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Structural and Functional Properties of Neocartilage Construct Engineered in Poly (Lactic-*co*-Glycolic Acid) (PLGA) based Scaffolds

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Abstract: The study aims to assess the structural and functional properties of in vitro three-dimensional (3D) PLGA-based hybrid scaffolds seeded with chondrocytes, particularly in terms of the production of specific cartilaginous extracellular matrix (ECM). The PLGA scaffolds were incorporated with atelocollagen and/or fresh fibrin and assigned to four groups; PLGA only as control, PLGA-fibrin (PF), PLGA-atelocollagen (PA) and PLGA-atelocollagen-fibrin (PAF). The resulting PLGA hybrid scaffolds were characterized based on gross appearance, attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy analysis, porosity and swelling tests as well as cytocompatibility analysis using cell proliferation (MTT) assay. All scaffolds seeded with cells were cultured for three weeks in vitro. Macroscopic changes were recorded using photographs. Microscopic evaluation of 'cells-scaffolds' construct was done using Haematoxylin and Eosin (H&E), Safranin O, Alcian Blue, Toluidine Blue and scanning electron microscopy (SEM). The production of cartilage specific ECM was measured using sulphated glycosaminoglycan (sGAG) assay. Based on physical characterizations, PLGA-based hybrid scaffolds have been successfully manufactured and showed no cytocompatibility issues. The PAF exhibited cartilaginous tissue morphology better than other scaffold groups, grossly and microscopically. On SEM, the presence of branching fibers that produce a web-like network on the surface of PLGA-based hybrid scaffolds indicated ECM secretion. This is supported by the manifestation of glycosaminoglycan and as well as proteoglycan through histology and sGAG assay. This present study indicated that PLGA-based hybrid scaffolds promote formation of neocartilage in vitro.

Keywords: tissue engineering, neocartilage, extracellular matrix, bioscaffold, PLGA, atelocollagen, fibrin

1. Introduction

Tissue engineering principle aims at its three key working components namely biomaterial scaffolds, cells sources and signaling molecules [1]. Balance between this triad is necessary to achieve efficient natural regeneration of tissue and/or organ for clinical application. Biologically, ECM is found at the outer part of cells. It is secreted by the cells that form the tissue. The ECM provides structural composition and biochemical support to maintain cells and tissues integrity. It plays an important role in connective tissue type such as cartilage in providing mechanical strength. The other functions of ECM are to facilitate for cellular interaction, cell adhesion, and cell differentiation [2]. The components of ECM are mainly water, collagen and proteoglycans. There are also glycoproteins present in lesser

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amount [3] as well as other non-collagenous proteins. These components help to retain water within the ECM of articular cartilage hence contribute to the mechanical support for the cartilage.

Biomaterial scaffold provide temporary framework for the tissue-engineered in vitro [4]. There are several criteria that need to be considered for the determination of an excellent biomaterial scaffold. The good biomaterial scaffold will be able to facilitate the cell affinity for the tissue morphogenesis [5,6]. Having favourable microenvironment i.e. interconnected pores is one of the scaffold requirements to provide enough space for the cell intrusion within the scaffold [7], cell attachment, cell proliferation and cell growth [2, 8]. Designated scaffold must be biocompatible and must not provoke the immune response during post-implantation phase [9]. Despite of having the controlled degradation rate, bioscaffold must also present with adequate biomechanical support for the newly formed tissues before they are able to secrete their own supportive matrix in the cultured setting.

The types of biomaterial used plays a crucial factor in determining the quality of newly regenerated tissue. PLGA is a synthetic polymeric scaffolding material widely used in tissue engineering application. It is an FDA-approved biomaterial applied for various clinical application such as surgical sutures [10] and grafts for prosthetic devices. However, despite of its controllable degradation rate, PLGA, a copolymer of PLA and PGA encounter problem with acidic by-product. The lactic acid, a by-product resulted from the degradation of PLGA has been shown to provoke undesired inflammatory response [11]. Therefore, PLGA is often used in combination with natural biomaterial for instance atelocollagen and fibrin. Previous studies have successfully indicated that PLGA in combination with atelocollagen and/or fresh fibrin (PAF) facilitated for neo-tissues formation comparable to that of native tissues [12] and provide good microenvironment for cell growth and adhesion [13, 14]. Hence, this current study is to characterize the structural and functional properties of hybrid biomaterial scaffold to form neo-cartilage like tissue in monolayer culture. This study involves the incorporation of PLGA-based scaffold with atelocollagen and/or fibrin. The PLGA-based scaffolds were evaluated for structural properties and functionality using histology, biochemical and biomechanical analyses.

2. Methodology

2.1 3D PLGA-based Hybrid Scaffolds Formation

The previous stepwise procedure of PLGA-based hybrid formation with some modifications was adapted in this study [12, 14, 15]. The disk-shape 3D PLGA (65:35) scaffolds with 7mm (diameter) × 3mm (height) were formed using salt leaching and solvent casting method (mole ratio 65:35, molecular weight 24,000 – 38,000 g/mole, Resomer® RG 653 H, Evonik, Boehringer Ingelheim, Germany). Sieved sodium chloride (NaCl, Merck, Germany) with the size approximately 355-400 µm were mixed with methylene chloride solution. The mixed solution was then poured into silicone mould. The cylindrical disk shaped PLGA scaffold were soaked in ultrapure water for overnight to form porous scaffold. The scaffold was then freeze dried for 24 h. PLGA and atelocollagen scaffold was prepared using 1% atelocollagen type I (Cosmobio, Japan) which were crossed linked with 40 mM 1-ethyl-(3-3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, Thermofisher Scientific, USA) and 20mM *N*-hydroxysuccinimide (NHS) in 2-ethenesulfonic acid (Thermo Fisher Scientific, Waltham, Massachusetts, United States) for 1 h, and freeze dried for 24 h. PLGA-fibrin/PLGA-atelocollagen-fibrin scaffolds were prepared by soaking scaffold in fibrin and cell suspension, before polymerized with calcium chloride ($CaCl_2$) (Green Cross P.D. Company, Yongin, Korea). Prior to seeding, all scaffolds were sterilized with 70% ethanol and washed with PBS three times.

2.2 Chondrocytes Harvest and Isolation

With the approval from Animal Care and Use Committee International Islamic University Malaysia (IIUM) (IACUC), IIUM/IACUC-2018 (2), articular cartilage samples were obtained from a 2-year old rabbits [14, 15]. Following 6 h of sample collection, the cartilage samples were dissected and washed with phosphate-buffered saline (PBS; pH 7.2) (Gibco, Grand Island, NY) supplemented with 10% of Antibiotics and Antimycotic (AA) (Gibco). Each sample were minced and digested using 0.6% Collagenase II (Gibco) before incubated in an orbital incubator (Stuart Scientific, Redhill, UK) at 37°C. After 6 h of digestion, chondrocyte suspensions were centrifuged at 6 000 rpm for 5 minutes (Jouan, Duguay Trouin, SH). Cells pellet were washed with PBS, centrifuged and resuspended with PBS for total cell counting using haemacytometer (Weber Scientific International, Ltd., Middlx, England) determined by trypan blue dye exclusion test (Gibco). With the initial seeding of 50 000 cells/cm^2, harvested chondrocytes were plated in 6-well-plates (Falcon, Franklin Lakes, NJ). Chondrocytes were cultured and maintained in FD complete media under controlled environment of 37°C, 5% CO_2 incubator (Thermo Fisher Scientific, Waltham, Massachusetts, United States).

2.3 ATR-FTIR Spectroscopy Analysis

The ATR-FTIR Spectroscopy analysis was completed to investigate the incorporation of atelocollagen or fibrin on PLGA scaffolds for the prepared 3D hybrid scaffolds. The Spectrum Two FTIR Spectrometer (Pelkin, USA) with the spectral range of 4000–400 cm^{-1} and 4 cm^{-1} resolution was utilized for the measurement of the infrared spectra. Freeze-dried samples of each scaffold groups were placed on platform and gently pressed down with pressure tips. The spectrum

produced was normalized by comparing to the ambient air spectrum as the background. The presence of amide bond indicated the positive incorporation of atelocollagen and fibrin in the scaffolds.

2.4 Morphology Observation of PLGA-based Scaffolds and "Cells-Scaffold" Constructs

The interior architecture structures of the PLGA-based scaffolds with (cultured in vitro for 3-weeks) or without cells, were visualized microscopically using scanning electron microscope (ZEISS EVO 50 field emission SEM, Jena Germany). The scaffolds without cell-seeded were observed immediately. Meanwhile, the scaffolds seeded with cells (constructs) were fixed overnight in 2.5% glutaraldehyde (Agar Scientific Ltd, Essex, United Kingdom). The 0.1M phosphate buffer were used to wash the constructs 10 mins each for 3 times prior to post fixed process at room temperature using 1% osmium tetroxide (Agar Scientific Ltd, Essex, United Kingdom) for 2 h. All the constructs were then undergone dehydration process using graded series of ethanol (50%, 75%, 95% and 100%). The constructs were air dried for 24 h inside the fume hood. Upon the process of viewing, the constructs were coated with the gold (JFC-1600 auto fine coater) and viewed under SEM at the voltage of 10 kV.

2.5 Total Porosity Study using Gravimetric Analysis

The simple gravimetric analysis was utilized for the investigation of scaffolds total porosity that analyzed the weight difference of the scaffolds for the before and after immersion in fluid solution. Firstly, the dried scaffolds from all groups were weighed. The scaffolds were then completely immersed in absolute ethanol (Leica Biosystems, USA) for 1 h and the weighed were recorded. To enhance the permeation of the absolute ethanol solution into the scaffolds, ultrasound frequency was implemented using the ultrasonicator bath. The scaffolds porosity was calculated using the formula stated in Equation 1:

$$\text{Porosity} = \frac{(M_2 - M_1)}{\rho V} \times 100 \quad (1)$$

where M_1 is the weight of dried scaffolds, M_2 is the weight of scaffold after immersion with ethanol, ρ is the density of ethanol (0.789 g/cm^3), and V is the volume of scaffold = πr2h = (3.1416 × 0.352 cm^2 × 0.3 cm) = 0.1155.

2.6 Swelling Test

The swelling test was conducted by immersing the scaffolds in 15 ml water for 24 h at constant temperature of 37°C to analyze the scaffolds capacity to absorb water. The dried and wet weight of the scaffolds after immersion was recorded. The formula for the water absorption percentage (SWA) of the scaffold was portrayed in Equation 2:

$$S_{WA} = \left[\frac{(W_{24H} - W_0)}{W_0}\right] \times 100 \quad (2)$$

where, W_{24H} is the scaffolds wet weight after 24 h immersion and W_0 is the scaffolds dried weight. The water absorption percentage (S_{WA}) values were expressed as mean ± standard deviation (SD) (n = 6).

2.7 Microscopic Observation using Basic and Special Histology Staining

All scaffolds were fixed in 10% neutral buffered formalin (Leica, Biosystems, Wetzlar, Germany), processed embedded and sectioned to prepare 5 µm thick sections (Leica Microsystems, Wetzlar, Germany). The sections were stained with Hematoxylin and eosin (H & E) to access tissue histoarchitecture, Safranin O staining to evaluate the proteoglycan-rich matrix production, Alcian blue staining to detect the GAG accumulation and Toluidine blue for the distribution of proteoglycan. All sections were visualized using a light microscope (Olympus).

2.8 Cytocompatibility Analysis using Modified 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) Assay

Cell viability was measured using MTT assay (MTT, Merck, Germany) with some modification at day 7, 14 and 21 respectively. The 100 µL of MTT solution (5 mg/mL stock in PBS) was added into each sample and incubated for 4 h at 37°C. The resulted formazan crystals were solubalized using 1 mL of dimethyl sulfoxide (DMSO, Merck, Germany) each construct. The absorbance intensity was measured using microplate reader (Versa Max, Molecular Devices, USA) at 570 nm wavelength. The cell viability value was calculated accordingly using standard curve equation.

2.9 ECM Production Analysis using Sulphated Glycosaminoglycan (sGAG) Production Assay

All construct was digested with papain digestion solution (125 µg/ml of papain, 5 mM L-cystine, 100 mM Na_2HPO_4, 5 mM EDTA, at pH 6.8) at 60°C for 16 h. sGAG content were quantitatively analyzed using Alcian Blue assay. Each sample (50 µl) was mixed with 750 µl Alcian Blue working solution and measured at 620 nm wavelength. The standard curve was constructed using the range of 12.5-400 µg/ml. The total sGAG content was presented in percentage and normalized with the wet weight of the sample respectively.

2.10 Statistical Analysis

Statistical analyses of quantitative data were performed using one-way analysis of variance (ANOVA) with 95% of confidence level. For the data that were not normally distributed, Kruskal–Wallis test (non-parametric test) were done. The differences were considered significant when $p < 0.05$. The data were expressed as mean ± standard deviation (SD).

3. Results

3.1Secondary Structure Assessment of Proteins by ATR-FTIR Spectroscopy

No peak of amide bond was detected for negative control group (PLGA scaffold only). The two notable peaks of stronger and weaker formation of amide bond was presented indicating the positive incorporation of atelocollagen and fibrin into PLGA (results not shown).

3.2 Macroscopic and Microscopic Morphology of PLGA-based Scaffolds and "Cells-Scaffold" Constructs

The macroscopic morphology of "cell-scaffold" construct for all groups were accessed in terms of shape, size, gross appearance and mechanical strength of the construct throughout the 3-week culture. Based on the results summarized in Fig. 1 below, all "cell-scaffold" construct groups maintained their disk-shaped after 3-weeks in culture. There were some discrepancies in terms of the size of the construct, but the differences were not significant. The gross surface appearance was comparable for all groups. However, the PAF and PF groups presented with a smoother and glossier surface than the other groups. To access the mechanical strength of the construct, a simple palpation was conducted by a blinded evaluator. PAF group was reported to has firmer structure compared to PA, PF and PLGA only group.

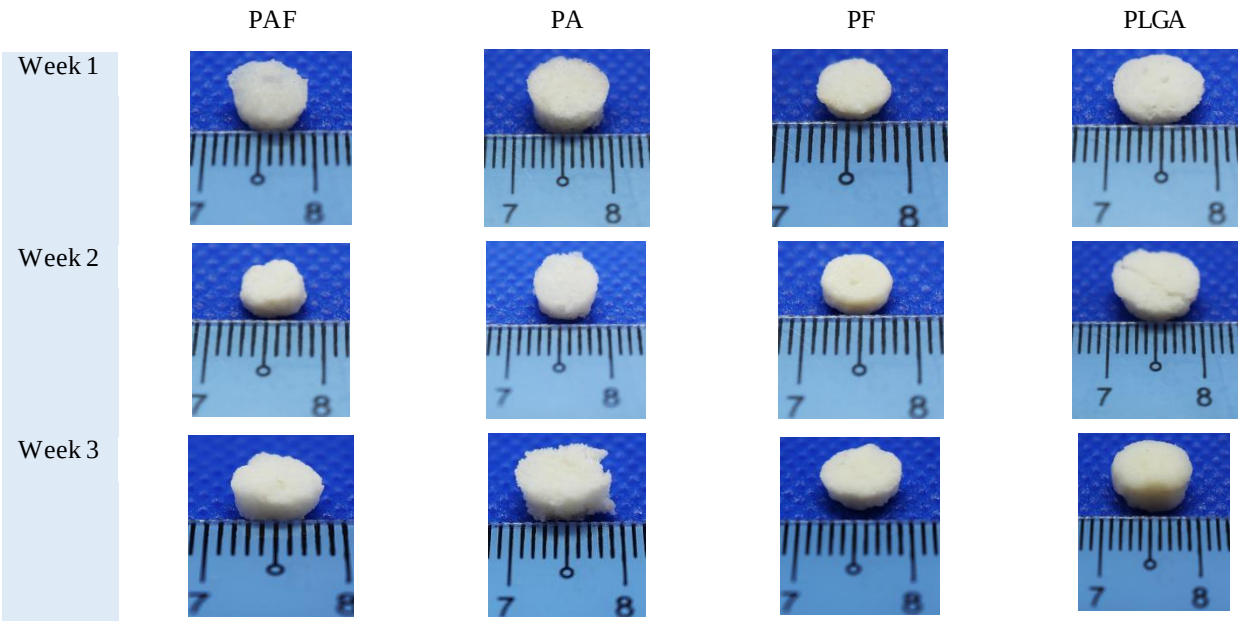


Fig. 1- Macroscopic morphology evaluation of in vitro "cell-scaffold" construct for PAF, PA, PF and PLGA throughout 3-weeks culture.

Based on the SEM micrographs for PLGA-based scaffolds (without cells), it showed that the microscopic pore structures appeared interconnected for all scaffold groups. At the end of 3-weeks culture, the cells occupied within the internal micropores as well as on the surface of the construct. However, in terms of cells distribution, there are some variations between the hybrid scaffold groups and the PLGA only group. The hybrid scaffold groups provide better cell adhesion and facilitate cell spreading and extension compared to other groups. The ECM secretion as indicated by the web-like fibers network substance were detected in the PAF and PA group only groups. The results are summarized in Fig. 2 below.

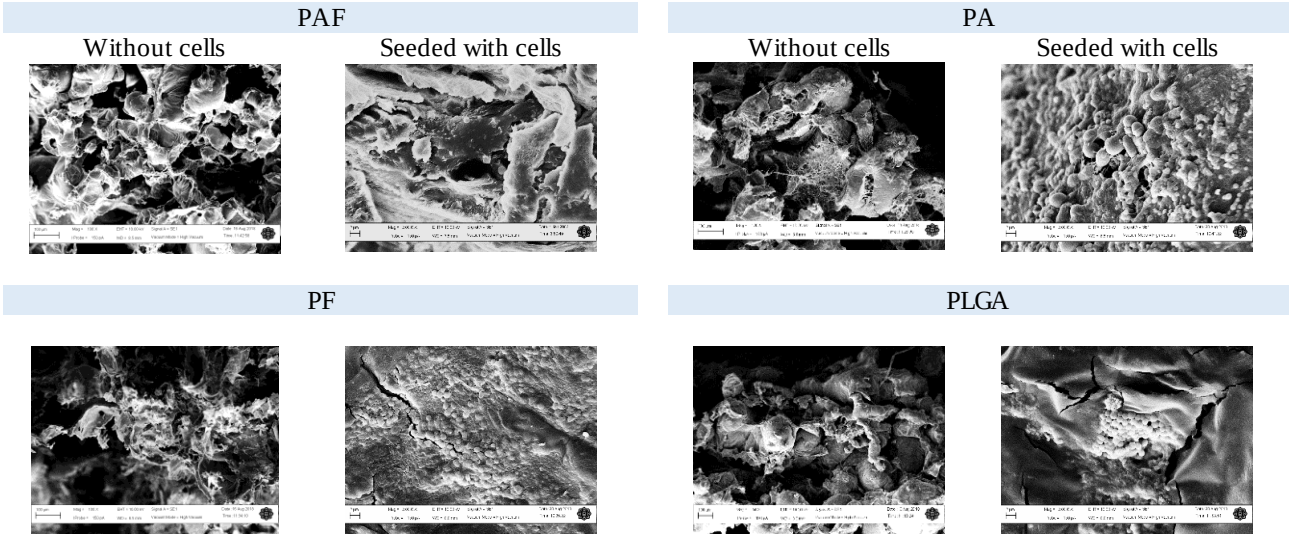


Fig. 2 - Microscopic observation of PAF, PA, PF and PLGA without cells observed using SEM (left) and "cells-scaffold" constructs at the end of week 3 in vitro culture (taken at 2 kX magnification) (right).

3.3 Porosity Measurement using Gravimetric Analysis

The gravimetric analysis was used to evaluate the total porosity of each scaffold. From the result as shown in Fig.3, there was no statistical differences on the porosity when incorporated with/without atelocollagen and/or fibrin into PLGA based scaffold. (PAF, 61.03 ± 11.80; PA, 56.37 ± 9.74; PF, 62.22 ± 13.37; PLGA, 59.93 ± 5.42)

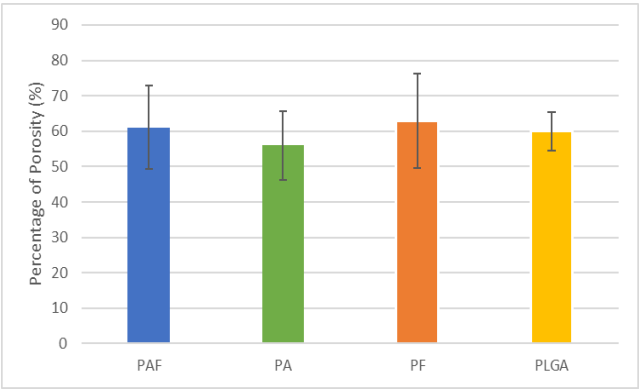


Fig. 3- Porosity analyses of the PLGA based scaffold groups. There is no significant effect found in this study between the hybrid scaffold and PLGA only group scaffold. However, the incorporation of atelocollagen and/or fibrin into PLGA scaffold indicated the decreases of total porosity.

3.4 Swelling Index Capacity

The overall results for swelling ratio of the PLGA based scaffold was in the range of 400%–680% as showed in Fig.4. PLGA only group scaffold presented the highest swelling ratio, PLGA, 680.02% ± 174.61%, followed by PA, 545.23% ± 33.96%; PF, 481.84% ± 50.606% and PAF, 441.38% ± 70.12%. The differences of swelling behavior between groups were not statically significant.

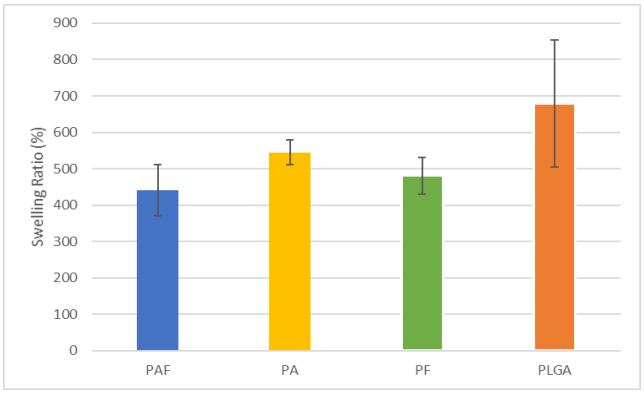
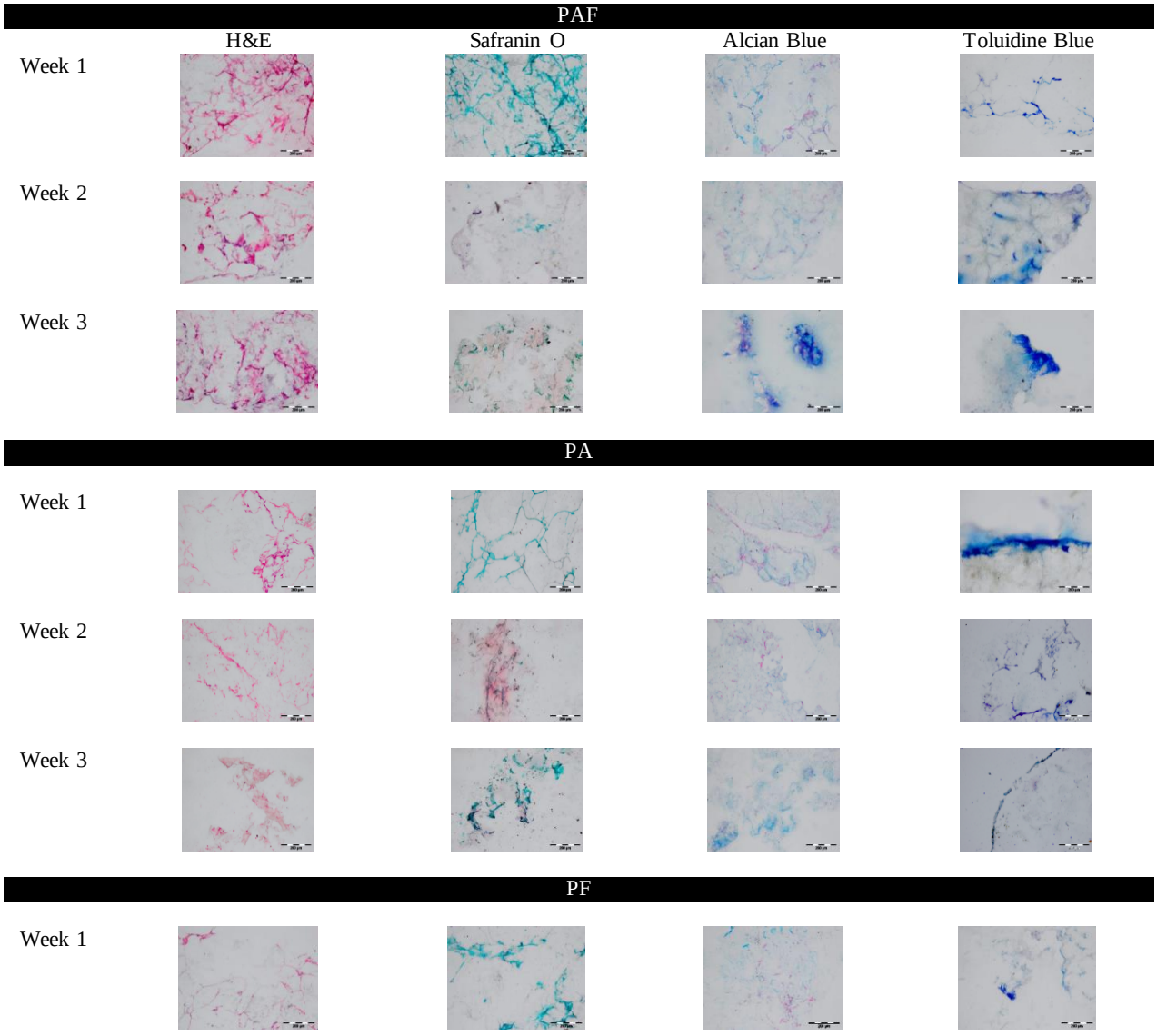


Fig. 4- Swelling index capacity of PLGA based scaffolds during immersion in water for 24 hours ranging from 400% to 680%. There was no significant differences between the groups.

3.5 Microscopic Morphology of "Cell-Scaffold" Constructs

There were some variations in terms of cartilaginous tissue formation in vitro between groups. There was no prominent cartilaginous characteristic showed in all groups at week 1. However, there were minimal ECM secretion seen in PAF group during week 3 in vitro culture tabulated in Fig.5.



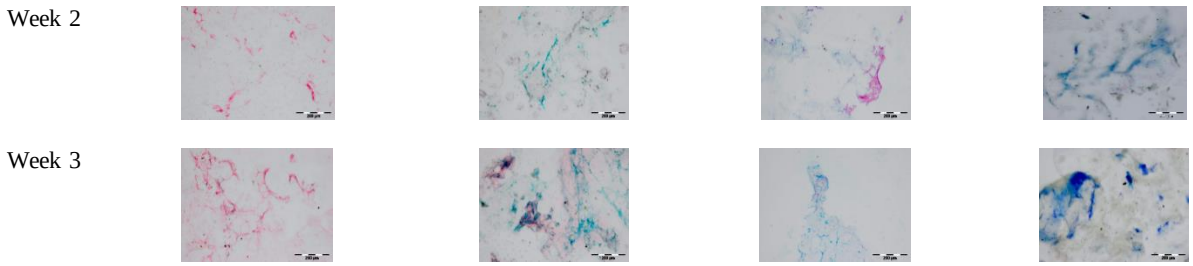


Fig. 5- Histological evaluation of in vitro "cells-scaffolds" construct for 3-weeks in vitro culture (×100 magnification).

3.6 Cell Viability Evaluation

Cell viability was measured to evaluate cell proliferation in different type of constructs groups at week 1, 2 and 3 in vitro. All groups except PA group showed similar increment trend in terms of cell viability for the first two week (PAF, 2,338,319 ± 203,462; PF, 1,209,652 ± 29,361; PLGA, 682,763 ± 45,991), but reduce on week 3 (PAF, 1,142,985 ± 87,940; PF, 913,763 ± 32,144; PLGA, 385,207 ± 51937.23). Meanwhile, PA group demonstrated constant increment of cell numbers throughout 3-week of in vitro culture as presented in Fig. 6 below.

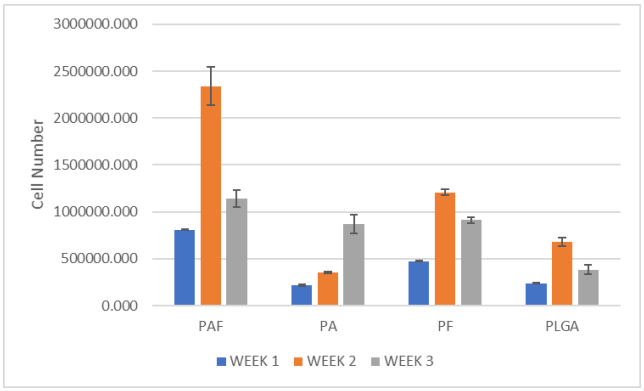


Fig. 6- Cell viability evaluation for PLGA based scaffold in 3-week culture consecutively. PAF showed highest cell viability throughout the 3-week of culture when compared to the other groups.

3.7 Specific Cartilage ECM Formation based on sGAG Level

To access the function of the construct in vitro, the production of cartilage specific ECM (sGAG level) were conducted. The cumulative sGAG content over wet weight was presented in percentage (%) after 3- week in vitro culture for PAF, 73.5 ± 5.557; PA, 56.3 ± 6.113; PF, 52.0 ± 8.856; and PLGA only, 39.1 ± 7.744 (Fig. 7).

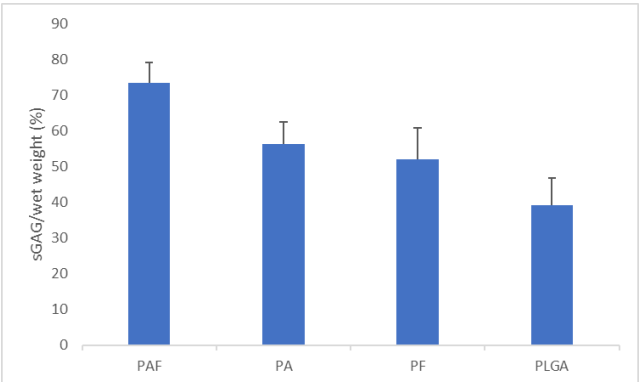


Fig. 7- The cumulative sGAG content over wet weight in percentage (%) after 3- week in vitro culture. There is no statistical difference in terms if total sGAG production over wet weight in different groups of "cell-scaffold" construct.

4. Discussions

The incorporation of atelocollagen and fibrin into PLGA based scaffold were investigated through the ATR-FTIR. From the ATR-FTIR spectra (result not shown), two notable peaks have been detected indicating the presence of amide bond. The amide bond demonstrated the linking between PLGA carboxyl group and atelocollagen amine group [12]. EDC/NHS, a cross-linker which is crucial to prevent the leaking of materials from scaffold has facilitate to the successful integration of atelocollagen and/or fibrin into PLGA.

In the formation of bioscaffold, the structural formation of pores play an important role. The interconnection of pores crucial to accommodate cellular penetration of cells [16, 17]. The presence of pores also facilitates a good exchange between the nutrients for cell growth [18] and the excretion of waste products within the scaffolds [19]. A conventional method has been applied in this study using salt leaching and solvent casting method. The formation of pores inside the PLGA spaces are the result of hydrated NaCl and the sublimation of the solvent [20]. In this study, NaCl (350-400 µm) were used to produce interconnected microscopic pores within the internal structure of the scaffolds. However, this technique was unable to control the structural uniformity of the pore architecture [7].

One of the characteristics of a good scaffold is it can provide enough mechanical strength as an initial support for the neo-tissue formation [21]. This is perhaps due to the atelocollagen that promotes the secretion of collagen within the cells [22]. The secretion of ECM contributes to the mechanical strength of the scaffolds [19]. This has been depicted with the presence of reticulated-like fibers network structure blanketing the cell clusters on the scanned surface of PAF and PA bioscaffold groups at the end of the 3-weeks culture. The use of atelocollagen has been reported to encourage the production of ECM [12, 23].

Based on the result, PAF presented with firmer structure and showed better cartilaginous appearance as indicated by glossy surface and cartilage-like appearance when compared to the other groups. Based on the result, it has been suggested that hybrid scaffolds promote better template for the cell affinity as compared to PLGA only scaffold [13, 14, 15, 21]. This is perhaps to the presence of atelocollagen [12, 23]. The properties of fibrin that act as glue to the cells [24, 25], provide better attachment of cells [26] and act as a good transport for cells delivery [27] also contribute to the promotion of cell growth within the framework of scaffolds. Hence, the incorporation of atelocollagen and fibrin to PLGA may provide better mechanical strength through the ECM production as well as adequate mechanical support to maintain the disk-shaped of scaffold throughout 3-weeks in culture. This result is supported by the previous studies which also presented with the similar result [12, 28]. By contrast, PLGA scaffold group has been found out to be brittle and presented with rough surface. PLGA has been known to has hydrophobic properties which can lessen the capacity of the cell adhesion [29] on its surface. Thus, delay the formation of cartilaginous tissue.

Some analytical procedures and method validation involving swelling index capacity test and porosity measurement using gravimetric analysis has been conducted to test the physical properties PLGA based scaffolds. The total porosity for all groups of scaffolds ranging from 56% to 62% is considered acceptable. It is meaningful to evaluate the porosity of the bioscaffolds as it is closely related to the interconnectivity of the pores within scaffold [16, 17] as well as biomechanical strength [21]. However, different PLGA mole ratio used also affect the bioscaffold total porosity range [30].

The swelling test was conducted to evaluate the water uptake capacity of the PLGA-based scaffolds. From the result, the water uptake capacity for all bioscaffold groups still within the scope which ranging from 400% to 680%. PLGA only group exhibited the highest water uptake compared to the other hybrid bioscaffolds. Meanwhile, the water uptake capacity of PLGA hybrid scaffolds showed lesser percentage compared to PLGA alone group may be due to the atelocollagen which filling the porous structure of bioscaffold. The addition of self-aggregated fibrin protein into the scaffolds also may render the efficiency of water absorption capacity of the scaffold [31].

It is crucial in materials science and tissue engineering that the basic association of a biomaterial scaffold is instrumental in deciding its properties. Essentially, natural tissues are described by remarkable structures that able to dictate the specific function. Hence, basic histological staining using H&E and specific staining using Safranin O, Alcian Blue and Toluidine Blue has been applied. The staining is used to inspect the overall histoarchitecture of the PLGA-based scaffold as well as to evaluate the production of cartilage specific matrix including glycosaminoglycan and proteoglycan. The overall result showed that, PAF "cell-scaffold" construct presented with positive staining of H&E indicating better cartilaginous histoarchitecture compared to other groups. The presence of proteoglycan rich matrix was minimally stained with Safranin O red coloured, supported by positive Alcian blue and Toluidine blue staining affirming glycosaminoglycan (GAG) secretion at week 3 for PAF group. In contrast, it has been reported in previous studies that PLGA only group did not exhibit any cartilaginous tissue formation even at 3-week of in vitro culture [12, 13]. The sGAG production was further verified quantitatively measured by Alcian blue assay. The cumulative sGAG content revealed that PAF encourage ECM secretion better compared to the other PLGA-based group based on the highest sGAG production compared to other PLGA-based groups [12, 28].

5. Conclusions

Based on physical characterizations, PLGA-based hybrid scaffolds have been successfully manufactured and showed no cytocompatibility issues. The PAF exhibited cartilaginous tissue morphology better than other scaffold groups, grossly and microscopically. On SEM, the presence of branching fibers that produce a web-like network on the surface of PLGA-based hybrid scaffolds indicated ECM secretion. This is supported by the presence of GAG and proteoglycan-rich matrix through histology and sGAG assay. To conclude, the 3D porous PLGA-based "cell-scaffold"

construct incorporated with atelocollagen and fibrin may offer a better cell delivery vehicle to regenerate tissue-engineered cartilage for 3D in vitro culture system.

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