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Collagen Structural Hierarchy and Susceptibility to Degradation by Ultraviolet Radiation

Olena S. Rabotyagova, Peggy Cebe, and David L. Kaplan*

Departments of Chemistry, Biomedical Engineering and Physics, Tufts University, Medford, Massachusetts 02155 USA

Abstract

Collagen type I is the most abundant extracellular matrix protein in the human body, providing the basis for tissue structure and directing cellular functions. Collagen has complex structural hierarchy, organized at different length scales, including the characteristic triple helical feature. In the present study, the relationship between collagen structure (native vs. denatured) and sensitivity to UV radiation was assessed, with a focus on changes in primary structure, changes in conformation, microstructure and material properties. A brief review of free radical reactions involved in collagen degradation is also provided as a mechanistic basis for the changes observed in the study. Structural and functional changes in the collagens were related to the initial conformation (native vs. denatured) and the energy of irradiation. These changes were tracked using SDS-PAGE to assess molecular weight, Fourier transform infrared (FTIR) spectroscopy to study changes in the secondary structure, and atomic force microscopy (AFM) to characterize changes in mechanical properties. The results correlate differences in sensitivity to irradiation with initial collagen structural state: collagen in native conformation vs. heat-treated (denatured) collagen. Changes in collagen were found at all levels of the hierarchical structural organization. In general, the native collagen triple helix is most sensitive to UV-254nm radiation. The triple helix delays single chain degradation. The loss of the triple helix in collagen is accompanied by hydrogen abstraction through free radical mechanisms. The results received suggest that the effects of electromagnetic radiation on biologically relevant extracellular matrices (collagen in the present study) are important to assess in the context of the state of collagen structure. The results have implications in tissue remodeling, wound repair and disease progression.

Keywords

collagen; radiation; structure; conformation; tissue remodeling

1. Introduction

Synthetic and natural polymers exposed to electromagnetic radiation undergo photo-aging and photo-degradation [1]. Degradation of biological materials (mainly proteins) leads to the loss of material structure and function. One of the main components of the electromagnetic radiation is ultraviolet (UV) radiation. UV radiation in sunlight is divided into three regions

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*Corresponding author: David L. Kaplan, Department of Biomedical Engineering, Tufts University, 4 Colby Street, Medford, MA 02155, USA, Phone: (617)626-3251, Fax: (617)627-3231, david.kaplan@tufts.edu.

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dependent on wavelength (λ), UVC ($\lambda = 200\text{--}280\text{ nm}$), UVB ($\lambda = 280\text{--}320\text{ nm}$), and UVA ($\lambda = 320\text{--}400\text{ nm}$). UVC has the highest energy, ϵ , ($\epsilon = hc/\lambda$, where c is the speed of light in a vacuum, h is Planck's constant) and is the most biologically damaging region of solar radiation [2].

Collagen has a unique structure, it is the most abundant extracellular matrix protein in the animal kingdom [3], and it is a major stress-bearing component of all connective tissues, including bone [4], cartilage [5], tendon [6], and skin [7]. Three polypeptide II-like helical chains are closely packed in a triple helix which is stabilized by glycine at every third residue [8]. Gly-X-Y is the characteristic repeat found in all collagens, where X and Y are often proline (Pro) and hydroxyproline (Hyp), respectively. The triple helix imparts mechanical integrity [9]. Physical properties of collagenous materials directly correlate with the primary sequence that impacts conformation and fibril assembly [10]. Collagen is a non-toxic, biocompatible polymer that guides cell behavior. Up-to-date collagen properties are widely utilized in biomedical and material science applications [11].

Collagen type I consists of three chains two alpha one chains (α_1) and one alpha two chain (α_2). Together three chains form a left-handed triple helix [12]. The collagen triple helix is sensitive to UV radiation. Electromagnetic (e.g. UV) radiation causes structural damage to collagen, which include the following actions: structural changes to phenylalanine to generate tyrosine (structural scission of $-\text{OH}$), decarboxylation (structural scission of $-\text{C}=\text{O}$), hydrogen abstraction (structural scission of $-\text{N}-\text{H}$), thermal denaturation, and general oxidative degradation [13]. Any chain scission, which occurs in collagen, has the potential to initiate aging effects or to hasten degradation, leading to changes in collagen bioactivity [14].

Since collagen has a complex structural hierarchy with mechanical and biological features impacting at different length scales, it is important to examine the interplay between collagen architecture (native state – collagen in triple helical conformation vs. denatured state – random coil conformation) and susceptibility to UV radiation. The goal of the current study was to identify how UV irradiation impacts the structure and functions of collagen materials, based on the starting structure of the material. We also proposed that radiation-induced changes in collagen impact free radical reactions; therefore, potential mechanisms of free radical reactions in collagens related to chain scission were also explored. The current research provides a foundation for future mechanistic insight into irradiated collagens and the impact on cellular responses.

2. Experimental Part

2.1 Preparation of collagen films

Sterile rat tail derived collagen type I (Roche, Indianapolis, IN) was dissolved at 3 mg/ml in 0.02M acetic acid to maintain native conformation [15]. Sixty microliters of a rat-tail collagen type I suspension was applied to a 5×5 silicon slide (TedPella, Redding, CA). This slide was placed in desiccator at ambient temperature and pressure to remove solvent. Under these conditions the film was dried to form native collagen fibers with characteristic triple helical structure. Denaturation of collagen was accomplished by incubating native collagen solution for 8 hours in a 50°C water bath [15]. Denatured collagen films were prepared in a manner similar to native collagen films.

2.2 Irradiation of collagen films

The collagen films were irradiated in air at room temperature using a UV lamp (Spectroline, model R-51A, Spectroline Corporation, Westbury, NY). The UV lamp emitted radiation at 254 nm wavelength with intensity of 7.3×10^{-3} watt/cm². The source of radiation was

located 25cm from samples and positioned horizontally. Samples of native and heat-denatured collagen were irradiated for different time periods during the study.

2.3 Material Characterization

The infrared spectra of collagen films, before and immediately after irradiation were recorded using a FTIR spectrometer Equinox 55 (Bruker Instruments, Billerica, MA). Spectra were scanned in absorption mode at 4 cm^{-1} resolution. The amide bands in the FTIR spectra were deconvolved using OPUS software from Bruker Instruments.

Atomic force microscopy (AFM) was used to determine changes in mechanical properties of collagen films after exposure to UV-254 nm. All imaging was performed in tapping and nanoindentation modes on a Dimension 3100 Nanoscope IV equipped with RTES probes (Nanodevices, Santa Barbara, CA). RTES probe cantilevers were $125\text{ }\mu\text{m}$ long with a resonant frequency of $300\pm 50\text{ kHz}$. Nanoindentation was performed at 0.05 nN force, with scratch rate of 0.1 Hz [9].

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli, using 4%-12% NuPAGE Bis-Tris polyacrylamide gels and Tris-glycine polyacrylamide gels (Invitrogen, Carlsbad, CA). Fifteen microliters of collagen solution, with a concentration of 5 mg/ml was applied and electrophoresed with molecular weight markers (See Blue Plus2, Invitrogen, Carlsbad, CA). Gels were stained with SimplyBlue SafeStain Kit (Invitrogen, Carlsbad, CA) for microgram detection and with SilverXpress Silver Staining Kit for nanogram detection (Invitrogen, Carlsbad, CA).

3. Results and Discussion

3.1. Preparation of collagen solutions

A concentration of 3 mg/ml for rat tail collagen solutions for film preparation was chosen based on a Roche current protocol. The denaturation procedure selected for the collagen solutions, 8 hrs at 50°C was based on our prior studies that demonstrated loss of the triple helical feature, without loss of molecular weight of the chains, under these conditions [15]. Films were selected as the material format for the present study to obtain collagen in its monomeric form that is widely used for cell studies [16].

3.2 FTIR spectroscopy

The amide A ($3330\text{--}3325\text{ cm}^{-1}$), amide B (3080 cm^{-1}), amide I (1650 cm^{-1}), and amide II (1550 cm^{-1}) bands are related to the peptide linkages of collagen. When comparing the behavior of collagen in native and denatured conformations under exposure to electromagnetic radiation, the amide I region is a useful area for focus since changes in the relative intensity is conformation dependent [17]. Deconvolution of the amide regions is required to understand the qualitative and quantitative contributions of different structural components of collagen to the peaks in amide regions.

3.2.1 The behavior of the amide A band components—The peak centered at 3330 cm^{-1} is an amide A component with a complex contour. Eight components of this band were determined by peak deconvolution of the $3600\text{--}2800\text{ cm}^{-1}$ region. The components of the amide A and B area are described below (Fig. 1). The amide A band of collagen is associated with NH-stretching [18]. The frequency of the amide bands depends on the conformation of collagen; the less structural order the protein has the lower the frequency of these amide bands [19]. After exposure to UV-254 nm, there was a shift of the band at 3324 cm^{-1} to longer wave number, likely associated with either scission of backbone amide bonds or the side chain amides. This is consistent with the fact that UV-254 nm is capable of

breaking C–N bonds and causing the scission of peptide bonds [20]. Our results suggest that photodegradation of collagen (both conformations) occurs with scission of peptide bonds. It is known that frequencies of the amide bands depend upon collagen conformation [19]. In general, when protein structural order decreases, the frequency of the amide bands decrease as well. UV-254 nm radiation causes a shift of the collagen amide A band to lower frequency, suggesting the thermally-induced disruption of hydrogen bonds leading to changes in collagen structural order. The changes in the position of the amide A components and the assignment of peaks are shown in Tables 1 and 2, respectively.

The band at 3217 cm^{-1} , representing asymmetric $-\text{CH}_2-\text{N}=\text{}$ mode, showed no changes during the exposures. The bands at 2877 cm^{-1} and 2956 cm^{-1} represent the CH_3 -asymmetric vibration mode and CH_2 -asymmetric vibration mode, respectively. The band at 2877 cm^{-1} shifted to a lower frequency, indicative of alteration of the bonds by the UV irradiation. The asymmetric CH_2 -component of glycine at 2956 cm^{-1} did not change, suggesting that UV radiation did not affect this structure. The amide B band of collagen, usually found at 3080 cm^{-1} [18], showed no changes after exposure to UV radiation.

Several components of the amide A part of the spectrum have direct relationships with changes in collagen triple helix and hydrogen bonding patterns. The bands centered at 3500 cm^{-1} and 3430 cm^{-1} relate to hydrogen bonded water [19]. After UV treatment the position of the bands shifted to lower frequency. The shift was caused by thermal evaporation of water molecules, evident at 3500 cm^{-1} (intermolecular water), and the disruption of hydrogen bonds that support the collagen helix, at 3430 cm^{-1} (intramolecular water). It was suggested that water molecules released from binding sites by loss of hydrogen bonds migrated to the surface of the collagen film by diffusion [19]. Water molecules bound in shallow regions of a film leave the surface earlier than those molecules bound in deeper locations. Loss of hydrogen bound water is a first step towards collagen degradation. The collagen triple helical conformation changes to random coil with increase a dose of UV radiation.

The behavior of the water related peaks in the $3500\text{--}3400\text{ cm}^{-1}$ region of the FTIR spectrum is complicated due to various water bridges involved in the aggregation of collagen molecules, as well water molecules involved in triple helix formation and stabilization. Four types of water bridges have been investigated by Berman and coworkers in collagenous peptides: (a) α (intrachain bridge between the Hyp(Y) C=O group and the Gly C=O group), (b) β (interchain bridge between the Hyp(Y) C=O group and the Gly C=O group), (c) γ (intrachain bridge between the Hyp(Y) OH group and the Gly C=O group), and (d) δ (interchain bridge between the Hyp(Y) OH group and the Hyp(Y) C=O group) bridges [21]. Kawahara and co-workers [22] designated three other types of water bridges from a (Hyp-Hyp-Gly)₁₀ peptide: (a) interchain bridge between the Hyp(X) OH groups and the Gly C=O groups, named the κ bridge; (b) an intrachain bridge between the Hyp(X) OH groups and the Hyp(Y) OH groups, named the λ bridge; and (c) the μ bridge -an interchain bridge between the Hyp(X) OH groups and the Hyp(Y) OH groups [22]. Different numbers of water molecules are involved in each water bridge. This adds complexity in the protein behavior and responses to UV, as well as in the interpretation of the amide A region of the FTIR spectrum, since the disruption of hydrogen bonds varies with different types of water bridges. According to Kawahara [22], one to three water molecules are involved in each intrachain bridge and two or three are involved in each interchain bridge. For example, two or three water molecules form the κ_2 and κ_3 interchain bridges. The interchain and intrachain water bridges in collagen are illustrated in Fig. 2.

3.2.2 The behavior of Amide I band components—The amide I band located between 1600 and 1700 cm^{-1} is useful for infrared spectroscopic analysis of the secondary

structure of proteins [17]. The amide I band represents the vibration of amide carbonyls along the polypeptide backbone. The complexity of this peak is due to the coupling of carbonyl stretching modes and heterogeneity among the backbone carbonyl groups [18]. The decomposition of the collagen amide I band revealed five components (Fig. 3A and Fig. 3B). Both native and denatured forms of collagen possess these band components centered at 1631 cm^{-1} , 1646 cm^{-1} , 1660 cm^{-1} , 1674 cm^{-1} and 1690 cm^{-1} . Despite the fact that both forms of collagen possess the same components bands, the behavior differs when treated with UV-254 nm. Figure 3C provides a comparison of FTIR spectra of the amide I and amide II regions for native and heat-denatured forms of the rat tail collagen type I before exposure to UV-254 nm. The main difference in the spectra is band intensity. Native collagen possesses an intense band at 1660 cm^{-1} , whereas heat-denatured collagen possesses an intense band at 1635 cm^{-1} . The band at 1660 cm^{-1} relates to the collagen native triple helix [17].

When native and heat-denatured forms of collagen were subjected to various doses of UV-254 nm, the components of the amide I region underwent changes related to structural modifications in the molecules. Changes in the amide I band of native collagen and heat-denatured collagen films are shown in Table 3. The amide I component at 1690 cm^{-1} has been attributed to helices of aggregated collagen-like peptides [23]. According to Muyonga [23] and Brodsky [24], this peak vanishes with hydration of collagen or gelatin. After treatment with UV, the 1690 cm^{-1} component became prominent and the peak area increased for both native and heat-denatured collagens. This observation suggests that dehydration of collagen takes place upon exposure to UV-254 nm, leading to the disruption of the aggregated collagen fibrils.

The band centered at 1674 cm^{-1} is attributed to β -turns of the C- and N-telopeptides in collagen [17]. The β -turn conformation is important related to the accessibility of lysine for hydroxylation and subsequent crosslink formation. The ionic region of the α_1 N-telopeptide (7DEKS_{10}) forms a β -turn fold that puts K_9 into a conformation in which it can be a substrate for hydroxylation by lysyl hydroxylase and further oxidation by lysyl oxidase. In the α_2 chain of collagen, the N-telopeptide has lysine in the sequence 3DAKG_6 that adopts a β -turn conformation so lysine can be hydroxylated [17]. After treatment of native collagen with UV-254 nm radiation, the band intensity at 1674 cm^{-1} increased, suggesting distortion of the ordered state. Denatured collagen also showed a slight increase in absorbance of this band after irradiation with UV-254 nm. At the same time, the position of this band shifted slightly to the higher wave number.

UV treated collagen demonstrated an increase in peak area at 1646 cm^{-1} . This peak is assigned to random coil structure [23]. With an increase of a radiation dose the integrated area of the band at 1646 cm^{-1} increased, suggesting helix-coil transformation of collagen. The native amide I peak at 1660 cm^{-1} represents a helix-related hydrogen-bonded set of carbonyls. According to Payne and Veis [17], the highest frequency carbonyl peak in native collagen represents the weakest H-bonded system, the $\text{C}_2=\text{O}_2$ group ($\text{C}_2=\text{O}_2$ of $\text{X}---\text{H}_4-\text{N}_4$ of Gly). When collagen undergoes heat denaturation, $\text{C}_2=\text{O}_2$ is hydrogen bonded to water, leading to the reciprocal enhancement of the $1633/1635\text{ cm}^{-1}$ band and depletion of the peak at 1660 cm^{-1} . These H-bonds in the denatured state are considerably stronger than in the native state. In the present study, the intensity of the peak at 1660 cm^{-1} decreased after initial irradiation. However, with an increase of dose of UV radiation the integrated area of the peak increased. This can be explained based on the observation that the average random chain contribution of the non-Pro, non-Hyp amino acid residues in the X and Y positions of collagen generate a peak at 1660 cm^{-1} [17]. At the same time, the intact α -helices are known to have their absorption maxima at $1650\text{--}1655\text{ cm}^{-1}$. Upon disruption to the random chain form the absorption maximum shifts to higher frequency, 1665 cm^{-1} . This can also

contribute to peaks at 1660 and 1674 cm^{-1} as observed in the FTIR spectra. The assignment of amide I band components is shown in Table 4.

The peak at 1633 cm^{-1} can be assigned as an amide band in the α -helix, since its integral absorbance decreased. According to Payne and Veis [17], a band at 1633 cm^{-1} represents a collagen left-handed helix in denatured state that has bound water. After UV treatment, water is no longer bound to the α -helix and the depletion of the integral absorbance of the helical peak is observed for both forms of collagen.

3.2.3 The behavior of Amide II band components—Amide II absorption consists of amide N–H bending vibrations (60%) and C–N stretching vibrations (40%) [23]. Deconvolution of the amide II bands reveals four distinct components. The amide groups of the triple helical state are associated with a peak at 1549 cm^{-1} . With increase of dose of UV radiation, the absorbance of the amide II peak at 1546 cm^{-1} decreased [18]. Hydrogen abstraction or structural scission of –N–H was assigned to this event [19]. The assignments of the band components for amide II are shown in Table 5. The changes in integral absorbance of the amide II band components are shown in Table 6. The peak at 1530 cm^{-1} represents the disordered structure of collagen [25]. The 1530 cm^{-1} originates from the formation of imide bonds through free radical mechanisms. With an increase of irradiation dose the band area at 1530 cm^{-1} increased, suggesting helix-random coil transformations. Carboxyl groups from glutamic and aspartic acids contribute a peak at 1565 cm^{-1} . With increased irradiation time decarboxylation of carboxylate anions related to these glutamic and aspartic acids occurs. However, due to free radical reactions that take place when the collagen is exposed to UV radiation, carbonyl groups are also formed and contribute to the peak at 1565 cm^{-1} . Tyrosine ring vibrations are located at 1515 cm^{-1} in the amide II region. The increase in intensity at 1515 cm^{-1} is attributed to the formation of tyrosine from phenylalanine. Formation of tyrosine is an initial step in the photo-aging of collagen [20]. Thus, the changes in amide II bands are also impacted when collagens were subjected to UV-254 nm radiation.

3.3 Polyacrylamide Gel Electrophoresis

The SDS-PAGE images of the native collagen type I depicts several distinct bands that reflect collagen composition as a heterotrimer composed of two identical α_1 -chains and one α_2 -chain with molecular weights around 139 kDa and 129 kDa, respectively [26]. Migration of reduced collagen type I generates three bands on a gel; namely, the monomeric α_1 and α_2 bands with a 2:1 ratio, the β -band, and the γ -band [26]. The β -band of collagen is located at 250 kDa and is a dimer that represents α_1 and α_2 -chains together or two α_1 -chains (Fig. 4A). A γ -band of collagen type I appears at the top part of the gel and represents three α -chains together [27]. SDS PAGE stained with SimplyBlue SafeStain (Fig. 4B) and SilverXpress (Fig. 4A and Fig. 5B) showed normal migration patterns for the native and heat-denatured collagens, while the irradiated samples had different migration patterns reflecting a gradual reduction in the α -band, β -band, and γ -bands. The γ -band appeared only in unirradiated samples indicating that the crosslinks providing trimeric bands underwent cleavage first. Furthermore, the presence of two α -bands and a β -band in the lanes of the irradiated specimens did not appear to be as strong as in the unirradiated controls, despite similar amounts of solubilized collagen loaded in all lanes. This result indicates that the collagen molecules were cleaved along their backbones so that the number of intact collagen molecules was diminished. The lanes of irradiated specimens exhibited a smear indicating extensive cleavage along the collagen backbone. Heat denatured collagen showed a reduced number of high molecular weight bands before irradiation with UV-254 nm (Fig. 4C). Since heat denatured collagen is not in the triple helix conformation, the γ -band does not appear on the gels. With increase in radiation dose extensive backbone cleavage occurs, resulting in

the generation of peptides with low molecular weights and the disappearance of the two α -bands and one β -band. The results indicate that when native collagen was heat-denatured before exposure to UV-254 nm it became more susceptible to peptide scission reactions, resulting in extensive cleavage of the collagen backbone.

3.4 Atomic Force Microscopy

To assess changes in mechanical features as a functional evaluation of impact of the irradiation, unirradiated and irradiated samples of native and heat-denatured collagen films were investigated using Atomic Force Microscopy (AFM). A series of parallel lines were patterned via an AFM tip and imaged in tapping mode at a scan rate of 1.0 Hz. A force of 0.50 nN was applied on the films to conduct the scratch tests. Fig. 5 shows topographic and phase images of native and heat-denatured collagen films before treatment with UV radiation. The topographic images provide information on the distance between the scanner and each lateral data point of a sample, while the phase images distinguish between domains with different surface properties [28]. Differences in phase indicate differences in material properties between irradiated vs. native collagen films [29]. When irradiated collagen was present in its native conformation the average line width was 117 ± 2.3 nm and the average height was 8.24 ± 0.3 nm. In contrast, when the native collagen films were irradiated with UV-254 nm for 80 hours (187 J/cm^2) different line patterns were observed. The average line height did not change significantly and was 7 ± 0.4 nm, whereas the average line width in the irradiated sample decreased to 83.7 ± 12 nm. Heat-denatured collagen showed similar scratching patterns when compared with the native collagen films. Before irradiation the average line width was 119 ± 4.2 nm and average height was 4 ± 2 nm. The scratched lines on the heat denatured unirradiated collagen films differed when compared to the native unirradiated collagen. These results suggest that with same amount of force applied to the AFM tip, it was more difficult to scratch the unirradiated material. However, when the same sample of heat denatured collagen was exposed to UV-254 nm with a radiation dose 134 J/cm^2 , the depth of the scratched lines increased to 14 ± 6 nm, while line width decreased in line width (84.6 ± 11 nm). Table 7 summarizes the surface characteristics of the scratched lines on the various collagen films. The AFM phase and topological data revealed differences among unirradiated and irradiated samples treated under thermal and ultraviolet conditions, indicating that collagenous materials become weaker from a mechanical perspective after treatment.

3.5 Reactions leading to collagen damage

3.5.1 Generation of free radicals—The experimental results suggest that intermolecular hydrogen bonds break first, followed by collapse of intramolecular hydrogen bonds involved in the triple helix conformation. The loss of hydrogen bonds is accompanied by hydrogen abstraction through free radical mechanisms [30]. Fig. 6 depicts these stages of collagen degradation. The result of dehydration is the separation of three α chains of collagen, which then assume random coil conformations. Peptide bond scission occurs through free radical mechanisms that lead to degradation. Free radicals are generated in chemical and biological systems by either direct cleavage of bonds or by electron transfer reactions. The former process predominates when the system is exposed to energetic radiation (e.g. γ -radiation, UV light). In most biological systems radicals are generated by electron transfer reactions [30]. Radicals are generally short-lived and react rapidly with a number of targets, often generating other radicals. The reactive free radicals and reactive oxygen species involve the formation of superoxide anion ($\text{O}_2^{\cdot-}$), non-radical hydrogen peroxide (H_2O_2), and hydroxyl radical ($\text{HO}\cdot$). For example, hydroxyl radicals are non-selective strong oxidizers and attack organic compounds through hydrogen abstraction in alkenes or alcohols, or by adding to double bonds in the case of aromatic compounds [31]. Radicals undergo variety of reactions including hydrogen abstraction, electron transfer (oxidation or reduction of the substrate),

addition, fragmentation and rearrangement, dimerization, disproportionation and substitution (concerted addition and elimination) with amino acids, peptides and proteins [32].

A wide range of different radicals can be formed upon the reaction between a protein and an attacking radical. This is due to the varied nature of the amino acid side chains which offer multiple sites of attack, in addition to attack on the peptide backbone. The nature of the radicals formed in peptides and proteins depends on the attacking radical. Thus, electrophilic radicals such as hydroxyl and alkoxy radicals, preferentially oxidize electron-rich sites, whereas nucleophilic species (such as phenyl and many other carbon-centered radicals) attack electron-deficient sites. Since collagen is rich in glycine, this amino acid is favorable for the formation of a secondary α -carbon radical which is more stable than the tertiary radicals formed from other amino acids [32]. However, theoretical calculations have shown that the stability of α -carbon radicals varies with secondary structure as a result of the constraints that such structure plays on the geometry of the α -carbon radical [33]. These species are therefore less stable, and attack at the α -carbon would be expected to be less favorable, when present in a sheet or helix conformation. Furthermore, secondary and tertiary structures may play a significant role in blocking access of radicals present in bulk solution to backbone sites as a result of the outward protrusion of the side chains [34]. Thus side chain reactions may play a more important role in the chemistry of intact globular or sheet proteins than in the chemistry of disordered structures or small random coil peptides, or analogous to the present study – native vs. denatured states of collagen.

3.5.2 Propagation of radical damage in collagen—The reaction of most radicals with peptides or proteins results in the initial formation of carbon-centered radicals at side chains or α -carbon sites [30]. These species can be formed via hydrogen abstraction from C-H bonds (side chain or α -carbon) or from radical addition to an aromatic ring. Carbon-centered radicals can also be generated via secondary reactions of other species, such as reactions of alkoxy, peroxy, or nitrogen-centered radicals. Next, most carbon-centered radicals react rapidly with O_2 to generate peroxy species [34]. Peroxy radicals with α -substituted heteroatoms can undergo rapid unimolecular elimination of $HOO\cdot/O_2^{\cdot-}$ [35]. Unimolecular elimination occurs with side chains containing either α -hydroxyl or α -amino groups. For example, carbon-centered radicals formed at C-6 on lysine side chains react rapidly with O_2 to give peroxy radicals which readily eliminate NH^+4 and $HOO\cdot$ to yield α -aminoadipate- δ -semialdehyde [30].

Similar reactions occur with α -amides involved in backbone damage [36]. The elimination reactions may be the key to protein chain oxidation, as they result in damage to one amino acid and the release of the radicals that can propagate further damage. Alkoxy radicals can be generated from peroxy radicals via tetroxides or one-electron reduction of alkyl hydroperoxides [36]. Alkoxy radicals undergo rapid addition and hydrogen abstraction reactions, as well as unimolecular fragmentation and rearrangements [37]. Most primary and secondary alkoxy radicals undergo a rapid 1,2-hydrogen shift, which results in the formation of α -hydroxyalkyl radicals. 1,2-hydrogen shift competes with intra-1,5-hydrogen shift and intermolecular hydrogen abstraction to generate alcohols [30]. β -fragmentation reactions occur with tertiary alkoxy radicals, where 1,2-hydrogen shift reactions cannot take place, and with primary or secondary alkoxy radicals where particularly stable carbon-centered radical and aldehyde or ketone are formed [38]. α -carbon hydrogen abstraction accounts for more than 90% of the radicals formed from series of alanine-derived peptides upon reaction with hydroxyalkyl radicals. α -carbon radicals have greater stability over the primary alkyl radicals formed by hydrogen atom abstraction of methyl side chains [33]. The same statement is true for the glycine residues which are found in collagen. However, the yield of such backbone-derived radicals decreases markedly when there is a presence of side chains that form stabilized radicals and behave as steric factors [34]. α -Carbon radicals

decay mainly by dimerization in the absence of oxygen. When oxygen is present peroxy and alkoxy species are generated [36]. α -carbon peroxy radicals undergo complex series of reactions resulting in backbone cleavage. These species have been assumed to rapidly eliminate peroxy radicals to generate acyl imines that subsequently react with water to form the corresponding amides and carbonyl compounds. Hydrogen atom abstraction by backbone α -carbon peroxy radicals yields α -carbon hydroperoxides, whereas cross-termination reactions with oxygen and peroxy radicals yield alkoxy radicals via reactions analogous to those that take place on side chains [35]. The hydroperoxides can undergo both thermal or UV catalyzed decomposition to give further alkoxy radicals that undergo rapid β -scission (fragmentation), leading to the formation of carbonyl groups and acyl radicals of the partial structure $\cdot\text{C}(\text{O})$ [39]. When C-terminal α -carbon alkoxy radicals participate in reactions, $\text{CO}_2\cdot^-$ (or $\cdot\text{C}(\text{O})\text{NH}_2$ in the case of C-terminal amides) will be released. The alkoxy radical pathway can be important in the generation of free radical species. Fragments with new N-termini, as opposed to products with blocked N-termini that arise via the imine pathway, have been detected with oxidized proteins; these materials may be alkoxy radical β -scission products [30]. Fig. 7 summarizes the major reactions on collagen α -chains contributing to the degradation based on the above mechanistic interpretations from literature.

Conclusion

All levels of collagen structural organization undergo changes after exposure to UV-254nm radiation. The native collagen triple helix is the first structural target of UV-254 nm radiation. During the early stage of collagen degradation, characterized by transition from collagen triple helix to random coil conformation and associated with water loss; the triple helix helps protect single collagen chains against peptide scission. Once the collagen triple helix is destroyed, extensive peptide bond cleavage occurs via free radical reactions (Fig. 7). This process is confirmed by comparing degradation rates of native collagen vs. heat-denatured collagen. The free radicals interact with other collagen molecules and water, and propagate impact on collagen degradation. Active hydroxyl radicals derived from water surrounding the collagen molecules and hydroxyl groups along the collagen backbone interact with the collagen macromolecules and generate new radicals and macroradicals. Radiolytically generated hydroxyl species in the presence of oxygen cause damage for all biological molecules.

FTIR, AFM and SDS-PAGE studies confirmed the correlation between collagen structural hierarchy and the negative effects of UV radiation. The results suggest that the harmful effects of electromagnetic radiation on biologically relevant extracellular matrices (collagen in the present study) are important to assess in the context of the polymer structural state – with implications in tissue remodeling, wound repair and disease progression.

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Fig. 1.
The components of collagen FTIR spectra (2800–3500 cm^{-1} region).

Fig. 2.

Types of water bridges (from Kawahara et al., 2005). (A) Interchain κ_2 bridge between the Hyp(X) OH group and the Gly C=O group; (B) Intrachain λ_1 bridge between the Hyp(X) OH group and the Hyp(Y) C=O group. Dotted lines (yellow) designate the hydrogen bonds. Water molecules (blue) which are involved in the water bridge are labeled by W.

Fig. 3. (A) The deconvoluted amide I and amide II absorbance bands of native collagen films; (B) The deconvoluted amide I and amide II absorbance bands of heat-denatured collagen films; (C) Comparison of amide I and amide II absorption bands of native and heat-denatured collagen films.

Fig. 4.

(A) SDS tris-glycine gel of native (NC) collagen type I stained with SilverXpress before and after UV254nm irradiation (hours). (B) SDS bis-tris gel of native (NC) collagen type I stained with SimplyBlue. (C) SDS tris-glycine gel of heat-denatured collagen type I stained with SilverXpress before and after UV254 nm irradiation (hours). SeeBlue Plus2 was used as a protein ladder.

Fig. 5. AFM topology (A,C) and phase (B,D) images of native (top) and heat-denatured (bottom) collagen films before treatment with UV254 nm. Each image is 5 microns along the edge.

Fig. 6.
Potential mechanism of collagen degradation by UV-254.

Fig. 7.
Potential mechanisms of collagen backbone cleavage.

Table 1

Changes of the frequencies of bands contributing to the 2800–3500 cm^{-1} region

Peak locations and percent area contributions of deconvoluted bands of the 2800–3500 cm^{-1} region																
Dose J/ cm^2	cm^{-1}	%	cm^{-1}	%	cm^{-1}	%	cm^{-1}	%	cm^{-1}	%	cm^{-1}	%	cm^{-1}	%	cm^{-1}	%
	1		2		3		4		5		6		7		8	
0	2877	1	2946	5	3080	6	3217	25	3322	41	3431	11	3508	6	3577	3
16	2878	2	2947	5	3079	6	3217	23	3317	40	3419	9	3476	5	3528	3
52	2875	1	2947	6	3080	9	3219	24	3319	39	3414	10	3473	6	3524	3
170	2871	3	2949	6	3078	7	3215	23	3316	41	3417	10	3478	6	3536	4
222	2871	2	2947	7	3079	8	3219	24	3317	41	3413	9	3470	5	3521	3

Table 2

Assignment of bands of the 2800–3500 cm⁻¹ region

Region	Position cm ⁻¹	Assignment	Comments
Water	3500–3470	Intermolecular hydrogen bounds	Intermolecular water
	3450	NH stretch coupled with H-bond from H ₂ O	Hydrogen bonds of retain water. The desorption of water is a two stage process: H ₂ O is released from the binding sites by breakage of H-bonds, and than H ₂ O reaches the surface of collagen by diffusion.
Amide A	3330–3325	N-H stretch	The smaller the structural order the lower frequency.
	3217–3180	Asymmetric –CH ₂ -N=	The shift is attributed to disruption of H- bonds and scission of amides or broken N-H bonds from side chains.
Amide B	3080	N-H stretch	Pro and Hyp side chains
	2946	CH ₂ - stretching of methylen	N-H bonds
		CH ₃ - stretching of methyl (assimetric)	Gly backbone and proline side chains
			Side chains of amino acids

Table 3

Changes in the relative integral absorbance of the amide I band components of native collagen (NC) and heat-denatured collagen (DC)

DOSE	Component peak location (cm ⁻¹), relative mean of percent area (%) contribution of total band, and standard deviation of a mean																								
	J/cm ²	cm ⁻¹	%	cm ⁻¹	%	cm ⁻¹	%	cm ⁻¹	%	cm ⁻¹	%	cm ⁻¹	%	cm ⁻¹	%	cm ⁻¹	%	cm ⁻¹	%	cm ⁻¹	%	cm ⁻¹	%	cm ⁻¹	
	NC	DC	NC	DC	NC	DC	NC	DC	NC	DC	NC	DC	NC	DC	NC	DC	NC	DC	NC	DC	NC	DC	NC	DC	
0	1631	55	1628	38	1646	17	1646	27	1660	20	1660	33	1674	7	1672	13	1690	1	1685	3	1672	13	1690	1	1685
5	1631	52	1628	45	1646	22	1644	23	1661	19	1661	25	1675	7	1676	5	1691	0	1690	1	1675	7	1676	5	1691
47	1630	38	1630	30	1645	22	1644	25	1661	24	1661	32	1675	12	1675	10	1690	4	1688	3	1675	12	1675	10	1690
94	1631	36	1631	27	1647	24	1646	28	1660	24	1661	31	1674	12	1673	11	1690	4	1686	3	1674	12	1673	11	1690
187	1630	11	1631	25	1645	29	1645	27	1660	32	1659	24	1674	20	1669	15	1689	8	1681	9	1674	20	1669	15	1689

Table 4

Assignment of the band components of the amide I region

POSITION cm ⁻¹	Assignment
1690	Intermolecular association. Helices of aggregated collagen-like peptides
1675	β-turns
1660	Collagen in triple helix, with contribution from α-helix and beta-turns (protein amide stretching vibration of C=O) Random chain contribution of the non-proline and non-hydroxyproline
1646–1650	Random coil conformation: imide residues (amide I in random coil)
1630	Left-handed 3–10 helix in denaturated state
	Partially beta-sheet region

Table 5

Assignment of the band components of the amide II region

POSITION	Assignment	Comments
1565	Carboxyl groups	Side chains of D and E
1549	N-H bending vibration and C-N stretching vibration Amide II in triple helix	Collagen in triple helix
1530	Amide II in a random coil	Disordered structures due to denaturation
1515	Tyrosine side chain	Transformation of F to Y

Table 6

Location, percent area contribution and standard deviation of the amide II components before and after exposure to UV radiation

DOSE J/cm ²	Component peak location and percent area contribution of total band											
	1			2			3			4		
	cm ⁻¹	%	SD	cm ⁻¹	%	SD	cm ⁻¹	%	SD	cm ⁻¹	%	SD
0	1514	5	3	1532	34	7	1549	38	4	1565	23	5
5	1516	6	4	1531	43	20	1548	28	5	1564	23	24
47	1515	7	2	1532	39	4	1550	31	14	1566	21	11
94	1515	9	0	1530	36	7	1547	31	5	1562	23	21
187	1516	13	3	1532	42	7	1550	24	4	1565	21	13

Table 7

Dimensional changes in the scratching lines on the collagen films before and after exposure to UV-254 nm

SAMPLE	Dose J/cm²	Width nm	S.D.	Height nm	S.D.
Native collagen	0	117	2.3	8	0.3
Native collagen	187	84	13.0	7	0.5
Denatured collagen	0	120	4.3	4	2.7
Denatured collagen	134	85	11.2	14	6.8