Dual function of proanthocyanidins as both MMP inhibitor and crosslinker in dentin biomodification: A literature review

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Proanthocyanidin, a natural phytochemical bioactive agent, simultaneously can silence the activity of dentinal proteases and cross-link the collagen matrix; both of these phenomena would be the fundamentals for bio-stability of resin-dentin interface which is essential for a promising adhesive dentistry. This review provides an overview of the data developed by different groups of researchers and highlighted topics are proanthocyanidin chemistry, natural resources and the unique interactions between proanthocyanidin-collagen and proanthocyanidin-MMPs in dentin. Besides, clinical applications of proanthocyanidin in the form of proanthocyanidin-containing adhesives, preconditioners and etchants have been reviewed. One hundred and twelve studies have been published in peer-reviewed journals from 1981 to 2017, all were comprised in this review, some of them have been actually proven to be promising from clinical point of view and others need further assessment before their adoption as clinically practicable protocols.

Keywords: Proanthocyanidin, Cross-linking, MMP inhibitor, Dentin biomodification

INTRODUCTION

Contemporary adhesive dentistry, despite its great improvements, still has shortcomings specific to resin-dentin bonds in both etch & rinse and self-etch adhesives. The problem is a rapid time-related loss of resin-dentin bond strength which is attributed to the unstable and permeable nature of the formed hybrid layer that will undergo water sorption, collagen matrix hydrolytic degradation and resin leaching. Ionic state and hydrophilicity of the adhesives are responsible for this problem. On the other hand, host derived enzymes, which are trapped as proteases in dentinal matrix, will be activated due to caries or mild acidic pH conditions. These enzymes composed of matrix metalloproteinases (MMPs) and cysteine cathepsins, both play significant roles in destruction of exposed collagen fibrils in hybrid layer. Suboptimal resin infiltration through demineralized collagen matrix, which is more common in etch and rinse (E&R) adhesive systems, and occur to a lesser extent in self-etch (SE) adhesives, will cause collagen fibrils to remain denuded, especially at the bottom of the hybrid layer which is susceptible to enzymatic degradation.

Nowadays, two strategies have been utilized to challenge this enzymatic degradation: 1; using MMP inhibitors in etchants, adhesives or directly on dentin prior to adhesive application, 2; using cross-linkers in adhesives, etchants or directly on dentin. Crosslinking improves dentin mechanical properties and cause its resist against enzymatic degradations. Some agents such as glutaraldehyde (GD), carbodimide and proanthocyanidin (PA) are supposed to have both cross linking and MMP inhibitory effects. Some drawbacks such as high cytotoxicity, mismatched mechanical properties and suboptimal long-term stability have been attributed to synthetic cross-linkers. Some natural cross linkers, such as PA and genipin seems to overcome some of these drawbacks. Besides, PAs have shown faster cross-linking induction rate in comparison with genipin. With these in mind, the objective of this review is summarizing the features of PA, as a natural dentin cross-linker and a non-specific MMP inhibitor.

PA NATURAL RESOURCES AND CHEMISTRY

PAs represent a major group of phenolic compounds that occur ubiquitously in woody and some herbaceous plants. The presence of these polyphenolic compounds in plants are probable for their defense mechanisms. They belong to condensed tannins category, highly hydroxylated structures capable of forming an insoluble complex with carbohydrates and proteins. The resultant complexes derived from the interaction of PA and collagen are believed to be stabilized primarily by hydrogen bonding between the protein amide carbonyl and the phenolic hydroxyl and also covalent and hydrophobic bonds. These condensed tannins do not hydrolyze easily.

The building blocks of PAs are flavan-3-ol oligomers which have 3 rings (ring A: triketide, ring B: phenylpropanoid, ring C: pyran which is formed by condensation). The main flavan-3-ol units in grape seed extract (GSE) PA are catechin (C), epicatechin (EC), catechingallate (CG) and epicatechingallate (ECG) whilst the main monomeric flavan-3-ol unit in cocoa seed extract (COE) is EC unit (Fig. 1). The chemical structure of flavan-3-ols units that is available in grape seed and COE dictates their physical properties, reactivity and their interactivity with collagen. Besides, just some PA-molecules are able to interact with collagen peptide and not all of them. Flavan-3-ol subunits are detectable by high performance liquid chromatography and mass spectrometry (HPLC/
The most abundant flavan-3-ol units in grape seed extract proanthocyanidin. Hydrogen bonds between phenolic hydroxyl groups and collagen carbonyl amid are responsible for primary stabilization of PA-collagen complexes.

PAs are divided into different classes according to their hydroxylation patterns in A and B rings of flavonoid skeleton, their 3-D structure, spatial arrangement and linked positions of monomers\textsuperscript{17}. Flavanyl units in B-type PA, are linked via only one inter-flavanoid carbon-carbon bond (C-4 to C-8 or C-4 to C-6), but analogs of the A-type possess an unusual second ether linkage between C2\textsubscript{β} to O to C-7 or C2\textsubscript{β} to O to C-5\textsuperscript{17,26}. These components are responsible for antioxidant activity and free-radical scavenging effect of PAs\textsuperscript{27}. Based on PAs variation, the chemical properties of apparently similar structures must be considered before interpreting their biologic efficacy and function.

PA-COLLAGEN FIBERS INTERACTIONS

The matter of the reinforcing effect of natural cross-linkers, especially GSE PAs, on dentinal collagen has engrossed interests increasingly since 2007. Some studies are concentrated on PA capability for modifying demineralized dentin mechanical properties\textsuperscript{28,29} and the others were mainly focused on PA efficacy on dentin collagen stability against enzymatic biodegradation\textsuperscript{13,14,30}.

Dentin organic matrix is composed of 90% fibrillar type I collagen and 10% non-collagenous proteins such as phosphoproteins and proteoglycans\textsuperscript{31}. Type I collagen as a heterotrimeric molecule is composed of one \(\alpha_2\) chain and two \(\alpha_1\) chains\textsuperscript{32}. Both inter- and intramolecular physiological cross-linking are the key factors responsible for stability, strength and viscoelasticity of dentinal collagen matrix\textsuperscript{33-35} (Fig. 2). The quantity and type of cross-linking affect collagen thermal stability and resistance to degradation. If the cross-links available in the length of collagen fibrils are cleaved into peptide chains/fragments, they will be solubilized. So, inserting additional cross-links between collagen microfibrils via exogenous cross-linker agents, not only can improve the collagen matrix modulus of elasticity and its stiffness, but also may insure hybrid layer long-term stability via increasing its collagen matrix resistance against biodegradation by proteases. PA can increase the resistance of collagen against collagenases via masking the cleavage sites of collagen matrix. The other attitude around this probable insurance of hybrid layer stability is the additional cross-links ability to inhibit the long rod-like collagen molecules from sliding past each other under routine mechanical stresses\textsuperscript{35,36}. Although, the exact mechanism of cross-linking is not completely understood, however, four different theories explain PA interactions with proteins which include covalent\textsuperscript{37}, hydrogen bonding\textsuperscript{38}, ionic\textsuperscript{39} and hydrophobic\textsuperscript{15} interactions. Hydrogen bonds between the protein amide carbonyl and phenolic hydroxyl groups are considered as the crucial forces for stabilizing PA-treated collagen fibrils. Moreover, the 3D structure of collagen triple helix, allows the hydrogen bonding to the carbonyl oxygen of the peptide backbone so more readily\textsuperscript{40}.\n
Fig. 1 The most abundant flavan-3-ol units in grape seed extract proanthocyanidin.

Fig. 2 Schematic picture illustrating physiological and exogenous cross-links induced as intramolecular and intermolecular cross-links. (A) cross-links between collagen fibrils, (B) a more detailed view from the dashed line area in A, showing individual collagen molecules composed of single \(\alpha\) chains which are cross-linked by additional exogenous cross-links.
Fourier Transform Infrared Spectroscopy (FTIR) demonstrated that PA can displace water between collagen microfibrils and create some new hydrogen bonds between the fibrils, so in this way it would aggregate the fibrils and protect the collagen triple helix. Also, higher PA concentrations can form a denser collagen matrix which can inhibit water seepage and decrease vapor permeability of collagen/PA films\(^4\). Miles et al. showed that collagen cross-linking can dehydrate the collagen and in this way, it will be responsible for improved collagen biological stability\(^4\). According to FTIR analysis, PA-collagen interactions are not time dependent and can occur even in exposure times as short as 10 s and it is supposed that the formation of imine C=N bonds can be the probable mechanism\(^3\).

In bonding procedures of E&R adhesive systems, in the cases of vigorous drying of dentin or when no water enters into the spaces between demineralized collagen fibers, hydrogen bonds will be formed between collagen fibrils and consequently this will cause collagen collapse and limit resin penetration, so in order to avoid this unwanted collapse, collagen hydration is essential\(^4\). It is assumed that stiffening effect induced by cross-linkers may reduce the risk of collagen collapse after demineralization and dentin overdrying, and hence, may permit more hydrophobic adhesives to enter into interfibrilar spaces\(^4\). As a rule, a stiffer dentin matrix is a so more suitable collagen substrate for hybridization\(^5\).

Bedran-russo et al. demonstrated that GSE PA can increase the stiffness and ultimate tensile strength of demineralized dentin in a concentration-related and time-dependent manner\(^2\). Castellan et al. found that increased stiffness induced by both GSE and COE derived PA in demineralized dentin is in a direct relation with exposure time to PA, furthermore, it strongly depends on the PA origin and the used solvent\(^6\). In a study conducted by Maciel et al.\(^7\), the acetone group showed lower swelling values compared to distilled water group, the probable causes of this phenomenon can be the higher ability of the acetone-saturated collagen fibrils to form interpeptide bonds and the absence of the plasticizing effect of water in the acetone group. In another study, Castellan et al. reported that both GSE and COE derived PA can decrease the swelling ratio and water sorption rate of collagen matrix\(^8\). In this study, no statistically significant changes were occurred in UTS values of dentin matrix treated with either GSE or COE whilst both of these groups, showed a considerable difference in UTS values in comparison with the control group. It is reported that PA-treated demineralized dentin samples had lower swelling ratio compared to distilled water and acetone treated samples\(^9\). Lower swelling ratios can cause lower collagen biodegradation via diminishing the collagenase absorption\(^10\).

With this concept in mind that hydrogen bonding between phenolic hydroxyl and protein amid carbonyl groups are the main supposed force for PA-collagen interactions, it should be noticed that the solvent used for preparing a PA solution can affect PA-collagen interactions. Hansen solubility parameter (\(\delta H\)) of ethanol and acetone, which is the indicator of the amount of probable hydrogen bonding, is lower compared to distilled water. So if acetone or ethanol are used as PA solvents, more hydrogen bonding sites will be remained available on PA molecule for interaction with collagen\(^11\). According to Hagerman and Klucher report\(^12\), ethanol can stimulate PA-collagen interactions and protract its stability via diminishing the dielectric constant of the media, so it can be concluded that ethanol may be the solvent of choice for PA-containing powders.

Negatively charged proteoglycans available in intra-fibrilar spaces of demineralized dentin can form a hydrogel acting as a “molecular sieve”, so that, it can prevent the complete diffusion of high molecular weight, hydrophobic resin monomers into the fibrilar spaces containing residual water. It is demonstrated that PA can cause significant drops in glycosaminoglycan (GAG) content in demineralized dentin, that consequently will alter hydration pattern and molecular sieve effect in dentin. All these factors would enhance mechanical properties of resin-dentin inter-diffusion zone\(^13\).

Furthermore, there are several reports indicating that the susceptibility of dense PA treated collagen matrix to creep rupture or cyclic fatigue rupture, would be declined after prolonged intraoral function\(^14,15\).

**PA-DENTINAL MATRIX METALLOPROTEINASE INTERACTIONS**

For the first time in 2004, Pashley et al. found some evidence about slow enzymatic degradation of demineralized dentin in the absence of bacteria which can create a weak link in the adhesive interface\(^16\). Nowadays, it is demonstrated that MMPs are responsible for collagen matrix breakdown during dental caries\(^17,18\) and periodontal diseases\(^19\). They can also have some roles in resin-dentin bond interface degradation\(^16\). Several synthetic and natural MMP inhibitors have been introduced to silence the MMP activity. PAs are known as non-specific MMP-inhibitors. Before entering into the details of PA-dentinal MMPs interactions, first some reminders about MMPs are reviewed here: MMPs are categorized as calcium and zinc dependent endopeptidases which produce aszymogens or proenzymes during tooth development and entrapped in dentin\(^20,21\). These enzymes are capable to destruct all types of extracellular matrix (ECM) and basement membrane (BM) proteins\(^22\). Once the initial proenzymes expose to a mild acidic environment, the one created by an E&R or SE adhesive resins, they convert to active proteinases. 37% phosphoric acid used in E&R systems, due to its very low PH (0.7) can denaturize and deactivate MMPs\(^23,24\), and also etching and rinsing can elute calcium and zinc ion essential for MMP activity\(^25\). However, this passivation is only transient and MMPs will be reactivated by milder demineralization pattern and higher acidic pH of E&R and SE adhesives. Moreover, it has been shown that pH values between 2.3 and 5 are effective in activation of salivary gelatinases\(^26\).
MMP family has 23 members. This family is divided into 6 groups which are as follows:

- **Collagenases** (MMP-1, MMP-8 and MMP-13),
- **Gelatinases** (MMP-2 and MMP-9),
- **Stromelysins** (MMP-3, MMP-10 and MMP-11),
- **Matrilysins** (MMP-7 and MMP-26),
- **Membrane-type matrix metalloproteinases (MT-MMPs)** (MMP-14–17, MMP-24 and MMP-25),
- **Others** (MMP-12, MMP-19, MMP-20, MMP-21, MMP-23, MMP-27, MMP-28) (Table 1).

### Table 1: Family of Matrix Metalloproteinases

| Group                                      | MMP                                      |
|--------------------------------------------|------------------------------------------|
| Collagenase                                | MMP-1, MMP-8 and MMP-13                  |
| Gelatinases                                | MMP-2 and MMP-9                         |
| Stromelysins                               | MMP-3, MMP-10 and MMP-11                |
| Matrilysin                                 | MMP-7 and MMP-26                        |
| Membrane-type matrix metalloproteinases    | MMP-14–17, MMP-24 and MMP-25             |
| Others                                     | MMP-12, MMP-19, MMP-20, MMP-21, MMP-23, MMP-27, MMP-28 |

According to zymography analysis, MMP-2 and MMP-9 are the most abundant types available in dentin67,68. However, these are gelatinases, not true collagenases. They can take away the voluminous globular telopeptides from collagen molecules and deliver the hydrolytic sites of collagen to true collagenases69.

The majority of MMPs have a domain sequence composed of a signal peptide, the propeptide domain with a cysteine residue, the catalytic domain with a zinc ion and C-terminal hemopexin-like domain70. In inactive enzyme, zinc ion of catalytic domain bond to cysteine residue of propeptide domain (autoinhibitory prodomain) and hence the enzyme structure will be stabilized and prevented from binding and cleavage of collagen fibers. This prodomain-catalytic zinc bridge is so called “cysteine switch” mechanism71. If this cysteinized linkage breaks, the prodomain will be destabilized or removed, so, the active site becomes available to cleave substrates. In other words, the enzyme will be activated72. In most MMP-family members, a hemopexin domain is attached to their C-terminal by a flexible hinge. This hemopexin domain has a four-bladed β-propeller structure that intercede protein–protein interactions. Also, this domain is responsible for proper substrate recognition, activation of the enzyme, protease localization, internalization and degradation70.

At first try of an active collagenase MMP with collagen, collagenase bound to triple helix of collagen through both catalytic and hemopexin domain, however, small cleft of catalytic domain will prevent it from cleavage in its native state, so it can not cleave the triple helix of collagen. Further, unwinding of triple helix by a gelatinase MMP and hemopexin domain, render a single α-chain of collagen to the active site of catalytic domain of collagenase MMP. Then collagenase will hydrolyze 3 single chains into 1/4 and 3/4 fragments73.

Although the exact mechanism of MMP inhibition is not fully understood, but the most accepted theory is that MMP inhibitors exert their inhibitory effect through the divalent chelation of metal ions, especially the catalytic zinc atom, so they must have some functional groups for chelating the zinc ion of catalytic domain74. In this way, MMP inhibitors will prevent MMPs from binding to collagen substrate and its further cleavage.

PA is a non-specific MMP inhibitor which some conflict of interest is available in describing its inhibitory mechanism of action. Busenlehner and Armstrong reported that cross-linkers may interfere with MMPs and probably other exogenous proteolytic enzymes via conformational changes in the enzyme 3-D structure75. Sela-Passwell theoretically supposed that cross-linkers as antagonist molecules are targeted to the catalytic domain or exert their effect through allosteric inhibition of alternative enzyme domains and make some irreversible conformational changes there76. In addition to create covalent cross-links in collagen, cross-linkers are capable to cross-link the protease enzymes directly and interfere with their molecular mobility77. The other supposed paradigm is that PA may indirectly interfere with production and activation process of proteases by modulating host immune responses78.

There are some reports about PA MMP inhibitory effects somewhere in human body aside from dentin, as the case of point, cranberry PA can inhibit production and secretion of MMP-1 and MMP-9 by macrophages in response to periodontal pathogens79. There is also a report on inhibitory effect of the Amazonian medicinal plant Sangre de grado (Croton palanostigma) as a rich source of PA, on gelatinolytic activity of MMP-2 and MMP-9 from synovial fluid in osteoarthritis patients80. Some small flavan-3-ol units such as C and ECG demonstrate diverse values of collagenase inhibitory effect77,78. Epainghe et al. studied the effect of GSE PA on human demineralized dentin and demonstrated that PA can inactivate more than 90% of soluble recombinant MMP-2, -8 and -9 and around 70–80% of cysteine cathepsin B and K. However, most of dentin MMPs are not soluble, but they bound to the collagen matrix, so, in order to perform a more precise assessment of PA MMP inhibitory effect, loss of dry mass and collagen solubilization from demineralized dentin was measured as a representative of endogenous collagenase activity. Demineralized dentin beam pretreated with GSE PA...
had lesser loss of dry mass and reduced hydroxyproline (HYP) release in the medium over time, in comparison with chlorhexidine treated beams.80

According to generic total-MMP activity screening assay, 1 min pretreatment of demineralized dentin by 5% GSE PA lowered the enzyme activity up to 64% compared to the baseline values. Besides, 5 min dentin cross-linking by 1% GSE could exert 69% MMP inhibition, it is interesting that despite the lower concentration, 1% GSE PA had higher MMP inhibitory effect in comparison with 5% GSE PA in the same treatment time. Multiplex analysis demonstrated a downturn in the amount of released MMP-8, -2 and -9 in the extracts of GSE PA pre-treated demineralized dentin.80 It has been demonstrated that 5% grape seed proanthocyanidin can exert its MMP inhibitory effect until 6 months, but such as this long-term effect not only is cross-linker dependent but also is dose-dependent.81

Several proline residues available in MMP-9 have a high tendency to polyphenol binding sites geometrically.82 Despite the fact that MMP-2 just has one proline residue, however, it has been reported that both of these enzymes are affected by polyphenolic compounds similarly, the proline content and the amino acid sequence of the enzymes are responsible for these phenomena.83 Seseogullari-Dirihan et al. showed that the inhibitory effect of GSE proanthocyanidins on cathepsin K is so more better compared to their effect on the larger MMPs in dentin.84

PA-INTEGRATED DENTAL ADHESIVES

As a new delivery method to preserve PA in the vicinity of hybrid layer and maintain the steady release of the active compound for a longer period of time, the issue of incorporating PAs into dental adhesives and its pros and cons has been assessed in several studies. For the first time in 2010, Green et al.85, added 5 wt% GSE PA into a model adhesive similar to single bond plus adhesive (3M ESPE, St. Paul, MN, USA)86 so that final PA-incorporated model adhesive was composed of solvent (ethanol)/monomer/PA mass ratio of 37/60/3. It was reported that despite the dark color of PA, there was no considerable color difference between PA-incorporated model adhesive and pure adhesive. Demineralized dentin specimens bonded by pure and PA-incorporated model adhesives were subjected to 0.1% collagenase solution for zero, one and six days. According to preceding studies, it is anticipated that almost all of collagen fibrils are digested after 1 day exposure to collagenase, but interestingly after six days of collagenase treatment, the samples in PA-incorporated model group, demonstrated the collagen fibrils with intact and normal cross-banding, organization and dimensions. However, the quality of the adhesive component of the hybrid layer created by PA-incorporated adhesive was slightly lesser than pure adhesive which is probably due to lower collagen encapsulation owning to decreased degree of conversion (D.C). FTIR analysis showed that D.C of pure sample adhesive was around 86%, while this percent was approximately 68% in PA-incorporated model adhesive. Despite this significant drop in the amount of D.C, it is yet within the acceptable range for commercially acceptable adhesives.87 The outcome of decreased D.C would be the elution of some monomers/oligomers from hybrid layer which will cause more uncapsulated collagen fibrils.88 So, based on this study, PA incorporation into dental adhesives can reinforce the collagen fibrils deviating of resin encapsulation to defy against collagenase solution. In another study, the effect of PA incorporation into Bis-GMA/HEMA model adhesives with three different photo-initiator systems, including the CQ/amine, CQ/amine/iodoium salt, and TPO systems was tested. It was demonstrated that regardless of photo-initiator system, PA can alter the monomer conversion and polymerization kinetics in all the adhesives. However, according to FTIR analysis and D.C values, TPO system had the best fit with PA concentrations as high as 5% in the adhesives.89 Decreased D.C may be reparable via modifying the type and concentration of the photo-initiators; however, these details are beyond the scope of this review. A 52-week in vitro study90 incorporated 3.75% w/w GSE PA into both primer and adhesive, however, ethanol was as the solvent in model adhesive, whereas primers were solved in distilled water. The study indicated that the initial lower values of μTBS were due to PA effect on the adhesive and it did not exemplify PA impression on dentinal collagens. Despite the declined D.C. due to PA addition which can compromises the initial μTBS values, it may allow proper mobility essential for continuing the polymerization reaction and make it last for a longer period of time before reaching diffusion limitation in termination process.92 Several studies have shown that even just incubation in water or buffer can significantly decrease μTBS values prior to any collagenase treatment,93 this trial showed that both PA incorporation and storage time can affect μTBS values, but the storage medium will not afflict it. One of the demerits of this study was that the samples were soaked in PA-primer for 1 h which is not realistic and applicable in clinic.

Epasinghe et al.90 have shown that applied GSE PA concentrations lesser or equal to 2% in the adhesive will not have any adverse effect on the immediate μTBS of resin-dentin interface, whilst, the adhesive with 3% PA can exert significant drops in μTBS values. Besides, they found that adhesive containing 3% PA, predominately showed adhesive failure in resin-dentin interface. This adhesive also showed the greatest nano-leakage in the hybrid layer, dentinal tubules and adhesive layer, whereas adhesives containing 2% PA or lower had mixed and cohesive failures and the least nanoleakage at the base of the hybrid layer. The author has announced that 3% PA can diminish the polymerization of resin and exert some adverse effects on bond strength and cause adhesive failure. Formation of linear chains is essential for resin polymerization reaction, however, in the cases of inserting PA as an additive into an
adhesive resin, PA concentrations up to 2% will be trapped within the linear chains after curing\textsuperscript{100}. Besides, higher PA concentrations, because of higher density of their molecules, will disrupt the linear chain formation, which consequently will result in inadequate resin polymerization, microvoids, more water channels and a weaker resin-dentin interface\textsuperscript{101}. Furthermore, resin polymerization, microvoids, more water channels formation, which consequently will result in inadequate density of their molecules, will disrupt the linear chain. Moreover, higher PA concentrations, because of higher PA concentrations in the adhesive resin, however, this release did not happen with a steady pattern, so that an abrupt and myriad PA release was happened for the first 48 h but after 5 days, the mean release rate reached a stable plateau, however, PA release was continued until the last day of the study i.e. 28th day. Because of the lack of data on the durability of this release in longer time periods, in another in vitro study\textsuperscript{102}, the long-term effect of 1, 2 and 3 wt% GSE PA-incorporated adhesives on resin-dentin inter-diffusion zone was assessed. In this study, three different storage methods were used which were 24 h indirect water exposure (IE), 6 M IE and 6 M direct water exposure (DE). After the designated period of time, it was shown that water exposure and PA concentration had a significant effect on bond strength and nano-leakage percentage, so that bond strength values of control and experimental groups decreased with PA concentrations and ageing. Contrary to the previously mentioned study which confirmed the short-term beneficial effect of PA-incorporated adhesives on the bond strength, the results of this study showed that incorporation of PA into a dental adhesive cannot inhibit the degradation of resin-dentin bond over time.

Because of the absence of the previous dentin surface demineralization with SE adhesives and available controversy regarding the role of these adhesives in MMP activation, this concept is announced that PA inclusion may not be applicable for SE adhesives. However, the literature lacks information on the issue of PA incorporation into SE adhesives, and so, the long-term effect of this inclusion on the stability of dentin bonding can be a matter of further investigations.

**TRANSIENT COLLAGEN CROSS-LINKING BY PA-CONTAINING PRECONDITIONERS**

PA, a natural phytochemical agent, has shown positive effects as a preconditioner in dentin biomodification. In this approach, PA will be rinsed off before adhesive application, and hence, it will have little or no effect on the curing behavior of the adhesive resin. Although this will burden an extra step to the bonding procedures going against operators preference for faster bonding protocols but in turn the probable gained benefits must be considered. It has been shown that resin-dentin bond strength in sound and carries affected dentin can be increased by GSE PA pre-treatment\textsuperscript{28,103}. Based on the PA concentration and treatment time, several studies have been conducted in laboratory conditions in recent years. However, in the eyes of an operator, a solution which exerts minimum discoloration in dentin and needs shorter application time is more desirable. It is logical that lower weight percent of PA in a solvent and shorter contact time with it, will create a lighter color and will be more user-friendly, provided that PA can exert its MMP-inhibitory and cross-linking behavior with these conditions simultaneously.

It is demonstrated that 72 h pre-treatment of root dentin by 0.5% PA can increase dentin resistance against enzymatic digestion and carries\textsuperscript{105}. Bedran-Russo et al. tested 0.65 and 6.5% GSE PA with exposure times of 10, 30 min, 1, 2 and 4 h on demineralized dentin and found that as PA concentration and exposure time increase, stiffness of demineralized dentin will be raised\textsuperscript{28}. Macedo et al. demonstrated that 6.5% GSE PA with 1 h application time can enhance microtensile bond strength and stability of dentin collagen in both caries-affected and sound dentin\textsuperscript{104}. Al-Ammar et al. found that dentin pretreatment with 6.5% GSE PA for 1 h can significantly increase dentin tensile bond strength\textsuperscript{105}. It should be noticed that in all studies mentioned in this part, PA was compared to GD, a synthetic cross-linker considering as a gold-standard cross-linker and both cross-linkers showed relatively resembling results.

Castellan et al. assessed the effect of different originated PAs solubilized in different solvents on dentin matrix. It was found that both 6.5% GSE or 6.5% COE PAs can increase mechanical properties and collagenase resistance of dentin. With 10 min PA pre-treatment of dentin, short term resin-dentin bond improved and the swelling ratio was decreased in comparison with the control group\textsuperscript{105}.

Increasing denaturation temperature (T\textsubscript{d}) values has a potential relation to the degree of cross-linking of biomodified tissues\textsuperscript{28}, in other words, the demineralized dentin with higher T\textsubscript{d} value will resist against heat diffusion through its collagen fibrils. It was reported that GSE demonstrated the highest T\textsubscript{d} regardless of PA concentration. Higher and multiple T\textsubscript{d} peaks observed in GSE group is a strong indicator for different degrees of collagen cross-linking, i.e. newly formed covalent and non-covalent bonds in collagen fibrils induced by PA can hold back heat diffusion through collagen molecule and enhance the stability of the biomodified collagen matrix. Micro-assay for GAGs and histological electron microscopy showed a significant decrease in proteoglycan content in GSE pre-treated samples while it did not alter in GD and control groups. As mentioned in PA-collagen interactions, hydrogel formed by GAGs can function as a molecular-sieve which limits resin penetration between collagen fibrils, so such diminishing effect on GAGs content will raise hybrid layer quality. HYP assay confirmed the reinforcing mechanism of GSE and GD on collagen fibrils, however there was an inverse relationship between GSE concentration and HYP release into the incubation media, while the same relationship was not found for GD group, probably due to the restricted covalent interactions of GD with dentin.
collagen. Moreover, plenty of chemical bonds induced by these two collagen biomodifiers are durable under hydrated conditions during the time. 

Some recent studies have assessed the effect of shorter and clinically applicable time periods of PA application. It was demonstrated that PA-based preconditioners applied for 60 and 120 s can increase the ultimate tensile strength and cross-linking degree of demineralized dentin with a concentration- and time-dependent manner, so 120 s application of the preconditioner contained 15% PA showed the highest UTS values.

Fang et al. evaluated some variables such as PA concentration, application time and solvent type in PA-based dentin preconditioners via analysis of bond strength, failure mode in the bond interface and the D.C. Five, ten and fifteen percentage GSE PA in clinically relevant times such as 30, 60 and 120 s were tested. In contrast with PA-incorporated adhesives, it was found that transient PA pretreatment of demineralized dentin before adhesive bonding will not compromise the D.C and curing behavior of the adhesive. Current study showed that in a same pre-treatment time, 15% PA group bonded with Adper TM Single Bond 2 (SB, 3M ESPE) showed higher µTBS values compared to GD-pretreated group. Regardless of the applied solvent, all of PA-pre-treated groups enhanced resin-dentin bond strength, however, according to the used adhesive system, some variations occurred, for example, in SB group, µTBS values of 10 or 15% PA-pre-treated samples, regardless of PA solvents (water or ethanol) was significantly higher than the values in non-treated controls in a concentration-dependent manner; whilst in NT group (Prime & Bond NT, Dentsply De Trey, Konstanz, Germany), µTBS was increased just in 15% PA-distilled water and 10% PA-acetone groups. SB as a water/ethanol-based adhesive showed better µTBS values in comparison with a water/acetone-based adhesive, i.e., NT. Several factors may be participated in this adhesive-related variation, but it seems that the discrepancy between PA and adhesive solvents probably has a negative effect on bond strength. Another study demonstrated that 2 min dentin pre-treatment with 6.5% GSE PA cannot improve the µTBS of self-adhesive cement to dentin interface whilst GD can increase the µTBS of G-Cem to dentin.

Weight loss percentage of the demineralized dentin collagen slabs treated by 3.75% GSE PA for 10 s and 1 min was measured as an indicator of PA efficacy on collagen resistance after collagenase treatment. Surprisingly, even 10 s exposure to PA could improve the biological stability of the collagen fibrils. In another study by Liu and Wang, it was shown that the bovine collagen samples treated by 2 wt% GSE PA or more, regardless of the treatment time, were significantly protected from enzymatic degradation due to collagenase solution. According to a recent in situ study, 60 s application of 6.5% PA caused the highest knoop hardness number (KHN) immediately and 14 days after degradation in a cariogenic oral environment (COE). µTBS values of resin-dentin interface were not diminished after 14 days in COE, moreover, PA treatment did not increase the nano-leakage in the interface.

According to the mentioned studies above, myriad novel advantages can be gained from PAs in dentin biomodification, however, the concern is its dark color which is sustained in dentin despite thorough washing, so it seems that isolating an uncolored fraction of PA must be a matter of future researches.

### PA INCORPORATED DENTAL ETCHANTS

As all operators prefer simplified techniques in bonding procedure, for omitting separate application and washing steps essential for PA pre-conditioners, the effect of PA incorporation into the formulation of the etchants was evaluated in some recent studies. For the first time, Liu et al. added 2% GSE to 5, 10 and 20% phosphoric acid. According to FTIR spectroscopy and digestion assay, with 30 s application of GSE-incorporated phosphoric acid etchant, demineralized dentin collagen was protected from bacterial collagenase digestion. However, because of unsynchronized penetration of phosphoric acid and PA, 20% acid may cause over etching and it is concluded that GSE-incorporated phosphoric acid concentrations lower than 20% can be utilized as collagen cross-linkers. Hass et al. compared 35% phosphoric acid with 2% proanthocynidin-containing 10% phosphoric acid for their effect on the properties of resin-dentin and enamel-resin interface and their MMP inhibitory effect. Relatively complete inhibition of MMP activity within the resin-dentin interface was observed after 2% PA/10% PhA etching, whilst severe MMP activity was available in 35% PhA group. After 6 M, µTBS values of resin-dentin interface was stable just in 2% PA/10% PhA group, however, for enamel-resin interface, the etchant type and the duration of storage period could not influence the bond strengths.

The routine dental etchant available for E&R bonding systems is 35–40% phosphoric acid which is essential for optimum enamel bonding, however, in mentioned studies above, lower percentage of the acid has been examined. Further studies should evaluate different aspects of this incorporation, so that, the objective of omitting the complexity of the bonding procedure does not jeopardize the optimal enamel bonding.

### CONCLUSIONS

Based on this review, the following can be concluded regarding PA as a dentin biomodifier:

1. PA via its cross-linking effect not only can enhance the mechanical properties of exposed dentinal collagen, but also is able to dehydrate the fibrils and in this way a more promising collagen substrate will be rendered for hybridization and eventually a bio-stable hybrid layer will be
formed.
2. In addition to cross-linking behavior, PA, as a non-specific MMP-inhibitor, can protect the exposed collagen fibrils in the hybrid layer from biodegradation resulting from dentin proteases.
3. Contrary to PA included pre-conditioners, PA-incorporated adhesives are incompatible with higher PA concentrations. Although, pre-conditioning exert an extra step to the bonding procedure, however, it will have some merits, i.e. it can deliver higher PA concentrations and it will not interfere with curing behavior of the adhesive resin significantly.

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