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Functional Modification through Crosslinking and Treatment with Short Laser Pulses of Collagen Derived Biomaterials for Application in the Regenerative Medicine

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Abstract. The search of new biomaterials with improved functionality, biocompatibility and low immunogenicity is a major trend of the regenerative medicine and the related interdisciplinary fields. The proteins of the collagen family comprise around 30% of the total protein mass in the body and naturally they are among the first choices as compound of such biomaterials. However the processing increases collagen solubility and decreases its mechanical strength. To combat this issue often crosslinking is applied, but many crosslinking agents increase toxicity and immunogenicity. Therefore here studied biomaterials are made of naturally occurring compounds as a prerequisite of better biocompatibility. The appropriate biomaterial texture and patterning is another important aspect of the successful regeneration, and laser based techniques gradually take their respective place in this field.

To evaluate newly synthesized collagen-derived crosslinked biomaterials upon laser texture modification with intent to improve their surface characteristics in regard to cell- and bio-compatibility and make them more suitable for use in the regenerative medicine.

The samples were prepared from tendon derived collagen and crosslinked with glucose in custom crosslinker. Short laser pulses were generated with Integra C ultrafast laser system and beams with different parameters sets were applied on the samples. The texture modifications were examined and characterized with optical and SEM microscopy.

Texture modifications with regimes and parameters of the beam for narrow energy confinement in these materials show more consistent results than the ones with wider confinement. This is reflected in the size as well in the shape of the resulted laser-induced modifications. Low-crosslinked samples show higher variability of the laser caused texture modifications when the irradiating beam is set with parameters producing wider energy confinement.

Laser treatment with beam parameters ensuring narrow energy confinement is better suited for these biomaterials especially when pattern directed cell attachment and growth is required for improved tissue regeneration.

1 Introduction

The search of new biomaterials with improved functionality, biocompatibility and low immunogenicity became a major trend of the modern regenerative medicine and the related interdisciplinary fields. The proteins of the collagen family comprise around 30% of the total protein mass in the body of the mammals [1] and naturally they are among the first choices as compound of such biomaterials. However during the extraction procedures and processing and the accompanying denaturation of the collagen its solubility increases and its mechanical strength decreases [2]. To combat this issue often crosslinking is applied [3-5], but many crosslinking agents especially the highly effective aldehydes as glutaraldehye, formaldehyde, and glyceraldehyde increase toxicity and immunogenicity [6,7]. Therefore the biomaterials studied here are made of naturally occurring compounds as a prerequisite of better biocompatibility [8].

The appropriate biomaterial texture and patterning is another important aspect of the successful regeneration [9]. In the last decades with the advance of the technology gradually were accumulated data and evidence showing that cell growth and biocompatibility of the biomaterials can be influenced and even modulated through modification of the surface and texture of the biomaterials at micro- to nano-scale range [10-12] for which purpose the laser-based techniques are especially useful [13,14]. Such modulatory effect is even more pronounced in the stem cell differentiation and the recently emerging stem cell techniques for tissue regeneration based on this phenomena [15].

In this study we aimed to evaluate newly synthesized collagen-derived crosslinked biomaterials upon laser texture modification with intent of improvement of the surface morphology and characteristics favouring cell- and bio-compatibility and thus make them more suitable for use in the regenerative medicine.

2 Methods

Biomaterials' preparation. The samples were prepared from collagen derived Wistar rats tail tendon. The collagen fibers were thoroughfully cleansed from muscle and lipid debris before further processing. Afterward the collagen fibers were cut in pieces in size less than 1mm on a

tabletop cutter. The crushed collagen fibers were placed in 3M acetic acid at 277 K and the mixture was continuously mixed with magnetic stirrer at low speed. After a week the solution of the extracted collagen along with the undissolved debris was centrifuged at 4000 g for an hour at 277 K. The supernatant was collected and the solubilized collagen was extracted with the saline gradient method [16]. The extracted collagen was dried at 277 K and kept at this temperature until further use.

Before the biomatrix preparation the collagen was mixed with water at weight ratio 1:2 and heated to 338K for 60 min. Afterward the solution was cooled to 310K and the glucose powder was added to achieve mass fraction of 5%, 10% and 20%. In order to facilitate the crosslinking the temperature was raised to 345K for few hours in thermostat custom crosslinker.

Laser system and irradiation. Short laser pulses were generated with a regeneratively amplified Ti:Sapphire laser (Integra-C FS, Quantronix, USA) with 790 nm center wavelength. The maximum pulse energy was 1 mJ and the maximal repetition rate (R) was 1 kHz, and 130 fs pulse duration. The averaged laser power delivered to the sample was measured with a calibrated laser power meter Molectron PM30 ((Molectron Detector, USA) placed after the focal plane of the focusing lens. The pulse control and isolation was done with Thorlabs optical chopper MC2000 (Thorlabs, USA) and Uniblitz shutter drive T132 (Vincent Associates, USA) controlled in LabView environment (National Instruments, USA). The samples were irradiated with pulse series with number of pulses from one to eight and time interval between them of 70 ms. The ablation threshold fluence for rich in collagens and water mediums is under 1.8 J/cm^2 [17] for femtosecond pulses so fluence above that value was used.

Laser texture modifications observation and estimation. The texture modifications were examined with optical dark field microscopy for coarse estimate of the results of the short pulse laser irradiation. The fine details of the laser texture modifications were explored with SEM microscopy Lyra XM Tescan, (Lyra, Tescan, Czech Republic) at 5–20 kV with magnifications up to 3000x.

Data analysis. The images of the samples recorded from Lyra XM SEM were processed with ImageJ [18]. The images were scaled according to the recorded scale and magnification by Lyra XM system. Afterward the micro-morphology of the laser induced surface textures and their common elements such as microcavities, bubbles etc. were measured. The data were grouped according to their type and size and were further statistically analyzed with Microsoft Excel (Microsoft, USA) and Origin Pro (OriginLab, USA).

3 Results and Discussion

The size of the ablation craters was with similar value of approx. 4×10^{-6} cm^2 with little impact of the set variations of the variable parameters of the laser beam. However the size of the elements of the surface morphology in the craters varied at higher extent. Dependence of the distribution by size of the micro- and submicro-sized cavities by the number of pulses was observed. For ablation with low number of pulses the percentage of the small sized (0.5–2 μm) microcavities is higher than the bigger ones (2–6 μm).

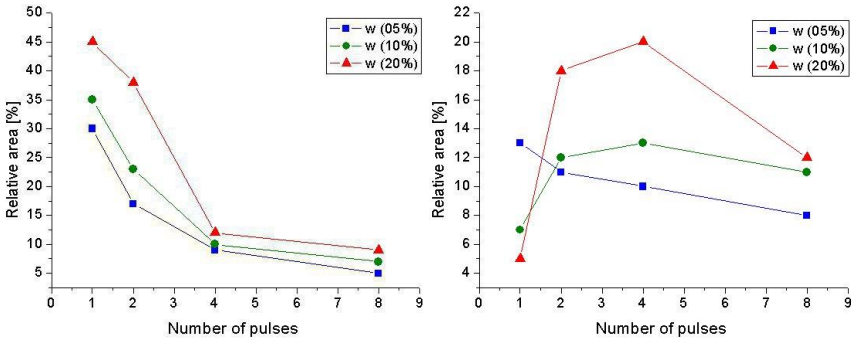

Figure 1: Relative area occupied by the surface morphology elements in the ablative craters grouped by size when irradiated with different pulse series of collagen derived biomatrices crosslinked with D-glucose with mass fractions 5%, 10% and 20%, and $f = 1$ J/cm^2: (Left) area occupied by the micro-cavities 0.5–2 μm; (Right) area occupied by the micro-cavities 2–6 μm.

The surface morphology differed for the biomaterial compositions different mass fractions of the crosslinking agent with gradual decrease of the relative area for the micro-cavities 0.5–2 μm. These differences were significant according to the results of the Kruskal-Wallis test at $P > 0.05$ with the following χ^2 values 23.48, 25.81, 16.30, 16.38 for $n = 1$, 2, 4, 8 accordingly.

The relation of the relative area for the micro-cavities $> 2\,\mu$m for the different mass fractions of the crosslinking agent and the number of pulses were more complex and statistically significant according to the results of the Kruskal-Wallis test at $P > 0.05$ with the following χ^2 values 24.58, 22.70, 24.82, 18.61 for $n = 1$, 2, 4, 8 accordingly.

The relative area of the micro-cavities with size over 2μm for the lowest mass fraction of the crosslinking agent followed similar pattern to the one for the smaller sized micro-cavities with gradual decrease with the

increase of the number of pulses. However for the higher mass fractions of the crosslinking agent there was a gradual increase of the relative area of the micro-cavities over 2μm with the increase of the number of pulses up to 4 afterward the area decreased.

Also surface smearing and lost of fine texture was observed at higher number of pulse series (4-8) at matrix compositions with high concentrations of crosslinking agent ($w = 10\%$ and 20%). This is reflected in the gradual reduction of the percentage of microcavities under 2μm and the reduction of the relative area of the microcavities over 2 μm at number of pulses above 4.

At low concentrations of the crosslinking agent ($w = 5\%$) the surface smearing was reflected in the gradual area reduction of the microcavities of both groups with the increase of the number of pulses (from 1 to 8).

At low energy depositions in single or two pulse regime small fragments of extruding material with micron or submicron thickness is observed at/or near the rim of the ablation craters. At irradiation regimes with higher number of pulse series almost no such fragments were observed a possible explanation is that due to the higher number of pulses more thermal energy was accumulated in the area adjacent to the holes which leads to local temperature increase and consequent melting of the extruded material.

Additionaly the SEM inspection of the ablative craters showed that almost no bubbles appear under the surface which is in contrast with the results of similar study [19] where pulses with longer durations (in the nanosecond range) were used and the bubbles were observed in a layer with thickness of several microns. So comparing the femtosecond ablation of this type biomaterials with the processing with longer pulses it seems that the first one can be advantageous and appealing in some areas of the regenerative medicine for which presence of gaseous phase inside the materials is undesirable and can have adverse effects.

Matter ejection, or ablation, becomes possible when the energy absorbed in a given volume exceeds the medium's cohesion energy. Since matter displacements do not have time to take place during the laser pulse time scale, the ablation threshold is completely characterized by the laser energy absorbed in the target at constant density [17] and the removed matter seems to be influenced by the cohesion energy of the medium.

In the presented results was shown that the surface morphology of the ablative craters differ depending on the mass fraction of the corsslinking agent. The used crosslinking agent cause two important changes in this type of biomaterials – increase of the mechanical strength due to inter collagen molecules covalent bonds and increase of the linear absorption at the wavewidth of the pulse [20]. Although these factors are

considered to play minor role in the ablative crater formation through femtosecond pulses [20] here we presented that they can tweak the fine texture of the ablative surface providing a valuable tool in the hands of the bioengeneers for who's field the microscale surface modifications can play crucial role.

4 Conclusion

Laser treatment with beam parameters ensuring narrow energy confinement is better suited for these biomaterials especially when pattern directed cell attachment and growth is required for improved tissue regeneration.

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