THE EFFECTS OF RADIO-FREQUENCY PLASMA DISCHARGE ON SURFACE PROPERTIES AND BIOCOMPATIBILITY OF POLYCAPROLACTONE SCAFFOLDS

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Abstract

The study is aimed at the assessment of surface modification of bioresorbable polymeric material (polycaprolactone (PLC) by radio-frequency magnetron discharge plasma with hydroxyapatite target sputtering. Plasma surface modification increases its roughness, the surface free energy, hydrophilicity and enhances the growth of PCL crystallites. An increase in plasma exposure times (30, 60, 150 s) increases the saturation of the polymer surface with sputtering target ions (calcium, phosphorus). The effects of plasma exposure times on the behavior of multipotent mesenchymal stem cells (MMSCs) were assessed. Plasma modification of PCL stimulates the attachment and subsequent proliferation of MMSCs, thus a 60-second plasma exposure period is considered to be an optimal modification mode. This type of plasma modification does not affect cell viability (apoptosis, necrosis).

Thus, the radio-frequency magnetron sputtering of a hydroxyapatite target appears to be a promosing method to modify bioresorbable polymer surfaces (PCL) in tissue engineering.

Keywords: *surface modification, polycaprolactone, roughness, free surface energy, hydrophilicity, biocompatibility*

1. INTRODUCTION

Tissue-engineering has the potential to eliminate physical or mechanical defects of tissues that have been damaged, to restore their functions, including biological (metabolic), opening the possibility for tissue regeneration. The advancement in tissue engineering aims to produce novel highly functional materials and to develop methods for their modification, enabling to obtain properties analogous of the living tissues.

Bioresorbable polymers, including synthetic and natural polymers, are the most promising materials, commonly used for tissue engineering. They are able to degrade into non-toxic products of low molecular weights in physiological fluids (carbon dioxide and water) and be substituted by own tissues during the degradation process (Nair &Laurencin 2007). Furthermore, biodegradable synthetic polymers exhibit satisfactory mechanical properties. These properties may be regulated by combining different polymers or using the copolymerization process according to research needs.

Polycaprolactone (PCL) due to its good properties is gaining increasing interest among researchers, developing tissue-engineering scaffolds. PCL is a semi-crystalline aliphatic polyester (the degree of crystallinity decreases with increase in molecular weight) having a relatively low melting temperature (+ 59-64 °C) and T_g (– 60° C) (Woodruff & Hutmacher 2010). PCL may be dissolved in various organic solvents and combined with other biodegradable polymers (Sun et al. 2006; Jones et al. 2002; Antonova et al. 2012). However, the use of unmodified PCL in tissue engineering is limited by its hydrophobicity, preventing cell adhesion, spreading and proliferation (Zheng et.al 2014). Modification of polymeric materials allows changing their surface properties, namely chemical composition, surface topography and hydrophilicity, assessed by contact angle and surface free energy measurements (Ma,

Mao & Gao 2007). Plasma modificaton increases the surface roughness, reduces the contact angle and stimulates the attachment and proliferation of various cell types (Yoshida et al 2013; Siow et al. 2006)

Non-thermal plasma treatments, namely corona discharge, dielectric barrier discharges, radio frequency and microwave discharges, are widely used for surface modification of biodegradable polymers (Desmet et al. 2009). However, there is practically no experience in surface modification of bioresobable polymer using the RF plasma discharge with sputter deposition of solid dielectric targets (RFMS). Surface modification of bioresorbable polymer using the RFMS allows modifying its surface composition, not only by changing the atmosphere in the process chamber, but also by altering the chemical composition of the sputtering target (Boyd, O'Kane & Meenan 2013; Tverdokhlebov et al. 2012).

The aim of the current study is to assess the effects of PCL surface modification by the RF plasma discharge with sputter deposition of a hydroxyapatite target and to evaluate the effects of plasma exposure times on the surface relief, surface free energies as well as on its adhesive and cytotoxic properties.

2. MATERIAL AND METHODS

2.1. Preparation of samples for research

The effects of radio-frequency discharge plasma on the surface properties of bioresorbable polymers were studied on polymeric films, produced from a 4% polymer solution in dichloromethane (Panreac, Spain). Policaprolactone PURASORB® PC 12 (Purac, Spain) was used as bioresorbable polymer. 12 ± 1 grams of the polymer solution was cast on the polished surface of the specially designed glass trough. The solvent was removed after 24 hours form the polymer; the trough was filled with distilled water to separate the formed film from the trough surface. The resultant film underwent thermal treatment at 35 ° C for 24 hours to remove residual moisture.

2.2. Surface modification

Magnetron sputtering system has been used for surface modification. The elongated magnetron sputtering with a target 330 × 120 × 6 mm in size, connected with the HF-generator (max power of 4 kW at 13.56 MHz), is positioned horizontally in the vacuum chamber. The target for sputter deposition was fabricated from hydroxyapatite powder ($Ca_5(PO_4)_3(OH)$). The modification process was performed under the following sputter parameters: base pressure within the chamber 5×10^{-5} Pa, Ar gas pressure (working pressure) 3×10^{-1} Pa; RF power level ~ 5 W / cm ^{2}, RF power 350 W; the distance between the target and substrate 16 cm, plasma exposure time - 30 s, 60 s, 150 s. Unmodified PCL samples were used as controls

2.3. The study of surface topography

High resolution topographic imaging of sample surface was obtained with atomic force microscopy (AFM) using a microscope «Solver-HV» (NT-MDT, Russia). The measurements were performed using the AFM cantilevers NSG11 [NT-MDT, Russia] with needle tip curvature radius of 10 nm, dopant concentration of 5×10^{20} cm^{-3} and scan region of 30×30 μm and 2×2 μm. All measurements were carried out under air (normal conditions) in semi-contact mode. The AFM images were processed using the Gwiddion 2.25 software package.

2.4. The study of the elemental composition of the surface

The study of the elemental composition of samples was carried out by X-ray fluorescence analysis (XRF) with a spectrometer Shimadzu XRF 1800 X-Ray Fluorescence Spectrometer under the following parameters: accelerating voltage of 40 kV, scan speed 8°/ min, step width 0,1 °. The content of carbon (C), oxygen (O), calcium (Ca), phosphorus (P), chlorine (Cl) was evaluated.

2.5. Study of surface wettability

Contact angle was measured with Contact Angle Measuring Module EasyDrop (Germany) using sessile liquid droplets method. For every measurement 3 ml of liquid was dropped on the sample surface by the syringe. The contact angle was calculated for 1 minute after placing a drop on the surface. Water and glycerin were used as the test liquid. In order to prevent contamination of the samples and the subsequent distortion of the results, all the measurements were performed immediately after the surface modification. The total surface energy, polar and dispersion components were calculated by the Owens-Wendt-Rabel-Kaelble (OWRK) method (Owens & Wendt 1969).

2.6. Evaluation of cell adhesion and viability

The efficiency of cell adhesion to the modified surface and their viability was assessed using second-passage multipotent mesenchymal stromal (MMSCs) cells derived from bone marrow. MMSCs were harvested from femurs of Wistar rats. The study was performed in vitro. The polymer films were cut into 1.8 cm^2 samples to cultivate MMSCs. The samples were seeded MMSCs at the concentration 2,5 × 10^5 cells per well and, then cultured for 5 days. The samples were placed in each well of a 24-well culture plate. Cells were grown using DMEM culture media (Gibco, USA) with 1% HEPES buffer, 10% fetal calf serum, 1% L-glutamine, 100 U/ml penicillin, 0.1 mcg/ml streptomycin, 0.1 .mcg / ml amphotericin B (Sigma Aldrich, USA) at a temperature of 37 ° C and 5% CO2.

MMSCs phenotype and viability were assessed before cell seeding and after being seeded onto the film surfaces by flow cytometric analysis using a FACS Calibur (Becton Dickinson). MMSC suspensions underwent fluorescent staining. Cell suspension was seeded onto the film surfaces or tissue culture plastic, trypsinized with 0.5% trypsin-EDTA (Sigma Aldrich, USA) for 10 min at 37 °C. After the incubation, the trypsin activity was quenched with warm growth media. The cells then were washed with phosphate-buffered saline. The phenotype of cells was determined using monoclonal antibody CD90 labeled with fluorescein isothiocyanate (FITC, FITC mouse anti-Rat CD90, BD Bioscience Pharmingen, USA), and CD45 labeled with phycoerythrin (PE, PE mouse anti-Rat CD45, BD Bioscience Pharmingen, USA). The samples were prepared according to the manufacturer's protocol.

Cell viability was preliminary assessed before cell seeding and after being seeded onto the film surfaces by flow cytometric analysis using the Annexin V: PE Apoptosis Detection Kit (BD Biosciences, USA), allowing co-staining of cell suspension with Annexin V and DNA dye, 7-amino-actinomycin D (7-AAD). After a 5- day incubation, the BM MMSCs were detached using trypsin from the unmodified film surfaces (Control Group 1) and from tissue culture plastic (Control Group 2), which is normally considered the gold standard for cell culture. The relative number of viable, necrotic and apoptotic cells (early and late apoptosis) were represented as a percentage (%) of total cells.

Fluorochrome PKH26 (Sigma Aldrich, USA) and Hoechst 33342(Sigma Aldrich, USA) nuclear dye were used to stain cells for fluorescence microscopy. The detection of labeled BM MMSCs on the scaffolds was performed using an inverted microscope Axio Observer Z1 (Carl Zeiss). The efficiency of cell adhesion to the scaffold surfaces was assessed by the number of adherent cells visible in ten complete microscope fields, counted and averaged per 1 mm^2.

2.7. Statistical treatment

The data were analysed using the methods of statistical description and statistical hypothesis testing available in the standard software package Statistica (version 6.0). The Kolmogorov-Smirnov test was used to assess the normal distribution of data. If the data have normal distribution, the statistical significance was assessed using the Student's t-test. The statistical significance of the variables with skewed distribution was estimated using non-parametric tests. Significant difference of three or more variables within one group was assessed using the Friedman test, pairwise comparison - the Wilcoxon signed rank test with Bonferroni correction. Data are presented as median and 25^{th} and 75^{th} quartiles (Me (25%, 75%). The confidence interval was 95%, $p < 0.05$.

3. RESULTS AND DISCUSSION

High-resolution AFM images of PCL sample surfaces before and after the RFMS modification after different exposure times are presented on Figure 1.

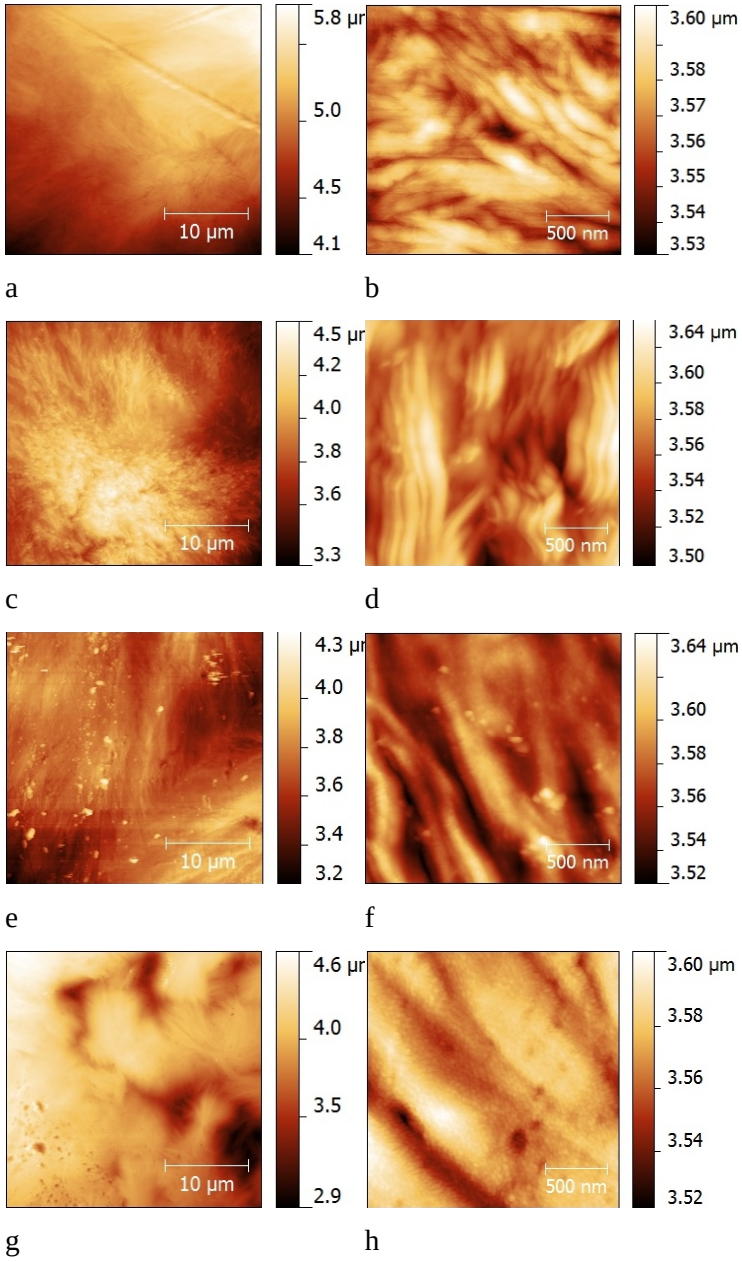


Figure 1. AFM imaging of PCL surfaces after different exposure times of plasma modification: a-b) non-modified; c-d) 30 s; e-f) 60 s; g-h) 150 s.

High-resolution imaging of the polymer surface has reported several changes in the surface morphology. An increase of PCL surface roughness is associated with increased time of plasma modification (Table 1).

Table 1. Surface roughness and hydrophilicity after different exposure times of plasma modification

exposure time (s)	Root Mean Square (RMS) roughness (nm)	Water contact angle θ (degrees)	Glycerin contact angle θ (degrees)	Surface free energy γ (mJ/m^2)	Dispersion component $a\gamma^d$ (mJ/m^2)	Polar component γ^p (mJ/m^2)
0	38.8±4.2	76.9±5.0	84.8±3.1	32.9±2.0	0.8±0.3	32.1±1.7
30	42.6±2.1	41.9±3.3	47.3±3.3	55.8±4.3	12.3±2.7	45.5±1.7
60	51.2±3.4	34.8±3.6	40.1±5.6	62.5±3.4	9.2±1.3	53.4±2.1
150	68.1±4.8	12.8±2.3	24.7±3.6	72.8±5.3	10.9±1.3	61.9±3.6

Unmodified surface of PCL have a spindle-shaped morphology with crystalline regions with an average length of 390 ± 130 nm and width of 105 ± 25 nm. The formation of the crystalline structure of PCL is caused by its high crystallization rate under rapid cooling (Chatani et al. 1970). A 30-second exposure time of the RFMS on the PCL films resulted in an increase in the spindle-shaped particles length to 850 ± 280 nm with an average width of 95 ± 31 nm. A 2-fold increase of exposure time resulted in the growth of crystallites. The length of the particles increased to 1350 ± 200 nm with the width to 280 ± 40 nm. A further increase in plasma exposure time caused melting of the crystallites and further formation of spherical-shaped beads of an average diameter 55 ± 15 nm on their surface.

The formation of relief surface morphology is associated with the following reasons. First, PCL surface modification with high-energy plasma particles (electrons, Ar^+ ions, sputtering target ions: CaO^+, Ca^{2+}, PHO^+, PO^+, P^+, $(PO4^-)_3$) inevitably decreases molecular weight of PCL. The reduction in PCL molecular weight enhances the degree of crystallinity (Engelberg & Kohn 1991). Second, the structure of crystalline polymers is known to be a heterogeneous system consisting of two ordered crystalline and amorphous phases. The interphase structure between the amorphous and crystalline phases ensures the structural integrity of the polymer system. Thus, amorphous regions are less stable than crystalline ones. As a result, plasma modification promotes predominantly the destruction of polymer amorphous regions (Slepička et al 2013). The resultant low-molecular products are removed by the vacuum system, contributing to the selective etching of the surface. Third, high-energy plasma particles during braking the surface of PCL lose their kinetic energy, causing intense heating of the surface. PCL amorphous regions are able to pass into the crystalline state under thermal effect; thus, the speed of this process is proportional to the crystallization temperature (Li & Jungnickel 1993). Since, the formed polymer crystal is characterized by a smaller volume than the amorphous region, it provokes surface "contraction" and promotes the formation of developed relief.

The analysis and semi-quantitative elemental composition of the studied samples was performed before and after modification (Table 2). Typical fluorescence spectra of calcium (Ca) and phosphorus (P) are shown in Figure 2.

Table 2. Concentrations of calcium (Ca) and phosphorus (P) in the samples

exposure time (s)	*Ca* (atomic %)	spectral-line intensity (arbitrary units)	background intensity (arbitrary units)	*P* (atomic %)	spectral-line intensity (arbitrary units)	background intensity (arbitrary units)
0	0	–	–	0	–	–
30	≥ 0.003	0.108	0.137	≥ 0.003	0.063	0.022
60	≤ 0.003	0.134	0.135	0.022±0.001	0.394	0.033
150	0.007±0.001	0.241	0.126	0.036±0.001	0.673	0.038

The concentration of the sputtering target elements on the polymer surface, namely calcium and phosphorus (Figure 2a and 2b) elevates with increased exposure time of plasma abnormal glow discharge. Apparently, spherical-shaped beads formation, occurred on the surface of PCL films after a 150-second plasma exposure (Figure 1h), are sites of the formation and further growth of calcium-phosphate coating. Previously, we have shown that the deposition of calcium phosphate coating formed by radio-frequency magnetron sputtering of a hydroxyapatite target on the surface of the thermoplastic polymer is caused by aggregation and coalescence of calcium phosphate concretions (Tverdokhlebov et al. 2012).

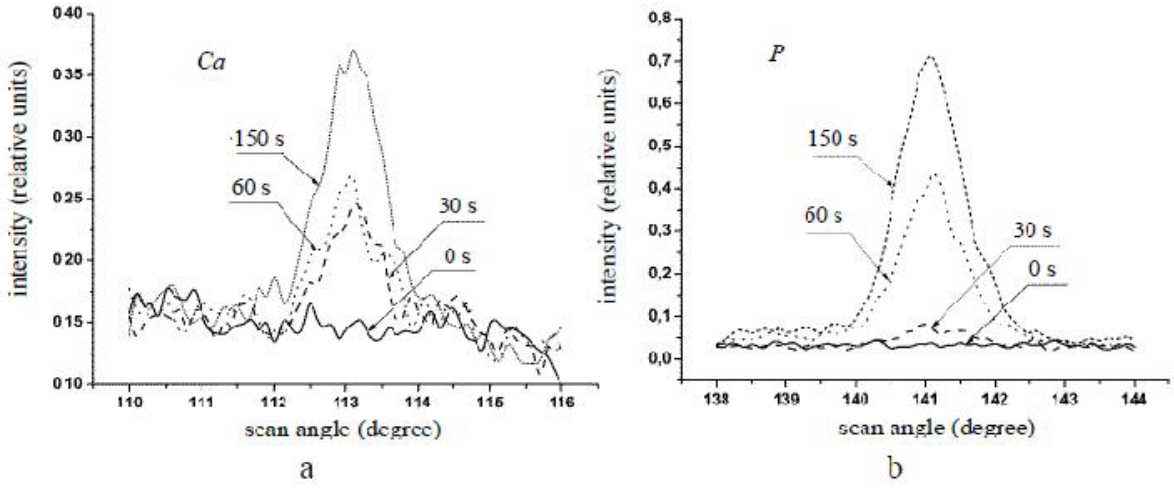


Figure 2. Fluorescence spectra of the PCL samples, modified with the RFMS after different exposure times: a) calcium (Ca); b) phosphorus (P)

Plasma modification of PCL surface gradually changes its properties from hydrophobic with the free surface energy of ~30 mJ/m^2 to more hydrophilic with γ of ~73 mJ/m^2 (Figure 3, Table 1). An increase in the surface free energy (SFE) by plasma modification is attributed to the dehydrogenation processes of PCL. Amorphous solids traditionally exhibit lower surface energy values compared to crystallines. Therefore, an increase in the surface energy confirms the presence of the PCL crystallization processes with the further formation of a bioresorbable polymer/ calcium-phosphate bioceramics.

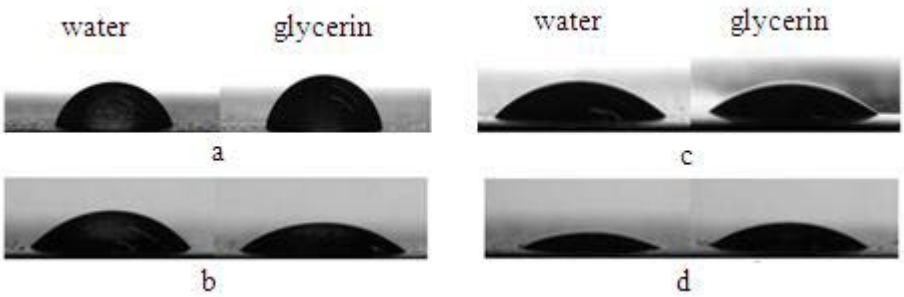


Figure 3. Liquid droplets on the PCL surface after different plasma exposure time: a) unmodified (0 s); b) 30 s; c) 60 s; d) 150 s.

A significant increase of the polar SFE component due to increased plasma exposure time is associated with strong intermolecular forces on the PCL surface. While an insignificant change of the

dispersion FSE component is attributed to poor effects, produced on PCL non-polar surface properties. First of all, this may be explained by the presence of local molecular domains in the PCL structure participating in radical formation, molecular cross-linking or C= bonds formation, caused by argon ion irradiation during plasma modification process. The second reason is the interaction between sputtering target ions and the polymer surface. Moreover, the interaction with oxygen ions results in the formation of C-O and O-C=O functional groups on the polymer surface as well as the surface saturation with heavier Ca and P ions.

Good cells adhesion, migration and differentiation require moderate hydrophilicity of biomaterial surface (Bacakova et al. 2011). Furthermore, the formation surface nanoscale roughness relief promotes cell adhesion (McMurray 2011; McNamara 2010). Plasma PCL modification has changed surface properties, potentially enhancing the interaction with cells: moderate increase hydrophilicity and roughness. Assess the effectiveness of the adhesion and cells viability after interaction with the surface was performed on MMSC. Analysis of the phenotype culture MMSC before seeding showed that 98.5% cells was CD90 + CD45-. The process of cultivation on a matrix cell has not changed cells phenotype

After a 5-day culture of MMSCs, a significant increase in cell adhesion on PCL modified surfaces has been reported (Table 3, Figure 4).

Table 3. MMSCs adhesion and viability, measured in the samples

Exposure time (s)	MMSC number /1 mm^2, Me (25%; 75%)	MMSCs relative number, Me (25%; 75%)			
		viable	early apoptosis	late apoptosis	necrosis
0 (Control 1)	111.5# (71; 166)	52.62# (52.59; 52.78)	39.3# (37.31; 41.23)	7.39# (6.0; 8.78)	0.72 (0.015; 1.13)
30	644.5*# (437; 720)	85.68* (83.65; 87.72)	13.62* (11.68; 15.55)	0.52* (0.37; 0.66)	0.18 (0.13; 0.23)
60	828.5* (749; 844)	88.98* (88.55; 89.41)	8.99* (8.38; 9.6)	1.29* (1.12; 1.45)	0.75 (0.73; 0.76)
150	833* (774; 877)	85.55* (84.5; 86.62)	13.12* (11.83; 14.4)	1.03* (0.96; 1.09)	0.3 (0.13; 0.46)
culture plastic (Control 2)	816* (777; 886)	79.17* (79.0; 79.33)	20.84* (20.67; 21.0)	0*	0

Notes: * - The significant differences between the experimental group and Control 1 ($p < 0.05$); # - The significant differences between the experimental group and Control 2 ($p < 0.05$)

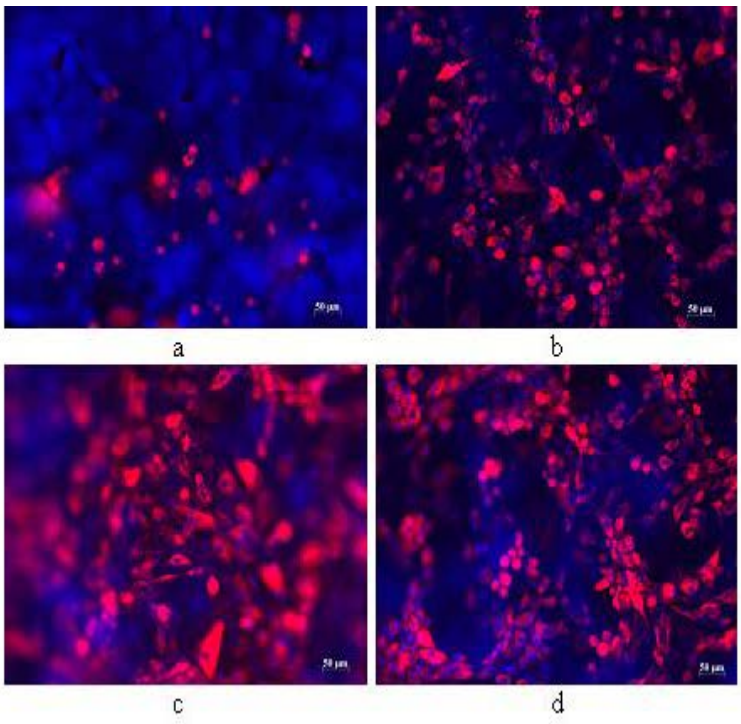


Figure 4. Imaging of MSCs (fluorescence microscopy) on the PCL after different plasma exposure time: a) non-modified (0 s), b) 30 s c) 60 s d) 150 s

There was a 4.3-fold increase in the total number of adherent cells on the modified surface compared to unmodified ones after a 30-second plasma exposure time. The modification process for 60 and 150 seconds has reported a 7.5-fold increase of the adherent cells ($p < 0.05$). After a 5-day period, the significant difference between the amount of MMSC on the modified surfaces (a 60-second and a 150-second exposure time) and tissue culture plastic with a formed confluent monolayer was not detected.Importantly, the degree of cell spreading suggests appropriate surface properties for cell/polymer interactions. The correlation between the degree of cell spreading and the number of cells on the film surfaces and their viability was defined (Table 3, Figure 4).

After a 5-day culture, cell viability of MMSCs on plasma modified samples (Table 3) increased 1.6-fold compared to Control 1 with unmodified film surfaces. Thus, there were no significant differences between the relative amount of viable MMSCs detached from modified film surfaces and tissue culture plastic. These results suggest that there is no cytotoxic effects, poduced by plasma surface modification, on MMSCs.

Modification of polymer surfaces for 30 s, 60 s and 150 s resulted in a 2.9-, 4.4 -, 3-fold decrease in the rate of early apoptosis among MMSCs compared to unmodofied film surfaces. The progression of late apoptosis in the cells detached from the modified PCL films significantly decreased compared to unmodified film surfaces (a 14.2-fold decrease for a 30-second plasma exposure times, a 5.7-fold decraese for a 60-second plasma exposure time and a 7.2-fold decrease for a 150-second plasma exposure time). The most suitable mode of modification was a 60-second plasma exposure time, because within this period the highest level of cell adhesion and viability has been achieved, while the number of early apoptotic MMSCs was lower compared to other modification modes and even tissue culture plastic.

Features of cell behavior (adhesion, spreading, proliferation and viability) on the surface of the samples confirm appropriate surface properties for cell/polymer interactions as a result of plasma modification.

4. CONCLUSION

The surface of bioresorbable polymer material (PCL) may be modified in plasma abnormal glow discharge, occured during the radio-frequency magnetron sputtering of a hydroxyapatite target. The RFMS increases the surface roughness, enhances the growth of PCL crystallites, saturates the polymer with sputtering target ions (calcium, phosphorus). An increase in plasma exposure times (30, 60, 150 s) increases the content of the sputtering target elements on the surface of the polymer material (calcium, phosphorus) resulting in higher surface free energies and hydrophilicity.

Plasma modification of PCL stimulates the attachment and subsequent proliferation of MMSCs and does not affect their viability (apoptosis, necrosis). A 60-second period of plasma exposure is considered to be an optimal among other modes. It provides the highest level of cell adhesion and viability, demonstrating the lowest rate of early apoptosis among the cells in comparison with other modification modes.

Thus, the modification of polymer surfaces (PCL) by plasma abnormal glow discharge during a radio-frequency magnetron sputtering of a hydroxyapatite optimizes the surface properties and improves its biocompatibility. This method of modification may be used in tissue engineering.

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