded low damping capacity to the dentin ECM (Fig. 3B), likely due to the strong covalent-binding [[24], [25], [26]] that could limit the sliding of collagen fibrils. Conversely, PACs either maintained or increased the viscoelasticity of the dentin ECM after biomodification. This is likely due to the formation of hydrogen bonding, suggested as the main interaction between the phenolic hydroxyl of PACs and the amide carbonyl groups of collagen [27,28]. To a lesser extent, ionic, hydrophobic, and/or covalent interactions may also take place through the association of aromatic rings with proline residues [9,10,27,[29], [30], [31], [32]]. However, the increase in tissue viscosity is determined by breakage and reformation of protein-solvent H-bonds during dynamic testing [3]. Such differences in the viscoelasticity range require the application of repetitive harmonic load.

A 12- to 16-fold increase in storage modulus was found with treatments using PAC-enriched extracts from pine bark (*Pinus massoniana*; PMe) and grape seeds (*Vitis vinifera*; VVe). Their high reactivity is due to the presence of OH groups in the PACs that interact with the dentin ECM through primary H-bonds [[28], [29], [30]]. Despite the reported stability of PAC-induced biomodification [7,12], an initial drop in the mechanical properties is observed within the first three months [33,34]. The applied protein destabilization model was set to work as an accelerator of the aging of crosslinks. Urea-induced protein destabilization involved the breakdown of hydrogen bonds [19] and was expected to generate irreversible mechanical changes in the dentin ECM. After urea immersion, dentin treated with PMe and VVe exhibited a significant reduction in storage (-50%) and loss moduli (-34 to -49%), which revealed a strong H-bond crosslinking nature. Interestingly, after one year, the storage modulus was stable, whereas the loss modulus gradually decreased (Fig. 3C and 3D). Minor variations in loss modulus can highly impact the damping capacity of tissues. While glutaraldehyde conferred viscoelastic stability, the damping capacity of PAC-treated dentin ECM progressively decreased and after 12 months matched that of glutaraldehyde-treated ECM. One possible reason, why the dynamic behavior shifted from viscous or fluid-like to more elastic-like is the decreased hydrophilicity of the PACs-treated dentin surfaces. Due to high-density exogenous crosslinks [31], lower hydrophilicity reduced the amount of bound water within the ECM [7], thereby decreasing viscoelasticity [35]. Moreover, hydrogen bonds that are not stabilized by adjacent hydrophobic bonds can be dissociated with aqueous buffers [30], shifting the tissue to a more elastic-like behavior. The lack of disturbance in glutaraldehyde-treated dentin ECM showed the specificity of urea to H-bond disruption [24], as well as the stability of exogenous collagen crosslinks. On the other hand, untreated dentin ECM showed a more fluid-like behavior after one year, likely due to collagen solubilization by endogenous proteases [34,36,37].

Spectroscopic analysis of PAC-treated dentin ECM evidenced a reduction in peak intensity of the amide bands, suggesting conformational changes related to the triple-helix structure of the collagen [38] due

to formation of exogenous collagen crosslinking. PMe and VVe PACs are structurally diverse [34], and chemical differences were expressed in the resulting spectra, which indicate variations in the dentin-interaction mechanisms. The partial reversal of these modifications observed after urea immersion supports the conclusion that the effectiveness of the destabilization protocol is connected to the nature of the formed cross-links. However, the detection of a new band (~1116 cm^{-1}) that remained constant after the urea-challenge confirmed that treatment with PACs results in permanent structural modification of dentin matrices, regardless of the source of PAC investigated. This stable feature could indicate the presence of covalent or chemical interactions that are as strong as covalent bonds between PACs and collagen [27].

In this study, the enrichment of an extract from *Vitis vinifera* is the result of excluding most higher-order oligomeric and polymeric PACs [7,17]. On the other hand, extract from *Pinus massoniana* contains trimers to higher-order oligomeric PACs [17]. PACs' structural features such as galloylation, interflavan linkages (IFLs), degree of polymerization, plant source, degree of hydroxylation, and positioning of the hydroxyl groups determine the type and density of cross-link interactions [7,11,13,15,34,39], which in turn may regulate the stability of the PAC-induced biomodification that is key for durable dental adhesive restorations [12]. B-type IFLs and galloylated moieties are mostly found in VVe, whereas A-type linkages and the lack of gallocatechin monomers and/or 3-*O*-gallates is more common in PMe. VVe and PMe can be considered the most potent sources to mechanically enhance the dentin matrix [15,34]. Although B-type IFLs and galloylated compounds are related to lower long-term mechanical stability, in this model, PMe-treated dentin ECM also showed a high reduction in viscoelastic properties after the destabilization. Variations in the chemical profile and dynamic mechanical behavior of the PMe and VVe-biomodified dentin revealed distinct plant-specific mechanisms of interaction. Due to the specificity of urea-mediated destabilization on H-bonds, results indicate that besides PACs' structural features (IFLs, galloylation, and degree of polymerization), the inter-molecular bonding interaction with the dentin is a key feature to estimate the stability of the PACs-mediated biomodification.

5. Conclusion

DMA is a reliable method to perform repeated bulk dentin viscoelastic characterizations and to elucidate the role of inter-molecular forces in the stability of newly formed collagen cross-links. The protein de-stabilization protocol resulted in reproducible accelerated breakage of hydrogen bonds. Permanent structural collagen modifications induced by glutaraldehyde and two distinct sources of PACs (VVe and PMe) enhanced the mechanical properties. Strong covalent-binding in the dentin ECM produced low damping capacity and mechanical stability.

Declaration of interests

None

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