

Multiscale characterization of solar radiation-induced structural degradation in Type I collagen

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Abstract

Collagen, the most abundant structural protein in animals, plays a crucial role in maintaining skin integrity, elasticity, and strength. Type I collagen, which predominates in the skin, is particularly vulnerable to environmental stressors, such as solar radiation. Prolonged sun exposure accelerates collagen degradation, driving skin aging and impairing tissue functionality. However, the molecular mechanisms governing these intricate processes remain unclear. In this study, we employed bovine Type I collagen as a model system to investigate the molecular alterations induced by solar radiation, focusing on changes in structure, morphology, and fibrillogenesis potential. Collagen samples were irradiated using a solar simulator that mimics the full solar spectrum to ensure standardized conditions. Structural changes at different levels, were analysed using classical spectroscopies, fluorescence lifetime imaging microscopy, and scattering-type scanning near-field optical microscopy (s-SNOM). This multimodal approach enabled both sensitive detection of molecular alterations and spatial mapping of local heterogeneities within collagen fibers. Results indicates partial destabilization of the triple-helical structure and a loss of cross-links and telopeptides, consistent with molecular misalignment. FLIM imaging on samples stained with Anilinonaphthalene-1-sulfonic acid (ANS), a gold standard fluorescent dye for the study of protein conformational transition highlighted increased sample heterogeneity and a reduction in hydrophobic regions, pointing to structural disruption which could be also related to the loss of self-assembly capabilities of collagen molecules.

Introduction

The skin is the largest organ of the human body, serving as a crucial interface between the body and the external environment. It is responsible for protecting underlying tissues from pathogens, harmful chemicals, and physical damage, while also playing vital roles in thermoregulation, sensory perception, and the synthesis of vitamin D[1]. The skin, constantly exposed to various environmental

factors, faces numerous external stresses that can significantly affect its structure and function. Among these, solar radiation is a primary driver of premature skin aging, primarily due to the photodamage sustained by various skin components that interact with light[2–4].

Among the most critical components affected by solar exposure is collagen, a key protein that provides structural integrity and elasticity to the skin[5–7]. Collagen is the most abundant protein in animals, accounting for approximately one-third of the total protein weight in the human body[7,8]. It is a large structural protein with a molecular weight of approximately 280 – 300 kDa, characterized by a hierarchical structure. Among the twenty-eight collagen types, the two most prevalent collagen types in skin are type I and type III[7,9]. Collagen type I plays a crucial role in preserving the structural integrity and mechanical strength of the skin. As the most abundant collagen type in the dermis, it constitutes approximately 80-90% of the total collagen content in this layer[7,9–11]. Its dense, fibrous network forms the backbone of the skin's extracellular matrix, providing resilience and tensile strength, which are essential for withstanding physical stresses and preserving skin elasticity.

Collagen is composed of three individual polypeptide chains, each with a distinct amino acid sequence. These chains are organized into a left-handed helix, entwined in a right-handed triple helix configuration, stabilized by interchain hydrogen bonds formed between the amino groups of glycines and the carbonyl groups of prolines[7–9]. The triple-helix structure is favored by the specific amino acid sequences of the three chains that compose it. The primary structure of each polypeptide chain consists of repeating amino acid triplets, typically in the form Gly-X-Y, where Gly represents glycine, and X and Y are often proline and hydroxyproline[7,8]. These amino acids are very frequent in the collagen molecular structure since they stabilize its triple helix both for a minimum steric effect of their residues (and glycine in particular due to the absence of a side chain) and for the capability to form intermolecular hydrogen bonds between the three polypeptide chains in the triple helix[7]. At the ends of the triple helix are the telopeptide regions, two non-helical domains constituted by the C-terminal and N-terminal of the polypeptide chains. The telopeptides are usually formed by a variety of amino acids different from the triads that almost exclusively form the triple-helix, and they have a crucial role in collagen fibrillogenesis[7,12]. In particular, the C-terminal of collagen molecules contains a hydrophobic core that interacts with specific binding sites in the triple helical structure, allowing an alignment between two adjacent triple helices[7,13]. This alignment favors the maturation of collagen fibrils and is responsible for the characteristic 67 nm D-band periodicity pattern, which can be observed via Atomic Force Microscopy (AFM) and Transmission Electron Microscopy (TEM)[14,15]. The formation and stabilization of collagen fibrils can also be attributed to the cross-links formed between adjacent collagen molecules. The telopeptides contain lysine and hydroxylysine residues that, following oxidation processes, form two pyridinium cross-link species: pyridinoline and deoxypyridinoline cross-links. These cross-links occur between the N- and C-terminals of adjacent molecules, binding two collagen molecules head-to-tail along the fibril[7,13].

Collagen cross-linking status is responsible for the chemical and mechanical properties of collagen fibers, and its modifications are associated with the onset of various pathological states in the human body[3,16–19]. Collagen organization may change in response to different stresses (e.g., chemical, mechanical, photochemical) applied to the tissues[8,20]. In particular, collagen type I is susceptible to photodamages from solar radiation[4,21,22]. The degradation of collagen caused by solar radiation can disrupt the balance of the extracellular matrix, leading to a range of harmful effects, including reduced skin elasticity and increased susceptibility to skin diseases, ultimately accelerating the aging

process[4,23,24]. The modifications can occur at different scales and, due to its characteristic hierarchical structure, alterations on the molecular level may have a cascade effect on its meso- and macro-scale structure, impairing its function. Alterations of collagen cross-links can translate into variations in the diameter and length of individual fibers [22,25,26], which irretrievably affect the organization and therefore the mechanical and chemical properties of the entire collagen network that sustains tissues and organs. However, the molecular mechanisms underlying the photodegradation of collagen caused by light on collagen remain yet partially unexplored. Thus, understanding the specific effects of solar radiation on Type I collagen is crucial for developing targeted interventions that address the core structural components most affected by solar exposure.

In this experimental study, we analyze bovine Type I collagen as a model to investigate the molecular modifications induced by solar radiation on collagen fibers and their impact on its morphology. A solar simulator replicating the solar radiation spectrum was employed to ensure standardized and controlled irradiation conditions. The spectroscopic properties of collagen were analyzed following irradiation, allowing us to gain insights into the structural and physicochemical modifications of collagen at the nano- and microscale in response to radiation. s-SNOM (scattering-type Scanning Near-field Optical Microscopy) analyses were also conducted, enabling the acquisition of IR spectra in selected ROIs with nanoscale spatial resolution. The combination of AFM measurements and IR spectra highlighted both morphological changes in the fibers and structural alterations in the triple helix after irradiation with the solar simulator. To investigate the modifications undergone by hydrophobic patches following irradiation, we utilized 8-Anilinonaphthalene-1-sulfonic acid (ANS), a well-known fluorescence probe in the literature for its ability to bind to hydrophobic regions of proteins[27–31].

Results show that, under the applied experimental conditions, collagen undergoes significant degradation due to solar radiation. This degradation results in the destruction of the telopeptide regions and partial destabilization of the triple helix, impairing collagen's ability to form fibrillar structures.

Materials and methods

Material: Bovine Collagen Type I was purchased by Advanced BioMatrix (5162). ANS (8-Anilinonaphthalene-1-sulfonic acid) was purchased by Merck (A-1028).

Sample preparation: The collagen suspension was prepared following the instructions provided by the manufacturer. 70 mg of collagen powder was reconstituted with 3.5 ml of cold milli-Q water in an ice bath, stirring continuously for 15 minutes at 1000 rpm, until the collagen was fully wetted. Following, 3.5 ml of cold HCl 0.02 M was added and the stirring increased to 1250 rpm for 25 minutes paying attention to keep the ice bath cold during the whole process. At this stage, the collagen appears to be fully suspended, forming a viscous suspension in which the larger insoluble collagen particles remain dispersed and do not settle at the bottom of the beaker. The collagen suspension thus prepared, consists of 10 mg/ml bovine collagen type I in 0.01 M HCL and it has a final pH of 2. This stock suspension was diluted with cold 0.01 M HCl to prepare suspensions at lower concentrations.

To analyze the effects of solar exposure, the sample was subjected to sunlight irradiation. The solar-treated sample was prepared by irradiating for five hours 3 ml of a 2 mg/ml collagen suspension in a quartz cuvette (1 cm pathlength), using a solar simulator lamp (Abet Technologies, model 10500), which mimics the solar spectrum. The sample was positioned at a distance from the lamp to maintain a constant intensity of 1 Sun (100 mW/cm^2). The spectrum of the solar simulator ranges from Near-Ultraviolet to Near-Infrared, having a relatively low emission in the Near-UV region, which grows at longer wavelengths, reaching its maximum in the visible range at about 500 nm, and then decreasing again in the Near-IR, according to the black body radiation of the solar spectrum.

During the irradiation, the sample was gently stirred to ensure uniform exposure. ANS was added at the selected concentration immediately before the fluorescence measurements.

Collagen films of untreated and solar-treated samples were prepared by mixing in an ice bath 50 μl of cold Milli-Q water, 200 μl of a 2 mg/ml collagen suspension, 50 μl of 10X PBS, and 2 μl of 0.5 M NaOH. The solution was then cast onto a glass coverslip, placed on ice for one hour, and subsequently incubated at 37°C overnight. Collagen films were rehydrated and stained with 13 μM ANS the day after their preparation and imaged the same day.

Fluorescence measurements: Fluorescence spectra were acquired using a Jasco-FP-8500 spectrofluorometer using a 1 cm path-length quartz cuvette. To acquire the collagen fluorescence spectra, the measurements were performed on 2 mg/ml untreated and solar-treated collagen suspensions. The excitation wavelength is 270 nm, and the acquisition range is 270 nm – 600 nm. The response time was set to 2 s, data interval to 0.5 nm, scan speed to 100 nm/min, and excitation and emission bandwidths to 5 nm.

To acquire the ANS fluorescence spectra, the measurements were performed on 1 mg/ml untreated and solar-treated collagen suspensions. The excitation wavelength is 280 nm and the acquisition range is 280 nm – 650 nm. The response time was set to 2 s, data interval to 0.5 nm, scan speed to 100 nm/min, excitation bandwidths to 10 nm, and emission bandwidths to 5 nm.

Turbidity measurements: The turbidity of the sample was evaluated by measuring the absorbance at 450 nm, a wavelength at which no absorption from the sample is expected, meaning that any non-zero signal can be attributed to the scattering of the sample. Spectra were acquired using a Jasco V-

770 spectrophotometer. Measurements were performed in 1 cm path-length quartz cuvettes on both untreated and solar-treated 2 mg/ml collagen suspensions. The absorption spectra were acquired in the range 200 nm - 500 nm. The bandwidth was set to 2 nm, the scan speed to 100 nm/min, the response time to 0.96 s, and the data interval to 0.5 nm.

CD (Circular dichroism) measurements: CD spectra were acquired using a Jasco J 1500 spectropolarimeter equipped with a Jasco CTU 100 using a 0.5 mm path-length quartz cuvette for the untreated sample and a 0.1 mm path-length quartz cuvette for the solar-treated sample. The measurements were performed on 1 mg/ml untreated and solar-treated collagen suspensions. The absorption spectra were acquired in the range 188 nm - 255 nm. The bandwidth was set to 2 nm, the scan speed to 100 nm/min, the digital integration time to 1 s, and the data interval to 0.2 nm. The CD absorption spectra were corrected by subtracting the blank and set to zero at 255 nm. Data were normalized to the minimum of the spectrum for a better comparison. Differential CD spectrum was obtained by subtracting the CD spectrum acquired for the untreated sample from the CD spectrum solar-treated one.

s-SNOM (scattering-type Scanning Near-field Optical Microscopy) measurements and analysis: s-SNOM measurements were performed at the SISSI-nano branch of SISSI beamline at Elettra Sincrotrone Trieste with the help of the Neascan microscope (Attocube) nano-FTIR instrument coupled to a DFG (Difference Frequency Generation) laser operating in the spectral region 600 cm^{-1} - 2000 cm^{-1} . To perform the measurements, 20 μl of collagen suspensions were cast onto gold-coated silicon wafers, allowed to adsorb to the surface, and left to dry for two minutes. The remaining liquid was then removed and discarded, and the sample was rinsed three times with 40 μl of Milli-Q water. A gentle nitrogen flux was used after the last washing in order to help the drying process and avoid residual water contributions. The washing cycles were necessary to avoid the interferences due to buffer salt crystals.

Measurements were performed in tapping mode thanks to the use of a conductive, 20 nm wide, specifically developed for s-SNOM measurements (neaspec). The tapping frequency was set to 265 kHz and all the measurements were performed with a free oscillation amplitude of about 100 nm. The setpoint was adjusted, after preliminary measurements, to 90 % of free amplitude. The mentioned settings ensured a penetration, at second harmonic decoupling of IR radiation around 100 nm[32,33]. Spectra were acquired and computed with neaspec postprocessing routines by using clean regions on the gold substrate as reference. Reference spectra were acquired at intervals of one hour to be able to compensate for possible dephasing of the source. Infrared spectra were acquired in the region 1350 cm^{-1} - 1800 cm^{-1} with a resolution of 4 cm^{-1} . Phase correction was manually applied on the Imaginary component of the optical signal before processing the phase spectra. Second harmonic of optical phase spectra were analyzed and used as representative of the nanoscale infrared absorption of the samples.

Two-photon microscopy: The imaging of the collagen films was performed using a Leica TCS SP5 confocal laser scanning microscope, with a 63 $\times$ /1.40 oil objective, and a scanning frequency of 400 Hz (Leica Microsystems, Germany). ANS fluorescence was excited at 780 nm (Spectra-Physics Mai-Tai Ti:Sa ultra-fast laser) and collected in the range 400 - 650 nm. The images were acquired at a resolution of 1024 $\times$ 1024 pixels.

Fluorescence Lifetime Imaging Microscopy (FLIM) measurements and analysis: FLIM measurements (time domain) were performed using a Leica TCS SP5 confocal laser scanning microscope coupled with picoHarp 300 TCSPC module (Picoquant, Germany). 256×256 pixels FLIM images were acquired with a $63\times/1.40$ oil objective and a scanning frequency of 400 Hz. ANS fluorescence was excited at 780 nm (Spectra-Physics Mai-Tai Ti:Sa, laser repetition rate is 80 MHz) and collected in the range 400 nm - 650 nm.

FLIM data were analyzed by means of the phasor approach, introduced by Digman and colleagues in 2008[34]. The phasor approach allows for a graphical representation of the fluorescence lifetime data acquired at each pixel of the FLIM image. This technique involves the mapping of the exponential fluorescence decay on a polar plot, also known as phasor plot, through the Fourier transform. The real (g) and the imaginary (s) parts of the Fourier transform are the x and the y-axis of the phasor plot, respectively. All the fluorescence decays lie within a semicircle centered at the point (0; 0.5) and with a radius of 0.5, called the “universal circle”. The universal circle extends from the point (1; 0), where shorter lifetimes ($\tau = 0$) are mapped, to the point (0; 0), where longer lifetimes ($\tau = \infty$) are mapped. The single-exponential (or single-component) decays lie on the circumference of the universal circle, while the multi-exponential (or multi-component) decays lie within it since phasors respect vector algebra.

The result of a FLIM image analyzed by the phasor approach is the formation of clouds of points in the phasor plot, called lifetime distribution, for each fluorescence lifetime measured. To spatially localize the fluorescence lifetime on the original image, these lifetime distributions may be selected by colored cursors that highlight in the same colors the pixels of the image associated with the selected phasor. This creates a new image, called “phasor map” which provides the spatial distribution of the fluorescence lifetimes.

FLIM analysis was performed by the SimFCS4 software developed at the Laboratory of Fluorescence Dynamics, University of California at Irvine (<http://www.lfd.uci.edu>). The fluorescence lifetime of fluorescein ($\tau = 4$ ns, single exponential[35]) was used to calibrate FLIM.

Result and discussion

To investigate the damage induced by solar radiation on Collagen (Type 1), we explore the molecular modifications that collagen undergoes upon irradiation using a solar simulator. The use of the solar simulator allowed us to standardize and control the experimental conditions at which the irradiation occurred (power of the source, distance, and angle of exposure). Specifically, we explored the modification in the molecular structure of collagen by analyzing its intrinsic spectroscopic properties and the fluorescence emission of 8-Anilinoanthracene-1-sulfonic acid (ANS), an extrinsic fluorescent probe used to investigate the hydrophobic patches in proteins[27–31].

Figure 1 shows the variation of spectroscopic properties of the collagen suspension upon irradiation to the solar simulator. Figure 1a shows the modification of the intrinsic fluorescence of the Collagen sample during a five-hour irradiation, and Figure 1b shows the absorbance of the sample at 450 nm, as a measurement of the sample turbidity. In Figure 1c the normalized CD spectra of the collagen before and after the five-hours of irradiation are reported. To highlight the variations in the CD spectra, the differential spectrum is shown below, calculated as the difference between the spectra of the solar-treated and untreated samples.

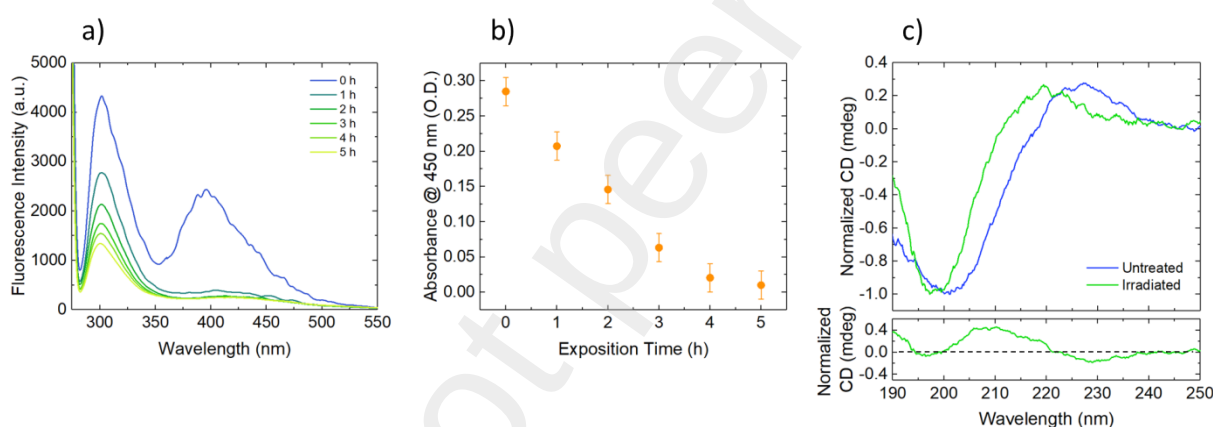


Figure 1: **a)** Fluorescence of the collagen sample during the irradiation of the solar simulator. The sample consists of 2 mg/ml Bovine Collagen Type 1 suspension in 0.01 M HCl. The fluorescence intensity of collagen decreases during exposure to the solar simulator. **b)** Absorbance of the collagen sample at 450 nm, as a measurement of its turbidity. During the exposure, the turbidity of the sample decreases. **c)** CD spectra of the untreated sample (blue) and the solar-treated sample (green) after five hours of exposure to the solar simulator. Below, the differential spectrum is calculated as the difference between the green and the blue spectra.

The fluorescence spectrum of collagen suspension before solar irradiation is shown in Figure 1a (0 h spectrum shown in blue). It shows characteristic features that resemble those reported in the literature, with two dominant peaks[36]. The first one is centered at about 300 nm, and it is associated with the aromatic residues[37], mainly tyrosines[38–40]. A second broad fluorescence peak, centered at about 400 nm, is associated with the collagen cross-link species. The fluorescent cross-link species in collagen are the pyridinolines and deoxypyridinoline[41–43], which are known to be characterized by a fluorescence emission at 400 nm[42], in line with our data. These species are formed by the covalent binding of lysine or hydroxylysine residues found in the telopeptides of collagen molecules. Thus, their formation links together adjacent type-I collagen triple helices at N and C terminals[44,45]. Similarly, tyrosine residues are almost exclusively found only in the telopeptide regions of the collagen[46–49].

Monitoring the changes occurring in the fluorescence spectrum at different time points during the irradiation provides information on the overall state of the collagen molecules and, in particular, on the telopeptide regions of the collagen chains.

During the irradiation process, the overall collagen fluorescence decreases, suggesting the possible alteration or disruption of the aromatic species responsible for its optical properties. Notably, the two fluorescence bands exhibit distinct behaviors: the intensity of the tyrosine-associated peak (300 nm) gradually declines, while the band linked to cross-linking species (400 nm) drops abruptly, nearly disappearing within the first hour and stabilizing after the third. This effect suggests that cross-link species are more sensitive to the solar treatment with respect to the tyrosine residues. Furthermore, the initial spectrum (shown in blue) is noisy due to the presence of insoluble supramolecular assemblies that diffuse within the sample. After the first hour of irradiation, the distortion attributable to large species diffusion is greatly reduced, suggesting the breaking of the larger insoluble species, due to the solar simulator irradiation.

The observed effects on collagen are a combination of photodegradation effects due to the Near-UV and the shorter visible wavelengths and the thermal effects due to the Near-IR and the longer visible wavelengths. As a reference, an analogous collagen sample was irradiated by a 270 nm UV light. The modifications in collagen fluorescence are reported in Figure S1. Unlike the solar-treated sample, the cross-link fluorescence of the UV-treated sample gradually decreases in parallel with the tyrosine fluorescence peak.

Turbidity measurements were acquired as the absorbance at 450 nm, i.e. where no absorption is expected from the sample. As shown in Figure 1b, the turbidity of the sample gradually decreases during irradiation with the solar simulator. This reduction, consistent with the previously hypothesized disintegration of collagen clusters within the sample. Irradiation may therefore enhance solubility and lead to an overall loss of the supramolecular structure. The increased solubility of the collagen structures in solution is in accordance with the literature that reports how the absence of telopeptides in collagen molecules increases its solubility[49], thus indicating that the solar radiation affects the non-triple helical region of the collagen responsible for the formation of the fibers.

CD spectra of the sample before and after the irradiation are shown in Figure 1c. The CD spectrum of the untreated collagen sample (shown in blue) presents the characteristic features of the collagen triple helix, showing a positive signal centered at about 225 nm and a negative peak at 200 nm[50–52]. At the end of irradiation treatment, the CD spectrum (green) undergoes significant modifications: the positive component shifts to 220 nm. A significant reduction of the negative component at 210 nm is observed, and the minimum peak shifts towards shorter wavelengths. These modifications reflect an alteration of the collagen triple helical structure, suggesting its partial substitution with a random coil structure[53–55]. Interestingly, the observed CD spectrum is different from the CD spectra of denatured collagen reported in the literature[56–58]. To highlight the differences between the untreated and the solar-treated samples the differential spectrum between the two CD spectra was calculated and reported in the lower panel of Figure 1c. From the differential spectrum, it is possible to observe the increase of a large positive component at 210 nm and a smaller negative component at 230 nm. The rise of the 210 nm component is in line with the modification of the secondary structure in favor of random coil, while the reduction of the signal at 230 nm can be attributed to a reduction of the aromatic patches[59], in accordance with the modification in the

telo peptide regions. In the present conditions not only collagen denaturation occurs but a rearrangement of the overall structure due to the reduction of aromatic packing also occurs. This being in line with what is observed in figure 1a).

To gain deeper insights into the molecular-level alterations occurring in the hydrophobic regions of collagen structures, the spectroscopic properties of ANS can be exploited. ANS is a small fluorescent probe that binds to protein hydrophobic regions in proteins[27,28] and it is known to bind to collagen[60,61]. In particular, Del Pozo and colleagues[60] observed that ANS primarily binds to collagen telopeptides, which are highly hydrophobic due to the presence of tyrosine residues[49]. Changes in ANS fluorescence intensity or spectral shifts may reveal subtle modifications in the local environment of the hydrophobic regions of proteins. In particular, a blue shift of the ANS fluorescence band denotes a more hydrophobic environment. At the same time, a spectral redshift may indicate that ANS experiences a more hydrophilic environment, being, for instance, more exposed to the solvent. Thus, monitoring ANS fluorescence variations can be correlated with specific molecular events.

In Figure 2 the results of titration experiments of ANS in collagen suspensions are reported. Figure 2a and 2b show the fluorescence of ANS at increasing concentrations, ranging from 0.4 μM to 11.7 μM , at fixed collagen concentrations, respectively in the untreated and solar-treated collagen suspension. Figure 2c and 2d respectively display the area and the centroid of the ANS fluorescence band as functions of its concentration for both the untreated and the solar simulator irradiated collagen sample.

ANS titration experiments were performed at a fixed collagen concentration for both the untreated and the solar simulator irradiated collagen suspension. In Figure 2a, the results of the experiment on the untreated sample are shown. Similar results were obtained for the treated sample reported in Figure 2b. The fluorescence band of ANS partially overlaps with the fluorescence band of collagen at 400 nm, as shown in Figure 1a. To isolate the contribution of ANS fluorescence from that of collagen, we carefully subtracted the collagen signal from all ANS spectra.

As the concentration of ANS increased, its fluorescence intensity also increased, reaching a maximum at 7.5 μM . Above this concentration, the fluorescence intensity remained nearly constant. Furthermore, as the ANS concentration increased, its fluorescence band exhibited a red shift. The maximum of the first ANS fluorescence spectrum, measured at 0.4 μM , is positioned at about 465 nm, while the maximum of the last spectrum, measured at 11.7 μM , shifted to around 480 nm, showing a 15 nm shift.

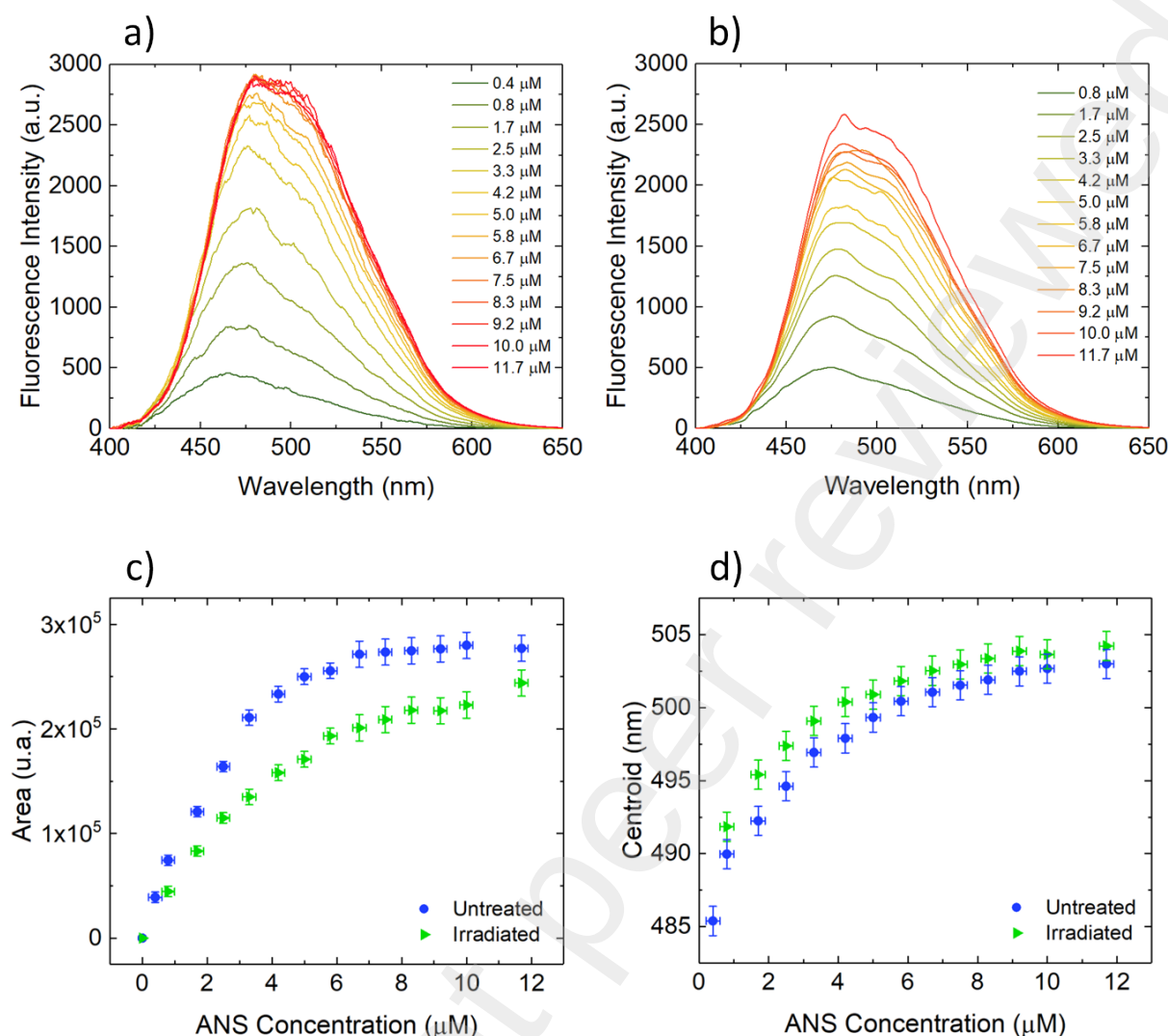


Figure 2: **a)** ANS fluorescence spectra acquired in 1 mg/ml untreated collagen suspension in 0.01 M HCl at increasing ANS concentration from 0.4 μM to 11.7 μM ($\lambda_{\text{exc}} = 280 \text{ nm}$, detection range = 280 nm - 650 nm). **b)** ANS fluorescence spectra acquired in 1 mg/ml solar-treated collagen suspension in 0.01 M HCl at increasing ANS concentration from 0.4 μM to 11.7 μM ($\lambda_{\text{exc}} = 280 \text{ nm}$, detection range = 280 nm - 650 nm). **c)** ANS fluorescence band area as a function of the ANS concentration for the titration experiment in the untreated (blue) and solar-treated (green) collagen suspension. Data present a linear region followed by a plateau region. The maximum fluorescence measured at the plateau region in the solar-treated sample is lower than that one in the untreated sample, denoting a reduction of binding sites number/affinity after the solar exposure. **d)** Centroid of the ANS fluorescence band as a function of the ANS concentration in the untreated (blue) and solar-treated (green) samples. ANS fluorescence bands redshift indicating that ANS experiences a more polar environment at increasing concentration in both samples.

The fluorescence peak intensity (measured as the peak area) is reported as a function of ANS concentration in Figure 2c. In the untreated sample, a linear trend is observable between 0 μM and 4 μM and then the signal reaches a plateau region above 7 μM of ANS. The irradiated sample presents reduced intensity at each concentration with respect to the untreated sample possibly indicating a reduction of the number of the binding sites and/or an overall lower affinity/accessibility of the solar-treated collagen for ANS, in line with the structural and chemical modifications in both the telopeptides and the triple helix regions of the collagen molecules after the irradiation.

The average positions (centroids) of the fluorescence peaks are reported in Figure 2d for the untreated and solar-treated samples. A similar red shift is observed, suggesting that at increasing concentrations, the dye experiences a more polar environment, possibly due to greater exposure to the solvent. This shift suggests that the increasing concentration of ANS results in the saturation of its binding sites on collagen. As reported by del Pozo and colleagues[60], collagen presents a single primary binding site for ANS, located in the hydrophobic regions of the telopeptides, which, however, presents different affinities. The collagen triple helix is predominantly composed of glycine, proline, and hydroxyproline, which are non-polar amino acids, arranged in a repeating triplet sequence (Gly-X-Y), where glycine (Gly) is the first amino acid in the triplet and X and Y are typically proline (Pro) and hydroxyproline (Hyp), respectively[7]. This arrangement forms the characteristic three-stranded helix, with glycine providing flexibility and allowing the tight packing of the three polypeptide chains. It is plausible that ANS may bind to the triple helix after saturating the primary binding site. However, no such interaction has been confirmed in the literature.

From Figure 2d it can be noted that the ANS fluorescence measured in the treated sample is consistently more red-shifted compared to the untreated sample, in line with a modification of the original binding sites towards the formation of less specific ones and the overall increase in polarity, in line with more soluble species. However, both samples display a similar trend, and above 7 μM , coinciding with the concentration where the plateau regions occur in both samples, no relevant red shift of the fluorescence band can be detected, indicating that all the binding sites are completely saturated. To clarify whether the differences in fluorescence intensity arise from actual modifications of the ANS binding sites or merely from a reduction in their number, the fluorescence lifetime of ANS was analyzed. For this purpose, FLIM (Fluorescence Lifetime Imaging Microscopy) images were acquired, and the results were analyzed using the phasor approach[34]. This method provides a graphical representation and an estimation of fluorescence lifetimes without the need for fitting or the use of an a priori model. Figure 3 shows the analysis of FLIM data using the phasor approach. Measurements were performed on the untreated sample, shown in Figure 3a - 3e, and on the treated sample, shown in Figure 3f - 3j. Two ANS concentrations were used to explore two different ANS-collagen binding regimes. The selected concentrations for this investigation were 2.5 μM , corresponding to the linear region, which is mainly associated with the more hydrophobic and more accessible binding site, and 15 μM , i.e. in excess with respect critical concentration characterizing the saturation region, to investigate the less hydrophobic regions of collagen. To improve the signal-to-noise ratio, the collagen concentration was doubled with respect to the bulk experiment, from 1 mg/ml to 2 mg/ml. To maintain a constant collagen-to-ANS ratio, the concentration of ANS was also doubled, resulting in concentrations of 5 μM (linear region) and 30 μM (saturation).

Figure 3a and 3c show 256x256-pixels representative fluorescence intensity images of the untreated collagen sample stained with 5 μM and 30 μM respectively. Figure 3b and 3d show the phasor maps for the same samples obtained from the phasor plot reported in Figure 3e. The collagen supramolecular structure which can be observed in the intensity images is characterized by a relatively low and homogeneous fluorescence intensity at both the ANS concentrations. As shown in the phasor plot, each sample is characterized by a broad fluorescence lifetime distribution, with its center shifting at different dye concentrations. In particular, the longer lifetime, closer to the point with coordinates (0,0), is highlighted with a red circle, while the shorter lifetime is marked in green. The corresponding pixels are mapped using the same colors in the phasor map. Notably, measurements on samples with

lower ANS concentrations show a uniform fluorescence lifetime distribution, corresponding to the red-selected region. In contrast, samples at higher concentrations exhibit a different lifetime, with treated collagen structures uniformly colored in green on the phasor map. The longer lifetime measured at lower concentration can be associated with a more hydrophobic and rigid environment[62,63] while the shorter to less hydrophobic one. Furthermore, the presence of a shorter fluorescence lifetime indicates that at higher ANS concentrations, ANS binds to less hydrophobic and more solvent-exposed regions of collagen triple helix.

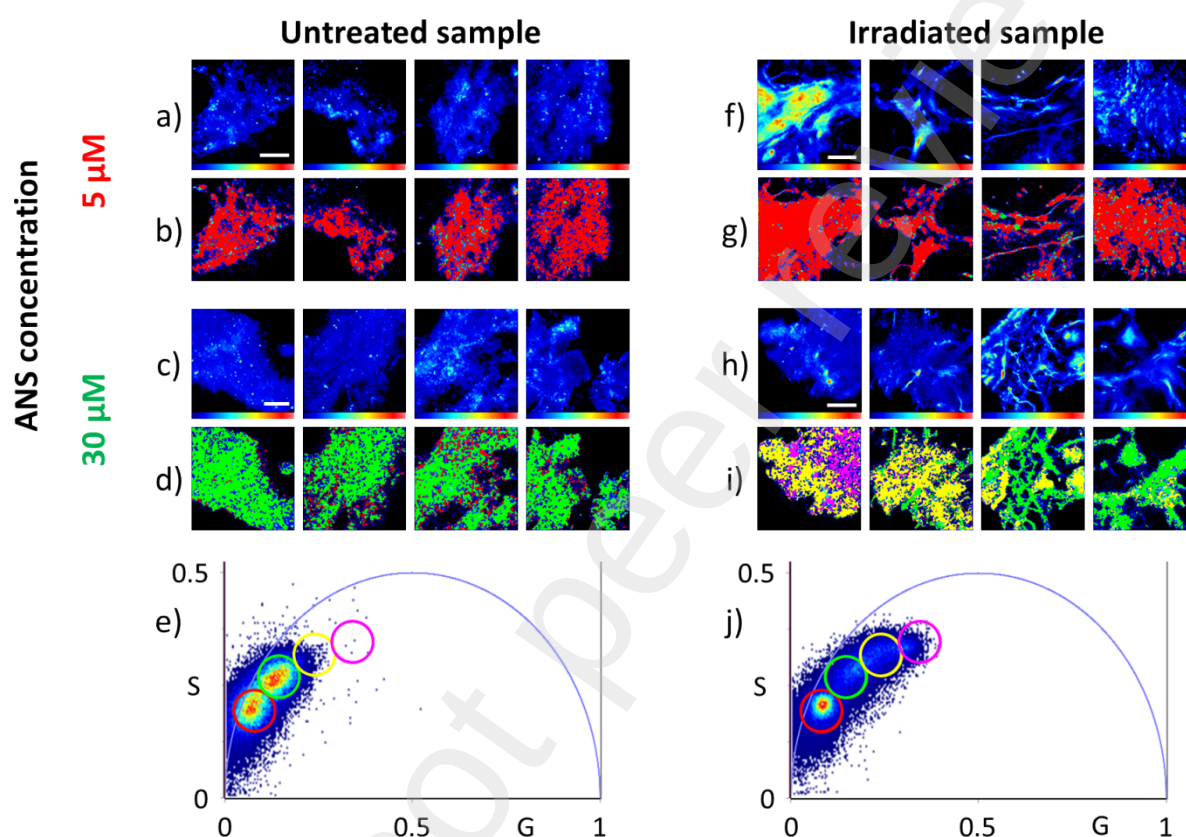


Figure 3: Phasor analysis of FLIM data on untreated and solar-treated samples. The samples consist of 2 mg/ml untreated (left) or treated (right) collagen suspensions in HCl 0.01M stained with 5 μ M (**a-b** and **f-g**) and 30 μ M (**c-d** and **h-i**). The measurements were acquired by means of two-photon microscopy ($\lambda_{exc} = 780$ nm, detection range 400 nm - 650 nm). **a-b**) 256x256 pixels intensity map and phasor maps of the untreated sample stained with 5 μ M ANS. **c-d**) 256x256 pixels intensity map and phasor maps of the untreated sample stained with 30 μ M ANS. **e**) Phasor plot revealing two lifetime distributions and colored cursors used to select them. **f-g**) 256x256 pixels intensity map and phasor maps of the treated sample stained with 5 μ M ANS. **h-i**) 256x256 pixels intensity map and phasor maps of the treated sample stained with 30 μ M ANS. **j**) Phasor plot revealing an elongated lifetime distributions and colored cursors used to select it.

Figure 3f and 3h show 256x256-pixels representative fluorescence intensity images of the collagen sample irradiated using the solar simulator in presence of ANS at 5 μ M and 30 μ M. Figure 3b and 3d show the phasor maps for the same samples obtained from the phasor plot reported in Figure 3e.

As it is evident upon irradiation ANS fluorescence lifetime distribution undergoes further broadening toward shorter lifetime. To quantify these changes, lifetime distribution was segmented using four colored circles to provide a spatial map of the fluorescence lifetime. The red and green cursors used for the analysis of the untreated sample are kept in the same position to aid the comparison between

the two samples. The red cursor selects the longer lifetime distribution, associated with the lower ANS concentration. Interestingly, at lower ANS concentration the lifetime distributions of treated and untreated samples are the same within the experimental error. Indicating that more hydrophobic regions are negligibly affected by irradiation. Since the lifetime distributions observed in both samples are identical within experimental error, the variation in fluorescence intensity reported in Figure 2 likely reflects a reduction in the number of ANS binding sites in collagen following irradiation. This result suggests that irradiation only affects a portion of internal hydrophobic patches which become no longer accessible to the ANS molecules at this concentration, indicating a variation of the hydrophobic patches. This effect is consistent with the reduction in native collagen fluorescence, the increased solubility, as shown in Figures 1.

At higher ANS concentrations, the sample's heterogeneity increases, accompanied by an overall reduction in fluorescence lifetime. This behavior suggests that the dye interacts with a less hydrophobic environment. The environment of the solar-treated sample is largely heterogeneous and characterized by different degrees of polarity, as suggested by the elongated fluorescence lifetime distribution. This is evident by the phasor map where a spatial correlation of the pixel color is not observed. The broadening of the lifetime distribution may reflect the destabilizing of the triple helix structure of collagen. Moreover, it is also not possible to exclude that at higher ANS concentrations the modified telopeptide regions that are not accessible in the low-concentration regime become accessible.

To gain deeper insight into the secondary structure of collagen fibers and to detect potential destabilization of the triple helix, we employed s-SNOM. The technique enables simultaneous acquisition of the topography and the infrared absorption spectrum of collagen with lateral resolution around 20 nm by taking advantage of the coupling of an atomic force microscope (AFM) to IR interferometry[64–67]. This approach enables the correlation of nanoscale morphological features with molecular structural changes, providing valuable information on the effects induced by irradiation.

Figure 4 summarizes the results we obtained on the untreated (shown in panels 4a - 4g) and solar-treated (shown in panels 4h - 4j) collagen samples.

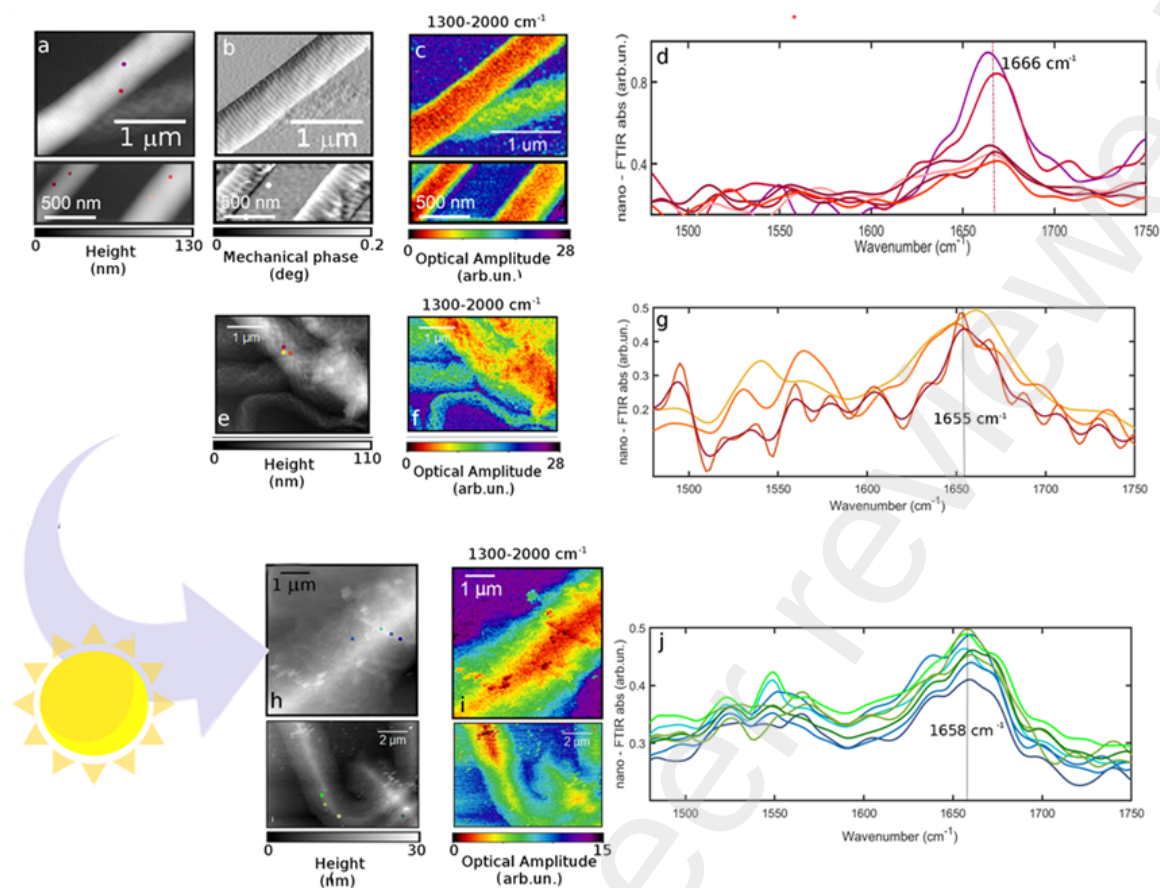


Figure 4: AFM and s-SNOM white-light imaging of untreated collagen fibers acquired with 20 nm lateral resolution (**a-c** and **e-f**). Two different fiber populations from the untreated sample are shown, displaying intact D-banding patterned fibers (**a-c**) and naturally degraded fibers with altered patterns (**e-f**). s-SNOM absorption spectra are shown at (**d**) and (**g**), acquired on the areas marked with the same color in panel **a**) and panel **e**). AFM and s-SNOM white-light imaging of solar-treated collagen fibers acquired with 20 nm lateral resolution (**h-i**). s-SNOM absorption spectra at (**j**), acquired on the areas marked with the same color are shown at (**h**). All spectra were acquired in the region of Amide II (1480 - 1580 cm^{-1}) and Amide I (1580 - 1710 cm^{-1}) bands with the average position of Amide I peak (calculated on $n > 5$ spectra) shown in the plot panels and marked with a vertical line.

Figure 4a and 4e show representative AFM height maps, Figure 4b shows the mechanical phase maps, and Figure 4c and 4f show the infrared optical amplitude (which is proportional to sample reflection) maps of the untreated sample. Figure 4d and 4g, show the punctual infrared spectra acquired on 20 nm wide areas highlighted by colored dots in panels 4a and 4e. Untreated collagen was found to be composed of an ensemble of two distinct populations. The first population, representing the majority of the fibers found in the sample, was composed of intact collagen fibers showing a thickness above 100 nm and having the typical D-banding pattern (with bands running perpendicular to the fiber axis) (see Figure 4a-c as a reference)[14,68]. Intact patterned fibers showed relevant AFM phase contrast, shown in Figure 4b from which we estimated the periodicity of the D-banding pattern to be between 55 and 70 nm, range in accordance with previous literature data[7,14,15]. The D-banding pattern was also detectable from the s-SNOM map in Figure 4c, suggesting the possible relation between the morphology and the structure of collagen proteins in the fiber. Figure 4d shows some of the spectra acquired on patterned fibers, specifically in the areas marked with dots of the same colors in panel a. As a first remark, we observed that Amide II band (1480 - 1580 cm^{-1}) intensity is extremely low and barely observable if compared to Amide I band (1580 - 1710 cm^{-1}). This observation indicates that the

chemical groups that participate to Amide I band are preferentially oriented along the polarization axis of the s-SNOM signal, the AFM tip axis. We can thus infer that the Amide I chemical groups are highly oriented perpendicular to the fiber axis[69] made up by polyproline-like helices. The Amide I band acquired at different positions (and also on different fibers) appeared quite reproducible with a peak that was around 1666 cm^{-1} (varying in the range $1662 - 1670\text{ cm}^{-1}$), as estimated through the average spectrum (see Figure S2), and a shoulder at about 1635 cm^{-1} . We associated the two spectral components at 1666 cm^{-1} and 1635 cm^{-1} with the two main triple helix features typically observed in the infrared spectrum of type I collagen, which reflect the vibrational state of the Proline-rich triplet responsible for the formation of the triple helix[70,71].

Alongside this collagen well-ordered fiber species, the untreated collagen sample is also composed of a second population of fibers that are found to a minor extent. This consists of thinner fibers with thicknesses around $30 - 40\text{ nm}$, which show a less regular pattern with bands running diagonally with respect to the fiber axis, as reported in Figure 4e - 4f. This pattern was previously observed in untreated collagen fibers, and it was associated with the natural degradation possibly occurring on fibers in low hydration and dry conditions[72]. The spectroscopic analysis performed on those fibers, shown in Figure 4g, where spectra are acquired on the areas marked with the same color in Figure 4e, revealed the increase in the intensity ratio between Amide II and Amide I band, possibly due to the misaligning of proteins in bands. More importantly, the Amide I main peak shifted to 1655 cm^{-1} .

Similarly to what is reported for untreated collagen, Figure 4h-4j shows the results obtained with s-SNOM on the solar-treated collagen sample. Figure 4h shows representative AFM height maps, and Figure 4i shows the infrared optical amplitude maps of the solar-treated sample. Figure 4j shows the infrared spectra acquired on $20 \times 20\text{ nm}$ wide ROIs that are marked with colored dots in panels 4h. AFM height maps (shown in Figure 4h) revealed the presence of thin fibers, with a height below 50 nm and an apparent diameter above $1\text{ }\mu\text{m}$. A close inspection of the sample allowed to spot the presence of possible residual patterning, as suggested by Figure 4h - 4i where stripes running diagonally with respect to the fiber axis can be sporadically observed in both height and s-SNIM maps. Spectroscopic analysis with s-SNOM is shown in Figure 4j where some of the spectra acquired on the areas marked with the same color code in Figure 4h are shown. As a first remark, we noticed that the intensity ratio between Amide II and Amide I peaks is similar to the one shown in Figure 4g, where naturally degraded fibers were measured, hinting at a breakage in the regular orientation of amide groups in the fiber, in line with the disappearance of the regular D-band. The Amide I band appeared slightly shifted if compared to intact collagen, peaked in average around 1658 cm^{-1} (see Figure 2S) with variability in the position between 1655 and 1660 cm^{-1} .

The Amide I band is found in the region $1670 - 1650\text{ cm}^{-1}$ (depending on the primary structure of the collagen, environmental conditions, and physical state)[70,71,73–75] and it has been alternatively associated by theoretical studies with Pro or Gly carbonyl groups[70,71]. As stated by Lazarev et al.[76] the position of the Amide I strictly depends on the structural equilibrium of the triple helix with surrounding structural water molecules and shifts to lower frequencies when the intermolecular bridge between the polypeptide and structural water is altered, eventually leading to the breakage of the helix with the simultaneous shift of Amide I main moiety to the region of unbonded chains (around 1655 cm^{-1}). By comparing the spectra acquired on untreated (panel d and g) and solar-treated fibers (panel j), we can assume that the Amide I band does not undergo any relevant broadening upon irradiation. Based on this and on the fact that a residual pattern is still present even after irradiation,

we suppose that rather than being due to a redistribution of secondary structures, the detected changes could instead reflect a destabilization of the structure of triple helix, with partial distortion of secondary and quaternary structural moieties in line with the CD data.

Collectively, the reported data leads to the conclusion that upon irradiation by solar simulator, the triple helix structure of collagen is strongly altered, and the collagen fibers lose their integrity. To test if this loss also affects the ability of collagen fiber to self-assemble into more complex microscale structures, films of untreated and treated collagen were prepared.

Figure 5 shows two representative 1024x1024 pixels images of collagen films prepared with untreated collagen (a) and solar-treated collagen (b), stained with ANS.

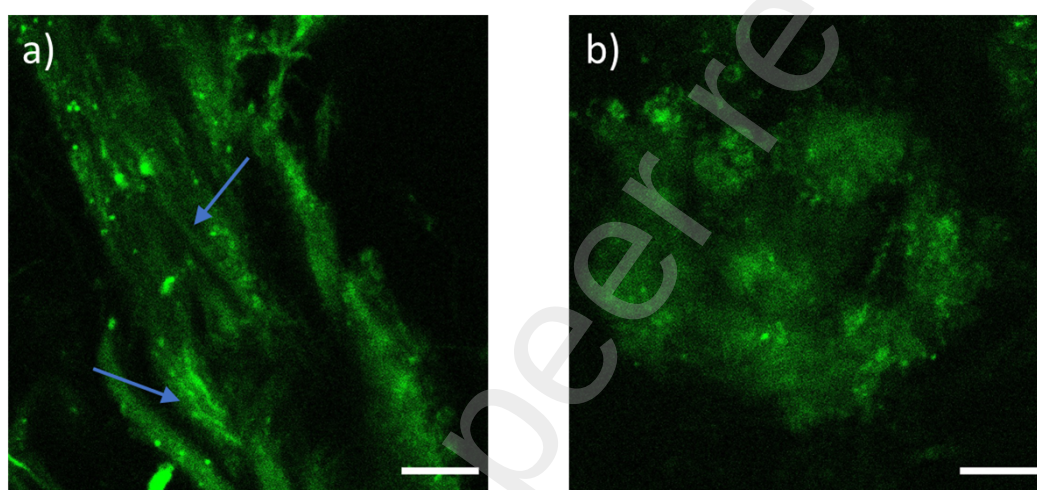


Figure 5: Representative 1024x1024 pixels two-photon microscopy images of untreated (a) and solar-treated (b) collagen films, stained with ANS. The films were prepared by mixing in an ice bath 50 μ l of cold distilled water, 200 μ l of 2 mg/ml untreated or solar-treated collagen, 50 μ l of 10X PBS, and 2 μ l of 0.5 M NaOH. Then the solutions were incubated at 37°C overnight and rehydrated and stained with 13 μ M of ANS the day after. The films were imaged the same day, via two-photon microscopy ($\lambda_{exc} = 780$ nm, detection range = 400 nm - 650 nm). Scale bar = 10 μ m. The two samples have different morphologies: **a)** the untreated collagen self-assembles in fibrillar structure on the micro-scale, and single collagen fibers can be detected (highlighted by blue arrows); **b)** the film prepared with solar-treated collagen presents an amorphous morphology and no fibrillar structures can be detected.

As is evident, the morphologies of the two films are significantly different. The untreated collagen film is organized in a fibrillar structure on a micron-scale level. The fiber bundle is well-oriented and single fibers are visible (highlighted by blue arrows). In contrast, the film prepared with the solar-treated sample lacks any fibrillar structure. Its supramolecular organization does not exhibit the ordered self-assembly seen in the collagen bundle shown in panel a, and no fibrillar features are apparent. The loss of the collagen fibril pattern and the formation of an amorphous arrangement of collagen molecules have already been reported for collagen molecules that do not present the telopeptides and it was associated with the roles of the C- and N- termina telopeptides in cross-linking and fibril formation[77,78].

Conclusions:

Solar radiation, while essential for life, poses significant risks to human health. Prolonged exposure to solar radiation accelerates photoaging, leading to structural and functional degradation of the skin's connective tissues. This degradation is primarily due to damage to Type I collagen, the main structural component of the dermis. In this paper, we analyzed the molecular modifications induced by solar radiation on Type I collagen. Solar radiation was reproduced using a solar simulator. The use of the solar simulator allows for standardized experimental conditions by operating at a constant power, thereby avoiding potential unwanted environmental effects. Following irradiation, we observed a decrease in the intrinsic fluorescence of collagen and an increase in its solubility. This effect indicates a loss of supramolecular structure due to the degradation of aromatic species (tyrosine residues and cross-link species) located in the non-helical regions of the telopeptides. These regions stabilize the fibrillar structure by facilitating the alignment of individual collagen molecules. The secondary structure was explored using CD spectroscopy and s-SNOM analysis, which revealed that solar-treated collagen exhibits partial destabilization of the triple-helical structure. These changes lead to the degradation of collagen fibers and a consequent loss of the D-band pattern, which remains clearly visible in untreated collagen fibers. This is likely due to the misalignment of collagen molecules, which are no longer well-aligned once the cross-links and telopeptides are degraded. From a mesoscale perspective, it was also observed that solar-treated collagen loses its ability to form fiber bundles.

Using a combination of steady-state fluorescence spectroscopy and time-resolved non-linear fluorescence microscopy, we observed increased sample heterogeneity upon irradiation, accompanied by an overall reduction in ANS affinity for collagen. Specifically, the more hydrophobic regions, corresponding to high-affinity binding sites, appear to be relatively protected from irradiation-induced damage. In contrast, the less specific and more solvent-exposed regions, likely associated with the triple helix structure, undergo substantial structural alterations, indicating significant disruption of the affected molecules.

These findings provide new insights into the molecular mechanisms underlying collagen degradation upon solar exposure, contributing to a deeper understanding of skin photo-aging. These results suggest that solar-induced damage significantly alters collagen fibers at both the micro- and nanoscale, propagating from molecular properties to the macroscopic characteristics of the entire matrix. We believe that these observed modifications may underlie changes in the fibrillar collagen organization, which are involved in skin photoaging. To gain a deeper understanding of the photoaging process, it would be beneficial to increase the complexity of the system under investigation. Future research could focus on studying matrices composed not only of collagen but also of elastin, glycoproteins, and other extracellular matrix components to better understand their role in modulating solar radiation-induced damage. Finally, exploring potential interventions, such as antioxidant treatments and collagen-stabilizing agents, could pave the way for novel therapeutic strategies to counteract photoaging and connective tissue disorders.

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CRedit authorship contribution statement

Declaration of Competing Interest

The authors declare that they have no competing interests.

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Data availability

The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used Chat GPT only in order to improve language writing. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.