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Nanothin polyelectrolyte layers for biotechnological applications

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ABSTRACT

Cell culturing is the first step in a multilevel process of novel materials acceptance for their further application in medicine, biomedical engineering or biotechnology. Therefore, it is particularly important to optimize implemented procedures and techniques, ensuring a high experiments repeatability. In this paper, the system of polyurethane membrane support modified with nanothin multilayered film of polyelectrolytes has been elaborated for controlled growth of adherent cells. Selected properties of a platform have been examined. Additionally, the impact of the designed system on cells have been evaluated in vitro. Cells function has been assessed, using flow cytometry and fluorescence microscopy. Furthermore, transport properties of proposed systems have been investigated using fluorescent agents. Finally, a surface morphology of the designed platform has been estimated, by means of scanning electron microscopy. The proposed system can be successfully applied in biomedical engineering or/and biotechnology.

Keywords: Polyurethane; Polyelectrolytes; Nanothin membranes; Cell culturing

1. Introduction

In recent years, cell culturing has gained high popularity not only in the field of life sciences but also in other disciplines. Indisputably, it is an effective tool, which is regularly applied in cytotoxicity and biocompatibility studies. Moreover, possible effects, which particular material might cause on cells, should be evaluated before its contact with human tissues. Thereby, cell culturing is the first step in a multilevel approval process, in which the suitability of novel materials is thoroughly examined before its application to medicine, biomedical engineering or biotechnology. As a result, inappropriate materials will be eliminated during the initial testing phase, without a need for conducting a relatively expensive in vivo studies.

The complexity of cell cultures controlling should be taken into account, due to the living organisms' sensitivity. Even a small external interference influences dynamically changing living cultures, what results in deterioration of their functions. Therefore, a modification of every material causes decrease of the efficiency of cell functioning.

In the case of adherent cells culturing, a support-substrate plays a critical role in both cells growth as well as their morphology. A great number of both natural and synthetic biomaterials have already been tested for their usefulness in such a system, due to an optimal preparation of support surfaces, which can cooperate with the cells [1–10]. An example of such synthetic biomaterials are polyurethanes. This group of materials is characterized by high bioresorbability and biocompatibility. Furthermore, they have good mechanical properties; thus, they are often used as a scaffold in tissue engineering. Nevertheless, despite their undeniable advantages, an exclusive application of polyurethane, as a substrate for adherent cells cultures, is not an optimal solution. Hydrophobic surface of these polymers is not preferred by the cells. For these reasons, various modifications of polyurethanes are

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applied. Firstly, the material can be doped with other polymers during synthesis, to obtain copolymers with different properties. Another idea is to modify the final slide of polyurethane by ultrathin polyelectrolyte (PE) shell. Covering the support with semi-permeable nanothin membranes ensures their efficacious separation from the living cells. Additionally, such layers are a barrier, as well as a kind of "storage area", which stores and releases active substances, which stimulate cells growth. Furthermore, an application of hydrophilic PE, as a final nanothin film, can increase cells adhesion to the surface.

The system of polyurethane membrane support modified with nanothin multilayered film of PEs has been elaborated for controlled growth of adherent cells. The selected properties of such a platform have been examined. Among them, biocompatibility, in terms of cytotoxicity and membrane transport behavior, can be enumerated. Cells of WEHI-164 cell line have been applied as model ones in these experiments. The impact of the designed systems on cells has been evaluated in vitro. Cells condition has been monitored microscopically. Moreover, flow cytometry has been implemented to quantitative assessment of cell viability. Transport properties of proposed systems have been investigated using fluorescent agents. A surface morphology of the designed platform has been assessed using scanning electron microscopy (SEM). It can be stated that the proposed system can be applied in biomedical engineering or/and biotechnology in the future.

2. Experimental

2.1. Materials

Reagents: Polyurethane (PU) (Sigma, USA), poly-L-lysine hydrobromide, PLL (MW 15-30 kD; Sigma, USA), poly(ethylenimine) (MW 60 kD; Aldrich, USA), sodium alginate (Alg; Sigma, USA), and Hoechst 33342 (Invitrogen, USA).

Media: RPMI-1640 (Biomed, UE) and newborn calf serum NCS (Biochorom, EU).

Culture media: RPMI-1640 supplemented 10% NCS (RPMI-1640/10% NCS).

Cell lines: WEHI-164 – mouse fibrosarcoma cell line.

2.2. Methods

2.2.1. PE solutions

The following PE solutions were applied for membrane forming: poly-L-lysine solution in 0.1 M NaCl at concentration 1 mg ml^{-1}, polyethyleneimine solution in 0.1 M NaCl at concentration 1 mg ml^{-1}, and alginate solution in 0.1 M NaCl at concentration 0.05%.

2.2.2. Support preparation

In order to achieve the membrane support, a casting method was applied. Previously solved at dimethylformamide (DMF) polyurethane was slowly cast on a glass plate, which was next immersed into the distilled water bath, where a polymer was cross-linked. After that, the obtained membrane was activated using ultraviolet radiation.

2.2.3. Support modification by PEs

Layer-by-layer technique was applied to prepare PE bilayers on the polyurethane support. The support, obtained accordingly to the procedure described above, was incubated in the poly-L-lysine solution for 4 min. Next, membranes were washed out in 0.9% NaCl solution, dried and incubated in alginate or polyethyleneimine solution for 4 min. After the incubation, the support was washed out with 0.9% NaCl, to remove the unadsorbed polymer and finally dried. Two different PE membrane layers were adsorbed: poly-L-lysine|alginate (PLL|Alg) or poly-L-lysine|polyethyleneimine (PLL|PEI).

2.2.4. Membrane cut-off

A mixture of carriers (alginate beads), encapsulated with evaluated PE layers, was placed into a solution of molecules of different sizes in order to define the membrane cut-off. Dextrans were applied as a model molecules. On the other hand, non-coated alginate beads were used as a negative control. Concentration changes were analyzed spectrophotometrically at a wavelength of 450 nm.

2.3. Cell line culture

WEHI-164 cell line (American Type Culture Collection, Rockville, MD, USA) was maintained in RPMI-1640 supplemented with 10% newborn calf serum and 1% penicillin and streptomycin. Cells were cultured to over 90% confluence and then washed with Dulbecco's Phosphate Buffered Saline (DPBS), without Ca^{2+} and Mg^{2+}, after which they were collected with 0.25% trypsin EDTA (PAA Cell Culture Company®, Cambridge, UK). Additionally, cells were counted using haemocytometer.

After the passage, the cells were cultured in the presence of modified surfaces.

2.3.1. Impact of designed membrane systems on WEHI-164 cell line

Two membranes (prepared accordingly to the above procedures) were assessed simultaneously: PU|PLL|Alg and PU|PLL|PEI. The WEHI-164 cells at concentration 0.5×10^6 cells ml^{-1} were cultured in the presence of each one of the systems. As the negative control I, cells without the systems were maintained. Additionally, cells with the presence of non-modified polyurethane support were cultured as a negative control II. The cells were maintained (37°C, 5% CO_2) in culture medium RPMI-1640|10% NCS for 72 h. The initial state of the cell population was evaluated using Scepter™.

2.3.2. Cytotoxicity of the proposed membrane system

A general state of cells, their morphology and a rate of adhesion to the membrane were assessed by microscopic observation. Furthermore, quantitative analysis of cells was taken. The viability of cells was evaluated after 2, 24 or 72 h of culturing in flow cytometer with propidium iodide (PI). An intercalating agent, PI, entered the cells with damaged cell membrane, binding to their nucleic acids, and thus forming red fluorescence of dead cells.

2.4. Visualization and results analysis

2.4.1. Scanning electron microscopy

The structure of constructed membrane systems was analyzed using SEM (scanning electron microscope TM-1000, Japan).

2.4.2. Fluorescence microscopy

Cell valiability was examined by cell staining using the fluorescent dye (Hoechst 33258). The light passing through the double filter FITC-TRITC, with a wavelength of 475–490 nm or 540–565 nm, was used (Olympus IX70) for fluorescence observation. Photographs were taken using a digital camera Olympus Camedia C-5050.

Microphotographs were projected, visualized and analyzed with DP-Soft 3.2 (Build 779; analySIS®) software produced by Soft Imaging Systems GmbH for Olympus or ImageJ (Java-based image processing program developed at the US National Institutes of Health).

2.4.3. Flow cytometry

The cytotoxicity of tested membrane systems were assessed using Canto II flow cytometer (Becton Dickinson Immunocytochemistry Systems, USA). The results were processed by the FACS Diva software system (Becton Dickinson, USA). Evaluated objects were separated from other events on light scatter characteristics.

2.4.4. Statistical analysis

Mean values and standard deviations, as well as statistical significance, were calculated in the Statistica 7.1 software. The values of $p < 0.05$ were assumed as significant.

3. Results and discussion

3.1. Surface modifications

Virgin polyurethane, the resin used to basic membrane manufacturing, is hydrophobic in its raw state. However, such a kind of surface is particularly not conducive to cells growth, causing their poor adhesion. Hence, the nanothin PE films were applied to modify the produced support. Materials with hydrophilic functional groups were used to obtain the polarized surfaces, which were more favored by the cells. Following PE membrane layers were adsorbed: poly-L-lysine|alginate (PLL|Alg) and poly-L-lysine| polyethyleneimine (PLL|PEI). The schematic diagram of surface modifications is showed in Fig. 1.

The attention should be given to the fact that the layer-by-layer technique has been applied to obtain desired films [1,2,11–13].

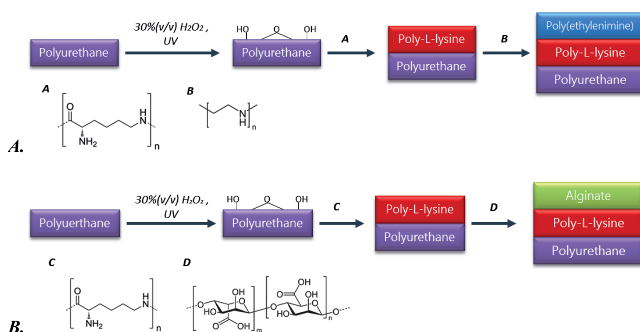


Fig. 1. Surface modification: (A) polyurethane support with adsorbed poly-L-lysine|alginate (PLL|Alg) nanothin film and (B) polyurethane support with adsorbed poly-L-lysine|polyethyleneimine (PLL|PEI) nanothin film.

3.2. Transport properties: assessment of the shells cut-off

The alginian beads coated with PE membranes were used to estimate the nanothin films cutoff values. In these assessments, the dextrans were used as the model particles. For both tested membranes: PLL|PEI and PLL|Alg, cut-off values were assessed on dextran 150 kDa level, which was sufficient for transport of both, basic nutrients and factors produced by immobilized cells, through the cell membrane.

3.3. Surface characterization

Microscopic observations have been taken to examine the developed membranes. Obtained images indicated that the resulting membranes did not constitute a compact planar structure (Fig. 2).

It has been confirmed that tested films have irregular microscale features, forming their corrugated surface. Moreover, the number of pores have been visible in their structure, as presented in Fig. 3. Therefore, in contrast to conventionally applied flat plastic polymer (e.g., polystyrene) surfaces, prepared films can better imitate a three-dimensional condition, occurring in a natural environment of living organisms (in vivo). Furthermore, implemented charged PEs can facilitate cells adhesion to the surface.

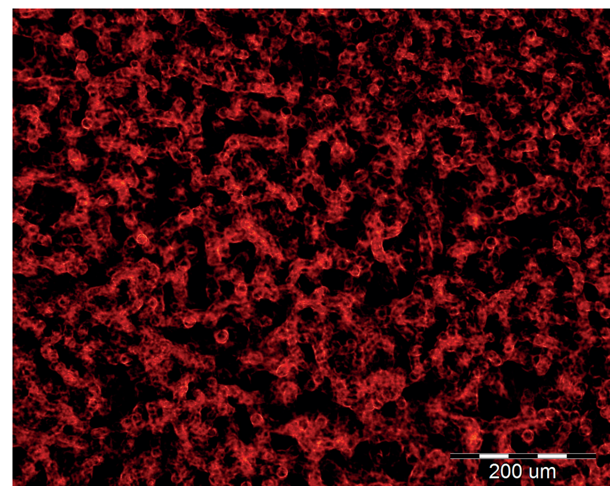


Fig. 2. Fluorescence image of the polyurethane support modified by nanothin poly-L-lysine|alginate (PLL|Alg) layers. Numerous of microcorrugation is observed on the surface of the obtained membrane.

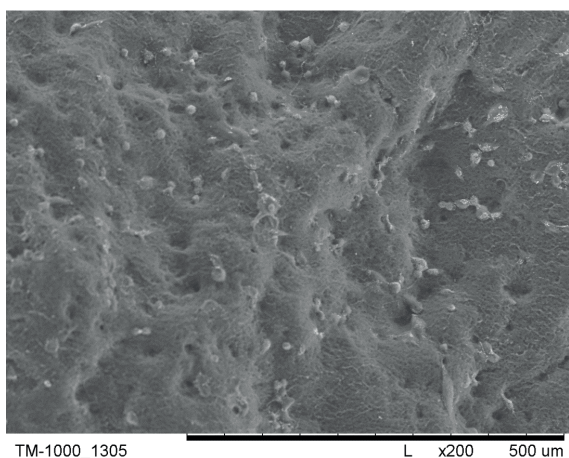


Fig. 3. SEM microphotography of WEHI-164 cultured in the presence of polyurethane support, modified by nanothin poly-L-lysine|alginate (PLL|Alg) layers. Irregular structure of obtained surface is visible.

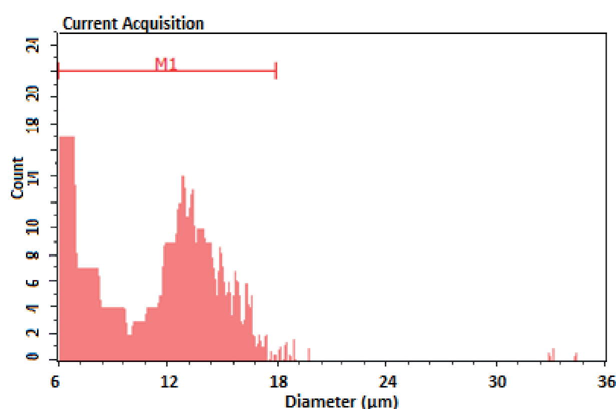


Fig. 4. An exemplary image of cell diameter distribution in measurement provided by Scepter™.

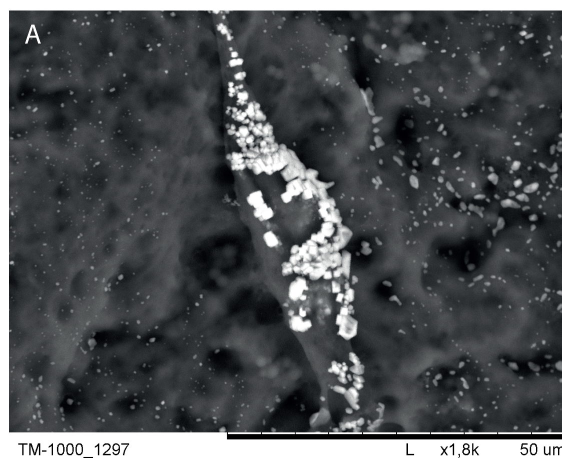
3.4. WEHI-164 cell line analysis

The initial state of the WEHI-164 cell line has been monitored. One of the markers of the cells state is its diameter. To ensure experiments repeatability, the cells diameter has been tested by Scepter™. An example of such an analysis is presented in Fig. 4.

3.5. Impact of designed membrane systems on WEHI-164 cell line

Cells' shape and appearance were regularly examined. It was observed that cells cultured in the presence of designed membranes were well flattened and had elongated shapes, which were characteristic for fibroblastic cells (Figs. 5(A) and (B)). Moreover, the cells grew while attached to the prepared substrates; however, the single cells were irregularly distributed on the films surface (Fig. 6).

It should be noted that in case of cultures, which maintained on the designed surfaces, the biggest number of cells was noticed in the membrane pores (Fig. 7.). The individual cells were free to grow up in the interior of pores, to build the



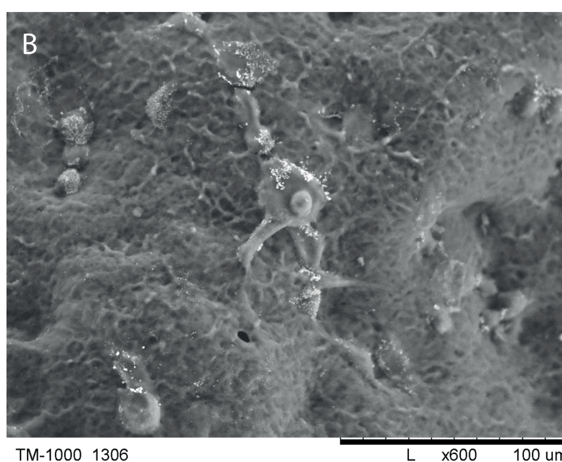


Fig. 5. SEM microphotography of WEHI-164 cultured in the presence of polyurethane support modified by: (A) nanothin poly-L-lysine|alginate (PLL|Alg) and (B) nanothin poly-L-lysine| polyethyleneimine (PLL|PEI) layers. Salt crystals are used to visualize cell shape.

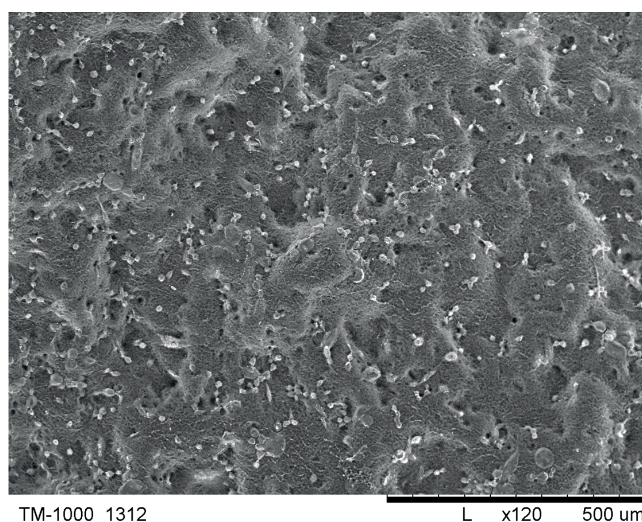


Fig. 6. SEM microphotography of WEHI-164 cultured in the presence of polyurethane support modified by nanothin poly-L-lysine|alginate layers. Irregular cells distribution is visible.

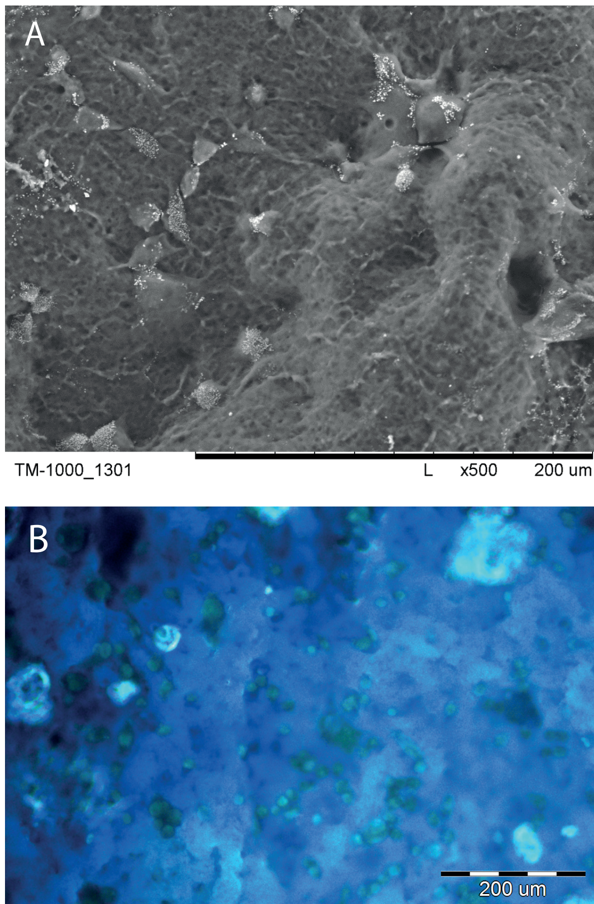


Fig. 7. WEHI-164 cultured in the presence of polyurethane support modified by nanothin poly-L-lysine|alginate (PLL|Alg) films. The biggest number of cells is noticed in the membrane pores: (A) SEM microphotography and (B) fluorescence image.

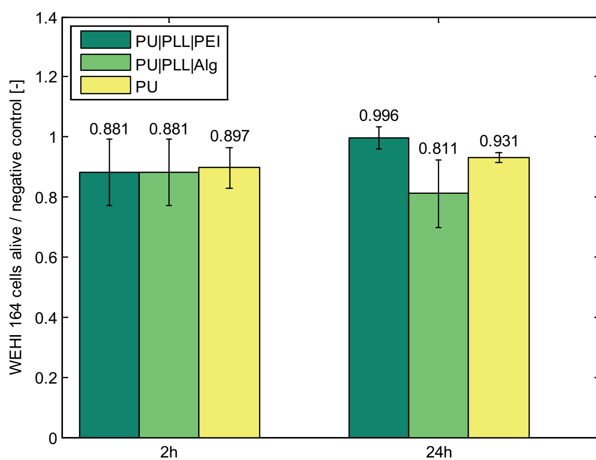


Fig. 8. The ratio of percentage share of live WEHI-164 during 24-h culture cultured in the presence of polyurethane support unmodified (PU) or modified by nanothin poly-L-lysine|alginate (PLL|Alg), nanothin poly-L-lysine|polyethyleneimine (PLL|PEI) films to the negative control (cells cultured without additions).

three-dimensional structures. Whereas, typically (in case of culturing on conventional, flat vessels) fibroblastic cells prefer to grow rather in clusters forcing a planar culture.

It should be stated that there were no significant differences observed in cell morphology cultured on both of the tested films.

3.6. Cytotoxicity of the proposed membrane system

Cells viability (quantitative analysis) was assessed using flow cytometry. The impact of applied membranes on evaluated eukaryotic cells was assessed during a 24-h experiment. It was observed that the modified polyurethane films did not exhibit cytotoxic impact on the evaluated cells (Fig. 8.). There was no statistical difference in cell viability between the cells cultured in the presence of both modified membranes, in reference to a negative control (culture without additives) ($p = 0.0010 < 0.05$).

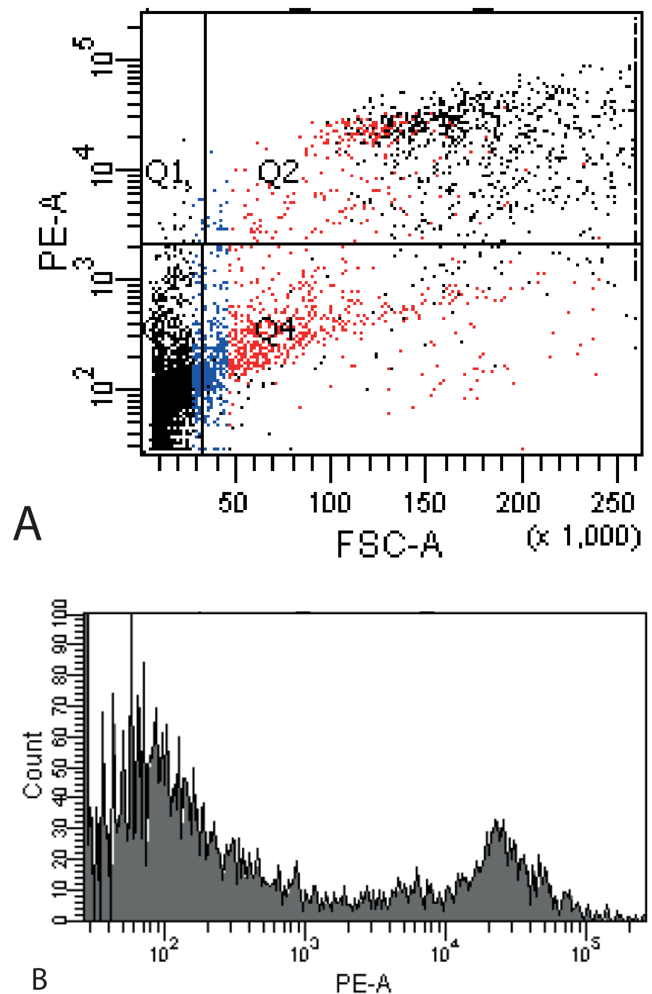


Fig. 9. Exemplary cytometric image of WEHI-164 cells after 24-h culturing in the presence of polyurethane support modified by nanothin poly-L-lysine|polyethyleneimine (PLL|PEI) layers. There were about 76.3% living cells (cytogram A, quadrant Q4). The dead cells (exhibiting red fluorescence) may be observed in quadrant Q2 (cytogram A). The expression of red fluorescence is presented in histogram (B).

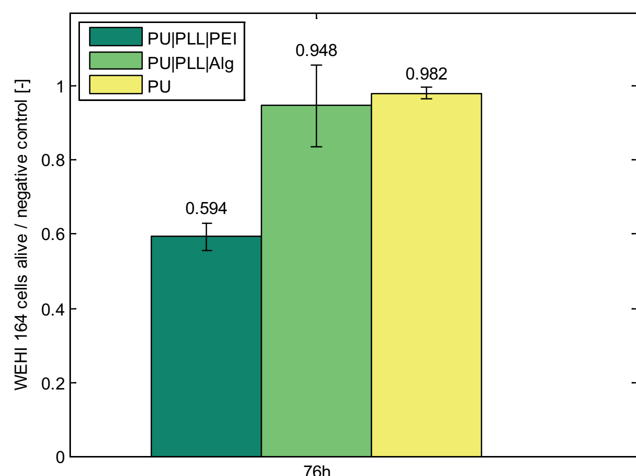


Fig. 10. The ratio of percentage share of alive WEHI-164 during 72-h culture cultured in the presence of polyurethane support modified by nanothin poly-L-lysine|alginate (PLL|Alg), nanothin poly-L-lysine|polyethyleneimine (PLL|PEI) films or unmodified polyurethane support to the negative control (72-h culture cultured without additions).

In Fig. 9, an exemplary cytometric pictures of evaluated cells after 24-h culturing in the presence of PU|PLL|PEI membrane is presented. There were about 76.3% living cells.

3.7. Over 24-h culturing

The excessive cell growth was noted after 24-h culturing of cells, which adhered to the designed PU|PLL|PEI surface, what resulted in limited access to nutrients, demonstrating the membrane ability to enhance cell growth (Fig. 10 shows cell condition after the third day of culture). Therefore, carrying out the long-term fibroblasts culture in the presence of developed membranes requires cells cyclic transfer into new substrate.

4. Conclusion

Presented studies demonstrated that the designed systems of the porous PU support, modified with PE layers assisted growth and functioning of evaluated fibroblastic cells. Especially, PU|PLL|PEI can be recommended for this purpose.

It can be stated that proposed platforms can facilitate biomedical engineering, especially in the field of a construction of the three-dimensional setups for fibroblasts culturing. Described materials configurations can also be implemented for biotechnological applications. Conceivably, they can be used in systems, which release biologically active substances. Their unique properties, like high biocompability, are highly desired in such systems.

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