Quantitative method for the analysis of cell attachment using aligned scaffold structures

F Tian\textsuperscript{1}, H Hosseinkhani\textsuperscript{2}, G G Estrada\textsuperscript{3}, H Kobayashi\textsuperscript{1,4}
\textsuperscript{1}Bio-functional materials research group, National Institute for Materials Science (NIMS) 1-1, Namiki, Tsukuba-shi, Ibaraki 305-0044, Japan
\textsuperscript{2}International Center for Young Scientists (ICYS), National Institute for Materials Science (NIMS) 1-1, Namiki, Tsukuba-shi, Ibaraki 305-0044, Japan
\textsuperscript{3}Max Planck Institute for Metals Research, Heisenbergstr. 3, Stuttgart 70569, Germany
\textsuperscript{4}Institute of Biomaterials and Bioengineering, Medical and Dental University, Tokyo 113-8549, Japan

KOBAYASHI.Hisatoshi@nims.go.jp

Abstract. This paper presents a new quantitative method that evaluates the cell attachment affinity to aligned scaffold structures composed of poly (glycolic acid) PGA/collagen. The structures were fabricated by the electrospinning method. We analyzed the relationship between the number and length of attached cells to fibers of different diameters under different concentrations of PGA/collagen. The findings are three fold. Firstly, the fibers fabricated on the order of nano-scale significantly enhanced the number of attaching cells compared with those fibers fabricated on the order of micro-scale. Secondly, the cell morphology is affected by both the amount of collagen in PGA/collagen hybrid fibers and the cells adhesion affinity to fibers. Finally, PGA/collagen hybrid fibers on the range of 0.5 \textmu m with a concentration of 67% collagen provided the best cell adhesion. This study provides an attractive technique to fabricate suitable diameters and collagen concentrations for tissue engineering applications.

1 Introduction

Nano-scaffolds have provided good conditions for cell adhesion and are currently being applied to many different types of implantable medical devices [1]. However, there is no clear consensus regarding which method and variables are most relevant to evaluate the cell adhesion affinity with scaffolds. For example, one study compared the attachment of osteoblasts to micro-scale polyethylene terephalat (PET) fibers of different diameters [2]. Other authors compared the adhesion of NIH 3T3 fibroblasts to poly (p-dioxanone-co-L-lactide)-block-poly (ethylene glycol)(PPDO/PLLA-b-PEG) nanofibers [3]. Nowadays, the non-woven structure still inhibits the real reason that effects of cell adhesion on scaffolds. It is thus necessary to set up a quantitative model to study the cell adhesion to scaffolds. Several approaches have been considered for the fabrication of the alignment of polymeric nano-fibers [4-7]. Those approaches make it possible to evaluate the cells adhesion on aligned fibers.

An interesting feature of native ECM (Extracellular Matrix) is its nanoscale structure. In native
tissues, collagen fibers range from 50-500 nm in diameter and are very uniform in size [8]. In order to study the effect of collagen concentration upon cell adhesion, we evaluated two parameters: collagen composition and fiber diameter. This study evaluates the cell adhesion to regular scaffolds that were fabricated with different composition of fibers of different diameters. A new method is presented to study the adhesion of NIH 3T3 cells on these scaffolds. Electrospinning was employed to reproduce fibers with different diameters.

2 Materials and Methods

Solutions for electrospinning were prepared by dissolving 100 mg/ml collagen with 67 mg/ml PGA in 1,1,1,3,3,3 Hexafluoro-2-2 Propanol at different concentrations ranging from 16% to 93%. The polymer solution was delivered by a programmable pump (Harvard Apparatus, MA) to the exit hole of the electrode (Spinneret with a hole diameter of 0.7 mm). The flow rate was set to 5 ml/h. A high-voltage power supply (Nippon-stabilizer industry, Co., Ltd.) was used to provide the voltage of 26 kV. The diameter’s drum was 15 cm, with a hole of 2 cm in diameter at the center. A stepper motor (Eyela Mazela Z., Tokyo, Rikakikai Co., Japan) was used to control the speed of the drum. The grounded platform for collecting the fibers was a silicone. The mandrel was rotated at 1200rpm to generate aligned fibers. Figure 1A shows some typical aligned fibers collected on the substrate.

NIH 3T3 fibroblasts were cultured at 37 °C in 95% air and 5% CO₂ atmosphere. Dulbeccos’ Modified Eagle’s Medium (DMEM) supplemented with 10% bovine calf serum (GIBCO, BRL) was changed every two days. The scaffolds were sterilized in ethylene oxide gas (EOG) for 22h and then washed 3 times in sterile Phosphate buffer saline (PBS, pH 7.4) to remove traces of 1,1,1,3,3,3 Hexafluoro-2-2 Propanol. NIH 3T3 cells at different concentrations (10^2 – 10^4 cell/ml) were seeded on the fibers for 1h without serum and gently washed with serum-free DMEM. NIH 3T3 cells were allowed to attach for 8h to DMEM, supplement with 10% bovine calf serum, for cell shape observations. Cells were assessed by the following immunocytochemical analysis [9]. The samples were fixed with methanol for 20 min. They were washed with PBS, and permeated with 0.2% Triton X-100 for 5 min at room temperature, and incubated in 1% normal bovine serum in PBS for 1h. Samples were hybridized with the primary antibody, i.e. mouse IgG anti vicullin (1:2000 dilution, Sigma). Samples were then washed with PBS, incubated with sheep anti-mouse-Cy3 antibody (1:200). The cultures were mounted on N-propyl/gallate/glycerol, and examined under exciting filters of 530-550nm and 460-495nm (Zeiss fluorescent microscope, Germany). All reagents and antibodies were obtained from Sigma Chemical Co. (St. Louis, MO). The number of cells and their length were quantified for aligned fibers of 2 mm long and different diameters.

Differences between samples and the control were evaluated using the statistical analysis package SPSS 11. Statistically significance was set to p<0.05. A one-way analysis of variance (ANOVA) was used to test the differences between the subgroups of collagen concentrations and fiber diameters. Standard box plots and five number summaries were calculated to obtain the quartiles. Outliers were detected by looking at points far beyond first and third quartiles, i.e. outliers in Tukey’s sense.

3 Results and Discussion

The number of attached cells to fibers is related to cell concentration in the medium. Figure 1B shows the number of attached cells to the fibers after 1h in a DMEM medium without serum. The boxplot, Figure 1C, shows an increasing trend between cell concentrations and the number of adherent cells to fibers. This method can be used to compare the cell attachment to fibers of different diameters.
The cells spread on the fibers after an incubating time of 8h in DMEM medium supplemented with 10% bovine calf serum. The length and number of attached cells to fibers were analyzed on separated aligned scaffolds fabricated by a different composition of fibers with different diameters. NIH 3T3 cells were counted on fibers with different diameters (Figure 2 A, B and C). Analysis of Variance (ANOVA) tests were used to analyze the number of attaching cells. There is a very significant difference ($p<0.01$) between the five groups of concentrations. However, there is non-significant difference between the numbers of cells attached to fibers with diameter of 10 μm or 3-5 μm over all collagen concentrations used in this study (figure 3). A closer analysis to the groups did reveal striking, quantitative differences between the collagen concentrations, in which the group of 67% collagen introduced the variance found by ANOVA. Moreover, a within group analysis shows that the smaller diameter tested (0.5 μm) is significantly different ($p<0.01$) to any other diameter among the three diameters in the five groups of concentrations. Our results clearly indicate that there is strong relationship between collagen concentration, fiber diameter and number of cells attached to fibers. In particular, the highest cell attachment was observed for PGA/collagen hybrid fibers with a concentration of 67% collagen and fiber diameter of 0.5 μm. The cells present a more rounded morphology when they adhered to scaffolds with larger diameters. NIH 3T3 cells followed the PGA and collagen direction, and aligned on the longest fibers. The cells appear more spread and bio-polar in their morphology on the scaffolds of smallest diameter. The number and average length of attaching cells were much higher on nano-fibers than in the micro-fibers. It is known that cells attaching to nano-fibers appear more spread and exhibit a smooth muscle-like morphology [10,11]. Our observations are consistent with these findings.

Figure 2. Light microscopy photographs of cells attached to fibers of different diameter scaffold, with a concentration of 67% collagen, after an incubating time of 8 hours in 10% serum mediums: (a) 10μm; (b) 3-5 μm; (c) 0.5 μm.
Figure 3. Relationship between the number of attaching cells, collagen concentrations and fiber diameters. An ANOVA test shows that there is a very significant difference between the five groups of collagen concentrations and the number of attaching cells, \( p<0.01 \). The source of variance is identified as a concentration of 67% collagen and fibers with diameter of 0.5\( \mu \)m, \( p<0.01 \). These combined factors are associated to the highest average number of attaching cells.

The correlation between the cell behavior and collagen concentrations in scaffolds has not yet well studied [2]. Figures 2C show that those fibers with diameter of 0.5 \( \mu \)m and collagen concentration of 67\% provided the highest average length \( (p<0.01) \) of cells attached to the fibers. The combination of statistical analyses and experimental observations help us to correlate: the highest number of attaching cells and the longest average cell length to collagen concentration (67\%, see Figure 3). There is a bell-shape relationship between the cell adhesion and the collagen composition among all fibers with different diameters, with 67\% at the center of the distribution. The number and average length of attaching cells increase following the concentration of collagen increase at the concentration under 67\%. The number and average length of attaching cells decrease when the collagen concentration is over 67\%. Collagen provides necessary substance for cell attachment. However, Collagen has poor Young's Modulus comparing with PGA[11]. The high concentration of Collagen decreases the strength in fibers for cell adhesion. PGA complements the weakness of Collagen. Aligned scaffold structures can help to quantify the cell adhesion. Therefore we expect that this method can be employed to provide information to select scaffolds for tissue engineering applications.

References
[1] Ma Z W, Kotaki M, Inai R and Ramakrishna S 2005 Tissue Engineering 11 101-09
[2] Takahashi Y. and Tabata Y. 2004 J. Biomater. Sci. polymer Edn. 15 41-57
[3] Bhattachar R R, Bhattacharai N, Yi H K, Hwang P H, Cha D L and Kim H Y 2005 Biomaterials 25 2595-2602
[4] Katta P, Alessandro M, Ramsier R D and Chase G G 2004 Nano Lett. 4 2215-8
[5] Kameoka J, Verbridge S S, Liu H, Czaplewski D A and Craighead H G 2004 Nano Lett. 4 2105-8
[6] Wnek G E, Carr M E, Simpson D G and Bowlin G L 2003 Nano Lett. 3 213-6
[7] Baker S C, Atkin N, Gunning P A, Granville N, Wilson K, Wilson D and Southgate, J 2006 Biomaterials 27 3136-46
[8] Rho K S, Jeong L, Lee G, Seo B M, Park Y J, Hong S D, Roh S, Cho J J, Park W H and Min B M 2006 Biomaterials 27 1452-61
[9] Tian F, Cui D, Schwarz H, Estrada G G and Kobayashi H 2006 Toxicology in Vitro. 20 1202-12
[10] Yang F, Murugan R, Wang S, Ramakrishna S 2006 Biomaterials 26 2603-10
[11] Li M, Guo Y, Wei Y, MacDiarmid A G, Lelkes P I 2006 Biomaterials 27 2705-11

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