Research Paper

The role of proteoglycans in the nanoindentation creep behavior of human dentin

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ABSTRACT

Attempts to understand the mechanical behavior of dentin and other mineralized tissues have been primarily focused on the role of their more abundant matrix components, such as collagen and hydroxyapatite. The structural mechanisms endowing these biological materials with outstanding load bearing properties, however, remain elusive to date. Furthermore, while their response to deformation has been extensively studied, mechanisms contributing to their recovery from induced deformation remain poorly described in the literature. Here, we offer novel insights into the participation of proteoglycans (PG) and glycosaminoglycans (GAG) in regulating the nanoindentation creep deformation and recovery of mineralized and demineralized dentin. Accordingly, after the enzymatic digestion of either PGs and associated GAGs or only GAGs, the nanoindentation creep deformation of dentin increased significantly, while the relative recovery of both the mineralized and demineralized dentin dropped by 40–70%. In summary, our results suggest that PGs and GAGs may participate in a nanoscale mechanism that contributes significantly to the outstanding durability of dentin and possibly other mineralized tissues of similar composition.

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1. Introduction

Studies concerning the regulation of deformation response in human mineralized tissues, including dentin and bone, have been primarily focused on the role of their more predominant constituents, namely collagen and hydroxyapatite. A number of nanoscale deformation mechanisms have been proposed to explain the effective load bearing ability of mineralized tissues, including void and crack formation (Gupta et al., 2005), mineralized collagen fibril-matrix shear (Burr et al., 1997), and nanogranular friction between mineral particles (Tai et al., 2006), among several other mechanisms (Fratzl, 2008a, 2008b; Sugiyama et al., 2007; Gupta et al., 2006; Jager and Fratzl, 2000; Koester et al., 2008). Despite these extensive efforts, the evidence of mineralized tissues' ability to *recover* from deformation, and thereby prevent successive accumulation of plastic or non-linear strain remains virtually non-existent in the literature.

Dentin is a load bearing mineralized composite that represents the largest component of tooth structure, sharing great compositional similarities to bone (Bertassoni et al., 2009, 2012). Its primary biomechanical role is to provide a tougher foundation for the brittle enamel (Imbeni et al., 2005), thus playing a major role in the durability of teeth. Similar to bone, dentin is primarily composed of type I collagen fibrils, water and nanocrystallites of carbonated hydroxyapatite (Bertassoni et al., 2012). Although these more abundant components have received considerable attention regarding their contributions to the mechanical properties of mineralized tissues, the specific role of the structures responsible for their mutual interaction in the matrix have been underestimated thus far, particularly the organic components interconnecting the collagen fibrils in the matrix.

Proteoglycans (PGs) and their glycosaminoglycan (GAGs) side chains represent a major group of noncollagenous structures with known structural and mechanical relevance (Goldberg and Takagi, 1993; Bertassoni and Swain, 2014; Bertassoni and Marshall, 2009) for vertebrates. Other matrix proteins, such as phosphoproteins and γ-carboxyglutamate-containing proteins are believed to be largely involved in mineral–matrix binding events (Linde, 1989), although they have also been associated with important mechanisms of dissipation of mechanical energy (Adams et al., 2008; Fantner et al., 2007). Decorin and biglycan are the two members of the small-leucine-rich-proteoglycan (SLRP) family predominantly expressed in dentin (Goldberg and Takagi, 1993). The GAGs most frequently found in dentin, in turn, are chondroitin 4-sulfate and a relatively lower content of chondroitin 6-sulfate (Goldberg and Takagi, 1993).

PGs have widely been shown to contribute extensively for nearly all connective tissues to resist against deformation under loading (Scott et al., 2004; Scott, 2003), particularly soft and non-minerlized tissues. Loss of specific PG family members has been linked to skin fragility, pulmonary emphysema, heart valve diseases, osteoarthritis (Melrose et al., 2008; Stanton et al., 2005; He and Swain, 2009) and several other conditions affecting the mechanical properties of multiple tissues (for a review refer to Ameye and Young (2002). Despite extensive evidence of the relevance of PGs to the extracellular matrix of nearly all vertebrates, knowledge of the mechanical role of these structures (Scott, 2003) in mineralized tissues, including dentin and bone, remains limited (Bertassoni et al., 2014; Xu et al., 1998). To address this knowledge gap we sought new insights into the roles of PGs and associated GAGs in the nanoindentation creep behavior of dentin. More specifically, we tested the hypothesis that, since PGs and GAGs interconnect collagen fibrils in dentin, they would function as key regulators of the viscoelastic response of healthy and demineralized dentin by hindering nanoindentation creep deformation and regulating deformation recovery.

2. Materials and methods

2.1. Specimen preparation and demineralization

Permanent and healthy human third molars ($N=8$) were obtained according to protocols approved by the Sydney South West Area Health Service and the Royal Prince Alfred hospital's bioethics committee on human research. Extracted teeth were thoroughly cleaned, rinsed in saline with 0.1% thymol and stored at room temperature. Roots and the top of the enamel crowns were cut using a low speed saw (Isomet; Buehler, Lake Bluff, IL, USA) under water irrigation in the mid-coronal region perpendicular to the tubule direction, thus exposing the remaining dentin on both sides. Dentin blocks measuring 3.5 mm in length and width and 2 mm in thickness were cut and subsequently ground and polished on silicon carbide papers of 600 and 1200 grits, followed by 9 μm and 1 μm polishing diamond paste on a rotary polishing machine (Buehler, Lake Bluff, IL, USA). Each specimen surface was partially covered with a masking tape to provide a reference area of mineralized dentin and the remaining surfaces were exposed to a demineralizing solution of 10% (v/v) citric acid for 2 min following demineralization protocols described elsewhere (Balooch et al., 2008).

2.2. Enzymatic digestions with chondroitinase-ABC and trypsin

Following demineralization, specimens were rinsed thoroughly with deionized water and the masking tape was removed. One set of specimens was immersed in a chondroitinase-ABC (C-ABC) solution ($N=4$) and another set was immersed in a trypsin solution ($N=4$). C-ABC was used to digest the chondroitin/dermatan sulfate GAG side-chains of PGs (Ho et al., 2005). Trypsin, on the other hand, was used primarily to cleave the protein core of proteoglycans following previously established protocols (Rapraeger and Bernfield, 1985). C-ABC solution contained 0.1 U/mL C-ABC from *Proteus vulgaris* (Sigma-Aldrich, St. Louis, MO), 50 mM Tris, 60 mM sodium acetate and 0.02% (w/v) bovine serum albumin at pH 8.0. Specimens were stored for 48 h in 37 °C under constant stirring and solutions were exchanged after 24 h. Trypsin solution contained 1 mg/ml TPCK-treated trypsin (Sigma–Aldrich, St. Louis, MO) with 0.2 M ammonium bicarbonate at pH 7.9. Specimens were stored for 48 h at 37 °C under constant stirring and solutions were also exchanged after 24 h. As our trypsin and C-ABC treatments aimed primarily at

digesting PGs and GAGs, respectively, the treatment groups will be referred to as 'PG digested' and 'GAG digested' in the following sections.

2.3. Western Blotting

To confirm the digestion of PGs and GAGs, dentin samples ($N=6$) were pulverized using a hand drill and subjected to the same enzymatic protocols described above. After two changes of the enzyme solutions, samples were extracted using 4 M Guanadine HCl (which denatures and unfolds proteins enabling their extraction) in 50 mM Tris with 0.5 M EDTA (to de-calcify and inhibit metalloproteinses) at pH 7.4 and 4 °C for 2 days with one change at 24 h. The combined 48 h supernatant was dialyzed against deionized water using a 3 kDa MWCO membrane at 4 °C for 24 h, and lyophilized for another 24 h to remove the Guanadine HCl used in first step which otherwise interferes with electrophoresis. Dentin extracts (3 μg) were dissolved and run in SDS-PAGE gels, where the electrophoretic mobility of the denatured proteins separates proteins of different molecular weights in different bands. Extracts were run on a 12.5% 0.75 mM non-reducing SDS-PAGE gel and transferred to a nitrocellulose membrane, for specific antibody recognition and blotting. After blocking with 5% skim milk, TBS and azide, to block non-specific binding, extracts were probed with a rabbit monoclonal antibody recognizing biglycan (0.5 μg/mL, Abcam 109369) for 3 h at room temperature. After washing in TBST 3 times, 10 min each, the membrane was incubated with secondary goat anti-rabbit antibody (1:2000) for 2 h at room temperature, and developed for 30 min in 15 mL of developing solution (0.2 M Tris pH 9.5 and 2 mM MgCl$_2$ and 50 μl of Bio-Rad developing solution A and B).

2.4. Nanoindentation

All samples were hydrated for at least 24 h before testing and had their surfaces indented while fully immersed in deionized water, perpendicular to the tubule direction, using a UMIS indenter system with a calibrated Berkovich tip of 170 nm nominal radius. Indentations were performed using a one-step loading to a maximum load of 5 mN (Fig. 2A, inset), followed by a hold period of 30 s. This was followed by a one-step unloading and hold of 30 s at a minimum load of 1 mN. A maximum load of 5 mN was chosen to ensure that the maximum depth of penetration of the indenter was about 10% of the total depth of demineralization (15–20 μm) in the acid-etched dentin. A short hold period of only 30 s was chosen to more closely match clinical situations where teeth are subjected to continuous loading, such as in clenching and bruxism, which typically last for about 9–30 s (Clarke et al., 1984).

The normalized creep, h'_{max}, was calculated by subtracting the initial depth of penetration at the onset of the holding period at a maximum load, h_{i-max}, against the total depth of penetration at the maximum load, h_t. The normalized creep recovery, h'_{min}, on the other hand, was calculated by subtracting the initial depth of penetration at minimum load, h_{i-min}, by the final depth of penetration, h_f. The relative creep recovery was calculated as the percentage of normalized
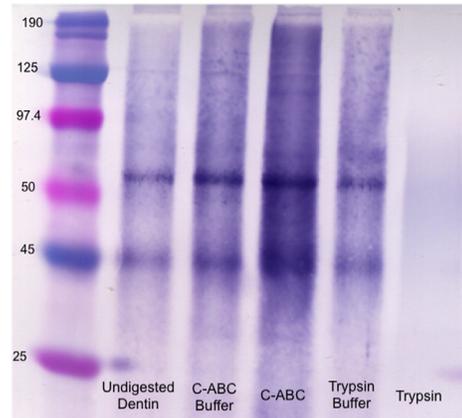

Fig. 1 – Biglycan Western blot of extracts from undigested, C-ABC, and trypsin treated dentin. The migration of pre-stained molecular mass markers is shown in lane 1 (on the left). The second lane is undigested dentin, not exposed to any incubation as a control. C-ABC and trypsin buffers (3rd and 5th lanes, respectively) represent samples that were exposed to the respective digest buffer solutions under the same conditions but without enzymes. Lane 4 shows a representative blot of C-ABC treated dentin where bands at 42 and 55 kDa show notably higher intensities of deglycosylated biglycan core protein than when incubated in buffer alone, confirming removal of GAG. In contrast, trypsin treated dentin (lane 6) does not show any bands, consistent with degradation of the biglycan core protein.

recovery at a minimum load of 1 mN, h'_{min}, relative to the normalized deformation at a maximum load of 5 mN, $h'_{max◆}$.

A total of 15 indentations were performed per specimen for each variable tested and indents were separated by a minimum of 40 μm to avoid the influence of residual stresses from adjacent impressions. Data was analyzed using ANOVA and a Tukey's test at a significance level of 5% to represent the uncertainty of the comparison between the digested groups (either GAGs or PGs), the mineralized (untreated) and demineralized control groups.

3. Results and discussion

The primary objective of this study was to gain new insights into the participation of PGs and associated GAGs on the nanoindentation creep behavior of human dentin. Lack of specific bands for biglycan following trypsin digestion (Fig. 1) demonstrates efficient digestion of the core protein. In contrast, C-ABC enzyme preserved the PG protein core, as demonstrated by the biglycan bands, which appeared as a doublet at about 42 and 55 kDa. The notably higher intensity in the biglycan bands of the C-ABC treated group demonstrates the efficient removal of GAG chains reducing the molecular mass and allowing a previously glycosylated biglycan core proteins to enter the resolving gel and accumulate at their respective bands with a higher intensity than in other groups. The C-ABC digestion method has been extensively utilized to remove GAGs from dentin, and further confirmation of its effective
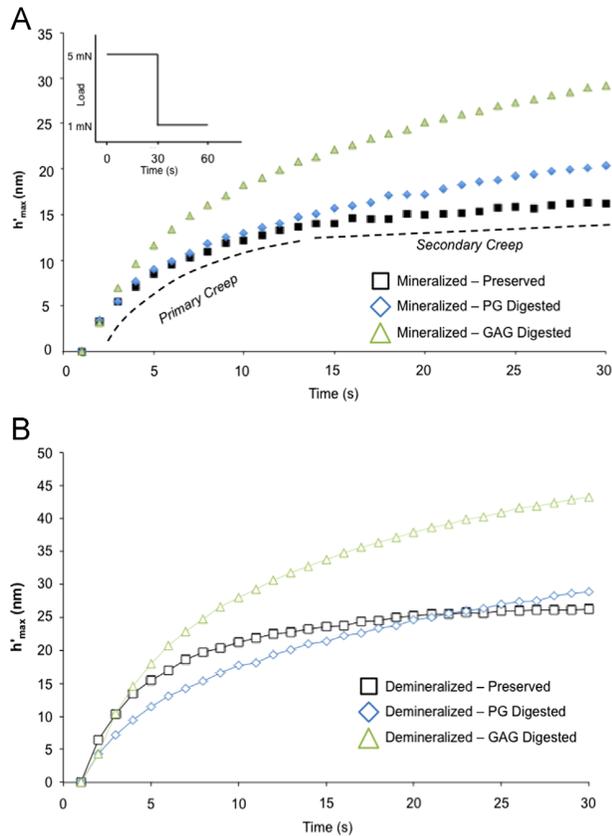
Fig. 2 – Normalized depth of penetration of indenter during creep of (A) mineralized and (B) demineralized dentin, before and after digestion of PGs ad GAGs. (A) illustrates the stages of primary and secondary creep.

GAG-removal ability has been illustrated via TEM (Scott, 1992) and AFM (Ho et al., 2005) microscopy previously.

Restorative dental procedures rely extensively on the interaction of dental materials with the demineralized tissue matrix, therefore we examined both fully mineralized dentin and acid etched specimens. Moreover, we were interested in testing whether our digestion protocols would have a similar effect in the mineralized matrix compared to the demineralized tissue, where PGs and GAGs would be putatively more easily accessible to the digestive enzymes. Our data shows that a similar trend resulting from of the removal of PGs and GAGs in both mineralized and demineralized dentin occurred, yet specific mechanical changes were observed for individual groups. Fig. 2 shows an increase in the normalized creep deformation of the digested samples in both mineralized (Fig. 2A) and demineralized groups (Fig. 2B). The depth of penetration of the indenter during creep deformation of the mineralized dentin (16.1 ± 2.2 nm) showed nearly a two-fold increase after removal of GAGs (30.6 ± 4.7 nm) ($p < 0.0001$) and less after removal of PGs (20.9 ± 4.5 nm) ($p < 0.05$). Mineralized dentin after GAG digestion also deformed significantly more than PG digested dentin ($p < 0.0001$). In general, the demineralized specimens presented a higher normalized creep deformation than the mineralized dentin ($p < 0.05$), which is consistent with the increased viscoelastic behavior

of mineral-free organic scaffolds as determined previously (Bertassoni and Swain, 2012; Angker et al., 2004). The creep deformation of demineralized dentin was of 26.1 ± 3.3 nm, and nearly doubled (45.3 ± 4.2 nm) after the digestion of GAGs ($p < 0.0001$). PG digestion also caused an increase in the creep deformation of the demineralized specimens leading to a normalized creep indentation depth of 28.9 ± 2.7 nm ($p < 0.0001$). Similar to the mineralized tissue, the difference between GAG digested and PG digested demineralized dentin was also significant ($p < 0.0001$). These results suggest that both components of the proteoglycan structure, the PG protein core and the GAG side-chains, contribute significantly, and perhaps distinctly, to the ability of dentin to resist against creep deformation.

Interestingly, both mineralized and demineralized dentin samples with preserved PGs and GAGs (untreated) showed an earlier onset of secondary creep, which was particularly evident for the demineralized group. Therefore, based on the mechanical data we found, PGs and GAGs appear to function as damped 'springs' within the dentin matrix, a behavior that has been well documented in non-mineralized tissues (Scott, 2003), but poorly characterized in dentin and bone. The viscous response of natural biological materials is known to play an important role in their function to bear and distribute mechanical force. Therefore, PGs and GAGs, although a very small percentage of the tissue volume, could be linked to preventing accumulation of sub-micrometer deformation and non-linear strains within the tissue matrix. Consequently, considering that dentin is known to form a tougher foundation for the brittle enamel (Imbeni et al., 2005), this mechanism may, to a certain extent, contribute to prevent teeth from undergoing catastrophic failure at earlier stages in life.

Fig. 3 shows the creep recovery of dentin at minimum load. Both mineralized and demineralized dentin suffered nearly a two-fold reduction in normalized recovery after removal of GAGs and PGs in comparison to the mineralized and demineralized control groups. The normalized recovery of mineralized (Fig. 3A) and demineralized (Fig. 3B) dentin with preserved GAGs and PGs was 11.9 ± 1.4 nm and 17.6 ± 2.8 nm, respectively. After digestion of GAGs, the time dependent recovery of mineralized dentin dropped to 8.0 ± 3.9 nm ($p < 0.0001$), and demineralized dentin to 10.0 ± 5.1 nm ($p < 0.0001$). PGs digestion, in turn, decreased the creep recovery of mineralized dentin to 9.5 ± 2.1 nm ($p < 0.0001$) and of demineralized dentin to only 5.4 ± 2.8 nm ($p < 0.0001$). The difference between the creep recovery of GAG and PG digested demineralized dentin was also significant at ($p < 0.05$).

Analyses of the relative recovery of mineralized and demineralized dentin specimens (Fig. 4), which represents how much of the creep deformation was recovered after the loads were partly removed, are shown in Fig. 4. These results are indicative of the ability of the matrix molecules to re-arrange back to a pre-stressed organization over time. There was little contribution of the mineral phase to the relative recovery of creep deformation, with no significant difference between mineralized ($73.6 \pm 16\%$) and demineralized dentin ($67.4 \pm 16.8\%$) as shown in Fig. 4. In contrast, after GAGs digestion the recovery ability of mineralized and demineralized specimens suffered a drastic drop down to
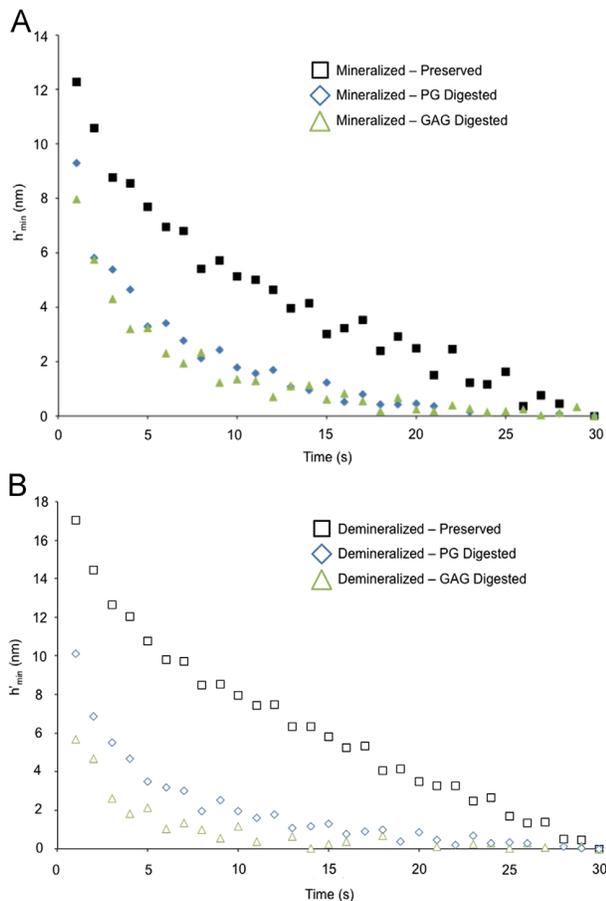
Fig. 3 – Normalized depth indenter during creep recovery of (A) mineralized and (B) demineralized dentin before and after digestion of PGs ad GAGs.

Fig. 4 – Relative recovery of dentin before and after digestion of GAGs and PGs.

only $25.9 \pm 15.7\%$ and $22.1 \pm 12.9\%$, respectively ($p < 0.0001$). Similarly, PG digestion in mineralized dentin yielded a relative recovery of $45.4 \pm 17.7\%$ ($p < 0.0001$). PG digested demineralized dentin, on the other hand, recovered only $18.7 \pm 10.9\%$ ($p < 0.0001$), showing a decrease of nearly 75% in comparison to the demineralized dentin group with preserved PGs and GAGs. A summary of all nanoindentation creep properties is presented in Table 1.

It is interesting to note a greater effect on nanoindentation properties of GAG-removal compared with digestion of the PG core proteins with trypsin. While the precise mechanisms underlying this observation are unclear, trypsin also digests many other noncollagenous proteins in the dentin matrix. We have previously shown that this may also affect the nanostructure of collagen intrafibrillarly (Bertassoni et al., 2012). Therefore, in contrast to GAG-digested dentin where only interfibrillar chondroitin and dermatan sulfate are broken down, a much more complex set of deformation events may occur in the trypsin treated matrix both intra- and interfibrillarly, some of which may counteract the loss of GAG. The mechanisms underpinning these phenomena are currently being investigated in our laboratory. Possible interpretations for the time dependent behavior we observed require a critical appreciation of the structure of collagen, PGs and GAGs
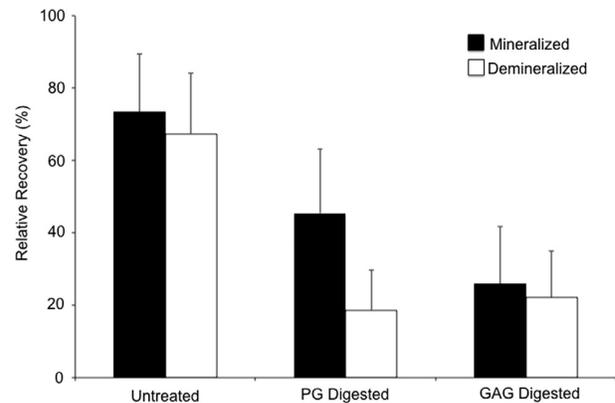
in the dentin matrix. On a molecular scale, the protein core of the most abundant PGs in dentin, biglycan and decorin, adopt a folded helical configuration stabilized by intramolecular H-bonds and electrostatic interactions that have been suggested to interact with the collagen surface via an array of H-bonds (Orgel et al., 2009). Highly negatively charged GAG chains, that have been shown to form anti-parallel interfibrillar co-aggregates primarily stabilized by electrostatic interactions, are believed to guarantee the mutual orientation and separation of collagen fibrils in the matrix (Scott, 1992). At a tissue level, PGs and GAGs are known to bind water and change the osmotic pressure within the tissue (Screen et al., 2011). Therefore, upon their removal, the strain recovery associated with the permeability-induced pressure against the indenter and potentially the backflow of fluid into the deformed volume under the area of indentation is greatly diminished (Bertassoni et al., 2009). It is also known that during loading, the tissue below the area of contact during deformation undergoes shearing (Gupta et al., 2007) of parallel fibrils interconnected with PGs, GAGs and noncollagenous proteins (Screen et al., 2011). Together, these structures may lead to (a) PG protein core elongation (a known mechanism of folded proteins like biglycan and decorin), (b) slippage between the interconnecting GAGs (Scott, 2003, 1992) and (c) disruption of sacrificial bonds within the PG protein core and the associated noncollagenous proteins (Fantner et al., 2007, 2005; Thompson et al., 2001). Therefore, our findings support the assertion that mechanical energy dissipation within the mineralized tissues may be largely regulated by structurally dependent electrostatic interactions, as suggested in previous reports (Fantner et al., 2007, 2005; Thompson et al., 2001). These latter events may potentially be influenced by GAGs, while recovery may be regulated by reconfiguration of these electrostatic and H-bonding interactions back to a pre-stressed arrangement.

It is noteworthy, however, that creep properties of biological materials are also dependent upon other factors besides the composition of the tissue in question, such as the indentation loading rates, temperature, and other testing factors (Stanton et al., 2005), which represents a limitation of our study. Further analyses to account for these specific effects form the basis for future work. Moreover, the mechanical behavior of dentin beneath a sharp indenter, where the

Table 1 – Summary of the creep properties of mineralized and demineralized dentin.

	Mineralized			Demineralized		
	Untreated	PG digested	GAG digested	Untreated	PG digested	GAG digested
$\epsilon'r$ (%)	73.6 ± 16	45.4 ± 17.7	25.9 ± 15.7	67.4 ± 16.8	18.7 ± 10.9	22.1 ± 12.9
h'_{max} (nm)	16.2 ± 1.4	20.9 ± 4.5	30.6 ± 4.7	26.1 ± 3.3	28.9 ± 2.7	45.3 ± 4.2
h'_{min} (nm)	11.9 ± 1.4	9.5 ± 2.1	8.0 ± 3.9	17.6 ± 2.8	5.4 ± 2.8	10.0 ± 5.1

strains are comparatively exaggerated in comparison to an *in-vivo* loading condition beneath the tooth cusp and protected by the surrounding enamel, limit the direct correlation of our results with a clinical setting. Despite these limitations, to the best of our knowledge, this work provides the first evidence for the participation of PGs and GAGs in the time dependent mechanics of dentin of other mineralized tissues of similar composition.

In summary, these results point to a novel interpretation of a nanostructural mechanism regulating the mechanical behavior of dentin and possibly other collagen-composed load bearing mineralized tissues. Interestingly, contrary to most load bearing muscoskeletal tissues in the body, such as cartilage and bone, mineralized tooth structures do not retain mechano-sensitive cell bodies that respond to successive loading by stimulating tissue remodeling. Therefore, the longevity of teeth depends almost exclusively on structural mechanisms preventing permanent and/or catastrophic deformation events. Consequently, the ability to recover from nanometer scale deformation in a time dependent manner, as regulated by PGs and GAGs, may be an important mechanism contributing to the improved longevity of dentin and teeth.

4.   Conclusion

Our results indicate that the digestion of PGs and GAGs has a significant effect in the time dependent behavior of dentin as determined by nanoindentation. Although PGs and GAGs represent only a small percentage (2–3%) of the entire tissue volume, these structures may be important contributors to the time dependent mechanics of dentin, and possibly other load bearing mineralized tissues of similar composition.

Acknowledgements

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