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The effect of elastin on chondrocyte adhesion and proliferation on poly (ε-caprolactone)/elastin composites

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ABSTRACT

The aim of this study was to demonstrate the effect of elastin on chondrocyte adhesion and proliferation within the structure of poly (ε-caprolactone) (PCL)/elastin composites. The homogenous 3D structure composites were constructed by using high pressure CO_2 in two stages. Porous PCL structures with average pore sizes of 540 ± 21 μm and a high degree of interconnectivity were produced using gas foaming/salt leaching. The PCL scaffolds were then impregnated with elastin and cross-linked with glutaraldehyde (GA) under high pressure CO_2. The effects of elastin and cross-linker concentrations on the characteristics of composites were investigated. Increasing the elastin concentration from 25 mg/ml to 100 mg/ml elevated the amount of cross-linked elastin inside the macropores of PCL. Fourier transform infrared (FTIR) analysis showed that elastin was homogeneously distributed throughout the 3D structure of all composites. The weight gain of composites increased 2-fold from 15.8 ± 0.3 to 38.3 ± 0.7 (w/w) % by increasing the elastin concentration from 25 mg/ml to 50 mg/ml and approached a plateau above this concentration. The presence of elastin within the pores of PCL improved the water uptake properties of PCL scaffolds; the water uptake ratio of PCL was enhanced 100-fold from 0.030 ± 0.005 g liquid/g polymer to 11.80 ± 0.01 g liquid/g polymer, when the elastin solution concentration was 50 mg/ml. These composites exhibited lower compressive modulus and energy loss compared to pure PCL scaffolds due to their higher water content and elasticity. *In vitro* studies show that these composites can support primary articular cartilage chondrocyte adhesion and proliferation within the 3D structures. These results demonstrate the potential of using PCL/elastin composites for cartilage repair.

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1. Introduction

Reconstruction and regeneration of organ function often require three dimensional (3D) porous scaffolds as a template. Porosity and pore interconnectivity within the scaffold are critical for cell adhesion, proliferation, and the diffusion of nutrients and oxygen throughout the 3D constructs [1,2]. The scaffold should also provide appropriate mechanical properties to support the regeneration of damaged tissue [3]. Synthetic biodegradable polymers such as poly (ε-caprolactone) (PCL) have superior mechanical properties compared to natural polymers and can be easily processed [4,5]. However, their applications in tissue engineering are limited because their intrinsic hydrophobicity and absence of cell-recognition sites hinder cellular penetration, adhesion, and growth into the porous structures [6,7]. Synthetic polymers are combined with natural polymers such as chitosan [6,7], collagen [8], and elastin [9] to overcome these drawbacks.

Elastin-based biomaterials have great potential for use as hydrogel scaffolds for the regeneration of damaged cartilage [10]. Recently, it has been demonstrated that elastin networks play mechanical and biological roles in cartilage repair [11]. In the uppermost zone of articular cartilage, where few cells are present, elastin fibers and microfibrils form a dense 3D extracellular network with thickness varying from 10 μm to 200 μm [11]. This abundant elastin-rich foundation means that elastin-containing scaffolds can be useful for the construction of a new generation of cartilage substitutes. The results of *in vitro* studies show that chondrocytes [10,12] and progenitor cells [13] encapsulated in elastin-like polypeptides (ELPs) undergo cartilage matrix synthesis. In addition, ELPs sequences are native to musculoskeletal tissues, and elicit no known antigenic response from the host, when implanted subcutaneously [14,15]. Although ELPs provide a physical environment for chondrocyte differentiation and cartilage matrix synthesis, a major

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limitation of elastin-based biomaterials, like other natural hydrogels, is their inability to support the significant loads experienced by cartilaginous tissue *in vivo* [12,16]. Efforts have been made to increase the mechanical strength of elastin-based hydrogel scaffolds through enzymatic or chemical cross-linking: a dynamic shear modulus of less than 5 kPa was achieved using a transglutaminase (Ttg) [12] cross-linker, and compressive stiffness up to 50 kPa was obtained using β[tris(hydroxymethyl)phosphino]propionic acid (THPP) [17] as a cross-linking agent. However, the compressive stiffness of elastin-based hydrogel scaffolds is still lower than the target value for articular cartilage (~500–1000 kPa) [18]. Elastin has recently been combined with synthetic polymers to produce hybrid electrospun biomaterials with improved mechanical properties for vascular applications where the tensile modulus is greater than 400 kPa [19,20].

Scaffold structural properties of high porosity and interconnected pores enhance cell proliferation by allowing nutrient and oxygen diffusion [1]. Such characteristics are particularly important for avascular cartilage tissue replacement constructs. Gas foaming technique has been used to create porosity in hydrophobic polymers such as poly(lactic acid) [2] and hydrophilic polymers such as elastin [22,23]. The advantages of using high pressure CO_2 for the construction of porous scaffolds include: decreasing process time, creating homogenous 3D structure, and eliminating the use of organic solvents [6,8,21]. In this study, dense gas CO_2 was used to fabricate porous 3D structures of PCL/elastin composites. The effects of elastin and cross-linker concentrations on the characteristics of fabricated composites were investigated. *In vitro* cell studies were conducted to demonstrate the potential of using PCL/elastin composites for chondrocyte growth and proliferation.

2. Materials and methods

2.1. Materials

α-elastin extracted from bovine ligament was obtained from Elastin Products Co. (Missouri USA). PCL (MW = 80 kDa, Tm = 60 °C, Tg = −60 °C), glutaraldehyde (GA), and sodium chloride (NaCl) were purchased from Sigma (Australia). Food grade carbon dioxide (99.99% purity) was supplied by BOC. Primary ovine articular cartilage chondrocytes were generously provided by C.B. Little and M.M. Smith, Raymond Purves Bone and Joint Research Labs, Kolling Institute of Medical Research, Institute of Bone & Joint Research, University of Sydney. Cells were maintained in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F-12 Ham) supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin and streptomycin. All tissue culture reagents were obtained from Sigma. [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium (MTS) reagent was purchased from Promega (Australia).

2.2. Construction of PCL/elastin composite scaffold

2.2.1. Formation of porous PCL scaffold

Gas foaming/salt leaching processing was used to make porous 3D PCL scaffolds. A homogenous mixture of PCL and NaCl particles was first prepared by melt mixing; the mixture was placed in a mould, gas-foamed using carbon dioxide (CO_2) as a blowing agent, and soaked in water to leach out salt particles. PCL scaffolds with average pore sizes of 540 ± 21 μm were produced using CO_2 at 65 bar, 70 °C, processing time of 1 h, depressurization rate of 15 bar/min, and addition of 30 wt% salt particles (500–700 μm).

2.2.2. Composite PCL/elastin scaffolds

High pressure CO_2 was used to impregnate elastin into the 3D structure of PCL scaffold. A PCL scaffold was placed in a Teflon mold inside a high pressure vessel; an elastin solution containing GA was then injected into mold. After the vessel was sealed and allowed to reach thermal equilibrium at 37 °C, the system was pressurized with CO_2 to 60 bar, isolated and maintained at these conditions for 1 h. The system was depressurized at 15 bar/min and the sample was collected. The resulting construct was washed repeatedly in PBS (10 mM phosphate, 150 mM NaCl; pH 7.4), and then placed in 100 mM Tris in PBS for 1 h to quench the cross-linking reaction. After Tris treatment, the composite scaffold was washed twice and stored in PBS for further analysis.

The effects of α-elastin and GA concentrations on the characteristics of the composites were assessed. Various concentrations of α-elastin solution including 10, 25, 50, 75, 100 mg/ml and two different concentrations of GA (0.25 and 0.5% (v/v)) were used. The amount of elastin embedded in the 3D PCL scaffolds was determined by gravimetric analysis using the following equation

$$\text{Weight gain} = \frac{(W_{Composite} - W_{PCL})}{W_{PCL}} \times 100$$

2.3. Scanning electron microscopy (SEM)

SEM images were acquired using a Philips XL30 scanning electron microscope (15 kV) and used to determine the pore characteristics of the composites and to examine cellular infiltration and adhesion. Lyophilized scaffolds were mounted on aluminium stubs using conductive carbon paint, then gold coated prior to SEM analysis.

Cell-seeded scaffolds were fixed with 2% (v/v) GA in 0.1 M Na-cacodylate buffer with 0.1 M sucrose for 1 h at 37 °C. Samples underwent post-fixation with 1% osmium in 0.1 M Na-cacodylate for 1 h and were then dehydrated in ethanol solutions at 70%, 80%, 90% and 3 times 100% for 10 min each. For drying, the samples were immersed for 3 min in 100% hexamethyldisilazane then transferred to a desiccator for 25 min to avoid water contamination. Finally they were mounted on stubs and sputter coated with 10 nm gold.

2.4. Fourier transform infrared (FTIR) spectroscopy

FTIR analysis was used to qualitatively characterize the functional groups of elastin and PCL, and to confirm the 3D penetration of elastin into the PCL scaffolds. FTIR spectra were collected at the resolution of 2 cm^{-1} and signal average of 32 scans in each interferogram over the range of 1900–1400 cm^{-1} using a Varian 660 IR FTIR spectrometer. Composite scaffolds with thickness of 3 mm were used for FTIR analysis. The depth of elastin penetration into the PCL scaffolds was evaluated by performing FTIR analysis on the top surface and two layers cut from within the composites (approximately 1 mm and 2 mm below the surface).

2.5. Water uptake properties

The water uptake ratio of the PCL/α-elastin composite scaffolds was evaluated at 37 °C in PBS. The scaffolds were lyophilized prior to use and were weighed dry. The samples were then soaked in 10 ml PBS for 24 h. The excess liquid was removed from the wet samples and the water uptake ratio was calculated based on a ratio of the increase in mass to that of the dry sample. The reported data at each condition was the average measurement for at least three scaffolds.

2.6. Mechanical characterization: Compressive properties

Uniaxial compression tests were performed in an unconfined state using an Instron (Model 5543) with a 500 N load cell according to the testing procedure described previously [22,23]. Compression tests were performed on PCL scaffold, PCL/α-elastin composite, and pure α-elastin hydrogel. Prior to mechanical testing, the samples were soaked for 2 h in PBS. The thickness (3 ± 0.1 mm) and diameter (12.5 ± 0.7 mm) of each sample was then measured using digital callipers. The compressive properties of the samples were tested in the hydrated state, in PBS, at room temperature. The samples were subjected to a loading and unloading cycle and the compression (mm) and load (N) were collected at a cross speed of 30 μm/s and 40% final strain level. The compressive modulus was obtained as the tangent slope of the stress-strain curve between 10% and 20% strain level. In addition, for all samples, the energy loss based on the compression cycle was computed. Three specimens were tested for each sample type (PCL scaffold, PCL/α-elastin composite, and pure α-elastin hydrogel). The composite PCL/α-elastin was prepared by subjecting a PCL scaffold soaked in an aqueous solution containing 50 mg/ml elastin and 0.25% (v/v) GA to high pressure CO_2 as described above.

2.7. In vitro cell culture

The ability of cells to grow into the 3D structure of the PCL/α-elastin composites and pure PCL scaffolds was assessed using MTS and SEM analysis. Scaffolds made using 50 mg/ml elastin and 0.25% (v/v) GA were transferred into a 48-well plate and washed twice with ethanol to sterilize the materials. The scaffolds were then washed at least twice with culture media to remove any residual ethanol and equilibrated in culture media (DMEM/F-12 Ham, 10% FBS, pen-strep) at 37 °C overnight. The cells were then seeded onto the scaffolds at 1.6 × 10^5 cells/well. Unseeded hydrogels located in adjacent wells were used for comparison. The scaffolds were kept in a CO_2 incubator for 7 days at 37 °C, after which they were fixed to assess cell penetration and growth using SEM analysis.

2.7.1. Proliferation/cytotoxicity assay

MTS analysis was performed at time intervals of 1, 4, and 7 days to determine cellular proliferation and viability within the scaffolds. Following culturing for the appropriate time the cell-seeded scaffolds were moved to a new 48-wellplate where 250-μl fresh medium and 50-μl Cell Titer 96 Aqueous One reagent were then added into each well. The scaffolds were kept in a CO_2 incubator at 37 °C for 1 h then read at

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