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Effect of the Polymer Modification on the Cytocompatibility of Human and Rat Cells

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***Abstract*—** Tissue engineering includes combination of materials and techniques used for the improvement, repair or replacement of the tissue. Scaffolds, permanent or temporally material, are used as support for the creation of the "new cell structures". For this important component (scaffold), a variety of materials can be used. The advantage of some polymeric materials is their cytocompatibility and possibility of biodegradation. Poly(L-lactic acid) (PLLA) is a biodegradable, semi-crystalline thermoplastic polymer. PLLA can be fully degraded into H_2O and CO_2. In this experiment, the effect of the surface modification of biodegradable polymer (performed by plasma treatment) on the various cell types was studied. The surface parameters and changes of the physicochemical properties of modified PLLA substrates were studied by different methods. Surface wettability was determined by goniometry, surface morphology and roughness study were performed with atomic force microscopy and chemical composition was determined using photoelectron spectroscopy. The physicochemical properties were studied in relation to cytocompatibility of human osteoblast (MG 63 cells), rat vascular smooth muscle cells (VSMC), and human stem cells (ASC) of the adipose tissue *in vitro*. A fluorescence microscopy was chosen to study and compare cell-material interaction. Important parameters of the cytocompatibility like adhesion, proliferation, viability, shape, spreading of the cells were evaluated. It was found that the modification leads to the change of the surface wettability depending on the time of modification. Short time of exposition (10-120 s) can reduce the wettability of the aged samples, exposition longer than 150 s causes to increase of contact angle of the aged PLLA. The surface morphology is significantly influenced by duration of modification, too. The plasma treatment involves the formation of the crystallites, whose number increases with increasing time of modification. On the basis of physicochemical properties evaluation, the cells were cultivated on the selected samples. Cell-material interactions are strongly affected by material chemical structure and surface morphology. It was proved that the plasma treatment of PLLA has a positive effect on the adhesion, spreading, homogeneity of distribution and viability of all cultivated cells. This effect was even more apparent for the VSMCs and ASCs which homogeneously covered almost the whole surface of the substrate after 7 days of cultivation. The viability of these cells was high (more than 98% for VSMCs, 89-96% for ASCs). This experiment is one part of the basic research, which aims to easily create scaffolds for tissue engineering with subsequent use of stem cells and their subsequent "reorientation" towards the bone cells or smooth muscle cells.

***Keywords*—**Poly(L-lactic acid), plasma treatment, surface characterization, cytocompatibility, human osteoblasts, rat vascular smooth muscle cells, human stem cells.

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I. Introduction

An important task of tissue engineering is the study of proliferation of cells obtained from various parts of the body while maintaining their specific activities. It was found the unique properties of nanomaterials may not only affect cell adhesion and interconnectivity throughout the cellular structure of the sample surface, but also represent a significant factor for structural and organizational stability of cells [1]. Some natural and synthetic materials are used to form the substrates for cells cultivation. These substrates form a suitable environment for grow, differentiation and formation of the cultured cells. The cells create the tissue while the substrates are degraded (absorbed). Chitosan [2], [3], polyglycolic acid (PGA) [4] or polylactic acid (PLLA, PLA) [5] belong to group of these materials.

The interaction between the cell and studied substrates is strongly dependent on the physical and chemical properties of the substrate. The main parameters that decide about the colonization of the material surface with cells are surface polarity, wetting, electric charge, roughness and morphology. Equally important is the surface chemical structure, namely the presence of various functional groups and biomolecules on the surface of these materials [6], [7].

Cell affinity of biomaterials plays an important role in tissue engineering which is related to the surface properties of biomaterials. Cellular affinity includes two aspects; cell attachment and cell growth. The cell attachment belongs to the first phase of cell/materials interactions and the quality of this phase will influence the cell's capacity to proliferate and to differentiate itself on contact with the implant. The cell adhesion is a first step before events like cell spreading, cell migration and differentiated cell function [8], [9]. The cells are "connected" to the substrate surface via the adhesive points, which creates a connection between the substrate surface and cell cytoskeleton. Formation of these interfaces is not only influenced by the surface chemistry of the substrate (e.g. an important role is played by the presence of ligands), as well as electrostatic charge, wettability (surface polarity) and modulus of elasticity of the polymer substrate [10], [11]. Cell adhesion is influenced by the surface properties of biomaterials. It is known that the properties such as wettability, surface charge, roughness or topography can significantly influence and control adhesion and subsequent proliferation of the cells [9].

There are many materials potentially usable for replace or repair tissue in tissue engineering. However, parameters of these materials need to be modified to increase their suitability for cell colonization and the formation of new tissue [6]. The present research deals with the creation of substrates

supporting adhesion, proliferation and differentiation of cells in a controlled manner. The substrates can be formed as planar (two-dimensionally) or spatial (two-dimensionally) scaffolds and subsequently used for cells colonization and creation of vascular prostheses, endothelial layer bone implants [6], [12]-[14], or skin substitutes formed from the polymeric substrate and layer of fibroblasts covered by keratinocytes [15].

Advanced tissue damage therapies are concentrated on stem cells, which can be used for direct application to the damaged sites or for tissue engineering using appropriate scaffolds. Stem cells can be obtained from specific locations in the adult organism, such as bone marrow, blood, skin, skeletal muscle and particularly fat [16]. Adipose tissue is relatively abundant in many patients and is relatively easily accessible without considerable donor site morbidity due to its subcutaneous localization [17]. In comparison with the other sources of stem cells in the human body, ASCs in the adipose tissue are present in much larger quantities [18], and have a higher proliferation capacity [19] and delayed senescence [20]. In addition, ASCs can be induced *in vitro* to differentiate into other mesodermal cell types, such as osteoblasts, chondroblasts, skeletal myocytes, smooth muscle cells and endothelial cells, and they are also able to transdifferentiate into cells of ectodermal origin, e.g. neurons and epithelial cells, and cells of endodermal origin, such as hepatocytes and pancreatic islet cells [16], [20], [21].

Many potential materials for construction of tissue repair have corresponding parameters for integration with surrounding tissue in the body and need to be significantly modified in order to improve their attractiveness for cell colonization and the formation of new tissues.

In this work, the influence of the plasma modification of PLLA on the adhesion, proliferation, differentiation and migration of various cell types (stem cells derived from adipose tissue (ASC), human osteoblasts (MG 63 cells) and rat VSMC) was studied. PLLA polymer film was modified by argon plasma under different conditions (such as variables were chosen performance plasma discharges and modification time). Subsequently, the surface was analyzed by various methods and characterized. The suitability of a substrate for culturing cells was determined *in vitro*. The aim of this experiment is to determine the appropriate type of modification PLLA enabling "ideal" interaction between the substrate and the cell. This experiment will serve as a basis for subsequent research directed towards targeted "specialty" (differentiation) stem cells toward specific cell type, in our case the vascular cells or bone cells.

II. Experimental Section

A. Material

All experiments were carried out on PLLA acid foils (density and thickness of the sheets were 1.25 $g \cdot cm^{-3}$ and 50 μm, purchased from Goodfellow, UK). Circular samples in final diameter of 2 cm were cut from the polymer sheets.

B. Modification

The PLLA foils were modified by an Ar^+ plasma discharge on Balzers SCD 050 device (Baltec, UK). The time of modification was 50-480 seconds, the discharge power was 3 W. The processing parameters were: DC, gas purity 99.997%, Ar flow 0.3 l s^{-1}, Ar pressure 10 Pa, electrode area 48 cm^2, the inter-electrode electrode distance of 50 mm.

C. Surface Characterization

The surface wettability was determined by goniometry - by measuring the contact angle (CA) using the static water drop method. The analysis was performed on at least 2 samples from each modification or pristine PLLA. The measurement was performed using distilled water on 8 different positions on each sample. The measurement was carried out at room temperature on DSA 100 (KRÜSS GmbH, Germany).

CA of the plasma treated samples strongly depends on the time from modification. Determination of the CA began 20 minutes after modification; measurements were carried out over the next 20 days. After this time, the surface was already stable.

The presence of the oxygen (O), carbon (C) and nitrogen (N) atoms in the pristine and modified PLLA surface layer was proved by XPS method using Omicron Nanotechnology ESCAProbeP spectrometer with monochromated X-ray source (1486.7 eV) with the step size of 0.05 eV. Detection angles of photoelectrons were (i.e. perpendicular position of the detector to the sample). The analyzed areas were 2x3 mm^2. The elemental composition was determined from the individual peaks areas using CasaXPS software.

Study of the changes in surface morphology and roughness of the pristine and modified samples were examined by atomic force microscopy (AFM) using a VEECO CP II device in tapping mode. It was probe RTESPA-CP, spring constant 20-80 $N \cdot m^{-1}$. By repeated measurements of the same region (10 × 10 μm^2), it was proven that the surface morphology did not change after three consecutive scans. The surface roughness value (R_a) represents the arithmetic average of the deviation from the centre plane of the samples.

D. Cytocompatibility and in vitro Experiments

The PLLA samples were sterilized with 70% ethanol for one hour. The samples were inserted into 12-well plates (TPP, Switzerland, diameter 2.1 cm) and all samples were air-dried. After the drying process, the samples were fixed to the bottom of the culture wells by plastic rings in order to prevent the samples floating in the cell culture media. 3 types of cells were seeded on the samples. Vascular smooth muscle cells (VSMC, isolated by an explantation method from rat aorta), human osteoblasts (MG 63) and adipose stem cells (ASC, obtained from adipose tissue using liposuction method) were used. All cells were seeded with the density 17 000 cells per cm^2 into 3 ml of Dulbecco´s Modified Eagle Minimum Essential Medium (DMEM, Sigma) supplement with 10% fetal bovine serum (FBS, Sebak). The cells were cultivated for 24, 96 and 168 hours at 37 °C in a humidied air atmosphere containing 5% CO_2.

The analysis by "randomly chosen fields" was used for determine the number of cells, morphology and spreading of the cultivated cells. The samples used for this analysis were stained for 40 minutes with a combination of fluorescent membrane dye Texas Red C2-maleimide (Molecular probes, Invitrogen) and a nuclear dye Hoechst #33342 (Sigma). The number, morphology and distribution of cells on substrate surface were evaluated from photographs taken under an Olympus Ix 51 microscope using an Olympus DP 70 digital camera. The number of cells was determined using image analysis software NIS Elements. Adhesion was determined by the number of initially adhered cells 24 h after seeding, the proliferation 98 and 168 h after seeding. The viability of the cells was determined by Cell Viability analyzer (Vi-Cell XR, Beckman Coulter) based on the trypan blue exclusion test.

III. Results and Discussion

A. Goniometry, CA

It was observed that plasma treatment leads to cleavage of macromolecules, surface ablation and changes of chemical composition. All these effects resulted in changes of surface properties e.g. surface wettability and CA. The change of the surface within the specific time interval from the plasma modification is known as aging. The dependence of the CA on aging time for plasma modified PLLA by different modification times is shown in Fig. 1. The wettability of surface increases after plasma treatment. Exposure by plasma discharge leads the creation of free radicals and subsequent creation of oxygen groups and oxidation of the layer exposed to the air [22], [23]. CA measured within 10 days after modification increased and reached a saturated value. Restoration of the CA is connected with the rearrangement of degraded macromolecules and creation of new functional groups on the plasma treated surfaces. From Fig. 1, it is obvious that CAs of plasma treated samples strongly increase with prolonging exposure time. CA of all samples increased with increasing time from the plasma modification. Aged PLLA substrates modified by plasma for shorter time (50 or 120 s) had lower values of the CA compared with untreated PLLA. Aged PLLA substrates modified by plasma for longer time (300 or 480 s) had higher values of CA compared with untreated PLLA.

B. Surface Morphology and Roughness

Effect of plasma modification on the surface morphology and roughness of the samples was determined by AFM. In Fig. 2 are shown AFM scans and roughness values of the pristine and plasma modified PLLA.

It is evident that the surface morphology of samples is dependent on the time of plasma modification. Short exposure time (50 s) leads to the formation of small amounts of crystallites on the surface of the samples. Increasing time of modification caused significant increase of amount of the crystallites and consequently led to an increase in surface roughness of the samples.

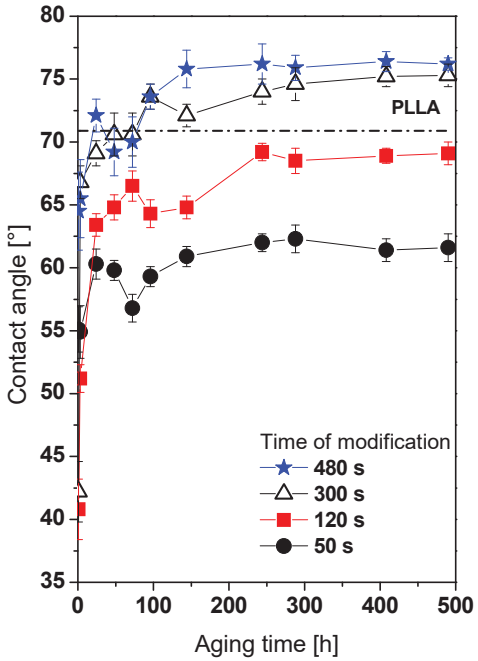

Fig. 1 Dependence of the CA of plasma treated PLLA on the aging time (after modification); Plasma exposure time was 50-480 s, power 3 W; CA value for pristine PLLA is presented by dotted line

C. Surface Chemistry

Plasma discharge of PLLA samples leads to oxidation of the surface layer and formation of new chemical groups on the polymer. Changes of chemical composition of PLLA surface layer caused by plasma treatment were studied by XPS analysis. The carbon (C), oxygen (O) and nitrogen (N) concentration of the surface layer (thickness of 6-8 atomic layers) of pristine and plasma treated PLLA is shown in Table I. It is evident that the plasma modification of PLLA leads to a decrease in oxygen concentration in the surface layer compared to the pristine PLLA. For modified samples it was also detected the presence of nitrogen in the surface layer. The presence of nitrogen may be explained by reacting the activated surface with the residual air in the sputtering apparatus (reactions during modification) or with air atmosphere after finishing of modification.

D. Cytocompatibility of PLLA

One of the aims of this work was to enhance the attractiveness of the PLLA surface and create surface supporting the cells adhesion, proliferation and spreading. In this work were 3 different types of cells studied: human osteoblast (MG 63 cells), rat VSMCs and human stem cells from adipose tissue (ASC).

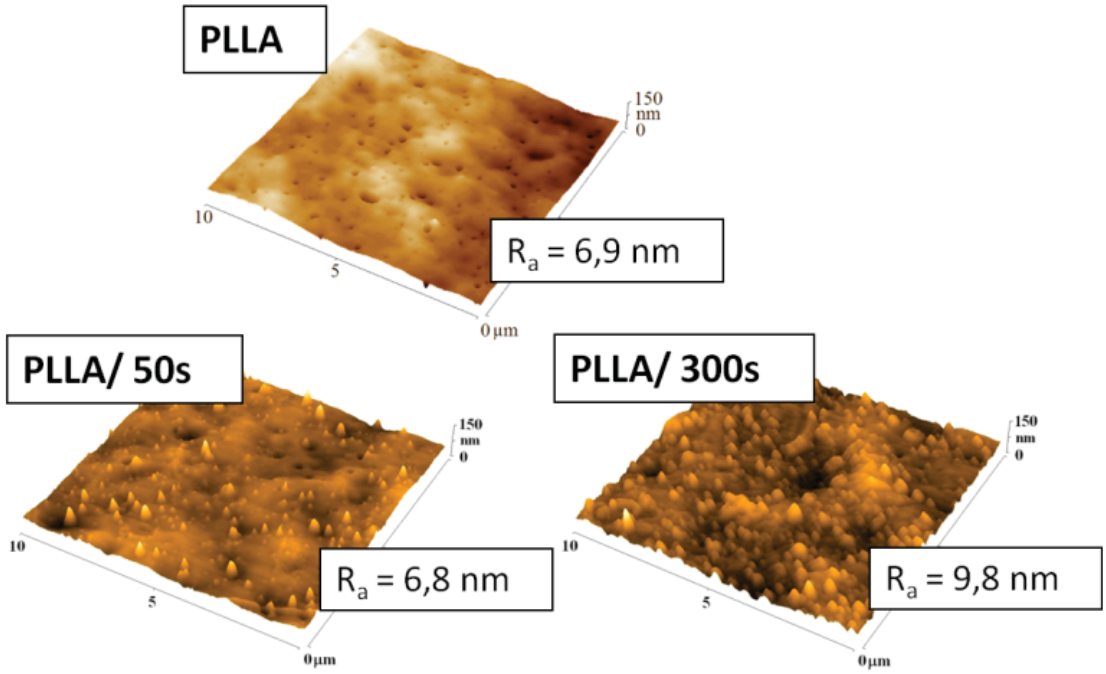

Fig. 2 AFM images of pristine PLLA and plasma modified PLLA. Plasma exposure time was 50 (PLLA/ 50s) and 300 s (PLLA/ 300s), power 3 W. Mean roughness value (R_a; in nm represents the arithmetic average of deviations from the centre plane of the sample

TABLE I
THE ATOMIC CONCENTRATION OF THE CARBON (C(1S)), OXYGEN (O(1S)) AND NITROGEN (N(1S)) IN THE POLYMER SURFACE LAYER OF PRISTINE PLLA (PLLA) AND PLLA TREATED IN PLASMA DISCHARGE FOR 50 S (PLLA/ 50S), 120 S (PLLA/ 120S) AND 300 S (PLLA/ 300S)

Sample	Atomic concentration [at. %]		
	C (1s)	O (1s)	N (1s)
PLLA	63.4	36.6	-
PLLA/50s	65.3	34.5	0.2
PLLA/120s	67.3	32.1	0.6
PLLA/300s	66.5	32.6	0.9

1. Adhesion and Proliferation of Human Osteoblasts

The number of adhered (24 h after seeding) and proliferated (96 and 168 h after seeding) human osteoblasts (MG 63 cells) is shown in Fig. 3. On day 1 after seeding, the cells on all tested PLLA substrates adhered in numbers similar. The 3rd day after seeding, the cell number on pristine PLLA became even lower than on plasma modified PLLA samples. Significantly higher number of proliferated cells was detected 7 days after seeding. The more pronounced cell growth was observed on the plasma modified samples but in this case, the effect of plasma modification on cytocompatibility of PLLA in not extensively large. The cells on the tested samples covered the surface of samples homogeneously. The cells on pristine PLLA were smaller in comparison with the cells cultivated on PLLA modified for 50 or 120 s. The cells formed clasters on some pristine PLLA samples. The viability was 89-92%. The cells proliferated on plasma modified PLLA were very well spreaded, also viability was higher than 98%. The number of adhered and proliferated cells on both modified PLLA was comparable within the error.

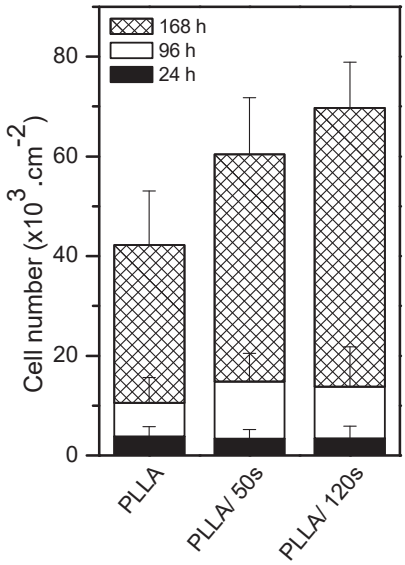

Fig. 3 Dependence of the number of adhered (24 h after seeding) and proliferated (96 and 168 h after seeding) MG 63 cells on the pristine and plasma treated PLLA. Plasma exposure time was 50 and 120 s, power 3W

2. Adhesion and Proliferation of Rat VSMCs

The number of adhered (24 h after seeding) and proliferated (96 and 168 h after seeding) rat VSMCs is shown in Fig. 4. It is evident that 24 h after seeding, the number of adhered cells on the pristine PLLA and PLLA modified for 50 s is very low. Significantly higher number of proliferated VSMCs was detected after 96 h of cultivation. The cells cultivated on modified PLLA were spreaded and evenly placed on tested substrates. The number of VSMCs cultivated for 168 h on

pristine PLLA was small, the cells remained poorly spreaded and covered the surface of the sample unevenly. The cells cultivated on both modified PLLA were uniformly distributed, they had their typically form and did not form into the clumps, viability of these VSMC was 98-99% as compared with viability cells cultivated on pristine PLLA - 82 %.

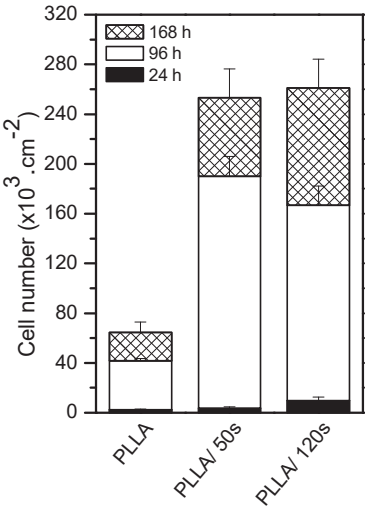

Fig. 4 Dependence of the number of adhered (24 h after seeding) and proliferated (96 and 168 h after seeding) VSMCs on the pristine and plasma treated PLLA. Plasma exposure time was 50 and 120 s, power 3W.

3. Adhesion and Proliferation of ASC

The number of adhered (24 h after seeding) and proliferated (96 and 168 h after seeding) ASC is shown in Fig. 5. The results show that the pristine PLLA is for the cultivation of ASC unsuitable. ASC cultured on the pristine PLLA did not have correct physiological shape and their viability was low (ca 60-70%).

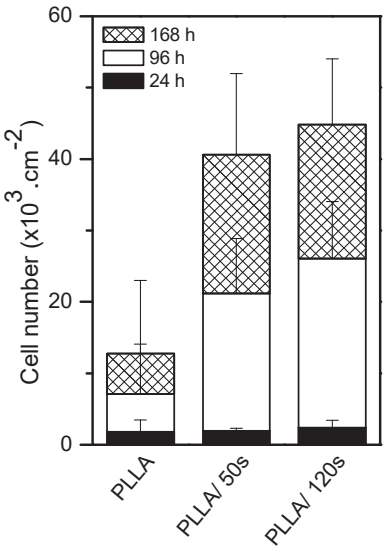

Fig. 5 Dependence of the number of adhered (24 h after seeding) and proliferated (96 and 168 h after seeding) ASC on the pristine and plasma treated PLLA. Plasma exposure time was 50 and 120 s, power 3W

The cells formed clusters and created sites that were not covered by cells. Also directional orientation was not observed. The significantly higher amount of adhered proliferated ASC was observed on modified substrates, cells viability was between 89-96%. These cells homogeneously covered the entire surface modified PLLA and were spreaded uniformly. In the case of cells cultivated on PLLA modified for 50 s, in some places, the overlap cells were detected, cells "migrated" random direction, in most cases it was not observed preferential orientation. The cells cultivated on PLLA modified for 120 s were preferentially oriented. From the results, it is evident that the PLLA modified for 120 s is the most appropriate substrate for further experiments and ASC cultivation.

Based on these results, we summarize that the plasma treatment leads to creation of surface suitable for cells cultivation. The cells adhesion maybe mediating by ECM molecules, e.g., vitronectin, fibronectin, collagen and laminin, present in the serum supplement of the culture media or synthesized by cells, which could be adsorbed on these surfaces in an appropriate amount [23], [24]. The PLLA surface modified by Ar plasma discharge probably enables or improves the adsorption these molecules from culture medium and thus forming a surface attractive for cell adhesion.
This experiment will serve as a basis for subsequent research directed towards the targeted differentiation of stem cells towards a particular cell type (e.g. vascular or bone cells).

IV. Conclusion

It was found that the application of plasma modification can significantly affect the surface properties of the PLLA film. Plasma treatment leads to decrease (short exposure time) respectively increase (long exposure in plasma PLLA) of CA of aged PLLA depending on the conditions of modification. Exposure to plasma discharge leads to creation of crystallites on PLLA surface. With increasing time of exposure, the amount of the crystallites and the surface roughness of the sample increases.

Plasma treatment positively influenced cell adhesion and proliferation. In the case of rat VSMCs and ASC, the significant increase in cell proliferation was detected. Also, positive effect on cell spreading and homogeneous coverage of PLLA surface was found.

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