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Effects of polar solvents and adhesive resin on the denaturation temperatures of demineralised dentine matrices

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SUMMARY

Objectives—To measure the denaturation temperature (Td) of demineralised dentine matrix as a function of infiltration with water vs polar solvents vs adhesive resins.

Methods—Small disks of normal dentine were completely demineralised in 0.5 M EDTA. Dried demineralised specimens were placed in water, methanol, ethanol, acetone, η -butanol or HEMA. Additional specimens were infiltrated with Prime & Bond NT and polymerised. All specimens sealed in high pressure pans and scanned using differential scanning calorimetry (DSC).

Results—Demineralised dentine saturated with water showed a Td of 65.6°C that increased with saturation by methanol, ethanol, acetone, η -butanol or HEMA to 148.5°C. These increases in Td were inversely related to the molar concentration of the solvents and to their Hoy's solubility parameter for hydrogen bonding (δ_h , $p < 0.01$), as well as directly related to the cube root of their molecular weights ($p < 0.001$). The presence of adhesive resins also increased the Td of demineralised matrices to even higher values depending if the resin bonded dentine was measured after 24 h of water storage (166.8°C) or dry (172.7°C) storage.

Conclusions—Solvents and monomers with low δ_h values (i.e. 100% HEMA) increase the Td of demineralized dentin above that produced by solvents with higher δ_h values such as methanol and water.

Keywords

Collagen; Dentine; DSC; Glass transition temperature; Solvents; Resin

1. Introduction

There is growing interest in the thermal stability of mineralised and demineralised dentine matrices. Poorly irrigated high speed dental burs can literally burn mineralised dentine. In endodontics, root canal dentine is exposed up to 300°C during warm vertical compaction of thermoplastic gutta-percha root filling material. That may be sufficient heat to thermally

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denature adjacent mineralised or partially demineralised dentine matrices, causing a loss in mechanical strength¹. In adhesive dentistry, smear layers are removed from bur-prepared dentine using 32-40% phosphoric acid. This also removes all minerals from the top 5-10 μm of dentine, thereby exposing the collagen fibril matrix without denaturation^{2,3}. Presumably, the loss of apatite crystallites would make the collagen matrix of demineralised dentine more susceptible to thermal denaturation. However, few reports are available on the denaturation temperatures of mineralised or demineralised collagen matrices⁴⁻¹⁰. In the latter report, demineralised dentine matrices saturated with water had a denaturation temperature (T_d) of 65.6°C. As water was removed, the T_d rose to 176.1°C. That value was not significantly different from the T_d value of hydrated mineralised dentine (170.4°C)¹⁰. Mineral crystals increase, while demineralization lowers the T_d of dentine matrices. The major source of tensile strength in native collagen matrices saturated with water is the presence of covalent cross-links between collagen peptides. During adhesive bonding, liquid comonomers in various solvents are infiltrated into the demineralised matrix. After solvent evaporation, the monomers are polymerised. While it is likely that resin-infiltration may stabilise the collagen fibrils of the dentine matrix from thermal challenges, it may be due more to their organic solvents dehydrating the matrix, than to the resins.

The mechanical stability of collagen seems to depend on the presence of covalent cross-links between collagen molecules^{1,11}. The thermal stability of collagen molecules represents the resistance to unfolding as a result of heat. This unfolding is believed to occur first by the separation of triple helices into individual helices, due to the disruption of hydrogen bonds between the polypeptide chains⁷⁻¹⁰. Individual helices then unfold into random coils due to disruption of intrahelical hydrogen bonds¹².

Previous reports on the T_d of rat tail tendon and calf skin found that as when tissues were dehydrated¹³, the T_d of collagen increased^{13,14}. The mechanism(s) responsible for this effect are not completely understood. The loss of water seems to collapse the collagen fibrillar matrix into a denser state where there is steric hindrance to molecular vibrations at elevated temperatures^{14,15}. Miles and Ghelashvili¹⁵ proposed that the higher T_d of insoluble collagen relative to soluble collagen was due to the stabilising influence of adjacent collagen molecules. They suggested that this stabilisation increased as water was lost by bringing collagen fibrils closer together. Another interpretation of their data is that adjacent dried collagen peptides can form new, intrinsic associations due to weak forces such as hydrogen bonding¹⁶, in addition to the covalent intermolecular cross-links^{17,18} that are critical for maintenance of the stability of the triple helices¹⁹. These new hydrogen bonds stiffen²⁰ and strengthen the collagen matrix²¹, making it more stable to elevations in temperature, in the absence of water. When they are rehydrated, there is a rapid loss in stiffness, indicating that it is reversible and due to weak, noncovalent forces²⁰.

An interesting new model was developed that directly measures changes in the dimensions of water-saturated demineralised dentine matrices when they are dried, using a contact LVDT probe²²⁻²⁷. Using solvents with known solubility parameters (δ) for hydrogen bonding forces (δ_h), as “liquid energy probes”²⁸, solvents with δ_h values below 18 (J/cm^3)^{1/2} could not expand dried collapsed demineralised dentine matrices^{23,25}. Conversely, those with δ_h values greater than 18 (J/cm^3)^{1/2} could expand the matrices²⁷. These results indicated that the interpeptide hydrogen bonding forces of the dentine matrix (primarily type I collagen) had an intermolecular “cohesive strength” of about 18 (J/cm^3)^{1/2}.²² The rate and extent of matrix expansion was determined by the difference between the interpeptide hydrogen bonding “strength” and the solubility parameter for hydrogen bonding (δ_h) of the solvent, with water, with a δ_h value of 40 (J/cm^3)^{1/2}, giving the greatest expansion²⁵. Solvents such as ethanol, with a Hoy’s δ_h value of 20 (J/cm^3)^{1/2}, that are only slightly higher than the intrinsic δ_h of collagen at 18 (J/cm^3)^{1/2}, only break some, but not all of the

interpeptide hydrogen bonds that form in dehydrated collagen²⁵. Another common solvent for dental adhesives is 2-hydroxyethyl methacrylate (HEMA), which has an δ_h of 16.4 (J/cm³)^{1/2}. This value is below that of the δ_h of collagen, so HEMA cannot break interpeptide hydrogen bonds and cannot expand collapsed, dried collagen matrices^{23,27}. The similarity of the changes in T_d on immersion of collagen matrices in ethylene glycol (MW = 62) vs those of water (MW = 18) suggested the role played by hydrogen bonding of ethylene glycol molecules ($\delta_h = 29.8$ (J/cm³)^{1/2}) to the collagen molecules in disrupting interpeptide hydrogen bonds¹⁶.

In the presence of water, the strongest hydrogen bonding solvent known, little interpeptide hydrogen bonding can occur²⁷. At the other extreme, in matrices completely dehydrated by methanol, ethanol, acetone, *n*-butanol or HEMA, progressively more interpeptide hydrogen bonding can occur between adjacent collagen peptides. This should increase the thermal stability of collagen and increase T_d values. Infiltration of collagen by adhesive resins should also stabilise collagen and raise T_d . Thus, our test hypothesis is that the denaturation temperature of demineralised dentine matrices is determined, in part, by the amount of intrinsic hydrogen bonding between collagen peptides.

2. Materials and methods

2.1 Teeth

Fifteen extracted third molars were obtained from young patients (18-23 years). These teeth were extracted only for clinically appropriate reasons using a protocol approved by the Institutional Review Board of the University of Iowa (IRB Approval #200010082). The extracted teeth were stored at 4°C in a phosphate buffered saline (PBS) for no more than 1 month, with a calcium phosphate saturation ratio of 11.52 for hydroxyapatite²⁹.

2.2 Demineralised dentine specimens

Dentine disks 1 mm thick cut from mid-coronal dentine using an Isomet saw (Buehler Ltd., Lake Bluff, IL, USA) were completely demineralised in 0.5 M ethylenediamine tetra-acetic acid (EDTA; pH 7.4) with constant stirring for 14 days at 25°C. The completely demineralised dentine disks were rinsed free of EDTA with water for 1 hr, and then cut into 1 mm × 1 mm × 1 mm blocks with a razor blade. Two 1 mm³ blocks were prepared from each of the 15 teeth, resulting in a total of 30 blocks. After these blocks were cut, they were mixed together and stored in 4°C PBS containing 0.01% sodium azide to inhibit microbial growth. Three blocks were randomly assigned to each of the ten groups designated for the measurement of denaturation temperatures.

2.3 Measurement of denaturation temperature

Specimens sealed in high pressure pans were placed in a DSC (Model DSC-7, Perkin Elmer Life and Analytical Sciences, Inc., Wellesley, MA, USA) and scanned from 25°C to 200°C at a rate of 10°C/min. Subsequent cooling and reheating confirmed that the collagen denaturation was irreversible¹⁶. The DSC was calibrated prior to use with iridium standards.

2.4 Calculation of Hoy's solubility parameters of the solvents

The Hoy's triple solubility parameters (δ_d , δ_h , δ_p) of the solvents was calculated by summing the molar attraction constants for the constituents of each substance according to the method of Van Krevelen³⁰, using a commercially available software (Computer Chemistry Consultancy, Singen-Friedingen, Germany).

2.5 Experimental design

2.5.1 Effects of polar solvents on the T_d of demineralised dentine—The demineralized dentine blocks were weighed (Mettler Toledo AB104-A, readability 0.0001 g, Columbus, Ohio, USA) wet and then placed in sealed jars containing anhydrous calcium chloride (Drierite, Fisher Scientific, Chicago, IL, USA) for 2 hrs to obtain constant dry weight. They were then individually placed in separate capped glass vials containing 10 mL of water, methanol, ethanol, acetone, η -butanol or HEMA for 12 hrs. All chemicals were ASC grade (i.e. contained <0.5 vol% water) and were purchased from Sigma-Aldrich Chemicals, St. Louis, MO, USA). The specimens were removed, blot-dried and immediately sealed in tared high-pressure DSC pans and scanned in the DSC.

2.5.2 Effects of adhesive resin infiltration on the T_d of demineralised dentine—Completely demineralised (1 mm³) blocks of wet coronal dentine from young teeth were placed in sealed vials containing 0.125 mL (6 drops) ml of a commercially available dental bonding system, Prime&Bond NT (PBNT, Lot#031124, L.D. Caulk, Milford, DE, USA). These vials were covered with aluminum foil to prevent light from penetrating. The adhesive was allowed to infiltrate into the water-saturated matrix at 25°C for 60 min permitting the acetone solvent to chemically dehydrate the matrix. The adhesive infiltrated dentine blocks were gently blotted with a piece of lint-free tissue to remove excess resin and then the residual solvent specimen was placed on an analytical microbalance to permit evaporation by compressed air until the specimen ceased losing mass (after about 5 min). Then the resin was light-cured with a quartz-tungsten-halogen light-curing unit (470 nm) at 600 mW/cm² for 30 sec on each side. The specimens were randomly divided amongst wet and dry storage. After 24 hrs of storage, and gentle air drying for the wet storage group, specimens were individually sealed in DSC pans and scanned.

2.6 Statistical analyses

The data were divided into two sets: resin-infiltrated specimens and solvent-treated specimens for examining the effect of resin infiltration and solvent treatment on denaturation temperature (T_d). For each data set, the normality (Kolmogorow-Smirnoff test) and homoscedasticity (Levene test) assumptions of the data appeared to have been violated. Thus, each data set was separately analysed using one-way ANOVA on ranks and post-hoc Dunn's multiple comparison tests at the 0.05 level of significance.

3. Results

The results of this study are summarised in the Table. The table is divided into two sections depending upon the dentine treatment. In the first section, when water-saturated demineralised dentine specimens were fully infiltrated with Prime&Bond NT (60 min in the dark to prevent light-curing) prior to polymerisation, the mean T_d value was 173°C after dry storage or 167°C after soaking in water over night followed by blot-drying. A similar mass of dry PBNT resin gave a glass transition temperature (T_g) of the polymeric resin of 191.3°C (Table). Since dentin was not involved in this group, only T_g of the resin was reported instead of the denaturation temperature of dentins (T_d). There was no statistical significance among the T_d results from the two PBNT-infiltrated dentine groups and the T_g of the dry PBNT resin ($p>0.05$).

In the second section, when T_d values were measured in dry demineralised dentine that had been soaked in the various polar solvents for 12 hrs (Table), the lowest T_d was obtained with water ($65.6 \pm 2.9^\circ\text{C}$). When methanol was used to solvate the matrices, the T_d increased to $92.4 \pm 0.7^\circ\text{C}$ ($p<0.05$). Immersion in ethanol and acetone produced further increases in T_d to 108 and 123°C, respectively ($p<0.05$). Specimens soaked in *n*-butanol or HEMA had even

higher T_g values (139 and 149°C, respectively). When these T_d values were plotted against the Hoy's solubility parameter for hydrogen bonding forces (δ_h) of these solvents (Table), a significant ($R^2=0.85$, $p<0.01$) inverse correlation was found (Fig. 1). This significant correlation was largely contributed by the inclusion of the water-saturated demineralised dentine group. Lower, less significant correlations were found between T_d and Hoy's solubility parameters for dispersive forces (δ_d), polar forces (δ_p) or total cohesive forces (δ_t) (data not shown). When T_d was plotted against the cube root of the molecular weight of these solvents (Fig. 2), a significant ($R^2=0.97$, $p<0.001$) positive correlation was found. An even better negative correlation ($R^2=0.99$, $p<0.001$) found between T_d and the molar concentration of the neat solvents (Fig. 2, insert).

4. Discussion

The results of this study were similar to those obtained by others from partially mineralised turkey leg tendons⁵, synthetically mineralised bovine hides⁶, and mineralised dentine¹⁰, showing that the presence of apatitic mineral crystallites within and around collagen fibrils of the matrix provide thermal stability to the collagen. The T_d of demineralised dentine was dependent on fractional water content, increasing with decreases in water content (Table). In the native state, collagen peptides are stabilized by covalent cross-links. Few intermolecular hydrogen bonds can form in the presence of water.¹⁰ Our speculation is supported by the recent observation³¹ that removal of water from mineralised dentine using water-free polar solvents produced a reversible increase in fracture resistance, strength and stiffness of the mineralised dentine associated with lower hydrogen bonding solvents (acetone, ethanol and methanol). Those authors performed that experiment to demonstrate that dehydration of mineralised dentine matrix can alter resistance to fracture. This has no clinical significance because treatment of roots *in vivo* with water-free polar solvents would be reversed within several hours by water in body fluids penetrating through root cementum and dentine to displace ethanol or acetone with water that would plasticise the collagen and return it to its original strength and stiffness. Those authors also performed ultraviolet Raman spectroscopy to show that the height of the amide I bond ($\sim 1640\text{ cm}^{-1}$) was highest for acetone, intermediate for ethanol and lowest for methanol, correlating well with their Hoy's δ_h values of 11, 20 and 24 (J/cm^3)^{1/2}, respectively (Table). They attributed these changes in the mechanical properties of mineralised dentine to changes in interpeptide hydrogen bonding³¹. That is, such bonding is minimal in the presence of water with a Hoy's $\delta_h = 40$ (J/cm^3)^{1/2}, and gradually increases to a maximum in acetone, where there was a large amount of interpeptide hydrogen bonding. It is remarkable that hydration effects on the collagen matrix of mineralised dentine can have detectable effects on the mechanical properties of mineralised dentine³¹.

When dried demineralised dentine was immersed in various water-free alcohols, or HEMA, there was a progressive decrease in T_d that was inversely related to Hoy's δ_h values (Fig. 1), directly related with the cube root of the molecular weight (Fig. 2), and inversely with the molar concentration of the solvents (Fig. 2, insert). In a previous study, when the swelling pressure of dried demineralised dentine matrix was tested with the same solvents, a similar relationship was found³². In that study, solvents with Hansen's δ_h values above 18 (J/cm^3)^{1/2} produced swelling pressures that were directly related to their Hansen's δ_h values³².

The interfibrillar spaces between collagen fibrils are 20-30 nm wide in fully hydrated demineralised dentine³³, but can be virtually absent in air-dried matrices²⁵. Thus, the molecular size of potential solvents is important, along with their δ_h values³². Molecular size correlates well with the cube root of the molecular weight for small molecules. The amount of molecular agitation that can be thermally induced is inversely related to molecular size. Similarly, small molecules such as water have a higher molar concentration (55.6 moles/L

for water vs. 7.7 moles/L for HEMA) than larger molecules. We speculate that the higher molar concentration (Table) of low molecular weight solvents increases the number of solvent molecules that can thermally interact with the matrix proteins. This would permit smaller molecules to transfer more thermal kinetic energy to collagen than larger solvent molecules. In the presence of water, there is little interpeptide hydrogen bonding, and the spaces between the collagen fibrils are maximal. Water fills the interfibrillar spaces^{25,32} and easily penetrates the collagen peptides where it hydrogen bonds to functional groups. Ethanol can only partially break the interpeptide hydrogen bonds, permitting the residual interpeptide hydrogen bonds to stabilise the matrix (Fig. 1). Ethanol has a larger size and lower molar concentration than water (Table). The amount of kinetic energy that ethanol can transmit to the collagen matrix at any given temperature is less than that of water, hence higher temperatures are required to reach T_d . In dry demineralised dentine (very low hydration state IV, Table, $T_d = 176.1^\circ\text{C}$), there is maximum interpeptide hydrogen bonding that stabilises the collagen molecules, and there is no solvent available to transmit the thermal energy from the pan to collagen to disrupt its structure. Instead, higher temperatures must be used to directly thermally agitate the weakest portion of the collagen matrix. Type I collagen in dentine self-assemble into aggregations of molecules to form microfibrils that, in turn, form fibrils. Each molecule is a superhelix of three independent alpha chains stabilised by covalent cross-links^{1,17,18}. At the C-terminus of each triple helix, there is a 65 residue-long teleopeptide called the thermally labile domain. This domain has a lower thermal energy requirement for denaturation than does the helical portion³⁴. This is where it is believed that hydration with water or other polar solvents exerts their effect. In the absence of water, more interpeptide hydrogen bonding can occur in this domain and stabilise it. In the presence of water, much less hydrogen bonding can occur between these adjacent peptides. At the T_d , it is thought that this thermally-labile domain in the globular (i.e. nonhelical) terminal teleopeptide dissociates from one or more of the three adjacent teleopeptides, allowing an unzipping of the triple helical collagen molecule to occur³⁴. This structural derangement requires energy. The DSC quantifies this endothermic energy as a peak on the time/temperature curve that is defined as a denaturation temperature (T_d). This is analogous to the glass transition temperature for synthetic polymers. In glassy polymers such as Prime & Bond NT, the temperature associated with “melting” the stiff matrix into a soft flowable melted polymer is called the glass transition temperature or T_g . This is also an endothermic process. The temperature at which this thermally-labile peptide domain dissociates is dependent on the proximity of other microfibrils, with collapsed or close packing of microfibrils providing steric hindrance to dissociation¹⁴. This close proximity would also increase the amount of interpeptide hydrogen bonding that could also contribute to structural stability.

The T_d of soluble, newly formed, uncross-linked collagen is about $40\text{--}41^\circ\text{C}$, while that of new assembled collagen aggregations that form collagen fibrils is about 67°C ¹⁹. There are two alternative explanations for this phenomenon. As collagen fibrils are essentially polymers, Flory and Garrett³⁵ opined that the transition from helix to coil is a melting process, in which the melting point of polymers is depressed in the presence of a solvent. This is analogous to the contribution of solutes in lowering the freezing point of solvents. The less the solvent concentration, the higher is the melting point. That theory predicts that T_d would vary inversely with the molar concentration of solvents. The alternative explanation is based on the polymer-in-a-box theory of Doi and Edwards³⁶. That theory argues that collagen fibrils aggregates stabilise themselves and their nearest neighbours by spatially confining their molecular mobility. Unfortunately, our results support both theories. The significant dependence of T_d on solvent concentration and cube root of the molecular weight (Fig. 2) support the Flory and Garrett theory³⁶. However, the ability of polymerised adhesive resins to raise the T_d of dentine collagen argues strongly for the polymer-in-the-box theory because the resins seem to provide spatial confinement of the collagen.

If adhesive comonomers and their solvents can displace water from collagen and can penetrate between collagen fibrils and coat the thermally-labile domains of collagen molecules, then the T_d of resin-bonded demineralised dentine may increase. When Prime&Bond NT (PBNT) solvated in acetone was used to infiltrate demineralised dentine, and was subsequently polymerised, the T_d of dentine increased from 65.6°C (Table) to 173°C. It is unclear how much of this increase in T_d was due to the acetone solvent in PBNT, that might have removed enough water from the matrix to increase T_d to 123°C (acetone-saturated demineralised dentine, Table) versus how much was due to PBNT. When polymerized PBNT only was heated at the same rate as resin-infiltrated dentine, an endothermic peak was seen at 191.3°C in the polymer. This peak is not a denaturation temperature (T_d) but is a glass transition temperature (T_g) or polymer melting temperature. The fact that the resin-infiltrated demineralised dentine had a T_d of 166.8°C even after soaking in water for 24 h, suggests that water uptake may have plasticized the PBNT resin so that it had a glass transition temperature that was super-imposed on that of the collagen. In this case, the adhesive resin would offer no protection to collagen. Clearly, more research should be done in this area.

5. Conclusion

Within the limits of this study, it may be concluded that the presence of water-free organic solvents increased the thermal stability of demineralised dentine collagen matrices. Interpeptide hydrogen bonding seems to stabilise collagen to thermal challenge. Water molecules associate with functional groups in collagen that are capable of forming interpeptide hydrogen bonds make collagen more susceptible to thermal denaturation.

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Fig. 1. Denaturation temperature (T_d) of dentine matrix collagen was inversely proportional to the Hoy's δ_h (J/cm^3)^{1/2} of the solvents ($R^2 = 0.85$, $p < 0.01$).

Fig. 2. Denaturation temperature (T_d) of the dentine matrix collagen was directly proportional to the cube root of the molecular weight ($R^2 = 0.97$, $p < 0.001$) of the solvents. INSERT: A highly significant ($p < 0.001$) inverse relationship was found between T_d and the molar concentration of the solvents.

Table

Denaturation temperature (T_d) of resin-bonded demineralised dentine and solvated demineralised dentine.

Dentine condition/treatment	T_d	Hoy's δ_h	MW	$\sqrt[3]{M}$ W	moles/ L
A. Resin-bonded demineralised dentine					
PBNT-infiltrated, 24 h dry storage	172.7 ± 6.3 (3) ^a	--	--	--	--
PBNT-infiltrated, 24 h wet storage	166.8 ± 19.4 (3) ^a	--	--	--	--
PBNT-resin only, dry	191.3 ± 0.6 (3) ^a	--	--	--	--
B. Solvent effects on demineralised dentine					
Water-saturated demineralised dentine	65.6 ± 2.9 (3) ^A	40.4	18	2.62	55.56
Methanol-saturated demineralised dentine	92.4 ± 0.7 (3) ^B	24.0	32	3.17	24.72
Ethanol-saturated demineralised dentine	108.3 ± 6.4 (3) ^C	20.0	46	3.58	17.15
Acetone-saturated demineralised dentine	123.1 ± 1.2 (3) ^D	11.0	58	3.87	13.64
η -butanol-saturated demineralised dentine	139.2 ± 3.8 (3) ^E	15.4	74	4.19	10.95
HEMA-saturated demineralised dentine	148.5 ± 4.5 (3) ^F	16.1	130	5.06	8.25

Values are mean T_d ± SD in °C (number of specimens). Data identified by different superscript lower case letters are significantly different ($p < 0.05$).