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A highly organized three-dimensional alginate scaffold for cartilage tissue engineering prepared by microfluidic technology

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ABSTRACT

Osteoarthritis is a degenerative disease and frequently involves the knee, hip and phalangeal joints. Current treatments used in small cartilage defects including multiple drilling, abrasion arthroplasty, mosaicplasty, and autogenous chondrocyte transplantation, however, there are problems needed to be solved. The standard treatment for severe osteoarthritis is total joint arthroplasty. The disadvantages of this surgery are the possibility of implant loosening . Therefore, tissue engineering for cartilage regeneration has become a promising topic. We have developed a new method to produce a highly organized single polymer (alginate) scaffold using microfluidic device. Scanning electron microscope and confocal fluoroscope examinations showed that the scaffold has a regular interconnected porous structure in the scale of 250 μm and high porosity. The scaffold is effective in chondrocyte culture; the cell viability test (WST-1 assay), cell toxicity (lactate dehydrogenase assay), cell survival rate, extracellular matrix production (glycosaminoglycans contents), cell proliferation (DNA quantification), and gene expression (real-time PCR) all revealed good results for chondrocyte culture. The chondrocytes can maintain normal phenotypes, highly express aggrecan and type II collagen, and secrete a great deal of extracellular matrix when seeded in the alginate scaffold. This study demonstrated that a highly organized alginate scaffold can be prepared with an economical microfluidic device, and this scaffold is effective in cartilage tissue engineering.

1. Introduction

Because of the limited capacity for self-regeneration, minor trauma to articular cartilage may lead to progressive damage and degeneration [1]. Osteochondritis dissecans and chondral defects are focal lesions which are frequently found in young patients as a result of sports injury [2]. A chondral defect is confined to the cartilage itself without penetrating into the subchondral bone, and spontaneous healing is limited due to the lack of blood supply in the cartilage. Osteochondral lesions involve the subchondral bone and the mesenchymal chondroprogenitor cells that come to repair the lesions. However, the new cartilage is composed of type I collagen and increased fibronectin which lacks the lower friction and elastic properties [3]. Current treatment for small chondral defects includes multiple drilling and abrasion arthroplasty, but the results vary. Mosaicplasty and autogenous chondrocyte transplantation are new options, but gap existence and fibrocartilage formation are still problems [4].

Tissue engineering, a field integrating biology, medicine and engineering, provides a promise of cartilage regeneration. The scaffold serves as an extracellular matrix (ECM) to provide a three-dimensional conformation and orientation to cells, and has a vital role in tissue engineering [5]. In general, chondrocyte cultured as a monolayer, as in Petri dishes and tissue culture flasks, loses the chondrogenic phenotype and the re-differentiated potential with culturing passages [6]. On the other hand, cells delivered into

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a three-dimensional (3D) structure with some growth factor can maintain the phenotype and increase cell proliferation [7]. For cartilage tissue engineering, various materials, including gelatin, collagen, alginate, and some derivatives, are used to fabricate scaffolds as hydrogels and porous structure forms [8–11].. However, poor biomechanical and handling characteristics are still the shortcomings of this kind of hydrogel scaffold.

Conventional methods for producing 3D porous scaffolds include freeze drying, electrospinning, phase separation, gas foaming, solvent casting and particulate leaching [12–16]. However, the diameter of the pores may not be of equal size and the inter-pole may not connect consistently within the scaffold. Previous study reveals that if the pore size has high variability, this will create impediments to cell seeding and growing. To decrease the effect of the structure, highly ordered and uniform spatial structures are preferable [17]. New solid freeform fabrication techniques such as photolithographic patterning and layering, direct writing, and two-photon stereolithography have been proposed to prepare 3D ordered scaffolds [18–21]. Though they obtain highly organized scaffolds, these methods involve expensive robotic control and time-consuming pixel-by-pixel writing. A reliable and economic method for scaffold preparation may benefit cartilage tissue engineering.

A self-assembly approach by templating colloidal crystals was reported by Kotov to prepare scaffolds with inverted crystal structure [22]. Regarding this procedure, scaffolds are fabricated in the form of solid foam, which is formed by rapid solidification of liquid foam. Solid foams are classified into open-cell or closed-cell foams depending on whether the cellular faces of its liquid parent are retained. On the other hand, liquid foams are colloidal dispersions of gas bubbles in a liquid in which the bubbles are in contact. They are thermodynamically meta-stable. Monodisperse foams self-assemble into crystalline phases and exhibit stronger mechanical strength and longer stability than polydispersed foams. Microfluidics can manipulate fluidic flow on microscales and provide a new means to generate monodispersed droplets. Microfluidic methods have been employed in flow focusing, cross flowing, and co-flowing of liquid and gas streams [23–26]. In this study, the microfluidic device was used to generate monodispersed alginate droplets. The alginate droplets were gelated to form a highly-organized scaffold; the feasibility of the use of this scaffold in cartilage tissue engineering was demonstrated.

2. Materials and methods

2.1. Microfluidic device and alginate scaffold fabrication

The microfluidic device is a two-channel fluid jacket microencapsulator-bubble formation equipped with a micropipette (Fig. 1). The micropipette (inner diameter: 45 μm, and outer diameter: 95 μm) was prepared by a micropipette puller (P-97, Sutter Instrument, USA). 1.5% alginate (A2158, Sigma-Aldrich, St. Louis, MO, USA) with 1% Pluronic® F127 (P6866, Invitrogen, UK) surfactant prepared and filtered with a 0.22 μm filter (Millex-GV, Millipore, USA) for sterilization. The alginate solution was put into a 20 cc syringe and dropped by the syringe pump (PHD 22/2000, Harvard Apparatus, USA). A rubber pipe connected the syringe to the outer micropipette of the microfluidic device, and another rubber pipe was connected from nitrogen gas bottle to the inner micropipette. Nitrogen gas and aqueous alginate solution with surfactant were pumped through the inner and the outer channels, respectively. The hollow droplets were generated under the controlled flow rate (300 μl/min) and gas pressure (6psi) by the syringe pump with a digital pressure indicator (PM, Heise, USA) [27].

The droplets were injected into a 2% calcium chloride ($CaCl_2$, C2661, Sigma-Aldrich, St. Louis, MO, USA) solution, and gelated as empty microspheres by ionic bonding via Ca^{2+}. The microsphere in the $CaCl_2$ solution was verified by a stereoscope (Leica EZ4, Switzerland). If the array of the microsphere was adequate with high organization, the microsphere would be continued for further procedure. After 2 h, a gelated alginate scaffold was obtained. The scaffold was then put in the vacuum system overnight for removing air bubbles and synthesizing the interconnecting pore.

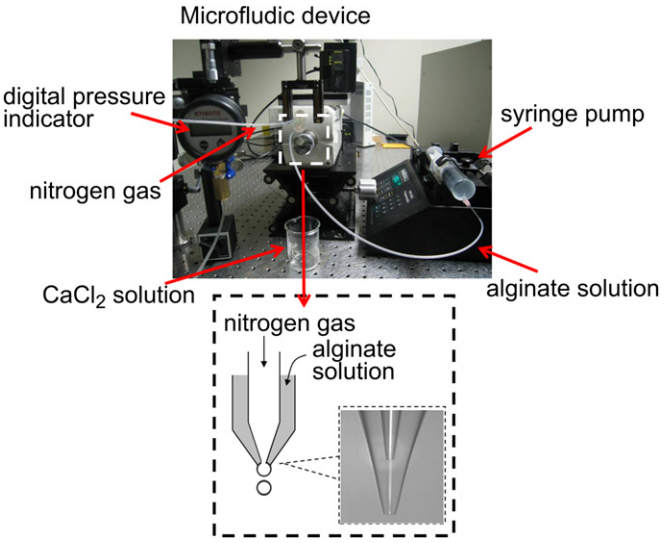

Fig. 1. The microfluidic device.

A dermal punch originally designed for skin tumor biopsy was used to cut the scaffold blocks as uniform cylindrical scaffolds (4 mm in diameter and 4 mm in length). Finally, the alginate scaffolds were immersed in 5% penicillin-gentamycin-streptomycin (P4083, Sigma-Aldrich, St. Louis, MO, USA) 1 h for disinfection.

2.2. Scanning electron microscope (SEM) and confocal laser scanning microscope observation for the alginate scaffold

The microstructure of the alginate scaffold was examined using a scanning electron microscope (SEM) (S-800, Hitachi, Japan). Samples were fixed in a 10% formalin solution (533998, Sigma-Aldrich, St. Louis, MO, USA) of neutral buffer, followed by dehydration in a graded ethanol series, critical point drying (Sousimis, PVT-3B critical point dryer), sputter-coating with gold ion by an ion sputter (Joel, JFC-1100E Ion sputtering device, 1100E), and examined.

For confocal microscope observation, 0.2 mg/ml fluorescein isothiocyanate (FITC, F7250, Sigma-Aldrich, St. Louis, MO, USA) was added to the alginate solution first. The alginate solution containing FITC was used to prepare scaffolds as previously described and observed using a confocal laser scanning microscope (TCS-SP5, Leica, Bannockburn, IL).

2.3. Swelling ratio, porosity and compressive strength of the scaffold

The swelling ratio and porosity of the new alginate scaffold were demonstrated. The wet alginate scaffolds were weighed (Wt) first, and the scaffolds were dried in a freeze dryer. Dried scaffolds were weighed (W_0) again. The swelling ratio Q was defined as W_t/W_0.

The porosity of the scaffolds was measured according to Archimedes' principle [28]. The porosity was calculated according to the following formula: porosity $=((W2\text{-}W1)/(W2\text{-}W3))\times 100\%$, where $W1$ is the weight of the sample in air, $W2$ is the weight of the sample with water, and $W3$ is the weight of the sample suspended in water.

Compressive strength of alginate scaffolds were tested according a previous study with an Instron 4505 mechanical tester with 10 kN load cells following the guidelines in ASTM D5024-95a [29]. The crosshead speed was set at 0.4 mm/min for the Instron tester, and load was applied until the specimens were compressed to approximately 30% of the original thickness. Compressive modulus was calculated as the slope of the initial linear portion of the stress-strain curve. Four independent samples were analyzed in this series of tests.

A traditional alginate scaffold was prepared and carried out identical analyses for comparative purposes [30]. Briefly, 1.5% alginate solution was frozen at −20 °C. The frozen solution was immersed in aqueous ethanol solution of $CaCl_2$ at −20 °C for gelation. Finally, the frozen alginate was lyophilized for 24 h to obtain the scaffold.

2.4. Chondrocyte harvest, culture, and seeding

Porcine chondrocyte was used in this study. Cartilage was harvested from pig aged 10–12 months; the porcine hind leg was disinfected initially with alcohol and then betadine after scrubbing. Sterilizing and drapping of the knee joint was then carried out. Medial parapatellar arthrotomy was performed to open the knee joint. After subluxing the patella, the knee joint was fully exposed. The cartilage from the femoral condyle, tibial plateau and patella was cut into thin slices using a scalpel. The slices of cartilage were collected and washed with sterilized phosphate buffered