THE USE OF FIBRIN AND POLY(LACTIC-CO-GLYCOLIC ACID) HYBRID SCAFFOLD FOR ARTICULAR CARTILAGE TISSUE ENGINEERING: AN *IN VIVO* ANALYSIS

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Abstract

Our preliminary results indicated that fibrin and poly(lactic-co-glycolic acid) (PLGA) hybrid scaffold promoted early chondrogenesis of articular cartilage constructs *in vitro*. The aim of this study was to evaluate *in vivo* cartilaginous tissue formation by chondrocyte-seeded fibrin/PLGA hybrid scaffolds. PLGA scaffolds were soaked carefully, in chondrocyte-fibrin suspension, and polymerized by dropping thrombin-calcium chloride ($CaCl_2$) solution. PLGA-seeded chondrocytes were used as a control. Resulting constructs were implanted subcutaneously, at the dorsum of nude mice, for 4 weeks. Macroscopic observation, histological evaluation, gene expression and sulphated-glycosaminoglycan (sGAG) analyses were performed at each time point of 1, 2 and 4 weeks post-implantation. Cartilaginous tissue formation in fibrin/PLGA hybrid construct was confirmed by the presence of lacunae and cartilage-isolated cells embedded within basophilic ground substance. Presence of proteoglycan and glycosaminoglycan (GAG) in fibrin/PLGA hybrid constructs was confirmed by positive Safranin O and Alcian Blue staining. Collagen type II exhibited intense immunopositivity at the pericellular matrices. Chondrogenic properties were further demonstrated by the expression of gene encoded cartilage-specific markers, collagen type II and aggrecan core protein. The sGAG production in fibrin/PLGA hybrid constructs was higher than in the PLGA group. In conclusion, fibrin/PLGA hybrid scaffold promotes cartilaginous tissue formation *in vivo* and may serve as a potential cell delivery vehicle and a structural basis for articular cartilage tissue-engineering.

Key Words: articular cartilage, chondrocytes, tissue engineering, scaffold, fibrin, poly(lactic-co-glycolic acid)

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Introduction

Autologous chondrocyte implantation (ACI) has emerged as a successful viable alternative treatment in orthopaedics for arthroscopic intervention or joint replacement surgery (Brittberg *et al*., 1994; Mandelbaum *et al*., 2007; Peterson *et al*., 2003). Recently, further development of original ACI has led to the invention of *in vitro* three-dimensional (3D) cartilage implants that enabled easier surgical handling and facilitated cartilaginous tissue formation (Munirah *et al*., 2005; Munirah *et al.*, 2007; Willers *et al*., 2005). This was supported by numerous investigations of the potential of full-thickness cartilage restoration, using 3D implants, in various animal models such as rabbit (Ito *et al*., 2005; Shao *et al*., 2006), goat (Niederauer *et al*., 2000), dog (Breinan *et al*., 2001) and sheep (Munirah *et al.*, 2007). Since most studies reported implantation by means of arthrotomy, future improvement could be achieved by shifting this open surgery procedure to arthroscopy during the implantation of tissue-engineered construct.

We have successfully performed an autologous 'chondrocyte-fibrin' construct (ACFC) implantation to restore osteochondral defects in a sheep model (Munirah *et al*., 2007). Although the 3D nature of ACFC has allowed easier surgical handling and produced good results, at 12 weeks post-transplantation, the implantation was still carried out by means of open surgery. Besides, since ACFC was too soft to stay in the defect independently, periosteum – which was reported to induce formation of hypertrophic tissue (Peterson *et al*., 2000) – was used to secure ACFC into the osteochondral defect. Another trial on the restoration of osteochondral defects in patellar groove of rabbits, utilising the press-fit method and fibrin sealant, conducted by Uematsu *et al.* (2005), demonstrated that in some implants, consisting of PLGA-seeded cells, it had extruded from the defect. Therefore, in a search for ways to apply minimally invasive surgery for cartilage restoration, basic research is still necessary to develop its full potential. We believe a better outcome will be obtained by improving the scaffold materials of tissue-engineered constructs rather than by using currently available technique.

Hybridisation of various synthetically derived and naturally derived biodegradable polymers has been widely used for scaffold tissue engineering (Fan *et al.*, 2006; Khang *et al.*, 2003; Kim *et al.*, 2006; Kim *et al*., 2007; Ko *et al*., 2007). Advantages of synthetically derived

biodegradable polymers include controllable degradation rate, high reproducibility and ease of fabrication into specific shapes. Naturally derived biodegradable polymers usually mimick key elements of normal tissue (Khang *et al.,* 2004; Park *et al.,* 2005). Poly(lactic-co-glycolic acid) (PLGA) is bioabsorbable and biocompatible material, making it a promising material in the context of regenerative medicine. Numerous attempts have been made for successful tissue reconstruction using PLGA-based scaffold, either by PLGA itself (Park *et al*., 2005; Uematsu *et al.,* 2005) or by incorporation of PLGA with natural polymers such as collagen (Chen *et al.,* 2006a; Chen *et al.,* 2006b), extracellular matrix scaffolds – namely small intestinal submucosa (Kim *et al.,* 2006; Lee *et al.,* 2004) and demineralised bone particles (Jang *et al*., 2005).

In the present study we aimed to evaluate *in vivo* formation of cartilaginous tissue in PLGA, utilizing a fibrin carrier matrix, after implantation in nude mice. Our preliminary results indicated that fibrin was an ideal cell transplantation matrix and enhanced *in vitro* chondrogenesis of rabbit articular chondrocytes (Munirah *et al*., 2008a) and intervertebral disc cells (Munirah *et al*., 2008b) in PLGA scaffolds. Fibrin, clinically applied as fibrin glue, is a natural polymer that forms during blood coagulation. Fibrin has been widely used for cartilage reconstruction purposes (Munirah *et al*., 2007; Munirah *et al*., 2006). Recently, Endres *et al*. (2007) showed that the 3D arrangement of human articular chondrocytes in human fibrin glue and resorbable polyglycolic acid (PGA) scaffolds, cultured in the presence of human serum, is an excellent system for the maturation of cartilage grafts in articular cartilage regeneration. Several previous studies also demonstrated that the fibrin immobilisation techniques promoted homogeneous cell distribution and bone formation, when using human periosteum-derived progenitor cells in PLGA (Zheng *et al.,* 2006), PLGA-TCP composites (Arnold *et al.,* 2002) and PLGA-polydioxanon fleeces (Perka *et al.,* 2000).

This report describes the formation of *in vitro* and *in vivo* cartilaginous tissue in a fibrin/PLGA scaffold. The results that demonstrated the feasibility of fibrin/PLGA as an ideal cell transplantation matrix for chondrogenesis and a structural basis for tissue-engineered articular cartilage are presented.

Materials and Methods

Harvest of cartilage, chondrocytes isolation and monolayer culture expansion

Articular cartilage was aseptically dissected from the femoral condyles and patellae of 6 weeks-old New Zealand White rabbits (n=6). Each sample was placed in normal saline and processed within 6 to 12 hours post-surgery. Cartilage was washed with phosphate buffered saline (PBS 1X, pH 7.2) (Gibco, Grand Island, NY, USA) containing 100 µg/ml penicillin and 100 µg/ml streptomycin (Gibco). Sample was then minced into small fragments and digested with 0.6% collagenase A (Roche Applied Science, Mannheim, Germany) at 37°C for 6 hours, for chondrocyte isolation. The resulting cell suspension was centrifuged at 1,500rpm, 5 min, 37°C (BHG, Hermle Z 360 K, Wehingen, Germany). The supernatant was decanted and the cell pellet was washed with PBS to remove the remaining enzyme. After final centrifugation, the cell pellet was resuspended in PBS, for total cell count with a haemocytometer (Hausser Scientific, Horsham, PA, USA). Cell viability was determined using the trypan blue dye exclusion test (Gibco). Harvested chondrocytes were then seeded in 6 well-plates (Falcon, Franklin Lakes, NJ), with the initial seeding of 5,000 cells/cm^2 in the primary passage (P0).

Chondrocytes were cultured in the mixture of equal volume of F12 nutrient mixture (F12) and Dulbecco's Modified Eagle Medium (DMEM) (Gibco), supplemented with 10% foetal bovine serum (FBS) (Gibco) with the presence of antibiotics and antimycotic (Gibco), 200 mM L-glutamine (Gibco), 50 µg/ml of ascorbic acid (Sigma) and 15 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES) buffer 1M (Gibco) – as previously described (Ruszymah *et al*., 2007a; Ruszymah *et al*., 2007b). All cultures were maintained in a 5% CO_2 incubator (Optima Model 560, Optima Inc, Tokyo, Japan) at 37°C, with the medium changed every other day.

Preparation of microporous 3D PLGA scaffolds

PLGA copolymer (mole ratio 50:50, molecular weight 33,000 g/mole, Resomer RG 503 H) was purchased from Boehringer Ingelheim Pharma GmbH (Ingelheim, Germany). Micro-porous 3D PLGA scaffolds were fabricated by solvent casting/salt leaching technique using methylene chloride (CH_2Cl_2) (JT Baker, Baker Analyzed® A.C.S reagent, Selangor, Malaysia), as previously described (Kim *et al*., 2006; Khang *et al*., 2003). In this method, sieved sodium chloride (NaCl) particles (approximately 180 µm) were dispersed in a polymer/solvent solution (0.2% w/v concentration of PLGA in methylene chloride), which was then cast to make a cylindrical disk scaffold using silicone moulds (7 mm in diameter and 3 mm in thickness). The salt particles were leached out by continuous soaking in deionised water for 48 hours. This selective dissolution produces highly porous polymer. The 3D PLGA scaffolds were freeze-dried for 24 hours in a freeze-dryer (IlShin Lab Co., Seoul, South Korea), to remove any remaining solvent. The average of pore diameter; 139.4 µm and porosity: 85.2% was determined by the mercury intrusion porosimeter; an AutoPore II 9220 (Micromeritics Co., Norcross, GA, USA). PLGA scaffolds were sterilised with 70% ethanol and washed three times with PBS before use.

Formation of *in vitro* constructs

Manufactured scaffolds were assigned into two experimental groups – cultured chondrocytes were seeded into (1) PLGA scaffolds with fibrin (fibrin/PLGA) and (2) PLGA scaffolds without fibrin. Cultured chondrocytes from the primary passage (P0) were trypsinised and sub-cultured (P1) in 75 cm^2 culture flasks (Falcon) at a density of 5,000cells/cm^2. After the cells reached confluency, the P1 cells were harvested, counted for total cell and viability. Approximately, one million cells per scaffold were

incorporated and resuspended in the (1) commercially available fibrin glue kit from Greenplast® (Green Cross P. D. Company, Yongin, Korea) and (2) culture medium. Greenplast fibrin glue kit consists of four vials – vial (1) lyophilized fibrinogen, (2) aprotinin solution, (3) lyophilized thrombin and (4) calcium chloride ($CaCl_2$) solution. Greenplast fibrinogen (65–115 mg/ml) was diluted in culture medium and the thrombin concentration (400–600 units/ml) was adjusted, using calcium chloride solution, in vial 4 to obtain a final working solution containing approximately 30-60 mg/ml fibrinogen and 10-50 units/ml thrombin – as suggested by the manufacturer. PLGA scaffolds were soaked carefully in the chondrocyte-fibrin suspension and polymerized, by dropping thrombin-$CaCl_2$ solution on to the scaffolds. For control group, PLGA scaffolds were soaked in chondrocytes suspended in culture medium. All constructs were cultured for 3 weeks *in vitro*, prior to implantation *in vivo*. Cell viability was assessed by a modified 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium-bromide (MTT, Sigma, St. Louis, MO, USA) method that measured the reduction of a tetrazolium component (MTT) into an insoluble formazan product, by the mitochondria of viable cells. At a defined day, all constructs were transferred into new plates with 1 ml of new medium. 100 µl of MTT solution (5 mg/ml stock in PBS) was added into each well for 4 hrs incubation at 37°C. Next, all constructs were transferred into another new plate and the crystals formed were dissolved by using 1 ml dimethylsulfoxide (DMSO, Sigma) per scaffold. 100 µl of solubilised mixture was transferred into 96-well microtiter plates and then the absorption intensity was analyzed using an ELISA plate reader at 590 nm (E-max, Molecular Device, MDS Analytical Technologies, Sunnyvale, CA, USA). Data were expressed as mean ± standard error of mean (SEM) of 6 samples (n=6).

Implantation of the constructs

Athymic nude mice were anaesthetized upon surgery. Minor skin incision was made at the dorsum of nude mice. Four constructs were implanted into the unconnected subcutaneous pockets of each nude mouse. Eighteen mice were used in this experiment (total implants, 72: PLGA/fibrin = 36, PLGA only = 36). The resulting *in vivo* constructs were harvested at 1, 2 and 4 weeks post-implantation. At time of explantation, all constructs were dissected free from the nude mice, weighed and processed for histological analysis, gene expression study and sulphated glycosaminoglycan (sGAG) content.

Macroscopic observation, histological evaluation and immunohistochemistry analysis

Each construct was observed grossly at room temperature, without any fixation, and palpated with forceps clinically to assess mechanical rigidity. Samples were then prepared for histological analyses. After fixation with 10% formalin for 24 hours, specimens were embedded in paraffin blocks and sectioned with a Leica microtome. All sections were then deparaffinised, rehydrated and stained with Haematoxylin and Eosin (H&E) to assess tissue morphology, Safranin O staining, to identify the presence of proteoglycan-rich matrix, and Alcian blue staining, to detect accumulation of GAG, were applied.

Immunohistochemistry analysis was performed in accordance to the manufacturer's protocol (UltraTek HRP Kit, Immunotech, Marseille, France). All slides were deparaffinised, rehydrated and pre-treated with 0.4% pepsin (v/v 0.01N Hydrochloric acid) (Sigma-Aldrich, Inc. USA) at room temperature for 30 minutes. Slides were washed with PBS (Gibco). To reduce non-specific background staining, due to endogenous peroxidase, slides were incubated with hydrogen peroxide for 10 minutes. All sections were then treated with peroxidase block (Super Block, UltraTek HRP Kit; ScyTek Laboratories, Logan, UT, USA) for 10 minutes prior to antibody incubation. Monoclonal mouse anti-rabbit collagen type II (Calbiochem® EMD Biosciences Inc., La Jolla) and monoclonal mouse anti-rabbit collagen type I (Sigma Aldrich) antibodies were diluted (1:1000) with antibody DILUENT (DAKO Cytomation) and were applied to the sections for 60 minutes. After washing with PBS, UltraTek anti-polyvalent biotinylated antibody (UltraTek HRP Kit) was applied to the slides for 10 minutes at room temperature. Slides were then incubated with streptavidin-peroxidase (UltraTek HRP Kit) for 10 minutes. After washing with PBS, the signal was finally visualized as a brownish precipitate, using freshly prepared chromogen substrate 3-amino-9-ethylcarbazole (AEC) (UltraTek AEC Kit). Sections were counterstained with Mayer's Haematoxylin and mounted, using permanent aqueous mounting medium (SkyTek Laboratories).

Total RNA isolation, cDNA synthesis and conventional PCR

Total RNA was extracted from *in vitro* and *in vivo* constructs to build up a profile of changes in gene expression. Approximately, $1.0x10^5$ cells were homogenised using 1ml TRIzol reagent (Invitrogen, Carlsbad, CA). Cell lysate was centrifuged at 12,000 rpm, at 4°C for 5 minutes (Mega 17R, Hanil Science Industrial Co, Inchon, Korea). The supernatant was then transferred into a 15 ml centrifugal tube (Falcon). Chloroform (Sigma-Aldrich) was added into the tube containing the supernatant and was then shaken vigorously for 10 seconds. After 10 minutes incubation at the room temperature, the mixture was then separated into an aqueous phase, containing RNA, an interphase and organic phase by centrifugation at 12,000 rpm for 15 minutes at 4°C. The aqueous phase containing RNA was carefully transferred into a new 15 ml centrifugal tube. Isopropanol (Sigma-Aldrich) was then mixed homogeneously into the tube, to precipitate the total RNA. 5 µl of polyacryl carrier (Molecular Research Center, Cincinnati) was added to the mixture to facilitate RNA precipitation. The resulting total RNA was washed with 75% ethanol and air-dried. Yield and purity of the extracted RNA was determined spectrophotometrically (BioRad, Hercules, CA, USA).

Reverse transcription was carried out using Superscript™ II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. Total RNA (100-ng) was reverse transcribed in

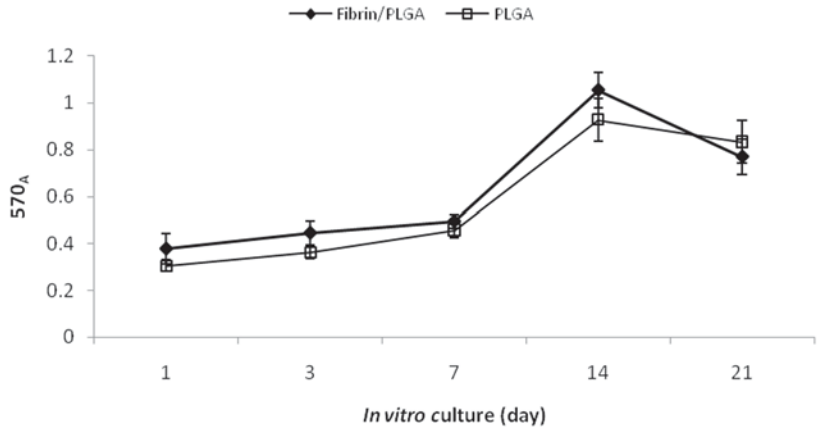


Figure 1. Fibrin/PLGA and PLGA group exhibited similar cell growth pattern *in vitro* (n=6). The MTT assay showed cell proliferation was gradually increased from day 1 until day 7, with fibrin/PLGA showing significantly higher cells proliferation activity ($p< 0.05$) compared to PLGA at day 3. Next, cell proliferation activity in the fibrin/PLGA and PLGA was significantly increased by day 14. The proliferation activity then declined by day 21 in both groups.

a 31µl reaction volume containing oligo(dT)12-18 (500 µg/ml), dNTP mix (10 mM), sterile distilled water, 5X First-strand buffer (250 mM Tris-HCl, pH 8.3 at room temperature, 375 mM KCl; 15 mM $MgCl_2$), 0.1 M DTT, RNaseOUT™ (40 units/µl and Superscript™ II RT under the following conditions: 65 °C for 5 minutes, 42 °C for 2 minutes, 42 °C and 70 °C for 50 minutes and 15 minutes respectively). Polymerase chain reaction protocol was carried out in a 20 µl reaction volume containing 10 µl of 2x concentrated PCR master mix (Roche Applied Science, Germany), 3 µl of reverse-transcribed reaction mixture, 10 µM primers and sterile PCR water (Roche Applied Science) using the Takara Thermal Cycler (Takara Bio Inc. Japan). After initial denaturation of the templates at 94°C, polymerase chain reaction was performed for 25 cycles at 94°C for 30 seconds, at 52° C for 30 seconds, at 72°C for 30 seconds and final extension at 72°C for 5 minutes. The PCR primers for detecting collagen type II, aggrecan core protein and collagen type I were designed from previous sequences as reported elsewhere (Ha *et al.* 2006; Yamazaki *et al*. 2002). Primer sequences were as follows: collagen type II: forward: 5'-gcacccatggacattggaggg-3' / reverse: 5'-atgttttaaaaaatacgaag-3' (Ha *et al.*, 2006). Aggrecan core protein: forward: 5'-atcaacagagacctacgatgt-3' / reverse: 5'-gttagggtagaggtagaccgt-3' (Yamazaki *et al*., 2002). Collagen type I: forward: 5'-gatgcgttccagttcgagta-3' / reverse: 5'-ggtcttccggtggtcttgta-3' (Ha *et al*., 2006). The gene for rabbit β-actin was used as an endogenous control. The sequences for rabbit β-actin primer set (Yamazaki *et al*., 2002) were: forward: 5'-ccggcttcgcgggcgacg-3' / reverse: 5'-tcccggccagccaggtcc-3'. All primers were prepared by GenoTech. Corp. (Daejeon, Korea). 6 µl of the amplified PCR products were separated by 1.5% agarose gel electrophoresis (SeaKem® LE Agarose, Cambrex Bio Science, Rockland, ME, USA), stained with SYBR® Green Nucleic Acid Gel stain (Cambrex Bio Science) and visualised by UV transillumination, using a gel documentation system EDAS 290 Kodak (Viber Lourmat, Touray, France).

Sulphated glycosaminoglycan (sGAG) production assay

All samples were lyophilised and treated with papain digestion solution (125 µg/ml of papain, 5 mM L-cysteine, 100 mM Na_2HPO_4, 5 mM EDTA, pH 6.8) at 60°C for 16 hours. The sGAG contents were analyzed using a 1,9-dimethylmethylene blue (DMMB) assay (Whitley *et al*., 1989). Each sample was mixed with the DMMB solution and the absorbance was measured at 590 nm wavelength. Total sGAG of each sample was extrapolated using a standard plot of shark chondroitin sulphate (Sigma Aldrich) in the range of 0-50 µg/ml and normalized by the dry-weight of each sample (n=6), as a relative sGAG content (%). The dry-weight of each sample was determined after lyophilisation. Data was expressed as mean ± standard error of the mean (SEM) of 6 samples (n=6). Results were analyzed using Student's *t*-test and the difference are considered significance when $p<0.05$.

Results

Cell proliferation and viability

After cell seeding, cells adhered to the scaffolds, proliferated and produced cartilaginous matrices to fill void spaces in the scaffold. The fibrin/PLGA hybrid construct and PLGA (control) constructs exhibited similar cell growth pattern, as determined by means of MTT assay (Figure 1). From the chart, it can be seen that cell proliferation increased gradually from day-1 until day-7. The fibrin/PLGA hybrid construct showed a significantly higher cell viability and proliferation activity, compared to the PLGA at day-3. Next, by day-14, cell proliferation in the fibrin/PLGA and PLGA had increased significantly by 2.13x and 2.03x, respectively. Cell proliferation then declined by day-21, in both groups. It has been indicated that the early stage of chondrogenesis involves the activity to establish cell-to-cell communication and cell-to-matrix interaction, with regards to new cartilaginous tissue

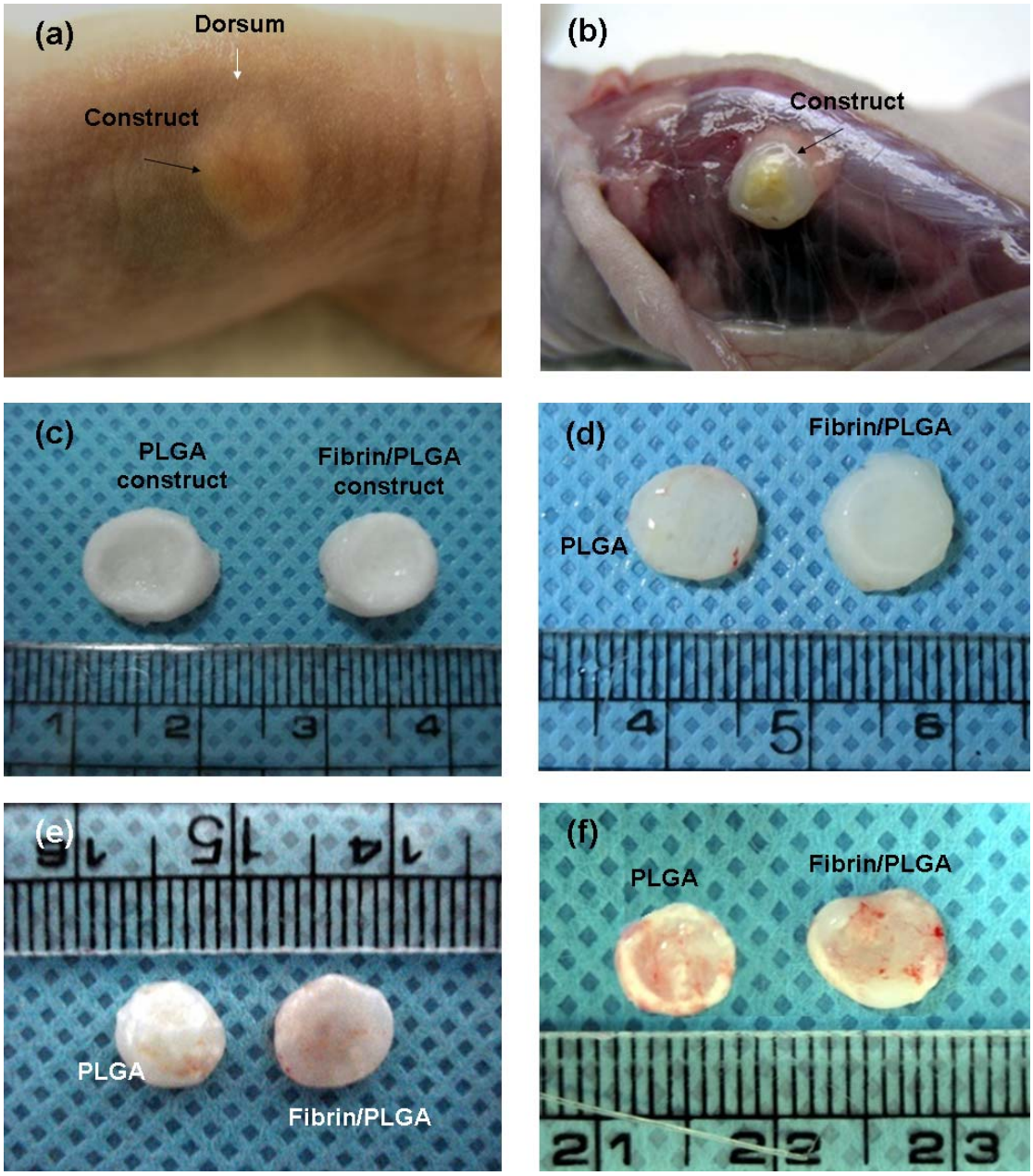


Figure 2. Figure 2a represents the subcutaneously implanted construct at the dorsum part of athymic nude mice. No evidence of superficial infection or fistula formation was demonstrated in the nude mice (Figure 2b). Figure 2c represents *in vitro* fibrin/PLGA and PLGA constructs before implantation. At time of explantation, gross examination showed no evidence of malignant invasion in any of the specimens. A thin vascularised capsule was observed surrounding the constructs. All fibrin/PLGA and PLGA constructs exhibited smooth and glistening appearance at each time point of 1 week (Figure 2d), 2 week (Figure 2e) and 4 weeks (Figure 2f).

formation, which causes cellular proliferation to become less active. This may be a possible explanation to a significant reduction of cell proliferation by 1.37x in the fibrin/PLGA construct after 21 days.

Macroscopic observation of *in vivo* constructs

After being cultured for 3 weeks *in vitro*, fibrin/PLGA hybrid and PLGA constructs were implanted subcutaneously at the dorsum of athymic nude mice (Figure 2a). After 1, 2 and 4 weeks implantation, no evidence of superficial infection or fistula formation was demonstrated in the nude mice. All constructs were easily dissected from the subcutaneous tissue (Figure 2b). After implantation, both fibrin/PLGA and PLGA constructs maintained their original rounded cylindrical shape, with a slight reduction in the size when compared to *in vitro* specimens. However, no significant variation was found between fibrin/PLGA and PLGA. Figure 2c represented *in vitro* fibrin/PLGA hybrid and PLGA constructs before implantation. All fibrin/PLGA hybrid and PLGA constructs exhibited smooth and glistening cartilage-like properties at each time point of 1 week (Figure 2d), 2 weeks (Figure 2e) and 4 weeks (Figure 2f) after implantation. Gross examination showed no evidence of malignant invasion in any of specimens. A thin vascularised capsule was observed surrounding the construct. Fibrin/PLGA hybrid and PLGA constructs preserved their original rounded shapes throughout the 4 week implantation.

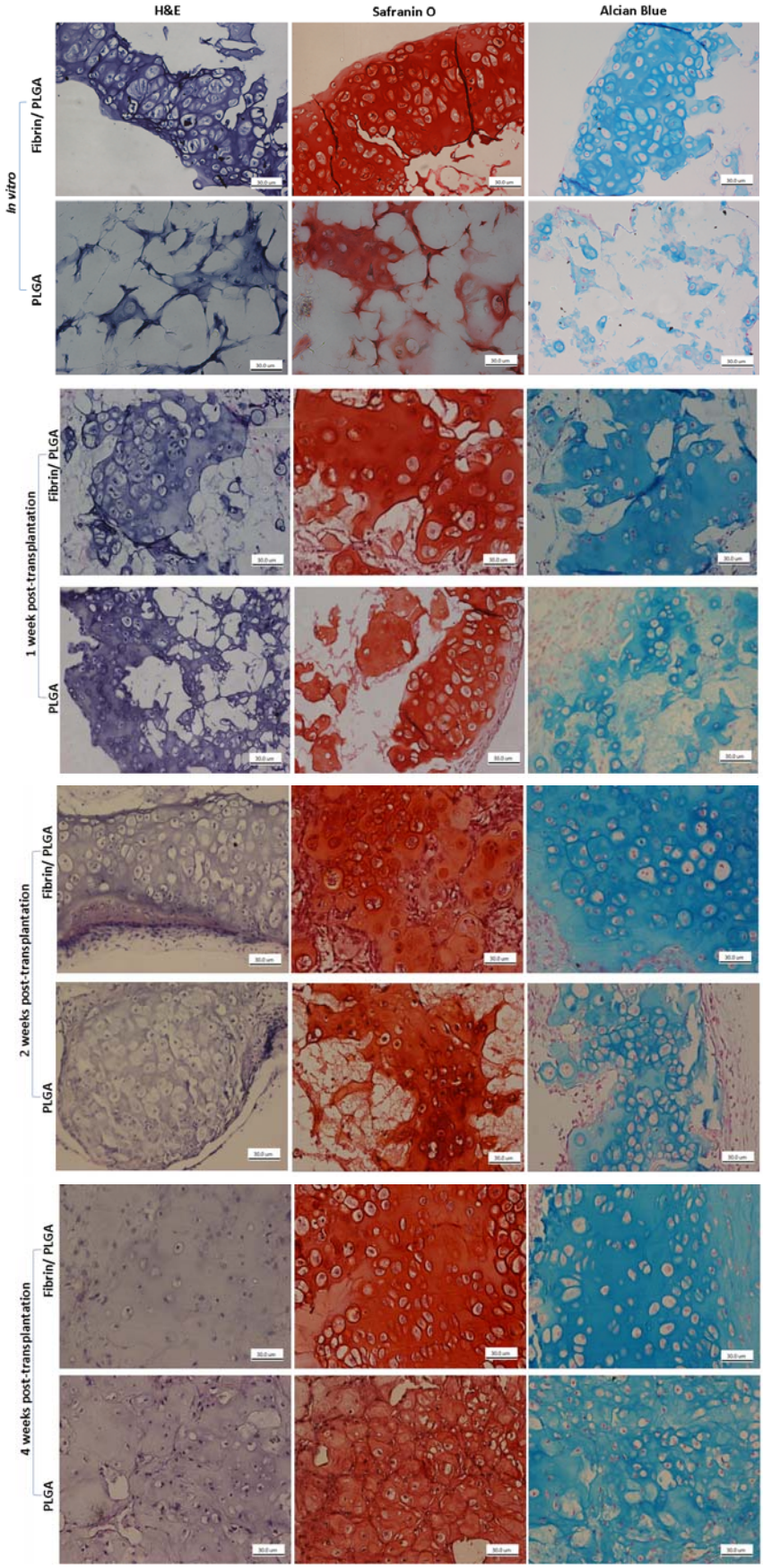


Figure 3. Before implantation (*in vitro*), a greater number of round morphological chondrocytes embedded in basophilic ECM were observed in fibrin/PLGA than in PLGA. The pericellular and inter-territorial matrix was strongly stained by Safranin O, to indicate presence of proteoglycan-rich matrix. The deposition of accumulated GAG was further demonstrated by positive Alcian blue staining. After 1, 2 and 4 weeks implantation, the *in vivo* fibrin/PLGA construct demonstrated superior cartilaginous histoarchitecture properties, when compared to PLGA, in terms of cells distribution, cells-matrix organization and overall ECM production.

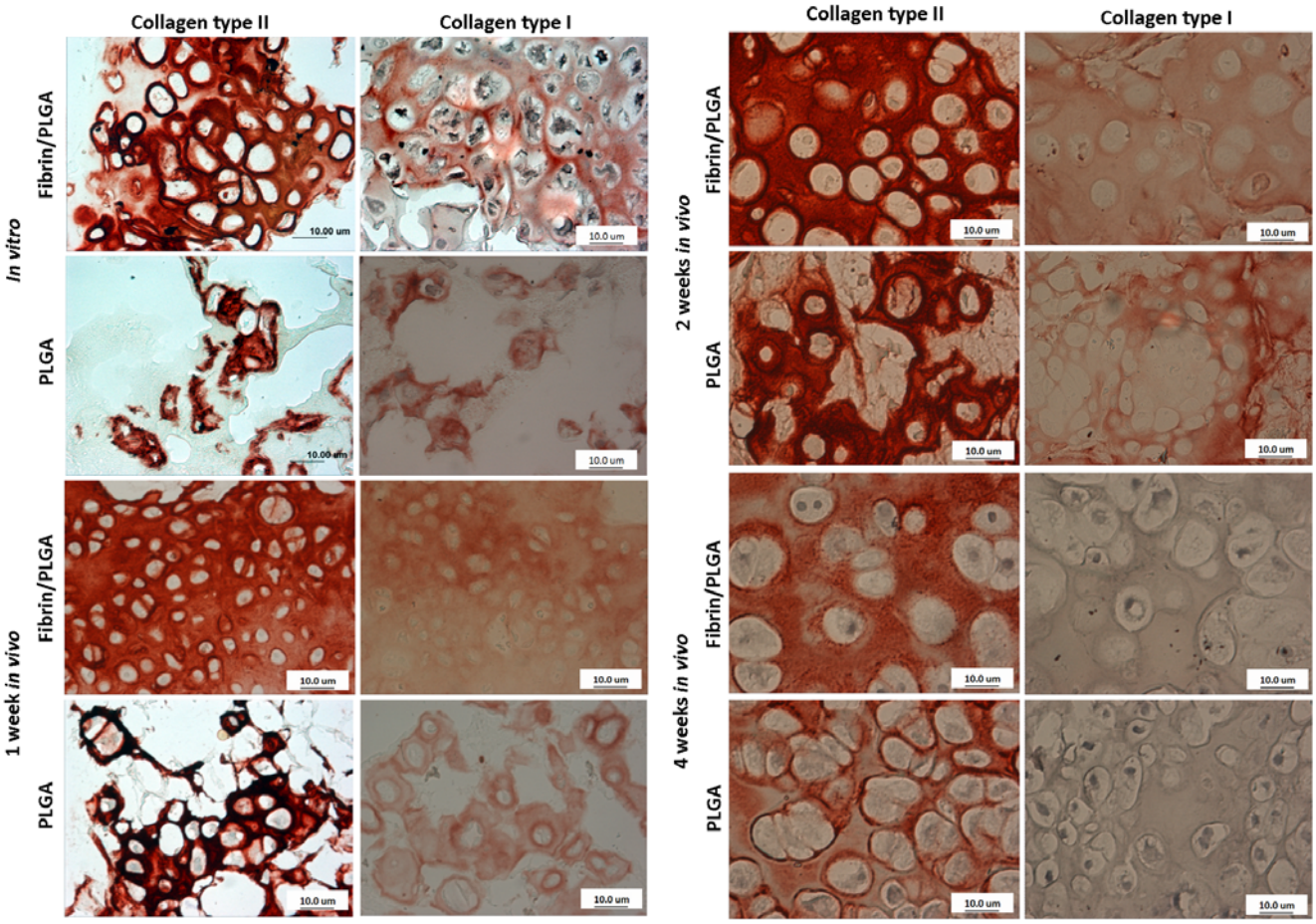
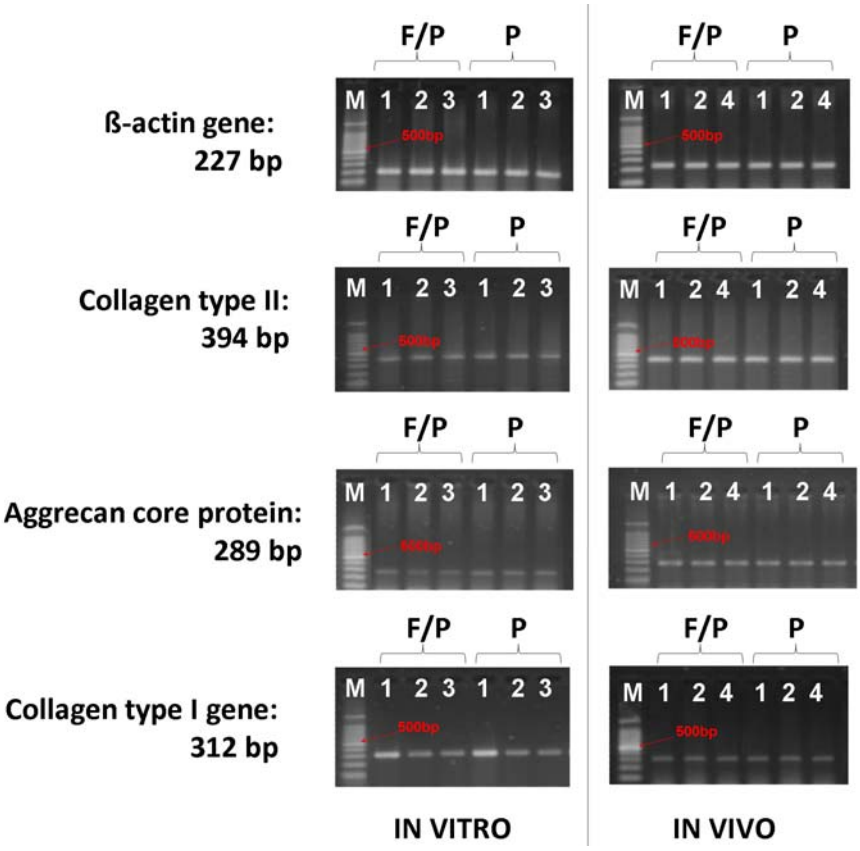


Figure 4. Collagen type II exhibited strong immunopositivity in the *in vitro* fibrin/PLGA constructs, mainly localised surrounding pericellular and inter-territorial matrix. After implantation, fibrin/PLGA showed more homogeneous distribution of collagen type II than PLGA. Collagen type I in fibrin/PLGA showed moderate immunopositivity throughout the ECM of specimens. Interestingly, after 4 weeks implantation, collagen type I showed either very weak or no expression in both fibrin/PLGA and PLGA.

Figure 5. Gel photos are representative of the mRNA expression analysis of *in vitro* and *in vivo* fibrin/PLGA (n=6) and PLGA constructs (n=6) using RT-PCR. The expression of genes encoded cartilage-specific markers; collagen type II and aggrecan core protein was gradually expressed in all constructs. Collagen type I was down-regulated in fibrin/PLGA and PLGA group after implantation. β-actin gene was steadily expressed in all samples; to verify the analysis was reliable and successful.

Notes:
M: 100bp marker
F/P: fibrin/PLGA
P: PLGA

IN VITRO:
1: week 1, **2**: week 2, **3**: week 3
IN VIVO:
1: week 1, **2**: week 2, **4**: week 4



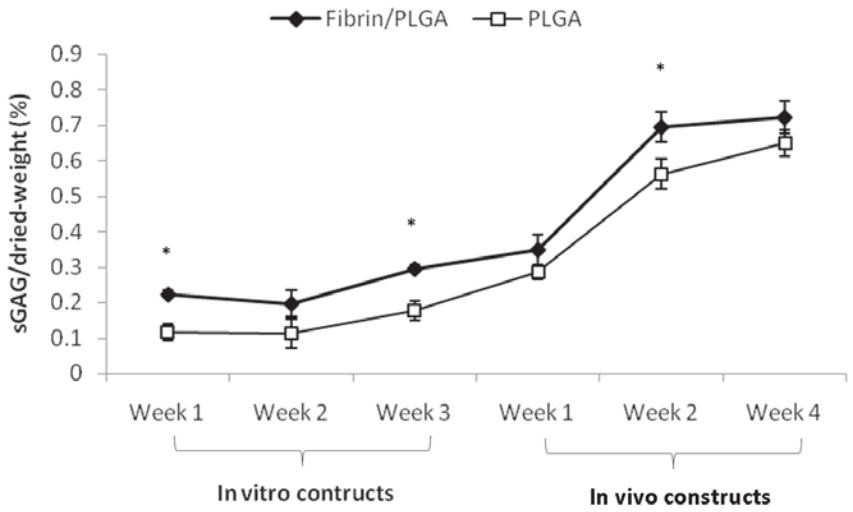


Figure 6. sGAG production in fibrin/PLGA was better than in PLGA at each time point before and after implantation (n=6). The relative sGAG contents (%) were significantly higher (p<0.05) in fibrin/PLGA compared to PLGA at week 1 and week 3 *in vitro* and after 2-week implantation (*).

Histological evaluation of *in vitro* and *in vivo* constructs

The morphology of cells and distribution of cartilaginous ECM in the fibrin/PLGA hybrid and PLGA scaffolds were examined by means of histological staining; namely H&E, Safranin O staining and Alcian blue staining (Figure 3). Before implantation, H&E staining showed that chondrocyte clusters and ECM had filled void spaces of fibrin/PLGA hybrid scaffold. *In vitro* fibrin/PLGA constructs exhibited superior histo-architectural characteristics of cartilage-like tissue, when compared to the PLGA group. Closely-packed cartilage-isolated cells were homogeneously distributed in the basophilic ground substance. The pericellular and inter-territorial matrix was strongly stained by the characteristic red of Safranin O, to indicate presence of proteoglycan-rich matrix. Cartilaginous ECM deposition was further demonstrated by positive Alcian blue staining to confirm presence of accumulated GAG. Next, formation of cartilaginous tissue in fibrin/PLGA hybrid constructs was remarkably evidenced at each time point of 1, 2 and 4 weeks after *in vivo* implantation. The difference between fibrin/PLGA hybrid construct and control group was clearly visible in term of overall cartilaginous tissue formation, cells organization and ECM distribution in the specimens. Fibrin/PLGA constructs exhibited good quality cartilage-like tissue, when compared to the control group.

Immunohistochemistry analysis

We analysed collagen type II and collagen type I immunolocalization in the fibrin/PLGA hybrid construct, and compared it with PLGA group at 1, 2 and 4 weeks post-implantation (Figure 4). Collagen type II was detected in all specimens. Collagen type II exhibited strong immunopositivity at the specific region of *in vitro* fibrin/PLGA hybrid construct, mainly localized at the pericellular and inter-territorial matrix. After *in vivo* implantation, fibrin/PLGA hybrid construct showed more homogenous collagen type II distribution than in the PLGA scaffold, at each time point. Analysis of collagen type I in fibrin/PLGA hybrid constructs showed moderate immunopositivity throughout ECM, at each time point of 1 and 2 weeks post-implantation. Interestingly, after 4 weeks implantation, 75% of implanted constructs showed no collagen I expression, whilst the rest showed very weak expression.

Cartilage-specific phenotypic expression analysis

When the mRNA expression of the fibrin/PLGA hybrid construct and PLGA group were compared, both showed comparable potential in sustaining the specific chondrogenic phenotype. The expression of gene encoded cartilage-specific markers, collagen type II and aggrecan core protein, was steadily observed in *in vitro* culture – whereas collagen type I, the cartilage dedifferentiation marker, exhibited down-regulation pattern after 2 weeks *in vitro*. After implantation, collagen type II and aggrecan core protein mRNA expression was better than *in vitro* specimens. Collagen type I was detected in all *in vivo* specimens, but the expression was weaker than in the *in vitro* samples. The house-keeping gene, β-actin was steadily expressed in all specimens, to verify that the analysis was reliable and successful. Results were summarised in Figure 5.

Sulphated glycosaminoglycan (sGAG) production assay

Normalised by dry-weight of each sample (n=6), the relative sGAG content (%) was significantly higher (p<0.05) in fibrin/PLGA hybrid constructs compared to control at 1 week and 3 week *in vitro* culture. With 0.223±0.010 relative sGAG content, fibrin/PLGA hybrid constructs exhibited 1.92x higher sGAG production than the PLGA group; 0.116±0.025 at week 1. At week-2 there was no significant difference between the two groups, with the relative sGAG content in fibrin/PLGA hybrid constructs being 0.197±0.037 and PLGA group 0.113 ±0.042. By week 3, fibrin/PLGA hybrid constructs exhibited 0.296± 0.011 relative sGAG content, which was 1.67x higher than the relative sGAG content in the PLGA group; 0.177± 0.027. After implantation, the relative sGAG

content (%) in fibrin/PLGA hybrid construct was given by 0.349 ± 0.043 (1 week), 0.696 ± 0.042 (2 weeks) and 0.723 ± 0.045 (4 weeks). The relative sGAG content in fibrin/PLGA hybrid group increased significantly by 2 weeks post-implantation by 1.99x. While, in PLGA construct, the relative sGAG content (%) was 0.286 ± 0.021 (1 week), 0.562 ± 0.042 (2 weeks) and 0.650 ± 0.038 (4 weeks). Results were summarised in Figure 6.

Discussion

Tissue-engineered cartilage constructs should meet certain criteria in order to improve surgical handling and to withstand mechanical loading. Since synthetically derived biodegradable polymers can be moulded easily into the desired shapes with proper mechanical strength, they have been used as a skeleton for a mechanically weak natural polymer to withstand tissue collapse. The hybrid polymers used in this study are a Food and Drugs Administration (FDA) approved copolymer, lactic acid and glycolic acid, and the commercially available natural polymer fibrin from Greenplast®. Fibrin was reported to be an ideal cell carrier (Eyrich *et al*., 2007), to provide homogenous cells distribution without significant cell loss, during cell seeding procedures (Zheng *et al*., 2006). In this study, we evaluated the formation of *in vivo* cartilaginous tissue using rabbit articular chondrocytes seeded in fibrin/PLGA hybrid scaffold at each time point of 1, 2, and 4 weeks post-implantation.

Our *in vitro* results indicated that chondrocytes-seeded in fibrin/PLGA secreted cartilage-specific ECM molecules and developed chondrocyte-chondrocyte interaction, to form clusters of various sizes, while preserving the original shapes of cells. Immobilization of cells in fibrin resulted in homogenous cell distribution in PLGA scaffolds. Moreover, the deposition of newly developed cartilaginous ECM in fibrin/PLGA was histologically superior to that of PLGA group. This phenomenon may be due to higher cell seeding efficiency and homogeneity in fibrin/PLGA than in PLGA. Homogenous distribution of cells was also observed during the osteogenesis of human periosteum-derived progenitor cells in fibrin/PLGA (Zheng *et al*., 2006), while Lee *et al*. (2005) reported superior uniformity of chondrocytes, distributed in fibrin-filled polyurethane scaffold, when compared to polyurethane scaffold without fibrin.

After implantation, both fibrin/PLGA and PLGA constructs maintained their original rounded cylindrical shape, with a slight reduction in the size when compared to *in vitro* specimens. However, no significant variation was found between fibrin/PLGA and PLGA. All constructs showed cartilage-like properties with a white, smooth and glistening appearance, as reported elsewhere (Munirah *et al*., 2005, 2007). Fibrin/PLGA showed histologically mature chondrocytes and extensive development of cartilaginous ECM, indicated by presence of an abundant proteoglycan-rich matrix and accumulated GAG. Conversely, the PLGA group exhibited minimal cartilage histoarchitecture *in vitro* – indicated by few rounded cells in the void spaces of the scaffold. The entire PLGA scaffold was covered with newly developed cartilaginous ECM after only 4 weeks implantation. The incorporation of fibrin into the scaffold visibly facilitated formation of high quality cartilaginous tissue (Endres *et al*., 2007). Conversely, Jin *et al.* (2007) reported that *in vitro* differentiated human adipose-derived stem cells, seeded in PLGA, rapidly lost their chondrocytic phenotype and failed to produce cartilaginous tissue *in vivo*. Only fibrous tissues and angiogenesis invasions were observed after 4 weeks implantation.

Although there were remarkable histological differences between fibrin/PLGA and PLGA groups, there were no significant variations in the semi-quantitative gene expression for collagen type II, aggrecan core protein or collagen type I. Cartilage-specific chondrocyte phenotype was maintained in both groups. However, the expression of collagen type II and aggrecan core protein, improved after *in vivo* implantation, suggesting a mature cartilaginous phenotype. Interestingly, collagen type I expression demonstrated a down-regulation pattern after 2 and 3 weeks *in vitro*. However, the expression remained visible after 4 weeks implantation. On the contrary, immunohistochemistry against collagen type I denoted that 75% of implanted constructs showed no collagen I expression after 4 weeks implantation. This contradictory observation may have been due to the discrepancy between mRNA level and protein level analysis. Sasano *et al*. (1999; 2001) reported chondrocytes that rat tibial articular cartilage synthesised collagen type I and accumulated protein in ECM, during articular cartilage development. Absence of appropriate mechanical stimuli in the subcutaneous dorsum is believed to elicit some unfavourable effects on the maturation of neo-cartilage (Chen *et al.*, 2004). Accordingly, the arrangement of articular chondrocytes in fibrin and PLGA scaffolds is an excellent system for maturation of cartilaginous tissues. Significant elevation of sGAG after implantation suggested progress towards development of cartilaginous tissue in fibrin/PLGA. If this result is applicable for future clinical use, a better outcome would be obtained than by using the currently available treatment methods.

Conclusions

Fibrin/PLGA scaffold promotes cellular proliferation and cartilaginous tissue formation of rabbit articular chondrocytes. This study suggests that fibrin/PLGA hybrid scaffold may serve as a potential cell delivery vehicle and a structural basis for tissue-engineered articular cartilage. Future study utilising autologous *in vivo* system in big animal model is necessary to validate the feasibility of fibrin/PLGA hybrid scaffold and cells composite for articular cartilage restoration.

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Discussion with Reviewers

S. Grad: The pore size of 90-180μm appears to be relatively small. A proper cell seeding procedure is therefore crucial to achieve a uniform cell distribution. It is unclear how the cells were seeded, since the descriptions in the abstract and methods section vary. Were different seeding methods evaluated? Also, a common observation in chondrocyte-seeded scaffolds is the development of a prominent layer rich in extracellular matrix at the edges of the constructs. Was this phenomenon also noticed in PLGA and/or fibrin/PLGA constructs?

Authors: The reviewer is correct. Initially, we seeded the cells directly onto scaffold but, as the reviewer mentioned above, a common observation in chondrocyte-seeded scaffolds is the development of a prominent layer rich in extracellular matrix at the edges of the constructs. Therefore, we tried to soak the scaffold carefully in cells-fibrin admixture and cell suspension in culture medium. To achieve a uniform cell distribution, we introduced some mechanical force, using a pipette, to aid cellular infiltration throughout the scaffold.

S. Grad: How do the amounts of sGAG/dry weight compare to values of native articular cartilage?

Authors: The aim of this study was not to regenerate a complete native cartilage tissue, that would be ideal for replacing articular cartilage loss, but more towards developing a cell delivery system for effective cartilaginous tissue formation from chondrocytes, as well as to demonstrate the applicability of fibrin-cells-PLGA complex. This is the reason why we only compared fibrin/PLGA and PLGA.

D. Eglin: At the concentration of fibrinogen used, the viscosity of the solution is very high and by dropping the thrombin-$CaCl_2$ on the top of the scaffold, without mixing, it is likely that the homogeneity of the gel in the PLGA scaffolds may not be optimal and the gel formation (e.g., cross-link density) may also be heterogeneous. (more cross-linking on the surface than in the bulk of the PLGA scaffolds. Did the authors check the fibrin gel homogeneity in the PLGA scaffolds?

Authors: 65–115 mg/ml and 400–600 units/ml is a working concentration of fibrinogen and thrombin recommended by the manufacturer for general procedure during surgery. However, since we were using cells, fibrinogen and the thrombin-$CaCl_2$ solution concentrations had to be modified. The fibrinogen concentration was diluted to approximately 30-60 mg/ml, with culture medium, and the thrombin solution was diluted to 10-50 units/ml, with $CaCl_2$ solution, to achieve at least 15 to 30 sec clotting time – as suggested by the manufacturer. This has been included in the text. We did not check the fibrin gel homogeneity in PLGA, as such, but we did notice a homogenous formazan crystal distribution in the fibrin/PLGA construct as well as the PLGA construct, during the MTT assay. We believe this phenomenon was very much associated with cells-fibrin homogeneity in PLGA. Furthermore, histological evaluation of *in vitro* constructs demonstrated a homogenous cells distribution in fibrin/PLGA scaffold.