



# The potential of 3-dimensional construct engineered from poly(lactic-co-glycolic acid)/fibrin hybrid scaffold seeded with bone marrow mesenchymal stem cells for *in vitro* cartilage tissue engineering



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## ABSTRACT

Articular cartilage is well known for its simple uniqueness of avascular and aneural structure that has limited capacity to heal itself when injured. The use of three dimensional construct in tissue engineering holds great potential in regenerating cartilage defects. This study evaluated the *in vitro* cartilaginous tissue formation using rabbit's bone marrow mesenchymal stem cells (BMSCs)-seeded onto poly(lactic-co-glycolic acid) PLGA/fibrin and PLGA scaffolds. The *in vitro* cartilaginous engineered constructs were evaluated by gross inspection, histology, cell proliferation, gene expression and sulphated glycosaminoglycan (sGAG) production at week 1, 2 and 3. After 3 weeks of culture, the PLGA/fibrin construct demonstrated gross features similar to the native tissue with smooth, firm and glistening appearance, superior histoarchitectural and better cartilaginous extracellular matrix compound in concert with the positive glycosaminoglycan accumulation on Alcian blue. Significantly higher cell proliferation in PLGA/fibrin construct was noted at day-7, day-14 and day-21 ( $p < 0.05$  respectively). Both constructs expressed the accumulation of collagen type II, collagen type IX, aggrecan and sox9, showed down-regulation of collagen type I as well as produced relative sGAG content with PLGA/fibrin construct exhibited better gene expression in all profiles and showed significantly higher relative sGAG content at each time point ( $p < 0.05$ ). This study suggested that with optimum *in vitro* manipulation, PLGA/fibrin when seeded with pluripotent non-committed BMSCs has the capability to differentiate into chondrogenic lineage and may serve as a prospective construct to be developed as functional tissue engineered cartilage.

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## 1. Introduction

Articular cartilage is a simple but highly organized structure that is avascular, aneural and has little ability for self-regeneration. The possibility for spontaneous self-repair will be lacking if any of the chondral lesions do not penetrate the underlying subchondral bone

(Yang et al., 2012). Clinically, significant debilitation can result from the wear and tear or even minor injury to the articular cartilage. Over time, osteoarthritis may develop in the afflicted joint, which is ultimately requiring knee replacement or other invasive treatment (Nehrer et al., 2008).

Early intervention may help to prevent damage from spreading to surrounding healthy cartilage and to prevent further deterioration to the joint surface. Although current concepts of clinical treatment for cartilage injuries are quite promising, e.g. drilling or microfracture (Steadman et al., 2001), abrasion arthroplasty

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(Johnson, 2001), mosaicplasty (Bentley et al., 2003), autologous chondrocytes implantation (ACI) (Brittberg et al., 2001) and prosthetic joint replacement (Hunziker, 2002), yet no definitive treatment has resulted in complete satisfactory restoration of the articular cartilage to a normal state (Hunziker, 2002; Nestic et al., 2006).

In concordance to the current treatment, tissue engineering has emerged as an alternative approach in restoring cartilage defect. The principles of tissue engineering involve three major components which include the utilization of quality cell sources, biocompatible scaffolds and conducive signalling molecules (Langer and Vacanti, 1993).

An ideal cell source for cartilage tissue engineering should be easily accessible and able to produce rich extra-cellular matrix compound especially the collagen type II and aggrecan (Rozlin et al., 2013). Being a committed sole cell type of articular cartilage, chondrocytes is an ideal cell source that is proven to be clinically safe and efficient (Roelofs et al., 2013). It has been used in autologous chondrocyte implantation for more than two decades (Mobasher et al., 2014). However, additional surgery is needed to harvest the chondrocytes from the non-load-bearing area of the same joint. On top of that, it is technically difficult to acquire enough number of cells during harvesting (Roelofs et al., 2013) and chondrocytes tend to lose their phenotype during culture-expansion (Schnabel et al., 2002). Because of the limitations related to chondrocytes, many researchers are now looking for other cell sources possibilities. One of many cell sources, mesenchymal stem cells (MSCs) has been widely used as an important and potential cell source in many studies (Haugh et al., 2011; Kim et al., 2013; Zhai et al., 2011). MSCs are multipotent stem cells that can divide into a number of cell types including; chondrocytes, osteoblasts and adipocytes (Eca et al., 2009). Interestingly, accessible and high capacity of *in vitro* expansion makes MSCs as an attractive source of cells for cartilage tissue engineering (Vinatier et al., 2009). Even though the use of chondrocytes for cartilage tissue engineering is prevalent, concerns related with cell differentiation, the limited life span of these cells and donor site morbidity have brought the usage of MSCs to the prominence of such applications (Tuli et al., 2003).

Biomaterial scaffolds in tissue engineering have been developed using natural and synthetic polymers. Among the few synthetic polymers approved by the Food and Drug Administration (FDA) which has extensively been exploited clinically is poly(lactic-co-glycolic acid) or PLGA. It is widely been utilized in tissue engineering field and used as a drug slow-release biomaterial owing to its biodegradability, biocompatibility, low immunogenicity, good mechanical strength and general stability (Yao et al., 2009). It was indicated that the application of PLGA promotes chondrogenic morphology and phenotype in *in vitro* and *in vivo* cultures similar to the native cartilage (Baek and Ko, 2006; Baek et al., 2002). PLGA/fibrin scaffolds were also reported to facilitate cells growth, matrix production and early chondrogenesis in *in vitro* environment (Sha'ban et al., 2008). Our previous studies for articular cartilage restoration focused on engineering autologous cartilage construct using human chondrocytes (Munirah et al., 2010, 2008a, 2005) or ovine chondrocytes (Munirah et al., 2007) incorporated with the autologous fibrin as biomaterial. Fibrin is believed to be an excellent cell transporter as it provides homogenous distribution of cells with minimal cell lost during seeding technique (Munirah et al., 2008b,c; Sha'ban et al., 2008). Unlike synthetic polymer, fibrin is a natural and biologically active polymer involves in wound healing process. It promotes cell adhesion and it is used as a mechanical and structural stable scaffold in tissue engineering research.

The third important aspect in tissue engineering is the signalling factors. The governing principle of signalling factors is that, the fate of cell is affected by cell's interactions with their microenvironment components (Rozlin et al., 2015). These may include soluble

and immobilized factors, the extracellular matrix, as well as signals presented by the adjacent cells. The two major types of signalling factors namely biochemical factors (e.g. nutrients and growth factors) and, physical stimulation (e.g. compression and tension) are essential to guide cell growth and differentiation. Likewise, it is believed that the cell fate has strong association with culture conditions (Munirah et al., 2005). In cell culture, defined culture media will promote cell growth and induce differentiation by providing main regulatory or growth factors (Munirah et al., 2010). Cells require optimal physiological and physical conditions for example oxygen, temperature, pH, three dimensional microenvironment and adequate cell to cell contact. Inadequate signalling factors will lead to loss of specialized function, cells senescence or aging and, eventually cell death (Rozlin et al., 2015).

Therefore, in this study we aimed to evaluate and explore the quality of the *in vitro* engineered construct composed of rabbit bone marrow mesenchymal stem cells (BMSCs) seeded on PLGA/fibrin hybrid scaffold and cultured in a commercially available chondrogenic differentiation media. We hypothesized that the PLGA/fibrin-BMSCs construct will have the ability to support the appropriate cellular activity, in order to optimize tissue regeneration.

## 2. Materials and methods

### 2.1. Cell isolation and culture

Approval for harvest of rabbit bone marrow was granted by the International Islamic University Malaysia Research Ethics Committee (IREC) (reference number: IUM/305/20/4/10). Bone marrow mesenchymal stem cells (BMSCs) were aseptically aspirated from the iliac crest of 16-week-old New Zealand White rabbits ( $n=6$ ). Gradient centrifugation method using Ficoll Paque™ (Ficoll-Paque™ Plus, GE Healthcare, Bio-Science AB, Sweden) was used to isolate mononuclear cells in accordance with the manufacturer protocol. Single cell suspension was then enumerated and evaluated for cell viability using Trypan Blue dye (Gibco, Invitrogen, USA). Cells were plated with the initial seeding of 5000 cells/cm<sup>2</sup> in 6-well plates (Thermo Scientific, Nunclon Delta Surface, Denmark) supplemented with the commercially available chondrogenic culture medium (ChondroEnhance™, TELA Technology, Malaysia). The cells were maintained for 2–4 days before the first medium change. All cultures were maintained in a standard culture conditions of 37 °C and 5% humidified CO<sub>2</sub> (Thermo Scientific, Barnstead Lab Line, USA). The primary culture was subcultured until passage two with the medium changed every 2 days.

### 2.2. Preparation of fresh plasma-derived fibrin

Alcohol swab (Kendall Webcol, Kendall Healthcare, USA) was applied to the aural artery of rabbit's ear. Blood was drawn using multiple sample needles and collected into buffered sodium citrate tube (BD Vacutainer, BD, USA). The blood collected was left at room temperature for 30 min–1 h. It was then transferred into a new Eppendorf tube (Eppendorf, Eppendorf Ag, Germany) and spun at 4800 rpm for 6 min at 4 °C (Mikro 220R, Hettich Zentrifugen, Germany). The clear uppermost layer was carefully collected and filtered using sterile filter membrane (Minisart, Sartorius Stedim Biotech, Taiwan), transferred into another new Eppendorf tube upon usage.

### 2.3. Fabrication of three dimensional (3-D) microporous poly(lactic-co-glycolic acid) (PLGA) scaffolds

PLGA (Sigma–Aldrich) co-polymer (mole ratio 50:50, molecular weight 33 kg/mol, Resomer RG 503 H) was purchased from

Boehringer-Ingelheim Pharma (Ingelheim, Germany). Microporous 3-D PLGA scaffolds were fabricated by solvent casting/salt leaching technique using methylene chloride ( $\text{CH}_2\text{Cl}_2$ ) (Merck, Merck Schuchardt OHG, Germany) as previously described (Khang et al., 2003; Munirah et al., 2008a,b,c). In this method, sieved sodium chloride (NaCl) (Merck, Merck KGaA, Germany) particles (300–350  $\mu\text{m}$ ) were dissolved in a polymer/solvent solution (0.2% w/v concentration of PLGA in methylene chloride) which then casted into a cylindrical disk scaffold using a silicone mould (7 mm in diameter and 3 mm in thickness). The salt particles were leached out by continuous soaking in ultra-pure water for 48 h. The scaffolds were then freeze-dried for 24 h using freeze-dryer (EyeLa FDU-1200, Tokyo Rikakikai, Japan) to remove any remaining solvent. The 3-D PLGA scaffolds were sterilized with 70% ethanol (Leica Microsystems, Surgipath®, USA) and washed three times with phosphate buffered saline (Gibco, Invitrogen™, USA) before use.

#### 2.4. In vitro three dimensional (3-yD) construct formation

The scaffolds were assigned into two experimental groups—cultured BMSCs were seeded onto (1) PLGA scaffolds with fibrin (PLGA/fibrin) and (2) PLGA scaffolds without fibrin. Cultured BMSCs from passage 2 (P2) were used. Cells were counted for total cell and viability using haemocytometer ((Silverlite, Rohem Instruments, India). Approximately, 100,000 cells per scaffold were incorporated and resuspended in the (1) plasma-derived fibrin and (2) culture medium. PLGA scaffolds were soaked carefully in the BMSC plasma-derived fibrin suspension and polymerized, by dropping Calcium Chloride ( $\text{CaCl}_2$ ) (Green Cross P.D. Company, Yongin, Korea) solution on to the scaffolds. For control group, PLGA scaffolds were carefully soaked in BMSC suspended in culture medium only. All constructs were placed in a 5%  $\text{CO}_2$  incubator at 37 °C, with the medium changed every day. The commercially available chondrogenic culture medium (ChondroEnhance™) supplemented with  $10^{-7}$  M dexamethasone (Calbiochem, EMD Chemicals, USA) and 1% insulin transferrin selenium (Gibco, Life Technologies, USA) were used as to promote chondrogenic differentiation at this time of point. All constructs were cultured for a maximum of 21 days *in vitro*, in which the constructs were later harvested and evaluated at each time of 1, 2 and 3 weeks.

#### 2.5. Macroscopic observation, histological evaluation and immunohistochemistry analysis

Each construct was observed at room temperature for its gross appearance, shape, colour and was palpated with forceps to assess its mechanical rigidity. All samples were then prepared for standard histological procedure. The samples were fixed with 10% buffered formalin (Surgipath, Leica Biosystems, USA), dehydrated and paraffin embedded. Sections were prepared by cutting at 8  $\mu\text{m}$  thickness stained with Haematoxylin and Eosin (H&E) in order to assess the tissue histoarchitecture, Alcian Blue staining to identify accumulation of glycosaminoglycan (GAG) and Safranin O staining to distinguish proteoglycan-rich matrix.

Immunohistochemistry analysis was performed in accordance to the manufacturer standard protocol (UltraTehc HRP Kit, Immunotech, France). All slides were deparaffinised, rehydrated and pre-treated with 0.1% of Proteinase K (Vivantis, Vivantis Technologies, USA) at room temperature for 30 min. After that, all sections were treated with peroxidase block (Protein Blocking Agent, UltraTehc HRP Kit, Beckman Coulter, France) for 10 min prior to antibody incubation and the slides were washed once with PBS. The primary antibody, monoclonal mouse anti-rabbit collagen type II (Calbiochem® EMD Biosciences Inc., La Jolla) were diluted with antibody DILUENT (1:1000) (DAKO Cytomation, DAKO North

America, USA) and monoclonal mouse anti-rabbit collagen type I (Sigma, Sigma–Aldrich, USA) antibodies were diluted with antibody DILUENT (1:2000) (DAKO Cytomation, DAKO North America, USA) and both were applied to the sections for 90 min. After washing with PBS for 3 times, UltraTek anti-polyvalent biotinylated secondary antibody (UltraTek HRP Kit, Beckman Coulter, France) was applied to the slides for 20 min. The slides were incubated with streptavidin-peroxidase reagent (UltraTek HRP Kit, Beckman Coulter, France) for another 20 min. The slides were washed in PBS for 4 times and after that, the signals were finally visualized as a brownish precipitate, using freshly prepared chromogen substrate 3-amino-9-ethylcarbazole (AEC) (UltraTek AEC Kit, Beckman Coulter, France). Finally sections were counterstained with Mayer's Haematoxylin (Sigma, Sigma–Aldrich, USA) and mounted in permanent aqueous mounting medium (Shandon Immu-Mount, Thermo Scientific, USA).

#### 2.6. Cell proliferation assay

Cell viability was measured at day-1, day-7, day-14 and day-21 of *in vitro* by using 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium-bromide (Merck, Merck KGaA, Germany) that measures the decrease of tetrazolium component into a non-soluble formazan product by the mitochondria of live cells. At each time point, all constructs were transferred into Eppendorf tubes (Eppendorf, Eppendorf Ag, Germany) with 1 ml of new medium. One hundred microlitre of MTT solution (0.5 mg/ml in PBS) was added to all Eppendorf tubes and incubated for 4 h at 37 °C. After that, all construct were transferred into another new tubes and 1 ml per scaffold of dimethylsulfoxide (DMSO, Merck, Germany) was added to solubilise the resulted crystal. One hundred microlitre of solubilised mixture was then pipetted into a 96-well microtiter plates (Nunclon™ Delta Surface, NUNC, Denmark) and the triplicate readings were recorded by using ELISA plate reader (Versamax Microplate Reader, Molecular Devices, USA) at 570 nm—yielding absorbance as a function of viable cell number. The results were analyzed by using Student's *t*-test and the difference was considered significant when  $p < 0.05$ . Data was expressed as mean  $\pm$  standard error of mean (SEM).

#### 2.7. Two-step reverse transcriptase polymerase chain reaction

Total RNA was extracted from 100,000 to 1 million BMSCs isolated from the tissue engineered construct using TRIzol reagent (Invitrogen, Life Technologies, USA). Cell suspension in the TRIzol reagent was centrifuged at 12,000 rpm for 5 min at 4 °C. The liquid phase formed was transferred into a new microcentrifuge tube (Purepak™, Molecular Bioproducts, Inc., USA). Chloroform (Sigma, Sigma–Aldrich, USA) was added and left for 10 min at room temperature. The mixture of liquid phase and chloroform was then centrifuged 12,000 rpm for 15 min at 4 °C resulting into three distinct layer which consist of RNA, protein layer and DNA. The upper most layer containing RNA was transferred carefully into another microcentrifuge tube. Isopropanol (Sigma, Sigma–Aldrich, USA) was added and mixed thoroughly. Poly acryl carrier (Molecular Research Centre, Inc. Cincinnati, OH) was used to help precipitate the total RNA. The total RNA was washed with 75% ethanol and air-dried. Expression of cartilage specific markers namely the collagen type II, collagen type IX, aggrecan core protein, sox-9 and also cartilage dedifferentiation marker collagen type I were evaluated by using a two-step reverse transcription polymerase chain reaction (PCR) technique. Specific primers used are shown in Table 1.  $\beta$ -actin serves as an internal control. The reverse transcription protocol was carried according to the manufacturer's protocol using Superscript™ II Reverse Transcriptase (Invitrogen, Life Technologies, USA). For PCR, after the initial denaturation of the template

**Table 1**  
Primers used for RT-PCR.

Gene name	Primer sequences	Product size (bp)
Collagen type II (GenBank Accession No. XM.002723439.2)	F:5'-CAACAACCAGATCGAGAGCA-3' R:5'-CCAGTAGTACCCTCTTCC-3'	112
Collagen type I (GenBank Accession No. NM.001195668.1)	F:5'-GGTCCTCAGGTCTTCTTGG-3' R:5'-CACCAGGAGCACCGTTGACT-3'	184
$\beta$ -actin (GenBank Accession No. NM.001101683.1)	F:5'-GCCATCCTGCGTCTGGACCT-GGCT-3' R:5'-GTGATGACCTGGCCGTGAG-GCAGC-3'	227
Sox-9 (GenBank Accession No. XM.008271763.1)	F:5'-GGTGCTCAAGGGCTACG-ACT-3' R:5'-GGGTGGTCTTCTTGTGCTG-3'	273
Collagen type IX (GenBank Accession No. XM.008263089.1)	F:5'-ACTGGAGCAATCGGCTTCA-3' R:5'-CCCTTTCACCTTGTGTC-3'	158
Aggrecan core protein (GenBank Accession No. XM.008251726.1)	F:5'-ATCAACAGAGACCTACGATGT-3' R:5'-GTTAGGGGTAGAGGTAG-ACCGT-3'	290

at 94 °C, polymerase chain reaction was performed for 38 cycles at 94 °C for 30 s, at 52 °C for 30 s, at 72 °C for 30 s and final extension at 72 °C for 5 min. The amplified PCR products were separated by 1.5% agarose gel (Vivantis, Vivantis Inc., USA) via electrophoresis (EC 300 XL, Thermo Scientific, USA) stained with ethidium bromide (EMSURE®, Merck KGaA, Germany) and visualized by UV transillumination using gel documentation system Alphamager ® HP (Alpha Imager HP System, Alpha Innotech, USA).

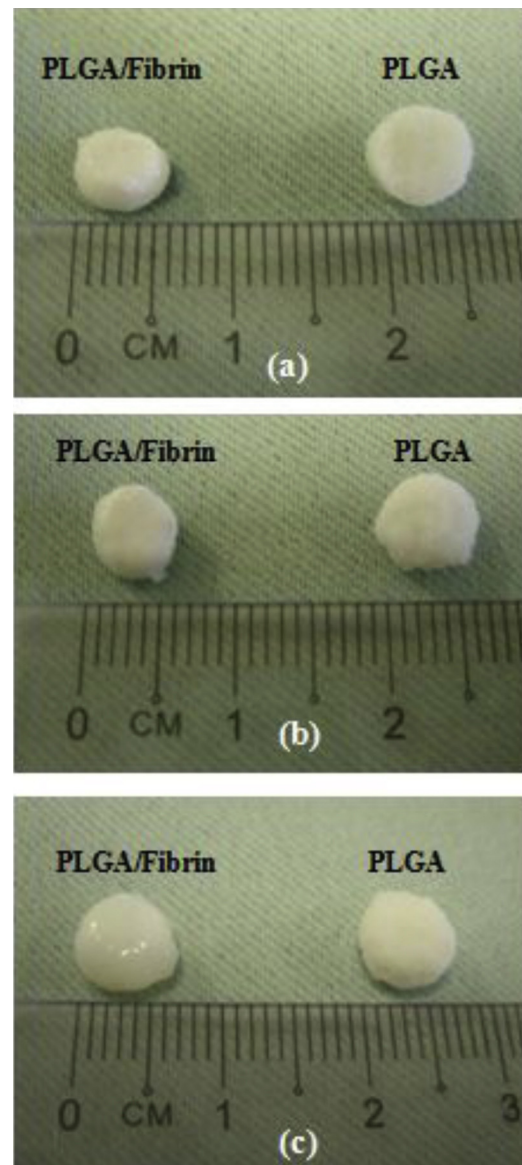
### 2.8. Sulphated glycosaminoglycan (sGAG) production assay

All samples were lyophilised and treated with papain digestion solution (125 µg/ml of papain, 5 mM L-cysteine, 100 mM Na<sub>2</sub>HPO<sub>4</sub>, 5 mM EDTA, pH 6.8) at 60 °C for 16 h. The amount of sGAG content was qualified by using K-Assay® sGAG assay kit (K-Assay®, Kamiya Biomedical Company, USA) following the manufacturer's protocol. The absorbance was measured in 96-well plates using an ELISA plate reader at an absorbance wavelength of 570 nm. Total sGAG of each sample were extrapolated using a sGAG standard solution supplied by the manufacturer and normalized by dried-weight of each sample ( $n=6$ ) as a relative sGAG content (%). The dried-weight of each sample was determined after lyophilisation. Data was expressed as mean  $\pm$  standard error of the mean (SEM). Student's *t*-test was used with  $p < 0.05$  was considered statistically significant. All statistical analysis was performed using statistical package for the social sciences (SPSS) version 15.0.

## 3. Results

### 3.1. Gross morphology of constructs

Fig. 1 shows the gross morphologic changes of the PLGA/Fibrin and PLGA constructs during 1, 2 and 3 weeks of *in vitro* culture. Both constructs demonstrated a slight reduction in the size but maintained the original cylindrical shape throughout the culture period. At week 1 of *in vitro* culture (Fig. 1a), both constructs showed quite similar morphological appearance. At week 2 however, PLGA/Fibrin construct started to demonstrate slightly smoother surface when compared to PLGA construct (Fig. 1b). Towards the end of week 3 (Fig. 1c), the *in vitro* PLGA/Fibrin construct appeared to be whiter, glossier and smoother than PLGA construct.

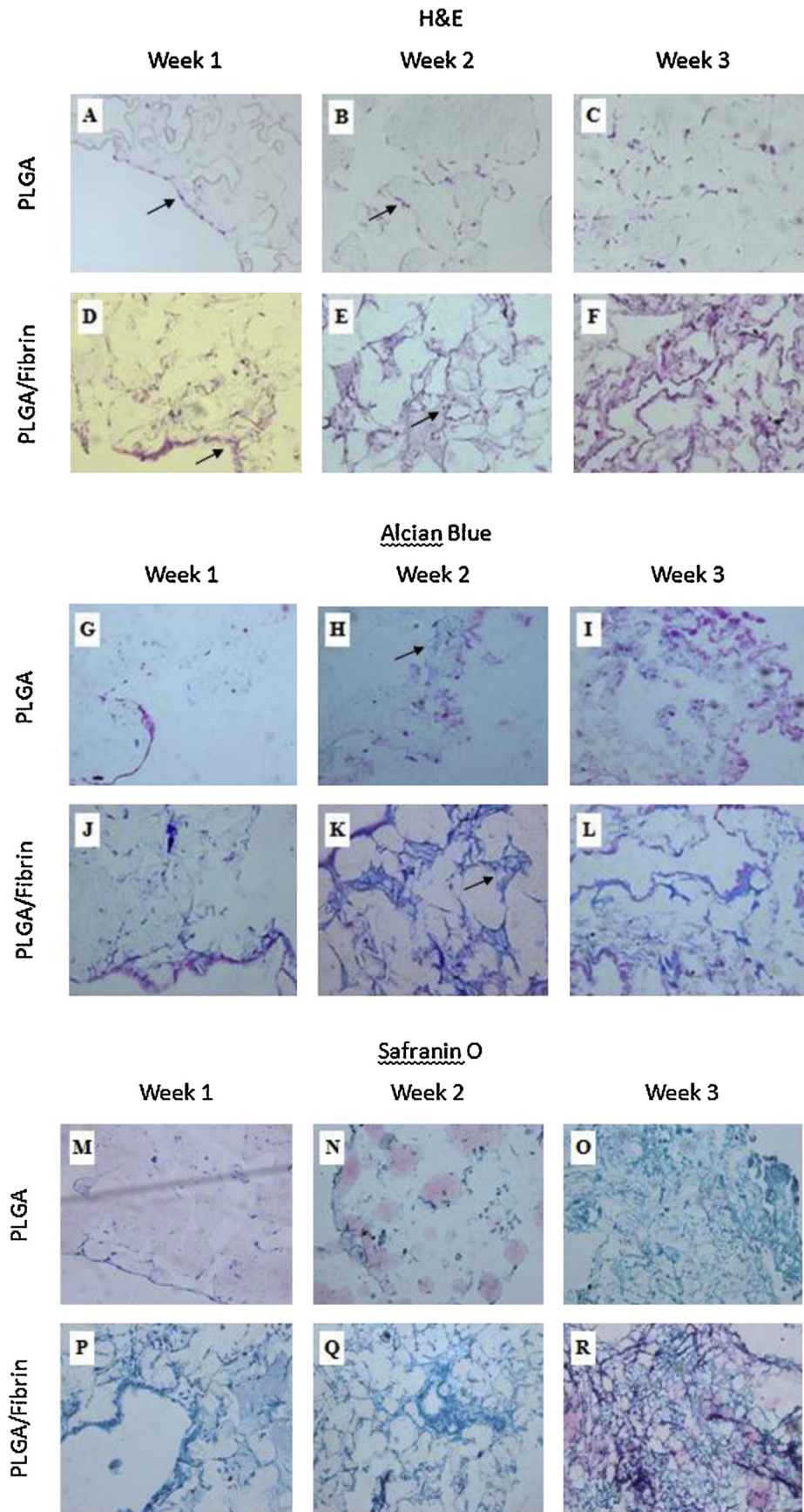


**Fig. 1.** Gross morphology of constructs. Both PLGA/Fibrin and PLGA constructs showed almost similar morphological appearance at week 1 of *in vitro* culture (a). At week 2, PLGA/Fibrin construct demonstrated slightly smoother surface when compared to PLGA construct (b). Towards the end of week 3, the *in vitro* PLGA/Fibrin construct appeared to be whiter, glossier and smoother than PLGA construct (c). Both constructs however appeared to have a slight reduction in size throughout the culture period.

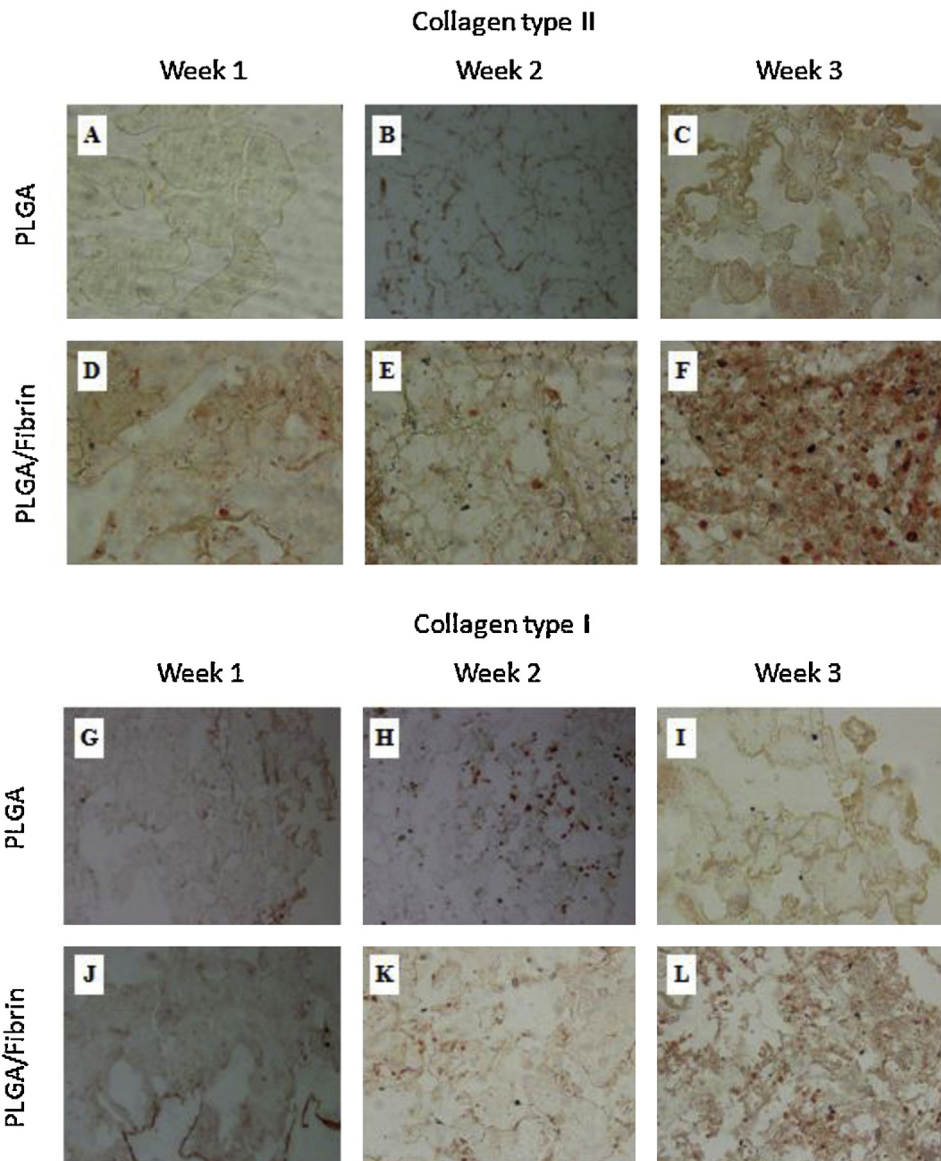
glossier and smoother resembling cartilage-like tissue morphology compared to PLGA construct. On top of that, PLGA/Fibrin construct was noted to be firmer than PLGA construct when palpated with forceps.

### 3.2. Histological evaluation of *in vitro* constructs

The cell morphology and extracellular matrix distribution in PLGA/Fibrin and PLGA constructs were analyzed via histological staining. During the initial culture period for H&E staining (Fig. 2A–F), both constructs demonstrated almost the same extent of cell attachment at week 1 whereby the cells were seen mainly on the peripheral part of the scaffold. However, from week 2 onwards, the cells proliferation rate in PLGA/Fibrin construct appeared to be more superior to PLGA construct in terms of histo-architecture property with increasing time point. Cells were noted to spread



**Fig. 2.** Histological evaluation of the *in vitro* constructs. Histological observations of PLGA/Fibrin and PLGA constructs *in vitro*. H&E (A–F), Alcian Blue (G–L) and Safranin-O (M–R) staining are shown (magnification of 10× and scale bar: 100 μm). Arrow demonstrated the cells (A, B, D and E) and extracellular matrix (H and K).



**Fig. 3.** Immunohistochemistry evaluation of *in vitro* constructs. Immunohistological observations of PLGA/Fibrin and PLGA constructs *in vitro*. The sections were stained with collagen type II (A–F) and collagen type I (G–L) staining (magnification of 10 $\times$  and scale bar: 100  $\mu$ m).

evenly throughout the PLGA/Fibrin construct and produced higher cell density. On the other hand, cells in PLGA construct showed minimal cell proliferation and less cell migration. Cartilaginous extracellular matrix deposition was further visualized by positive Alcian blue staining (Fig. 2G–L) in PLGA/Fibrin construct which confirmed the presence of accumulated GAG. From the second week, cartilaginous matrix noted to fill the void spaces in the PLGA/Fibrin constructs compared to PLGA constructs. Surprisingly the characteristic red staining of Safranin-O (Fig. 2M–R) on the pericellular and interterritorial matrix was not seen in both groups which indicated the non-presence of proteoglycan-rich matrix. Nevertheless the cell organization and extracellular matrix distribution were indeed far more enhanced in PLGA/Fibrin construct.

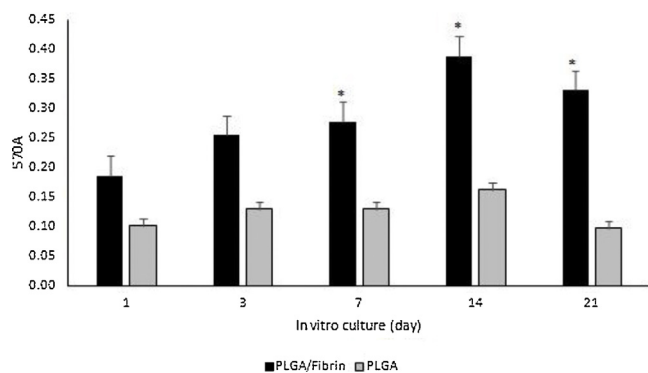
### 3.3. Immunohistochemistry evaluation of *in vitro* constructs

Immunolocalization of collagen type I and type II was compared between PLGA/Fibrin construct and PLGA constructs at week 1, 2 and 3 of *in vitro* culture. As shown in Fig. 3D–F, the PLGA/fibrin construct demonstrated a strong immunopositivity reaction indicated

by the expression of collagen type II and the amount of collagen type II matrices increased with time. On the other hand, a weaker coloration for collagen type II staining appeared in the PLGA construct (Fig. 3A–C). For collagen type I, the PLGA/Fibrin construct exhibited a moderate immunopositivity from week 1 until week 3 (Fig. 3G–I).

### 3.4. Measurement of cell proliferation assay of *in vitro* construct

The cell growth exhibited normal growth curve for both groups. The PLGA/Fibrin construct and PLGA construct demonstrated identical growth pattern throughout the *in vitro* culture with PLGA/Fibrin construct showed superior proliferation activity than PLGA. The cells remained metabolically active and continued to slowly proliferate in both scaffolds from day-1 of *in vitro* culture before entering an exponential phase by day-3 until day-14 and then started to decline by day-21. Overall, the increase in cell proliferation was noted to be significantly higher in PLGA/Fibrin construct compared to PLGA construct on day-7, day-14 and day-21 ( $p < 0.05$ ). The results are summarized in Fig. 4.



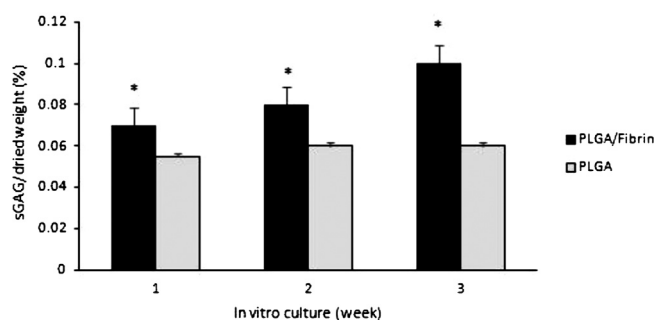
**Fig. 4.** Cell proliferation activity of *in vitro* construct. Proliferation of BMSCs seeded on PLGA/Fibrin and PLGA scaffolds during the cultivation time of 21 days of *in vitro* culture. PLGA/Fibrin construct showed significantly higher cell proliferation on day-7, day-14 and day-21 compared to PLGA construct ( $p < 0.05$ ).

### 3.5. Two-step reverse transcriptase polymerase chain reaction

The seeded BMSCs were allowed to undergo chondrogenic differentiation for up to 3 weeks. Gene expression analysis in Fig. 5 shows the expression of chondrogenic markers. Collagen type II, Collagen type IX, sox9 and aggrecan core protein were both detected in the PLGA/Fibrin and PLGA construct with PLGA/Fibrin group exhibited more intense expression for all markers compared to PLGA. The cartilage dedifferentiation marker, collagen type I was detected both in the PLGA/Fibrin and PLGA constructs but demonstrated a down-regulation pattern from week 2 onwards. The house-keeping gene,  $\beta$ -actin was steadily expressed in all samples verifying that two-step reverse-transcriptase PCR was consistent and reliable.

### 3.6. Sulphated glycosaminoglycan (sGAG) production assay

The relative sGAG content was normalized by dry-weight of each samples ( $n = 6$ ). Fig. 6 shows the PLGA/Fibrin constructs revealed greater sGAG content than PLGA constructs at each time point. PLGA/Fibrin constructs demonstrated significantly higher relative sGAG content ( $p < 0.05$ ) compared to PLGA constructs

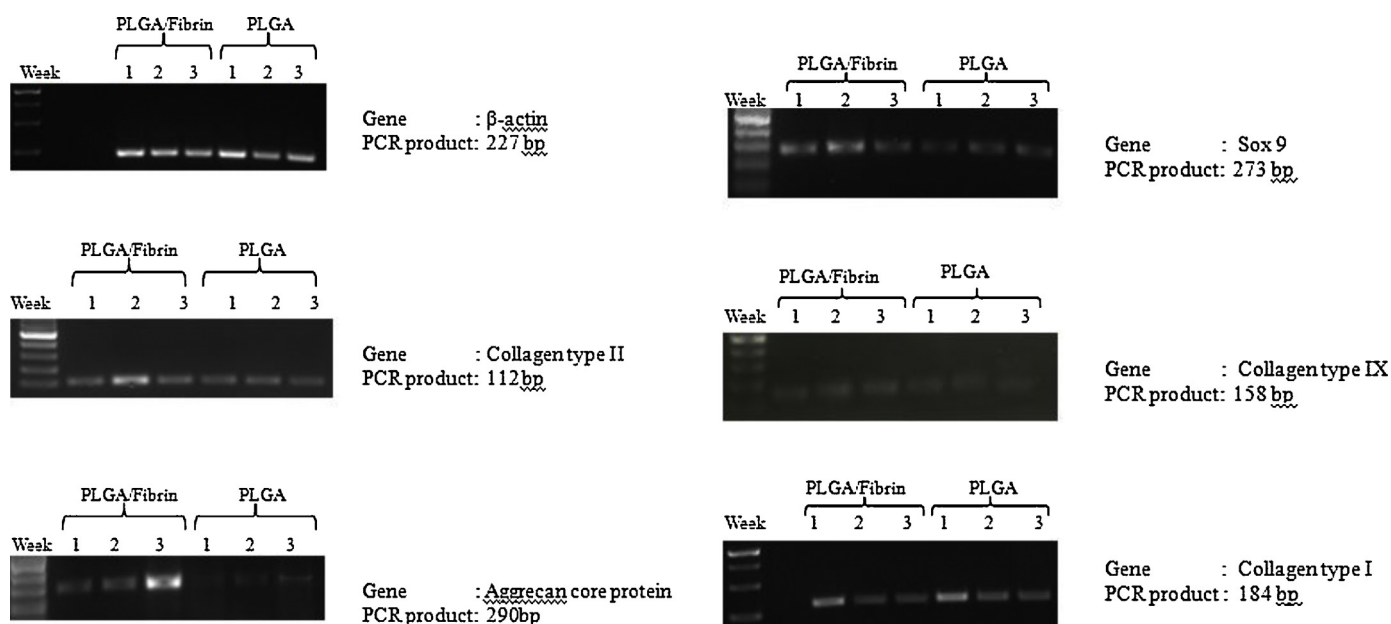


**Fig. 6.** Sulphated glycosaminoglycan (sGAG) production assay. Relative sulphated glycosaminoglycan (sGAG) contents (%) after 1, 2 and 3 weeks of *in vitro* culture. The symbol "\*" indicates a statistical significance ( $p < 0.05$ ).

at week-1, week-2 and week-3 of *in vitro* culture. At week-1, PLGA/Fibrin constructs demonstrated a relative sGAG production of  $0.069 \pm 0.071$  which is 1.23 times higher than PLGA constructs;  $0.056 \pm 0.045$ . At week-2, the relative sGAG content for PLGA/Fibrin constructs and PLGA constructs were  $0.079 \pm 0.060$  and  $0.06 \pm 0.029$  respectively. By week-3, PLGA/Fibrin constructs exhibited superior relative sGAG production with  $0.093 \pm 0.057$ , a 1.48 times higher production than PLGA constructs;  $0.063 \pm 0.063$ . The results are summarized in Fig. 6.

## 4. Discussion

In this study, we fabricated a combination of natural and synthetic three-dimensional PLGA/Fibrin scaffold seeded with BMSCs which has the ability and the potential to support tissue regeneration in the *in vitro* environment. PLGA was chosen as the preferred synthetic construct due to its well-known biodegradable and biocompatible characteristic. For the past two decades, PLGA has been intensely studied and has become an attractive candidate to be used in tissue engineering (Park et al., 2005). With respect to its design, PLGA is a copolymer of poly lactic acid (PLA) and poly glycolic acid (PGA). The degradation time for PLGA depends on the monomers' ratio whereby a higher content of glycolide units will give a shorter degradation time (Stevanović and Uskoković, 2009).



**Fig. 5.** Two-step reverse transcriptase polymerase chain reaction. RT-PCR analysis of the mRNA expression of collagen type II, aggrecan, Sox9, collagen type IX and collagen type I in the *in vitro* PLGA/Fibrin and PLGA constructs at 1, 2 and 3 weeks of culture.

For this study, the PLGA used has a 50:50 monomer's ratio (50% lactic acid and 50% glycolic acid) which has been shown to demonstrate the fastest degradation time (Makadia and Siegel, 2011). Due to its excellent performance of PLGA biopolymer, many researchers still used PLGA in their study, but with some modification on fabricating PLGA to improve this potential biopolymer in cartilage tissue engineering (Chang et al., 2013; Nanda et al., 2014).

We also used fibrin to be incorporated together with BMSCs and seeded onto the PLGA scaffold. Fibrin was reported to minimize cells lost during *in vitro* seeding due to the homogeneous cell distribution by immobilizing the cells (Sha'ban et al., 2008). Fibrin can also be useful as a cell matrix material, used as a combination of cell matrix material with a polymeric scaffold, and as a vehicle for growth factors or other healing agents (Eyrich et al., 2006). Several studies indicated that fibrin facilitates chondrogenic differentiation of MSC (Haasper et al., 2008; Ho et al., 2010; Im et al., 2006). Indeed, the use of fibrin alone as scaffold have been examined thoroughly in many of our previous studies (Munirah et al., 2010, 2008b, 2005). However, due to the limited resources, we had to limit the scope of the this present study by excluding the fibrin scaffold only group and compared the applicability of PLGA/Fibrin/BMSCs as a construct in its own complex environment with PLGA/BMSCs. In addition, the aim of the study was not to look at the role of fibrin as scaffold per se but, more towards developing a cell delivery system for effective cartilaginous tissue formation. On the same account, our previous *in vitro* and *in vivo* studies using the ectopic model on the use of PLGA/fibrin hybrid scaffolds seeded with articular chondrocytes showed promising findings for cartilage tissue engineering purpose (Munirah et al., 2008a,c; Sha'ban et al., 2008).

Despite numerous advantages, the acidic degradation of PLGA has been much of a concern because they are often linked to as the sources of inflammatory reactions in the body (Lee et al., 2014; Kum et al., 2013). This is not good for clinical application. While fibrin has been indicated to unable to aid the chondrogenic differentiation of the 3D construct *in vitro* (Munirah et al., 2010, 2008b, 2005). Moreover, in pre-clinical evaluation, as scaffold, fibrin appears too soft to hold onto the implantation area independently and thus requires additional fixation during surgery (Munirah et al., 2007). However, when we combine PLGA and fibrin, these hybrid scaffolds indicated good results in view of the fact that they lessen the inflammatory reaction and promoted both *in vitro* and *in vivo* cartilage formation (Munirah et al., 2008a,c; Sha'ban et al., 2008). Therefore, the combination of PLGA with natural polymers, such as fibrin, might overcome the limitations of synthetic and naturally derived polymers alone and produce a material with properties beneficial for cartilage reconstruction (Wang et al., 2013).

In addition to the many selections of biomaterial, successful cartilage regeneration also depends on the appropriate selection of cell sourcing. Hence, instead of choosing the committed chondrocytes, we used BMSCs as the preferred cell source due to their multipotentiality and easy accessibility. In this current study, we evaluated the *in vitro* formation of rabbit BMSCs seeded in PLGA/Fibrin construct at each time point of 1, 2 and 3 weeks.

It was noted that macroscopically, PLGA/Fibrin construct exhibited a superior gross features which is smoother and glistening appearance, somewhat similar to the morphology of hyaline cartilage-like tissue compared to PLGA construct towards week-3 of *in vitro* culture. The H&E staining of the 3-D *in vitro* construct for both PLGA/fibrin and PLGA showed the cells were initially segregated or mainly found on the superficial part of the scaffold at week-1 of culture. But from week-2 onwards, cells in PLGA/Fibrin constructs were noted to migrate and proliferate markedly compared to PLGA constructs. This can be contributed by higher cell seeding efficiency and the fact that fibrin helps in the immobilization of cells and distributed them homogeneously (Lee et al.,

2005). Of course another study by Xue et al. (2012), also indicated that the cartilage-like tissue which contained lacunae structures were observed clearly in the PLGA/fibrin construct compared to PLGA construct.

The cell proliferation assay demonstrated a higher cell proliferation in PLGA/Fibrin constructs from day-1 until day-14 of culture and subsequently declined by day-21. However, based on the histological finding, the PLGA/Fibrin constructs showed an increase in the cell number with time, which is contrary to the cell proliferation assay result. This present finding may be due to the fact that during the late culture stage, the cells are in the secretory phase, and no more in the differentiation phase, whereby the constructs are now starting to form tissues. Therefore, the foundation block for cartilage is the matrix synthesis. Other possible explanation perhaps, the cells have reached the plateau phase and entered a stable resting zone. This finding is consistent with Sha'ban et al. (2008) whom used PLGA/Fibrin scaffold seeded with chondrocytes.

Our present PLGA/Fibrin constructs exhibited a strong Alcian blue and collagen type II staining starting from week-2 of *in vitro* culture. This indicates that the BMSCs were capable of producing major chondrogenic matrices (Leyh et al., 2014; Mauck et al., 2006; Miao et al., 2009; Zhang et al., 2014). Initially, we anticipated that collagen type I would gradually decline towards week 3 as demonstrated by the collagen type I expression on RT-PCR. However, the immunohistochemistry staining showed quite the opposite finding. This result is quite similar to Kosher et al. (1986) study that demonstrated a relatively high collagen type I at the end of the culture period. This event could be explained by the synthesis of collagen type I by the pre-chondrogenic BMSC during the initial differentiation phase before the initiation of collagen type II production. It is also suggested that transcription of collagen type I are never inactivated (Focht and Adams, 1984). A gene's mRNA level does not usually predict its protein level (Kendrick, 2014). Hence, in this study, to have additional collagen IX, Sox9, and aggrecan immunohistochemistry staining is good to compliment the gene expression profile since it will give different perspectives on chondrogenesis. However, again, due to limitation in resources, this present study is dedicated to evaluate immunohistochemistry for collagen type I and II only. Furthermore, many previous papers are showing on immunohistochemistry for collagen type II only (Cao et al., 2011; Elsler et al., 2012; Feng et al., 2011; Song et al., 2013; Wu et al., 2014; Yang et al., 2012).

Although there was evidence of accumulated GAG, surprisingly there was no positive Safranin-O staining to indicate presence of cartilage specific proteoglycan-rich matrix. Compared to the previous study (Sha'ban et al., 2008), PLGA/Fibrin seeded with chondrocytes showed cartilage histoarchitecture superior to PLGA group; the chondrocytes were homogeneously distributed in the ECM and exhibited rounded morphology with lacunae embedded in basophilic ground substance. They also reported that the Safranin-O staining was intensely stained at 3 weeks of *in vitro* culture when compared to PLGA construct (Sha'ban et al., 2008). Cao et al. (2011) also successfully demonstrated a positive Safranin-O staining when BMSCs transfected with Adv-Sox9 and seeded on polyglycolic acid (PGA) scaffold.

BMSCs are multipotent cells which has the prospective to differentiate into multiple cell lineages. In view of that, BMSCs have distinct differences in their extracellular matrix character, organization and composition (Connelly et al., 2008). We suspect that the constructs were lacking of proteoglycan rich matrix due to the fact that probably the undifferentiated BMSCs that we harvested was a mixture of non-homogeneous population of mesenchymal progenitors and that not all of the BMSCs were undergoing chondrogenesis despite being cultured *in vitro* for 3 weeks under the influence of chondrogenic media. There are reports that demon-

strated regardless of a long culture period for up to 10 weeks, the level of proteoglycan produced by BMSCs were still far less lower than articular chondrocytes (Mauck et al., 2006). Perhaps some other options to increase the likelihood of expressing more proteoglycan by the BMSCs are to increase the number of BMSCs progenitors in the initial population by cell sorting or immune-selection, applying external stimuli or use different growth factors (i.e. TGF- $\beta$ 1 or TGF- $\beta$ 3) in various concentration or different exposure pattern or use co-culture (chondrocytes and BMSCs) as the cell sources.

On the contrary, although our histologic findings presented low qualitative evidence, the synthesis of cartilaginous extracellular matrix compound and the deposition of proteoglycan was observed from gene expression level. The gene expression profiles for collagen type II, aggrecan core protein, Sox9 and collagen type IX were expressed in both constructs with PLGA/Fibrin demonstrated better expression in all occasions. Interestingly, the cartilage dedifferentiation marker, collagen type I was also observed in both of the constructs and showed a down regulation pattern. Collagen type II can be found in every stages of cartilage development and plays an essential role as a specific cartilaginous marker. During the early onset of chondrogenesis, collagen type I will initially be present but later when maturity sets in, it becomes undetected and overshadowed by the expression of collagen type II, which eventually will be replaced again by collagen type I during cartilage degradation (Morrison et al., 1996). Therefore, our findings were in concert with the above statement and we expect that both collagen type II and I were co-expressed because the engineered cartilage is still considered a young developing tissue. Sox9 was noted to be expressed in both constructs with PLGA/Fibrin showed greater expression than PLGA. Sox9 is a well-known potent activator of type II collagen expression (Wang et al., 2014). It is the “master regulator” of chondrocyte phenotype which acts as a vital transcription factor for chondrogenesis (Cao et al., 2011). Sox9 was shown to express few cartilage specific ECM compound including aggrecan and collagen type II, IX and XI. Having said that, Kyriotou et al. (2003) found that Sox9 could not restore the chondrocyte phenotype on its own and will inhibit the expression of collagen type II (COL2A1) when over expressed at high level at whatever differentiation state of chondrocytes.

One of the characteristic of native articular cartilage is to have abundant aggrecan which is the major proteoglycan as a load-bearer. Like any other proteoglycans, aggrecan has a core protein that binds with the sulphated glycosaminoglycan (sGAG) chains (Kiani et al., 2002). In concert with the gene expression, we managed to demonstrate that the relative sGAG content in PLGA/Fibrin constructs was significantly higher compared to PLGA construct in all three weeks of *in vitro* culture. The production of sGAG in both constructs could also be influenced by the culturing time. At each time point, the amount of sGAG content was evaluated and showed gradual increment. Previous study on the effect of culture time on the properties of 3-D woven PCL scaffolds seeded with human MSCs found that cartilaginous tissue formed at 3 weeks and new formed ECM were observed at the interface after 7 weeks in chondrogenic medium culture (Nooeaid et al., 2012). Hence, it was believed that the sGAG amount depended on culture time, which means the production of sGAG was improved with increasing culture duration (Kock et al., 2012). As expected based from our result, even when our 3-D constructs were able to produce sGAG, the production by BMSCs was still considered at a lower level compared to construct that uses articular chondrocyte as the cell source. This statement is confirmed by a study done by Connelly et al. (2008) that found BMSC constructs exhibited less collagen II and aggrecan compared to the articular chondrocyte constructs that was seeded on agarose gels and cultured under identical conditions. Even after a long culture period for up to 10 weeks, bovine BMSCs were noted

to generate low production of sGAG compared to articular chondrocytes (Mauck et al., 2006).

In this present study, the findings revealed that the PLGA/Fibrin constructs seeded with BMSCs has a better-quality chondrogenic property characterized by higher cell proliferation, better distribution on collagen type II and Alcian blue staining for glycosaminoglycan, superior expression of chondrogenic genes (collagen type II, collagen type IX, aggrecan core protein and Sox9) as well as significantly higher sGAG production. Our data mirrors the findings of Sha'ban et al. (2008) that demonstrated that PLGA/Fibrin constructs promotes early *in vitro* chondrogenesis using chondrocytes and findings from Li et al. (2011) that found PLGA scaffolds co-culture system could promote chondrogenic differentiation using goat's BMSCs. As for the histological findings, we assumed that the lacking in the histoarchitecture property does not hinder the fact that PLGA/Fibrin construct seeded with BMSCs are capable to be considered as a potential cell delivery vehicle because other parameters especially the gene expression profile were in comparable measures.

Our previous studies on fibrin scaffold had consistently indicated that the *in vitro* 3-D constructs made from 'chondrocytes-fibrin' mixture had similar gene expression pattern to that of serial passages or, the monolayer cultured chondrocytes (Munirah et al., 2010, 2008b, 2005). Gradual loss of chondrocytic phenotype in monolayer cultured chondrocytes was thought as the contributing factors that cause the inability of 'chondrocytes-fibrin' constructs to spontaneously express and form mature cartilage *in vitro*. Since we employed a similar *in vitro* culture approach, it is believed that the lack of some cartilage attributes in this present study is also due to the non-dynamic nature of the static *in vitro* culture environment. Apparently, the static culture does not mimic the actual microenvironment of the body. It provides only minimal cells-cells interaction. Although the cells were pooled together closely in the 3-D form using the biomaterial scaffolds, they may perform no specific function because of the minimal exertion or forces from the surrounding milieu. It is suspected that those constructs were passive and rely solely on the diffusion of nutrients from the culture media for their basic nutrients needs (Munirah et al., 2010). However, after *in vivo* implantation, increased in expression level of cartilage specific markers was evident in the 'chondrocytes-fibrin' constructs (Munirah et al., 2010, 2008b, 2005).

Development of vertebrae skeletal system involves a cellular event which is represented earliest by the process of chondrogenesis (Goldring et al., 2006). During this early phase of mesenchymal stem cell aggregation, there will be cell adhesion which is being promoted by the cell-cell interactions together with the surrounding matrix (Tuan, 2004). Thereafter, there will be mesenchymal condensation which will lead to chondrogenesis through different factors such as signaling pathways and the involvement of various growth factors including fibroblast growth factor (FGF) and bone morphogenetic protein (BMP) (Goldring et al., 2006). It has been shown that supplementary growth factors or corticosteroids has the prospect to improve *in vitro* differentiation and promotes chondrogenesis (Derfoul et al., 2006; Im et al., 2006). In this study, we used the commercially available chondrogenic media that contains Transforming Growth Factor Beta-2 (TGF-B2), Insulin-like Growth Factor-1 (IGF-1) and basic Fibroblast Growth Factor (bFGF) which were studied comprehensively (Chua et al., 2010) and are known to promote cells proliferation and quality cartilage formation (Munirah et al., 2010). The TGF-B family is known to be an excellent cartilage inducing factor (Derynk et al., 2008). Apparently Seyedin et al. (1986) showed that TGF-B1 is a more potent regulator for chondrogenesis compared to TGF-B1 and TGF-B3. Therefore, future work should include TGF-B1 as IGF-1 and TGF-B1 has additive effect for chondrogenic differentiation in BMSCs

(Longobardi et al., 2006). Further optimization is necessary in terms of manipulating a specific culture condition in order to train and achieve the wanted chondrogenic lineage from the non-committed BMSCs. Since chondrogenesis is a dynamic process, continuous cell–cell and cell–matrix communications is essential in every stages of cartilage development.

## 5. Conclusion

Overall, this study depicted that a three-dimensional PLGA/Fibrin scaffold seeded with BMSCs has the potential implication for future work in cartilage tissue engineering. It is suggested that although BMSCs could generate the PLGA/Fibrin construct with substantial cartilaginous features, an optimum signaling factor is needed to provide an architectural support and cue to differentiate the BMSCs to chondrocyte-like cells. The *in vivo* experiment is already underway and currently the results are being written as a continuation from this study.

## Conflict of interest

None.

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