COMPARISON OF PDMS MICROTOPOGRAPHICAL PLATES AND PLANAR NANOFIBER SCAFFOLDS FOR IN VITRO CULTIVATION OF CARDIAC CELLS

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Abstract

PDMS microchambers and microchannels were evaluated in many studies of the specific *in vitro* cultivation. Modern microtopographical methods bring new possibilities how to prepare transparent planar blocks with isotropic lamellas. On the other hand, the planar nanofiber sheets are another type of modern culture substrate. Neonatal myocytes and the cardiac muscle cell lineage HL-1 were used for tests of the cell adherence, cell survival and cell orientation on pure or modified polydimethylsiloxane (PDMS) or polycaprolactone (PCL). PDMS with fibronectin coating was shown to be the material with the best cell adherence, viability and isotropic cell orientation from all tested variants.

Keywords: Nanofibers, microlithographical methods, *in vitro* models, tissue engineering, cardiomyocytes

1. INTRODUCTION

The transplantation of cardiomyocytes in the form of a “cell suspension“ did not lead to good results in previous animal experiments. To develop successful cardiovascular tissue regeneration methods and also to develop an *in vitro* model mimicking the real cardiac tissue, the preparation of large-scale planar compact cell sheets are needed. The new technologies should be introduced to improve the preparation of the myocardial tissue *in vitro* and to cultivate the typical myocardial structure with an isotropic orientation of cells and with numerous intercellular connections.

The previous experiments on cardiomyocytes carried out over the last 20 years have shown that the microstructure and coating of surfaces of *in vitro* chambers are the key factors which can control the cellular adherence, cellular orientation and cellular connection, and enhance the long-term confluency and maturation of cardiomyocytes. For example, PDMS is a non-toxic material, which was used in many *in vitro* systems as a planar surface, mostly for endothelial cells. Its surface microstructure can be modified to obtain a model mimicking the basal structure of extracellular scaffolds in the real tissue *in vivo*. On the other hand, the PCL prepared by the electrospinning method is another versatile way how to produce a biocompatible material simulating the structure of the extracellular matrix *in vivo* [1, 2]. The purpose of the work presented here was to provide a quantitave comparison of these substrates and their different coatings in terms of the cardiac cell adhesion and survival.

2. METHODS

2.1. Fabrication of microtopographical plates

Creating of PDMS layer needs some solid polymer material as a master substrate. After several tests, silicon (prepared from 6333097 nanopowder - Sigma Aldrich) was chosen as the best master substrate. All steps of the fabrication are illustrated in **Figure 1**. Silicon was deposited from hexamethyldisilazane (HMDS) vapours in N_2 gas at 100 °C for 60 s (for silicon substrate hydrophobicity to increase the photoresist adhesion). Photoresist S1813 (DOW Electronic) was spin-coated on silicon at 1800 RPM for 60 s (final creation of 3 µm thick layer of the photoresist). The phototresist was heated at 115 °C for 60 s. The pattern was exposed to direct writing by DWL at a power of 4.8 mW. The lines obtained were 3 µm wide and distanced 10 µm one from another (or two alternative variants with distanced 4 µm and 30 µm). The photoresist was developed in AZ 726MIF (AZ Electronic Materials USA Corp.). The master silicon with the patterned photoresist was rinsed in DI water and dried under N_2 gas. The master was coated by HMDS vapours in N_2 gas at 100 °C, again. PDMS was prepared by a mixture of silicone elastomer and a silicone elastomer curing agent (Dow Corning Europe S.A.). PDMS elastomers were well mixed and degassed in a vacuum chamber to remove bubbles, poured on HMDS-treated master and cured at 80 °C for 90 min. The PDMS substrate was then peeled off and cut to the shape desired. The final PDMS substrates had lamellas 3 µm tall and 3 µm wide, separated by 4, 10 or 20 µm (**Figure 2**). The final PDMS culture plates had geometrical size of one well in a standard 24-well plate. Each variant of the PDMS plate (variants 3 µm - 4 µm, 3 µm - 10 µm, 3 µm - 20 µm) was also prepared in a subvariant with additional fibronectin coating (F1141, Sigma-Aldrich) or collagen coating (Collagen I Rat, Invitrogen).

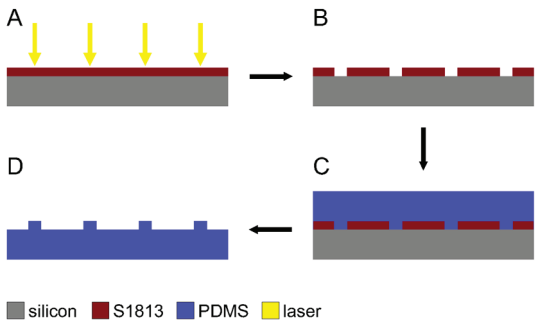

Figure 1 Fabrication process of PDMS substrate with lamellas, (A) silicon substrate coated by S1813 and exposed to laser, (B) development of photoresist, (C) pouring PDMS on master, (D) peeling off PDMS

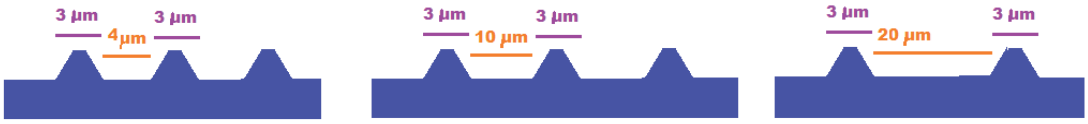

Figure 2 Final shape of PDMS surface prepared for our testing of cell cultivation.

2.2. Fabrication of nanofibers sheets

PCL (Mn = 80 kDa, Sigma-Aldrich) were dissolved in the HFIP for 24 hrs at room temperature and sonicated. The aligned PCL nanofiber yarns were prepared by an enhanced wet−dry electrospinning process - fiber forming technology based on drawing a submicron fiber from a solution or melt. A collection electrode was used for depositing the prepared fibers and preparation of parallel fiber structure with a significant isotropicity nature. Electron microscopy images (by Hitachi SU6600) revealed the structure, where a significant amount of parallel fibres are parallel with one main direction (**Figure 3**). The final nanofiber sheets were adjusted to the size of one well in a standard 24-well plate.

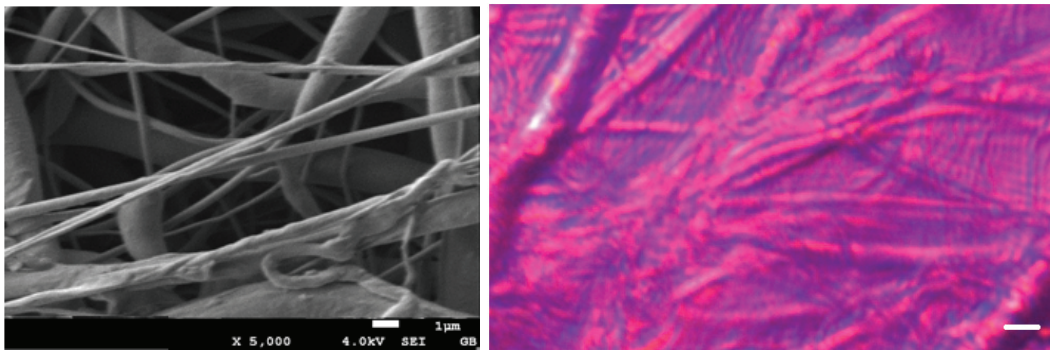

Figure 3 SEM image for control of significant isotropic structure of fibers orientation (scale bar = 1 µm) and illustrative image of isotropy of nanofibers from light microscope (scale bar = 10 µm)

The nanofibers planar plate was also prepared in a subvariant with an additional incubation in the fibronectin solution (F1141, Sigma-Aldrich) or collagen solution (Collagen I Rat, Invitrogen) for 2 hours at 37°C.

2.3. Neonatal myocardial cell preparation and test of culture plates

Neonatal rat cardiomyocytes were isolated from Wistar neonatal rats aged 2-5 days. Hearts were removed and washed in a salt solution containing 20 mM HEPES, 120 mM NaCl, 1 mM NaH_2PO_4, 5.5 mM glucose, 5.4 mM KCl, and 0.8 mM $MgSO_4$ (pH 7.3-7.4). Cardiomyocytes were isolated by the collagenase digestion, after which the cells were resuspended in a medium containing Iscove's modified Dulbecco's medium (IMDM) (Sigma-Aldrich) and Medium 199 (Sigma-Aldrich) (4:1) supplemented with horse serum (10 %) and fetal calf serum (5 %). The separation of non-myocardial cells (heart fibroblasts) and selecting the non-adhesive cells (cardiomyocytes) were done in accordance with protocol presented in [3].

Cardiomyocytes were resupended in the culture medium IMDM and transferred (in form of 100 µL drops) to all types of culture PDMS and PCL plates, which were prepared as described above (Chapters 1.1. and 1.2). Cells were seeded at density 5×10^4 cells/cm^2. The cells were incubated under 21 % O_2 and 5 % CO_2 at 37 °C. The cultivation medium was removed after 48 hrs and replaced by a medium containing IMDM and Medium 199 (4:1) with penicillin. The ratio of the adhered cell and non-adhered cells was quantified using Leica Dmi8 (20 field of view from each sample). The viability of the cells was quantified by using a Calcein viability kit (Biotium Ltd.) and Leica TCS SP8X. Statistical

2.4. HL-1 caridac cell preparation and test of culture plates

HL-1 cells were obtained from Sigma-Aldrich in cryoconserved form. The cells were defrosted, washed and cultivated on a plastic petri dish with fibronectin coating in accordance with the manufacturer´s instruction. After 3 passages, the cells were washed and resuspendend in Claycomb medium (JRH Biosciences) supplemented with 10 % fetal bovine serum (Sigma/Aldrich) and 2 mM L-glutamine (Invitrogen).

HL-1 cells were transferred (by 100-uL drops) to all types of culture microplates, which were prepared as described above (Chapters 1.1. and 1.2). The drops were counted to provide a density 5×10^4 cells/cm^2. The cells were incubated under 21 % O_2 and 5 % CO_2 at 37 °C. The cultivation medium was removed after 48 hrs and replaced by fresh DMEM medium with 5 % FBS (Sigma-Aldrich). The ratio of adhering / non-adhering cells was counted by using Leica Dmi8 or Leica TCS SP8X. The viability and function of the cells were quantified by using a Calcein viability kit (Biotium Ltd.)

2.5. Cell orientation and average isotropy index

The cell cultures on different substrates were recorded by Leica TCS SP8X (fluorescence mode 500/520 nm) and all images of 200 randomly selected cells in each culture was used for the calculation of the "average

isotropy index“ (AII). This index was computed by Matlab utility (mathematical methods described on example in **Figure 4**).

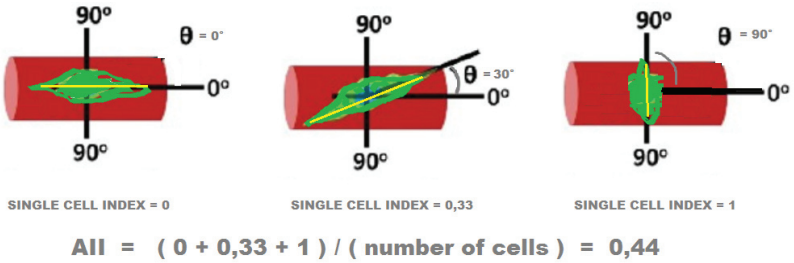

Figure 4 Description of software methods for calculating average isotropy index (AII) for certain set of three cells. Red line (direction 0°) highlights the basal direction of lamellas or main direction of sets of nanofibers.

3. RESULTS

The image of all PDMS substrates displayed a high ratio of adherent cells 24 hrs after seeding. Their viability was higher than 90 % in all variants. The detailed statistical analysis of the effectivity of the cell adherence and AII is summarized in **Table 1**. The most effective adherence and production of an isotropic cell culture was found in substrates “3-10 + fibronectin coating” and “3 μm - 10 μm + collagen coating”. For illustrative image of two types of cells in different subvariants of PDMS with coating see **Figures 5 and 6**.

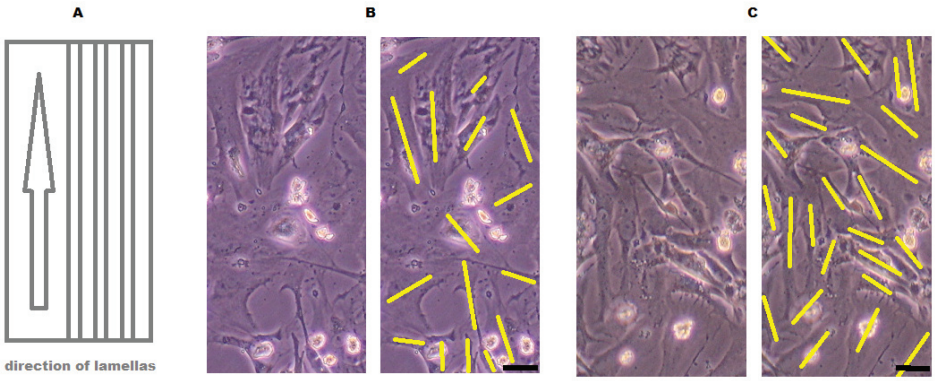

Figure 5 Neonatal myocyte adhesion and orientation on PDMS plate; (A) Direction of lamellas, (B) myocytes on PDMS (variant 3-10) with fibronectin coating (left: wide field microphoto, right: microphoto with marks of main axis of cells), (C) myocytes on collagen (variant 3-10)

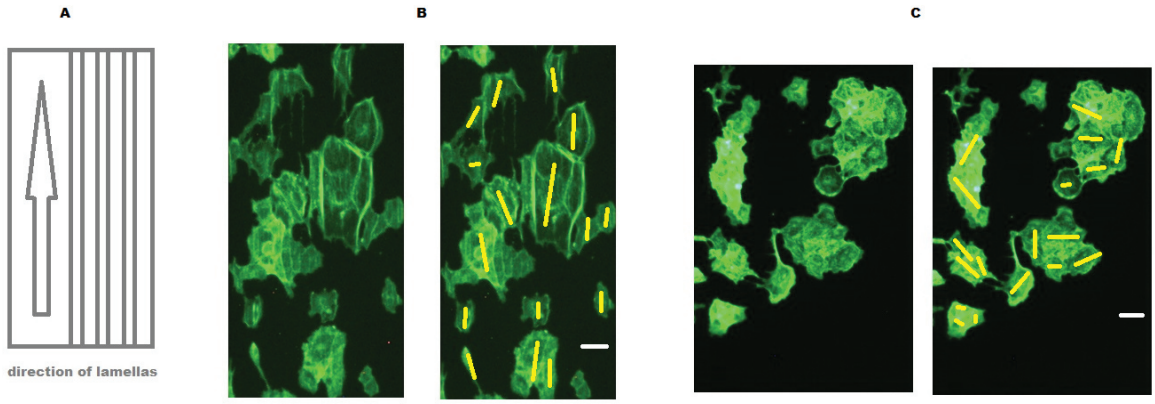

Figure 6 HL-1 myocyte adhesion and orientation on PDMS plate; (A) Direction of lamellas, (B) myocytes on PDMS (variant 3-10) with fibronectin coating (left: wide field microphoto, right: microphoto with marks of main axis of cells), (C) myocytes on control PDMS plate (plate without lamellas - anisotropic orientation of cells)

Table 1 Quantification of cell adhesion and cell isotropic orientation on different PDMS microstructures. Average from 3 independent cell samples.

Profile of plates (lamellas, their distance, their coating)	HL-1 cells		Neonatal myocytes	
	Cell adherence (%)	AII (rel. unit)	Cell adherence (%)	AII (rel. unit)
Triangle basis 3 μm Distance 4 μm	35 ± 5	0.36	12 ± 4	0.52
Triangle basis 3 μm Distance 10 μm	44 ± 8	0.24	18 ± 5	0.33
Triangle basis 3 μm Distance 20 μm	21 ± 6	0.44	14 ± 5	0.43
Triangle basis 3 μm Distance 4 μm + fibronectin	59 ± 16	0.19	36 ± 8	0.16
Triangle basis 3 μm Distance 10 μm + fibronectin	74 ± 15	0.17	38 ± 15	0.14
Triangle basis 3 μm Distance 20 μm + fibronectin	62 ± 20	0.28	37 ± 11	0.24
Triangle basis 3 μm Distance 4 μm + collagen	42 ± 16	0.34	52 ± 8	0.35
Triangle basis 3 μm Distance 10 μm + collagen	40 ± 12	0.31	67 ± 21	0.23
Triangle basis 3 μm Distance 20 μm + collagen	39 ± 11	0.48	59 ± 18	0.38

The microscopic image of all nanofiber substrates displayed a very low ratio of adherent cells 24 hrs after seeding. Their viability was higher than 90 % in all variants. A detailed statistical analysis of effectivity of cell adherence and AII index is summarized in **Table 2**.

Table 2 Quantification of cell adhesion and cell isotropic orientation on different nanofibers

Subvariant of nanofibers	HL-1 cells		Neonatal myocytes	
	Cell adherence (%)	AII (rel. unit)	Cell adherence (%)	AII (rel. unit)
Unmodified	5 ± 2	0.45	2 ± 1	0.52
Fibronectin add.	14 ± 8	0.19	8 ± 3	0.34
Collagen add.	8 ± 1	0.33	4 ± 3	0.29

4. CONCLUSION

The results show that developing of ideal substrates, which would cause the 100 % adhesion and 100 % isotropy of cardiomyocytes after seeding, is still a big challenge. PDMS with fibronectin or collagen coating exerted a significantly higher ratio of adherent cardiomyocytes (both HL-1 and neonatal cells) after seeding, higher than pure PDMS and higher than PCL nanofiber structures. Cardiac cells used in our experiments had a very low adherence to the PCL system compared to data in some recent communications [4,5], where another cells (stem cells or fibroblasts) were tested on similar PCL fibers. Geometrical variants of PDMS lamellas show a significant impact on the isotropy of final cultures of cardiomyocytes, the best variant for effective unification of cell direction in cultures being variant “3-10 + fibronectin” (triangle basis 3 μm, distance 20 μm, fibronectin coating). The positive results will be used for next additional optimising of surfaces created from PDMS and PCL nanofibers in our lab and could accelerate the development of new devices for cardiovascular medicine in near future.

ACKNOWLEDGEMENTS

The work was supported by the project by the project FEKT-S-17-4487 and by the project MPO TRIO FV20422 (Czech Ministry of Industry).

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