INFLUENCE OF SURFACE TOPOGRAPHY AND MORPHOLOGY OF PU/PLA FILMS ON FIBROBLASTS PERFORMANCE

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Keywords: *contact angle, roughness, morphology, cell adhesion, fibroblasts*
[Engineering of Biomaterials, 116-117, (2012), 120-122]

Introduction

Interfaces between engineered materials and cells play a crucial role in biomedical applications where the interaction between cells and the material surface dictates cell performance and therefore, the success of the implanted device [1]. Extracellular matrix (ECM) components and peptides, topographical features, support cells and growth factors have been extensively studied for the creation of biomimetic materials that control cellular responses such as adhesion, morphology and/or differentiation [2-5]. Substrate topography can influence cell morphology, its behaviour, and cell-substrate interactions, as well as cell-cell interactions [6]. Effects of topography at all scales have been studied on many different cell types including cells of the central and peripheral nervous systems [7]. Surface hydrophilicity/hydrophobicity have been widely reported to be a key factor in behaving of proteins and cells-surface interactions [8]. It is well known that both highly hydrophilic and highly hydrophobic surfaces are not favourable for cell attachment. Surfaces with moderate wettability are able to adsorb proper amounts of adhesive proteins, and, at the same time, enable to preserve their natural conformations, what stimulates positive cell response [9].

Polymer blending is an effective method of modifying material properties as well as surface properties [10]. In practise, obtaining homogenous mixture of two or more polymers is rather difficult because of their high molecular weight. Nonetheless, the method is considered to be one of the easiest, cheapest, and the most useful. Polylactide is a biodegradable thermoplastic and because of its good mechanical properties, biodegradability and non-toxic degradation products, it is being used for number of biomedical applications [11]. Because of their biocompatibility and possibility to obtain a broad range of mechanical properties polyurethanes have been extensively investigated as both biostable and biodegradable biomaterials. They could be applied as long lasting medical implants, such as cardiac pacemakers and vascular grafts [12] or biodegradable cardiovascular implants, artificial skin, cancellous bone graft substitutes and scaffolds for tissue engineering [13].

In our studies blends of polylactide and biodegradable polyurethane were investigated. The aim of this work was to evaluate cytotoxicity of the polymer mixture, and assess the influence of the surface parameters of the polymer films on fibroblasts adhesion, proliferation and secretion.

Materials and methods

Materials

Mixture of polylactide and polyurethane was tested in this research. Both used polymers were of biomedical grade and

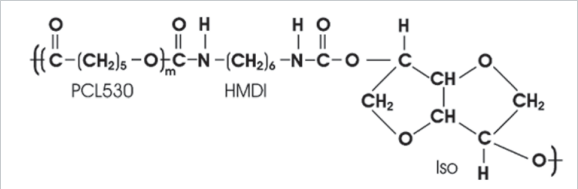

FIG. 1. Chemical structure of polyurethane used in the study.

were used without further purification. Polyurethane (PU) was purchased from GOY Bayer (Germany). PU molecules are built up of hexamethylenediisocyanate (HMDI) rigid segments, polycaprolactone (PCL) flexible segments and isosorbitol as a chain extender (FIG.1). Polylactide (PLA), consisting of 80% poly-L-lactide and 20% poly-DL-lactide was purchased from Purac (Netherlands). The N,N–dimethylformamide (DMF) of analytical grade was purchased from POCh (Poland).

Preparation of polymer free-standing films

Blends were prepared by dissolving both polymers in dimethylformamide to obtain a 10 wt.% solution. Weight ratio of PU to PLA was 4:1. The mixture was stirred with a magnetic stirrer for at least 48h at ~50°C, and then cast on glass Petri dishes. The films were dried at 50°C under vacuum for 48 hours. The films were sterilised with the use of the H_2O_2 cold plasma technique (Sterrad, ASP, J&J, USA).

Determination of physical properties of the films

The water contact angle of obtained PU/PLA films was measured using sessile drop method on Drop Shape Analysis System (DSA Mk2, Krüss, Germany). Ten measurements on each side of the film were accomplished. The data presented are average of ten measurements (± standard deviation).

Prior to taking roughness measurements a glass slide with the thin PU/PLA films was fixed onto a mount with a double-sided adhesive tape to prevent the samples from moving during the test. Roughness was measured with using a profilometer (Hommelwerke, Germany), equipped with cone shaped diamond tip (radius of 5 µm). The velocity of the moving cone was 0,50 mm/s. Each sample was measured ten times on both sides. The parameters calculated were surface average roughness (R_a), the maximum height of the profile (R_t) and the ten-point height of irregularities of surface (R_z). All the given values are presented as average of ten measurements (± standard deviation).

Determination of biological properties of the films

The sterilized films were placed in 24-well plate (Nunclon, Denmark), both surfaces of the films (top surface, i.g. air-cured, and bottom surface, i.g. glass-cured) were tested and as a control the bottom of the well tissue culture polystyrene-TCPS was used. NIH 3T3 mouse embryonic fibroblast cells were cultured on the studied materials in DMEM (PAA, Austria) supplemented with 10% FBS, 1% penicilin/streptomycin at 37^0C under 5.0% CO_2 atmosphere for 24 hours and 7 days. Initial cell density was $2.5 \cdot 10^4$ cells per well.

MTT test was used for cells viability measurements. At each time point MTT (Sigma Aldrich, Germany) solution (mg/mL) was added to the wells with tested films and incubated for 3h, after that the reaction was stopped with dimethyl sulfoxide (DMSO). The absorbance was measured on Multiscan FC Microplate Photometer (Thermo Scientific, USA) at 540 nm. To calculate the cell number calibration curve was prepared from by analyzing known number of

cells (from$1.0 \cdot 10^4$ to $5.0 \cdot 10^5$).

The total content of protein and nitric oxide (NO) level in the cells' supernatant was carried out by BCA protein assay and the Griess reaction, respectively. The BCA (bicinchoninic acid) reagent, prepared just before the assay, was mixed with CuSO solution (4%) in the proportion of 1:50. Then 10µl of the supernatant and 200 µl of BCA reagent were added to a 96 well-plate. After 30 min in the dark absorbance was measured at 570 nm. The Greiess reaction is based on the Griess reagent A–0.1% naphthalenethylenediamine dihydrochloride in water and the Griess reagent B–1% sulfanilamide in 5% H_3PO_4 mixed 1:1, v/v. Mixed reagents A and B in volume 100 µl were added to 100µl of the supernatant placed in 96-well plate and the optical density was determined at 540 nm.

The results were expressed as average and standard deviation for three independent samples. Statistical significance was evaluated according to t-test.

Morphology of the cells was observed under fluorescence microscopy (Zeiss Axiovert, Carl Zeiss, Germany). The cultured cells were fixed in 4% paraformaldehyde for 1h, then washed in PBS and stained with acridine orange solution (1mg/mL) to visualize the nucleic acids.

Results and discussion

The PU/PLA films analyzed in this study were handled and processed with due consideration for the differences between the polymer surfaces. The surface that had been in contact with glass during casting was denoted the "bottom" surface, while the other, which had been exposed to the gas phase, a mixture of air and residual solvent vapor, was denoted the "top" surface (FIG.2).

The water contact angle measurements were carried out to evaluate the wettability of thin PU/PLA films. The water contact angle of the top surface was 103±4°, but for the bottom surface was about 108±6°, both values are significantly different at $p<0.01$. This difference is due to the technique of samples preparation. When polymer is dissolved in polar

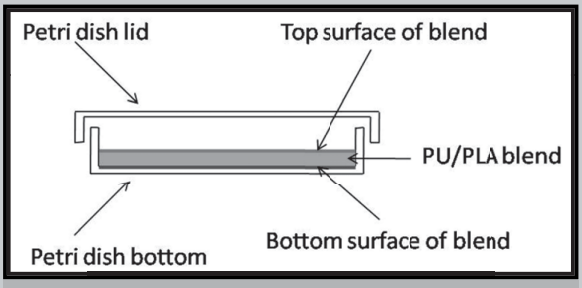

FIG. 2. Schematic diagram of the Petri dish used to cast the polymer films. Top denotes surface–air interface; Bottom denotes surface-glass interface.

liquid and then cast on glass trays, the water contact angle values of the top surface are expected to be lower than the bottom ones [14]. The relationship between different kind of cells and water contact angle for a variety polymer surfaces has been investigated for years. This investigations indicated that cell adhesion appears to be maximized on a surface with intermediate wettability, from 60° to 90° [15].

Another important factor which affects cell adhesion on polymer surface is its roughness. The results of profilometry measurements of PU/PLA blends are presented in TABLE 1. From the obtained results, it is noticeable that all three surface parameters (R_a, R_t, R_z) are slightly lower for the top than for the bottom surface, but the results are not statistically significant.

TABLE 1. Water contact angle (θ) and roughness (R_a, R_t, R_z) of top and bottom surface of PU/PLA films (average ± standard deviation, n=10).

	θ [°C]	R_a [µm]	R_t [µm]	R_z [µm]
TOP	103±4	1.8±0.7	17.5±2.9	10.3±0.2
BOTTOM	108±6**	2.8±0.2	22.1±2.5	16.1±1.5

**** Asterisks indicate a statistical significance between top and bottom surface: **p<0.01**

The MTT test was performed to evaluate viability of fibroblasts on PU/PLA films. One can notice that there are no significant differences after 24h of culture (FIG.3) and cell number is very similar for the top and the bottom surfaces of samples as well as for TCPS. It seems that in this case cell attachment and adhesion are independent on material surface properties. After 7 days the cells growth can be observed. However, the cell number on the top surface of PU/PLA is significantly higher than on the bottom surface. It indicated that fibroblasts adhere and proliferate more favourably on more hydrophilic and smoother surface. That fact has been also reported by other researchers [16]. Despite the fact that the highest cell number is achieved for the control material - TCPS, the PU/PLA blend still can be considered for biomedical applications.

The data from protein content are presented in FIG.4A. The total amount of proteins in supernatant after 24h is slightly lower for both the top and the bottom of PU/PLA film compering to TCPS. After 7 days the situation has changed and higher numbers were observed for both sides of PU/PLA than for TCPS. Comparing between the top and the bottom surfaces of PU/PLA we observed that the cells, which were seeded on the bottom surface, synthesised higher amounts of protein. This can be explained by the fact that fibroblasts produce more collagen when contacting with a more hydrophobic surface [15]. The nitric oxide (NO) is a important factor in evaluating of cytotoxicity of materials, the higher content, the more cytotoxic material may be. NO level was the same for all samples as shown in FIG.4B, no statistical differences between samples were found.

Morphology and distribution of NIH 3T3 cells cultured on both surfaces (top and bottom) of the PU/PLA films as well

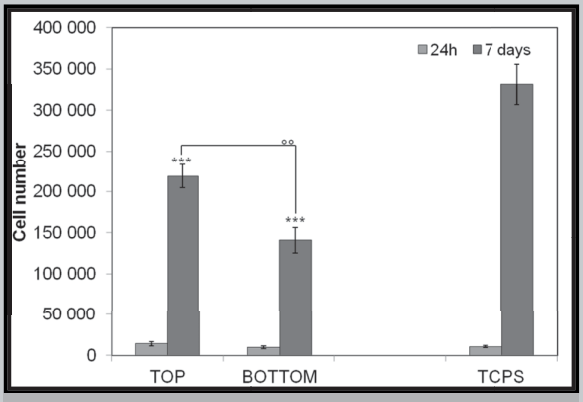

FIG. 3. Result of MTT test for top and bottom surfaces of PU/PLA film and for the control TCPS, after 24h and 7 days. Data are expressed as averages ± standard deviation, n=3. Asterisks indicate a statistical significance in correspondence to the control TCPS group: ***p<0.001. Circles indicate a statistical significance between the top and the bottom surface; °°p<0.05.

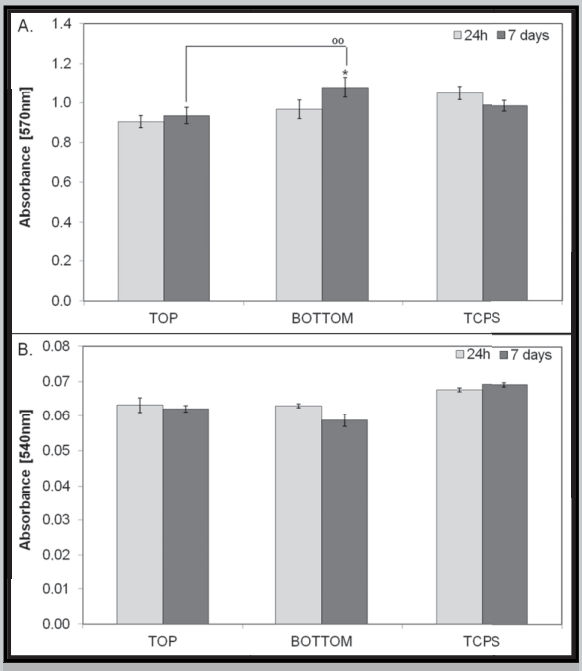

FIG. 4. Level of protein content (A.) and nitric oxide level (B.), both detected in supernatants from cell cultured for 24h and 7 days, on the top and the bottom surface of PU/PLA film and control TCPS. Data are expressed as average ± standard deviation, n=3. Circles indicate a statistical significance between top and bottom surface; $^{oo}p<0.05$.

as on the reference TCPS are presented in Fig. 5. After 24 h numbers of cells is low (FIG.5 A÷C) but the cells are well spread, polygonal or spindle-shaped and their morphology is similar to that on control TCPS. After 7 days higher numbers is observed (FIG.5 D÷E). It can be seen that the number of cells on PU/PLA films is lower than on the control TCPS. The results show also that number of cells depend on film surface, and on the more rough side (bottom) cells are distributed inhomogenously and their number is significantly lower than on the smoother side (top) (FIG.5 D,E). The results obtained from fluorescent microscopy correspond well to the MTT test.

Conclusion

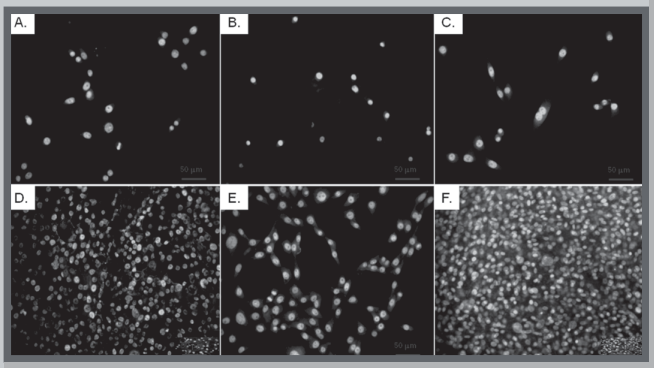

FIG. 5. Morphology of NIH 3T3cells cultured on the PU/PLA films: top surface (A, D), bottom surface (B,E) and reference samples TCPS (C,F) after 24h (A÷C) and 7 days (D÷F); fluorescence staining acridine orange, evaluation under fluorescence microscope.

PU/PLA films were produced by stirring the polymers in the solution. Their surface properties and their influence on cell adhesion, proliferation and secretion were evaluated. It was demonstrated that the top and the bottom surfaces show different wettability and roughness parameters. Top surface is more hydrophilic and smoother than the bottom one, therefore fibroblasts grew more likely on it. This was confirmed by the MTT tests and microscopic observations. Although, cell proliferation on the bottom surface of PU/PLA films was relatively low, the material can be considered nontoxic as confirmed by measurements the amounts of the evolved NO.

Acknowledgement

This work was supported by Polish Ministry of Science and Higher Education (Grant No. 2011/01/B/ST8/07426.

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