Modification of polylactic acid surface using RF plasma discharge with sputter deposition of a hydroxyapatite target for increased biocompatibility

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Abstract

Surface modification of polylactic acid (PLLA) by plasma of radio-frequency magnetron discharge with hydroxyapatite target sputtering was investigated. Increased biocompatibility was demonstrated using studies with bone marrow multipotent mesenchymal stromal cells. Atomic force microscopy demonstrates that the plasma treatment modifies the surface morphology of PLLA to produce rougher surface. Infrared spectroscopy and X-ray diffraction revealed that changes in the surface morphology are caused by the processes of PLLA crystallization. Fluorescent X-ray spectroscopy showed that the plasma treatment also changes the chemical composition of PLLA, enriching it with ions of the sputtered target: calcium, phosphorus and oxygen. It is hypothesised that these surface modifications increase biocompatibility of PLLA without increasing toxicity.

Keywords: polylactic acid, biomaterial, biocompatibility, magnetron sputter deposition, calcium-phosphate

Introduction

A biomaterial is a material intended to interface with biological systems to treat, enhance or replace any tissue, organ or function of the body [1]. Currently polylactic acid (PLLA) is one of the most widely used biomaterials [2].

PLLA is a polymer with the degree of crystallinity dependent on the molecular weight and polymer treatment parameters. PLLA is biocompatible and degrades into non-toxic components with a well-described degradation rate *in vivo* and has been used as degradable surgical sutures for a long time. It has gained US Food and Drug Administration approval for clinical use [3].

The surface properties of polymer materials play a crucial role in determining the overall biocompatibility, since the surface of materials comes first in contact with biological environment [4]. Surface morphology and its physiochemical properties have a major influence on the attachment of cells, they determine cell topology, spatial orientation of cell's cytoskeleton components and many other important parameters [5-7].

PLLA is chemically inert and has no reactive side-chain groups, which makes it challenging to modify its surface and bulk. PLLA is comparatively hydrophobic, with a static water contact angle of about 80 °. This leads to low cell affinity and can provoke an inflammatory response from the living host upon direct contact with biological fluids [8, 9].

Non-thermal plasma treatments (plasma corona discharge, dielectric barrier discharge, etc.) are often used for inserting chemically-reactive functional groups on polymeric substrates to increase the biocompatibility [10]. While non-thermal plasma surface treatments are preferred for simplicity, modification of PLLA under thermal plasma conditions remain less popular owing to inherent difficulties associated with identifying appropriate plasma conditions and complimenting target material(s) for a specific (bio)-material surface treatment [11].

Radio-frequency magnetron sputtering (RFMS) of a solid dielectric target is a common way to create coatings with high biocompatibility on metal surfaces and biostable polymeric materials such as polyethylene, silicone and polytetrafluoroethylene [12-18]. The RFMS method is based on the sputtering of material in vacuum due to the bombardment of the target surface with the working gas ions which are formed in the abnormal glow discharge plasma when a magnetic field is applied. Thus, the application of the RFMS method allows modifying the plasma

composition in a wide range not only by changing the atmosphere in the vacuum chamber, but also by changing the chemical composition of the sputtered target [19-23], which opens up new possibilities for the modification of PLLA surface.

Until now only few papers investigated the PLLA surface modification by using RFMS method [24, 25]. Such situation limits the application of the RFMS method as a universal technique for modifying various types of polymeric materials and narrows the range of possible methods of surface modification of biodegradable polymers for biomedical applications.

We have previously shown that the application of this method for modifying PLLA surface allows increasing the free energy and the biocompatibility of the surface [26]. In the present paper we continue the study of the effect of the RFMS modification on biocompatibility and chemical composition of the treated films. The mechanisms of the formation of highly rough surface during the plasma treatment were further investigated.

Materials and methods

Polymer films were prepared from a 4% solution of the polymeric material (Poly (L-lactide) PURASORB® PL 38, Purac) in dichloromethane (CH_2Cl_2 , Panreac Química S.L.U.). The polymer solution of 12 (± 1) g was placed in a specially designed glass bath with a polished bottom and left at room temperature. After 24 hours when most of the solvent had vaporized, the polymer films were removed from the bath using milli-Q water. The formed polymer films were then placed into a vacuum chamber with initial pressure of 10^{-3} Pa and temperature of 25 °C for 24 hours to remove the residual solvent.

The PLLA films were treated on the custom made magnetron device (Fig. 1) developed in Dr. Tverdokhlebov's lab. The magnetron has an elongated electrode that is placed horizontally into the vacuum chamber and was used with a target that is made of the hydroxyapatite powder ($\text{Ca}_5(\text{PO}_4)_3(\text{OH})$) with the size of 330×120×6 mm. The device has RF generator with a maximum power of 4 kW and an operating frequency of 13.56 MHz. For treatment of PLLA films specific RF power was set to 5W/cm² to maintain electron density within 10^9 – 10^{10} pp cm³ range and target-to-substrate distance was extended to 16 cm to facilitate enhanced coagulation of elemental plasma and target species under collision plasma conditions. Treatment duration was 30, 60 and 150 sec.

(a)

(b)

Fig. 1. RFMS device (a) and its elongated electrode (b).

High resolution atomic force microscopy (AFM) was used to investigate the surface morphology of samples. AFM microscope Solver-HV with cantilevers NSG11 (NT-MDT, Russia) was used. Processing of the images was performed using Gwyddion 2.25 software.

The chemical structure of the samples was studied using the Attenuated total reflectance Fourier transform infrared (FTIR) spectroscopy, using a Nicolet 6700 system (Thermo Scientific, USA) in the range of 800 to 2000 cm^{-1} with a resolution of 4 cm^{-1} .

The investigation of the crystal structure of the samples was conducted by X-ray diffraction (XRD) analysis using a Shimadzu XRD 6000 diffractometer. The samples were irradiated with monochromatic $\text{Cu } K\alpha$ radiation ($\lambda=1.54 \text{ \AA}$) produced using the accelerating voltage of 40 kV and the beam current of 30 mA. The scanning angle range, scanning step size and signal collection time were 6–35 °, 0.02 ° and 1.5 s respectively. The average size of the crystals (l_c) of the samples was calculated using the Debye-Scherrer equation:

$$l_c = \frac{k\lambda}{\cos\theta\sqrt{\beta^2 - \beta_r^2}}, \quad (1)$$

where λ is the wavelength of the incident radiation, β the width of the reflection at half height, β_r is the broadening reflex of the apparatus, θ is the angle of diffraction and $k = 0.9$.

X-ray fluorescence (XRF) analysis using spectrometer Shimadzu XRF 1800 was used to investigate the elemental composition of the samples. The accelerating voltage, scanning speed

and scanning step were set to 40 kV, 8 °/min and 0.1 ° respectively. Studies were conducted via a carbon (C), oxygen (O), calcium (Ca), phosphorus (P) and chlorine (Cl) channels.

Multipotent mesenchymal stromal cells from bone marrow (BM MMSCs) at second passage were used for cell adhesion investigation and were obtained from the Bank of Stem Cells Ltd (Tomsk, Russia). Efficiency of the cell adhesion to the modified surface was studied using fluorescent microscopy method with the inverted microscope Axio Observer Z1 (Carl Zeiss). The efficiency was evaluated by measuring the number of adherent cells visible in ten fields of view of the microscope, which were averaged and normalised to 1mm².

Preliminary estimation of the viability of these cells was performed by combined staining with Annexin V (BD Biosciences, USA), labelled with phycoerythrin (PE, BD Biosciences, USA) in combination with the 7-amino actinomycin D (7-AAD, BD Biosciences, USA). Phenotypic analysis of obtained cells was performed with monoclonal antibodies (anti-CD90 conjugated with Fluorescein Isothiocyanate and anti-CD45 conjugated with PE) on cytometer FACS Calibur (Becton Dickinson, USA).

Films were cut as disks with area of 1.8 cm² for the BM MMSCs cultivation. Discs were seeded with MMSCs, placed on to 24-set culture plate (concentration of 2.5×10⁵ cells per set) and cultivated for 5 days. Cells cultivation was carried out in culture medium DMEM (Gibco, USA) containing 1% of HEPES buffer, 10% of foetal bovine serum, 1% of L-glutamine, 100 U/ml of penicillin, 0.1 µg/ml of streptomycin, 0.1 µg/ml of amphotericin B (Sigma Aldrich, USA) at 37 °C and 5% of CO₂.

The study of cellular viability on the modified surface was carried out with FCM technology on cytometer FACS Calibur (Becton Dickinson, USA). Cells were detached from the film surface by using a 0.5% solution of trypsin-EDTA (Sigma Aldrich), centrifuged for 10 min at 716 g, resuspended in 1 ml of the culture medium and stained with Annexin-V, labeled PE, in combination with 7-AAD (BD Biosciences). Samples with non-modified PLLA surface were used as control.

The data was analyzed using the methods of statistical description and statistical hypothesis testing available in the standard software package Statistica (version 6.0). For the analysis of the data the hypothesis of normal distribution (Kolmogorov-Smirnov test) was used. In the case of the normal distribution the statistical significance was evaluated using the Student's *t*-test. When analyzed parameters had abnormal distribution, the estimation of the accuracy differences was determined using non-parametric criteria. To evaluate the accuracy of differences of three and

more indicators within the same group the criterion of Friedman was used. In case of pairwise comparisons Wilcoxon test with Bonferroni correction was applied. Data was presented as a median and 25th and 75th percentiles (Me (25%, 75%)). The significance level was at least 95% ($p < 0.05$) for all types of the statistical analysis.

Results and discussion

Fig. 2 shows high resolution AFM images of the surface of the investigated samples for different plasma treatment times. The AFM study of the surface shows the change in morphology and the increase in PLLA surface roughness with increasing the plasma treatment time. Specifically, Fig. 2(a)–(b) shows that non-modified PLLA surface has no significant cavities and protrusions. Fig. 2(c)–(d) shows that after the interaction with plasma for 30 seconds a significant number of round formations with an average diameter of 88 ± 22 nm appears on the surface. Fig. 2(e)–(f) shows that with increasing the treatment time to 60 seconds the formations' diameter increases to 240 ± 40 nm and smaller formations with an average diameter of 35 ± 4 nm are apparent on the surface of the large ones. At 150 seconds of the plasma treatment (Fig. 2 (g)–(h)) the surface of PLLA has a brain-like appearance with a higher surface roughness.

Fig. 2. AFM images of the PLLA surface at different plasma treatment times: 0 sec (non-modified) (a)–(b), 30 sec (c)–(d), 60 sec (e)–(f) and 150 sec (g)–(h).

There are two possible mechanisms responsible for the formation of the highly rough surface. The first "chemical" one is due to the PLLA degradation processes. It is known that the PLLA structure is a heterogeneous system consisting of ordered crystalline and amorphous parts. Amorphous regions located between the crystalline ones bind them together ensuring the structural integrity of the polymer system [27]. During the interaction of the PLLA surface with high-energy plasma particles (electrons, Ar^+ , ions of sputtered target (CaO^+ , Ca^{2+} , PHO^+ , PO^+ , P^+ , $(\text{PO}_4^-)_3$) amorphous regions are less stable than crystalline. As a result, there is a destruction of predominantly amorphous regions of the polymer under the plasma influence. Degradation products are removed by the vacuum system, resulting to a selective etching of the surface. Thus, the short time plasma treatment of the polymer leads to the formation of highly rough surface primarily due to the degradation processes of amorphous regions. The second "physical" mechanism is mostly connected with the processes of PLLA crystallization. High-energy plasma particles lose their kinetic energy by damping on PLLA surface which causes intense surface heating. It is known that amorphous regions of PLLA are able to transform into the crystalline state as a result of a thermal impact, and this process rate is proportional to the crystallization temperature [28]. The effect of the increasing PLLA crystallinity degree under the temperature influence was described for isothermal crystallization of films [29] and nonwoven materials [30]. Since the formed polymer crystal occupies a smaller volume than the amorphous region the surface "contraction" occurs, which leads to the formation of the rough surface.

Previous studies of PLLA crystallization processes with the help of IR spectroscopy revealed the bands in the spectra sensitive to changes in the crystal structure of PLLA macromolecules (Table 1). Therefore, IR spectra can be used to estimate changes in PLLA crystal structure.

Table 1

Relevant IR bands associated with different phases of PLLA (modified from [30]).

IR frequency (cm^{-1})	Crystalline form	Ref. №
860	Amorphous	[31]
871	α	[32]
908	β	[33]
921	α	[33]
955	Amorphous	[34]
1268	Amorphous	[34]
1358	Semicrystalline	[35]
1749	α	[36]

Fig. 3 shows the IR spectra of the samples where we can observe an increase in the degree of PLLA crystallinity with the increasing of the plasma treatment time. Thus, there is an increase of

the 1749 cm^{-1} band intensity in the 1810–1710 cm^{-1} region (the C=O stretching band region). There is an increase in the intensity of the band 1358 cm^{-1} with simultaneous decrease in the intensity of the band 1268 cm^{-1} in the region 1200–1500 cm^{-1} (the CH_3 , CH bending, and C–O–C stretching band region). The region of 840–960 cm^{-1} (the skeletal stretching and CH_3 rocking band region) has an increase in 921 cm^{-1} band intensity and a decrease at 955 cm^{-1} . There is band shift from 860 cm^{-1} to 871 cm^{-1} . The absence of the band at 908 cm^{-1} characteristic for PLLA in β -crystal form is noteworthy. It is known that the α -crystal modification of PLLA is easily formed from the melt [37, 38], therefore, the increase in the intensity of the absorption bands characteristic for the α -crystal structure of PLLA is an indication that the process of PLLA crystallization is caused by heating of the polymer surface as a result of the interaction with high-energy plasma particles.

PLLA film is in mostly amorphous state before the treatment which is evidenced by a vast halo observed at 15–25 ° (Fig. 4). The mostly amorphous state is due to a low crystallization rate of the PLLA solution at room temperature [33]. Fig. 4 shows changes of the reflections for treated films. The increase of the reflection (110/200) intensity at 16.5 ° is connected with the growth of the polymer crystal in the direction (110/200). The crystal size for 30 sec treatment is less than 10 nm, for 60 and 150 sec treatment times the crystal size is 16.6 ± 0.4 and 21.1 ± 0.3 nm respectively. There is a shift of several reflexes: (110/200) to the region of 16.6–16.7 °, reflex (203) from 18.6 to 18.8°. There is an increase in the intensity of minor, but noteworthy reflections at 12.1 (004/103), 14.7 (010) and 22.3 (015).

The obtained dependences are the evidence of PLLA crystallization mechanism “amorphous state $\rightarrow \alpha' \rightarrow \alpha$ ” described in [28, 39]. It is known that this crystallization mechanism is observed in the temperature range 110–170 °C. Transition “amorphous state $\rightarrow \alpha' \rightarrow \alpha$ ” detected by XRD is an indication of the significant increase in the PLLA surface temperature due to the interaction with plasma. Thus, PLLA crystallization processes in the formation of the surface relief play an important role when the time of the interaction between PLLA surface and plasma is significant. The formation of the brain-like appearance after the surface treatment for 150 sec (Fig. 2(g)–(h)) is caused by the processes of the fusion of α' type polymer crystals (formed on the surface during the short time plasma treatment) and the formation of highly ordered α type crystals formed because of the surface heating.

Fig. 5 shows the typical fluorescence spectra of the investigated samples for the following elements: carbon, oxygen, chlorine, calcium and phosphorus.

(a)

(b)

(c)

Fig. 3. FTIR results of the investigated samples: 800–2000 (a), 820–980 (b) and 1200–1500 cm^{-1} (c) regions.

Fig. 4. XRD results of the investigated samples.

Fig. 5. Fluorescent spectra of the investigated samples for the following elements. Channels of C (a), O (b), Cl (c), Ca (d) and P (e).

Results of semi-quantitative elemental analysis of the investigated samples before and after the plasma treatment are shown in Table 2. Increasing the treatment time to 150 seconds gives the decrease of chlorine (Cl) content by more than 18 times in comparison with non-modified film. This phenomenon may be observed due to the increase of the free surface on the films after treatment which allows an easier diffusion of dichloromethane residual from the bulk to the surface of the film and its subsequent removal into the vacuum system of the sputtering facility. We believe that the slight decrease of the carbon content (on average 2.5%) with the simultaneous increase of the oxygen content (on average 6%) can be explained by the formation of C–O and O–C=O functional groups with additional oxygen ions from the sputtered hydroxyapatite target.

Table 2

Semi-quantitative analysis of elemental composition of the investigated samples.

Treatment time, sec	Content the element, wt. %				
	Ca	P	C	O	Cl
0	<0.01	<0.01	63.22 ± 2.24	32.23 ± 1.74	4.54 ± 1.22
30	0.02 ± 0.02	<0.01	62.88 ± 2.68	35.92 ± 2.22	1.17 ± 0.43
60	0.07 ± 0.04	≈0.01	61.73 ± 2.12	37.59 ± 1.83	0.61 ± 0.32
150	<0.02	<0.01	60.58 ± 2.54	39.17 ± 1.68	0.25 ± 0.24

The content of calcium and phosphorus in PLLA films reached maximum value at 60 sec plasma treatment time and then decreased for 150 sec treatment. When the treatment time is relatively small (e.g. 30 sec), bombardment of the PLLA surface by ions of the sputtered target results in formation of a thin amorphous ((bio–)ceramic/(bio–)resorbable polymer layer. For longer treatment time (60–150 sec) PLLA surface heats up, leading to the increase of the thermochemical degradation rate of PLLA surface. Degradation also happens because the treated surface is bombarded by O²⁻ sputtered from the hydroxyapatite target. As a result, the surface is enriched with PLLA degradation products (low molecular weight polymers, oligomers, etc.). As these products are weakly bounded to the surface, this leads to their removal by the vacuum system installed on the sputtering facility together with ions of calcium and phosphorus.

Table 3 shows the results of the studies of the effect of PLLA surface treatment on BM MMSCs. Fig. 6 shows the images of fluorescently labelled cells on the investigated PLLA surfaces.

Table 3

The results of biological tests of the investigated samples.

Treatment time, sec	Amount of BM MMSCs on 1 mm ² of the surface, Me (25%; 75%)	Relative amount of BM MMSCs, (Me (25%; 75%))			
		Viable, %	Early apoptosis, %	Late apoptosis, %	Necrosis, %
0	112 (105; 174)	44.9 (42.4; 47.6)	11.7 (6.5; 16.9)	17.0 (15.4; 18.7)	26.3 (16.9; 35.7)
30	482 (370; 614)	66.1 (60.5; 71.8)	29.7 (26.6; 32.8)	3.12 (1.10; 5.14)	1.00 (0.53; 1.47)
60	707 (683; 829)	71.0 (62.7; 79.2)	24.4 (14.0; 34.9)	1.95 (1.07; 2.83)	2.65 (1.37; 3.93)
150	715 (654; 791)	71.7 (68.0; 74.6)	24.4 (19.8; 29.8)	2.07 (1.07; 3.07)	1.79 (1.10; 2.47)

(a)

(b)

(c)

(d)

Fig. 6. Images of fluorescent-labelled cells on the investigated surfaces: non-modified (a), after treatment for 30 sec (b), 60 sec (c) and 150 sec (d).

The significant increase in cell adhesion to modified PLLA surface is evident after 5 days of BM MMSCs cultivation (Table 3, Fig. 6). The total number of adherent cells on the surface modified for 30 sec was 4.3 times higher compared to the non-modified surface. The modification for 60 and 150 sec has given similar significant increase (about 6.3 times) in the adherent cells compared to non-modified surface ($p < 0.05$). Thus, there was no significant difference between the amount of BM MMSCs on the PLLA films treated for 60 and 150 sec.

The analysis of BM MMSCs culture on all the investigated surfaces using FCM demonstrated that 98.5% are CD90+ CD45– cells. Therefore, the surface properties do not cause the change of cellular phenotype during 5 days of BM MMSCs cultivation.

The investigation of cell viability of BM MMSCs, cultivated for 5 days (Table 3), demonstrated that the surface modification increased the number of viable BM MMSCs by 1.5–1.6 times ($p < 0.05$) compared to the non-modified PLLA surface. Early apoptosis of BM MMSCs was less frequent for non-modified surface in comparison with plasma treated one (2.5, 2.1 and 2.1 times less for 30, 60 and 150 sec treatments, respectively; $p < 0.05$). However, a significantly lower percentage of late apoptotic and necrotic cells was found on the modified PLLA surface comparing with non-modified ones. This observation raises a question regarding the genesis of the high proportion of BM MMSCs early apoptosis. It is known that cell activation level can have a significant effect on the increase of early apoptosis. The cell activation level promotes the emergence of phosphatidylserine molecules on surface of cells, for example as a result of active cell proliferation. This emergence may result in the increase of phosphatidylserine binding with Annexin-V [40]. Thus, the data for early apoptotic BM MMSCs suggests two possible mechanisms of early apoptosis: the first mechanism is the beginning of programmed cell death, whereas the second one is the active cell proliferation.

The rate of late apoptosis decreased by about 6 to 9 times on the modified surfaces comparing to non-modified surfaces, which indicates more favorable conditions for BM MMSCs cultivation on modified PLLA surfaces. The total rate of cell necrosis was 10 to 26 times lower on modified PLLA surface comparing to non-modified samples surface ($p < 0.05$).

Conclusions

It was shown that the RFMS plasma treatment with the solid hydroxyapatite target sputtering increases the biocompatibility of PLLA surface by stimulating processes of attachment and differentiation of MMSC pool. The FCM method revealed that the plasma treatment does not cause an adverse cellular response (apoptosis, necrosis). This surface modification can therefore

enhance PLLA usability as a biomaterial. It is hypothesised that the increased surface roughness of PLLA, which was demonstrated with AFM studies, plays a major role in the increased biocompatibility. FTIR, XRD studies of the surface demonstrated that the formation of the rough surface morphology was caused by the processes of PLLA crystallization. XRF studies also have confirmed that PLLA surface was significantly enriched by calcium and phosphorus from the hydroxyapatite target at longer treatment times. This enrichment can also contribute to improvements in the biocompatibility of the PLLA surface.

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