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Removal of Dentin Non-collagenous Structures Results in the Unraveling of Microfibril Bundles in Collagen Type I

Luiz E. Bertassoni^{1,2,3} and Michael V. Swain^{3,4}

¹Division of Biomaterials and Biomechanics, Department of Restorative Dentistry, School of Dentistry, Oregon Health and Science University, Portland OR, USA

²Center for Regenerative Medicine, Oregon Health and Science University, Portland OR, USA

³Bioengineering Laboratory, Faculty of Dentistry, University of Sydney, Sydney, NSW, Australia

⁴Bioclinical Sciences Department, Faculty of Dentistry, University of Kuwait, Kuwait

Abstract

Aims: The structural organization of collagen from mineralized tissues, such as dentin and bone, has been a topic of debate in the recent literature. Recent reports have presented novel interpretations of the complexity of collagen type I at different hierarchical levels and in different tissues. Here we investigate the nanostructural organization of demineralized dentin collagen following the digestion of non-collagenous components with a trypsin enzyme.

Materials and Methods: Dentin specimens were obtained from healthy third-molars, cut into small cubes and polished down to 1 μ m roughness. Samples were then demineralized with 10% citric acid for 2 min. Selected specimens were further treated with a solution containing 1 mg/ml trypsin for 48 hours at 37°C (pH 7.9 – 9.0). Both untreated and trypsin digested samples were analyzed using SDS-PAGE, Field Emission Scanning Electron Microscopy (FE-SEM), and nanoindentation, where surface hardness and creep properties were compared before and after treatments.

Results: FE-SEM images of demineralized dentin showed the banded morphology of D-periodical collagen type I, which upon enzymatic digestion with trypsin appeared to dissociate longitudinally, consistently unraveling ~20 nm structures (microfibril bundles). Such nanoscale structures, which we call microfibril bundles, to the best of our knowledge, have not been characterized in dentin previously. Mechanical characterization via nanoindentation showed that the unraveling of such microfibril bundles affected the creep displacement and creep rate of demineralized dentin.

Conclusion: In summary, our results provide novel evidence of the organization of collagen type I from dentin, which may have important implications for the interaction of dental materials with the organic dentin matrix and the mechanical properties of mineralized tissues.

Keywords

Dentin; Collagen; Microfibril; Proteoglycans; Trypsin

1. Introduction

Dentin is the largest component of the tooth. It offers structural support to the outer enamel and shares great compositional similarities to bone. Like most musculoskeletal connective human tissues, the dentin organic matrix is primarily constituted of type I collagen fibrils interconnected with noncollagenous macromolecules (30%) and water (20%), while the inorganic component consists of carbonated hydroxyapatite nanocrystals (50%) [1]. Although evolving models of the hierarchical organization of native collagen fibrils have emerged in the past decades [2–6], the structure and organization of collagen from mineralized tissues, such as dentin and bone, has been a topic of extensive debate in the literature [2, 7, 8]. It is generally accepted that collagen type I from mineralized tissues consists of two $\alpha 1$ and one $\alpha 2$ chains intertwined into a triple-helix. Every five adjacent triple-helical molecules, in turn, form a higher-order supramolecular structure that resembles a helix, called microfibrils, which in dentin, are believed to co-assemble into concentric bundles, better known simply as 'collagen fibrils', measuring about 100 nm in diameter [1].

Despite this well accepted organization, our understanding of the complexity of the collagen structure continues to evolve with the development of new characterization tools. For instance, recent reports have presented new evidence that interrogates the well-established D-spacing of 67 nm in collagen type I from different tissues [9–12]. Similarly, recent research has presented novel perspectives on the heterogeneous orientation of dissimilar collagen networks in human bone [13]. Specifically regarding the hierarchical organization of collagen type I, an increasing number of reports have shown the existence of substructural features that fall between the microfibrillar and fibrillar levels of collagen in bone [8], tendon [14–16], sclera [17], cornea [17] and *in vitro*-assembled fibrils [18]. Generally these structures have appeared as dissociated constituents of larger D-periodical fibrils. However, their dimensions (10–25 nm) have been too large to be characterized as microfibrils, defined as 5-mer repeats of laterally assembled triple-helical molecules measuring about 4–5 nm in diameter [3], and too small to be simply characterized as collagen fibrils, which in dentin have a typical diameter of 80–100 nm [19].

Early examples of these structures, which we will refer to simply as *microfibril bundles* from here on (considering that they are invariably constituted of multiple individual microfibrils), have been reported earlier by Scott [14], which has shown that collagen from rat tail tendon disintegrated longitudinally to reveal structures measuring 12–25 nm in diameter upon immersion in a sodium acetate-buffer. Images from collagen derived from cornea and sclera treated with 0.1 M acetic acid has also shown similar structures [17] with comparable dimensions to the ones described by Scott [14]. More recently, SEM and TEM studies of collagen type I from native bone and reconstituted bovine collagen have shown nanoscale structures in the range of 10–15 nm in diameter [8], which supported previous results from Raspanti et al. [18] showing the lateral aggregation of 12–15 nm structures during

fibrillogenesis of acid-soluble collagen *in vitro* to form larger collagen fibrils. These observations have increasingly suggested that type I collagen fibrils may have thinner building blocks constituted of multiple individual microfibrils that self-organize and – stabilize, thus potentially representing a level of structural hierarchy between the fibrillar (100 nm) and microfibrillar levels (5–6 nm) that has been poorly explored so far.

Here, we report new evidence of such microfibril bundles in collagen type I from dentin which were visible upon digestion of non-collagenous proteins and proteoglycans from demineralized dentin using a trypsin enzyme [20]. These structures are consistent with the partial longitudinal disaggregation of collagen similar to those previously described from arthritic cartilage [21] tendon [7, 17], sclera and cornea [17], but that have never been reported in dentin before. We discuss these observations in light of the potential organization of the observed nanostructures within the assembled type I collagen fibrils. We also address their putative correlation with the recently characterized molecular interaction of collagen with proteoglycans, a key molecule associated with collagen structural stability. We believe these findings may provide an improved understanding of the structural organization of collagen from dentin and provide new insights into the interaction of dental restorative materials with the dentin matrix.

2. Materials and Methods

2.1 Specimen preparation

Permanent human third molars (N=12) were obtained according to protocols approved by the Sydney Southwest Area Health Service and the Royal Prince Alfred Hospital's Bioethics Committee on human research. Roots and the occlusal portion of the tooth crowns were removed using a low speed saw (Isomet; Buehler, Lake Bluff, IL, USA) under constant water irrigation. One dentin block was cut from the mid-coronal regions of each tooth while preserving the exposed surfaces perpendicular to the tubule direction. The specimens were hand ground and polished on silicon carbide papers (600 and 1200 grits), followed by 9 µm and 1 µm polishing diamond pastes on a rotary polishing machine. Specimens were then exposed to a demineralizing solution of 10% (v/v) citric acid for 2 min, following previously established protocols [22, 23].

2.2 Trypsin digestion

After demineralization, specimens were rinsed thoroughly with de-ionized water. A trypsin solution previously reported to digest noncollagenous proteins and proteoglycans from human dentin [20] was used to treat the specimens. In brief, a solution containing 1 mg/ml TPCK-treated trypsin (Sigma–Aldrich, St. Louis, MO) and 0.2 M ammonium bicarbonate at pH 7.9 – 9.0 was prepared. Specimens were stored in solution for 48 hours at 37 °C under constant stirring and solutions were exchanged after 24 hours.

2.3 SDS-PAGE

To inspect the digestion of dentin non-collagenous molecules, samples (N=6) were pulverized and subjected to the enzymatic protocols described above. Next, samples were extracted using 4M Guanidine HCl in 50 mM Tris with 0.5 M EDTA 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 molecular weight cut out membrane at 4°C for 24 h, and lyophilized to remove the Guanidine HCl. Dentin extracts (3 µg) were dissolved and run in a 12.5% 0.75 mM non-reducing SDS-PAGE gel.

2.4 Microstructural analyses - FE-SEM

After demineralization and the trypsin treatment, samples (N=3) were thoroughly washed with deionized water and specimens were gradually dehydrated by immersing in acetone solutions of 25, 50, 70% (v/v) for 5 min each, and then in 80, 90, 95 and 100% (v/v) for 15 min each, followed by 2 consecutive immersions in 100% (v/v) acetone for 30 min each. Next, samples were immersed in hexamethyldisilazane (HMDS) overnight and permitted to evaporate under a protected hood. HMDS is a volatile organosilicon compound that further dehydrates the matrix and cross-links proteins acting as mild fixative solution. Samples were then coated with platinum (Pt) and imaged using an FE-SEM (Ultra Zeiss Oberkochen, Germany) at 1–5 kV using an *in lens* detector. Images were taken from a longitudinal perspective of the cross-sectioned samples (parallel to the tubular orientation), where the collagen fibrils had their near-radial arrangement preserved.

2.4 Mechanical characterization - nanoindentation

In order to gain insights into the effects of the collagen structural changes on the mechanical behavior of dentin, specimens (N=3) were nanoindented using an Ultra Micro-Indentation System (UMIS-2000; Fisher-Cripps Laboratories Pty. Limited, Sydney, Australia) with a calibrated Berkovich indenter having a nominal radius of approximately 170 nm, as described previously [24]. Nine indentations were performed per sample. Tests were first performed on demineralized specimens, and then after the enzymatic treatments. We then evaluated the nanoindentation creep behavior of the samples before and after trypsin treatments. Nanoindentation creep tests used a one-step loading to a maximum load of 5 mN followed by a hold period of 30 s to measure the creep deformation. All indentations were performed while the specimens were fully immersed in water. The normalized creep deformation, h'_{max} , was calculated by subtracting the initial depth of penetration at the onset of a holding period at a maximum load, h_{i-max} , against the total depth of penetration at the maximum load, h_f . The creep rate was then obtained by dividing the indenter displacement velocity, $\dot{h}$, by the plastic depth of penetration h , where $\dot{h} = dh/dt$. Data was analyzed using a Student t-test with a confidence level of 95%.

3. Results

SDS-PAGE of extracts from undigested and trypsin treated dentin showed a visible difference between groups. Although the coomassie blue staining used in the SDS-PAGE method does not reveal the entire composition of the dentin non-collagenous proteins, and no additional protein-specific binding assay was performed, the lack of bands in the trypsin treated lane in Figure 1 indicates absence of any detectable molecules, which is consistent with effective removal of most non-collagenous proteins and proteoglycans from the dentin matrix.

A longitudinal perspective of the dentin tubules and the intertubular region before the trypsin treatment revealed a more compact matrix (Figure 2A, 2C and Supplementary Figure S2A), where the tubules appeared to be covered with a membrane-like material, called *lamina limitans* [23]. These membrane-like structure has been proposed to surround the entire length of the tubules and to form a boundary between the intertubular and peritubular dentin [23]. Following the trypsin treatment (Figure 2B, 2D and Supplementary Figure S2B) a much more porous matrix was visible, which was more easily visualized within the tubules, where the near-radial organization of the collagenous network was preserved. It is important to highlight that in other surface areas where the collagen had been sectioned by the low-speed saw during sample preparation, it was very difficult to observe the longitudinal orientation of the collagen, either preserved or disrupted.

Images of the trypsin digested dentin showed striking topographical changes on the collagen structure (Figure 2D, 3A and 3B). Figure 3A shows several collagen fibrils where some of the repeating D-banding pattern of gap and overlap zones is partially visible. Other regions, however, showed narrower structures measuring approximately 20 nm in diameter (Figure 2D and 3B, arrows), as though the larger fibrils “untwisted” to unravel thinner nanoscale structures. Additional representative image of the trypsin treated dentin shows what appears as the longitudinal ‘splitting’ of a single collagen fibril (Figure 4A and 4B), also unraveling approximately 20 nm structures both superficially and within the core of the larger fibril (Figure 4B, arrows).

We then hypothesized that the increased porosity and abnormal collagen morphology could be associated with changes in the mechanical properties of demineralized dentin. The surface hardness of the demineralized tissue was greatly diminished in comparison to healthy dentin, but no significant changes were associated with the enzymatic treatment (Supplementary Figure S1). The nanoindentation creep behavior of the treated dentin, on the other hand, was significantly altered. Figure 5 shows a comparison of the normalized creep displacement of the demineralized dentin versus the demineralized dentin after trypsin digestion. Demineralized dentin had an average creep displacement of 26.5 ± 6.3 nm and the trypsin treatment yielded a significant increase to 53.5 ± 12.7 nm ($p < 0.0001$). A double logarithmic plot of creep rate versus time (Figure 6), suggests that there appears to be two components for the creep deformation of demineralized dentin. This response is illustrated by fitting a power-law curve to the initial third of the creep response and then the remainder of the creep rate versus time curve. Although the creep rate of the demineralized tissue drops rapidly during the second and third components, the estimation of the trypsin treated group (dotted line) suggests that, after collagen disruption, the tissue continues to creep at a steadier rate for longer periods of time.

4. Discussion

In this study, we report the structural and mechanical characterization of demineralized dentin following removal of non-collagenous proteins and proteoglycans with a trypsin enzyme. Our FE-SEM images revealed nanoscale structures that are consistent with the partial longitudinal disaggregation of collagen type I, as previously reported for cartilage [21], tendon [14, 15], cornea and sclera [17].

An important aspect that requires clarification prior to discussing the unraveling of these structures, which we refer to as microfibril bundles, is the confusion in the characterization and nomenclature of collagen ‘microfibrils’ in the current literature. For clarification, ‘microfibrils’, which have also been extensively referred to as subfibrils until the late 90s, are the 4–5 nm diameter lateral aggregates of 5 triple-helical molecules [3]. We argue that the structures we report here dissociate from larger (~100 nm) collagen fibrils [1] and appear to be formed by bundles of 3–5 microfibrils. Of note, these structures are too small to represent the known 80–100 nm D-periodical collagen fibrils found in dentin, and too large represent the 4–5 nm microfibrils, which fall far beyond the resolution limit of the FE-SEM instrument used in this study. Hence their definition as microfibril bundles.

3.1 Microscale morphological changes following the trypsin digestion

The trypsin digestion protocol we utilized has been previously demonstrated to remove proteoglycans and noncollagenous proteins from dentin and other collagenous tissues [15, 20, 25, 26]. Our SDS-PAGE results indicate that a broad range of dentin non-collagenous molecules, including its most abundant proteoglycans (decorin and biglycan) was extracted after trypsin digestion (Figure 1). Although most dentin non-collagenous proteins have been primarily linked to mineralization events or extracellular molecular regulation and signaling, the glycosaminoglycan component of proteoglycans has been shown to form interfibrillar aggregates that are responsible for the interconnection between fibrils in the extracellular matrix [1, 27, 28]. Therefore, we attribute the increase in the microporosity of the dentin matrix following the trypsin treatment (Figure 2B and 2D) to the removal of these interfibrillar structures, which is in agreement with recent reports using trypsin treatment to digest dentin non-collagenous proteins and proteoglycans [20, 23, 25, 29].

3.2 Nanoscale morphological changes – unraveling of collagen microfibril bundles

Trypsin is known to hydrolyze the peptide bonds at the carboxyl side of lysine and arginine residues in a collagen molecule; hence the C-telopeptide ends of molecules exposed to the enzyme may play an important role in the events we found [30–32]. The C-terminal telopeptide contains intermolecular cross-link to the adjacent molecule’s helical region [33]. Therefore, if the C-telopeptide ends of individual molecules are lost due to trypsin digestion, then the cross-linking points holding adjacent molecules together may also vanish. In which case, collagen fibrils would tend to unravel into much smaller filaments. Individual molecules have a diameter of approximately 1.5 nm. Collagen microfibrils, which consist of 5 five adjacent triple-helical molecules twisted into a supramolecular structure, have a diameter of approximately 4–5 nm. As stated above, both of these structures fall far beyond the resolution limit of the FE-SEM technique used in this study. Therefore, we propose that the structures we found fall between the microfibrillar (2nd) and fibrillar (3rd) hierarchical levels, and represent bundles of multiple laterally packed microfibrils. An extrapolative explanation for the phenomena we saw is that there may exist crosslinks within the microfibril bundles that are more resistant to the trypsin degradation than the crosslinks between them, which is a question that remains to be experimentally addressed in future work.

It is also relevant to note, however, that trypsin cleavage of the telopeptide ends of molecules in collagen fibrils will, at least in part, be a structure-limited mechanism. This is because the enzyme must first access the telopeptide end of each collagen molecule for cleavage to occur. According to the most recently accepted model [3], in the assembled D-periodical fibril, only one of two (N- or C-terminus) telopeptide ends of the molecule may have direct access to the enzyme. This is especially true for the outermost molecules in an assembled fibril. According to this organization, one telopeptide end of the molecule will be positioned at the gap zones, while the other will be partially covered by the helical region of the adjacent molecule - a consequence of the natural staggered pattern of molecules in a fibril. Furthermore, in the preserved D-periodical collagen fibril, the gap region is partially covered by the proteoglycan protein core [34], which further reduces accessibility for molecular cleavage of the C-telopeptides. Therefore, actual digestion of the assembled type I collagen fibril by trypsin - similar to other MMPs [35] - appears to be a much more complex and structurally regulated event, which remains to be better understood.

3.3 Correlation of microfibril bundles in collagen from dentin and other connective tissues

An increasing number of reports have highlighted the existence of collagen nanostructures between the microfibrillar and fibrillar levels in different connective tissues. The morphological similarities between the 20 nm microfibril bundles we found with the structures observed in collagen from cornea and sclera treated with 0.1 M acetic acid [17] are striking. Earlier studies on the morphogenesis of tendon also provided evidence that self-assembled collagen treated with trypsin unraveled collagen nanostructures beyond the fibrillar level that remained laterally aggregated in solution where individual microfibril bundles could still be easily distinguished [15]. Moreover, recent work by Antipova and Orgel using polyclonal antibodies against the proteoglycan biglycan showed extensive tissue destruction that may be analogous to that of rheumatoid arthritis where the collagen shows a remarkable similarity pattern of disruption to the ones we report here [21]. Additionally, a noteworthy study by Raspanti et al [18] also revealed individual 12–15 nm structures that aggregated to form larger D-periodical fibrils during fibrillogenesis *in vitro*. These observations support a fibrillogenesis model whereby collagen is formed following a sequence of coalescence and growth of subunits, as described previously [11]. Accordingly, fibrils nucleate near the cell-surface, grow in the axial direction followed by side-to-side and tip-to-tip fusion assisted by proteoglycans. Therefore, a plausible hypothesis is that the 20 nm structures we found may represent the more stable collagen precursors that are secreted by odontoblasts during dentinogenesis, which at a later stage, coalesce to form thicker fibrils aided by proteoglycans. The trypsin digestion, therefore, could have favored the disruption of the weaker bonds between the microfibril bundles, while the more stable precursors remained preserved.

Earlier electron microscopy works by Weinstock and Leblond [36, 37], however, have elucidated the process of intra- and extra-cellular transport of procollagen, and their transition into polymerized tropocollagen molecules. Accordingly, procollagen molecules assemble into parallel aggregates in the Golgi apparatus in odontoblasts, are transported within secretory granules to the odontoblast process and released by exocytosis in the

predentin region, where they give rise to tropocollagen molecules, which polymerize into collagen fibrils. This process would occur across the length of the dentin matrix, from the dentin-enamel junction trough to the pulp chamber. Likely, during such stage of tropocollagen polymerization, the intrafibrillar crosslinks and collagen-proteoglycan interactions believed to assist in the permanent fibril stability (that prevent the 20 nm microfibril bundles from separating) start to take place. This is consistent with the notion proteoglycan and glycosaminoglycan secretion and subsequent degradation take place at the transition of the pre-dentin zone and mineralization front. We contend, however, that such mechanics analyses relative to tissue development fall beyond the scope of our experimental design and objectives.

Of note, the solubility of collagen in mineralized tissues, such as dentin, is likely to differ significantly from collagen in other tissues, especially in non-mineralized ones. Therefore the mechanisms involved in the dissolution or disaggregation of thicker fibrils and consequent unraveling of thinner microfibril bundles in tissues other than dentin may be a consequence of different events. Moreover, it is unclear how much of the structural changes observed in our SEM images actually occur in dental disease processes, such as tooth decay and hybrid layer degradation. We highlight, however, that the observation of a new level of complexity in the intrafibrillar structural organization of collagen type I from dentin is likely to influence our understanding of the interaction of dental materials with the dentin matrix. The interface of dental materials with the tooth is formed primarily via their close interaction with the dentin collagen, and thus better predictability of an effective treatment can only be achieved once a thorough understanding of the tissue itself is achieved.

Considering that the effective interaction of dental polymers with the dentin matrix, particularly of current dental adhesives, relies on the putative complete impregnation and enveloping of the dentin collagen fibrils with monomers of high viscosity, a concept which we have challenged recently [1], it is natural to hypothesize that the increased complexity of the internal organization of collagen fibrils, as we report here, represents another level of difficulty for effective bonding to dentin. The majority of current dental adhesive procedures, especially the so-called etch-and-rinse and earlier versions self-etch systems lacking functional monomers, rely on simple diffusion of the monomer molecule into the collagen internal structure. The driving mechanisms transporting these molecules into the collagen structure, therefore, are very limited. Hence, the conjecture that dentin collagen fibrils consist of tightly packed microfibril bundles, in addition to the known twisted-packing and staggered arrangement of collagen molecules, represents another limitation to the concept of adhesive dentistry.

3.4 The potential involvement of proteoglycans in microfibrillar bundle stability

After collagen, proteoglycans and their glycosaminoglycan side chains represent the organic components with greater structural relevance [38] in the dentin matrix [39]. Decorin and biglycan are the most prominent proteoglycans in mineralized tissues, and their relevance in collagen fibrillogenesis has been extensively described in the literature. Interestingly, it has been shown that decorin embraces multiple collagen molecules of several microfibrils on the surface of collagen type I [21, 34], thus potentially contributing to the securing of microfibril

bundles as assembled fibrils. This conjecture is supported by the perspective that, *in vitro*, purified collagen remains as assembled fibrils in the presence of decorin, while the digestion of these assembled fibrils with trypsin, causes disaggregation and unraveling of thinner fibrils similar to ones we found [15]. Similarly, knockout mice with targeted disruption of decorin leads to abnormal collagen morphology [40], where the fibril outline closely resembles the disaggregated collagen we found here. Therefore, it is possible that the unraveling of microfibril bundles could be associated with the loss of surface proteoglycans due to the trypsin treatment [20].

3.5 Mechanical implications of trypsin digestion and unraveling of microfibril bundles

We have previously supported the assertion that during indentation the material beneath the contact area of the indenter may be regarded as an expanding cavity with a hydrostatic core, where the elastic/plastic boundary continues to expand as a function of the fluid flow within a porous matrix [24, 41]. Our interpretation is two-fold, firstly the increase in porosity of the trypsin treated dentin may have accounted for more significant displacement of the fluid within the matrix. Secondly, after the disruption of the collagen, the twisted arrangement of microfibril bundles that might offer resistance against intra-fibrillar shearing is diminished, and thus creep displacement is increased. This could also provide further insights into the fragility of diseased tissues where collagen and proteoglycans are affected [42]. These observations are consistent with reports that the targeted disruption of biglycan leads to an osteoporosis-like phenotype in genetically modified mice, which had bones with significantly lowered yield energy and failure loads compared to the wild-type counterparts [43]. Interestingly, targeted disruption of a comparable proteoglycan, decorin, also leads to tissue fragility. These comparisons are highly extrapolative at this stage, but it is reasonable to suggest that the preserved packing arrangement of collagen type I is fundamental to the ability of collagenous tissues to dissipate mechanical energy and resist against mechanical loading.

Moreover, proteoglycans are known to regulate the hydrostatic and osmotic pressure in hydrated tissues via the binding of their glycosaminoglycan side chains to water molecules. Loss of these interfibrillar and associated mechanisms, therefore, is likely to have contributed to a decrease in hydrostatic and osmotic pressure, which results in an increase in the creep deformation of the treated dentin, as we showed recently [29]. We have also recently described a set of nanoscale mechanisms where proteoglycans are proposed to undergo molecular folding and unfolding at the protein core level, whereas glycosaminoglycans undergo intermolecular sliding and intermolecular stretching to regulate the mechanical deformation of several mineralized tissues, such as dentin, cementum and bone [44]. Lack of these micro and nanoscale energy dissipation mechanisms support the mechanical changes that we found.

4. Conclusion

In summary, here we report novel evidence of collagen microfibril bundles in demineralized dentin as a result from the enzymatic digestion of dentin with trypsin. Our images showed approximately 20 nm structures that appeared to consistently unravel from thicker D-

periodical collagen type I. These results support previous reports showing organized nanoscale structures of similar dimensions that appear to self-organize hierarchically to form larger collagen fibrils in various tissues and may further elucidate the complexity of collagen type I in dentin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

5. Acknowledgements

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6. References

- [1]. Bertassoni LE, Orgel JP, Antipova O, Swain MV. The dentin organic matrix - limitations of restorative dentistry hidden on the nanometer scale. *Acta biomaterialia*. 2012;8:2419–33. [PubMed: 22414619]
- [2]. Fratzl P Collagen: structure and mechanics. Boston, MA: Springer; 2008.
- [3]. Orgel JP, Irving TC, Miller A, Wess TJ. Microfibrillar structure of type I collagen in situ. *Proceedings of the National Academy of Sciences of the United States of America*. 2006;103:9001–5. [PubMed: 16751282]
- [4]. Orgel JP, Miller A, Irving TC, Fischetti RF, Hammersley AP, Wess TJ. The in situ supermolecular structure of type I collagen. *Structure*. 2001;9:1061–9. [PubMed: 11709170]
- [5]. Petruska JA, Hodge AJ. A Subunit Model for the Tropocollagen Macromolecule. *Proceedings of the National Academy of Sciences of the United States of America*. 1964;51:871–6. [PubMed: 14173005]
- [6]. Smith JW. Molecular pattern in native collagen. *Nature*. 1968;219:157–8. [PubMed: 4173353]
- [7]. Katz EP, Wachtel E, Yamauchi M, Mechanic GL. The structure of mineralized collagen fibrils. *Connective tissue research*. 1989;21:149–54; discussion 55–8. [PubMed: 2605938]
- [8]. Li Y, Aparicio C. Discerning the subfibrillar structure of mineralized collagen fibrils: a model for the ultrastructure of bone. *PloS one*. 2013;8:e76782. [PubMed: 24086763]
- [9]. Erickson B, Fang M, Wallace JM, Orr BG, Les CM, Banaszak Holl MM. Nanoscale structure of type I collagen fibrils: quantitative measurement of D-spacing. *Biotechnology journal*. 2013;8:117–26. [PubMed: 23027700]
- [10]. Fang M, Goldstein EL, Matich EK, Orr BG, Holl MM. Type I collagen self-assembly: the roles of substrate and concentration. *Langmuir : the ACS journal of surfaces and colloids*. 2013;29:2330–8. [PubMed: 23339654]
- [11]. Fang M, Goldstein EL, Turner AS, Les CM, Orr BG, Fisher GJ, et al. Type I collagen D-spacing in fibril bundles of dermis, tendon, and bone: bridging between nano- and micro-level tissue hierarchy. *ACS nano*. 2012;6:9503–14. [PubMed: 23083115]
- [12]. Beniash E, Traub W, Veis A, Weiner S. A transmission electron microscope study using vitrified ice sections of predentin: structural changes in the dentin collagenous matrix prior to mineralization. *Journal of structural biology*. 2000;132:212–25. [PubMed: 11243890]
- [13]. Reznikov N, Almany-Magal R, Shahar R, Weiner S. Three-dimensional imaging of collagen fibril organization in rat circumferential lamellar bone using a dual beam electron microscope reveals ordered and disordered sub-lamellar structures. *Bone*. 2013;52:676–83. [PubMed: 23153959]

- [14]. Scott JE. Proteoglycan:collagen interactions and subfibrillar structure in collagen fibrils. Implications in the development and ageing of connective tissues. *Journal of anatomy*. 1990;169:23–35. [PubMed: 2384335]
- [15]. Graham HK, Holmes DF, Watson RB, Kadler KE. Identification of collagen fibril fusion during vertebrate tendon morphogenesis. The process relies on unipolar fibrils and is regulated by collagen-proteoglycan interaction. *Journal of molecular biology*. 2000;295:891–902. [PubMed: 10656798]
- [16]. Holmes DF, Graham HK, Trotter JA, Kadler KE. STEM/TEM studies of collagen fibril assembly. *Micron*. 2001;32:273–85. [PubMed: 11006507]
- [17]. Yamamoto S, Hashizume H, Hitomi J, Shigeno M, Sawaguchi S, Abe H, et al. The subfibrillar arrangement of corneal and scleral collagen fibrils as revealed by scanning electron and atomic force microscopy. *Archives of histology and cytology*. 2000;63:127–35. [PubMed: 10885449]
- [18]. Raspanti M, Viola M, Sonaggere M, Tira ME, Tenni R. Collagen fibril structure is affected by collagen concentration and decorin. *Biomacromolecules*. 2007;8:2087–91. [PubMed: 17530890]
- [19]. Habelitz S, Balooch M, Marshall SJ, Balooch G, Marshall GW, Jr. In situ atomic force microscopy of partially demineralized human dentin collagen fibrils. *Journal of structural biology*. 2002;138:227–36. [PubMed: 12217661]
- [20]. Bedran-Russo AK, Pereira PN, Duarte WR, Okuyama K, Yamauchi M. Removal of dentin matrix proteoglycans by trypsin digestion and its effect on dentin bonding. *Journal of biomedical materials research Part B, Applied biomaterials*. 2008;85:261–6. [PubMed: 17910048]
- [21]. Antipova O, Orgel JP. Non-enzymatic decomposition of collagen fibers by a biglycan antibody and a plausible mechanism for rheumatoid arthritis. *PloS one*. 2012;7:e32241. [PubMed: 22427827]
- [22]. Balooch M, Habelitz S, Kinney JH, Marshall SJ, Marshall GW. Mechanical properties of mineralized collagen fibrils as influenced by demineralization. *Journal of structural biology*. 2008;162:404–10. [PubMed: 18467127]
- [23]. Bertassoni LE, Stankoska K, Swain MV. Insights into the structure and composition of the peritubular dentin organic matrix and the lamina limitans. *Micron*. 2012;43:229–36. [PubMed: 21890367]
- [24]. Bertassoni LE, Swain MV. Influence of hydration on nanoindentation induced energy expenditure of dentin. *Journal of biomechanics*. 2012;45:1679–83. [PubMed: 22537569]
- [25]. Pereira PN, Bedran-de-Castro AK, Duarte WR, Yamauchi M. Removal of noncollagenous components affects dentin bonding. *Journal of biomedical materials research Part B, Applied biomaterials*. 2007;80:86–91.
- [26]. Berg A, Singer T, Moser E. High-resolution diffusivity imaging at 3.0 T for the detection of degenerative changes: a trypsin-based arthritis model. *Invest Radiol*. 2003;38:460–6. [PubMed: 12821861]
- [27]. Scott JE. Supramolecular organization of extracellular matrix glycosaminoglycans, in vitro and in the tissues. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 1992;6:2639–45. [PubMed: 1612287]
- [28]. Scott JE. Elasticity in extracellular matrix ‘shape modules’ of tendon, cartilage, etc. A sliding proteoglycan-filament model. *The Journal of physiology*. 2003;553:335–43. [PubMed: 12923209]
- [29]. Bertassoni LE, Kury M, Rathsam C, Little CB, Swain MV. The role of proteoglycans in the nanoindentation creep behavior of human dentin. *J Mech Behav Biomed Mater*. 2015;55:264–70. [PubMed: 26600409]
- [30]. Risteli L, Risteli J, Moniz C. Measuring collagen degradation. *Eur J Clin Invest*. 1993;23:339–40. [PubMed: 8344333]
- [31]. Sassi ML, Eriksen H, Risteli L, Niemi S, Mansell J, Gowen M, et al. Immunochemical characterization of assay for carboxyterminal telopeptide of human type I collagen: loss of antigenicity by treatment with cathepsin K. *Bone*. 2000;26:367–73. [PubMed: 10719280]
- [32]. Sassi M, Jukkola A, Riekkki R, Hoyhtya M, Risteli L, Oikarinen A, et al. Type I collagen turnover and cross-linking are increased in irradiated skin of breast cancer patients. *Radiother Oncol*. 2001;58:317–23. [PubMed: 11230894]

- [33]. Tjaderhane L, Buzalaf MA, Carrilho M, Chaussain C. Matrix metalloproteinases and other matrix proteinases in relation to cariology: the era of 'dentin degradomics'. *Caries Res.* 2015;49:193–208. [PubMed: 25661522]
- [34]. Orgel JP, Eid A, Antipova O, Bella J, Scott JE. Decorin core protein (decoron) shape complements collagen fibril surface structure and mediates its binding. *PloS one.* 2009;4:e7028. [PubMed: 19753304]
- [35]. Perumal S, Antipova O, Orgel JP. Collagen fibril architecture, domain organization, and triple-helical conformation govern its proteolysis. *Proceedings of the National Academy of Sciences of the United States of America.* 2008;105:2824–9. [PubMed: 18287018]
- [36]. Weinstock M, Leblond CP. Formation of collagen. *Federation proceedings.* 1974;33:1205–18. [PubMed: 4134092]
- [37]. Weinstock M, Leblond CP. Synthesis, migration, and release of precursor collagen by odontoblasts as visualized by radioautography after (3H)proline administration. *The Journal of cell biology.* 1974;60:92–127. [PubMed: 4129079]
- [38]. Iozzo RV. Matrix proteoglycans: from molecular design to cellular function. *Annual review of biochemistry.* 1998;67:609–52.
- [39]. Goldberg M, Takagi M. Dentine proteoglycans: composition, ultrastructure and functions. *The Histochemical journal.* 1993;25:781–806. [PubMed: 7507908]
- [40]. Danielson KG, Baribault H, Holmes DF, Graham H, Kadler KE, Iozzo RV. Targeted disruption of decorin leads to abnormal collagen fibril morphology and skin fragility. *The Journal of cell biology.* 1997;136:729–43. [PubMed: 9024701]
- [41]. Angker L, Nijhof N, Swain MV, Kilpatrick NM. Influence of hydration and mechanical characterization of carious primary dentine using an ultra-micro indentation system (UMIS). *European journal of oral sciences.* 2004;112:231–6. [PubMed: 15154920]
- [42]. Ameye L, Young MF. Mice deficient in small leucine-rich proteoglycans: novel in vivo models for osteoporosis, osteoarthritis, Ehlers-Danlos syndrome, muscular dystrophy, and corneal diseases. *Glycobiology.* 2002;12:107R–16R.
- [43]. Xu T, Bianco P, Fisher LW, Longenecker G, Smith E, Goldstein S, et al. Targeted disruption of the biglycan gene leads to an osteoporosis-like phenotype in mice. *Nat Genet.* 1998;20:78–82. [PubMed: 9731537]
- [44]. Bertassoni LE, Swain MV. The contribution of proteoglycans to the mechanical behavior of mineralized tissues. *J Mech Behav Biomed Mater.* 2014;38:91–104. [PubMed: 25043659]

Figure 1. SDS-PAGE of extracts from undigested 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; that is dentin not exposed to any incubation as a control. Trypsin buffers (3rd lane) represents samples that were exposed to the trypsin digest buffer solutions under the same conditions, but without enzymes. Trypsin buffers (4th lane) represents dentin treated with the active trypsin enzyme solution. The lack of bands in lane 4 indicates

absence of detectable molecules, which is consistent with effective removal of non-collagenous proteins and proteoglycans by trypsin.

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Figure 2.

FE-SEM image of demineralized dentin from a cross-section of a longitudinal tubule. A) Tubule wall of demineralized dentin covered with the lamina limitans (500 nm scale bar). B) Exposed fibrillar network of the demineralized dentin after trypsin treatment (400 nm scale bar). C) The D-banding pattern of collagen fibrils in demineralized dentin is preserved. Collagen network is covered by lamina limitans. (500 nm scale bar) D) Trypsin treated collagen fibrils showing the unraveling of smaller filaments, which we name microfibril

bundles, along the axis of the fibrils. Cracks in (A) and (C) are artifacts due to dehydration during sample preparation.

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Figure 3.

Higher magnification FE-SEM images of demineralized dentin after treatment with trypsin.

A) The D-periodic pattern of the fibrils is visible in several fibrils. An overlap-zone is shown with a white arrowhead, while the adjacent gap zone is pointed-out with an unfilled arrowhead. At this magnification the untwisting phenomenon is clearly seen (white arrow) and the resulting thinner (~20 nm) microfibril bundles are also visible with a greater degree of detail. Although the larger collagen fibrils appeared to untwist, the gap-overlap zones are still relatively preserved. B) A similar pattern of fibril untwisting unraveling thinner (~20 nm) microfibril bundles (white arrow) is seen. (200 nm scale bars)

Figure 4.

(A) A representative image of an area exhibiting a single fibril that appeared to split longitudinally unraveling thinner microfibril bundles internally after the treatment with trypsin. The white arrowhead shows a single microfibril bundle resulting from the untwisting of a larger collagen fibril, while the unfilled arrowhead indicates a region where the collagen appeared to separate longitudinally. The white pointed arrows indicate the inner edges of the 'splitting-fibril'. (B) A contrast enhanced representation of the region marked by the yellow dotted corners showing the 'splitting' phenomenon with greater detail. The pointed white arrows show individual microfibril bundles, both at the surface and within the innermost regions of the fibril. The asterisks represents structures that run perpendicular to the fibril direction, which could possibly be artifacts or disrupted and non fully-degraded PGs and noncollagenous proteins that previously interconnected adjacent fibrils. (200 nm scale bars)

Figure 5. Normalized creep displacement, h'_{max} , of demineralized dentine comparing the effect of trypsin treatment. The difference was statically significant ($p < 0.0001$).

Figure 6. Log of creep rate versus log of time for demineralized dentine comparing the effect of trypsin treatment.