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The interaction between bone marrow stromal cells and RGD modified three dimensional porous polycaprolactone scaffolds

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Abstract

We previously established a simple method to immobilize the Arg-Gly-Asp (RGD) peptide on polycaprolactone (PCL) two-dimensional film surfaces that significantly improved bone marrow stromal cell (BMSC) adhesion to these films. The current work extends this modification strategy to three-dimensional (3D) PCL scaffolds to investigate BMSCs attachment, cellular distribution and cellularity, signal transduction and survival on the modified PCL scaffold compared to those on the untreated ones. The results demonstrated that treatment of 3D PCL scaffold surfaces with 1,6-hexanediamine introduced the amino functional groups onto the porous PCL scaffold homogeneously as detected by a ninhydrin staining method. Followed by the cross-linking reaction, RGDC peptide was successfully immobilized on the surface of PCL scaffold. Although the static seeding method used in this study caused heterogeneous cell distribution, the RGD modified PCL scaffold still demonstrated the improved BMSC attachment and cellular distribution in the scaffold. More importantly, the integrin-mediated signal transduction FAK-PI3K-Akt pathway was significantly up-regulated by RGD modification and a subsequent increase in cell survival and growth was found in the modified scaffold. The present study introduces an easy method to immobilize RGD peptide on the 3D porous PCL scaffold and provides further evidence that modification of 3D PCL scaffolds with RGD peptides elicits specific cellular responses and improves the final cell-biomaterial interaction.

1. Introduction

Scaffolds with designed microstructures provide structural support and adequate mass transport to guide the tissue regeneration [1]. In tissue engineering, the scaffold also serves as a three-dimensional template for cell adhesion, proliferation, differentiation, extracellular

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matrix (ECM) formation and provides an appropriate environment for the newly formed tissue. Generally, the ideal scaffold for tissue regeneration should possess the properties of good biocompatibility, biodegradability with controllable degradation kinetics, easy fabrication and sufficient mechanical properties.

The synthetic, biodegradable poly(ϵ -caprolactone) (PCL) has received considerable attention for tissue engineering, especially for bone and cartilage regeneration since it has appropriate mechanical properties, is less expensive and is easily fabricated into complicated shapes with appropriate porosity [2–4]. The major limitation of PCL, however, is that it does not provide a desired environment for cell adhesion due to the lack of biological recognition sites and its intrinsic hydrophobicity [5,6]. Since cell adherence to the substrate is an early fundamental step leading to positive cell-substrate interaction enabling cell proliferation, migration and differentiation on the surface of materials [7,8], many strategies have been developed to modify PCL surfaces to improve the cell-substrate interaction and therefore increase its potential application.

One of the most commonly used surface modification techniques for biopolymer substrate surfaces is attachment of extracellular matrix (ECM) components or their derived synthetic peptides. Since the derived peptides possess higher stability, easier characterization and cost effectiveness, it has more potential in material surface modification [9]. Arg-Gly-Asp (RGD) is the most effective and often-employed peptide sequence for stimulating cell adhesion on synthetic material surfaces. This peptide sequence is present in many ECM proteins and can interact with the integrin receptors at the focal adhesion points. Once the RGD sequence is recognized by and binds to integrins, it will initiate an integrin-mediated cell adhesion process and activate signal transduction between the cell and ECM, thus influencing cell behavior on the substrate including proliferation, differentiation, apoptosis, survival and migration [10,11].

We previously established a simple method to immobilize Arg-Gly-Asp (RGD) peptide on PCL two-dimensional film surfaces and demonstrated that rat bone marrow stromal cell (BMSC) adhesion was significantly improved on the RGD modified PCL films in a serum-free culture condition [12]. Since three-dimensional scaffolds have larger surface area and highly interconnected porous structure with suitable porosity and pore size, modification of scaffold surface to improve the interaction between cell and scaffold surface would have more potential in tissue engineering. In the present work, we introduced a similar strategy to immobilize RGDC peptide on 3D PCL scaffold surfaces and tested BMSCs behaviors including attachment, cellular distribution, signal transduction and survival on the modified PCL scaffolds.

2. Materials and Methods

2.1. PCL films preparation and aminolysis

A procedure described previously was followed to prepare PCL films as well as to create the amine groups on the surface of PCL films [12,13]. Briefly, PCL films were prepared by dissolving PCL (CAPA 6501, Solvay Caprolactones, Warrington, Cheshire, UK; MW: 50,000 Da) powder in chloroform (Sigma) (10% w/v). PCL solution was then pipetted onto

glass coverslips and the solvent was allowed to evaporate slowly overnight and further dried under vacuum. Then the films were immersed into a 10% w/v solution of 1,6-hexanediamine (Sigma) prepared in isopropanol at 37°C for 1 h. After the exposure, the PCL films were thoroughly washed in deionized distilled water and were dried under vacuum at room temperature. The successful aminolysis of PCL films was detected by qualitatively ninhydrin staining or quantitatively ninhydrin analysis and as well as X-ray photoelectron spectroscopy (XPS).

The ninhydrin analysis method was employed to quantitatively detect the amount of NH₂ groups on the film [13,14] with minor modification. Briefly, the film was immersed in 1.0 mol/L ninhydrin/ethanol solution for 1 min and then was placed into a glass tube, followed by heating at 75 °C for 15 min in a water bath to accelerate the reaction between ninhydrin and amino groups on PCL film. After the adsorbed ethanol had evaporated, 2 mL of chloroform was added into the tube to dissolve the film. Another 2 mL of 2-propanol was added to stabilize the blue compound. The final solution was placed in a silica cuvette and the absorbance measured at 560 nm. A standard curve was prepared using solutions of known 1,6-hexanediamine concentrations.

The ninhydrin staining was developed from the ninhydrin assay in order to check the distribution of amine groups on the PCL films. Briefly, the films or scaffolds was immersed in 1.0 mol/L ninhydrin/ethanol solution for 1 min, then was transferred to glass Petri dishes with cover and incubated at 37 °C for 15 min to develop the color. Films were photographed with a Nikon digital camera.

The XPS analyses were performed on a Perkin Elmer model PHI 5400 XPS instrument with a monochromatized Al anode operated at 15 kV and 10 mA. The pressure during analysis was maintained at about 10⁻⁹ Torr. Survey spectra were obtained using a passing energy of 160 eV. High-resolution spectra were obtained using a passing energy of 20 eV. All binding energies were referenced to carbon C1s peaks at 284.0 eV.

2.2. PCL Scaffold Design, Fabrication

Three-dimensional (3D)-designed scaffolds (3mm height, 6.85mm diameter, 1mm spherical pores) were designed using custom written Interactive Data LanguageTM programs (IDL; Research Systems, Inc., Boulder, CO). Scaffolds were designed with 100% interconnection. Inverse wax molds of the designs were processed on a Solidscape T66 3D printer (SolidScape Inc., Merrimack, NH). Scaffolds were made by pressing inverse wax molds directly into melted polycaprolactone. Briefly, PCL pellets were placed into a Teflon mold, and melted (115°C, 1 Torr, 120 min). After melting and air bubble removal, the Teflon mold was pulled from the oven, and allowed to cool for 300 seconds at room temperature until it reached 80°C (just below the melting temperature of the wax molds). At this time, inverse wax molds were pressed into the melted PCL, and the entire construct was cooled overnight. The wax was then dissolved from the PCL using 100% EtOH.

Due to the less consistent size of the scaffold build by the methods abovementioned, for the quantitative data including cell attachment and proliferation in this study, the designed scaffolds were constructed by a laser sintering (LS) machine, Formiga 100TM (EOS, Munich,

Germany) in STL file format. Scaffolds were built layer-by-layer using a powder layer thickness of 100 μm . After LS processing was completed, the scaffolds were allowed to cool inside the machine process chamber for approximately 1 h and were then removed from the part bed. Excess powder surrounding the scaffolds was brushed off and the scaffolds were finally cleaned by blowing compressed air and physically removing unsintered powder from the scaffold interstices by insertion of a 1 mm diameter wire.

2. 3. PCL scaffolds aminolysis and RGDC immobilization

For the modification of 3D scaffolds, all the procedures were undertaken in the vacuum chamber to guarantee the solution penetrated through the whole scaffold and reacted fully. A similar aminolysis procedure as that on the PCL films was followed. The distribution of the created amine groups in the scaffold was detected by the ninhydrin staining. The aminated PCL scaffolds were pre-washed with activation buffer 3 times (0.1 M phosphate buffered saline contained 0.15 M NaCl, pH 7.2). For conjugation of RGDC peptides to the surface of aminated PCL scaffold, the heterobifunctional crosslinker sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) (Pierce Biotechnology, Rockford, IL) [15] (Hermanson et al., 1996) was used. 500 μL (4 mg/ml) of the sulfo-SMCC solution was pipetted onto aminated PCL scaffolds and incubated for 1 h at room temperature, followed by washing with conjugation buffer (activation buffer contained 0.1 M EDTA, pH 7.0). The RGDC peptide (Bachem California, Inc., Torrance, CA) was dissolved at a concentration of 0.125 mg/ml in conjugation buffer. 500 μL of the peptide solution was applied onto the sulfo-SMCC-treated PCL scaffold and incubated overnight at 4 $^{\circ}\text{C}$. Peptide conjugated PCL scaffolds were washed thoroughly with conjugation buffer twice and PBS for 3 times and dried under vacuum at room temperature. For all subsequent experiments, the following PCL samples were created and used: untreated PCL (PCL) and RGD-modified PCL (PCL-RGD).

The peptide surface density/amount as well as the homogeneity of the immobilized peptides on the PCL scaffolds was measured by amino acid analysis using HPLC. For the homogeneity measurement, the peptide content of upper and lower half of the scaffold was measured. we did not cut the scaffold into more pieces due to the measurement threshold of HPLC method. Briefly, the whole scaffold or the upper and lower half scaffolds were hydrolyzed in 6N HCl aqueous solution at 100 $^{\circ}\text{C}$ for 24 h. The hydrolyzed samples were analyzed on a Hewlett Packard AminoQuant System.

2.4. Cell culture study

2.4.1. Rat bone marrow stromal cell culture—Marrow donor animals (7- to 8-week-old Spargue-Dawley rats) were obtained from Charles River Laboratories (Wilmington, MA). Animals were caged under standard conditions and fed a laboratory diet and tap water ad libitum. Care and use of the laboratory animals followed the guidelines established by the University of Michigan Committee for the Use and Care of Animals. The femora and tibia of donor animals were excised, and adherent tissue was dissected. The marrow was expelled using a flushing stream of Hank's buffer salt solution (Gibco, Grand Island, NY,) delivered from a 10-ml syringe fitted with a 18-gauge needle. The cell suspension was centrifuged at 1000 rpm for 5 min at room temperature. A single-cell suspension was obtained by gentle

agitation through the syringe with 18 and 21-gauge needles. After centrifugation, bone marrow cells were re-suspended in alpha modified Eagle's medium (α -MEM; Gibco) supplemented with 10% fetal calf serum (FCS; Gibco), seeded onto T75 cell flasks and cultured in a humid atmosphere containing 5% CO₂ incubator at 37°C. On day 4, the medium was replaced with fresh medium. Bone marrow stromal cells at 2 to 4 passages were used in the experiments.

2.4.2. Cell seeding and distribution—The scaffolds were transferred to the sterile customized Teflon plate and sterilized in sterile 70% EtOH for 30 min. After aspirating the EtOH, the films were sterilized under UV light for another 30 min. The scaffolds were washed by PBS twice followed by α -MEM medium once (30 min each). Cell suspension was added drop-wise on the top of the scaffold at a density of 300,000 cells/scaffold in 30 μ l culture medium (α -MEM medium with 10% FBS) to allow for the initial cell attachment. 20 min later, 120 μ l of culture medium was carefully added to the base of the Teflon plate to cover the scaffolds. BMSCs distribution through the scaffolds was detected by H&E staining. Briefly, 4 h after cell seeding, the scaffolds were washed with PBS twice, and fixed in 4% PFA for 30 min at room temperature. The scaffolds were embedded in OCT and sectioned serially from top to the bottom using cyrosection. The slides were stained by Hematoxylin & Eosin staining.

2.4.3. Cell attachment and survival—After 4 h (for cell attachment) or 3 days (for cell survival and growth) of culture, the culture medium was aspirated and washed with PBS three times to remove non-adherent cells, and cell attachment was measured by a colorimetric method using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonphenyl)-2H-tetrazolium (MTS tetrazolium) compound (Cell-Titer 96 Aqueous One Solution Cell Proliferation Assay; Promega, Madison, WI). The amount of soluble formazan product produced by the reduction of MTS by metabolically active cells was measured by a 96-well spectrophotometer at 490 nm absorbance.

2.4.4. Focal adhesion kinase, Ark phosphorylation—Immunoblotting of phosphorylated-focal adhesion kinase (p-FAK) and phosphorylated Akt (p-Akt) activation was performed after 4 h of cell seeding. The detached cells were collected first. The attached cells were washed twice with ice-cold PBS, and scaffolds were cut into small pieces using a blade and pooled with the un-attached cells. After centrifugation, lysis buffer (25mM Hepes, 300 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.1% Triton X-100, 0.5 mM DTT, 20 mM β -glycerophosphate, 0.1 mM Na₃VO₄ and complete proteanase inhibitor cocktail tablet) was added to the pellet containing the crude cell extracts and the remained materials, followed by experiencing three times of frozen-thaw cycle and then incubated on ice for 15 min. Following centrifugation, the supernatants were saved and used for analyses. Protein assays were performed on each sample to normalized lysate content in each well. Equal amounts of protein were boiled for 5 min in Laemmli buffer and separated by SDS-PAGE on a 7.5 % gel, transferred to nitrocellulose membranes. After blocking with 5% w/v bovine serum albumin (BSA) solution in TBS-T (10 mM Tris, 150 mM NaCl, 0.1% v/v Tween 20), the blots were exposed to rabbit-anti-phosphorylated FAK (pY397) (Biosource, 1:1000), rabbit-anti pAKT1/2/3 (Ser-473) (Santa Cruze, 1:200) or mouse-anti beta-actin antibody (Biorad,

1:5000) and followed by reacting with HRP conjugated-secondary antibody. Immunoreactive bands were visualized using enhanced chemiluminescence detection.

2.5. Statistical Analysis

Data were expressed as mean of percentage $\pm$ SEM and were analyzed by t-test to determine the difference between each group at the same time point. A P value of <0.05 was considered statistically significant.

3. Results

When the PCL films were treated with 1,6-hexanediamine at 37 °C, free amino groups were introduced. The quantitative analysis of NH_2 using the ninhydrin quantitative assay shows that NH_2 density on the PCL film was $\sim 0.21 \mu\text{mol}/\text{cm}^2$ calculated by a standard curve using a known-concentration of 1,6-hexanediamine. XPS data demonstrates a significant N1s peak at 398 eV present on the PCL films after aminolysis. Ninhydrin staining demonstrates that after aminolysis, the PCL films were stained purple with homogenous distribution (Figure 1).

Because ninhydrin staining can demonstrate amino groups present on the 2D film after aminolysis, we therefore determined whether amine group was introduced onto the PCL scaffold using this staining method. Figure 2 shows that the scaffold was stained purple after reacting with 1,6-hexanediamine at 37°C for 1 h in the vacuum chamber. To know whether aminolysis reacted homogeneously in PCL scaffold, the scaffold was cut horizontally into 3 pieces. As shown in Figure 2 c–e, the upper, middle and lower layers of the scaffold were stained purple throughout.

Amino acid analysis shows that the amount of immobilized RGDC peptide on PCL scaffold was $0.0117 \pm 0.0023 \mu\text{g}/\text{mg}$, while the resulting immobilized RGDC density was $\sim 6.35 \times 10^{-10} \text{mol}/\text{cm}^2$. There was no difference of the amount of immobilized RGDC peptide on the upper and lower half of the scaffold (Figure 3).

At 4 h after cell seeding onto the scaffold, the cell distribution and cellularity was detected by H&E staining. The cell distribution at a given distance from the scaffold surface was determined with light microscope taken of serial cross-sections in the seeded scaffolds. Generally, the cells distributed heterogeneously within the PCL scaffold with or without RGD modification, with most of the cells distributed in the upper 1/3 scaffold (within 1 mm from the top). However, more cells attached and cell distribution was better on the PCL scaffold after RGD modification compared to the untreated PCL scaffold (Figure 4).

To determine whether the improved cell adhesion on the RGD modified PCL was through the specific integrin-mediated mechanism, we analyzed levels of FAK phosphorylation as a marker of integrin-mediated signaling and its downstream signaling Akt phosphorylation. Both phosphorylation of FAK and Akt on the untreated PCL scaffold were very weak. However, pFAK and pAKT expression were significantly increased on the RGD modified PCL scaffold compared to those on the untreated PCL (Figure 5A and 5B).

Cell attachment on the scaffolds with or without RGD modification was also quantified by MTS assay. Figure 6A shows that the adhered BMSCs cell numbers on the RGD modified PCL scaffold was 1.52 times higher than those on the untreated PCL films at 4 h time point. Lastly, Figure 6B shows that BMSC growth increased significantly at 3 days on the RGD-modified PCL films, which was 3.45 times higher, compared with those on untreated PCL scaffolds.

4. Discussion

Treatment of PCL surface with 1,6-hexanediamine introduced the amino functional groups onto PCL porous scaffolds homogenously as detected by the ninhydrin staining method. Followed by the cross-linking reaction, RGDC peptide was successfully immobilized on the surface of PCL scaffold. Rat bone marrow stromal cell attachment, cell distribution through the entire scaffold as well as cell proliferation was significantly improved in the RGD modified scaffold. In particular, the integrin-mediated cell signal pathway focal kinase (FAK)-PI3K-Akt pathway was significantly activated on the PCL scaffold with RGD modification.

Due to the lack of functional groups on the PCL surface for the peptide conjugation, the aminolysis method was first used to create the functional group (NH_2) on PCL surface. Several ways have been used to introduce the amine group on the polyester polymer surface including plasma treatment, aminolysis, co-polymerization or immobilization of a polymer with NH_2 -containing material such as chitosan [13, 16,17]. Among them, the aminolysis method may be the most applicable and easiest way to create the functional NH_2 group on the three-dimensional porous PCL scaffold surface since the reaction mainly relies on the extent of contact between the diamine molecules. Thus, the method may be implemented by fluid flow through the scaffold, and will be sufficient if the pores are interconnected.

The aminolysis method was developed based on the ester groups on the PCL backbone reacting with one amino group of 1, 6-hexanediamine to form a covalent bond while the other amino group remains free. As shown in this study as well as previous reports [12,14], the presence of NH_2 group on the PCL surface was confirmed by the quantitative ninhydrin assay and XPS characterization. Our present work also developed a qualitative staining method to detect the presence of NH_2 groups, named as “ninhydrin staining”. This staining relies on the same principle as the ninhydrin assay in which ninhydrin binds to amine groups present in the surface of the polymer producing a purple pigment [14]. This staining method made it possible to detect whether the aminolysis method can also be used in creating NH_2 group in the surface of three-dimensional scaffold and whether the created NH_2 functional group homogenously distributed inside of the scaffolds. Our data illustrated that after aminolysis, the whole PCL scaffold as well as the dissected scaffold was stained dark purple thoroughly, demonstrating the NH_2 group was successfully introduced onto the PCL scaffold. It is important to note that in the 2D films ($\sim 150\ \mu\text{m}$ in depth), the purple stain did not go through the entire film, however, in the 3D scaffold, the NH_2 purple stain seemed deeper than $150\ \mu\text{m}$ since the stain of scaffold walls was positive along the z-direction. Zhu et al reported that the aminolysis occurred mainly on the surface of PCL two-dimensional film no more than $50\ \mu\text{m}$ in z-direction [13]. The discrepancy of reaction depth of the

aminolysis method in 2D film and 3D porous scaffold may be caused by the fact that in this study, the structure of tested scaffold is inter-connected with high porosity, which is much easier for the solution to penetrate and react with material surface. Furthermore, the reaction condition is also slightly different in that all the procedure for the 3D scaffold was performed under vacuum chamber and the vacuum pressure also facilitated the 1,6-hexanediamine solution to penetrate much deeper in PCL polymer.

RGDC peptide was subsequently immobilized on the aminolyzed PCL scaffold cross-linked by Sulfo-SMCC. Sulfo-SMCC is a heterobifunctional crosslinker which specifically attaches the NH_2 group on the scaffold surface with one arm and the peptide's Cys residue at the C-terminus on the RGDC peptide with the other arm. The successful immobilization of RGDC peptide on PCL scaffold was confirmed by amino acid quantification. The measured density of RGDC immobilized on PCL scaffold was appropriately $6.35 \times 10^{-10} \text{ mol/cm}^2$, which was much higher than the proposed peptide density of $1 \times 10^{-14} \text{ mol/cm}^2$ for supporting cell adhesion and focal adhesion formation [18]. Due to the evidence there was no significant difference of the amount of RGDC peptide between in the upper and lower half scaffold, it could be assumed that the modification procedure that occurred in the PCL scaffold was homogeneous. Therefore, our current study developed a feasible method to modify PCL porous scaffold homogeneously with RGDC peptide.

Bone marrow stromal cells (BMSCs) are attractive for the bone tissue engineering due to their convenient harvesting, easy transfection with exogenous genes and multi-lineage differentiating potential [19,20]. We first investigated the BMSCs cellularity as well as cell distribution at 4 hrs after cell seeding on the PCL scaffold with or without RGD modification. It was found that cells distributed less homogenous within the scaffold, with most of the cells appeared in the upper 1/3 scaffold on both the untreated and RGD modified scaffolds. This may be due to the static cell seeding method used in this study. Several lines of evidence have shown that static seeding yields low seeding efficiencies and poor cell distribution throughout the scaffold porous network [21,22]. Nevertheless, our observation still showed more cells attached on the PCL scaffold after RGD modification than that on the untreated scaffold. In addition, our quantitative data of cell attachment also verified that the cell number attached on the RGD-modified PCL scaffold was significantly higher than that on the untreated ones. These qualitative and quantitative data demonstrated that BMSC attachment on the PCL scaffold could be improved by RGD modification. It is important to note that our recent data have demonstrated that the improvement of BMSC cell attachment on the RGD modified PCL films was through RGD functionalization since RGD pre-incubation could completely block this improvement, indicating the increase in cell attachment after modification was through RGD modification (data not shown).

It is well-known that once the integrin receptors located on the cell membrane recognize and bind to the RGD sequence on the substrate, the focal adhesions will be formed and subsequently activate focal adhesion kinase (FAK) by autophosphorylation at the tyrosine 397 site. The activation of FAK is considered as a marker of integrin-mediated signaling [23–25]. Our results demonstrated that the activated FAK p397 was significantly increased on the RGD-modified PCL scaffolds, indicating the RGD modification can initiate a specific integrin-mediated signal transduction between BMSC and the RGD modified PCL substrate.

The autophosphorylation of FAK at Tyr397 can create a binding site for c-Src, which therefore binds with phosphoinositide 3-kinase (PI-3K) and activates serine-threonine protein kinase (Akt) by phosphorylation. The activated Akt can phosphorylate a variety of substrates and plays a central role in the mediation of the downstream effects of PI3K pathway [26].

Our subsequent data showed that the activated form of Akt, phosphorylated-Akt (pAKT), was also significantly up-regulated on the PCL scaffold with RGD modification compared with that on the untreated PCL scaffold. The activation of Akt not only inhibit the pro-apoptotic factors such as caspase-9, it also activates the transcription of anti-apoptotic genes through the activation of the transcription factor NF- κ B, involved in the regulation of the key cellular functions including cell growth and survival [27–29]. This finding demonstrates that RGD modified PCL scaffold could activate BMSC FAK and therefore up-regulate PI-3K/Akt pathway, which may improve the cell survival and growth. The indication was verified by the cell survival data, which was significantly increased on the RGD modified PCL scaffolds compared to that on the untreated ones.

Because the main purpose of this study was to develop a surface modification procedure in the porous PCL scaffold and to test the BMSC behaviors, the simple static seeding method was used. Although the cell distribution in the scaffold was not homogenous due to this seeding method, the increases in cell attachment and proliferation as well as the activation of FAK-PI3K-Akt cell signaling pathway occurred in the 3D PCL scaffold after RGD modification demonstrated that functionalizing PCL scaffold with RGD peptides can improve the BMSC-PCL substrate interaction. Further studies using alternative seeding techniques such as dynamic seeding [30,31], centrifugal seeding [32,33] or using the perfusion culture system [34,35] (Holtorf et al., 2005; Wang et al., 2003) may improve cell seeding efficiency and cellular distribution.

5. Conclusions

RGDC was successfully immobilized on three-dimensional PCL scaffolds via aminolysis and a sulfo-SMCC cross-linker. Our results demonstrated that PCL with RGD modification promoted BMSC attachment and initiated the integrin-mediated signal transduction FAK-PI3K-Akt pathway which regulates subsequent increases in cell survival and growth. The present results indicate functionalizing PCL scaffold with RGD peptides improves the BMSC-PCL substrate interaction. Our present study, along with the published data, implied that the modification of PCL with RGD peptide advantageous for its practical application in tissue engineering.

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Fig. 1.

Quantitative and qualitative measurement of NH_2 group on the two-dimensional PCL films with or without aminolysis. Aminolysis was performed by treatment of 10% PCL films with 10% 1,6-hexanediamine at 37 °C for 1 h. A: Ninhydrin staining of PCL films without aminolysis; B: Ninhydrin staining of PCL films with aminolysis; C: XPS of PCL films with aminolysis and D: NH_2 content on the PCL films with or without aminolysis by ninhydrin quantitative assay.

Fig. 2.

Ninhydrin staining of NH_2 group on the three-dimensional PCL scaffold with or without aminolysis. Aminolysis was performed by treatment of PCL scaffolds with 10% 1,6-hexanediamine at 37 °C for 1 h in the vacuum chamber. A: PCL scaffold without aminolysis; B: PCL scaffold with aminolysis; C: The upper layer of PCL scaffold with aminolysis; D: The middle layer of PCL scaffold with aminolysis; and E: The lower layer of PCL scaffold with aminolysis.

Density (mol/cm ²)	Amount (μg/mg)
6.35±1.85 * 10 ⁻¹⁰	0.0117±0.0023

Fig. 3.

The immobilized RGDC amount in the whole or dissected scaffolds was measured by amino acid analysis using HPLC. The average data of 2–3 scaffolds were presented. The scaffold was aminolyzed at 37 °C for 1 h and subsequently immobilized with RGDC peptide using sulfo-SMCC crosslinker in vacuum chamber.

Fig. 4. BMSCs distribution through the scaffolds was detected by Hematoxylin and Eosin (H&E) staining at 4 h after cell seeding. The scaffolds were washed and fixed, cyro-protected and sectioned serially from top to the bottom using cyrostat. The depth showed in the figure was the distance from the top. The slides were stained by Hematoxylin and Eosin (H&E).

Fig. 5. Western blot analysis for phosphorylated-FAK (pFAK) and phosphorylated-Akt (pAKT) proteins in the cells cultured in the PCL scaffold with or without RGDC modification for 4 h. Beta-actin serves as a loading control. Similar results were obtained from two independent experiments. *, $P < 0.05$ and **, $p < 0.01$ compared with PCL groups.

Fig. 6.

MTS assay applied to determine rat BMSC cell attachment at 4 h after cell seeding (A), and survival and growth at 3 days after cell seeding (B) on PCL and PCL-RGD scaffolds. The scaffold was aminolyzed at 37 °C for 1 h and subsequently immobilized with RGDC peptide using sulfo-SMCC crosslinker (PCL-RGD) in vacuum chamber. Data were expressed as the percentage of PCL (n=6). *, $P < 0.05$ compared with PCL groups and **, $P < 0.01$ compared with PCL groups.