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Nanofabrication of Microbial Polyester by Electrospinning Promotes Cell Attachment

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Abstract: The biodegradable and biocompatible poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV), a copolymer of microbial polyester, was fabricated as nanofibrous mats by electrospinning. Image analysis of the electrospun nanofibers fabricated from a 2 wt% 2,2,2-trifluoroethanol solution revealed a unimodal distribution pattern of fiber diameters with an observed average diameter of ca. 185 nm. The fiber diameter of electrospun fabrics could be controlled by adjusting the electrospinning parameters, including the solvent composition, concentration, applied voltage, and tip-to-collector distance. Chondrocytes derived from rabbit ear were cultured on a PHBV cast film and an electrospun PHBV nano-fibrous mat. After incubation for 2 h, the percentages of attached chondrocytes on the surfaces of the flat PHBV film and the PHBV nanofibrous mat were 19.0 and 30.1%, respectively. On the surface of the electrospun PHBV fabric, more chondrocytes were attached and appeared to have a much greater spreaded morphology than did that of the flat PHBV cast film in the early culture stage. The electrospun PHBV nanofabric provides an attractive structure for the attachment and growth of chondrocytes as cell culture surfaces for tissue engineering.

Keywords: electrospun nanofiber, PHBV, chondrocyte, cell attachment, tissue engineering.

Introduction

The final goal of tissue engineering should be the functional recovery of damaged tissue *in vivo*, and *in vitro* reconstruction of tissue architecture whilst realizing exquisite tissue-specific functions.[1] One of the key factors of tissue engineering is to create a three-dimensional scaffold with suitable degradation rate. The porous scaffold for tissue engineering should have high porosity to be able to accommodate a large number of cells, as well as large interconnected pores to facilitate a uniform distribution of cells and the diffusion of oxygen and nutrients to them. Typically, biodegradable polymeric scaffolds are fabricated using particulate leaching,[2] gas forming,[3] high pressure gas expansion,[4] phase separation[5] and emulsion freeze-drying methods.[6]

It is well known that extracellular environment influences many aspects of cell behavior such as morphology, functionality and cell-cell interactions.[7] In natural tissues, cells are surrounded by extracellular matrix, which has physical structural features ranging from nanometer scale to micrometer scale. Hence, a nano-structured porous and large surface area is needed as an alternate to natural ECM. To restore native tissue structure as closely as possible, designing of scaffolds is very important. To mimic the natural ECM, many research groups tried to fabricate nanofibrous scaffold by phase separation,[8] self-assembly,[9,10] and electrospinning.[11-16]

The electrospinning method has been actively explored recently for potential applications because that offers ultrafine polymer fiber, high specific surface area and possibility of various modification in spite of simple process.[11] Many

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attempts for biomedical application were reported widely using synthetic and natural polymers as wound dressings,[17] drug delivery system,[18] vascular grafts, and scaffolds for tissue engineering.[7,12,14,15]

Especially for tissue engineering, ultrafine nanofibers have been fabricated from biodegradable natural or synthetic polymers, such as collagen,[12] fibrinogen,[17] silk fibroin,[19] poly(L-lactide)(PLLA),[20] poly(ε-caprolactone)(PCL),[21] poly (lactide-*co*-glycolide)(PLGA).[22] However, nanofiber has not been electrospun using poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)(PHBV) for tissue engineering application. PHBV, a copolymer of microbial polyester, is one of the promising materials for tissue engineering beside being biodegradable, biocompatible and inexpensive exhibit characteristics that are similar to conventional thermoplastics such as polypropylene.[23]

In this study, we prepared biodegradable PHBV nanofibrous mat for cell culture substrates by electrospinning method and chondrocytes' behavior on the nanofibrous mat and flat cast film surface have been investigated.

Experimental

Materials. Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) containing 5 mol% of 3-hydroxyvalerate with 680,000 molecular weight was purchased from Sigma Chemical Co. Chloroform and 2,2,2-trifluoroethanol (TFE) to prepare PHBV solution was purchased from Sigma-Aldrich Chemical and used as received without further purification.

Electrospinning. Electrospinning apparatus used in this study is shown in Figure 1. It consisted of an adjustable, regulated high voltage power supply (up to 40 kV), syringe pump, and collector units. PHBV was dissolved at various concentrations in TFE and chloroform, respectively. The PHBV solution is contained in a glass syringe controlled by syringe pump. A positive high voltage source through a wire was applied at the tip of a syringe needle. In this situation, a strong electric field is generated between PHBV solution and a collector. When the electric field reached a critical value with increasing voltage, mutual charge repulsion overcame the surface tension of polymer solution and electrically charged jet was ejected from the tip of a conical shape as the Taylor cone.[24]

Ultrafine fibers are formed by narrowing of the ejected jet fluid as it undergoes increasing surface charge density due to evaporation of the solvent.[11] The following variables including solution and process parameters such as concentration, applied electric potential, tip-to-collector distance and flow rate have been examined. Electrospun PHBV nanofibrous mat was carefully detached from collector and dried *in vacuo* for 2 days at room temperature to remove solvent molecules completely.

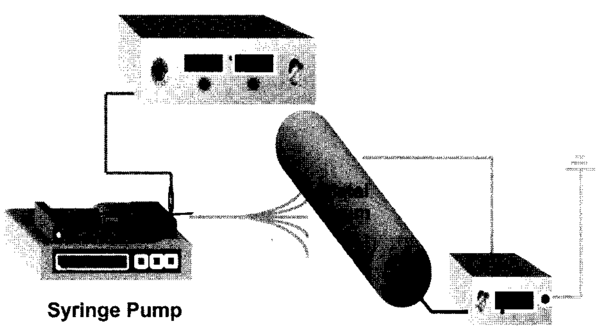

Figure 1. Schematic illustration of electrospinning apparatus used in this study.

Characterization. The diameter and morphology of electrospun nanofibers were determined by field emission scanning electron microscope (FE-SEM) and image analyzer (TDISE V3.1.73). The X-ray measurements were carried out to investigate the effect of electrospinning process on crystallization of PHBV. Electrospun PHBV fabrics were subjected to stress-strain analysis using a universal testing machine under an extension rate of 5 mm/min and 100 N load cell. Specific surface area of electrospun PHBV nanofibrous mat was determined by surface area & pore size analyzer (Nova 2000 & autosorb-1-C).

Cell Culture. Chondrocytes were selected and isolated from rabbit ear of New Zealand White as a model cell type of the anchorage-dependant tissue cells to examine the interaction of nanofibrous mat with cells. Separated chondrocytes were cultivated in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin G-streptomycin. To determine the effect of matrix structure on cell attachment, chondrocytes were evenly seeded at ca. 50,000 cells/cm^2 onto each surface of TCPS culture dish, electrospun PHBV nanofibrous mat and flat cast PHBV film prepared by solution casting method. The percentage of attached cells were determined by measuring lactate dehydrogenase (LDH) activity of cells lysed with Triton X-100.[25] Attached cell morphology was measured by FE-SEM, after 2 and 4 hrs of culture at 37 °C under a humidified atmosphere with 5% CO_2.

Results and Discussion

Fabrication and Characterization of Nanofiber. The PHBV nanofibrous mats were fabricated from PHBV solution of various concentrations in TFE and chloroform, respectively. The concentration of the polymer solution or the corresponding viscosity is one of the most effective parameters to control the fiber morphology. Extensive chain entanglements are necessary to produce electrospun fibers, the consequence being that lower solution concentrations lead to electrospraying rather than electrospinning.[26] From the above reason, the Brookfield viscosities of PHBV solutions in TFE and chloroform were determined to assist in choosing minimum concentrations for electrospinning (data not shown). The results suggest that PHBV begins to electrospin well more than 2 wt% TFE solution and 10 wt% chloroform solution, respectively. Electrospun fiber structures revealed randomly aligned fibers, ranging from 100 to 2,000 nm average diameters. The average diameter of electrospun PHBV fiber decreased with the decrease in solution concentration and the increase in electric potential and tip-to-collector distance.

FE-SEM micrographs of electrospun PHBV nanofibers having minimum fiber diameter produced from 2 wt% TFE and 10 wt% chloroform solutions are shown in Figure 2. The diameter of PHBV nanofibers electrospun from TFE solution is 10 times smaller than that of chloroform solution due to the higher dielectric property and conductivity of TFE than chloroform. Image analysis of the electrospun nanofibers fabricated from 2 wt% PHBV-TFE solution revealed an unimodal distribution of fiber diameters with an observed average diameter of 185 nm approximately (Figure 3). It could be controlled the fiber diameter of electrospun fabrics by adjusting electrospinning parameters, including solvent composition, concentration, applied voltage and tip-to-collector distance.

Figure 4 shows a photograph of an electrospun PHBV large mat for cell culture fabricated from 2 wt% PHBV-TFE solution that is approximately 70 × 70 mm with a thickness of 0.22 mm. Electrospun PHBV fabrics were subjected to stress-strain analysis using a universal testing machine. The tensile modulus and ultimate tensile stress were 110.5 ± 11.3 and 5.1 ± 0.5 MPa, respectively. Considering that the tensile modulus and ultimate tensile stress of human cartilage are 130 and 19 MPa,[27] PHBV nanofibrous mat prepared in this study shows relatively good mechanical properties as cell culture substrates. The X-ray measurements were carried out to investigate the effect of electrospinning process

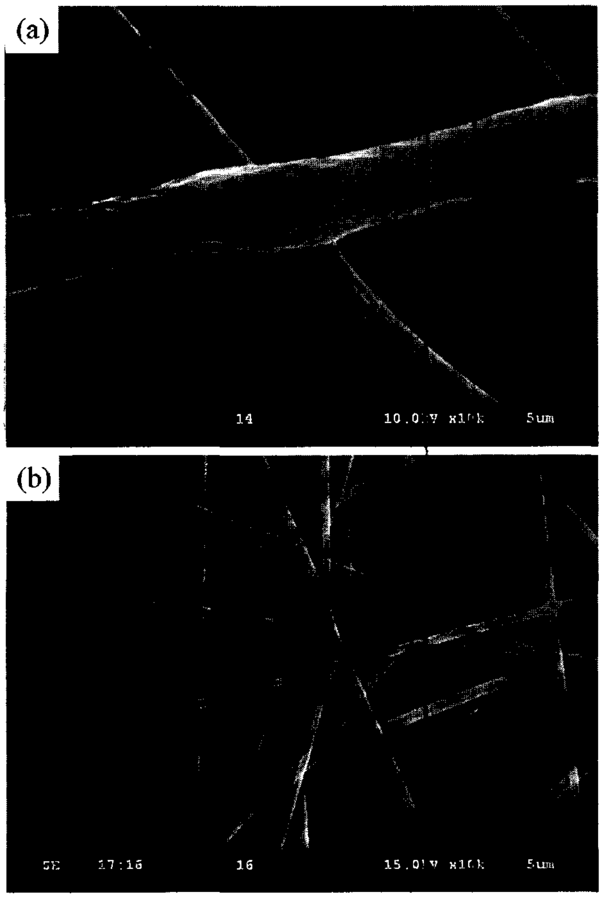

Figure 2. Electrospun PHBV nanofibers. FE-SEM micrographs of PHBV fabric having minimum fiber diameter electrospun from (a) 10 wt% chloroform solution under a voltage of 20 kV, tip-to-collector distance of 15 cm and (b) 2 wt% TFE solution under a voltage of 15 kV, tip-to-collector distance of 21 cm, respectively. In the preparation of these samples, the flow rate for polymer solution was 1 mL/h (Original magnification × 10,000).

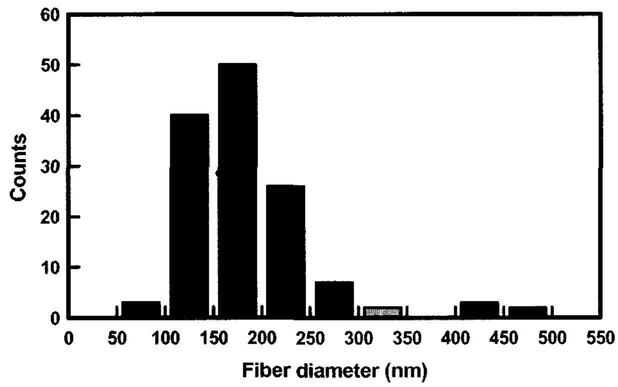

Figure 3. Fiber diameter analysis of PHBV nanofiber electrospun from 2 wt% PHBV-TFE solution. The average fiber diameter was 185 ± 70 nm.

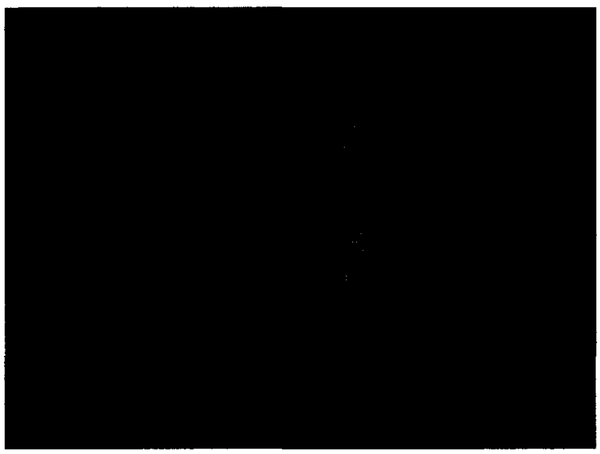

Figure 4. Example of electrospun PHBV nanofibrous mat (70 × 70 × 0.022 mm) obtained from 2 wt% PHBV-TFE solution.

on crystallization of PHBV. Crystallinity of PHBV is not significantly changed by the electrospinning process (data not shown).

For the application of nanofibrous mat in tissue engineering, porosity and surface area are very important factors. Surface area and pore size analysis demonstrated that the porosity of the electrospun PHBV nanofibrous mat from 2 wt% PHBV-TFE solution (Figure 4) was more than 70%. Specific surface area of electrospun PHBV nanofibrous mat (Figure 4) was approximately 140 m^2/g. It is clear that electrospun nanofibrous mat had high porosity and high level of specific surface area.

Cell Culture. To examine the interaction of nanofibrous mat with tissue cells, primary chondrocytes from rabbit ear were selected as a model cell type of the anchorage-dependant tissue cells. The percentage of attached chondrocytes on each surface of tissue culture polystyrene (TCPS) dish, flat PHBV cast film and PHBV nanofibrous mat after 2 h incubation was 29.5, 19.0 and 30.1 %, respectively.

The water contact angle of each PHBV films, PHBV nanofibrous mats and TCPS dishes determined by sessile drop method was 108°, 110° and 65°, respectively. This results indicate that PHBV shows very hydrophobic nature. Nevertheless their hydrophobic nature, more chondrocytes were attached on the PHBV nanofibrous mats than that of PHBV cast film. Chondrocytes adhesion behavior on PHBV nanofibrous mats in early culture stage is similar to TCPS dish which shows slightly hydrophobic (65°). This means that surface topography is very important factor to cell adhesion. Figure 6 shows FE-SEM micrographs of cultured chondrocytes on the surface of PHBV cast film and nanofibrous mat. On the surface of electrospun PHBV fabric, more chondrocytes were attached and showed much more spread morphology than that of PHBV cast film in the early culture stage.

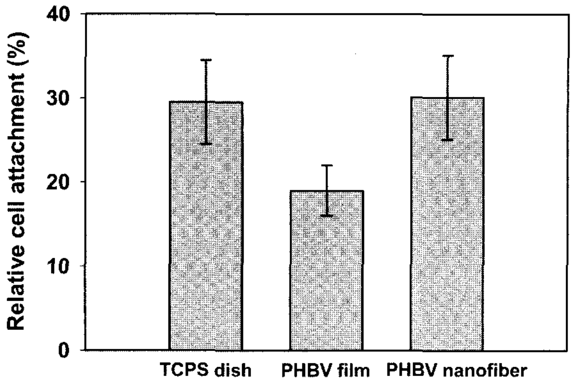

Figure 5. The percentage of attached chondrocytes on each surface of TCPS dish, PHBV cast film, and electrospun PHBV nanofibrous mat after 2 hrs incubation.

Attached and much more spread chondrocytes on nanofibrous mat proliferated more rapidly than that of flat film

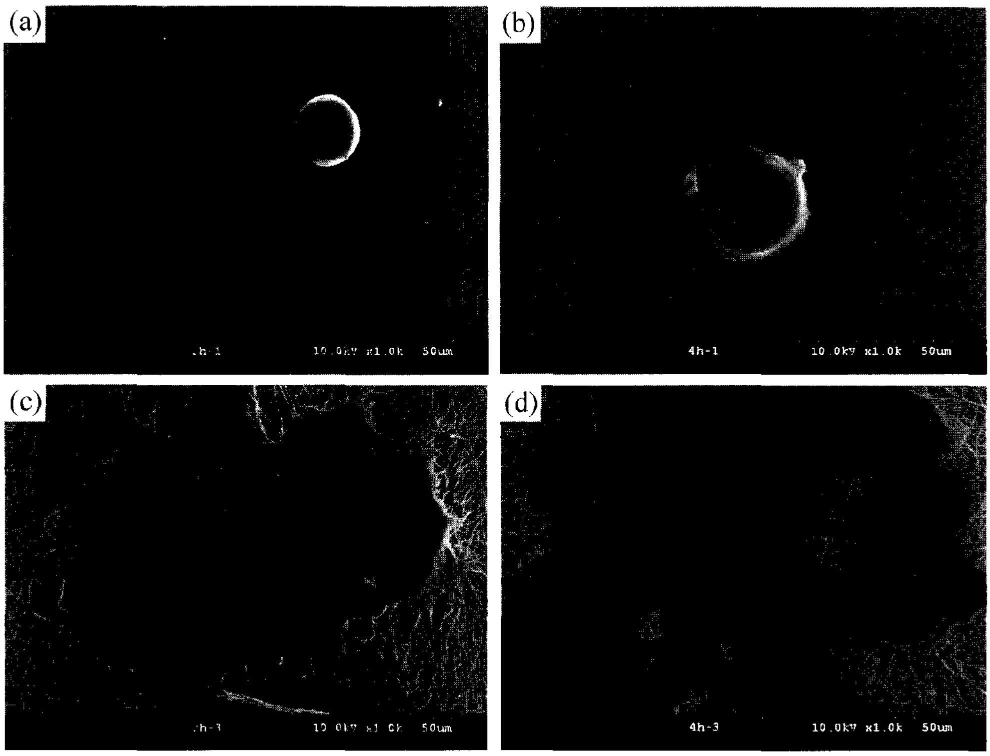

Figure 6. FE-SEM micrographs of chondrocytes attached (a, b) on the surface of PHBV film and (c, d) electrospun nanofibrous mat. Incubation times are 2 and 4 hrs for sample (a, c) and (b, d), respectively (Original magnification × 1,000).

surface (data not shown). This is probably because the nanofibrous structure provides a high level of surface area for cells to attach due to its three dimensional feature. Recently, it was reported that the nano-scale architecture built in three dimensional scaffold using phase-separation technique improved human serum protein adsorption.[28] Improved protein adsorption can enhance cell attachment on the substrate. From the biological standpoint, almost all of the tissue and organs are deposited in nanofibrous structures. Highly porous nanofibrous mat with high surface area offers a bio-mimicking structure during the process of tissue regeneration, more structural space for accommodation and attachment of cells, and enables the efficient exchange of nutrient and metabolic waste.

In this study, we fabricated biodegradable nanofibrous mat with average diameter of 185 nm by electrospinning of PHBV-TFE solution. Electrospun PHBV fabric provided an attractive structure for attachment and spreading of chondrocytes as tissue engineering scaffolds. We believe that the electrospun nanofibrous scaffold holds great promise for cell culture surface and tissue engineering applications.

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