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The potential of laser interferometry for a non-invasive assessment of biopolymer film structure and biological properties

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ABSTRACT

In the last years, there has been an increasing interest to the elaboration of new biocompatible and biodegradable medical polymers meant to contact the living body milieu. Amine-based biopolymer films with intrinsic biological activity have a significant potential for the synthesis of contemporaneous materials intended for surgery and tissue engineering. Our investigation is aimed to perform a non-invasive assessment of the structural characteristics and biological properties of biodegradable polymeric composites with anti-inflammatory activity, by means of ultra-high resolution laser interferometric microscopy.

Various samples of biodegradable polymers were studied with a phase-modulation laser interferometric microscope MIM-340 (Yekaterinburg, Russia) at a wavelength of 532 nm and magnification of x 20, with superficial plane resolution of up to 15 nm, vertical resolution of 0,1 nm and possibility to control the relief depth of up to 600 nm.

We have performed an *in vitro* non-invasive assessment of the impact of the structure, composition and modification conditions of the obtained biopolymer composites on the viability, adhesive properties and functional activity of the living blood cells (neutrophils, lymphocytes, and platelets). We propose a number of densitometry criteria to identify the most promising biopolymer samples for the development of medical products with characteristics maximally resembling the physiological ones.

Keywords: interference microscopy, phase thickness, biodegradable polymer films, viability, adhesive properties, blood cells

1. INTRODUCTION

Biopolymer films based on chitosan and fibroin hydrogels with intrinsic biological activity have a significant potential for the synthesis of contemporaneous materials intended for surgery and tissue engineering [1, 2]. Hydrogels are interesting biomaterials as their high water content makes them compatible with a majority of living tissues. Moreover, they are soft and bendable which minimizes the damage to the surrounding tissue during and after implantation in the patient [3].The mechanical properties of hydrogels tend to mimic those of the soft body-tissues which allows the gels to insure both functional and morphological characteristics of the tissue to be repaired [4,5].

To obtain stable hydrogels the crosslinking agents are introduced into the polymer solution. Hydrogels of amine-containing polymers in the form of films, fibers, and beads are obtained using cross-linking reagents: glutaraldehyde (GA) [6] or genipine [7]. It was shown during the past two decades that hydrogel matrices cross-linked by a natural reagent genipin have a smaller cytotoxic effect than those obtained with the widely GA cross-agent [8]. However, in a number of other studies, nontoxic hydrogels of chitosan crosslinked with GA have been obtained [9]. The conflicting data on the toxicity of reaction products of chitosan and GA may result from the use of samples with a high content of crosslinking agent in a number of studies as well as the use of traditional methods for assessing the cytotoxicity of materials using solutions of complex organic compounds and dyes: 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyl tetrazolium (4',6-diamidino-2-phenylindole dihydrochloride, propidium iodide.

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The prospective approach in medico-biological investigations is a non-invasive technology of the quantitative phase imaging (QPI) based on the principles of laser interferometry and holography [10, 11, 12]. Optic systems and apparatus-program complexes based on interferometer microscopy as compared with the contemporary methods has valuable advantages (non-invasiveness, fast-action, super-solution, etc.) considerably broadening the ways of their application [13, 14].

Our investigation is aimed to perform a non-invasive assessment of the structural characteristics and biological properties of biodegradable polymeric composites with anti-inflammatory activity, by means of ultra-high resolution laser interferometric microscopy.

2. METHODOLOGY

Chitosan with a molecular weight of 190 kDa and an 87% degree of deacetylation of the initial chitin (Roeper, Germany), the crosslinking reagents genipin (Sigma-Aldrich, USA) and glutaraldehyde (GA) (Merck, Germany) were used in the study. Gel formation in the chitosan solution–crosslinking agent systems was studied for different ratios of crosslinking agent to amino group of chitosan. Films were prepared by pouring chitosan 2% solutions chitosan in 2% acetic acid onto Petri dishes containing crosslinking agents followed by evaporation of the solvent at room temperature. The swelling of the chitosan films was studied by the gravimetric method. Samples of films before weighing were blotted with filter paper. The swelling capacity α (%) was calculated by the formula α = (mmoist − m0) ×100% m0, where m is the polymer sample weight after swelling and m0 is polymer sample weight before swelling. Due to the change ratios of crosslinking agent to amino group of chitosan and molecular weight of chitosan, gelation time may vary from a few minutes up to 5 hours. During the evaporation process of the solvent, the film acquired a blue color, the intensity of which increased with increasing of genipin content. The films do not dissolve in water and their swelling degree (400 - 2800%) decreases with increasing of concentration of the cross-linking agent and molecular weight of the polymer.
Various samples of biodegradable polymers were studied with a phase-modulation laser interferometric microscope MIM-340 (Yekaterinburg, Russia) at a wavelength of 532 nm and magnification of x20, with superficial plane resolution of up to 15 nm, vertical resolution of 0,1 nm and possibility to control the relief depth of up to 600 nm.

3. Results

Interferometer microscope allows creation of the phase height of the object depending on the object refractive index and its geometric dimensions as well as on the medium within which the object is located.

$$\Phi(x,y)=(nO-nB)\cdot z, \tag{1}$$

where nO and nB – the refractive indices of the object and the medium, accordingly, on the working wavelength of the device, z – geometric height of the object.

The mirrors together with the modulator provide linear-periodic modulation of the optical path difference which allows using the phase method for creation of phase image. To create the phase image, a series of interferograms are registered in shifting of the mirror from the reference arm of the interferometer. Distribution of the intensity in each point may be written in a form:

$$\begin{cases} I_0(x,y) = A(x,y) + B(x,y)\cos(\Phi(x,y)) \\ I_1(x,y) = A(x,y) + B(x,y)\cos(\Phi(x,y+kd)) \\ I_2(x,y) = A(x,y) + B(x,y)\cos(\Phi(x,y+2kd)) \\ I_3(x,y,t) = A(x,y) + B(x,y)\cos(\Phi(x,y+3kd(t))) \end{cases} \tag{2}$$

where $I_{0\text{-}3}(x,y)$ – distribution of irradiation intensity in the field of photodetector vision for the mirror position 0…3, $A(x,y) = I_1(x,y) + I_2(x,y)$, $B(x,y) = 2\sqrt{I_1(x,y)I_2(x,y)}$, I_1 and I_2 – intensity of the reference and measuring channels, k – wave number, d – the size of the reference mirror shift. The system of equations can be solved in relation to Φ(x,y) phase advance on the object.

Unknown quantity of phase difference is determined as follows:

$$\Phi(x,y) = arctg\left[\frac{\sqrt{\left[(I_1 - I_2) + (I_0 - I_3(t_0))\right] \cdot \left[3(I_1 - I_2) - (I_0 - I_3(t_0))\right]}}{I_1 + I_2 - I_0 - I_3(t_0)}\right] \quad (3)$$

where $I_3(t_0)$ – an instant value of the intensity determined by the exposure time of the photodetector.

Unlike the contemporary multi-step methods, phase calculation algorithm realized in the MIM microscope consists of the fact that the points of standing of the reference mirror and the law of its shift are chosen from considerations of minimizing the errors in the phase measuring. After automated processing, the results of the phase height calculating can be displayed in the form of three-dimensional and two-dimensional object profiles, graphs, and histograms.

The phase images of biopolymer samples were obtained which could be interpreted as a two-dimensional projection of their three-dimensional structure. The irregularities recorded on the interferograms are due to the peculiarities of the geometry of the biopolymers (uneven terrain) and/or differences in their composition and internal structure. The analyzed samples of biopolymers differed in the amount of the crosslinking agent: the stoichiometric ratio of genipin to 1M chitosan was 0.003, 0.004, and 0.005M. Table 1 summarizes the results of measurement of maximal and mean phase film thickness in the air medium. In these conditions, the minimal phase height was always equal to zero. It was established that increasing of crosslinking agent concentration led to practically linear growth of the mean film thickness (Figure 1).

Table 1. Phase height of the polymers relative to the substrate

Ratio of chitosan/genipin	maximum (nm)	average (nm)	standard deviation
chitosan /003 genipin	213,7	108,1	36,9
chitosan /004 genipin	265,1	118,5	70,7
chitosan /053 genipin	289,6	134,8	61,5

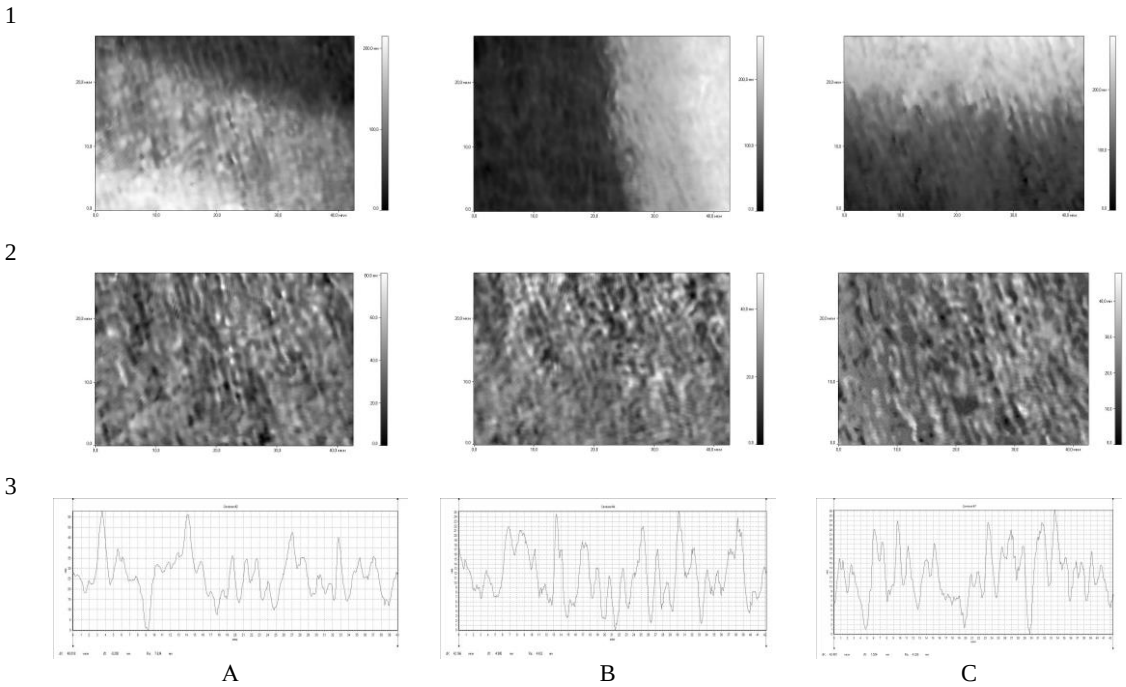

Figure 1. Films with genipin concentrations 0,003 (A), 0,004 (B) and 0,005M (C). 1 – the edge ; 2 – the plane; 3 – section of the plane.

The heterogeneity of the film structure was determined by measuring the standard deviation of the fluctuations in thickness of chitosan layer over the entire area of the survey, selected at a distance from the edge and having no extraneous inclusions. The measurement results are presented in Table 2. Increasing the genipin concentration from 0.003 to 0.004 M per 1 M chitosan reduced film heterogeneity by 1.5. However, further increase in genipin amount to 0.005 M did not lead to such significant changes in the film homogeneity: it increased only by 6.7%.

Table 2. Heterogeneity of the chitosan film structure

Ratio of chitosan/genipin	maximum (nm)	average (nm)	standard deviation
chitosan /003 genipin	213,7	108,1	36,9
chitosan /004 genipin	265,1	118,5	70,7
chitosan /053 genipin	289,6	134,8	61,5

We have performed an in vitro non-invasive assessment of the impact of the structure, composition and modification conditions of the obtained biopolymer composites on the viability, adhesive properties and functional activity of the living blood cells (neutrophils, lymphocytes, and platelets). Important aspect of using biopolymers is their possibility of acquiring properties as close to physiological as possible. For this purpose, the dynamics of morphofunctional alterations of blood cells parameters were evaluated in the process of interaction with the analyzed biodegradable composites.

4. CONCLUSION

We propose a number of densitometry criteria to identify the most promising biopolymer samples for the development of medical products with characteristics maximally resembling the physiological ones. It was shown that films based on chitosan crosslinked with GA and genipin are biocompatible.

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