Probing Collagen Nanocharacteristics after Low-Level Red Laser Irradiation

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Abstract— **The low level red light (LLRL) is increasingly being used in many fields of medicine, like wound healing and regeneration of damaged tissue. Although the positive effects of LLLT have been reported the effects of red light on nanoscale features of tissues still remain unknown and little is known about LLRL-collagen interactions. Thus, in this study, thin collagen films were formed as extracellular matrix (ECM) models and irradiated so as to investigate the influence of LLRL irradiation on collagen topography, mechanical properties and the influence in fibroblasts response. The alterations on topography and collagen Young's modulus were recorded using Atomic Force Microscopy (AFM). Furthermore, fluorescence microscopy was applied for studying the fibroblast response cultured on the collagen models. The results demonstrated that LLRL had minor effects on collagen topography. However, it was demonstrated that the LLRL altered the mechanical properties of the fibrils (the standard mechanical pattern due to the D – band periodicity was affected). Additionally, it was shown that fibroblasts cultured on LLRL-irradiated collagen thin films responded to LRLL. The above results provide new insights into the underlying nanoscale mechanisms of LLRL.**

Keywords— **Atomic Force Microscopy (AFM); Young's modulus; Fibroblasts; In Vitro; Low Level Laser Therapy (LLLT).**

I. INTRODUCTION

Nowadays, noninvasive therapies are increasingly being used in many fields of medicine, like wound healing and regeneration of damaged tissue [1-4]. A very promising noninvasive therapy is the Low Level Laser Therapy – (LLLT, typically 500–1100 nm) since its effects on tissue or individual tissue components is of increasing interest [5] [2] [6] [7]. LLLT has become increasingly popular for treating a wide range of medical conditions and the resulting reactions reduce tissue edema and inflammation, phagocytosis, collagen deposition, protein synthesis, and epithelialization, and improve the tensile strength of tissues [8]. Among the more promising applications are the bio-stimulation of wound healing [9] and the improvement of the healing process in a variety of pathological conditions, such as burns [10]. Although LLLT is now used to treat a wide variety of ailments, it remains somewhat controversial, because uncertainties remain concerning its fundamental molecular and cellular mechanisms, and a large number of dosimetry parameters must be controlled [8].

It has been reported that red light irradiation appears to have a wide range of effects from nanoscale to macroscale [1, 11-18]. Studying the effects of LLLT on extracellular matrix (ECM) elements, such as collagen, is important to understand how it affects the wound healing process. LLLT has been shown to contribute to increased expression of collagen and elastic fibers during the early phases of the wound healing process [11]. Also, it has been reported that red light irradiation contributed to better alignment of collagen fibers and as a result collagen reorientation had healing effects in injured tendons [19]. However, the influence of red light on collagen in the nanoscale needs further clarification. Although a large number of reports demonstrating the positive effects of LLLT in various in vitro, in vivo, and clinical studies have been published [20] [21] [22], little is known about the effect of laser light on collagen.

The investigation of the effects of red light on collagen in nanoscale due to the fact that most parts of the human body (e.g. skin, bone, cartilage, or tendons) contain collagen, and especially collagen type I, in the form of collagen fibrils is crucial as far as red light therapy is concerned. Collagen fibrils, depending on the tissue, are aligned laterally to form bundles and fibers, which among other properties offer to the tissue mechanical strength and stability [23-25]. The most characteristic structural feature of the type I collagen fibrils is their D – Band periodicity which is an alternating sequence of gap and overlapping regions, with a highly reproducible distance of 67 nm [26-28].

In this study, the nanoscale effects of red light irradiation on collagen fibrils were investigated. Atomic Force Microscopy (AFM) techniques were applied so as to quantify the surface modification of collagen induced by LLRL. AFM can provide a variety of information (from topography to mechanical properties) as far as a sample's surface characterization is concerned [26, 29-32]. Nanotopography characteristics and mechanical properties of collagen were recorded prior and post irradiation. For the mechanical testing, Young's modulus maps were created. Furthermore, irradiated and non-irradiated collagen films were used for culturing

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fibroblasts so as to access the indirect effects of irradiation on cells behavior. Fluorescence microscopy and image processing techniques were applied in order to quantify the possible alterations on fibroblasts. The results of the research aim to contribute towards a better understanding of the tissue – red light interaction mechanisms.

I. Materials and Methods

A. Collagen Thin Film Formation

A collagen stock solution was prepared as it has been described previously [32-34]. Briefly, type I collagen (Fluka 27662, Sigma-Aldrich) was dissolved in acetic acid and then it was homogenized. Part of the solution was diluted with phosphate-buffered saline (PBS) to a final concentration of 8 and 1 mg/mL. Collagen solution (50 μL) was flushed onto fresh-cleaved mica discs and was spin coated for 40 sec at 6000 rpm so as to form collagen thin films.

B. LLRL Irradiation of Collagen

A 661-nm diode laser system (GCSLS-10-1500m, China Daheng Group, Inc.) was used for irradiating collagen. For the collagen solution irradiation, the experiments were performed in 1-cm cuvettes and the solution was constantly stirred. The collagen solutions and films were placed 4 cm from the light. A laser thermal low power sensor (Ophir Laser Measurement Group) was used to measure the fluence rates, which adjusted to 4 mW/cm^2.

C. AFM Imaging

AFM imaging was performed in tapping mode using a commercial microscope (CP II, Vecco Bruker) with typical anisotropic AFM probes (MPP-1123, Bruker). Locator grids (G2761C, Agar Scientific) were used to map the surface. A scan rate between 0.5–1 Hz and fixed resolution (512 × 512 data points) was used.

D. AFM Force Scanning Technique

The mechanical characterization of the tested collagen fibrils was performed using the force scanning technique [31]. The tested fibrils were scanned several times with different set point forces (5nN, 10nN, 15nN and 17.5nN) and low scan rate (1Hz). The indentation values using the different set point forces were calculated by subtracting the height values of the topographical images from an arbitrary contact-point height z_0 [31]. Hence, arrays which consist of indentation values at every point of the scanned area were created. Subsequently, force – indentation curves for each point were created in order to assign every point of an image with is Young's modulus value. The Young's modulus values were calculated using the linearized Hertz model [31]. In particular, the linearized force indentation curves are expressed by the equation:

$$F^{2/3} = \left[\frac{4ER^{1/2}}{3\left(1-v^2\right)}\right]^{2/3} h \Rightarrow F^{2/3} = Sh \quad (1)$$

where F is the force, E is the Young's modulus, R is effective indenter radius, v is Poisson's ratio, h is the indentation depth and S is the slope of the linear $F^{2/3}$-h curve. Thus, by using a linear curve fitting, the slope of the curve $F^{2/3}$-h was calculated for each point of the scanned area and related to the Young's modulus as follows:

$$S = \left[\frac{4ER^{1/2}}{3\left(1-v^2\right)}\right]^{2/3} \quad (2)$$

In conclusion, the Young's modulus value for each point was calculated by transforming the equation (2) in the form:

$$E = \left[\frac{3\left(1-v^2\right)}{4R^{1/2}}\right] S^{3/2} \quad (3)$$

The Young's modulus values of the selected nanoregion prior and post the irradiation are expressed as mean value ± standard deviation.

E. Cell Culture and Seeding Cells on Films

A human dermal primary fibroblast cell line was cultivated in 25-cm^2 culture flasks in complete medium (Gibco, Life Technologies) containing Dulbecco's modified Eagle's medium supplemented with 10% Fetal Bovine Serum and 0.1% antibiotic-antimitotic. Cells were stored at 37°C in a 5% CO_2 humidified incubator, trypsinized, and re-seeded into fresh medium every 6–7 days. Cells were removed from tissue culture flasks by trypsinization and plated at a density of 2 × 105 cells/dish in a petri dish containing a substrate in complete medium. Cells were grown for three days before being imaged with fluorescence microscopy.

F. Fluorescence Microscopy Imaging of Cells

An Olympus BX-50 epifluorescent microscope and Rhodamine B (which labels cells' mitochondrial matrix) [35] was used to image the cells. Images were acquired with an Olympus 20×, UPlanFl, 0.50 NA. For *in vivo* microscopic observation of cells, a small perfusion chamber was used, as described previously [36].

G. Image Processing

Quantitative measurements from AFM images were acquired with the image analysis software that accompanied the AFM system, DI SPMLab NT ver.60.2 (Veeco) and the freeware scanning probe microscopy software WSxM 5.0 dev.2.1 [37]. The fluorescence microscopy images were analyzed with

ImageJ (NIH). The *Elongation (E)* factor of cells was calculated using the formula $E = \frac{Long\ Axis}{Short\ Axis} - 1$ [38]. The E factor is 0 for a circle and 1 for an ellipse with an axis ratio of 1:2 (values 0-0.5 indicates a spherical cell, 0.5-1 ellipsoid and higher that 1 elongate) [35]. The elongation of the nucleus was also assessed.

II. Results and Discussion

A. AFM Imaging of LLRL-Irradiated Collagen Films

With the applied protocols, thin collagen films, consisting of randomly oriented fibrils/fibers were formed (Fig. 1, a). In the images the characteristic D-band periodicity (~67 nm), which is a characteristic of the naturally self-assemply of collagen molecules [24], was clearly observed. In Fig.1b&c the same area after irradiation for 5&15 doses irradiation (each dose was lasted 45 seconds), is demonstrated. As it can be seen, the LLRL irradiation did induce any visible modifications on the collagen nanotopography. Measurements on fiber diameter, orientation, and average height demonstrated that there were not alteration after irradiation and surface roughness remained constant. Furthermore, the influence on the D-band was monitored. The results showed that for the used doses the D-periodicity did not altered (Fig. 1d-f).

B. Mechanical Properties of a Selected Nanoregion Prior and Post the Irradiation

In addition, collagen fibrils on collagen thin films with were irradiated for 0, 5 doses (5 x 45 sec), 15 doses (5 x 45 sec). The influence of the red light on the mechanical properties of collagen fibrils was investigated. Specifically, Young's modulus maps of a specific region on a collagen fibril were created prior and post the irradiation (Figure 2). The selected region was located at the center of the fibril due to the fact that only load – indentation data obtained near the center of a collagen fibril should be taken into account [28].

As it is observed there is a difference in Young's modulus values between overlapping and gap regions as it was expected. In particular, the Young's modulus values on the overlapping regions are greater than the corresponding values on the gap regions, as it has been previously reported [27]. This result is in the same order of magnitude with those obtained in previous studies [39].

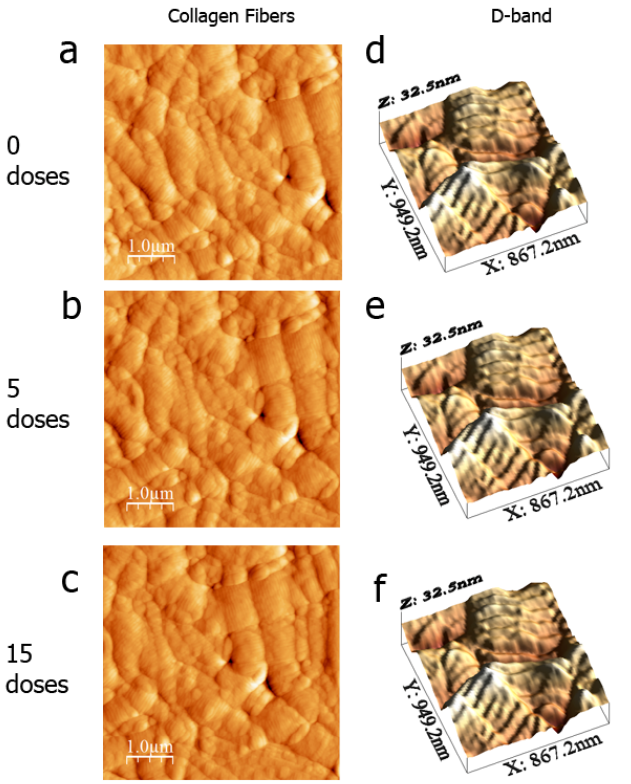

Fig. 1 Atomic force microscopy (AFM) topographic images of collagen films (a-c) and D-band (d-f) irradiated with different doses [0 doses (Fig. a, d), 5 doses (Fig. b, e) and 15 doses (Fig. c, f) of irradiation].

As it is presented in Figure 2, the Young's modulus of the selected nanoregion resulted in 0.38 ± 0.07 GPa prior the irradiation and 0.37 ± 0.08 GPa, 0.37 ± 0.10 GPa post the 5 and 15 doses. The results indicate that the red light irradiation did not alter the mean Young's modulus of the selected nanoregion but affected its mechanical heterogeneity.

For example the comparison of the Young's modulus values of two selected points (A, B in Figure 2) is presented. Both points were located on overlapping regions. Prior the irradiation the percentage difference of the Young's modulus of the two selected points resulted in 1.5%. After the 5 doses (5 x 45 sec) of irradiation the above result did not alter significantly (the percentage difference resulted in 2.1%). However, after the 15 doses (15 x 45 sec) of irradiation the percentage difference of the two selected points resulted in 14.6%. Hence, the irradiation procedure affected the standard mechanical pattern (due to the D – band periodicity) of the collagen fibril.

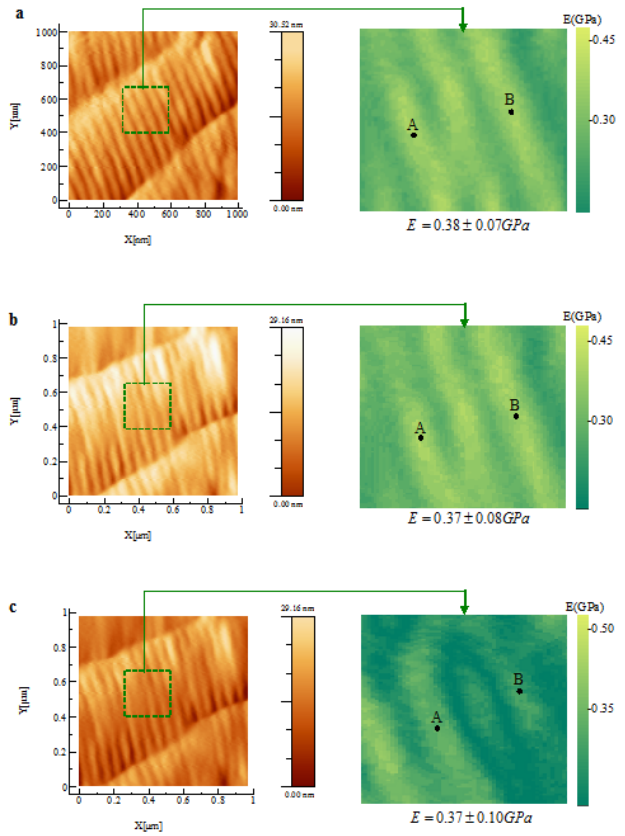

Fig. 2 Young's modulus maps of the same nanoregion prior and post the irradiation. The mechanical heterogeneity due to the D – band periodicity altered significantly after the 15 doses (15 x 45sec) of irradiation.

C. Culturing Cells on LLRL-Irradiated Collagen Films

In order to investigate the indirect influence of LLRL on cells, a primary human skin fibroblast cell line was culture on irradiated and non-irradiated collagen films. Fluorescence imaging and image analysis techniques were used to assess fibroblast morphology and alignment. From the fluorescence images it can be seen that fibroblasts on untreated films had had a more elongated structure (spindle-shaped), which is considered their native form (Fig. 3a). However, after the irradiation of the films, fibroblasts presented a more ellipsoidal or even spherical shape (Fig. 3b&c). By measuring the elongation of the it was found that prior to irradiation, the majority of the fibroblasts were the elongated type (59.0±1%), and only a small percentage of the fibroblasts were spherical (19.0±5.0%) (Fig. 3a). As irradiation of the collagen thin film increased, the percentage of elongated fibroblasts decreased. Simultaneously, the number of spherical cells increased.

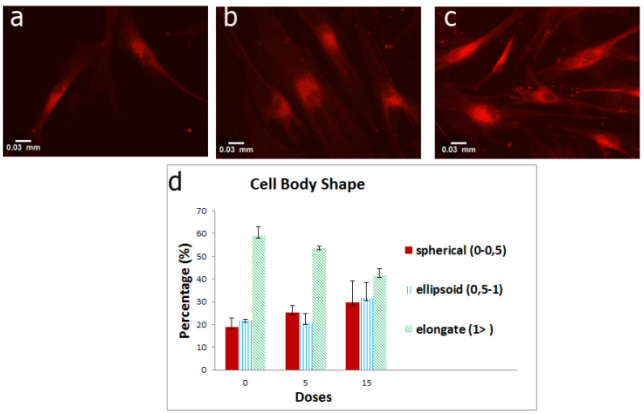

Fig. 3 Fluorescence images of fibroblast cultures on irradiated thin films (a,b,c). Chart presenting the elongation of cells on the different irradiated collagen films (d).

III. Conclusions

The red light is increasingly being used as a non invasive therapy for tissues' injuries and normal aging. In this paper, it was demonstrated that red light greatly affects the collagen fibrils' properties in the nanoscale. AFM nanocharacterization of collagen thin films demonstrated surface topography was not altered after irradiation, and the surface roughness of the films remained almost constant. Moreover, after irradiation the D-periodicity of collagen fibers did not show any significant modifications. The use of LLRL-irradiated collagen thin films as a cell substrate showed that increased irradiation repetition induced abnormal growth in fibroblasts. The bodies and nuclei of fibroblasts became more spherical. Additionally, the red light altered the mechanical pattern of the collagen fibrils due to the D – band periodicity. As a conclusion, the above research can be a step forward towards a better understanding of the tissue – red light interaction mechanisms and the investigation of the healing effects of red light in the nanoscale.

Conflict of Interest

The authors declare that they have no conflict of interest.

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