



Research article

Growth characteristics of human bone marrow mesenchymal stromal cells at cultivation on synthetic polyelectrolyte nanofilms *in vitro*Lyudmila M. Mezhevnikina^a, Dmitriy A. Reshetnikov^a, Maria G. Fomkina^{b,*}, Nurbol O. Appazov^c, Saltanat Zh. Ibadullayeva^c, Evgeniy E. Fesenko^a^a Institute of Cell Biophysics of the Russian Academy of Sciences, Pushchino, Moscow region, Russia^b Institute of Theoretical and Experimental Biophysics of the Russian Academy of Sciences, Pushchino, Moscow region, Russia^c Korkyt Ata Kyzylorda University, Kyzylorda, Kazakhstan

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ABSTRACT

This study examines the adhesive properties and cytotoxicity of polyelectrolyte nanofilms from polyethyleneimine (PEI), polyallylamine hydrochloride (PAH) and sodium polystyrene sulfonate (PSS) on human bone marrow mesenchymal stromal cells (h-MSCs) and mouse adipose tissue (m-MSC) *in vitro*. Films are formed on 24- and 96-well culture plates in the combinations: PEI, PAH, PEI-PSS, PEI-PSS-PAH, PEI-PSS-PEI. An analysis of the culture results show that direct contact of h-MSCs with the PEI surface promotes adhesion (93–95% of adhesive cells versus 40% in the control). On the PEI surface, h-MSCs are evenly distributed, form colonies and 80% monolayer after 72 h of culture, as in the control on culture plastic. On nanofilms from PAH and PEI-PSS-PAH, cells grow in the form of rosette-like colonies with long and thin processes similar to neurites. The cytotoxic properties of PSS were revealed in direct contact with h-MSCs (more than 40% of nonviable cells with damaged plasma membranes). On the PSS surface, cells lost their adhesiveness. To culture and stably grow the cell mass of h-MSCs, it is better to use monolayer nanofilms made of highly adhesive and non-toxic PEI polyelectrolyte, which can bind the growth factors of blood serum and platelet lysate, ensuring the growth of h-MSCs under *in vitro* deprivation conditions.

1. Introduction

Mesenchymal stromal cells (MSCs) of bone marrow, adipose tissue, umbilical cord and peripheral blood, and other tissues of animals and humans have found use as experimental models of gene and cell-based therapy, as well as medicinal drugs in the treatment of cardiovascular, neurodegenerative, immunity-related and oncological diseases [1, 2, 3, 4, 5]. The stimulating effects of MSCs are significantly associated with secretory activity [6, 7], as well as the ability to differentiate under the influence of the microenvironment *in vitro in vivo* into cardiomyocytes [8, 9], neurons [10, 11, 12, 13], endothelial and epithelial cells [14, 15, 16], adipocytes [17], chondrocytes [18], hepatocytes [19], bone tissue cells [20, 21].

For therapeutic use, a large amount of biomaterial is required, therefore, there is a problem of culturing MSCs preserving the biological properties and potential for multipotent differentiation after transplantation to patients. The complexity of the problem lies in the fact that the primary populations of MSCs from adult tissues are heterogeneous in

their composition. There are also individual and interspecies differences of MSCs linked to the source of origin which affects the growth characteristics *in vitro*. In particular, adhesion to the substrate is of key importance for MSCs. Adhesive cells are capable of realizing proliferative and differentiation potential in the direction of adipo-, chondro- and osteogenesis. The immunophenotype and differentiation of MSCs are influenced by the composition of the nutrient medium and culture conditions (culture age, starting density, number of passages performed). Some researchers also note that during the culture and growth of biomass for therapeutic purposes, the potential of MSCs to differentiation decreases [22, 23, 24].

Although MSCs grow and multiply on the negatively charged culture plastics in balanced nutrient media, their growth characteristics largely depend on the production of positively charged extracellular matrix proteins (collagen, fibronectin, laminin, polylysine, etc.) which enhance cell adhesion to the surface. Over the last years, synthetic polymers have become widespread as an alternative to natural adhesive polypeptides that can undergo hydrolysis by amide bonds under physiological

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conditions of culture [25, 26]. In addition, the effectiveness of extracellular matrix polypeptides as adhesive coatings depends on the presence of serum components in the nutrient medium [27]. It is shown in various cell cultures *in vitro* that in a serum-free medium, positively charged synthetic polyelectrolytes have advantages over the traditional adhesion factors such as collagen, polylysine and laminin [27, 28].

The purpose of our research is to examine the adhesive properties and cytotoxicity for human and mouse MSCs of such synthetic polymer

materials as polyethyleneimine (PEI), polyallylamine hydrochloride (PAH) and sodium polystyrene sulfonate (PSS). These polymers have found use in biomedical research for the manufacture of nanofilms and microcapsules to deliver macromolecules, pharmaceutical and diagnostic products to target cells [29, 30, 31]. In combination with extracellular matrix proteins, PEI, PAH, and PSS enhance adhesion, proliferation, and differentiation of primary and finite cell lines (e.g., fibroblasts, macrophages, neural cells, etc.). Since, to the best of our knowledge, the

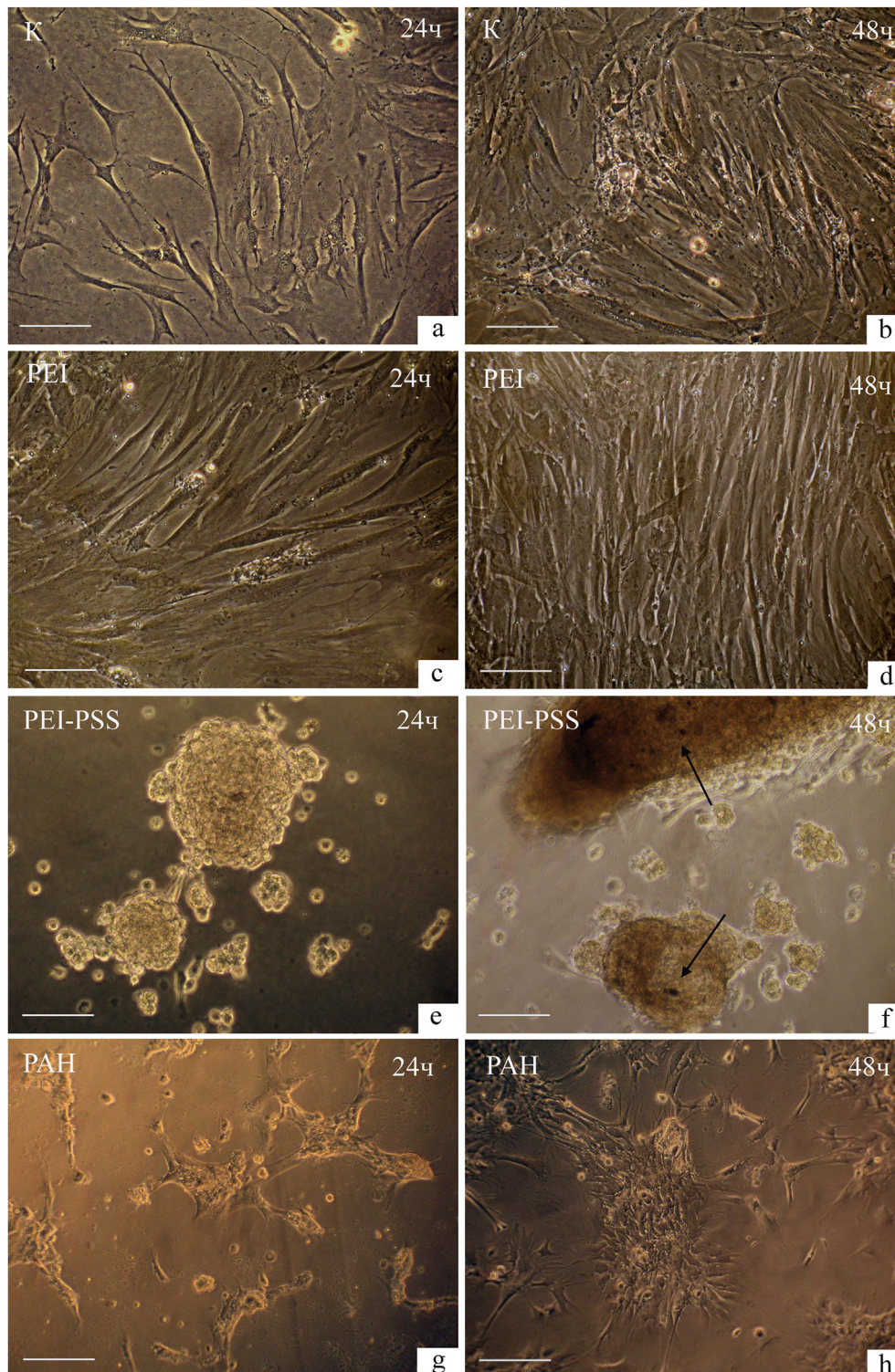


Figure 1. Morphology of h-MSCs after 24 and 48 h of culture in DMEM/F12 medium with 10% FBS in the control (a, b) and on polyelectrolyte films from PEI (c, d), PEI-PSS (e, f) and PAH (g, h), respectively. Objective magnification - 20 \times , scale - 100 microns.

investigation of primary cultures of h-MSCs has not been yet conducted, we are interested to study the effect of synthetic polymer materials on the adhesion and viability of these cells *in vitro*. We use PEI, PAH and PSS in various combinations. Films are deposited on the surface of culture plates by the layer-by-layer method of oppositely charged polyelectrolytes. h-MSCs and m-MSCs on culture plastic act as control. The adhesive properties and cytotoxicity of nanofilms, the morphology of the colonies and monolayer, and the viability and metabolic activity of h-MSCs and m-MSCs are then evaluated.

2. Material and methods

2.1. Objects of study

A primary culture of human mesenchymal stromal cells (h-MSCs) was obtained from the Federal Research and Clinical Center of Federal Medical-Biologic Agency (Moscow, Russia). Cells were isolated from a female volunteer donor and were characterized as positive for CD29, CD44, CD73, CD90, CD105 markers and as negative for CD34, CD45 and HLA-DRP. Courtesy to Dr. Belyavsky, mouse adipose tissue MSCs (m-MSCs) were obtained from the laboratory of stem and progenitor cells of the Institute of Molecular Biology of the Russian Academy of Sciences. All the experiments with the cell cultures of animal and human were performed in accordance with the Guidelines approved by the Federation of European Laboratory Animal Science Association (FELASA), and the Guidelines approved by the Ethic committee of the Institute of Cell Biophysics, RAS (Approval no. 57 dated December 30, 2011). Informed consent for the experiments was obtained from a female volunteer whose bone marrow cells were used in this work.

2.2. Culture of MSCs

High glucose DMEM/F12 medium (Gibco, USA) with the addition of 10% fetal bovine serum (FBS, Gibco, USA), 1 mM glutamine, 100 U/ml penicillin and streptomycin were used to culture h-MSCs and m-MSCs. Culture was carried out in a CO₂ incubator (Sanyo, Japan) at 37 °C and at high humidity in the atmosphere of 5% CO₂. MSCs were set on 24-, 48-, and 96-well plates (Nunc, USA) with nanofilms made of PEI, PAH, and PSS polyelectrolytes preliminarily deposited on the surface of wells. Cells growing on culture plastic without preliminary treatment with solutions of polyelectrolytes were used as the control. The culture duration of the test and control samples was 24–96 h depending on the conditions of the experiment. During this time, the change of the morphology of the colonies and the monolayer of MSCs was monitored under the inverted microscope at a 10× and 20× objective magnification (Axiovert 25, Zeiss, Germany).

h-MSCs and m-MSCs were passaged after reaching 80% of the monolayer. Before passaging, the DMEM/F12 nutrient medium was removed; the monolayer was washed twice in a phosphate buffer solution (PBS), after which the cells were removed from the surface of the culture plate or polyelectrolyte film with a solution of 0.25% trypsin-EDTA (NPP PanEco LLC, Russia). For a more complete detachment of the cell mass from the surface, the culture plates were placed for 5–10 min in the CO₂ incubator. Trypsin was inactivated by adding a nutrient medium with 10% FBS; the cells were pipetted several times in the medium with FBS and their number is counted in the Goryaev chamber; the cells were then plated at the desired concentration for experiments.

2.3. Assembly of nanofilms on culture plates

In this work, were used solutions of polyethyleneimine (PEI, 50 wt.% Soln. In water, MN: 60 kDa, MW: 50–100 kDa, MP Biomedicals Inc., USA), polyallylamine hydrochloride (PAH, MW: 60 kDa, Aldrich, USA) and sodium polystyrene sulfonate (PSS, MW: 75 kDa, Aldrich, USA). The layer-by-layer method of oppositely charged molecules was used to obtain single, double, and three-layer polyelectrolyte films (PEI, PAH,

PEI-PSS, PEI-PSS-PAH, PEI-PSS-PEI) on the surface of the culture plastic. To form a single-layer surface, a PEI or PAH polyelectrolyte solution with a thickness of about 3 nm was poured into the culture plastic. The solution was kept for 20 min at room temperature (20 °C) with gentle stirring relative to the horizontal surface 2–3 times. Then the solution was removed using an automatic pipette. The well was washed with distilled water (twice), directing a stream of water to the side walls of the well, so as not to disrupt the self-assembly of the polymer nanolayer. To obtain two- and three-layer films on the surface of plastic PEI-PSS, PEI-PSS-PAH and PEI-PSS-PEI, the procedure was repeated two or three times, successively adding solutions of the corresponding polyelectrolytes to the culture plastic.

The films were made under sterile conditions in the laminar box. To avoid contamination during subsequent culture of MSCs, before culturing, culture plates with nanofilms deposited on the surface of the wells were additionally sterilized for 3 h under a UV lamp.

2.4. *In vitro* cell analysis

Throughout the culture period, the morphology of cells, colonies, and a monolayer are evaluated using the inverted microscope (Axiovert 25, Zeiss, Germany). Test samples (culture on polyelectrolyte nanofilms) and control samples (culture on plastic) are recorded by photographing. They are then compared with each other according to morphology, cell adhesion and growth rate, mitochondrial dehydrogenase activity (MTT assay), and the number of nonviable cells with damaged plasma membranes. For each experiment conducted using nanofilms, 5–6 measurements are performed.

2.5. MSC viability

MSC viability was determined using a vital dye of 0.5% trypan blue (Fluka, Switzerland). The MTT assay was used to detect the activity of mitochondrial dehydrogenases. The ratio of viable and nonviable cells passing the vital dye through the plasma membranes was determined in the Goryaev chamber. For this purpose, 20 μl of the cell suspension was mixed with 20 μl of a 1% trypan blue solution; the chamber was filled; and the stained and unstained cells were counted according to the standard method. The cell multiplication rate (*V*) and population doubling time (*T*) under different conditions of MSC culture (on polyelectrolyte films and without using them) were calculated in the logarithmic growth phase according to the formula:

$$V = 3.32 \times \lg (X / X_0), \quad (1)$$

where *X*₀ is the initial cell concentration; *X* is the final cell concentration in the culture medium. In order to determine the population doubling time taking into account the duration of MSC culture, the following formula was used:

$$T = (\ln 2 \times d) / \ln (X / X_0) \quad (2)$$

2.6. MTT assay

The assay is based on the ability of mitochondrial dehydrogenases of viable cells to reduce the yellow water-soluble salt of 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazole bromide (MTT) into insoluble intracellular MTT-formazan crystals. The assay was performed on 96-well culture plates with polyelectrolyte nanofilms. An equal amount of MSCs was screened in the wells at the rate of 2.0–3.3 × 10⁴ cells per 100 μl of DMEM/F12 culture medium with 5 and 10% FBS or without FBS. At the end of the culture, 20 μl of MTT solution (Thiazolyl blue, MTT, Sigma, USA) was added to each well. The MTT solution was prepared immediately before use at a concentration of 5 mg/ml MTT salt (pH 7.4). The cells were incubated with MTT for 4 h at 37 °C in the CO₂ incubator; the

supernatant was removed; and the precipitate (reproduced dark blue formazan crystals generated by mitochondrial dehydrogenases) was dissolved in 150 μ l of dimethyl sulfoxide (DMSO, Sigma). The quantity of reproduced product was measured spectrophotometrically on a plate reader (Model 680 MicroplaReader S/N 20324) at a wavelength of 546 nm. The results were then compared with the optical density of cells in the control (culture on plastic without depositing polyelectrolyte nanofilms). The calculations were performed using the nonparametric Mann-Whitney u-test. Differences in optical density of more than 20% were considered reliable.

2.7. Determination of differential cell adhesion to the surface of nanofilms

The analysis was performed on a 48-hour culture of h-MSCs (9–10 passages). The cells were washed twice with PBS solution and trypsinized; their total number was counted. Next, they were distributed in equal volumes to 4-well embryological plates (Nunc, Denmark), three wells of which were pretreated according to LbL (layer-by-layer) method with polyelectrolyte solutions (PEI, PAH and PEI-PSS-PAH). One well remains free and served as control. h-MSCs were plated at a concentration of $5\text{--}7 \times 10^4$ cells/ml of DMEM/F12 culture medium with 10% FBS; then the plates were placed in the CO₂ incubator. After 1 or 3 h of incubation, the cells were thoroughly washed in PBS (twice) and in trypsin-EDTA solution (2–3 min at room temperature 20 °C); and nutrient medium with 10% FBS was added to inactivate the enzyme. The cells remaining attached to the surface were counted in 20 fields of view in each well at a 20 \times objective magnification (Olympus IX70, Japan). The number of attached cells was averaged according to three parallel experiments to detect differences in the adhesive properties of the polymers. For greater clarity and reliability of the results, h-MSCs were fixed with methyl alcohol (20 min at room temperature 20 °C) and stained according to Giemsa. The calculation of the number of attached cells was carried out using a cytophotometry image analysis software program (PhotoM).

2.8. Statistical analysis of the results

Statistical analysis of the obtained results was carried out by calculating arithmetic mean values and standard deviation using computer programs Sigma Plot and Microsoft Office Excel. To assess the statistical significance of the differences in the comparison groups, the nonparametric Mann-Whitney test was performed. p-value <0.05 is considered to indicate reliably significant differences between the two comparison groups.

3. Results

When culturing h-MSCs on PEI-PSS nanofilms, in which the PSS is in direct contact with cells, a general tendency is the formation of cell conglomerates of various shapes and sizes localized mainly in the center of the culture plate's well (Figure 1, e and f). The cells become tightly bound together but they interact weakly with the PSS surface. After 48 h of culturing, cell conglomerates become combined into larger 3-dimensional structures in which dark brown inclusions are found (Figure 1, f). A similar picture was revealed in the study of m-MSC, which were also collected in multilayer conglomerates in the center of the plate wells on PSS-PEI films.

This is possible due to the rapid release of PSS (3 min) into the culture medium with the formation of a bioadhesive gel-like viscous layer on the surface of PEI-PSS [32], on which, however, MSCs weakly adhere and therefore stick together. Comparing to the control and nanofilms from PEI and PAH, on the PEI-PSS films, weaker adhesion is observed (Figure 2). During manipulations (for example, medium change and passaging), a significant part of the cell mass is lost (about 40%) due to the lack of tight binding of cells to the PSS surface. The histological sign of cytotoxicity of PSS is the formation of specific crystals (Figure 1, f). As

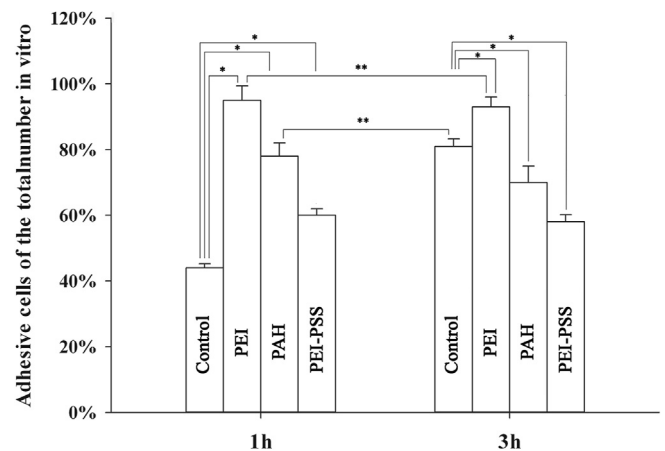


Figure 2. The adhesion rate of h-MSCs (% adhesive cells) to polyelectrolyte nanofilms and culture plastic (control) when culturing h-MSCs in DMEM/F12 medium with 10% FBS. The number of repetitions of each experiment $n = 5$. * - significant differences in cell adhesion rate on single-layer films of PEI, PAH and PEI-PSS ($P \leq 0.01\text{--}0.05$). ** - no statistical differences (NS) in the adhesive capacity of MSCs after 1 h and 3 h exposure on PEI films; between PAH (1 h of exposure) and control (3 h exposure) ($P \geq 0.05$).

shown by other researchers, PSS is an anion exchange resin that replaces sodium ions with potassium ions [33] and causes inflammation and tissue necrosis [34]. The adhesive properties of PEI, PAH, PSS polyelectrolytes were studied on the h-MSC culture (Figure 2).

The maximum adhesive capacity of h-MSCs is revealed on single-layer films of positively charged polyelectrolytes PEI and PAH (95 and 78%, respectively) during one hour of incubation at 37 °C (Figure 2). In the control, less than 50% of h-MSCs are attached during one hour, however, over longer time (3 h) of incubation, this indicator increases to 80%. The remaining h-MSCs remain unattached and do not participate in the formation of colonies and the monolayer. Unattached cells die relatively quickly with an increase in the duration of MSC culture up to 72 h as a result of the disturbance of the barrier properties of plasma membranes (Table 1).

The death of h-MSCs in the control is about 6%, which is twice as lower than cell death during the culture on single-layer PEI films (Table 1). However, despite the relatively high rate of spontaneous death of h-MSCs on PEI films (13.4%), stromal cells of human bone marrow are distributed more uniformly on the surface of PEI than on culture plastic in the control. In this case, the basic mass of cells was attached and spread on PEI during the first hour of incubation (Figure 2).

It is also established that fast adhesion and spreading of h-MSCs on the surface of the culture plate are critical for cellular proliferation (Table 1). A comparative analysis of the growth characteristics of h-MSCs on various surfaces shows that weak adhesion on double-layer PEI-PSS films correlates with a decrease in the rate of multiplication of h-MSCs and a significant increase in the proportion of damaged and nonviable cells (43.4%) with disturbed barrier properties of plasma membranes (Table 1). That indicates the cytotoxicity of PSS for this type of cells. At the same time, PEI and PAH polycations have less pronounced cytotoxic effects; however, in combination with PSS, they can also negatively affect the permeability of cell membranes (Table 1) and, therefore, *in vitro* cell viability (Figure 3).

It is noteworthy that during the culture of h-MSCs and m-MSCs on three-layer PEI-PSS-PAH films, a general tendency is observed that cell viability does not increase if compared to their viability in the control group or as it occurs on PAH, PEI, PEI-PSS-PEI films (Figure 3). On PEI-PSS-PAH films, a clear inhibition of cell growth is revealed (Table 1); this result is possibly due to differentiation of MSCs induced by the PAH polyelectrolyte (Figure 1, g, h). At the same time, the proportion of nonviable necrotic cells increases significantly (more than 30% against 6% in the control); and the morphology of the colonies and the

Table 1. Comparison of the efficiency of h-MSC culture on polyelectrolyte nanofilms and culture plastic.

Nanofilms	Colony and monolayer growth rate	Population doubling time, hour	Nonviable cells with damaged plasma membranes after 72 h of culture, %
Culture plastic (control)	2.18 ± 0.17	29.1 ± 4.23 (n = 4)	6.2
PEI	2.13 ± 0.15	22.7 ± 4.76** (n = 4)	13.4
PEI-PSS	1.37 ± 0.21*	28.6 ± 3.52 (n = 4)	43.4
PEI-PSS-PAH	1.66 ± 0.42*	25.5 ± 2.9 (n = 4)	31.6

Note. Cells were cultured in DMEM/F12 medium with 10% FBS for 72 h. Nonviable cells with damaged plasma membranes were detected using 0.5% vital trypan blue dye. * - significant differences in growth rate compared to culture in the control and on PEI films ($P \leq 0.01$) and ** - significant reduction in the population doubling time of h-MSCs on PEI film ($P \leq 0.01$).

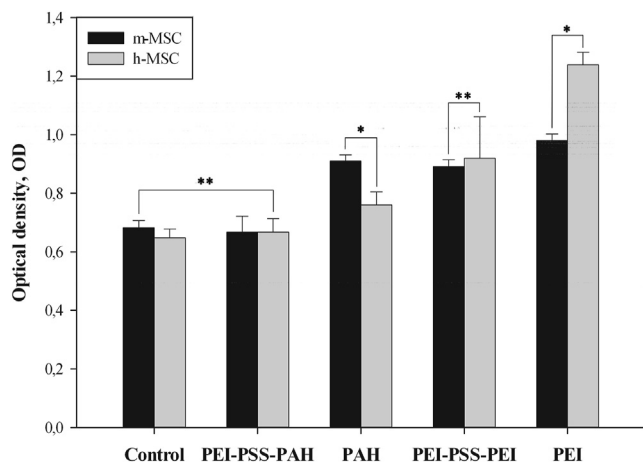


Figure 3. Viability of human and mouse MSCs *in vitro* (MTT assay). The culture duration in DMEM/F12 medium with 10% FBS was 48 h h-MSC — 7–9 passages after isolation from human bone marrow; m-MSC — mouse adipose tissue mesenchymal cells in 3–4 passages after thawing. * - significant differences between mouse and human MSCs in their survival rates on single-layer films from PEI and PAH films ($P \leq 0.05$); ** - NS between these two types of cells when cultured on plastic (control) and on three-layer PEI-PSS-PAH and PEI-PSS-PEI films ($P \geq 0.05$).

monolayer changes significantly too. Mesenchymal cells are distributed on the surface of PAH in the form of rosettes or star-shaped structures have long and thin processes, with the help of which contacts between individual colonies are formed (Figure 1, g). This is more pronounced for h-MSCs than for MSCs from mouse adipose tissue that retain a high level of viability on both single-layer and three-layer PAH films (three-layer PAH include the cytotoxic polymer PSS) (Table 1). It can be supposed that mouse adipose tissue MSCs are more resistant to the negative effects of PSS polyelectrolyte than human bone marrow MSCs. In direct contact

with the PSS surface, both m-MSCs and h-MSCs aggregate with each other into large multicellular structures weakly bound to the surface.

The best results on the adhesion and survival rate of different types of MSCs (mouse and human) *in vitro* are obtained on single-layer PEI nanofilms (Table 1, Figures 2 and 3). This polyelectrolyte establishes itself as a non-viral transfection agent. At physiological pH (7.2–7.4), it binds to DNA, RNA and proteins, penetrates animal and human cells. Our experiments show that on PEI nanofilms with immobilized factors of fetal bovine serum (FBS) and human platelet lysate (PL), h-MSCs grow and multiply under deprivation conditions (5% FBS) as well as without adding FBS to the nutrient medium (Table 2, Figure 4).

A comparative analysis of the culture results shows that in serum-free medium, h-MSCs retain the ability to adhere, grow and multiply as in the control with 10% FBS in the culture medium (Table 2). However, when using nanofilms PEI-5% FBS the cell growth rate is inhibited, and the population doubling time is increased to 48 h against 33.7 h in the control. The binding of PEI to 10% FBS or 5% platelet lysate provides a sufficiently high level of proliferative activity of h-MSCs in serum-free medium (Figure 4). This is also confirmed by the data on the increased survival of these cells under conditions of decreasing FBS concentration (5%) or complete absence of FBS in the culture medium (Figure 5).

The low viability of h-MSCs in the control on negatively charged plastic (Figure 5) is associated with a decrease in the concentration of serum in the nutrient medium from 10% to 5%. Under conditions of deprivation, a significant part of h-MSCs loses its adhesive capacity. To maintain these cells in viable state *in vitro*, exogenous growth factors FBS or PL, which are present in PEI nanofilms, are required (Figures 4 and 5). Obviously, MSCs themselves are not able to produce in sufficient quantities positively charged extracellular matrix proteins (collagen, fibronectin, laminin, etc.) that enhance their adhesive properties on negatively charged surfaces. If the culture plastic is preliminarily coated with a positively charged PEI polyelectrolyte, the adhesive properties and viability of MSCs are significantly increased in comparison with the control (Figures 2 and 3). When using PEI in combination with 5% PL or

Table 2. The effectiveness of culturing h-MSCs in serum-free medium on polyelectrolyte PEI nanofilms with immobilized growth factors.

Culture conditions	Number of cells in the medium after 48 h of culture (x104)	Growth rate	Population doubling time, hour
Control	8.8	1.4	33,7
*PEI-5% FBS	6.6	1.0	48,0
*PEI-5% PL	12.1	1.8	25,4
*PEI-10% FBS	12.1	1.9	25,7
*PEI-10% PL	9.9	1.6	30,4

Note. The initial number of h-MSCs explanted into the culture was 3.3–3.5 × 10⁴/ml. Control – culture on plastic in DMEM/F12 medium with the addition of 10% FBS. * - culturing h-MSCs in serum-free medium. h-MSCs were used at the passage 10 after isolation from bone marrow. * - the cultivation of MSC in serum-free medium DMEM/F12 on PEI films with immobilized growth factors: platelet lysate (PL) and fetal bovine serum (FBS). h-MSC is used at the 10th passage after isolation from the bone marrow. The number of repetitions n = 3.

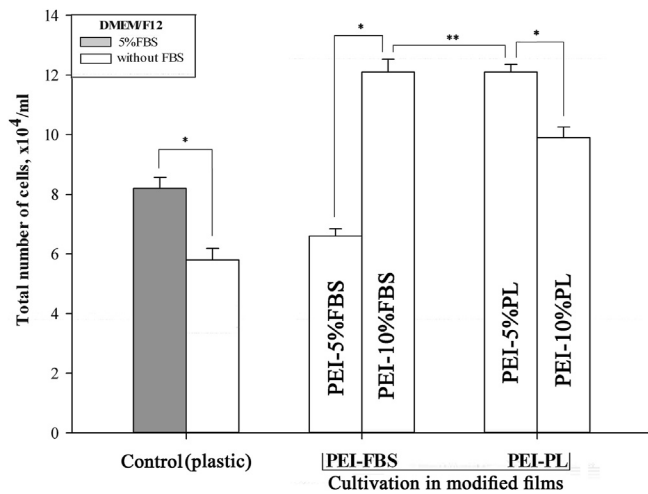


Figure 4. Proliferative activity of h-MSC (9 passages after isolation from bone marrow) when cultured in serum-free DMEM/F12 medium on PEI films with immobilized growth factors (5 and 10% FBS or 5 and 10% PL). h-MSCs were cultured at a concentration of 3.3×10^4 cells/100 μ l of DMEM/F12 medium without adding FBS. In the control (plastic), cultivation was carried out in the same DMEM/F12 medium without of FBS and with the addition of 5% FBS. The cultivation duration was 48 h. The experiments were carried out in 3 repetitions. * - Significant decrease in proliferative activity in the control during the cultivation of MSCs in a medium without serum, as well as on modified films PEI-5% FBS and PEI-10% PL compared with PEI-10% FBS and PEI-5% PL, respectively ($P \leq 0.01$); ** - NS in the efficiency of MSCs cultivation in serum-free medium on PEI-10% FBS and PEI-5% PL films ($P \geq 0.01$).

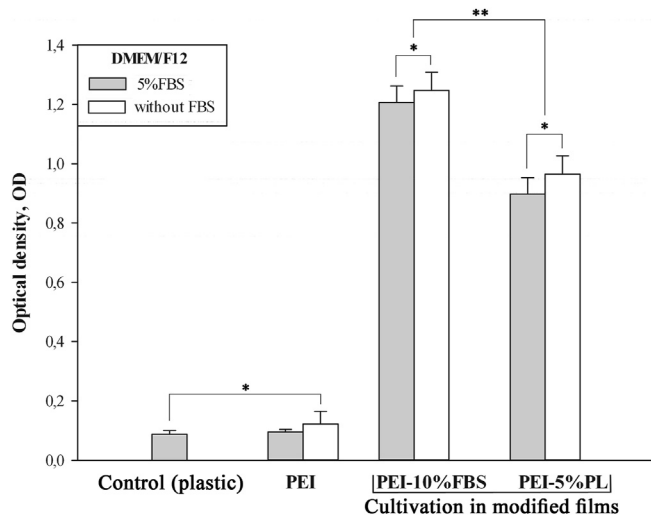


Figure 5. h-MSC viability after 48 h of cultivation on single-layer PEI films with immobilized growth factors of 5% PL and 10% FBS (MTT test). Culturing was performed on 96-well plates in DMEM/F12 medium with low content of FBS (5%) or without addition of FBS. h-MSCs were plated in wells in an equal amount on the basis of 2×10^4 cells per 100 μ l of nutrient medium. In the control, cells were cultured on untreated plastic with 5% FBS. MTT experiment carried out in 4 repetitions ($n = 4$). * - NS in h-MSCs survival in control and on unmodified PEI films PEI films in medium with 5% FSC and serum-free medium; between modified PEI films during MSCs cultivation under conditions of deprivation (5% FBS) and in serum-free medium ($P \geq 0.01$). ** - significant differences between the modified films, PEI and control ($P \leq 0.01$).

10% FBS (Figure 5), mesenchymal cells can be cultured in serum-free media or media with a reduced concentration of FBS. At the same time, MSCs remain viable *in vitro* which is important for the expansion of cell biomass for therapy. Films made of synthetic PEI polymer with immobilized growth factors have positive effects, as are extracellular

matrix regulatory proteins. They can be considered as a model for improving the culture methods of MSCs, which is especially important for h-MSC.

4. Discussion

Motivated by the research is based on a long-established fact for the vast majority of cell cultures, indicating that adhesion and spreading of cells on a substrate are necessary to start *in vitro* proliferation processes. We hypothesize that improvement of the adhesive properties of h-MSCs when cultured on polyelectrolyte nanofilms made from synthetic polymers PEI, PAH, and PSS promotes adhesion and, therefore, proliferation and growth of cell biomass for therapeutic purposes. These polyelectrolytes have adhesive properties for cell cultures and have long been used in various biomedical studies for the manufacture of nanofilms and microcapsules [27, 29, 33, 34]. In particular, the anchor properties of the PEI and PAH polycations are established on the cells of human embryonic kidney HEK-293 and pheochromocytoma of rat PC-12 as well as on the neural cells of animals and humans [27, 35]. Both polymers are not toxic to epithelial and myocytic cells, osteoblasts, and cortical neurons [27, 28, 35].

PEI and PAH polycations are used in combination with a negatively charged PSS polymer [33, 36]. PSS and PEI have isoelectric points 1 and 11, respectively [37]; therefore, the DMEM/F12 nutrient medium with pH 7.4 keeps the ionic force of the solution and the strength of both polyelectrolytes throughout the entire culture period. We use the PEI solution as a precursor for the subsequent application of the PSS solution. The results show that h-MSCs do not adhere and do not spread on the surface of PSS (Figure 1, e, f). To maintain the viability of h-MSCs *in vitro*, the adhesive properties of the culture surface are of paramount importance (Table 1, Figure 2). MSCs quickly (within 1 h) and firmly adhere to single-layer films of PEI polycation. Besides, doubling time of cell population reduces due to the high growth rate of the monolayer (23 h versus 29 h in the control without coating). The normal morphology of the monolayer is preserved on the PEI films (Figure 1, c, d), which favorably distinguishes PEI from the other two polyelectrolytes, PAH and PSS (Figure 1, e-h). The adhesive properties of PAH and PEI-PSS nanofilms differ little from the adhesive properties of untreated culture plastic (Figure 2). In addition, upon contact of h-MSCs with the surface of PAH and PSS, the number of irreversibly damaged cells increases by the necrosis mechanism (31.6 and 43.4%, respectively), which indicates the cytotoxicity of these polymers.

The toxicity of PAH is associated with weak polyelectrolyte properties and the presence of primary amines along the polymer molecular framework [38]. PSS films quickly dissolve (less than 3 min) upon contact with biological fluids to form a viscous and bioadhesive gel [32]. The rapid release of the active substance PSS from two and three-layer films PEI-PSS, PEI-PSS-PEI and PEI-PSS-PAH negatively affects the adhesion (Figure 2) and the viability of h-MSCs (Figure 3). Direct contact of MSCs with the PSS surface prevents adhesion and formation of a typical monolayer *in vitro* (Figure 1, e).

In recent years, many cases of the damaging effect of PSS on the epithelium of intestinal canal have been identified [39, 40, 41]. On histological preparations, PSS crystals, inflammation and necrosis are found. This polyelectrolyte penetrates the submucous membrane of intestinal canal and deeper tissues, causing necrotic damage. A similar situation is observed during the culture of h-MSCs on PEI-PSS films under *in vitro* conditions (Figure 1, h). After 72 h of incubation of h-MSCs on the PSS surface, cell death by the necrosis mechanism is more than 40%, which is several times higher than in the control (Table 1). Our results indicate that PEI-PSS films are highly toxic and not suitable for the culture of mouse and human MSCs.

Thus, the implementation of the cell adhesion mechanism that underlies the start of cell proliferation depends on the chemical composition of polyelectrolyte nanofilms and their surface charge. The three-layer polyelectrolyte films PEI-PSS-PEI and PEI-PSS-PAH proved to be less

suitable for the culture of h-MSCs as compared to the single-layer films PAH and PEI (Table 1, Figure 3). Our results also show that both polyelectrolytes (PAH and PEI) have good adhesive properties for h-MSCs and m-MSCs and relatively low cytotoxicity (Figure 3) but, in combination with PSS, can negatively affect cells.

For the culture of h-MSCs, it is better to use single-layer PEI films which ensure uniform distribution of cells on the surface, maximum adhesion, and a higher level of vitality *in vitro* compared to culture on negatively charged culture plastics. This polyelectrolyte does not have a noticeable negative effect on the morphology and proliferative activity of h-MSCs. Strong adhesive properties between MSCs and PEI are achieved, we believe, due to electrostatic attraction which is independent of the presence of FBS in the culture medium (Figures 4 and 5). Our experiments show, that at optimal concentrations (5% PL or 10% FBS), growth factors immobilized in PEI support the growth, adhesion and viability of h-MSCs in serum-free medium.

In conclusion, we would like to emphasize that the electrostatic assembly of PEI nanofilms on the surface of culture plastic is an attractive way to include biologically active growth factors of blood serum and platelet lysate into the composition of the polymer material. Single-layer PEI nanofilms with immobilized growth factors have positive effects and can be considered as a model for improving MSCs cultivation protocols and as a more cost-effective alternative to extracellular matrix components.

Declarations

Author contribution statement

Lyudmila M Mezhevikina: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Dmitriy A Reshetnikov: Performed the experiments; Analyzed and interpreted the data.

Maria Grigorievna Fomkina: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Nurbol O Appazov, Saltanat Zh Ibadullayeva, Evgeniy E Fesenko: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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