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Evaluation of cell proliferation and differentiation on a poly(propylene fumarate) 3D scaffold treated with functional peptides

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Abstract Synthetic polymers were used to fabricate a three-dimensional (3D) porous scaffold of poly(propylene fumarate)/diethyl fumarate (PPF/DEF). PPF-based materials are good candidates for bone regeneration, because of their non-toxic, biodegradable byproducts, and excellent mechanical properties. However, they exhibit hydrophobic surface properties that have negative effects on cell adhesion. To change the surface properties of a PPF/DEF scaffold, the authors used three peptide modifications (RGD, cyclo RGD, and RGD-KRSR mixture) to the scaffold and tested the effects on MC3T3-E1 pre-osteoblast adhesion, proliferation, and differentiation. The results indicated that peptide modification (particularly the RGD-KRDR mixture) altered the hydrophobic surface properties of the PPF/DEF scaffold, and promoted cell adhesion. Thus, it was suggest that peptide modification is a useful method for changing the properties of the PPF/DEF scaffold surface and may be applicable in bone tissue engineering.

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Introduction

Tissue engineering is an increasingly important research area in the field of tissue restoration and reconstruction. Many researchers have developed three-dimensional (3D) scaffolds that provide adequate mechanical strength and a suitable environment for cell adhesion, proliferation, differentiation, and extracellular matrix (ECM) formation for new tissue. 3D scaffolds also serve to enhance mass transport and guide tissue regeneration. Many synthetic materials have been developed for scaffold fabrication. Synthetic polymers have good biocompatibility and biodegradability properties, as well as suitable mechanical strength. However, their surface properties limit their use, primarily due to a lack of biological recognition sites and typically being intrinsically hydrophobic [1–4]. Because the surface properties of 3D scaffolds play an important role in determining the initial response of living cells to a biomaterial, and in controlling cellular events, such as adhesion, proliferation, migration, and differentiation, researchers have examined different methods of surface modification to enhance adhesion between cells and the scaffold. Notable approaches to improve scaffold surface properties include adsorption of an ECM protein (e.g., fibronectin, vironectin, or collagen), or direct material modification of cell recognition processes using RGD-modified synthetic peptides [2, 4]. ECM proteins were shown to influence integrin expression, subsequently expediting cell adhesion, proliferation, and differentiation. However, use of ECM proteins can produce an undesirable immune response and increases the risk of infection [3]. These problems can be overcome by presenting cell recognition motifs as small immobilized peptides. Peptide sequences present in many ECM proteins and cells bind to RGD through integrin receptors present in cell membranes.

Arg–Gly–Asp (RGD) has been shown to be present in fibronectin, and cell adhesive RGD sites have been identified in ECM proteins, such as vitronectin, fibrinogen, collagen, laminin, osteopontin, and bone sialoprotein. RGD peptides can stimulate cell adhesion and can also elicit-specific cell responses, such as initial adhesion, migration, and differentiation [2]. Also, KRSR (lysine–arginine–serine–arginine), part of the heparin binding site on ECM proteins, interacts with heparin sulfate proteoglycan on the cell membrane and promotes the selective adhesion of osteoblasts. It has been reported that enhanced osteoblast adhesion at higher RGD peptide surface density, and concentrated, surface-bound RGD may be needed for differentiation and mineralization [11–14]. As an indicator of improved cell adhesion, it was measured the peptide surface density of non-modified scaffolds and peptide-modified scaffolds. The authors fabricated 3D scaffolds using the biomaterial PPF/DEF, which retained its hydrophobic properties. It was made three different peptide modifications (RGD, cyclo RGD, and RGD-KRSR mixture) to the scaffolds, and tested for effects on MC3T3-E1 pre-osteoblast adhesion, proliferation, and differentiation processes. The data indicate that peptide modification was effective in changing the cell adhesion properties of the PPF/DEF scaffold.

Methods

Preparation of scaffold materials

Poly(propylene fumarate) was synthesized by condensation reaction according to Gerhart and co-workers [5–7] with the following modifications: 2.4 mol of fumaric acid (Sigma-Aldrich, St. Louis, MO, USA) and 3.0 mol of propylene glycol (KANTO Chemical, Tokyo, Japan) were placed in a triple-necked flask with an overhead electrical stirrer. During synthesis, the temperature of the solution was increased from room temperature to 180 °C. After 18 h, the reaction was terminated. The final product was a clear, light-yellow, very viscous liquid. The measured viscosity of the synthesized PPF, using an advanced rheometric expansion system (Rheometric Scientific, USA), was 738,000 cps at 26 °C. The molecular weight of the synthesized PPF was measured using a gel permeation chromatography (GPC) system. The weight average molecular weight (Mw) and the number average molecular weight (Mn) of the synthesized PPF were 1212 and 295 Daltons, respectively. To use PPF as a liquid polymer for microstereolithography (MSTL), diethyl fumarate (DEF: Tokyo Kasei Kogyo, Tokyo, Japan), a low-viscosity cross-linking agent, was added to reduce viscosity. Finally, the photoinitiator "Irgacure 819" (bis-acylphoshinoxides (BAPO); Ciba, Tokyo, Japan) was added at 1%, and the mixture was stirred continuously for 3 h. Figure 1 depicts the synthesis process of the PPF/DEF photopolymer.

Scaffold design and fabrication

MSTL, developed from a rapid prototyping process, can be used to fabricate free-form 3D microstructures by dividing a desired shape into several slices of a given thickness. During MSTL, an ultraviolet (UV) laser irradiates the free surface of a UV-curable liquid photopolymer, causing it to solidify. A focused laser beam, a few micrometers in diameter, is used to solidify a very small area of liquid photopolymer. To fabricate biodegradable scaffolds, PPF/DEF and PPF/DEF-HA were used as photopolymers. A heating device was installed at the bottom of the resin reservoir to decrease the viscosity of the photopolymer. A continuous-wave A diode laser ($\lambda = 375$ nm; Radius375, Coherent Inc., Santa Clara, CA, USA) and an x–y–z stage (ATS-100; Aerotech, Pittsburgh, PA, USA) with 500 nm resolution were used for scaffold fabrication. Figure 2 shows a schematic diagram of the MSTL system.

Surface modification using peptides

The authors modified the PPF/DEF scaffold surface using three different peptides. The peptides used as potential osteoblast adhesive factors were RGD, cyclo RGD, and an RGD-KRSR mixture. The RGD sequence was based on the integrin binding sequence of fibronectin. The KRSR sequence has been suggested to be a heparin sulfate binding peptide that promotes adhesion of osteoblasts. Cyclic RGD is known to be more stable against enzymatic degradation than linear RGD [8, 9]. The RGD sequence was purchased from Sigma-Aldrich (St. Louis, MO, USA), and cyclo RGD and KRSR sequences were synthesized by PEPTRON (Daejeon, South Korea). To investigate the effect of peptide coating, PPF/DEF 3D scaffolds were linked to carboxylic acid groups at the peptide's N-terminus. For the peptide surface coupling reaction, each scaffold was sterilized in 70% ethanol for 1 h and then rinsed twice with phosphate-buffered saline (PBS). Scaffolds were then immersed for 2 h at room temperature in a reaction solution containing 200 μL of treasyl chloride, 1 mL of pyridine, and 19 mL of tetrahydrofuran. After this gentle reaction period, the scaffolds were again rinsed with PBS. The scaffolds were then immersed in the 0.2 M sodium carbonate buffer (pH 9.6) mixed with one of the three peptides, and shaken for 24 h at room temperature. At the end of the reaction time, the scaffolds were rinsed twice with PBS. After rinsing, the peptide-modified scaffolds

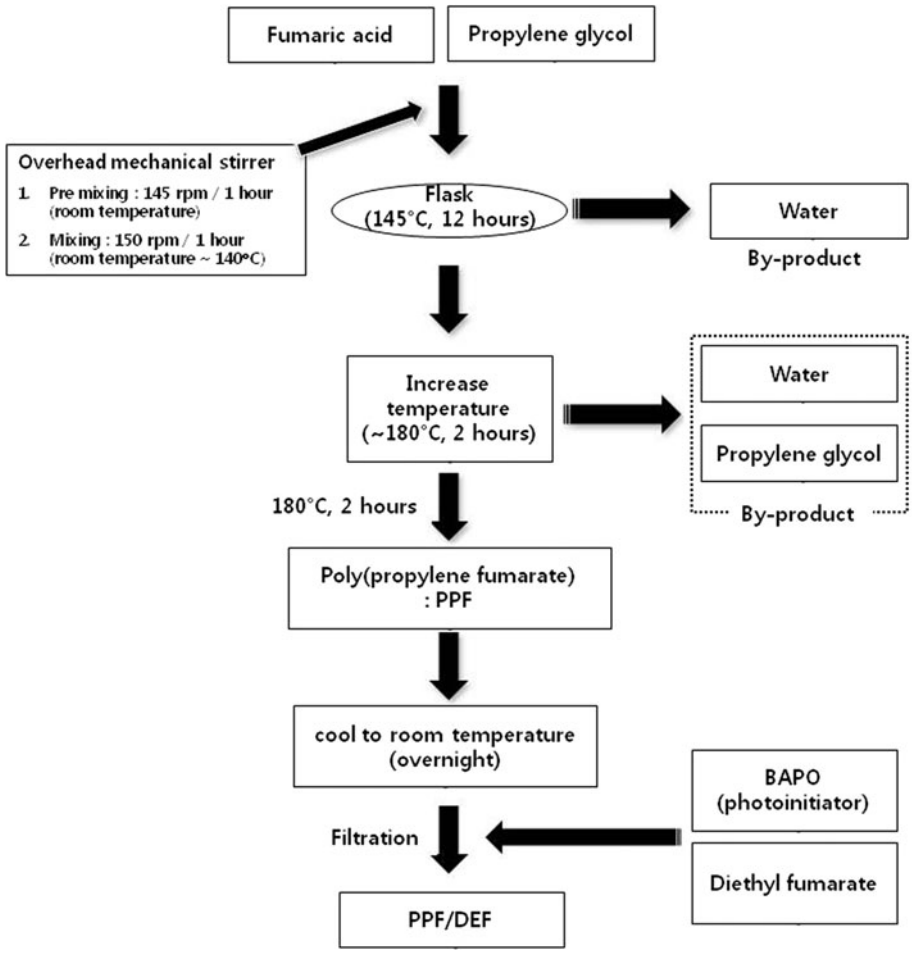

Fig. 1 A diagram for synthesis of PPF/DEF. The diagram describes each course of the experiment in regular sequence and the experimental conditions

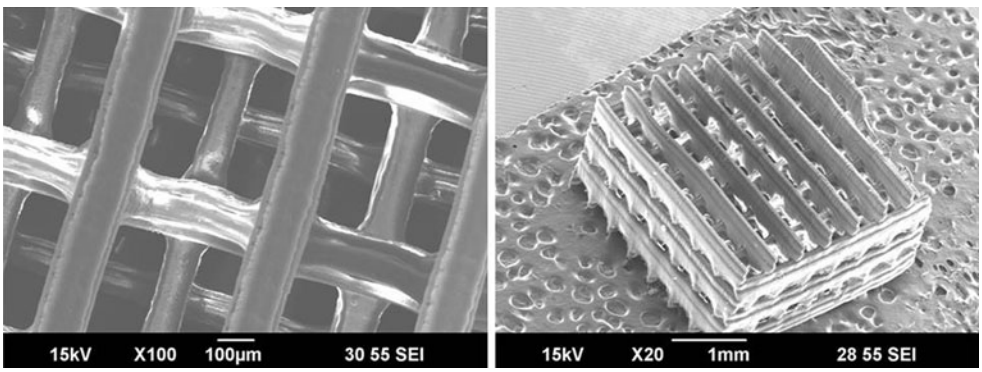

Fig. 2 SEM image of a PPF/DEF 3D scaffold using micro-stereolithography. The specimens were imaged with a JSM-5300 scanning electron microscope

were dried and sterilized by UV irradiation for 2 h before cell seeding.

Seeding of MC3T3-E1 pre-osteoblasts onto scaffold

MC3T3-E1 pre-osteoblasts were purchased from the RIKEN Cell Bank (Tsukuba, Japan). The cells were cultured at 37 °C in a 5% CO_2 humidified atmosphere in α-MEM medium containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco). The medium was changed every 2-3 days. When the cells reached confluence, they were removed from the culture dish using 0.25% trypsin-EDTA (Sigma-Aldrich), centrifuged, and

re-suspended in the α-MEM medium. For cell seeding onto the scaffolds, pre-wetted scaffolds were placed on a plate, then 10 μL (about 1×10^5 cells) of the modifying cell suspension was applied. The cells were allowed to adhere to the scaffolds for 2 h and then the scaffolds were washed twice with PBS to remove any unbound cells. The scaffolds were then transferred to 24-well plates (BD Falcon; BD Biosciences, Boston, MA, USA), and 1000 μL of medium was added to each plate. The scaffolds were maintained at 37 °C in a 5% CO_2 humidified atmosphere. The medium was changed every 2-3 days.

Cell attachment and viability assay

Cell attachment and viable cell levels were determined using the cell counting kit-8 solution (CCK-8, Dojindo, Japan), which generates an orange formazan product by cellular dehydrogenases. After incubating the cells with CCK-8 solution for 2 h, 100 μL of each sample was transferred to a 96-well plate, and measured at 450 nm with a micro-plate reader. Cell number was calculated from a calibration curve obtained from wells that contained a known number of viable cells [7].

Osteoblast differentiation

In order to assess the effect of the modified peptide treatment, osteoblast differentiation was measured. First, peptide-modified scaffolds were washed with PBS to remove unbound peptide. Next, MC3T3-E1 pre-osteoblasts (about 1×10^5 cells) were seeded onto the scaffold in serum-free medium and allowed to attach for 2 h. Cells were allowed to grow for 1 week before the culture medium was replaced with an osteogenic medium (α-MEM medium containing 10 mM of sodium glycerophosphate and 50 μg/mL of ascorbic acid). The cells were grown for an additional 3-14 days. The osteogenic medium was replaced every 3 days. After incubation, the scaffolds were washed three times with PBS to remove any remaining cell culture medium, and stored at −80 °C until assayed.

Alkaline phosphatase assay

Alkaline phosphatase (ALP) activity was used to scale osteoblastic differentiation. To prepare cell lysates, samples were soaked in 300 μL lysis buffer and sonicated in an ice bath for 1 min. To quantify cell lysates, we used a Quant-iT^{TM} protein assay kit (Invitrogen, USA). To estimate ALP activity, cell lysates were incubated in sodium ρ-nitrophenyl-2-phosphate at 37 °C for 1 h. After neutralizing the reaction with 1 M NaOH, the amount of ρ-nitrophenol released in solution was measured by light absorbance at 405 nm.

Results and discussion

Scaffold fabrication

A 3D scaffold was successfully fabricated using synthesized PPF/DEF at a ratio of 70:30, with a laser feed rate of 55 mm/min at a power of 280 mW. Under these conditions, the 3D scaffold consisted of a staggered arrangement of lines. A single layer had a thickness of 215 μm. Two layers were stacked together to fabricate the line structures, so final pore height was 430 μm. Ultimately, 12 layers were stacked together, producing a final scaffold height of 2.58 mm. After the final fabrication phase, the structures were cleaned with isopropyl alcohol in an ultrasonic cleaner. Figure 2 shows an SEM image of a fabricated scaffold. The lattice structure had a line width of 130-160 μm and a pore size of 330-360 μm. Although the scaffolds shrank 25% post curing, the direction of shrinkage was isotropic. Thus, the authors can predict and compensate for the shrinkage. The process resulted in highly accurate scaffold fabrication, offering the possibility of realizing a standardized 3D cell culture environment [1, 10]. The authors sterilized these scaffolds in 70% ethanol and rinsed with PBS. After this process, the scaffolds were looking normal with original shape.

Cell proliferation analysis with the peptides

The authors evaluated the initial adhesion of MC3T3-E1 pre-osteoblasts on PPF/DEF scaffolds using the CCK-8 assay for 1 day, and estimated the proliferation of cells at 3 days, 1 and 2 weeks. Initial cell adhesion and proliferation increased on peptide-modified PPF/DEF scaffolds, in comparison with non-modified scaffolds (Fig. 3). Modification using the RGD-KRSR mixture was significantly more effective in initial adhesion and proliferation than other peptide modifications. It is generally thought that KRSR present in bone is related to adhesive proteins and enhances proteoglycan-mediated osteoblast adhesion. The results indicate that KRSR promoted adhesion of MC3T3-E1 pre-osteoblasts, and scaffolds modified with an RGD-KRSR mixture may be good candidates for bone tissue engineering.

Analysis of peptide concentration on cell proliferation

The authors assessed the effect of RGD concentration on cell proliferation processes using unmodified PPF/DEF scaffolds, and scaffolds modified using 5 and 500 μg RGD peptide solutions. MC3T3-E1 pre-osteoblasts were seeded on the three scaffold types and cells were cultivated for 1, 3, 7, and 14 days. For each time period the authors evaluated cell proliferation using the CCK-8 assay kit.

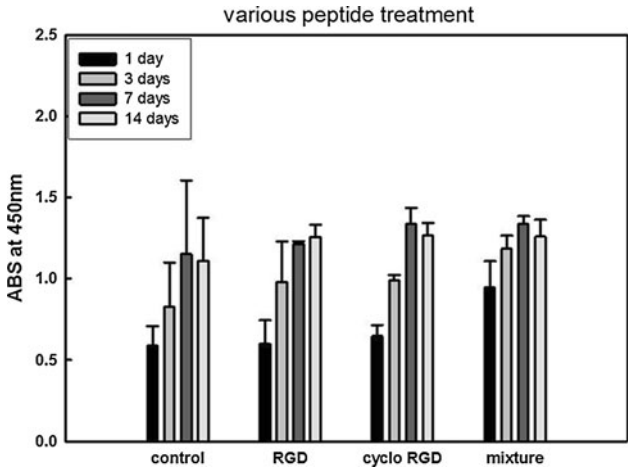

Fig. 3 Proliferation of MC3T3-E1 pre-osteoblast cells on a PPF/DEF scaffold modified with various peptide formulations. Initial adhesion of MC3T3-E1 preosteoblast on PPF/DEF evaluated by the CCK-8 assay for 1 day, and estimated the proliferation of cells at 3, 7, and 14 days

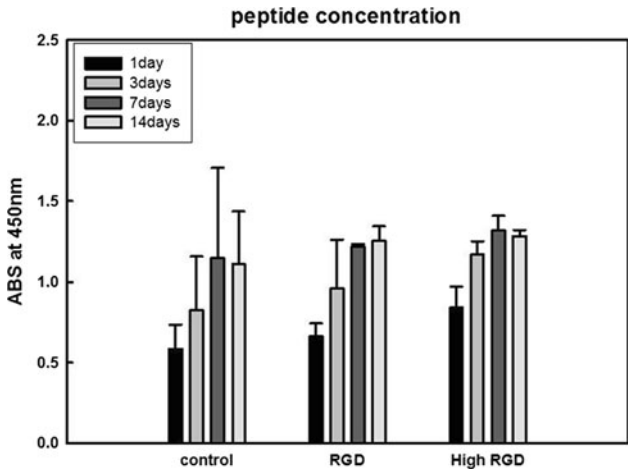

Fig. 4 Proliferation of MC3T3-E1 pre-osteoblast cells in a PPF/DEF scaffold varying a peptide concentration. PPF/DEF scaffolds were modified using 5 and 500 μg RGD peptide solutions and MC3T3-E1 pre-osteoblasts were seeded on three scaffold types (Control: non modified, RGD: 5 μg RGD peptide, High RGD: 500 μg RGD peptide)

RGD-modified scaffolds exhibited increased initial cell attachment and proliferation, in comparison with non-modified scaffolds (Fig. 4), with the high-concentration solution exhibiting the largest initial adhesion and proliferation response. However, cell proliferation after 7 days of cultivation did not increase, regardless of peptide mixture or concentration (Figs. 3, 4), likely due to cell saturation in the scaffolds.

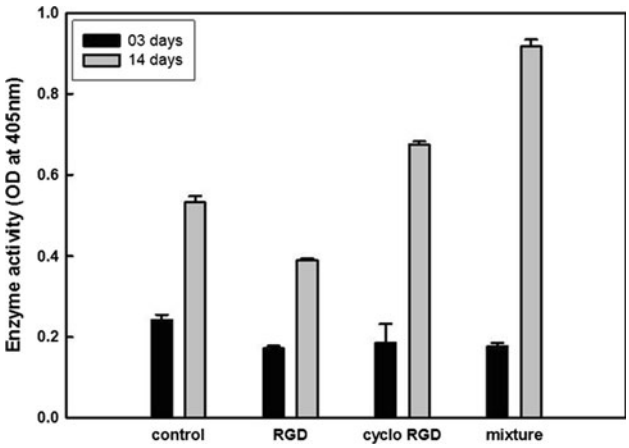

Fig. 5 Differentiation of MC3T3-E1 pre-osteoblast cells on a PPF/DEF scaffold modified with various peptide formulations. ALP activity, an indication of bone cell differentiation, was measured for different peptide mixtures after 3 and 14 days

Effect of various peptides on cell differentiation

The authors measured ALP activity, an indication of bone cell differentiation, for different peptide mixtures after 3 and 14 days of cultivation for unmodified and modified scaffold types. The initial (3 day) cell differentiation activity was low in the modified scaffolds (compared with unmodified), though at 14 days enzyme activity was highest in the RGD-KRSR modified scaffolds (Fig. 5). From these results, it was conclude that the RGD-KRSR mixture peptide treatment was most effective in cell differentiation.

Conclusions

The authors fabricated a 3D scaffold using PPF/DEF, suitable for use in bone tissue engineering. The PPF/DEF scaffolds have sufficient mechanical stability and are non-toxic, but their hydrophobic surface properties have negative effects on cell adhesion. It was modified the scaffold surface with various peptides to change the surface properties. MC3T3-E1 pre-osteoblast adhesion was increased in PPF/DEF scaffolds modified with any of the peptide solutions. The RGD-KRSR mixture produced the largest increase. Also, proliferation and differentiation of MC3T3-E1 pre-osteoblasts increased in the peptide-modified scaffolds. Peptides at higher concentrations increased initial cell adhesion and proliferation. These results indicate that peptide-modified PPF/DEF scaffolds do enhance MC3T3-E1 pre-osteoblast behavior, and their use is appropriate in biomedical applications.

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