Biomaterials 32 (2011) 7118–7126

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

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

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ARTICLE INFO

Article history:
Received 7 April 2011
Accepted 9 June 2011
Available online 2 July 2011

Keywords:
Alginate
Scaffold
Chondrocyte
Microfluidic device

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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doi:10.1016/j.biomaterials.2011.06.018

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.

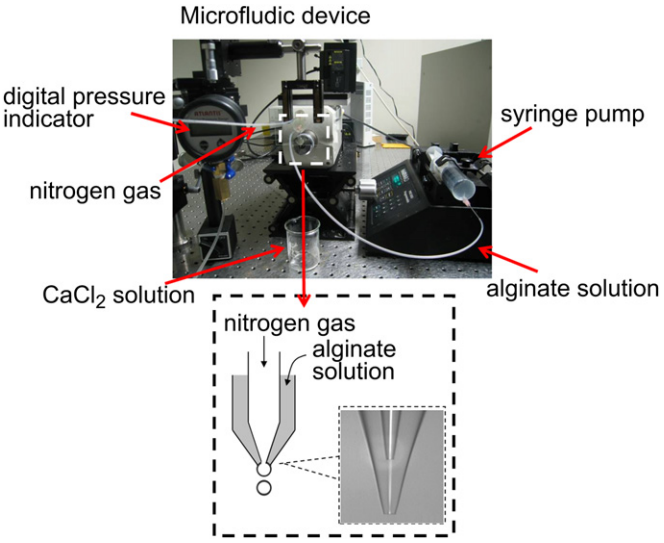

Fig. 1. The microfluidic device.

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

saline (PBS), then further immersed in 5% penicillin-streptomycin-neomycin for 15 min. After treatment, the tissues were washed with PBS to remove the residual antibiotic solution and were digested in 2% collagenase (C0130, Sigma-Aldrich, St. Louis, MO, USA) for 12 h. The digested cartilage was collected, washed and centrifuged to obtain a cell pellet. The cell pellet was then re-suspended in Dulbecco's modified eagle's medium (DMEM) (SH30003.01, Hyclone, Logan, UT) and seeded to a 10 cm culture dish at the cell density of 5 x 10^5 for cell proliferation. Chondrocytes were cultured in DMEM supplement with 50 μg/ml L-ascorbic acid (A5960, Sigma-Aldrich, St. Louis, MO, USA), 10% fetal bovine serum (100–106, Gemini Bio-Products, USA), and 1% antibiotic in an incubator set at 5% CO_2, 37 °C. The medium was changed every 2–3 days.

After 70–80% confluent cells were formed, the cells (passage 3–5) were detached by trypsin-EDTA (15400, Gibco, USA) and re-suspended in DMEM at the cell density of 5 x 10^6 cells/mL. Chondrocytes suspended in medium were seeded into the alginate scaffold using a 24 gauge needle; each scaffold contained 2 x 10^5 cells. The cells/scaffold construct was first placed in an incubator for 1 h for cell adhesion, and then transferred to a 12-well culture plate. Finally, medium was added, and then the culture medium was changed every 2 days. Chondrocytes cultured in the alginate scaffold were observed with an optical microscope.

2.5. Cell viability and cytotoxicity test

Twelve chondrocytes/scaffold constructs were used and separated into 2 subgroups (cultured for 1 and 3 days, respectively). The viability of the chondrocytes in the alginate scaffold was evaluated with WST-1 (K301-2500, Biovision, CA, USA) reagent. At pre-determined intervals, culture medium was aspirated out; and cells/scaffolds were washed with PBS. DMEM containing a 10% WST-1 agent was then added and incubated for a further 2.5 h. The result of WST-1 assay was measured using the spectrophotometer (Sunrise™, Tecan, Switzerland) at the wavelength of 460 nm.

Simultaneously, the DMEM that was initially aspirated out was reacted with a lactate dehydrogenase assay (LDH assay, G1780, Promega, USA) for the cytotoxicity test. 50 μl DMEM was added with 50 μl substrate solution and reacted for 0.5 h. Stop solution was added and the result of the LDH assay was determined by the spectrophotometer at the wavelength of 450 nm. The cell viability and cytotoxicity tests for the control group was the same quantity of chondrocytes cultured in 12-well culture plates for the same intervals and managements.

2.6. Live/dead staining

After being cultured for 7 days, chondrocytes in alginate scaffolds underwent live-dead staining in media containing 2 μmol/l of calcein-AM (17783, Fluka, Japan) and 2 μmol/l of EthD-1 (46043, Fluka, Japan) for 1 h to analyze cell survival. Calcein-AM was converted to green fluorescent indicators in active cells. The EthD-1, on the other hand, penetrated the membrane of the dead cells and bound to DNA and fluoresce red. After treatment, the chondrocytes/scaffolds were observed with the confocal microscope, and 3D cell images were reconstructed at 0.2 μm intervals.

2.7. Scanning electron microscope (SEM) observation for chondrocytes/scaffold constructs

The chondrocytes/scaffold constructs were fixed in a 10% formalin solution after being cultured for 7 days. After the procedure, samples were dehydrated in graded ethanol series, followed by critical point drying, sputter-coated with gold ion by an ion sputter, and examined using a SEM.

2.8. Total DNA quantification for cell proliferation

Twelve scaffolds cultured for 1, 2, and 3 weeks, respectively, were used for DNA quantification. The cultured specimens were digested in papain solution (P4762, Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 0.1 mg/mL with Hank's balanced salt solution (H2387, Sigma-Aldrich, St. Louis, MO, USA) at 60 °C for 16 h [31]. Total DNA of digested sample was extracted using a DNeasy Blood and Tissue kit (69504, QIAGEN, Germany). The DNA yield was quantified using an ultra violet/visible/near infrared (UV/VIS/NIR) spectrophotometer (DU 7500, Beckman, USA).

2.9. Glycosaminoglycan (GAG) content measurement

The extracellular matrix (ECM) of the cartilage is composed of glycosaminoglycan (GAG), collagen type II, hyaluronic acid, et. al.; GAG is a major component. The sulfated glycosaminoglycans (GAGs) production was evaluated by a 1,9-dimethylmethylene blue (DMMB, 341088, Sigma-Aldrich, St. Louis, MO, USA) assay. The DMMB solution was composed of 21 mg DMMB, 2 mg sodium formate (71541, Sigma-Aldrich, St. Louis, MO, USA), and 5 ml absolute alcohol in 1 L of distilled water, and the pH value was adjusted to 1.5 by 0.1 M formic acid (33015, Sigma, USA). The DMMB solution was stored at 4 °C and protected from light before usage.

In all, 12 chondrocytes/scaffolds were used for GAG content analysis after being cultured for 1, 2, and 3 weeks, respectively. The cultured specimens were digested in papain solution as previous description. 40 μl digested sample solution was mixed with 250 μl DMMB reagent and transferred to a 96-well microplate. Chondroitin-6-sulfate (C4384, Sigma Co., St. Louise, USA) was diluted sequentially and set up as a standard curve of the concentrations of 0–100 μg/ml. The absorbance was detected at a wavelength of 595 nm by a microplate reader.

2.10. RNA extraction and gene expression for chondrocytes (real-time PCR)

Total RNA of chondrocytes/scaffolds was extracted (RNA mini-kit of Quiagen), and RNA yield was quantified using a UV–VIS. The cDNA was synthesized from RNA using Superscript II RT (18064-014, Invitrogen) with a RT-PCR machine (PTC-200, MJ Research, USA). The cDNA was stored at −80 °C until further analyses.

Aggrecan, type I collagen, type II collagen, and type X collagen were chosen as target genes to analyze the chondrocyte gene expression (primer sequences are listed in Table 1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous housekeeping gene. The PCR single reaction mix included 4 μl of cDNA, 4 μl of 1 μM sense and antisense primer solution, 12.5 μl of 2X TaqMan® Universal PCR Master Mix (4304437, ABI, USA), and supplement with distilled water to the final volume of 25 μl. The content of cDNA was 50 ng per reaction. To correct for pipetting errors, each cDNA sample was run in triplicate. The PCR reaction consisted of an initial enzyme activation step at 50 °C for 2 min, samples were then denatured at 95 °C for 10 min. Subsequently, the samples were cycled 40 times in two stages, including a denaturation step at 95 °C for 15 s and a following annealing/extension step at 60 °C for 1 min. The PCR reaction was performed with an ABI PRISM 7900 Sequence Detection System with Sequence Detection Software 1.9.1. The relative expression of each target gene was examined using the $2^{-\Delta\Delta Ct}$ method.

2.11. Statistical analysis

Data was expressed as mean ± SEM. Statistical analyses of scaffold swelling ratio, porosity, compressive strength, cell viability, cytotoxicity, GAGs contents, total DNA, and real-time PCR were analyzed by ANOVA. Difference was considered significant when the p-value was less than 0.05.

3. Results

3.1. Characterization and observation of the alginate scaffold

Uniform alginate bubbles were generated by the microfluidic device (Fig. 2a). The droplets formed hollow beads and became 3D ordered array structures that were highly organized in the 0.1 M calcium chloride solution. After collection for 2 h, a sponge-type, multiple-layer alginate scaffold was formed (Fig. 2b). The disposition was like a hexagonal close-packed crystal structure, such as magnesium or zinc. The second array supplied 3 droplets to contact the alginate unit cell in the first array. The coordination number was 12 and the atomic packing factor was 0.74 [32]. The spontaneously self-assembled scaffold was a crystalline foam-like structure (Fig. 2c and d). After the vacuum degassing process, the alginate scaffold revealed a highly organized porous structure with interconnection. The pore size was uniform and consistent, seen from the surface and cross-section view. SEM showed that the size of the hollow microspheres was controlled in 250 μm for further cell culture (Fig. 2e).

Table 1
The primers designed for the real-time PCR.

Gene	Gene bank number	Primer Sequence	Size (basepair)
Type I collagen	AF201723	Sense CAGAACGGCCTCAGGTACCA Antisense CAGATCACGTCATCGCACAAC	101
Type II collagen	AF201724	Sense GAGAGGTCTTCCTGGCAAAG Antisense AAGTCCCTGGAAGCCAGAT	118
Type X collagen	AF222861	Sense CAGGTACCAGAGGTCCCATC Antisense CATTGAGGCCCTTAGTTGCT	117
Aggrecan	AF201722	Sense CGAAACATCACCGAGGGT Antisense GCAAATGTAAAGGGCTCCTC	107
GAPDH	AF017079	Sense GTCATCCATGACAACTTCGG Antisense GCCACAGTTTCCCAGAGG	103

GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

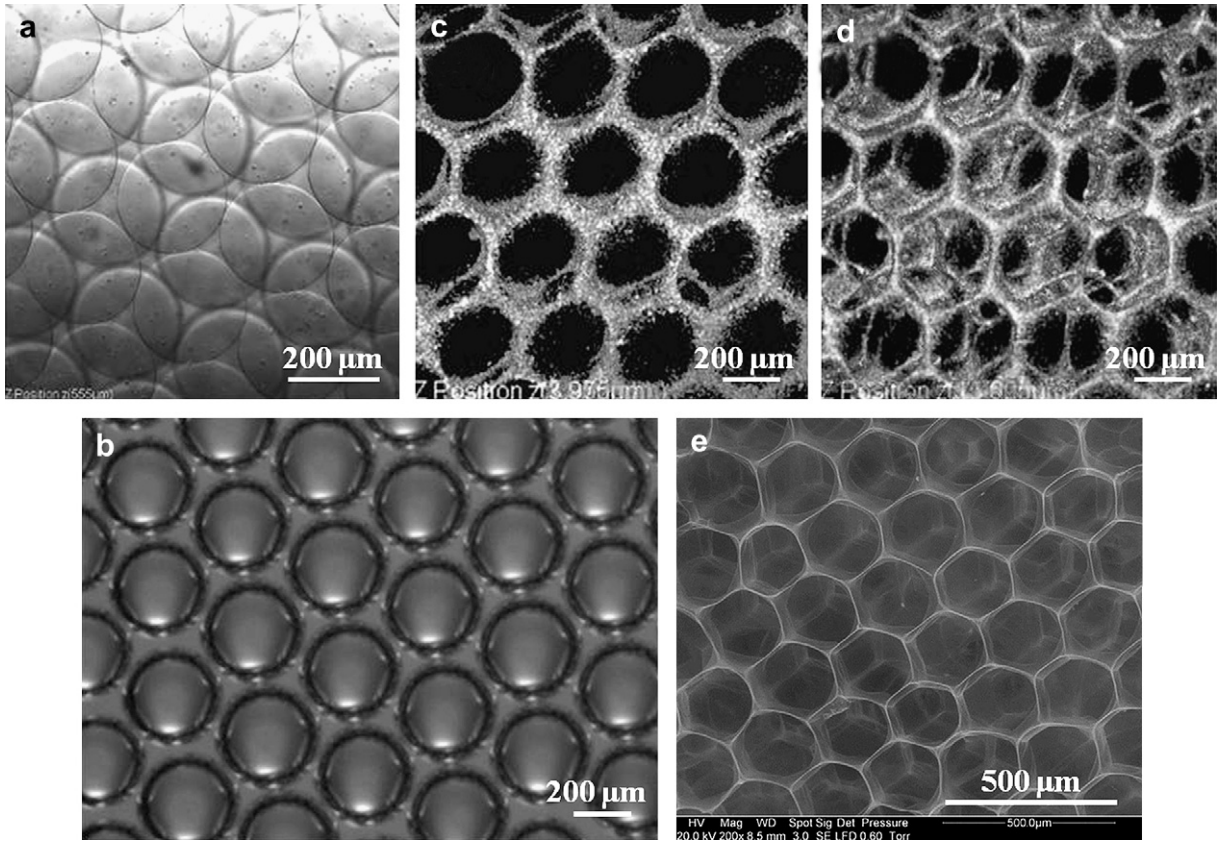

Fig. 2. Uniform alginate bubbles were generated by the microfluidic device (1a). The droplets formed a honeycomb structure after gelatin (1b). Confocal microscope showed a 3D ordered array structure with high organization (1c and 1d). After the vacuum degassing process, the alginate scaffold revealed a highly interconnecting porous structure with uniform pore size under SEM (1e).

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

The average swelling ratio was 91.83 ± 6.02% in the new alginate scaffold compared with 66.69 ± 0.87% in the traditional alginate scaffold ($n = 4$, $p < 0.01$, Fig. 3a). The average porosity was 86.90 ± 5.09% in new alginate scaffold, which was significantly higher than that of traditional alginate scaffold (66.63 ± 2.45%) ($n = 4$, $p < 0.05$, Fig. 3b).

Compression tests of the traditional and new alginate scaffolds were carried out to obtain the stress-strain relations. The compressive strength was 819 ± 50 Kpa for traditional alginate scaffold and 772 ± 37 Kpa for the scaffold prepared by microfluidic device, respectively. There was no significant difference ($n = 4$, $p > 0.05$).

3.3. Cell seeding, viability, and cytotoxicity test

Chondrocyte seeding in the scaffold was invested directly by optical microscope due to the transparent character of the scaffold. Fig. 4a represents cells seeded in the scaffold; after one day, single cells were observed. After 3 days, the cells proliferated and increased gradually (Fig. 4b). Fig. 4c represents cells seeded for 7 days; some cells were found to grow numerously and aggregate in the scaffold. The space of the scaffold was filled with cells after 14 days (Fig. 4d).

The results of the WST-1 assay revealed that the cell viability of chondrocytes cultured in the new alginate scaffold was increased as culture periods (Fig. 5a). There was no significant difference between cells cultured in the culture plate and in the alginate scaffolds ($n = 6$, $p < 0.05$). The cytotoxicity of the alginate scaffold to the chondrocytes is shown in Fig. 5b. Cell toxicity was also slightly increased as culture period, which presents the normal cell death under culture. There was also so significant difference between these two groups ($n = 6$, $p < 0.05$).

3.4. Live/dead staining

Live/dead staining showed the cells lived well in the scaffold. In Fig. 6a and e represent the bright views of the cells/scaffold; b and f in green represent live cells; e and g in red represents dead cells; and d and h are the combination pictures of the bright views/fluorescence pictures, indicating that the live cells and dead cells are not the same. On day 7, most cells were stained with green fluorescence, except the few cells that had died in the scaffold. The merged images reveals the live cells were distributed on the wall of the scaffold and showed good viability. Spreading cells (Fig. 6d) and the formation of cell aggregates (Fig. 6h) are simultaneously demonstrated.

3.5. Scanning electron microscope observation for chondrocytes/scaffold construct

SEM showed the chondrocytes were attached firmly with stretching pseudopodium on the scaffold (Fig. 7a). The cells grew well and secreted ECMs on the wall of the scaffold (Fig. 7b), and some cells were found to have migrated and attached in the interconnected pore. The lacunae of the scaffold also were filled

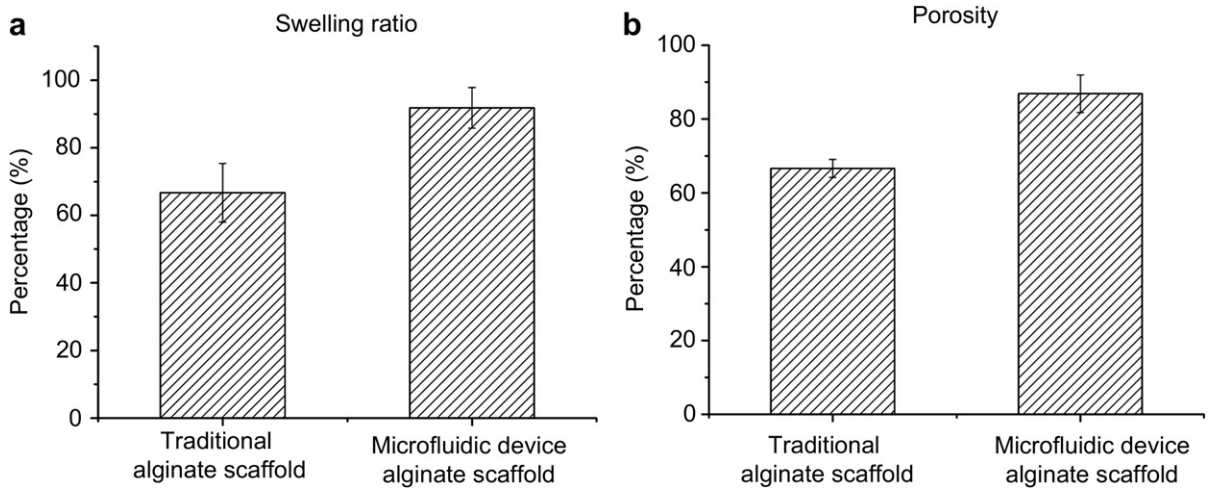

Fig. 3. a The alginate scaffold prepared by the microfluidic device had a higher swelling ratio (91.83 ± 6.02%) when compared with the traditional alginate scaffold (66.69 ± 0.87%). 3b The average porosity was 86.90 ± 5.09% in the newly alginate scaffold and was 66.63 ± 2.45% in the traditional alginate scaffold.

with cells (Fig. 7c). This examination reveals chondrocytes homogenously distributed in the porous alginate scaffold (Fig. 7d).

3.6. Cell proliferation and glycosaminoglycan (GAG) content

The amount of DNA could represent the cell amount. The DNA amount was increased from week 1 to 3 that reveals chondrocytes proliferated well in the alginate scaffold (Fig. 8a). This finding responded to the optical microscope observation, the chondrocytes increased as time.

DMMB assay showed that the amount of GAG content gradually increased that was in agreed with the result of DNA amount (Fig. 8b). This demonstrates that the highly organized alginate scaffold is suitable for cartilage tissue engineering and allows chondrocytes to secret ECM well.

3.7. Gene expression

Fig. 9 shows the mRNA expression patterns of the chondrocytes cultured in an alginate scaffold for 1, 2, and 3 weeks, respectively. The expressions of aggrecan and collagen type II were increased significantly. On the contrary, the collagen type I and X showed a decreasing trend. The results revealed the cells maintained their normal phenotypes as healthy chondrocytes in this new alginate scaffold.

4. Discussion

The scaffold serves as ECM to provide a 3-D conformation and orientation to cells and has a critical role in tissue engineering [33]. A porous structure supplies space for cell growth, signal

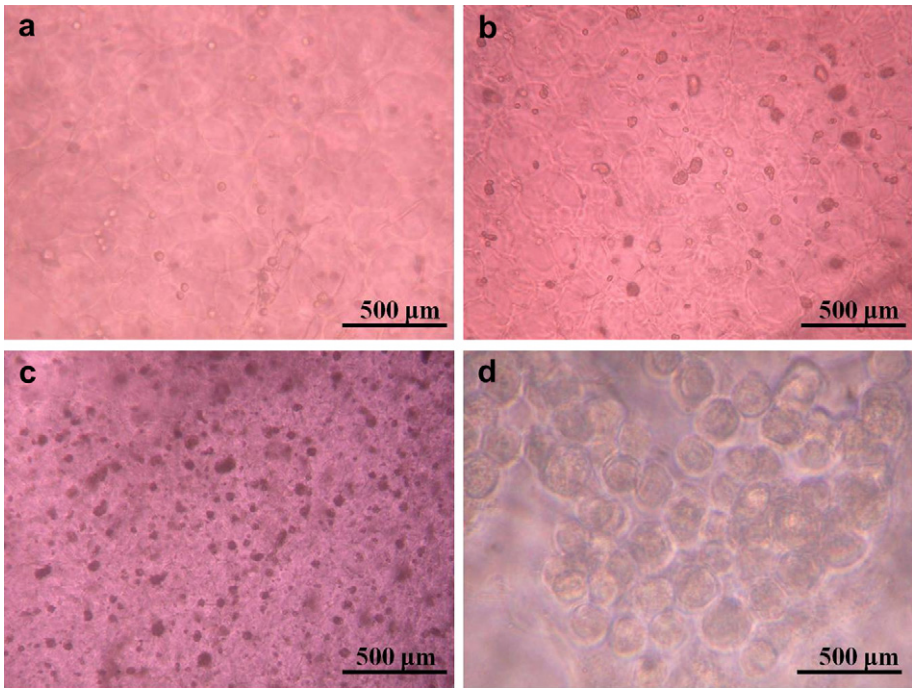

Fig. 4. a represents cells seeded in the scaffold after one day, single cells were observed. The cells proliferated and increased gradually at day 3 (4b). 4c shows some cells were found to grow numerously and aggregate in the scaffold after 7 days culture. The space of the scaffold was filled with cells after 14 days (4d).

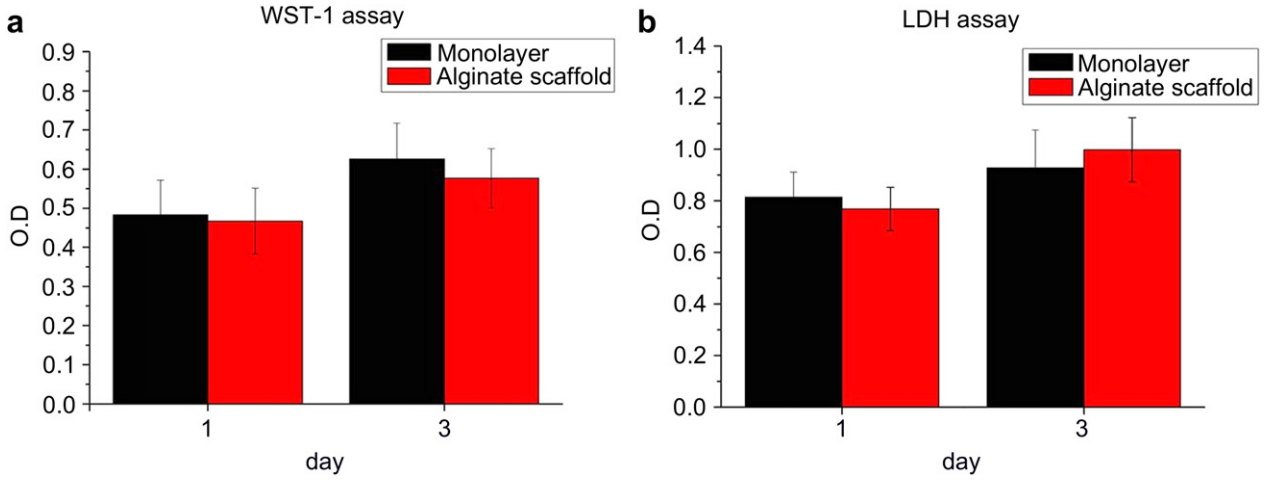

Fig. 5. a The cell viability of chondrocytes cultured in alginate scaffold was increased as culture periods when evaluated by WST-1 assay. There was no significant difference between cell cultured in the culture plate and in the alginate scaffolds ($n = 6$, $p < 0.05$). 5b shows the cytotoxicity of the alginate scaffold to the chondrocytes. Cell toxicity was slightly increased as culture period that presents the normal cell death under culture. There was also no significant difference these two groups ($n = 6$, $p < 0.05$).

transduction and regulation. Accordingly, computer-assisted designed printing, 2 photon confocal photolithography, and micromachining have been used to obtain a well-organized structure [18–21]. However, these methods are high-cost and complex in fabrication. In spite of some successes, these problems remain to be solved.

In this study, a microfluidic device, a relatively easy and low-cost approach, was used to fabricate a 3D highly organized structure. Alginate, a typical hydrogel biomaterial which can promote maintenance of the phenotype of the chondrocyte, was chosen as the raw material for scaffold preparation [34]. Regarding the microfluidic device, pore size is influenced and determined by fluid velocity, gas pressure and the diameter of the micropipette. The viscosity of solutions also influences pore size and bubble shape [27]. Bubble rupture during dropping down or before gelation was noted initially. Therefore, a higher concentration of alginate solution was chosen to prevent bubble rupture in the beginning. However, the tube of the device was easily obstructed due to the high viscosity alginate solution. To avoid the tendency of alginate bubble rupture at low concentrations, 1% Pluronic® F127 surfactant was added. The Pluronic® F127 surfactant has less cell toxicity than the others [35]. Carefully adjusting the fluid velocity and gas pressure, we successfully fabricated a 3D highly organized scaffold. This is an easily reproducible and practical method; and there is no

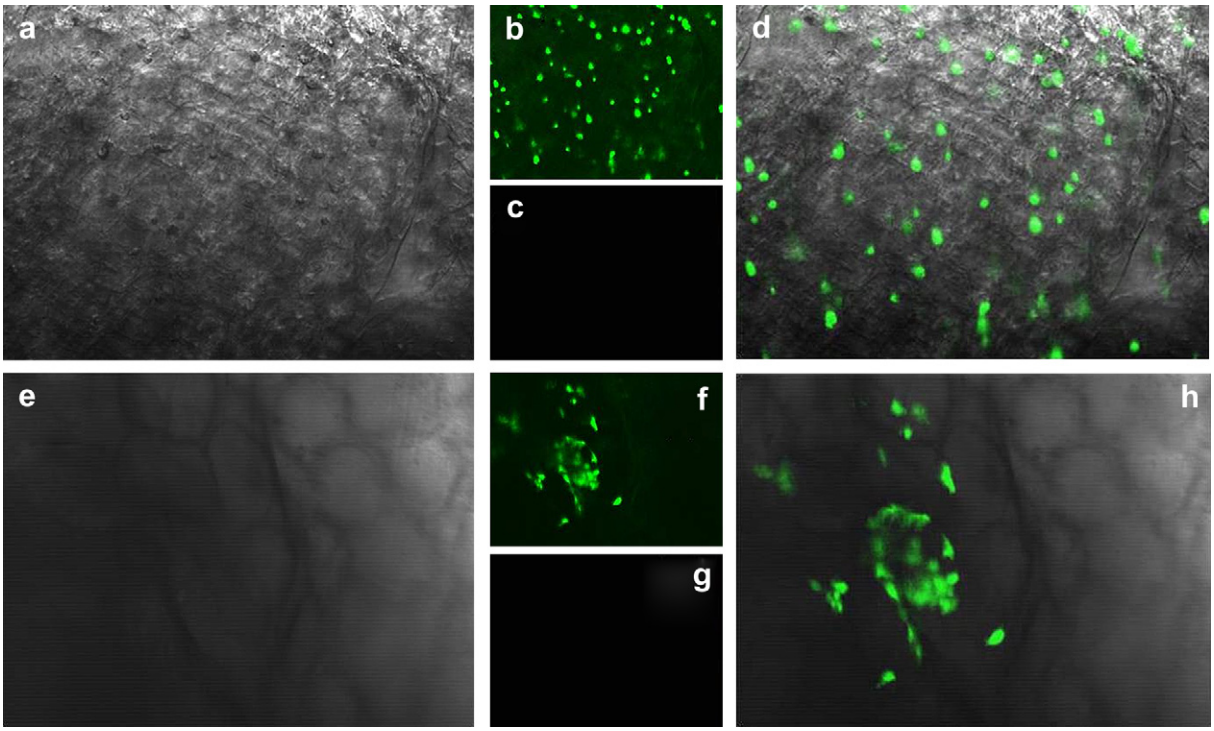

Fig. 6. Live/dead staining showed the cells lived well in the scaffold. On day 7, most cells were alive (green fluorescence). Both spreading cells (6d) and aggregates (6h) are simultaneously observed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

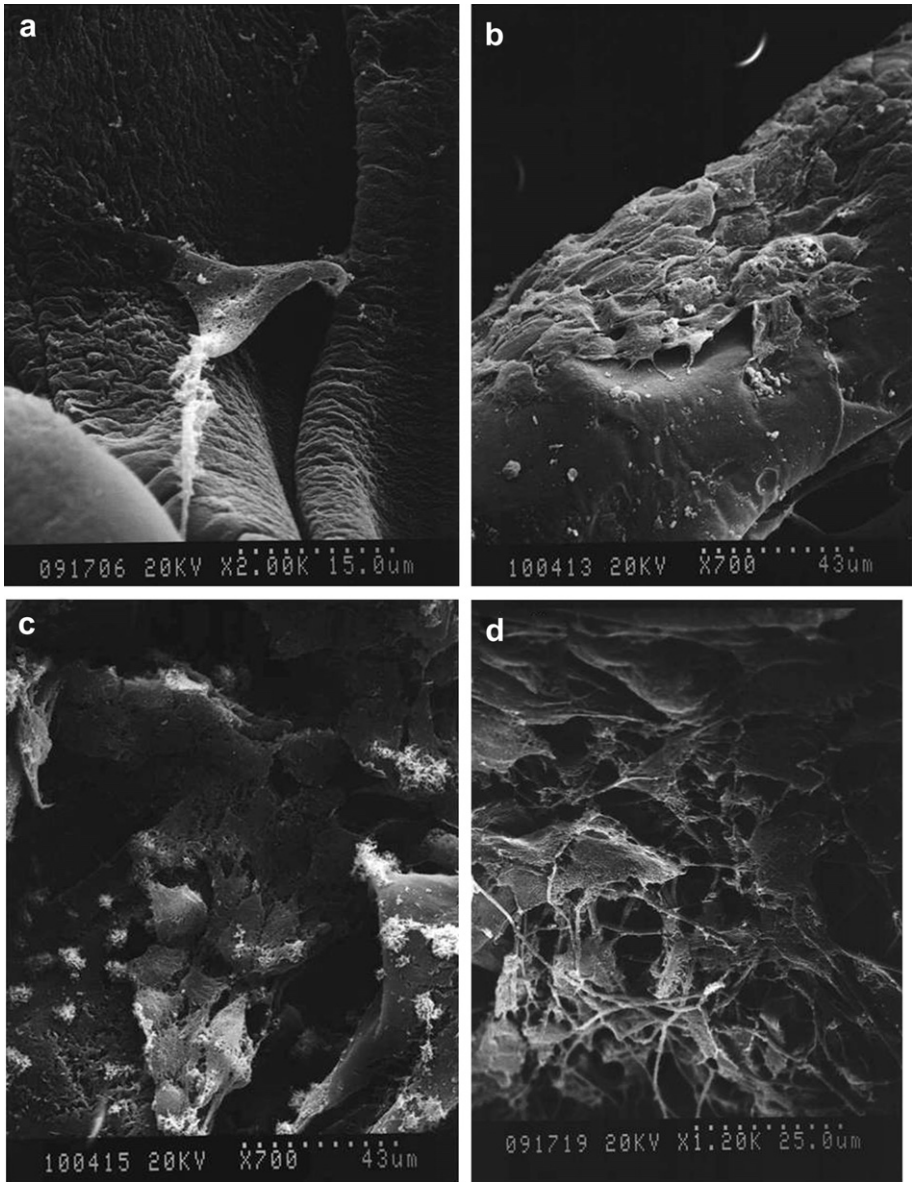

Fig. 7. SEM showed the chondrocytes were attached firmly with stretching pseudopodium on the scaffold (7a). The cells grew well and secreted ECM on the wall of the scaffold (7b). The lacunae of the scaffold also were filled with cells (7c). This examination reveals chondrocytes homogenously distributed in the porous alginate scaffold (7d).

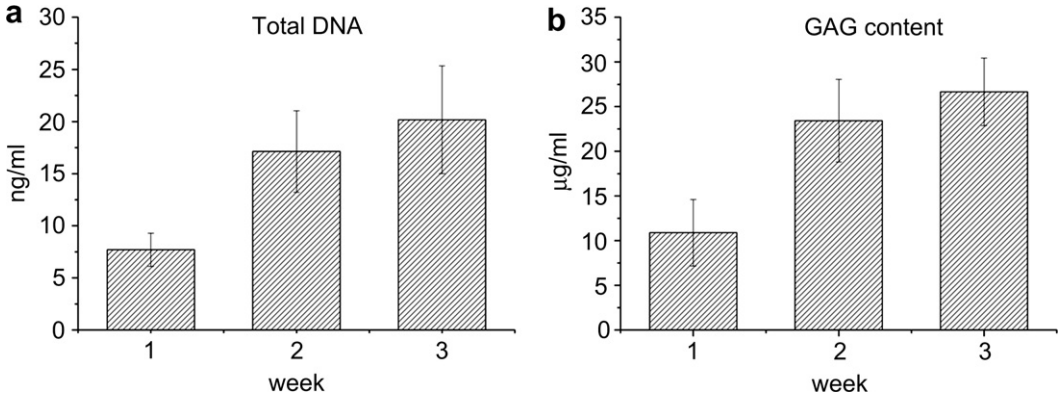

Fig. 8. Shows the DNA amount was increased from week 1 to 3 that demonstrates the chondrocytes proliferating well in the alginate scaffold (8a). The amount of GAG was gradually increased which reveals that the highly organized alginate scaffold is suitable for cartilage tissue engineering and allows chondrocytes to secret ECM (8a).

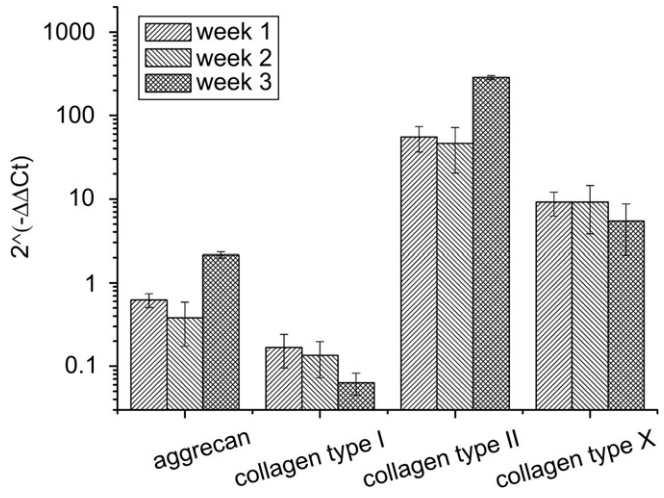

Fig. 9. Shows the mRNA expression patterns of the chondrocytes cultured in an alginate scaffold for 1, 2 and 3 weeks, respectively. The expressions of aggrecan and collagen type II were increased significantly. On the other hand, the collagen type I and X expressions showed a decreasing trend which reveals the cells kept normal phenotypes as healthy chondrocytes in this alginate scaffold.

need for complex methods or high technology to prepare the scaffold. Most important of all, this approach is much cheaper than others.

Fig. 2 shows that uniform alginate bubbles were obtained by this method. After gelation via Ca^{2+}, the pores of the alginate scaffold made with the microfluidic method were more uniform than the traditional alginate sponge. Previously, calcium alginate microspheres were prepared using emulsification method, and another study also using emulsification to prepare agarose microspheres for cell encapsulation [36]. However, the size was unequal and difficult to control in each time experiment. The microspheres were unequal in size, so they had difficulty contacting each other and could not form a regular array. Some studies used aldehyde to cross-link the alginate microspheres through the formation of bonds between the hydroxyl groups of neighboring alginate microspheres [37]. The disadvantage of using aldehyde is the cytotoxicity. However, we formed alginate beads of equal size and they became a 3D ordered array structure in the collecting well. The disposition was like a hexagonal close-packed crystal structure and was gelated by an ionic bond. Therefore, the cytotoxicity of aldehyde for cross-linking could be prevented, and this point of view could be verified by the cell viability and cytotoxicity assays (Fig. 5).

In addition, this method also overcomes the drawbacks such as inconsistent pore size that result with other methods. The size of the hollow polymer bead can be adjusted by air pressure and fluid velocity. It is also easily controlled by the micropipette tip diameter of the microfluidic device easily. As in a previous study, different cells have their different preferred pore size in 3D culture conditions [38,39]. Therefore, this device may also be applied in other areas of tissue engineering since the pore size is easy to adjust. Moreover, the highly organized structure was observed to have a resemblance to a bee honeycomb framework. The honeycomb structure is characterized by substantial rigidity in shear, high crushing stress, and has a light and relative insensitivity to local loss of stability that may be of benefit for further study [40].

It is reported that the alginate-based scaffold for cartilage tissue engineering induces foreign body giant cell reactions and immunological responses when implanted in experimental animals [41]. Some studies suggested alginate microspheres implanted in rodents would require low-dose immunosuppression [42]. To decrease the effect of immunological responses to alginate and the bone formation tendency of calcium chloride, less material for the scaffold preparation is worthy of consideration. Our scaffold was made by microsphere gelation and then vacuum-dried overnight to become a high porosity scaffold (Fig. 3). The thin wall of scaffold, the high swelling ratio and high porosity meant that the alginate and calcium chloride used in our method were much less than on the previous method. In spite the porosity of the scaffolds prepared by microfluidic device was significantly higher than the traditional scaffold, there was no significant difference in compressive strength. In general, high porous scaffold has relative lower mechanical property. The honeycomb structure of scaffold shall attribute to the well compressive strength. Besides, the high swelling ratio may allow the cells to exchange nutrients/metabolites easily, and also promotes signal transduction and message communication [43,44]. In general, the scaffold has a high water content also has better compatibility with tissues. The higher porosity may promote more cell migration and distribution in the scaffold.

The feasibility of this newly prepared scaffold for cartilage tissue engineering was further analyzed. Porcine chondrocytes were seeded into the alginate scaffolds. We found that the cells attached firmly and proliferated well (Fig. 4a), revealing that the scaffold has good biocompatibility. This finding is consistent with the result of the WST-1 assay. Fig. 5a shows the chondrocytes seeded in the scaffold had normal activity, similar to cells cultured on culture dishes, indicating that the scaffold did not affect the activity of chondrocytes. LDH assay also showed that the scaffold was not cytotoxic to chondrocytes (Fig. 5b).

For cartilage tissue engineering, the pore size of the scaffold was controlled at 250 μm in this study. Lien et. al. reported that chondrocytes showed an abnormal, de-differentiated form in the smaller pores, but normal phenotypes in larger pores, and cells preferred the group of scaffolds with a pore size between 250 μm and 500 μm for better proliferation and ECM production. They concluded that the size of the space for cell growth is a key factor for cell metabolism [45]. Therefore, an alginate scaffold with a size of 250 μm pore size was used for cell culture. It was reported that calcium from calcium chloride solution for gelation in alginate scaffold preparation has the tendency to induce chondrocyte differentiation toward osteogenesis [46]. Extracellular calcium regulates the matrix synthesis of chondrocyte, and the high calcium level induces chondrocytes to increase the expression of osteonectin, osteopontin, osteocalcin, and collagen type X [46,47]. Low calcium level decreases collagen type I expression whereas collagen type II expression remaining stable [48]. In spite the normal viability of the cells, real-time PCR was used to examine the gene expression patterns of chondrocytes in the alginate scaffold (Fig. 9). The expression of aggrecan and collagen type II was increased significantly and the expression of collagen type I and X decreased significantly. Aggrecan and collagen type II are the ECM of cartilage and collagen type I exists in connective tissue, such as tendon or ligament [49]. Collagen type X is expressed when chondrocytes hypertrophy. Generally, collagen type X is restricted to the deep cartilage zone and the adjacent calcified cartilage in adult articular cartilage [50]. A hypertrophic chondrocyte is a cell that has gradually differentiated toward osteogenesis [46,47]. Our results reveal the scaffold has the advantage of keeping the phenotypes of healthy chondrocyte without differentiating toward osteogenesis. This shall attribute to the calcium chloride used in our method is much less than traditional methods, and the normal phenotypes of chondrocytes can be preserved.

5. Conclusion

A 3D highly organized alginate scaffold with uniform pore size, high swelling ratio, and porosity could be prepared with

a microfluidic device economically. The scaffold was non-cytotoxic to chondrocytes; cells proliferated and functioned normally. Moreover, chondrocytes maintained their normal phenotypes within the scaffold. Animal study is ongoing and the scaffold may provide new possibilities for cartilage tissue engineering in the near future.

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