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**REVIEW**

# Variation in type I collagen fibril nanomorphology: the significance and origin

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**Although the axial D-periodic spacing is a well-recognized nanomorphological feature of type I collagen fibrils, the existence of a distribution of values has been largely overlooked since its discovery seven decades ago. Studies based on single fibril measurements occasionally noted variation in D-spacing values, but accredited it with no biological significance. Recent quantitative characterizations supported that a 10-nm collagen D-spacing distribution is intrinsic to collagen fibrils in various tissues as well as *in vitro* self-assembly of reconstituted collagen. In addition, the distribution is altered in *Osteogenesis Imperfecta* and long-term estrogen deprivation. Bone collagen is organized into lamellar sheets of bundles at the micro-scale, and D-spacings within a bundle of a lamella are mostly identical, whereas variations among different bundles contribute to the full-scale distribution. This seems to be a very general phenomenon for the protein as the same type of D-spacing/bundle organization is observed for dermal and tendon collagen. More research investigation of collagen nanomorphology in connection to bone biology is required to fully understand these new observations. Here we review the data demonstrating the existence of a D-spacing distribution, the impact of disease on the distribution and possible explanations for the origin of D-spacing variations based on various collagen fibrillogenesis models.**

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## Introduction

Type I collagen, composing 90% of the organic matrix of bone, has a crucial role in maintaining the structural integrity and functional properties of the bone. It modulates signal transduction of bone cells,[1] provides the framework for mineral nucleation and growth,[2] and contributes to the toughness and resilience of the bone.[3] Compositional and conformational changes of bone collagen have profound influence on the bone properties, particularly the mechanical performance. Some of the well-known examples are genetic mutations on type I collagen sequence resulting in *Osteogenesis Imperfecta* (OI) phenotypes with brittle bones;[4] nonenzymatic crosslinking of collagen resulting in accumulation of advanced glycation products and compromised bone strength.[5] Our understanding of bone collagen structure–property relationships at the micron to submicron scale is still sparse. Many questions remain to be answered regarding how microstructural organization, fibril orientation and fine details of collagen fibril nanomorphology influence bone properties.

Important aspects of collagen nanomorphological features have been extensively studied in tissues other than the bone. For example, tendon collagen fibrils exhibit a distribution of diameters, and fibril diameter was shown to influence the mechanical properties.[6,7] Fibril length in general can reach millimeters and tip-to-tip fusion further extends the length.[8] In this review we will focus on one of the most recognized and functionally important aspects of collagen nanomorphology, the axial gap/overlap D-periodic spacing. Bone collagen D-spacing provides open sites for mineral nucleation, proteoglycan binding and crosslinks to occur.[3] It also is an effective indicator of fibril strain during bone deformation.[9] Evidence from recent research has demonstrated the close relationship of collagen fibril D-spacing with bone micro-organization,[10] and significant nanomorphological changes in D-spacing related to bone diseases.[11,12] These findings highlight the significance of bone collagen D-spacing variations, and provide insight into the potential mechanisms for the variations in type I collagen fibril D-spacing. Some common features in collagen nano- and microstructures are shared among bone and other biological tissues and connections amongst these tissues will be elucidated.

## The Significance of Bone Collagen Nanomorphology

As a characteristic feature of collagen nanomorphology, the D-periodic spacing of collagen fibrils extracted from nerve, skin

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and cornea was shown in early electron micrographs,[13] and an estimated 64.6 ± 5.3 nm distribution of D-spacing was reported for human skin (**Figure 1b**).[14] The use of X-ray diffraction (XRD) in studying subfibrillar packing of collagen fibrils soon gained popularity and D-spacing values between 64 and 67 nm were reported.[15] In order to explain the periodic D-spacing, Hodge and Petruska proposed a parallel staggered fibril model.[16] A theoretical analysis by Hulmes *et al.*[17] demonstrated that maximal ionic and hydrophobic interactions occur when collagen monomers are offset by 234 ± 1 residues along the fibril axis, which roughly equals to 67 nm. In the following decades, XRD studies provided stunning structural details including 5-strand quasi-hexagonal lateral arrangement of microfibrils (**Figure 1a**), molecular kinking in the gap zone and longitudinal supertwist.[18–20] Nevertheless, techniques based on measurements of *individual* fibril's D-spacing continued to report a distribution of D-spacing values (**Table 1**); however, the physical and biological importance of D-spacing variation was not recognized. Textbooks and literature commonly introduce D-spacing as a single value of 67 nm.[3] Recently, the importance of the collagen D-spacing distribution was brought to light; significant alterations in bone collagen D-spacing distribution are shown in OI and long-term estrogen depletion.[11,12]

OI phenotypes are most commonly associated with mutations in genes encoding type I collagen or proteins involved in type I collagen posttranslational modification and intracellular trafficking.[21] On the molecular level, the detrimental effects of OI collagen mutations include slower folding of the triple helices, delayed intracellular trafficking and thus over modification,[22] destabilized tropocollagen molecules[23,24] and decreased mechanical stiffness of tropocollagen molecules[25] as well as the fibrils.[26] A recent study by Wallace *et al.*[12] showed changes in bone collagen nanomorphology as a result of Glycine 349 to Cysteine substitution in one *col1α1* allele. They used a heterozygous brtl/+ mouse model of type IV OI, hence a heterogeneous mixture of mutated and non-mutated collagen monomers. Interestingly, the resulting collagen fibril D-spacing is also more heterogeneous compared with the wild type (WT) animals. Although there was no significant difference between D-spacing means of WT and brtl/+, larger variations along the axial length of brtl/+ bones were noted, and the brtl/+ group contains only 55% of fibrils with D-spacings in 66–70 nm range, versus 75% in WT ($P = 0.001$).[11] In a subsequent study, Kemp *et al.*[27] demonstrated correlations between collagen fibril D-spacings and indentation-type nanomechanical properties with tendon fibrils of the brtl/+ OI mouse model. They found that

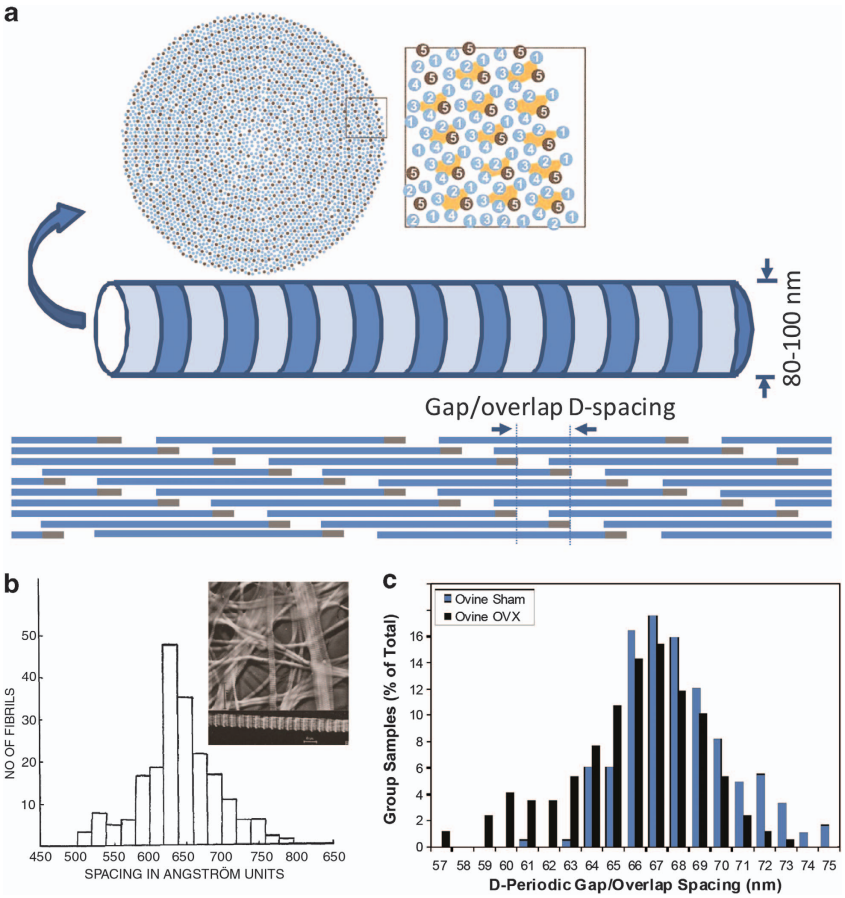


**Figure 1** Type I collagen fibril structure and nanomorphological heterogeneity. (**a**) The proposed quasi-hexagonal packing at fibril cross-section, adapted from Hulmes *et al.*[20] with permission. (**b**) TEM studies in the 1940s showing nanomorphological variations result in a D-spacing distribution, adapted from Gross and Schmitt[14] with permission. (**c**) Significant alteration of D-spacing distribution in the OVX ovine bone, adapted from Wallace *et al.*[12] with permission.

**Table 1** D-spacing variations reported in literature

| *Variations in D-spacing* | *Tissue and technique* | *Results and discussion* | *Reference* |
|---|---|---|---|
| 55–80 nm distribution<br>64.6 ± 5.3 nm[a] | Human skin<br>TEM | The 55–80 nm range of spacing is not unique to collagen, but also shared by neurotubules | Gross and Schmitt[14] |
| 64.6 ± 0.8 nm in cornea<br>67.7 ± 0.8 nm in tendon | Cornea and tendon<br>XRD | An 18° axial inclination in the cornea explains the D-spacing difference between the cornea and the tendon ($\cos\alpha = Dc/Dt$) | Marchini *et al.*[57] |
| 67.7 ± 0.9 nm in central zone<br>71.3 ± 0.4 nm in distal zone | Vitrified predentin<br>TEM | D-spacing differences in the two zones may be due to the presence of proteoglycans and ions that bind to collagen | Beniash *et al.*[72] |
| 54–75 nm distribution (predominantly 67–68 nm hydrated; 57, 62, 67 nm dehydrated) | Partially demineralized dentin<br>AFM | Reduced D-spacing may be due to dehydration-induced structure disorder and loss of crystallinity | Habelitz *et al.*[43] |
| 69.6 ± 2.9 nm | Rat digital tendon<br>AFM | Fibril D-spacing is preserved independent of the fibril diameter | Bozec *et al.*[70] |
| 63–73 nm distribution | Mice bone, dentin and tendon<br>AFM | A distribution of D-spacing exist in the bone, dentin and tendon, regardless of the presence of mineral, cellular origin, anatomical location or mechanical function of the tissue | Wallace *et al.*[39] |
| 68.0 ± 2.6 nm in sham;<br>65.9 ± 3.1 nm in OVX | Sham and OVX ovine radius bone<br>AFM | Estrogen depletion induces changes in type I collagen nanomorphology of bone ($P < 0.001$) | Wallace *et al.*[12] |
| 63–74 nm distribution in WT;<br>56–75 nm distribution in brtl/+ | WT and brtl/+ mice femur bone<br>AFM | D-spacing means between WT and brtl/+ are not different (67.6 nm vs 67.4 nm); D-spacing distributions between the phenotypes are statistically different ($P = 0.001$) | Wallace *et al.*[11] |
| 59–66 nm distribution in sham;<br>56–67 nm distribution in OVX | Sham and OVX ovine dermis<br>AFM | Estrogen depletion induces changes in type I collagen nanomorphology of dermis ($P < 0.001$) | Fang *et al.*[37] |
| 57–69 nm distribution | Ovine bone<br>AFM | Fibrils from one D-bundle share similar D-spacing; a distribution of values arises at the bundle level | Fang *et al.*[10] |

Abbreviations: AFM, atomic force microscopy; OVX, ovariectomized TEM, transmission electron microscopy; WT, wild type; XRD, X-ray diffraction.
[a]Estimated from the distribution histogram (**Figure 1b**).

modulus and indentation depth were correlated with brtl/+ fibril D-spacing in dried tendon fibrils, whereas energy dissipation was correlated with WT fibril D-spacing in hydrated tendon fibrils. Tensile stretching of an individual collagen fibril indicated correlation between fibril D-spacing and fibril mechanical properties; the nonlinear stress–strain curve suggests increased fibril modulus accompanying D-spacing elongation induced by tensile force.[28,29] In addition, higher elastic modulus in the overlap zone over gap zone has been demonstrated by atomic force microscopy (AFM) nanoindentation experiments.[30,31]

Alteration in collagen fibril nanomorphology has also been shown in long-term estrogen depletion. Estrogen deficiency in postmenopausal women results in increased bone resorption,[32] reduced bone quantity and mineral density,[33] changes in the micro-architecture and other material deterioration of the bone.[34] Unlike OI, which has a genetic origin directly linked to collagen, the knowledge on how estrogen deficiency may impact collagen is limited. Collagen crosslink content has been shown to decrease with osteoporosis.[35,36] The nanomorphology of collagen has been systematically compared between sham and ovariectomized (OVX) ovine bone and dermis.[12,37] In both tissue types, a higher percentage of fibrils with D-spacing values below mean minus one s.d. was associated with estrogen depletion (**Figure 1c**). Specifically, 28% of OVX bone collagen fibrils had D-spacings lower than 64 nm, whereas only 7% of such fibrils were found in the sham group.[12] Similar results were found in OVX dermis, further supporting the notion that estrogen deprivation affects collagen nanomorphology, regardless of the presence of mineral or not.[37]

The studies of collagen nanomorphology in OI and long-term estrogen depletion corroborated the important roles of collagen and the need for an effective method to evaluate the morphology of collagen in bone diseases. Note that in both cases only a subportion of fibrils exhibited abnormal nanomorphological values, indicating the importance of methods capable of a fibril-by-fibril analysis. Connecting collagen fibril nanomorphology with biochemical and mechanical properties of collagen fibrils will require combined techniques capable of nanometer-scale resolution such as AFM/nanoindentation and AFM/Raman. These methods will provide useful information pointing to the biological origin of the disease-induced collagen nanomorphology variations and the functional consequences on bone physiochemical and mechanical properties.

A few practical issues must be taken into consideration when studying bone collagen nanomorphology. Surface demineralization is required to reveal the underlying collagen matrix.[38] Gentle demineralization seems to have a minimal effect on

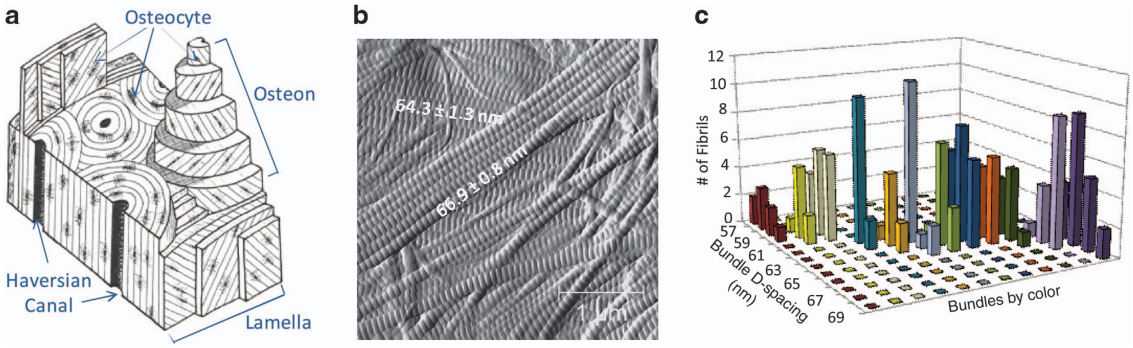


**Figure 2** Collagen nanomorphology in connection to micro-scale bone lamellar structure. (**a**) A schematic of bone micro-architecture, showing lamellae with collagen fibril bundles at different orientations. (**b**) AFM error images showing two layers of collagen bundles presumably from two lamellae, and different D-spacing values are associated with the two bundles. (**c**) Three-dimentional bar plot showing that bundle D-spacings occupy the full spectrum of distribution ranging from 57 to 69 nm, whereas fibril D-spacings within one bundle are narrow ( ± 1 nm). (**a**–**c**) are adapted from Fang *et al.*[10]

collagen D-spacing, as non-mineralized tissues such as the tendon and skin showed similar D-spacing distributions compared with the bone (see **Table 1**).[37,39] In addition, samples measured in air, and thus subject to some dehydration, showed minimal changes on D-spacing distribution of healthy and normal collagen fibrils.[40] Dehydration significantly impacts the mechanical behavior of bone tissue,[41] and the loss of water has been shown to affect the molecular packing of collagens within fibrils.[42] Dehydration could also have a role in varying D-spacing. For example, Kemp *et al.*[27] reported that air drying caused artifacts by removing low D-spacing values in the Brtl/+ phenotype, contrary to the conventional belief that dehydration causes fibril D-spacing shrinkage. Habelitz showed that in partially demineralized dentin, air drying changed the fibril D-spacing distribution from a unimodal distribution with a center at 67–68 nm and a range of 54–75 nm, to a distribution divided into three groups, centered at 57, 62 and 67 nm.[43] In another AFM-based investigation, where D-spacings of an identical set of 20 fibrils were measured in water vs air, no correlation in the small D-spacing shifts as a function of water vs air imaging was noted.[40] Although surface dehydration may have an impact on the D-spacing, it is unlikely that the 10-nm range distribution is an artifact of surface dehydration.

## Collagen Nanomorphology Associated with Tissue Hierarchy

Organization of collagen fibrils into various highly hierarchical structures in the bone matrix is a fascinating biological phenomenon. At the ultrastructural level, bone trabeculae and osteons are built by planar or cylindrical lamellar layers of collagen fibrils with different angular orientations between adjacent layers, known as the twisted plywood model.[44] Fibrils within one layer are aligned with each other as a bundle, similar to fibril bundles observed in the skin, tendon, cornea and aorta.[45] The birefringence of collagen bundles allows optical techniques such as polarized-light microscopy to visualize the different orientations of bone lamellae as alternating dark and bright bands.[46] Collagen fibril orientation is influenced by mechanical strain distribution and in turn enhances the mechanical property of bone.[47,48]

Collagen nanomorphology has a close connection with the aligned fibril bundle unit structure. Although a distribution of values ranging from 60 to 70 nm is frequently found in tissues, within a single collagen fibril bundle, the variation of D-spacing values can be within ± 1 nm, suggesting uniform axial packing of collagen monomers within a bundle.[10] Such fibril bundles with uniform D-spacing were named D-bundle. Different D-bundles, presumably belonging to different lamellar layers (**Figure 2**), could differ in D-spacing by up to 10 nm, which give rise to the full-scale distribution. A nested analysis of variance analysis partitioned the variance components at the animal, bundle and fibril level, and found that indeed the bundle-level variance accounted for 76% of the total variance.[10] In other words, fibril D-spacing variance nested within one bundle and variance of different animals are small compared with variance of different D-bundles. It should be noted that this characteristic of the D-bundle was also present in the dermis and the tendon.

The observation of narrow D-spacing values within a bundle and large differences across different bundles has important implications in current fibrillogenesis models. Studies carried out in the tendon clearly favor the hypothesis that cells have a dominating role in directing the alignment of fibrils.[49–51] Using transverse-sectioned transmission electron microscopy (TEM) imaging, Canty *et al.*[50] were able to trace collagen fibrils from extracellular bundles to deep within a fibroblast cell. The membrane protrusions of fibroblasts, also called fibripositors, were proposed as nucleation sites of collagen fibrillogenesis, and responsible for projecting collagen fibrils into parallel alignment. By this theory, a collagen bundle is formed by lateral association of fibrils excreted by one osteoblast and its orientation is determined by the direction in which the cell migrates. In this case, the bundle-to-bundle D-spacing difference could be due to cell-to-cell difference, such as the varying amount of minor collagens and posttranslational modifications. Currently it is unclear to what extent osteoblasts influence bone collagen orientation. Unlike the tendon, bone fibrils are orthogonally stacked in the twisted plywood spatial arrangement. *In vitro* osteoblast culture reproduces the orthogonal spatial arrangement of secreted collagen fibrils, however no evidence of osteoblast cells dominating the orthogonal fibril orientation was shown.[52] A different theory emphasizes the importance of the intrinsic liquid crystallinity of collagen.[53–55] Highly concentrated acid-soluble collagen has the characteristics of a cholesteric liquid crystal, which exhibits a striking resemblance to the twisted plywood structures in bone.[56] It is therefore plausible that high concentrations of

procollagen or tropocollagen are pre-aligned before fibrillogenesis at the bone resorption pocket, leading to the formation of a fibril bundle with uniform packing of individual collagen monomers, hence the narrow D-spacing within a bundle. To date, no one has observed the motion of osteoblasts in registration with the collagen fibrils they secrete or directly studied the liquid crystallinity of collagen during the *in vivo* process of fibrillogenesis.

## The Origin of Collagen D-spacing Distribution

For decades collagen D-spacing has been thought as a single value, either 64 nm in the skin and cornea, or 67 nm in the tendon and bone, based on X-ray diffraction data. Some have proposed a helicoidal fibril model to explain the discrepancy based on the observation of a 18° axial tilting of microfibrils in cornea using freeze etching technique. The 64 nm D-spacing in the cornea was rationalized as 67 cos(18°).[57,58] A distribution of D-spacings has only recently been reported with significant connections to bone diseases and bone tissue micro-architecture.[10–12,27,37,39,40,59] For now, our understanding of the origin and functions of a collagen D-spacing distribution is limited, potential biological and molecular bases for the D-spacing distribution are discussed in this section.

Many collagen-constituted tissues are also load-bearing tissues. Bone formation and resorption are stimulated by increased mechanical loading and disuse, respectively, as a part of bone functional adaptation.[60] It is plausible that variation in D-spacing is a reflection of changing local mechanical stresses. Although this hypothesis could explain the formation of different bundle D-spacings and narrow D-spacing within a bundle, experimental data to date suggest against the possibility of differences in mechanical loading causing a 10-nm distribution of D-spacings. Gupta *et al.*[9] studied the behavior of fibril strain over tissue strain of bone using small-angle X-ray scattering (SAXS) and noted that 0.7% macroscopic tissue strain corresponds with a 0.5% increase in D-spacing average as measured using SAXS. Puxkandl *et al.*[61] and Sasaki *et al.*[62] have demonstrated a similar effect in tendon. At the molecular level, the fibril strain is the direct result of triple-helix stretching; kinks in the gap zone are straightened causing the gap zone to move apart (see more details in the review by Fratzl *et al.*[63]). Intermolecular slippage and telopeptide unfolding also accompany mechanical loading.[64] In the post-yield regime, bone fibril strain does not increase with tissue strain, which Gupta *et al.*[9,65] suggest is to due to the interfibrillar shearing/sliding of noncollagenous components of the bone. Nevertheless, 0.5% fibril strain corresponds to 0.3 nm D-spacing increase, indicating that mechanical loading alone does not cause the 10-nm D-spacing variations. Another possibility is that the D-bundle variations could be due to different bundle relaxations induced by partial demineralization, depending on their initial levels of mechanical constraints within the bone matrix. However, the fact that similar bundle-to-bundle variations were also found in the dermis, which bears minimal mechanical loading, argues against such a possibility. Nevertheless, it does not rule out the possibility of fibril bundles with different D-spacings being formed by osteoblast cells that are under different mechanical stresses.

In addition to responses to mechanical cues, other potential cell-based factors include differential expression level of homotrimeric collagens and minor collagen type V/XI, and varying amount of posttranslational modification. Homotrimeric collagen is structurally more flexible than heterotrimers, which could have a profound influence on the fibril packing and properties.[66,67] For example, an *oim* mouse model, which uses homotrimeric type I collagen isoform ($\alpha1(I)_3$) instead of the normal heterotrimeric type I collagen ($\alpha1(I)_2\alpha2(I)$) as the organic building block of tissues, exhibits severe OI phenotypic properties. Full-scale atomistic simulation showed that homotrimeric collagen has a higher tendency of forming kinks, which leads to larger lateral intermolecular spacing.[67] This molecular level structural alteration could cause reduced intermolecular crosslinking and, consequently, weakened mechanical strength at the organ level. No change in the axial D-spacing was observed in the *oim* mice tendon, however, this observation was based on XRD measurement that only provides D-spacing average.[68] In addition to intracellular factors, extracellular factors such as crosslinks and proteoglycan binding are also potential factors that could lead to different packing density and thus D-spacing variations.

A recent study has shown that self-assembly of type I collagen *in vitro* produces similar distributions with those found in biological tissues.[69] Both mica-surface-mediated fibril assembly and fibrillar gel formed in confined glass capillary tubes exhibited a D-spacing distribution in the range of 60–70 nm. It suggests that D-spacing variation is intrinsic to type I collagen and its self-assembly, and it does not necessarily require cells, interfibrillar crosslinking and proteoglycan binding. The variations in fibril D-spacing may be a result of variant intrafibrillar interactions including hydrophobic interactions, electrostatic interactions, hydrogen bonding and crosslinks on hydroxylysines and hydroxyprolines. As an offset of 234 amino acids between collagen monomers maximizes the sum of these interactions, it is possible that D-spacing variations arise from various degrees of tilting or super coiling within a fibril, similar to the idea used to explain the 64 vs 67 nm D-spacing in different tissues. In addition, Bozec *et al.*[70] observed spiral and twisted rope features in digital tendon fibrils and proposed a classic n-plies rope model, which could also explain fibril D-spacing variation. More refined theoretical models incorporating collagen monomer and/or microfibril assembly should also be able to predict a D-spacing distribution.

## Summary and Perspectives

From the 2D Hodge-Petruska model in the 1960s[16] to the modern computer-simulated 3D model,[71] D-spacing has been a key aspect of collagen nanomorphology, yet the intrinsic heterogeneity of collagen D-spacing has rarely been emphasized. An axial D-spacing distribution arises at the bundle level and it is universal among bone and other tissues including the skin, tendon and dentin; it can also be reproduced by the self-assembly of type I collagen alone. The alteration of D-spacing distributions in bone diseases underscores the need to better understand the origin of D-spacing distribution and the biochemical/mechanical consequence of such nanomorphological changes. Different fibril D-spacings may have an impact on mineral nucleation and growth, binding with proteoglycans and fibril stiffness. Experimental investigations using combined instrumental analyses and theoretical modeling are required to elucidate the details of collagen structure–property relationship at nano- to micro-scale.

## Conflict of Interest

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