

Development and utilization of a bovine type I collagen microfibril model

Eleanor M. Brown^{*,1}

U.S. Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, 600 East Mermaid Lane, Wyndmoor, PA 19038, United States

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ABSTRACT

The structure of fibrous collagen, a long triple helix that self-associates in a staggered array to form a matrix of fibrils, fibers and fiber bundles, makes it uniquely suitable as a scaffold for biomaterial engineering. A major challenge for this application is to stabilize collagen structure by means that are acceptable for the end use. The bovine type I collagen microfibril model, built by computer assisted modeling, comprised of five right-handed triple helices in a left-handed super coil containing gap and overlap regions as well as the nonhelical telopeptides is a tool for predicting or visualizing chemistry to stabilize the matrix, insert an active agent, or otherwise modify collagen.

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1. Introduction

The shape and mechanical attributes of a vertebrate body are defined by its connective tissue, the cells of which are embedded in an extracellular matrix that is a complex mixture of proteins and carbohydrates and functions as a support for cellular materials. Collagen, the primary protein of connective tissue, is the most abundant protein in mammals, constituting about a quarter of the animal's total weight. Throughout history, collagen has played an important role in technology, serving as a basis for industrial biopolymers including leather, medical devices, food and adhesives. Animal hides and skins, major byproducts of the meat industry, are a rich source of collagen. The hides and skins are typically used for the production of leather, gelatin, and an array of biopolymer products. This raw material is largely type I collagen, one of the fibrous collagens that serve as scaffolds giving strength and form to the skin, tendons, bones, cornea and teeth. The structure of fibrous collagen, a long triple helix that further associates into fibrils, fibers and fiber bundles, makes it uniquely suitable as the basis for biomaterial engineering. Several recent reviews suggest numerous uses for collagen-based materials [1–5].

The structural stability and mechanical properties of collagen fibers in the living organism are largely functions of their hierarchical structure and the lysyl oxidase catalyzed crosslinks anchored

in the nonhelical telopeptides [6]. Once the hide has been removed from the animal, it must be stabilized to prevent degradation by microbes and loss of physical integrity when exposed to water or heat. The leather industry, through empirical methods over several centuries, has identified and perfected the use of tanning agents to stabilize the hide and produce beautiful and durable leathers with a variety of properties. Vegetable tanning with polyphenolic tannins, chrome tanning using salts of Cr(III), and glutaraldehyde tanning, which produces white leather, are examples.

A major challenge to the use of collagen in manufactured biomaterials is the identification of ways to isolate the collagen and stabilize its structure, which are acceptable and practical for the proposed end use. The resistance of the collagen triple helix to attack by enzymes other than collagenases, and very aggressive proteases, has led to the use of a mild pepsin digestion in the isolation and solubilization of collagen in preparation for biomedical and other industrial applications [7]. This treatment partially cleaves the nonhelical telopeptides, interrupting the native crosslinks while leaving the helical domain intact. The loss of natural crosslinks makes it essential to introduce new crosslinks or other stabilizing materials. Although materials from each of the categories of tanning agents have been used in the production of other biopolymer products, they are less than ideal for most biomaterial uses.

The design or selection of new crosslinking or stabilizing agents for collagen fiber matrices can be assisted by the use of models generated by computer modeling techniques in conjunction with spectroscopic data. Protein functionality follows from structure, and the stabilization of the collagen matrix involves the modification of the molecular structure. For globular proteins, in solution, secondary and higher order structure is typically determined by spectroscopic techniques that can also be used to monitor the

* Tel.: +1 215 233 6481; fax: +1 215 233 6795.

E-mail address: eleanor.brown@ars.usda.gov

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Table 1
MCF2012 development.

Stage 1	A = left handed helix (Gly-Pro-Pro) ₄ B = right handed triple helix 3(Gly-Xxx-Yyy) ₁₂ Xxx and Yyy from Fietzek 1976 [29] C = 5 × B left handed microfibril
Stage 2	D = D space microfibril scaffold 5[3(Gly-Pro-Hpr) ₁₀₅] E = D space microfibril 5[3(Gly-Xxx-Yyy) ₁₀₅ [29] overlap and gap, without telopeptides
Stage 3	F = E + telopeptides modeled on <i>in situ</i> data [25,26] G = F with AA sequence corrected [24]

effects of protein modification. The relative insolubility and hierarchical nature of collagen fiber structure limit the usefulness of these techniques. Nonetheless, the macroscopic structure of collagen fibers was established from X-ray diffraction patterns and electron micrographs more than half a century ago [8]. The conformation and conformational stability of the collagen triple helix was initially inferred from studies on collagen-like peptides in dilute solution using methods analogous to those for globular proteins [9]. The microfibril, a structure consisting of four to six tropocollagen molecules staggered lengthwise to produce the alternating gap and overlap regions observed in electron micrographs, was proposed as the limiting unit for three-dimensional arrangement in collagen fibers [10–12].

Computer-assisted molecular modeling is a useful tool for visualizing structure-function relationships in proteins, and for predicting the effects of proposed modifications to protein structure. Researchers with a variety of interests have developed models and synthesized crystalline collagen peptides to examine specific aspects of collagen structure. The perturbation of the triple helical structure of collagen peptides by benign or pathological mutations in the primary structure is an active area for research, reviewed recently by Brodsky et al. [13]. Several groups have explored the effects of hydration on the stability of collagen peptides [14–16]. At a higher level of complexity, the interdigitated quasi-hexagonal microfibril structure in the collagen fiber, identified by Orgel et al. [12] was used as a 3D template for the 2D map of medically relevant interaction sites on collagen [17], and for Buehler's [18] model of the mechanical characteristics of collagen fibers.

The objective of collagen modeling in this laboratory was to produce a tool that leather scientists and would find useful in studies of leather tanning mechanisms. This model would allow one to explore potential interactions of various types of tanning agents with collagen, as modifiers or interhelical crosslinkers. The model would also be amenable to use by scientists whose modeling resources may be limited to publically available software such as the Swiss-PdbViewer at <http://www.expasy.org/spdbv/> [19]. Over several years, a five-helix model of bovine type I collagen was developed [20–23], Table 1. Newer amino acid sequence data [24] and *in situ* conformations for telopeptides [25,26] are incorporated in the current model. Uses for the model that are relevant to collagen-based biomaterials in general are presented.

2. Model development

The bovine type I collagen monomer consists of two identical $\alpha 1(I)$ chains and one similar but non-identical $\alpha 2(I)$ chain. The amino acid sequence of the helical portion of each chain has the form of a repeating tripeptide (Gly-Xxx-Yyy)₃₃₈. About 25% of Xxx and Yyy are imino acids, proline and hydroxyproline respectively, although any of the typical amino acid residues except cysteine, tryptophan, and tyrosine (other than Tyr860 in the $\alpha 2(I)$ chain) can occupy these positions [24]. In the collagen fibril, helical domains are aligned end to end with a space between the C-terminus of one molecule and the N-terminus of the next. Neighboring molecules

are staggered so that striated patterns of alternating gap and overlap regions are observed in micrographs of collagen fibers. This alignment is directed and stabilized by telopeptides, at either end of the helical domain that extend into the gap. The telopeptides, which lack the tripeptide motif, are more flexible, allowing reactive side-chains to interact with adjacent triple helix chains in the formation of naturally occurring collagen crosslinks [27].

2.1. Model construction

The three-dimensional computer model of a bovine type I collagen microfibril was constructed using SYBYL molecular modeling software (Tripos Associates, St. Louis, MO, USA). In the first stage of construction [20], (Gly-Pro-Hpr)₁₂ peptides were constructed as left-handed helices and visually docked into right-handed triple helices 3(Gly-Pro-Hpr)₁₂, where individual chains were staggered by one residue with respect to each other. To produce a 36-residue long microfibril segment, four triple helical units 3(Gly-Pro-Hpr)₁₂ and one 3(Gly-Pro-Hpr)₁₀, to simulate the start of a gap region, were packed into a left-handed superhelix [8]. Energy minimization – via the AMBER force field [28], was performed at each step in the construction. Bovine type I collagen was simulated by substituting amino acid residues from published sequence data [29] for Pro and Hpr residues where appropriate.

A signature characteristic of type I collagen is the 67 nm D-space banding pattern, observed in micrographs, that represents one gap and one overlap region of the collagen molecule within a fiber. The second stage of model development [21] was to produce a model that included a full D-space by splicing shorter collagen segments together. Two triple helical segments 2[3(Gly-Pro-Hpr)_k] were aligned to overlap slightly. Overlapping atoms were deleted, and peptide bonds formed to create a longer helix 3(Gly-Pro-Hpr)_m, where *m* was less than 2*k*. This procedure was repeated until the model structure 3(Gly-Pro-Hpr)₆₉ was achieved. Five copies of this triple-helical model were then bundled such that the cross-section was a regular pentagon, to form a microfibril 5[3(Gly-Pro-Hpr)₆₉]. One triple helix was shortened to 3(Gly-Pro-Hpr)₂₆ to accommodate the gap region. Two modified 5[3(Gly-Pro-Hpr)₆₉] microfibrils were aligned face to face so that the gap regions of the two segments were contiguous, with overlap regions at each end capping the gap. These models were spliced together as described above to produce a 5[3(Gly-Pro-Hpr)₁₀₅] that when energy minimized, formed the structural scaffolding for the type I collagen microfibril model [21]. Single step substitution of the actual type I sequence proved to be impractical for a model of this size. Instead, side-chain groups were modified step-wise so that the number of non-hydrogen atoms was increased or decreased by one at each step of the modification with computational adjustment between steps. Thus bulkier side-chains were allowed to gradually grow into their sites within the protein, without major disruption to the backbone structure [21]. When the amino acid residues of this collagen type I helix were colored by type (acidic, basic, hydrophobic) to mimic the action of a negative microscopy stain (Fig. 1), a pattern analogous to those of collagen segment long spacing patterns appeared [30].

Telopeptides were initially constructed [22,23] using incomplete primary sequence data for bovine type I collagen [29], supplemented with sequence data for other mammalian telopeptides where the sequence was complete. Although conformational predictions [31] and data on isolated telopeptides in solution were available [32,33], data on telopeptide conformations within the fiber was lacking. Thus, telopeptide chains were initially built in random conformations, subjected to simulated annealing, and attached at the appropriate positions in the microfibril model. Molecular dynamics simulations followed by energy minimization allowed telopeptides to reach energetically favorable conformations relative to the microfibril. This model was used to evaluate

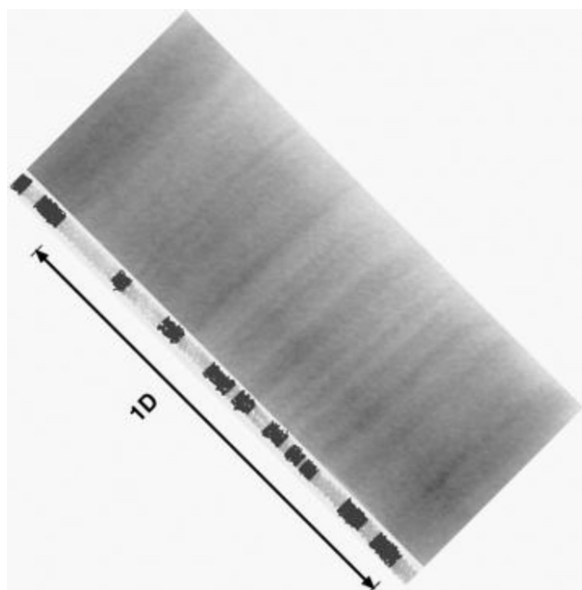


Fig. 1. Bovine collagen microfibril model, lacking telopeptides, colored to mimic negative microscopy staining, aligned with micrograph of calf skin collagen.

the possibility for interactions of telopeptide residues with residues in triple helical regions of neighboring chains, or with potential crosslinking reagents.

2.2. Model refinement

The current model includes several modifications to the earlier version. When the complete amino acid sequences for the bovine a1(I) chain (GenBank ID: AAI05185.1) and a2(I) chain (NCBI ID: NP_776945.1) became available [24], corrections were made to 39 residues, less than 1%, of the total. The initial conformations for both the N- and C- terminal telopeptides were reset to mimic the structures for docked telopeptides as described by Malone et al. [25,26]. Recent molecular dynamics simulations and energy minimizations have been performed with the Tripos force field [34] because of its facility with nonstandard amino acids (hydroxyproline and hydroxylysine) and nonprotein molecules such as crosslinkers and other modifiers.

This updated model contains 4360 amino acid residues, equivalent to 1.4 collagen monomers, representing a slice through a cylindrical assemblage of five triple helices. All parts of the bovine type I collagen sequence can be visualized in relation to neighboring helices. Molecular dynamics simulation, without constraints, was performed first at 300K for 10 ps followed by energy minimization to $-22,617$ kcal/mol, a value that changed by only 0.2% over 100 iterations. After a second molecular dynamics simulation at 350K for 10 ps, the structure was minimized as above, to $-24,441$ kcal/mol. Except for a 5% shrinkage in the nonbonded length of the structure, these operations had little effect on the general appearance of the microfibril structure (Fig. 2).

3. Model utilization

Although the utilization of collagen as the substrate for leather and other biomaterials has been practiced throughout history, there is still considerable uncertainty regarding the mechanisms by which collagen can be stabilized. The impetus for development of this model was to gain insight into the mechanisms of tanning animal skins to produce leather. The essence of tanning is to protect the skin from putrefaction and increase its hydrothermal stability. Tanning developed more as a craft than a science, with at

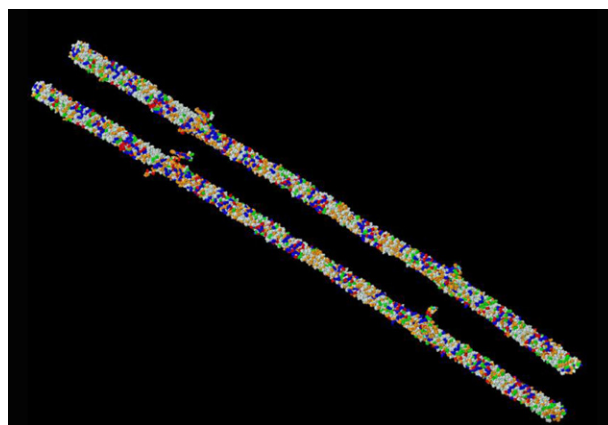


Fig. 2. Bovine microfibril model is shown with telopeptides, before (lower) and after (upper) image molecular dynamics at 350K without constraints. Residues are colored to reflect functionality, Acidic residues (Asp, Glu) are in red. Basic residues (Arg, His, Lys Hly) are in blue. Hydrophobic residues (Ala, Ile, Leu, Met, Phe, Tyr, Val) are in orange. Polar, neutral residues (Asn, Gln, Ser, Thr) are in green. Collagen template residues (Gly, Hpr, Pro) are in white. (For interpretation of the references to color in figure legend, the reader is referred to the web version of the article.)

least three categories of tanning agents becoming important. Basic chrome sulfate (BCS), a mixture of bi-, tri- and polynuclear Cr(III) salts is the primary tannage currently in use. Vegetable tannages using polyphenolic extracts from plant materials produce heavy leathers used in harnesses, belts and shoe soles. Derivatives of acrylamides, aldehydes and organic acids are used in the production of specialty chrome-free and white leathers. During the 20th century, the primary tanning mechanism was assumed to be the crosslinking of collagen sidechains on different fibers by tanning agents. The collagen microfibril model allows one to explore possible interactions of the fundamental components of these tanning agents with collagen monomers in a matrix that begins to approach fibrillar structure. With the assumption that tanning involves crosslinks anchored by either an acidic or a basic side chain on collagen, a three dimensional analysis of the relative locations of ionizable groups in a short segment of the microfibril model was performed [35]. The relative spacing of carboxylic acid and lysine sidechains in the microfibril provided an initial estimate of the crosslinking potential of binuclear metal complexes or complexes containing dialdehyde or diacid functionality [35].

3.1. Crosslinking reactions

An early success was an explanation for the finding that reaction of medium chain length, C-7 to C-12, dicarboxylic acids with collagen had a much greater effect on collagen thermal stability than did di-acids with either shorter or longer chains. Simulation of the acid molecules under experimental conditions gave an estimate of the potential crosslinking span of different chains, and showed that medium length chains were most appropriate for formation of inter-helical crosslinks [36]. Examination of the entire microfibril model, containing 124 lysine residues reveals 45 positions where the distance between two ϵ -amino lysine nitrogens is 0.7–1.1 nm, suitable for crosslinking by a C-7 to C-12 dicarboxylic acid. Eleven of these are potential interhelical crosslinks expected to stabilize microfibril structure.

In the chrome tanning process used to produce fine leathers, bi- or trinuclear Cr(III) sulfate complexes in BCS are most likely to form crosslinks between carboxyl group (Glu, Asp) side chains on collagen [37,38]. A carboxyl to carboxyl distance in the 0.6 to 0.8 nm range would be required to accommodate a hydroxide bridged bi- or trinuclear Cr(III) complex [39]. For effective stabilization, the participating carboxyl groups must be on different helices. Analysis

of the complete model, containing 115 Asp and 208 Glu residues, for carboxyl-to-carboxyl pairs separated by 0.6–0.8 nm showed 22 unique interhelix sites in the gap, and 18 in the overlap region. An additional three potential sites were eliminated on the assumption that because of steric hindrance, more than a single turn of helix should separate participating carboxyl groups. Second, because the model represents 1.4 molecules, the number of effective crosslinking sites was reduced to 26 on a molecular basis. The 52-carboxyl groups available for the formation of productive crosslinks is in excellent agreement with early estimates by Covington [40] that chrome tanning of collagen involves one in six acidic residues in collagen for a total of 54 residues, based on experimental data combined with an alignment of the collagen sequence to produce triple helices with hierarchical structures.

Glutaraldehyde and acrylamide derivatives are components of tannages used to produce white leathers, and also act as stabilizers for biopolymers in fields other than leather. They are thought to form crosslinks via the ϵ -amino groups of Lys residues [41]. Predicting optimum binding geometries for these materials is complicated by their tendency to self polymerize, characteristics that lead to a stabilizing mechanism whereby the initial step may be attachment of a monomer or oligomer to a Lys sidechain, followed by polymerization that may extend the effect across fibrils.

Genipin, a naturally occurring biocompatible crosslinking agent, isolated from the fruit of *Gardenia jasminoides* Ellis, is beginning to replace glutaraldehyde as a collagen stabilizer, especially in applications where its low cytotoxicity is an advantage. In acidic or neutral aqueous solution, genipin (Fig. 3a) undergoes a ring-opening reaction to form a dialdehyde that can polymerize via aldol condensation [42]. The extent of the polymerization varies, allowing for dimer, trimer, and larger crosslinking bridges between amino groups. Based on the analysis of Liang et al. [43] the most probable genipin polymers could bridge peptide chains at distances of 1.6–2.5 nm. At pH 7.5, 5% genipin was nearly as effective as glutaraldehyde at increasing the hydrothermal stability of bovine hide collagen [44]. The collagen microfibril model provides a basis for estimating the contributions of genipin bridges of different lengths. The model contains 124 Lys residues, about half of which show the potential for anchoring an interhelix bridge spanning a 1.6–2.5 nm range, on a molecular basis the potential is for a maximum of 22 such genipin bridges.

3.2. Reactions with polyphenols

Vegetable tanning is the most ancient of current tanning technologies, but from a mechanistic perspective, it is the least well understood. Polyphenolic vegetable tannin molecules are large, and have less potential for electrostatic or covalent bond formation with collagen than do metal salts, acids, or aldehydes. Tannins are proposed to interact with collagen via hydrophobic and hydrogen bonds, and to cluster between basic sidechains most probably in the gap region [45]. Interactions of catechin, a vegetable tannin monomer (Fig. 3b), with polar and charged side chains of a 24-residue collagen triple helix model were reported [46]. To examine the gap region of the microfibril for potential interactions of tannins with collagen, catechin molecules were docked into positions near hydroxyl groups on serine residues, and allowed to move freely under molecular dynamic simulations, at temperatures up to 400 K, while the microfibril was restrained [47]. When the change in distance between the tannin molecule and selected nearby sidechains that might participate in hydrophobic interactions or hydrogen bond formation was monitored during the dynamics simulation, the results suggested that hydrophobic interactions were at least as important as hydrogen bond formation [47].

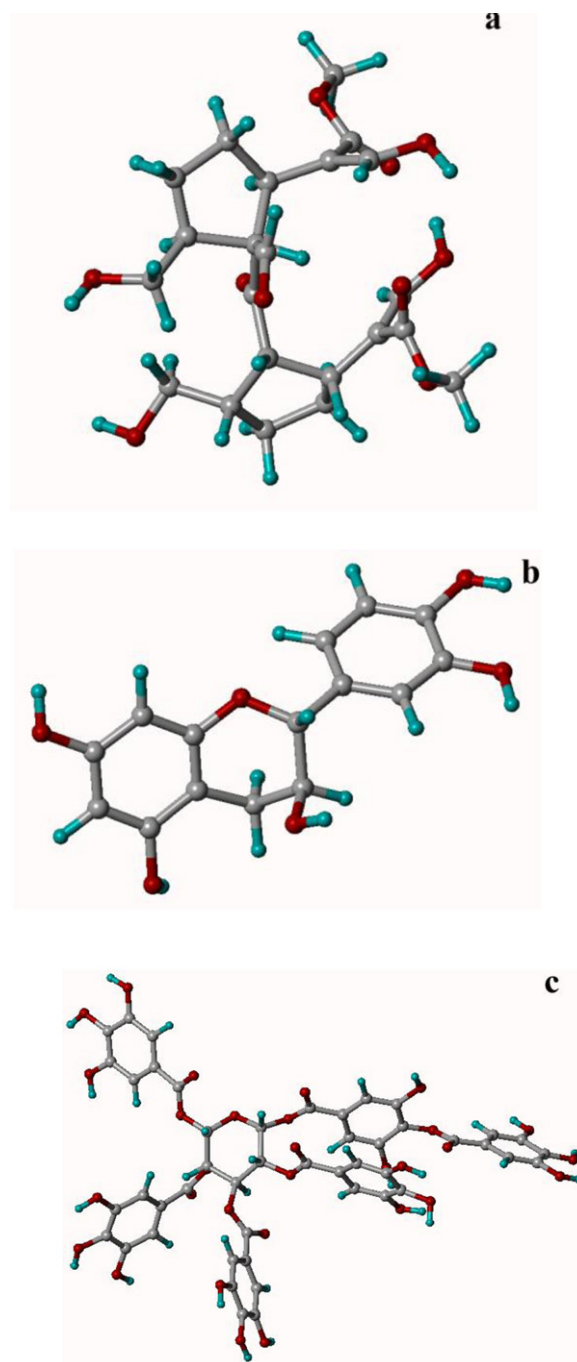


Fig. 3. Structures of leather related ligands for stabilizing collagen fiber structure: (a) digenipin, a plant derived protein crosslinker; (b) catechin, a model for vegetable tannins; (c) gallotannin, a vegetable tannin.

3.3. Role of water

To begin an exploration of the role of water in the interactions of vegetable tannins with collagen, segments of the overlap and gap regions of the microfibril model were excised and examined separately [48]. A model of gallotannin, a component of chestnut tannins (Fig. 3c), was constructed and energy minimized. This model was docked into several energetically favorable positions on each fragment of the microfibril model. The α -carbon backbone of collagen was kept immobile during molecular dynamics simulations for 10,000 fs (1×10^{-11} s) intervals at 400 K, 600 K and 800 K with and without an added layer of water, to identify possibly more favorable

interactions sites for the gallotannin molecules. Both inter- and intra-chain interactions were possible, and several potential sites for hydrogen bonding via Arg residues or hydrophobic interactions with Ala or Ile residues were identified [48].

The importance of water in the stabilization of the collagen matrix is well known. Raw hide is 60% water, and chrome-tanned leather is 40–50% water [49]. Models that explore the interactions of bound and free water on collagen peptides are common [50,51,16]. Although the computational expense of including explicit water in the full model is significant, studies using explicit solvent systems on various length microfibril models to simulate effects on collagen fibers have been reported [52–54]. Under molecular dynamics simulations, the microfibril conformation was shown to be less stable in water than in dilute solutions of formaldehyde or gallic acid/water [52]. The effects on collagen swelling with pH changes in different salt solutions, an aspect of leather processing, were reproduced [52–54].

3.4. Supramolecular effects

This model has provided a starting point for explorations by others [55–57] of collagen supramolecular structure as related to tanning mechanisms. Covington's link-lock mechanism [58] of collagen stabilization is the most recently proposed tanning mechanism. It postulates that reactions that increase the hydrothermal stability of collagen do so by attaching one reactant to the collagen triple helix, linking it into the surrounding water. A second reactant then locks the linked structure together, creating a macromolecular structure around the triple helices. Such a mechanism can potentially be tested with the microfibril model and if validated might serve as a basis for development of new types of collagen based biomaterials.

Although this model represents a five-helix microfibril, both intra- and inter-helical relationships should be independent of the number of helices chosen for the model. From a physiological perspective (Fig. S1 supplemental materials) shows that a pyridinoline type crosslink [59] can connect His92 on an $\alpha 2$ chain with Hly87 on an $\alpha 1$ chain while its tail points outward toward a C-terminal telopeptide. The cell interaction domain of type I collagen, identified by Sweeney et al. [17] falls within the overlap region. The slice of collagen represented by this model is an intact gap region with a partial overlap region at each end. Because the model extends beyond a single collagen monomer, residues 502–507, are included at both ends. By superimposing residues 502–507 from two copies of the model, aligned end to end, it was possible to visualize this physiologically important domain capped by the nearly equidistant N- and C-terminal intermolecular crosslinking sites (Fig. S2, supplemental materials).

4. Conclusions

Computer-assisted molecular modeling is a useful tool for visualizing structure–function relationships in proteins, and for predicting the effects of proposed modifications to protein structure. This bovine type I collagen microfibril model is a slice through a cylindrical assemblage of five triple helices containing gap and overlap regions as well as the nonhelical telopeptides. It represents one D-space, equivalent to 1.4 collagen monomers, and every residue of the bovine type I collagen sequence can be visualized in relation to neighboring helices. The model was developed as a tool for exploring mechanisms for interactions of tanning chemicals with collagen, and has proved useful in the analysis of crosslinking reactions. It is anticipated that the designers of collagen based biomaterials including new tannages for leather may find it useful.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijbiomac.2012.10.029.

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