Femtosecond-laser-assisted LIPSS generation on chitosan/hydroxyapatite thin layers for biomedical applications

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Abstract. In this work, chitosan/hydroxyapatite (Ch/HAp) thin composites were synthesized and examined before and after femtosecond laser irradiation in order to investigate the substrate response and surface changes occurring during the process. The zones of interaction formed after treatment by fs laser pulses were analysed by scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy (FTIR). The results revealed that the laser processing of Ch/HAp thin films leads to generation of well-defined laser-induced periodic surface structures (LIPSS) for a laser fluence $F = 2.07 \text{ J/cm}^2$ at 800 nm wavelength with pulse durations in the range of 130 fs. It is demonstrated experimentally that the femtosecond interaction regime results in the formation of ripples beneficial for the functionalization of biopolymer/ceramic samples for further tissue engineering applications. In the transmittance spectra, profile variations for the non-treated and laser-processed surfaces were noticed depending on the number of pulses applied.

1. Introduction
Traumas, fractures, surgery, infections, tumours, congenital abnormalities, progressive disease of bone and bone structure, are only some of the causes of injury to and deformations and loss of bone tissue [1], which is why reconstruction of bone tissue defects is a major challenge facing orthopaedists in their efforts to restore the normal functioning of the human body [2]. The most commonly clinically-used bone grafts are autologous and allogeneic bone tissue materials, which, apart from being biocompatible, non-immunogenic and carry no risk of disease transmission, have their drawbacks, such as increased morbidity, infection in the donor bone, a second surgery in the donor location, limited supply of nutrition and allograft rejection, which can cause neurosensory deficit [3]. As an alternative, bone graft materials, such as metals, polymers and ceramics are being applied to fill the bone defect and provide mechanical and structural support.

The essential elements needed for bone reconstruction in tissue engineering are cells, scaffolds, and growth factors. In recent years, temporary "platforms" of various materials have been extensively studied for seeding different types of cell cultures and improving cell adhesion, proliferation and/or differentiation. These scaffolds act as support structures for cell attachment and growth into bone...
tissues and, therefore, must have adequate mechanical properties. The clinical success of the scaffolds for bone regeneration could be ascribed to their combining inorganic and organic biomaterials.

Due to this fact, research on the field of bone tissue regeneration has been focused on hybrid biomaterials [4]. Such temporary "platforms" contain composites of hydroxyapatite, chitosan, collagen, stimulant molecules, etc. In order to avoid a reduction in the bioactivity and biocompatibility and to enhance the process of cell adherence and growth, it has been found appropriate to combine more than one element, such as a biopolymer and ceramics. The combination of hydroxyapatite (HAp) with chitosan for preparation of matrix substitutes has attracted interest in the field of tissue engineering as it combines the advantages of both materials and minimizes the drawbacks of using them separately – for example, the linear unbranched structure of chitosan and its high molecular weight makes it an excellent viscosity-enhancing agent in acidic environments, which gives scaffolds osteogenic and biodegradation properties [5, 6]; on the other hand hydroxyapatite is characterized by a high level of biocompatibility, so that it is used for fabrication of dense and porous bioceramics [7]. The strength of the scaffold is also associated with the level of porosity and high content of hydroxyapatite [8].

The design of the temporary scaffolds may include variety of conventional chemical or physical techniques, as solvent casting, particulate leaching, gas foaming, phase separation and freeze drying. However, these methods do not provide precise pore size, geometry, interconnectivity, and spatial distribution of the pores [9], which are all crucial factors for the scaffold creation and cells seeding.

Laser-induced surface texturing offers advantages over both the chemical and the other physical methods. It enables precise modification of certain surfaces that are difficult to treat with conventional chemical methods. As a result, the modified surfaces are free from contaminants [10]. The technique does not require special pre-treatment and improves cell adhesion, as it increases the roughness of the substrate. Control over the biomaterial’s characteristics might be achieved, as the interaction of ultra-short pulses with biological tissues results in reduced crack formation and heat diffusion, absence of molten zones, and reduced ablation thresholds.

The laser-induced periodic surface structures (LIPSS) or ripples are of interest in what concerns the surface structuring. Ripples formation was first observed in 1965 by Birnbaum upon ruby-laser irradiation of various semiconductor surfaces [11]. LIPSS appear due the interference of the incident laser beam with the light reflected from the material surface and could be used to modify the functional characteristics of the surfaces, such as wetting, optical properties, or friction control [12]. It has been shown that LIPSS evolution and properties are influenced by the wavelength \( \lambda \), the laser fluence \( F \), the optical properties of the material and the number of applied pulses \( N \). The LIPSS formation process is a non-contact phenomenon and provides possibilities of structuring the particular substance on a nanoscale. The process of ripples creation is being exhaustively investigated for many different biological applications due to the potential of improving the surface functionality and alter the wettability, both being important for the further cell behaviour on the material superficies [13].

2. Material and methods

2.1. LIPSS formation on Ch/HAp thin composites

Thin Ch/HAp composites were synthesized from a mixture of chitosan (medium molecular weight) and hydroxyapatite powder (with particle size 10 \( \mu \text{m}, \geq 100 \text{ m}^2/\text{g} \)) in a ratio 70:30, respectively, purchased from Sigma - Aldrich®. The solutions prepared were spread over 2x2 cm slides for further drying at room temperature for approximately 72 hours. The dried biopolymer/ceramic samples were fixed for further more precise experimental treatment. In order to obtain a clear visualization of the ripples (LIPSS) formation, a schematic representation of the basic steps of the process is given in Figure 1 below.
Figure 1. Schematic representation of the LIPSS (ripples) formation on synthesized Ch/HAp thin films.

In our experiments, we used a Ti:sapphire laser (Quantronix-Integra-C) delivering 130-fs pulses at a central wavelength of $\lambda = 800$ nm and a 50-Hz repetition rate. The beam profile has a Gaussian shape with $M^2 = 1.3$. The number of applied laser pulses ($N$) was controlled by a computer-driven fast mechanical shutter synchronized by controlling software. The experiments were performed in air with the laser beam focused to a focal spot with a diameter of approximately $40 \mu$m using a lens with a 10 cm focal length.

Figure 2. Experimental setup for femtosecond laser irradiation of Ch/HAp composites (a); image of the irradiation process of thin Ch/HAp layers (b).

The focusing lens was placed on a translation stage equipped with a micrometre screw for fine adjustment of the focus position on the specimen’s working surface. The sample was positioned perpendicular to the focused beam on a high-precision XYZ translation stage. The experimental setup was controlled by specially written LabView software. The samples were processed by scanning the focused laser beam over the material surface at precisely-defined separation intervals $d_x$ and $d_y$ in order to avoid a spatial overlap between the separate laser spots.

3. Results and discussion

Generally, it has been shown that LIPSS evolution and properties are influenced by the laser beam parameters. A significant point is the laser polarization, which has a strong effect on the process of ripples formation and has been investigated in detail by various researchers using linear, elliptical and circular polarization [13,14]. For example, Gräf et al. [12] reported results concerning the formation process and properties of laser-induced periodic surface structures (LIPSS) on different technically relevant glasses, including fused silica, borosilicate glass, and soda-lime-silicate glass, which were irradiated by 300-fs laser pulses at the wavelength $\lambda = 1025$ nm. Another research group, Rebollar et al. [14], described LIPSS formation on spin-coated thin films of several model aromatic polymers, including poly(ethylene terephthalate), poly(trimethylene terephthalate) and polycarbonate bisphenol upon femtosecond irradiation at 795 nm and 265 nm and fluences well below the ablation threshold.
We followed the laser energy density influence on the created Ch/HAp surfaces via scanning electron microscopy (Hitachi S570). To characterize the ripples creation on the thin films, we explored the effect of varying the number of applied laser pulses. In order to avoid mechanical damage and formation of micro-cracks, femtosecond, rather than nanosecond, laser pulses were applied to the biomaterial composites [15]. The use of pulses of femtosecond duration, i.e., high peak laser power, results in a nonlinear absorption process and non-thermal ablation, thus providing the attractive benefits of the capability of treating a wide variety of materials [16].

Figure 3. Scanning electron microscopy images of LIPSS formations on 70:30 Ch/HAp thin layers.

In our experiments, we observed formation of LIPSS when irradiating our samples by 5 and 10 laser pulses with duration 130 fs and \( F = 2.07 \text{ J/cm}^2 \) (Figure 3). We monitored the changes in the substrate surface; namely for \( N = 5 \) laser pulses, ripples were formed only at the periphery of the craters and smooth zones of molten substrate were clearly seen at the bottom of the craters; whereas with increasing the applied laser pulses to \( N = 10 \), the process of formation LIPSS was completed and ripples appeared throughout the craters. LIPSS were induced upon irradiation by linearly polarized laser radiation.

Additionally, Fourier transform infrared spectroscopy (FTIR, IR Affinity-1 “Shimadzu”) was used to obtain detailed information concerning the phase transformations and chemical bonds alterations in the Ch/HAp composite biomaterials before and after fs laser treatment (Figure 4). The measurements were conducted in a transmittance mode in the 4500 – 500 cm\(^{-1}\) range with a resolution of 4 cm\(^{-1}\) and 50 scan counts. The results illustrated significant differences in the spectra measured of the non-irradiated and laser-processed surfaces of the composite substrates. First, the FTIR spectra exhibited equal number and position of the various transmittance peaks, but with a major difference in the intensity of the peaks from the laser treated zones. Further, the shapes of the spectra from both processed areas (5 and 10 pulses) were similar, with the exception of a lower peak intensity of the saccharide structure of chitosan (around 900 cm\(^{-1}\)) in the matrix for \( N = 10 \) pulses.
In the spectrum of the non-treated surface, between 3400 cm$^{-1}$ and 3000 cm$^{-1}$, one characteristic group was observed, which corresponds to hydroxyl groups stretching in chitosan. The peaks near 1650 cm$^{-1}$ correspond to $\equiv C \equiv O$ stretching and $= N-H$ vibrations of amide structures I and II. Another peak noticed at about 1267 cm$^{-1}$ from the non-irradiated material surface is characteristic for the amide III structure. The band about 2880 cm$^{-1}$ is attributed to -CH "backbone" vibrations, while a maximum at about 1400 cm$^{-1}$ is assigned to -CH$_3$ and -CH$_2$ groups in plane deformation vibrations. The transmittance maxima identified at about 1030 cm$^{-1}$ to 1020 cm$^{-1}$ are commonly associated with the stretching of glucosamine in the chitosan structure. The groups observed in the range of about 1000 cm$^{-1}$ and 600-500 cm$^{-1}$ are attributed to the stretching and flexing vibrations of the PO$^4$$_3$- group.

The presence of chitosan in the non-irradiated Ch/HAp sample was clearly recorded, and, depending on the number of laser pulses, changes in the spectral shape occurred. This could be associated with the higher laser fluence and number of laser pulses applied in this case ($F = 2.07$ J/cm$^2$, $N = 5, 10$), which resulted in changes in the hydroxyapatite content in the matrix compared to our previous investigations of fs irradiation of pure chitosan thin films by 1 and 2 laser pulses with $F = 0.41$ J/cm$^2$, when discrepancies in the spectrum profile were not registered [17]. The results obtained confirmed the variation of the ablation thresholds due to the diversity of the materials considered.

4. Conclusion
The reported experimental results illustrated the generation of well-shaped LIPSS on chitosan/hydroxyapatite thin composites after femtosecond laser irradiation by laser pulses with wavelength 800 nm and duration 130 fs. The data was confirmed by SEM analysis, which clearly visualised structures throughout the laser-treated surface. FTIR was employed to acquire detailed information on the structural variations of the substrate content before and after laser treatment. We observed changes in the shapes of the transmittance spectra from the untreated and laser-processed surfaces depending on the number of laser pulses. No such changes occurred in our previous experiments on pure chitosan samples. The probable cause is the presence of hydroxyapatite for which the ablation threshold appears to be higher. The results obtained provide comprehensive evidence related to femtosecond laser processing of biomaterials and the boundary conditions for ripples formation as needed in view of improving the characteristics of the surface of scaffolds. The data confirm that fs laser processing is a technique suitable for material surfaces functionalization for further biomedical applications.
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