Laser treatment of chitosan/biopolymer materials of different molecular weight coated with ZnO for antimicrobial surface development

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Abstract. Creating novel temporary "platforms" for effective integration of engineered tissues has been extensively researched and innovated in order to obtain scaffolds fulfilling all requirements for seeding different types of cell cultures and improving the cells’ adhesion, proliferation and differentiation. A perfect scaffold should mimic the native porous environment of the cells – interconnected pores with well-defined sizes providing the normal functioning of the cells, as they can significantly influence not only the cells behavior, but also the integration of the implants with the surrounding "host" tissues. The biopolymer-based scaffolds still need additional modification in order to impart complete biological cellular functioning and communication. In this study, a femtosecond laser-based method for surface modification was applied to improving the morphological properties of chitosan-based ZnO magnetron sputtered blends and chitosan matrices of different molecular weight, thus achieving different levels of morphological structures for creation of enhanced antibacterial cell surface environment. The microstructured scaffolds were investigated by SEM, EDX and FTIR. Wettability measurements were performed in order to determine the hydrophilicity of the treated surfaces. Changes in the water contact angle (WCA) values were monitored in the range from 120° to 70° by introducing diverse laser patterning conditions. Modifying the topography/morphology of the sputtered biopolymer blends can essentially improve their bioactivity properties; moreover, creating hierarchical porosity will affect its antibacterial features, which will enable their successful applications in tissue engineering.

1. Introduction

Tissue engineering is emerging as a possible solution for regeneration of irreversible bone tissue defects. Its main “tools” are the artificially created cellular matrices. Chitosan is a natural polysaccharide characterized by high mechanical strength, biocompatibility, biodegradability and antimicrobial activity [1, 2]. Its linear structure and high molecular weight make it an ideal fibrous matrix that can be blended with other bactericidal metal compounds, such as ZnO, Ti, TiN, TiO, to form composite materials with...
even superior antimicrobial properties. Such blending forms cell scaffolds that combine the natural potent antimicrobial and photo-catalytic degradation properties of both components [3, 4]. Among these metal compounds, Zn$^{2+}$ ions have been widely used in bone repair bioconstructs, as they promote new bone formation and inhibit bone resorption [5, 6]. ZnO is famous for its antibacterial, antifungal and anti-inflammatory qualities, making it a desirable component in hybrid bone and dental scaffolds [7-9].

The “ideal” 3D-multicomponent matrices mimic the native porous environment of the cells – interconnected pores with well-defined sizes, ensuring the normal functioning of the seeded cells [10]. One of the main challenges in the design of tissue substitutes is the production of a customized implant with such interface properties but avoiding the risk of subsequent inflammation. Such porous structures, guaranteeing cellular functions such as adhesion, migration, survival, proliferation, differentiation, and communication, could be achieved by femtosecond laser modification – a non-contact technique providing hierarchical porosity and high levels of geometric complexity of the blended scaffolds, i.e., an innovative solution for development of customized, antibacterial implants surface mimicking the natural body environment. An interconnected pore structure with strictly defined and varying external sizes (1 – 500 μm) ensures the diffusion of oxygen and nutrients and cell viability and tissue ingrowth [11, 12]. Ultra-fast laser processing is providing highly precise treatment of the biomaterial surface and bulk, which allows a close control of the size and shape of the hierarchical interconnected porosity and chemical composition in order to further mimic the natural ECM structure. This technique changes not only the roughness of the treated material, but also affects its surface water contact angle properties, making it more hydrophilic. This has a positive impact on the cells’ adhesion, which is crucial for their viability [13].

On the other hand, magnetron sputtering is a low-temperature high-speed technique for preparing a uniform, strongly adhered film on the surface of polymers, ceramics and composite materials [14]. A thin film of sputtered metal compound, such as Zn, ZnO, Ti, TiO$_2$, Cu and Ag or Au nanoparticles, provides antibacterial properties, transition of hydrophobic to hydrophilic surface properties (ex. PLA), improved biocompatibility and increased roughness and cell adhesion, thus making the biomaterials suitable for biomedical applications and tissue engineering [15].

In this study, femtosecond laser-based surface modification was applied to improving the morphological properties of chitosan-based ZnO magnetron-sputtered blends and chitosan matrices of different molecular weight (50 kDa, 300 kDa, 500 kDa), achieving different morphology patterning and creating enhanced antibacterial cell surface environment. The microstructured scaffolds were investigated by SEM, EDX and FTIR. Water contact angle evaluation was performed in order to determine the hydrophilicity of the treated surface. The changes in the WCA values were monitored in the range from 120° to 70° by varying the laser patterning conditions. The analysis of the experimental results clearly shows that the femtosecond laser method could be applied for biomaterials surface functionalization with a high-level of precision.

2. Material and methods

2.1. Sample preparation

The chitosans used in the work had a molecular mass (MM) of 50 kDa, DD ≥ 90% (Pol-Aura, Gdansk, Poland), 300 kDa, DD ≥90% (“YuDa Chemicals”, Qingdao, China) and 500 kDa, DD 80.5% (“Bioprogress”, Moscow, Russia). The chitosan was dissolved in 1% lactic acid at room temperature overnight. The solution was poured in a Teflon mold and dried until the complete removal of the solvent. The films made of 300 kDa chitosan were subjected to modification by magnetron sputtering, namely, a thin layer of ZnO was deposited onto the chitosan substrate by high-frequency magnetron sputtering in the original installation of the Institute of Applied Physics with the following parameters: RF discharge frequency of 13.5 MHz, RF discharge power of 200 W, working gas (Ar) pressure of 1 Pa and a target-substrate distance of 7 cm.
2.2. Laser treatment
A Ti:sapphire laser (Quantronix-Integra-C) system (pulse duration \( \tau = 130 \) fs at a central wavelength of \( \lambda = 800 \) nm, repetition rate of 0.5 kHz, number of applied laser pulses \( N = 2, 10 \)) was used for femtosecond laser microprocessing of the prepared chitosan and chitosan-based ZnO magnetron sputtered blends. The experiments were performed in air with the laser beam focused on a spot with a diameter of 50 \( \mu \)m by a lens with a 10-cm focal length. The sample was positioned perpendicular to the laser beam on a high-precision XYZ translation stage and was processed by scanning the laser beam over the material surface in the X direction to produce a stripe-like modification. The scanning was performed at precisely-defined separation intervals in order to optimize the samples texturing and to avoid a spatial overlap between the individual laser spots. The experimental setup was controlled by LabView software.

2.3. SEM-EDX analysis
The surface morphology and elemental composition before and after fs laser treatment were evaluated by scanning electron microscopy (SEM) and energy dispersive X-ray analysis (EDX) using a SEM-TESCAN/LYRA/XMU apparatus. The samples were sputtered with gold (Au). Several images and the elemental percentage [wt. %] were taken from the laser-treated and non-treated zones of each sample.

2.4. FT-IR analysis
Detailed information about the chemical bonds alterations and phase transformations of the prepared biofilms before and after laser treatment were obtained in transmittance mode in the range 4500 – 500 cm\(^{-1}\) by an FTIR spectrophotometer (IR Affinity-1, Shimadzu, Kyoto, Japan).

2.5. WCA evaluation
The water contact angle evaluation was performed by a homemade installation in air with 1-\( \mu \)l distilled water droplet on the control and the laser-modified surfaces of the samples with the WCA evolution followed for 0.5 – 9 s. A reliable evaluation procedure was used – a contact angle goniometry controlled by ImageJ software with a contact-angle plug-in.

3. Results and discussion
Figure 1 shows representative SEM images of chitosan matrices of different molecular weight: 50 kDa (a), 300 kDa (b), 500 kDa (c) and chitosan-based ZnO magnetron sputtered blends (d), patterned by 10 and 2 fs-pulses and a fixed laser energy \( E = 0.124J/cm^2 \); the corresponding EDX data is presented in table 1 below. The SEM analysis shows the development of a granular morphology with the formation of pores on the samples surface (a-d) for both number of pulses applied. As a whole, as can be seen in Table 1, there is a slight variation in the C and O content [wt. %] with the number of pulses, which could be explained with breakage of hydrogen bonds; however, no uncommon elements are detected after laser modification of all samples.
Figure 1. SEM images (×5000 magnification) of chitosan matrices of different molecular weight 50 kDa (a), 300 kDa (b), 500 kDa (c) and chitosan-based ZnO magnetron-sputtered blends (d); control surfaces (first row), patterned with \( N = 10 \) fs pulses and fixed laser energy \( E = 0.124 \text{ J/cm}^2 \) areas (second row) and \( N = 2 \) fs pulses and \( E = 0.124 \text{ J/cm}^2 \) (third row).

Table 1. EDX data [wt. %] of chitosan matrices of different molecular weight 50 kDa (a), 300 kDa (b), 500 kDa (c) and chitosan-based ZnO magnetron-sputtered blends (d); control and fs modified surfaces, respectively.

| EDX                | C [wt%] | O [wt%] |
|--------------------|---------|---------|
| 50kDa Ch-control (a) | 58.89   | 41.11   |
| 50kDa Ch-fs modified N=10 (a) | 60.49   | 39.51   |
| 50kDa Ch-fs modified N=2 (a) | 60.17   | 39.83   |
| 300kDa Ch-control (b) | 58.75   | 42.25   |
| 300kDa Ch-fs modified N=10 (b) | 61.23   | 38.77   |
| 300kDa Ch-fs modified N=2 (b) | 58.89   | 41.11   |
| 500kDa Ch-control (c) | 57.86   | 42.14   |

| EDX                | C [wt%] | O [wt%] |
|--------------------|---------|---------|
| 500kDa Ch-fs modified N=10 (c) | 60.17   | 39.83   |
| 500kDa Ch-fs modified N=2 (c) | 61.21   | 38.79   |
| 300kDa Ch +ZnO mag, sputtered -control (d) | 51.43   | 48.57   |
| 300kDa Ch +ZnO mag, sputtered -fs modified N=10 (d) | 53.53   | 46.47   |
| 300kDa Ch +ZnO mag, sputtered -fs modified N=2 (d) | 53.94   | 46.06   |

The results of the FTIR spectroscopy complement the EDX analysis and confirm the lack of changes in the biochemical composition of the probes after fs treatment – figure 2. According to our previously work [16], the transmission peaks correspond to the bonds given in table 2. The shapes of the spectra from the processed areas (\( N = 10 \) and \( N = 2 \) pulses) were similar but with different intensity of the peaks.
Figure 2. FTIR spectroscopy of chitosan matrices of different molecular weight 50 kDa (a), 300 kDa (b), 500 kDa (c) and chitosan-based ZnO magnetron sputtered blends (d) after laser irradiation with \( N = 10, 2, \) and \( E = 0.124 \) J/cm\(^2\).

Table 2. Chemical bonds detected by FTIR transmittance spectra of chitosan and chitosan-based ZnO magnetron-sputtered thin films.

| Samples | Wave number (cm\(^{-1}\)) |
|---------|--------------------------|
|         | =N-H III | =CH\(_2\) | =CH\(_2\) | C=O | =N-H/II | CO\(_3^2-\) | C-H "backbone" | -OH |
| 50 kDa Ch-fs modified samples (a, b, c) | 1267 | 1400 | 1650 | 2363 | 2900-2880 | 2900-2880 | 3400-3000 |
| 300kDa Ch +ZnO mag. sputtered – fs modified (d) | 1267 | 1400 | 1650 | 2363 | 2900-2880 | 2900-2880 | 3400-3000 |

The hydroxyl groups stretching bonds in chitosan between 3400 cm\(^{-1}\) and 3000 cm\(^{-1}\) were observed in all spectra of the chitosan and chitosan-ZnO blends. The groups identified at about 1030 cm\(^{-1}\) to 1020 cm\(^{-1}\) are commonly associated with the stretching of glucosamine in the chitosan structure. The band at about 2990 – 2880 cm\(^{-1}\) is attributed to -CH "backbone" vibrations, while the peaks near 1650 cm\(^{-1}\) correspond to C=O stretching and =N-H vibrations of amide structures I and II. The maximum at about 1267 cm\(^{-1}\) is characteristic for the =N-H amide III structure, and the peak at about 1400 cm\(^{-1}\) is assigned to plane deformation vibrations of -CH\(_3\) and -CH\(_2\) groups.

Wettability measurements were performed in order to determine the hydrophilicity of the treated surface – a crucial factor for the cells’ normal functioning – the optimal surface conditions will allow the cells adhering to/in the scaffold and their further development [13]. Figure 3 shows representative images and data of the WCA evaluation of the 50 kDa Ch fs-modified thin films. Similar results were
obtained for all other tested samples. The average values of the droplet WCA 7 s – 9 s after application change in a wide range – from about 120° for untreated chitosan to about 30° for 50 kDa Ch fs-modified by $N = 10$. However, as can be seen from the figure, all samples become more hydrophilic with time (0.5 – 9 s) and with increasing the number of laser pulses applied ($N = 2, 10$). In their detailed work, Rosales-Leal et al. [17] demonstrate that changing the matrix wettability could allow one to control the cells’ adhesion and motion, better initial contact between the sample surface and the cells could be achieved and the total functionality of the cells implant could be further improved [13].

![Figure 3. WCA evaluation of fs-modified 300 kDa Ch samples (a, b) and image of the water droplet on the surface of 50 kDa Ch fs-modified by $N = 10$ after 0.5 s (c) and 9 s (d).](image)

4. Conclusion
Modifying the topography/morphology of sputtered biopolymer blends can substantially improve their bioactivity properties; moreover, creating a hierarchical porosity could affect its antibacterial features, which will enable their successful applications in tissue engineering. The data obtained confirms that fs-laser processing is a technique suitable for biomaterial surface functionalization for further biomedical applications. Changing scaffolds texture, in combination with improved wettability, could make the matrix created even more biocompatible and accepted by the cells.

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