Effects of water and water-free polar solvents on the tensile properties of demineralized dentin

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Abstract

Objectives. The aim of this study was to test the null hypothesis that the tensile properties of demineralized dentin are not influenced by the hydrogen bonding ability of anhydrous polar solvents.

Methods. Dentin disks 0.5 mm thick were prepared from mid-coronal dentin of extracted, unerupted, human third molars. ‘I’ beam and hour-glass shaped specimens were prepared from the disks, the ends protected with nail varnish and the central regions completely demineralized in 0.5 M EDTA for 5 days. Ultimate tensile stress (UTS) and low-strain apparent modulus of elasticity (E) were determined with the specimens immersed for 60 min in water, methanol, HEMA, acetone or air prior to testing in those same media. Apparent moduli of elasticity were measured on the same specimens in a repeated measures experimental design. The results were analyzed with a one-way ANOVA on ranks, followed by Dunn’s test at α = 0.05. Regression analysis examined the relationship between UTS or E and Hansen’s solubility parameter for hydrogen bonding (δh) of each solvent.

Results. The UTS of demineralized dentin in water, methanol, HEMA, acetone and air was 18(7), 29(7), 31(6), 41(13) and 146(27) MPa. Low-strain E for the same media were 11(7), 43(12), 79(21), 132(31) and 253(115) MPa. Regression analysis of UTS vs δh revealed a significant (p < 0.0005, r = −0.69, R² = 0.48) inverse, exponential relationship. A similar inverse relationship was obtained between low-strain E vs δh (p < 0.0001, r = −0.93, R² = 0.86).

Significance. The tensile properties of demineralized dentin are dependent on the hydrogen bonding ability of polar solvents (δh). Solvents with low δh values may permit new interpeptide H-bonding in collagen that increases its tensile properties. Solvents with high δh values prevent the development of these new interpeptide H-bonds.

Keywords: Collagen; Tensile properties; Modulus of elasticity; Ultimate tensile stress; Hydrogen bonding; Solubility parameters

1. Introduction

Although collagenous matrices are seldom exposed to anhydrous polar solvents in vivo, in adhesive dentistry, the collagenous matrix of dentin is inadvertently acid-etched during the removal of the mineral phase from the collagen fibril matrix. Adhesive monomers in polar solvents are then used to infiltrate resin around the fibrils to achieve micromechanical retention of composite resins [1]. Our previous work demonstrated that ethanol, hydroxymethacrylate (HEMA) and acetone stiffened demineralized dentin matrices by removing water [2]. Even greater stiffness could be produced by air-drying [2], a procedure known to increase the stiffness of rat-tail tendon [3] or reconstituted type I collagen [4]. We attributed the stiffening effect of dehydration to the ability of collagen peptides to develop weak associations (Van der Waals forces, hydrogen bonds, etc.) between neighboring peptides in the absence of water. More recently, we demonstrated that almost all anhydrous but water-miscible polar solvents induce shrinkage of demineralized dentin matrices as they dehydrate them [5]. Additionally, those polar solvents with low hydrogen bonding abilities produced more shrinkage than...
did those with high-hydrogen bonding abilities [5]. Hansen’s solubility parameter for hydrogen bonding ($\delta_h$) was used to rank the test solvents [6]. Those solvents that caused net shrinkage had $\delta_h$ values $< 19 (\text{J/cm}^3)^{1/2}$, while those that produced no net shrinkage had $\delta_h$ values $> 19 (\text{J/cm}^3)^{1/2}$. We speculated that $19 (\text{J/cm}^3)^{1/2}$ represented the $\delta_h$ equivalent of relatively dry demineralized dentin. That is, the functional groups between adjacent collagen peptides form H-bonds with a cohesive energy density equivalent of $19 (\text{J/cm}^3)^{1/2}$, thereby shrinking the collagen matrix. Water “plasticizes” collagen [2] by rapidly breaking interpeptide H-bonds because of its high $\delta_h$ value of $37 (\text{J/cm}^3)^{1/2}$, thereby dehydrating the matrix. Water can preferentially H-bond with collagen peptides, thereby preventing interpeptide H-bonding. If this theory is correct, then the modulus of elasticity and the ultimate tensile stress (UTS) of demineralized dentin matrices should be inversely related to the $\delta_h$ of anhydrous polar solvents used to dehydrate them.

The purpose of this study was to test the null hypothesis that the $\delta_h$ values of anhydrous polar solvents have no effect on the modulus of elasticity or the UTS of demineralized dentin matrices.

2. Materials and methods

2.1. Teeth

All specimens were prepared from mid-coronal dentin of extracted, unerupted human third molars. The teeth were stored at 4 °C in thymol-saturated isotonic saline, to inhibit microbial growth. They were used within 1 month of extraction. Using a diamond saw (Isomet, Buehler Ltd, Lake Bluff, IL, USA), the occlusal enamel was removed from the crown, and dentin disks 0.5 mm in thickness were prepared (Fig. 1). Either an hour-glass or an ‘I’ beam pattern were drawn on the disk with a pencil. The dentin outside the pattern was cut away with an ultrafine diamond bur in a high-speed handpiece using copious air–water spray, to leave the dentin in one of the two patterns (Fig. 1).

Both ends of the specimens were covered with two layers of nail varnish leaving the middle section uncovered and susceptible to 0.5 M EDTA (pH 7.4) demineralization for 5 days at 25 °C. The decalcification time was selected from microradiography data and by evaluation of decreases in the stiffness of the specimens on days 4 and 5 as described earlier [2].

2.2. Tensile testing

The UTS was measured after scraping the nail varnish from each mineralized end of the hour-glass shaped specimens using a scalpel, and then placing each mineralized end of the specimens into a friction grip device (Dirck device, Bioengineering, University of Iowa, Iowa City, IA, USA) in a Vitrodyne V1000 tester (Chatillon, Greensboro, NC, USA). Specimens were subjected to tensile load at 0.6 mm/min until failure to obtain the ultimate tensile load, which was converted to the UTS by dividing it by the cross-sectional area of the narrowest part of the hour-glass shape. This was generally 0.5 × 0.5 mm² but was measured to the nearest 0.01 mm with a digital micrometer (Sylae Ultra-Cal II, Fowler Inc., Newton, MN, USA) in each specimen prior to testing. All such measurements, as well as tensile testing, were done with the specimens immersed in 25 °C distilled water, or neat methanol, HEMA or acetone. There were four solvent groups: water, methanol, HEMA and acetone. Each group consisted of 10 specimens. The specimens were placed in the solvents for 60 min, prior to testing. A fifth group was included in which the specimens were removed from water and allowed to air dry for 60 min prior to testing.

The apparent modulus of elasticity (E) was measured on I beam-shaped specimens (Fig. 1) which had a gauge-length of 2.8 ± 0.2 mm. The mineralized ends of the specimens were held in the friction grips of a Vitrodyne tester. There were 15 specimens in this group. The low-strain apparent modulus of elasticity of each specimen was calculated at 4–5% strain after converting the load–displacement values to stress–strain values. Since the actual strain of the specimen was not measured with an extensometer, but only inferred from the displacement of the crosshead, the specimen stiffness values are termed apparent moduli of elasticity. The apparent modulus of elasticity was measured on each specimen after it was randomly equilibrated for 60 min in each of the four solvents. That is, each specimen was randomly exposed to water, methanol, HEMA or acetone, for 60 min. Between solvents, each specimen was immersed in 500 ml of water for 60 min with continuous stirring to extract the previous solvent and to rehydrate the matrix. At the end of these trials the specimens were allowed to dry in air (25 ± 1 °C, 42–44% relative humidity) for 60 min and the same low-strain apparent modulus of elasticity was measured in air. The rate of change in stiffness of three specimens was measured every 10 min for 80 min in three specimens that were randomly immersed in the four solvents.

2.3. Solvents

Acetone and methanol were 99.5% pure with a water content of <0.5% according to the manufacturers specifications (Fisher Scientific, Atlanta, GA, USA). They were certified American Chemical Society grade. Hydroxyethyl methacrylate was 99.5% pure, stabilized with 180 ppm hydroquinone monomethyl ester. It contained <0.5% water according to the supplier (Esschem Company, Essington, PA, USA). These solvents were used as they were received from the manufacturers, without any additional treatment. Since these are all hygroscopic solvents, they were purchased in 500 cm³ containers. Fresh containers were opened each working day to avoid contamination by atmospheric moisture.
Hansen’s solubility parameters for the solvents were obtained from a handbook [6].

2.4. Statistics

After calculating descriptive statistics, the distribution of the data was tested for normality using the Kolmogorov–Smirnov test [7]. The data were then analyzed by a Kruskal–Wallis one-way ANOVA on ranks to determine if there were any significant differences among the groups. Multiple comparisons were done to isolate and compare the significant results using Dunn’s test at \( \alpha = 0.05 \). Regression analysis was used to test the correlation of UTS and \( E \) with the \( \delta_h \) of the polar solvents.

3. Results

Table 1 lists the apparent modulus of elasticity and the UTS of the demineralized dentin specimens in water, methanol, HEMA, acetone and air. In water, the matrix had a UTS of about 18 MPa; specimens equilibrated with neat methanol, HEMA, acetone or air had increasingly higher UTS values. The low-strain apparent modulus of elasticity (\( E \)) of specimens in water was 10.7 MPa (Table 1), but increased when they were immersed in methanol, HEMA, acetone or were allowed to air dry (Table 1).

Use of the Kolmogorov–Smirnov test [7] on the UTS data indicated that they were normally distributed, but that the variance was not sufficiently equally distributed to justify the use of statistical tests based on normal distributions. A one-way analysis of variance on ranks was done using the Kruskal–Wallis method. It detected a highly significant difference among the groups (\( p < 0.001 \)). Multiple comparisons using Dunn’s test at \( \alpha = 0.05 \) isolated the differences as follows: The UTS of water-treated specimens was significantly lower than that of all other treatment groups, except methanol and HEMA (Table 1). Methanol, HEMA and acetone were not significantly different from each other (Table 1). Acetone and air were not different from each other although air was different from the rest of the treatments. Regression analysis of the UTS data vs Hansen’s solubility parameter for hydrogen bonding (\( \delta_h \)) of the solvents is shown in Fig. 2. Although the correlation coefficient (\( r = -0.69 \)) is relatively high and the relationship is significant (\( p < 0.001 \)), the \( R^2 \) value was only 0.48 (Fig. 2).

Fig. 3 shows the rate at which three randomly selected specimens increased stiffness when they were tested at 10 min intervals from 0 to 80 min in each of the four solvents. Acetone increased the stiffness of the matrix from 2 to 108 MPa within the first 10 min. HEMA increased stiffness at a rate about half as fast as acetone. Methanol increased stiffness even more slowly and water did not change the stiffness of the matrix over time.

The low-strain (ca. 5%) apparent modulus of elasticity (\( E \)) of the specimens in water, methanol, HEMA, acetone and air were 11(7), 43(12), 79(21), 132(31) and 253(114) MPa, respectively (Table 1). Analysis of variance

Table 1. Summary of the effects of polar solvents and air on the tensile properties of demineralized dentin

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Modulus (MPa)</th>
<th>UTS (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>10.7 (7.4) (15)</td>
<td>18.3 (6.5) (10)</td>
</tr>
<tr>
<td>Methanol</td>
<td>43.3 (12.2) (14)</td>
<td>28.8 (6.6) (10)</td>
</tr>
<tr>
<td>HEMA</td>
<td>78.9 (21.2) (15)</td>
<td>30.9 (6.4) (10)</td>
</tr>
<tr>
<td>Acetone</td>
<td>132.0 (30.9) (14)</td>
<td>40.8 (13.4) (10)</td>
</tr>
<tr>
<td>Air</td>
<td>253.4 (114.5) (12)</td>
<td>145.7 (27.3) (10)</td>
</tr>
</tbody>
</table>

Values are \( \text{mean} \pm \text{SD} \) (\( n \)). UTS = ultimate tensile stress. Groups identified by different lower case superscript letters are significantly different (\( p < 0.05 \)). Unequal group sizes were due to inadvertent breakage of specimens during gripping.

\[ E = 47.54 \times 0.077x, \quad R^2 = 0.48, \quad r = -0.69, \quad p < 0.0005 \]
viscoelastic specimens exhibit curvilinear stress–strain instead of the 4–5% strain used in the current study. These demineralized specimens were pulled to 15–20% strain (Table 1), although there was no significant difference between the stiffness of specimens in acetone vs air. Regression analysis of the low-strain modulus of elasticity data vs the \( \delta_h \) values for the solvents yielded a highly significant \( (p < 0.0001) \) inverse exponential relationship \( (r = -0.93, R^2 = -0.86) \). This is shown in Fig. 4 along with an insert that shows the response of a single specimen to the same variables.

4. Discussion

The results of this study demonstrate that the tensile properties of the demineralized dentin matrix in polar solvents are inversely related to the Hansen solubility parameter for hydrogen bonding (\( \delta_h \)) of those solvents (Figs. 2 and 4). Regressions of UTS and \( E \) vs \( \delta_d \) or \( \delta_h \) (dispersive forces or polar forces, respectively), gave lower correlation coefficients (data not shown). Similar increases in the low-strain (ca. 3%) stiffness of demineralized dentin had been reported when specimens were equilibrated with water, HEMA, acetone or air [2]. The present values were not significantly different from those values even though in the present study, the same specimens were measured repeatedly, while in the previous study, separate specimens had been used for each solvent. The present values for the apparent modulus of elasticity of demineralized dentin matrix in water (10.7 ± 7.4 MPa, Table 1) was much lower than previously reported values (250 ± 70 and 210 ± 60 MPa, respectively, [8,9]). In the previous work, the demineralized specimens were pulled to 15–20% strain instead of the 4–5% strain used in the current study. These viscoelastic specimens exhibit curvilinear stress–strain curves that gives higher apparent moduli of elasticity as they undergo more strain. Had the current specimens been strained to 15% in acetone or air, they would have been destroyed thereby preventing the desired repeated measures design.

It was speculated that dehydration of demineralized dentin by water-free but water-miscible polar solvents permitted weak forces to develop between adjacent collagen peptides (e.g. Van der Waals, hydrogen bonds, etc.) that could not occur in the presence of water [2]. More recently, polar solvents were found to induce shrinkage of hydrated dentin matrix that was also inversely related to the \( \delta_h \) of the solvents [5]. Methanol and ethylene glycol produced much less shrinkage of the matrix than did acetone or HEMA because their \( \delta_h \) values were much higher ca. 22 and 26 (J/cm\(^3\))\(^{1/2} \), respectively, than those of acetone or HEMA, 7 and 16.1 (J/cm\(^3\))\(^{1/2} \), respectively. Thus, polar solvents with high \( \delta_h \) values (i.e. methanol, water) apparently preferentially hydrogen bond with collagen peptides, thereby preventing the peptides from developing the additional interpeptide H-bonds that can occur when the matrix is immersed in solvents with \( \delta_h \) values below 19 (J/cm\(^3\))\(^{1/2} \), the postulated value for the \( \delta_h \) of relatively dry collagen [10]. The equation of the regression line in Fig. 4 shows that the apparent modulus of elasticity of the demineralized matrix would be 297 MPa when the \( \delta_h \) of the ‘solvent’ was zero. This value is very close to the apparent modulus of elasticity that was measured in air, 253 MPa (Table 1). It should be noted that the air-dried specimens probably contained bound water. At a relative humidity of 42–44%, the dentin matrix was only relatively dry. However, that relatively dry condition was sufficient to cause the largest changes in the tensile properties of the matrix (Table 1).

Collagen fibrils are made up of self-assembled aggregations of collagen molecules. Their mechanical properties are due to cross-links [11] and molecular intertwining [12]. That is, when pulled to failure, the collagen molecules do not break. Rather, they disaggregate or defibrillate [13]. Additionally, any weak forces that can develop between
adjacent peptides might contribute to the mechanical properties of collagen, but these are prevented from forming by the presence of water, the strongest hydrogen bonding solvent known. The 24-fold increase in the apparent modulus of elasticity and the eight-fold increase in the UTS of demineralized dentin matrix that occurred when specimens were treated in air vs water, indicates that weak forces can, under the right conditions, markedly strengthen the tensile properties of collagen.

Kato et al. [3] compared the tensile properties of wet vs dry rat tail tendons. Their moduli of elasticity increased from 570 to 2250 MPa and their UTS increased from 7.9 to 344 MPa, respectively, representing 3.9 and 9.3-fold 10 and 4.4-fold changes, respectively, [3]. Similarly, when the moduli of elasticity of reconstituted type I collagen was compared wet vs dry, the values increased from 58 to 1261 MPa. Their UTS values increased from 7.9 to 344 MPa, respectively, representing 22 and 44-fold increases [4]. The larger changes in the tensile properties of the reconstituted collagen were probably due, in part, to their initial lack of covalent cross-links, compared to those of tail tendon.

Previous work on the dimensional changes in the matrix of demineralized dentin [5] demonstrated that methanol can be used to dehydrate the matrix with little net shrinkage because of its high $\delta_h$ value and its ability to solvate collagen [10]. Methanol may be useful as a solvent for adhesive monomers and as a dehydrating solvent for collagen scaffolds to avoid shrinkage caused by solvents with low $\delta_h$ values. There may be concern over the use of methanol because it is toxic when ingested systemically. This is due to its metabolic conversion to formaldehyde. However, when used as a solvent for adhesives, perhaps 25–50 $\mu$l would be applied to a tooth, and 95% of it would be evaporated. This is analogous to the common use of 70% isopropyl alcohol on skin, even though isopropyl alcohol is toxic if ingested.

The results of this study provide a rationale for why collagenous structures become both stronger and stiffer when dehydrated. Indeed, this phenomenon can be extrapolated to explain increases in the tensile properties of paper [14,15] and silk [16,17] when they are dried. These are all fibrous polymeric structures composed of monomeric units that can hydrogen bond with adjacent fibers. The ability of polar solvents to break these hydrogen bonds has been ranked using their Hildebrand solubility parameters [10] or their ability to change the velocity of sound in wet paper [18]. Here, we ranked them by their Hansen’s solubility parameter for H-bonding forces. The correlations between the apparent moduli of elasticity of solvated dentin matrix or UTS and $\delta_h$ are remarkable. Weak forces, such as H-bonding, under dry conditions, can under special circumstances be responsible for most of the tensile properties of fibrous polymers. In acid-etched, demineralized dentin that is air-dried, these H-bonding forces are responsible for a collapse of the fibrillar meshwork [19,20]. However, in aqueous environments (e.g. biological conditions) we speculate that the high $\delta_h$ value for water, together with its high molar concentration (55 mol/l), prevents these H-bonds from forming between adjacent fibrils. The use of solubility parameters to rank the interaction of monomers with demineralized dentin was first done by Asmussen and his colleagues [21–23]. Miller et al. [24] calculated the Hansen’s triple solubility parameters of many monomers and solvents as well as of the collagenous matrix, and correlated these to bond strengths. Their more recent work examined the influence of the permeability of monomers on bond strength [25]. Utilizing the above published work as a foundation, we evaluated the interaction of the solvents that are commonly used in adhesive dentistry with wet vs dry demineralized dentin matrices [5]. It remains to be demonstrated that collapse of the matrix by solvents and/or comonomers leads to low resin–dentin bond strengths. Further research on that topic can test that hypothesis.

The results of this study require rejection of the null hypothesis that the $\delta_h$ values of anhydrous polar solvents have no effect on the apparent modulus of elasticity or the ultimate tensile strength of demineralized dentin matrix. What remains to be determined is the use of vibrational spectroscopy to confirm the “new” H-bonds in the dry vs wet matrix.

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References


