VASCULAR SMOOTH MUSCLE CELLS IN CULTURES ON LOW DENSITY POLYETHYLENE MODIFIED WITH PLASMA DISCHARGE AND BIOFUNCTIONALIZATION

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Abstract

Low density polyethylene (LDPE) was modified by an Ar plasma discharge and then grafted with glycine (Gly), bovine serum albumin (BSA) or polyethylene glykol (PEG). Some plasma-treated samples and samples grafted with BSA were exposed to a suspension of colloidal carbon particles (C, BSA+C). Pristine LDPE and tissue culture polystyrene dishes (PSC) were used as control samples. The materials were seeded with rat aortic smooth muscle cells and incubated in a medium DMEM with 10% of fetal bovine serum.

On day 1 after seeding, the cells on LDPE modified with plasma only, Gly, BSA and BSA+C adhered in similar numbers as on PSC, while the values on non-modified and PEG-modified samples were significantly lower. On day 5, the highest cell numbers were found again on LDPE with Gly, BSA and BSA+C. On day 7, the highest number of cells was found on LDPE modified only with plasma. The latter cells also displayed the largest cell spreading area. The increased cell colonization was probably due to the formation of oxygen-containing chemical functional groups after plasma irradiation, and also due to positive effects of grafted Gly, BSA and BSA in combination with colloidal C particles.

Key words: *Ar plasma discharge, biomaterials, low density polyethylene, cell adhesion, cell proliferation, grafting, tissue engineering, vascular smooth muscle cells.*

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Introduction

Synthetic polymers, such as polyethylene, polystyrene, polyurethane, polytetrafluoroethylene and polyethylene terephthalate, are commonly used in various industrial applications, as well as in biology and medicine. They not only serve as growth supports for cell cultures in vitro, but can also be used for constructing replacements for various tissues or organs, e.g., non-resorbable or semi-resorbable vascular prostheses, artificial heart valves, bone and joint replacements, and implants for plastic surgery (for a review, see [1-3]).

There are two approaches to the application of these materials. The first approach uses highly hydrophobic or extremely hydrophilic surfaces, which do not allow adhesion and growth of cells. This approach is used for creating bioinert blood vessel replacements, where permanent blood flow is necessary, and thus the adhesion of thrombocytes or immunocompetent cells is not desirable, due to the risk of restenosis of the graft (for a review see [2]). An alternative approach, widely accepted in recent tissue engineering, is to create surfaces that support colonization with cells and good integration of the replacement with the surrounding tissues of the patient's organism. This concept is used e.g. for constructing bone prostheses that will persist in the patient's organism for many years, and is being developed for creating bioartificial replacements of blood vessels, parenchymatous organs and even nervous tissue (for a review see [2,3]).

There are various ways of modifying the surfaces of materials to make them convenient for cell adhesion. For this purpose, surfaces have been exposed to ultraviolet (UV) irradiation [4], to a beam of various ions (e.g., oxygen, nitrogen, noble gases or halogens for biological applications [1-3]) or to a plasma discharge [5-7]. For more pronounced changes in the physicochemical properties of the modified surface, some of these processes can be realised in a gas atmosphere, e.g. in acetylene or ammonia [4]. The goal of these irradiation modifications is to create functional chemical groups containing oxygen or nitrogen, like carbonyl, carboxyl or amine groups, on the surface of the material. These groups increase the surface wettability, support the adsorption of cell adhesion-mediating extracellular matrix proteins and stimulate cell adhesion and growth [1-4].

An alternative and more exact approach can involve grafting the polymer surfaces directly with various biomolecules, which can influence the cell behavior in a more controllable manner. Therefore, in this study, low-density polyethylene, i.e. a material promising for biomedical use, was modified by an Ar plasma discharge and subsequent grafting with glycine (Gly), bovine serum albumin (BSA), polyethylene glycol (PEG) and/or colloidal carbon particles (C). On the modified polymer, we evaluated the adhesion and growth of vascular smooth muscle cells in cultures isolated from rat aorta.

Experimental

Preparation of the polymer samples.

The experiments were carried out on low-density polyethylene foils (LDPE) of the Granoten S*H type (thickness 0.04mm, density 0.922g▪cm^{-3}, melt flow index 0.8g/10minutes), purchased from Granitol a.s., Moravsky Beroun, Czech Republic. The foils were modified by an Ar^+ plasma discharge (gas purity: 99.997%) using a Balzers SCD 050 device. The time of exposure was 50 seconds, and the discharge power was 1.7W. Immediately after plasma modification, the samples were immersed in water solutions of glycine (Gly; Merck, Darmstadt, Germany, product No. 104201), bovine serum albumin (BSA; Sigma-Aldrich, Germany, product No. A9418) or polyethyleneglycol (PEG; Merck, Darmstadt, Germany, product No. 817018, m.w. 20 000). Some plasma-treated samples and samples grafted with BSA were exposed to a suspension of colloidal carbon particles (C; Spezial Schwartz 4, Degussa AG, Germany) [8]. Each substance was used in a concentration of 2wt.%, and the time of immersion was 12 hours at room temperature.

Cells and culture conditions.

The modified materials were cut into square samples 10▪10mm in size, sterilized with 70% ethanol for 1 hour, inserted into 24-well plates (TPP, Switzerland; well diameter 1.5cm) and seeded with smooth muscle cells derived from rat aorta by an explantation method [2,3]. The cells were used in passage 3 and seeded in a density of 17 000cm^{-2}.

The cells were cultivated in 1.5 ml of Dulbecco's Modified Eagle Minimum Essential Medium (Sigma, U.S.A.) supplemented with 10% foetal bovine serum (Sebak GmbH, Aidenbach, Germany) for 1, 2, 5 or 7 days (temperature of 37°C, humidified atmosphere of 5% of CO_2 in the air). For each experimental group and time interval, four samples were used. The cells on one sample were rinsed in phosphate-buffered saline (PBS), fixed by 70% cold ethanol (-20°C) and stained with a combination of fluorescent membrane dye Texas Red C2-maleimide (Molecular Probes, Invitrogen, Cat. No. T6008; 20ng/ml PBS) and a nuclear dye Hoechst # 33342 (Sigma, U.S.A.; 5µg/ml PBS). The number and the morphology of the cells on the sample surface were then evaluated on pictures taken under an Olympus IX 50 microscope, using an Olympus DP 70 digital camera. On the remaining three samples, the cells were rinsed with PBS, released with a trypsin-EDTA solution (Sigma, Cat. No. T4174) and counted in a Cell Viability Analyzer (Vi-CELL XR, Beckman Coulter). Non-modified LDPE and standard tissue culture polystyrene dishes (PSC) were used as control materials.

Statistics.

The results were presented as mean ± SEM (Standard Error of Mean). The statistical significance was evaluated by the ANOVA, Student-Newman-Keuls method. Values $p \leq 0.05$ were considered as significant.

Results and discussion

On the first day after seeding, the highest average number of initially adhered cells was observed on the PSC (17,096±3,034cells/cm^2). On the LDPE samples modified with plasma, and also on the plasma-modified samples grafted with BSA, BSA+C or Gly, the cell numbers ranged from 10,475±1,028cells/cm^2 to 10,951±1,027cells/cm^2. As revealed by ANOVA, these values were not statistically different from those obtained on PSC. In contrast, the cell population densities on pure LDPE and LDPE modified with PEG and C reached only 4,769±1,400cells/cm^2 and 9,048±388cells/cm^2, respectively, and these values were significantly lower than those on PSC (FIG.1). This relatively low initial cell adhesion can be explained by an antiadhesive action of PEG, based on its high hydrophilia and the mobility of its chain, which hamper stable adsorption of the cell adhesion-mediating proteins from the serum of the culture medium, mainly vitronectin and fibronectin (for a review, see [9]). Also carbon-modified surfaces, particularly those made

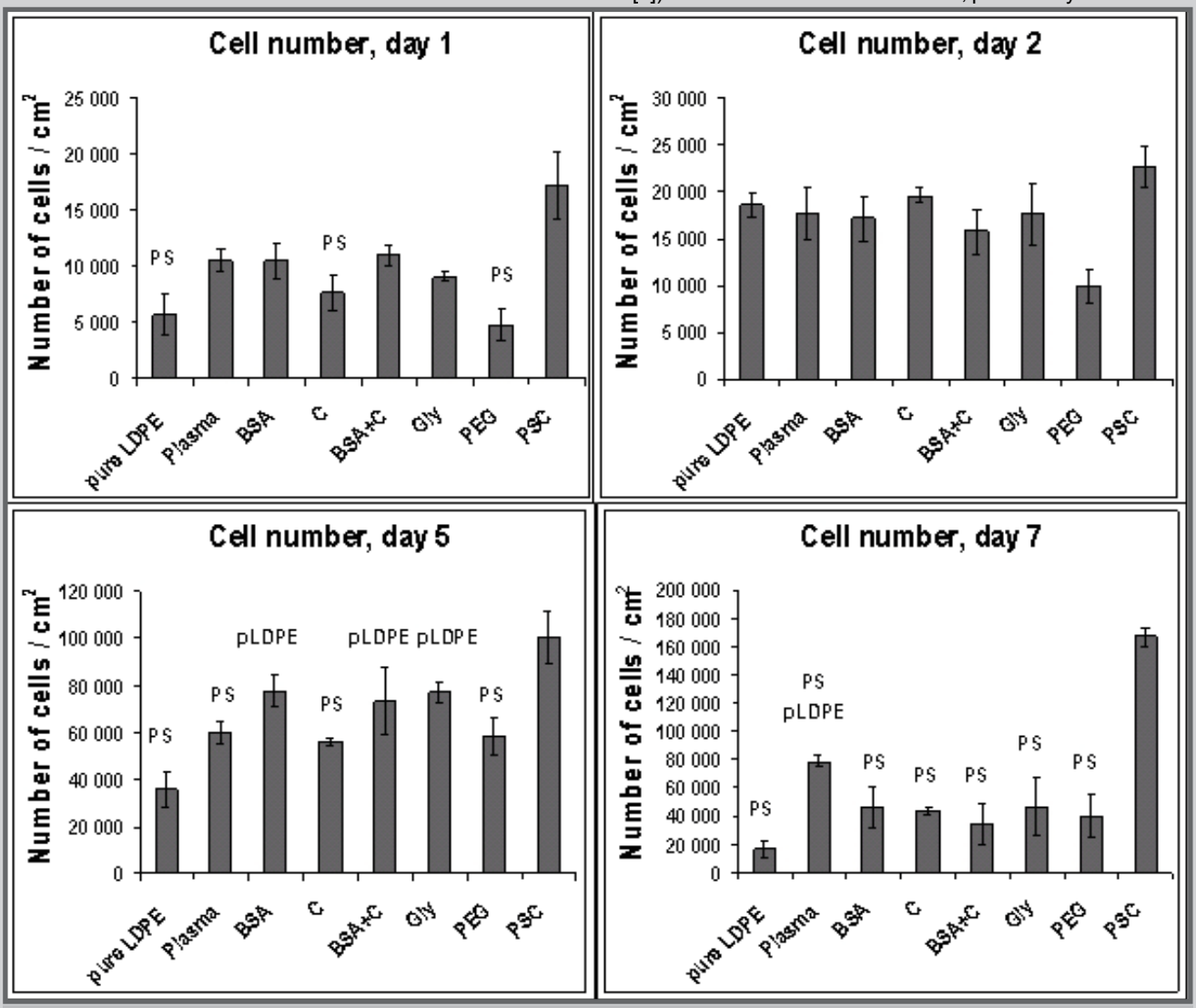

FIG.1. Number of rat aortic smooth muscle cells on day 1, 2, 5 and 7 after seeding on pure LDPE, LDPE modified by Ar plasma discharge (Plasma) and subsequently grafted with bovine serum albumin (BSA), colloidal carbon particles (C), BSA with subsequent exposure to colloidal carbon particles (BSA+C), glycine (Gly) or polyethylene glycol (PEG). A standard cell culture polystyrene dish (PSC) was used as a reference material. Mean ± SEM from 3 independent samples for each experimental group. ANOVA, Student-Newman-Keuls method. Statistical significance: PS, pLDPE: $p \leq 0.05$ compared to the values on pure LDPE and polystyrene dishes.

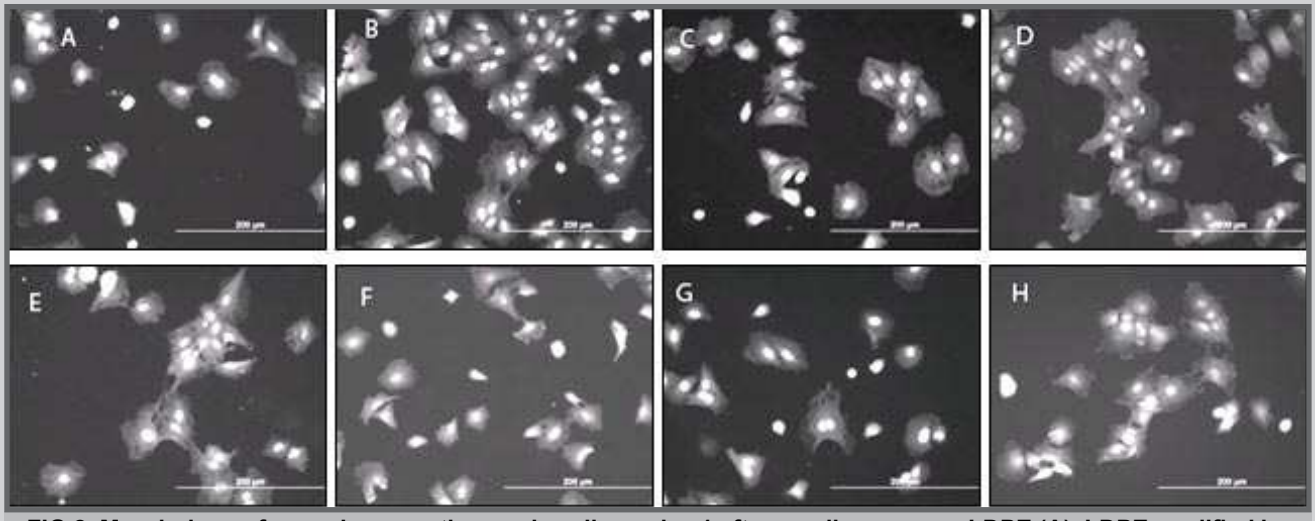

FIG.2. Morphology of vascular smooth muscle cells on day 1 after seeding on pure LDPE (A), LDPE modified by Ar plasma discharge (B), LDPE modified by Ar plasma discharge and subsequently grafted with bovine serum albumin (C), colloidal carbon particles (D), bovine serum albumin and colloidal carbon particles (E), glycine (F) or polyethylene glycol (G). H: cells on a polystyrene culture dish. Stained with Texas Red C2-maleimide and Hoechst #33342. Microscope Olympus IX 50, obj. 20, digital camera DP 70. Bar=200μm.

of amorphous hydrogenated carbon, have often behaved as rather bioinert and do not enhance cell adhesion. They have therefore been used for constructing blood-contacting and hemocompatible devices (for a review, see [10]).

On the second day of the experiment, the cell numbers on all tested samples became similar, although the highest average number of cells still persisted on the polystyrene culture dishes (22,670±2,234cells/cm^2), and the lowest average cell population density was found again on PEG (10,000±1,779cells/cm^2)

On day five after seeding, the highest numbers of cells were again obtained on PSC (100,741±10,926cells/cm^2), and similarly as on day 1, also on samples grafted with BSA (77,534±6,463cells/cm^2), Gly (77,058±4,200cells/cm^2) and BSA+C (73,253±14,462cells/cm^2). On these samples, the cell numbers were significantly higher in comparison with the value on pure LDPE (35,682±7,757cells/cm^2). In the case of BSA, this result is somewhat surprising, because, like PEG, this protein has been used for constructing surfaces that are non-adhesive for cells (for a review, see [9]). On the other hand, BSA has been reported to promote the adsorption of cell adhesion-mediating molecules in advantageous spatial conformations, supporting the accessibility of specific sites on these molecules (e.g., RGD-containing amino acid sequences) by cell adhesion receptors, such as integrins [11].

As for the beneficial action of glycine on cell adhesion, this molecule enriches the polymer surface with additional polar oxygen-containing groups and positively electrically charged amine groups, which also improve the adsorption of cell adhesion-mediating proteins in appropriate geometrical conformations for binding to cell adhesion receptors [12].

Seven days after seeding, the cell number on plasma-modified LDPE (78,960±4,479cells/cm^2) became significantly higher than the cell number on pure LDPE (16,658±5,600cells/cm^2). However, the cell population densities on all tested LDPE samples were significantly lower than on standard polystyrene cell culture dishes (16,6691±6,891μm$_c$). Nevertheless, the cells on all modified LDPE samples were able to form confluent layers.

In addition, the cells on the modified LDPE samples were usually better spread. The largest cell adhesion area (2,490±270μm$_c$), measured on day 1 after seeding, was found on plasma-irradiated LDPE, and was significantly larger compared to all remaining experimental groups, where the cell spreading areas ranged from 1,739±110 μm$_c$ (on pure LDPE) to 1,949±280μm$_c$ (on PSC). The cell spreading area on pure LDPE was significantly the smallest of the values obtained on all tested samples. The cells on all samples were mainly polygonal in shape, and this was more pronounced on the modified samples than on the unmodified LDPE samples (FIG.2).

The improved cell adhesion and growth of cells on samples modified by plasma discharge was most probably due to the creation of oxygen-containing functional groups on the polymer surface. Fourier transform infrared (FTIR) spectroscopy has indicated the presence of peroxide, ester, carbonyl, carboxyl, hydroxyl and amide groups and excessive double bonds in polyethylene modified with a plasma discharge [13]. Oxygen-containing groups are known to increase the surface wettability and improve the adsorption of cell adhesion-mediating extracellular matrix molecules from the serum of the culture medium in an appropriate amount, flexibility and spatial conformation, enabling good accessibility of specific sites on these molecules for cell adhesion receptors [1-3].

Conclusion

Treating low-density polyethylene with an Ar plasma discharge had positive effects on the adhesion and growth of vascular smooth muscle cells in cultures on this material. This improvement of the cell colonization was probably due to the formation of oxidized structures in the polyethylene surface layer and increased material wettability. The attractiveness of the material for cell colonization was further intensified by grafting the polymer surface with glycine, bovine serum albumin and bovine serum albumin with colloidal carbon particles. However, the exact mechanisms of the positive influence of these modifications on cell adhesion and growth need further investigation.

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COMPOSITE COLLAGEN-CALCIUM PHOSPHATE HYDROGELS FOR BONE SUBSTITUTION

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Abstract

Collagen is an interesting biomaterial for use as an injectable thermosenstive hydrogel whose gelation can be triggered after implantation by the patient's body temperature. Substances and particles can be incorporated during gel formation. For bone tissue engineering applications mineralizability of the collagen gel is desirable. One option is the incorporation of nanoparticles consisting of calcium phosphate (CaP) which should serve as nucleation sites for further mineralization. In this study, the feasibility of incorporating CaP particles in 3mg/ml collagen gels at CaP:collagen mass ratios of 4:1, 2:1 and 1:1 and their effect on the kinetics of gel formation and the gel mechanical properties were studied. Rheological studies confirmed that gels formed within 10 minutes and speed of formation was only slower at CaP:collagen = 4:1. Incorporation of CaP had no negative effect on gel mechanical strength. These results open the way for mineralization and biocompatibility studies.

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Introduction

Collagen is a biocompatible, biodegradable biomaterial which has been widely applied as a material in scaffolds for tissue engineering. Gels can be formed from acidic collagen solutions at 4°C by inducing fibrillogenesis through neutralization, adjustment of ionic strength and increase of temperature to 37°C. Thus collagen is an interesting material for use as an injectable thermosensitive hydrogel whose gelation can be triggered after implantation by the patient's body temperature. Indeed, collagen gels have been used as model systems to study the behavior of osteoblasts (1) in vitro, as drug delivery systems (2) and have been applied in the treatment of bone defects in vivo (3). Substances and particles can be incorporated during gel formation in order to alter the functional properties of the gel. For bone tissue engineering applications mineralizability of the collagen gel is desirable. One option to increase mineralizibility of hydrogels is functionalisation by incorporating nanoparticles consisting of calcium phosphate (CaP) which should serve as nucleation sites for further mineralization (4). In this study, the feasibility of incorporating CaP particles in 3g/ml collagen gels at CaP:collagen mass ratios of 4:1, 2:1 and 1:1 and their effect on the kinetics of gel formation and the gel mechanical properties were studied.

Materials and methods

Briefly, collagen I from rat tail was obtained from BD Biosciences, Netherlands. Gels were formed by neutralisation with 1M NaOH and addition of 10 x Phosphate Buffered Saline (PBS) and dilution with dd H_2O to achieve a final collagen concentration of 3mg/ml with final effective PBS concentration of 0.5. Ingredients were mixed at 4°C. Fibrillogenesis was initiated by raising the temperature to 37°C. Calcium phosphate was formed in the following way: 1.47g $Ca(OH)_2$ in 9.105ml was reacted with 0.805ml H_3PO_4 under agitation to yield 10ml 20% (w/v) $Ca_5(PO_4)_3(OH)$ solution, which was neutralised to pH 7.4. To form CaP-collagen composites,