โครงเลี้ยงเซลล์ชีวภาพเยือกแข็งสำหรับเพาะเลี้ยงเซลล์ไฟโบรบลาสต์ในหลอดทดลอง

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บทคัดย่อ

โครงเลี้ยงเซลล์ชีวภาพเป็นองค์ประกอบที่สำคัญในงานวิศวกรรมเนื้อเยื่อ โครงเลี้ยงเซลล์ที่เอื้อต่อการเจริญเติบโตของเซลล์ต้องมีโครงสร้างที่กระตุ้นการยึดเกาะของเซลล์ วัสดุชีวภาพจากธรรมชาติหลายชนิดถูกนำมาผลิตเป็นโครงเลี้ยงเซลล์เนื่องจากสามารถผลิตโครงเลี้ยงเซลล์ที่มีคุณสมบัติเลียนแบบ extracellular matrix ที่สามารถกระตุ้นการยึดเกาะของเซลล์ได้ การศึกษาครั้งนี้จึงมีวัตถุประสงค์เพื่อผลิตโครงเลี้ยงเซลล์จากวัสดุชีวภาพ อะกาโรส ไคโตซานและเจลาตินโดยใช้เทคนิคเยือกแข็ง จากนั้นนำโครงเลี้ยงเซลล์ที่ผลิตได้มาศึกษาคุณสมบัติด้านความสามารถในการอุ้มน้ำและอัตราการสลายตัว รวมทั้งศึกษาความเป็นพิษของโครงเลี้ยงเซลล์ต่อเซลล์ไฟโบรบลาสต์ของมนุษย์ด้วยการทดสอบ MTT นอกจากนี้ยังศึกษาลักษณะของเซลล์ไฟโบรบลาสต์ที่เจริญบนโครงเลี้ยงเซลล์ด้วยการย้อม hematoxylin and eosin (H&E) ผลการศึกษาพบว่าโครงเลี้ยงเซลล์ที่ผลิตได้ทั้งหมดมีความสามารถในการอุ้มน้ำสูงถึง 80% และมีอัตราการสลายตัวที่ 1 สัปดาห์ต่ำกว่า 11% ส่วนการทดสอบ MTT แสดงให้เห็นว่าโครงเลี้ยงเซลล์ที่ประกอบด้วยอะกาโรส 2-3% ไคโตซาน 2% และเจลาติน 3% สามารถกระตุ้นการเพิ่มจำนวนเซลล์ไฟโบรบลาสต์ได้ ผลการย้อม H&E พบเซลล์ไฟโบรบลาสต์เจริญอยู่ภายในโครงเลี้ยงเซลล์ ผลการศึกษาครั้งนี้บ่งชี้ว่าโครงเลี้ยงเซลล์ที่ผลิตจาก อะกาโรส ไคโตซานและเจลาตินสามารถนำมาผลิตเป็นโครงสำหรับเพาะเลี้ยงเซลล์ไฟโบรบลาสต์ของมนุษย์ได้ ซึ่งจะเป็นประโยชน์ต่อไปในงานด้านวิศวกรรมเนื้อเยื่อ

คำสำคัญ: โครงเลี้ยงเซลล์ชีวภาพ อะกาโรส-ไคโตซาน-เจลาติน ไฟโบรบลาสต์

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Freeze-Dried Biomaterials Scaffold as *in vitro* Model for Fibroblast Culture in Tissue Engineering

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Abstract

Scaffold materials are essential elements for tissue engineering. In order to facilitate cell proliferation, the scaffold materials should provide a good microenvironment for the adhesion of cells. Among various biomaterials, natural polymers have been used to construct the scaffolds due to their ability to mimic the extracellular matrix to guide the organization of the cells. The aim of the present study was to synthesize and evaluate a composite scaffold using natural polymers; agarose-chitosan-gelatin (AG-CH-GEL) by blending-freeze-drying procedure. The properties of the scaffold such as water uptake ability and biodegradation rate were studied. The cytotoxicity of the composite scaffold on human fibroblast was studied using MTT assay. Fibroblast morphology was assessed using hematoxylin and eosin stain. All scaffolds were able to uptake water up to 80% and degraded lower than 11% per week. The MTT assay demonstrated that the scaffold consisting of 2-3% agarose, 2% chitosan and 3% gelatin could support human fibroblast proliferation. H&E staining revealed the presence of fibroblasts in the scaffolds. These results indicated that the AG-CH-GEL composite scaffold could be used as the scaffold to support fibroblast growth and proliferation for tissue engineering.

Keywords: Biomaterials scaffold, Agarose-Chitosan-Gelatin, Fibroblast

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Introduction

The key factors for fabrication of scaffold that can support cell growth and differentiation are depending on materials that have desirable properties such as biocompatibility, biodegradability as well as three-dimensional porous structures. Numerous biomaterials have been already applied to develop scaffolds for tissue engineering.[1-3] One of the most widely used biomaterials is natural polymers such as agarose,[4-6] chitosan,[7-9] gelatin[10-12] and collagen.[13, 14] All of these materials reveal suitable properties for tissue engineering in terms of biodegradable and biocompatibility.

Agarose is a natural polysaccharide, which controls degradation and ability to maintain cellular phenotype. It has been demonstrated that agarose can be constructed into flexible scaffolds using a freeze-drying method.[4, 15] Moreover, agarose scaffolds did not cause inflammatory responses.[16, 17] The structure of chitosan composes of polysaccharide which shows good biocompatibility and biodegradability. Thus, chitosan is natural polymer that widely used for preparation of tissue engineering scaffolds, wound dressings as well as tissue adhesives.[3, 7, 18, 19] Gelatin is often used in tissue culture for tissue engineering because its structure is obtained via hydrolyzed collagen. Thus, gelatin shares structure as well as function with collagen.[12, 20, 21] In addition, Arg-Gly-Asp (RGD) sequence within the molecular structure of gelatin could support cell adhesion, proliferation, and differentiation.[22] These polymers can form 3D porous structures, which mimic the microenvironment in our bodies. However, they have some limitations. For example, a pure chitosan scaffold has poor mechanical properties.[23] Because of its inert polymer, agarose scaffold is not suitable for cell adhesion.[4] As well as gelatin scaffolds have a weak mechanical strength and have limited solubility in aqueous media.[24, 25] To decrease the limitation of natural polymer, the mixture of those materials is usually used in combination.[7, 9, 26, 27]

In our previous study,[28] we fabricated biomaterial scaffolds from agarose, chitosan, and collagen type I for application in tissue engineering. As mentioned above, chitosan has received particular attention due to its non-toxicity, biodegradability, antibacterial, and antimycotic activity.[8] Moreover, the polymer can be extracted from wasters such as shrimp shells and crab shells.[29, 30] Thus, we extracted chitosan from shrimp shells (*Penaeus monodon*) and mixed it with agarose and collagen to generate a biocomposite scaffold via freeze-drying technique.[31] The biomaterial scaffold consisted of an interconnected porous structure with high water uptake ability. In addition, fibroblast cells can grow and proliferate on the scaffold. In extension to our previous work, the aim of the present study was to synthesize a composite scaffold which was structured of agarose-chitosan-gelatin (AG-CH-GEL) via freeze-drying technique.

The study of Mao JS. *et al.* (2004)[32] demonstrated that the high positively charged chitosan inhibits fibroblast migration. In order to promote cell proliferation, adding gelatin to chitosan to decrease the positive charge of chitosan was performed in this study. Thus, adding gelatin to the chitosan scaffold may shield the highly positive charge of chitosan and improves cell proliferation. The developed composite scaffold was further characterized by H&E staining. The scaffold properties, including water-uptake ability, degradation rate, and cytotoxicity of the developed composites were assessed.

Materials and methods

Materials

Agarose (low EEO, gelling temperature at 38-40°C) was purchased from Sisco Research Laboratories (Mumbai, India). Gelatin was supplied from Himedia (Mumbai, India). MTT reagent (3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide) were purchased from Sigma-Aldrich (St Louis, MO). Human fibroblast cell line was purchased from the American Type Culture Collection (ATCC, CRL-1635, Rockville, MD, USA). Cell culture media, Dulbecco's modified Eagle's medium with high glucose (DMEM—HG), and penicillin/streptomycin were supplied from Gibco (Grand Island, NY, United States). Fetal bovine serum was purchased from HyClone (Cramlington, UK).

Preparation of AG-CH-GEL composite scaffolds

The preparation of the solution mixture was based on our previous study,[28] in which chitosan was extracted from the shrimp shell waste via the chemical method. Briefly, agarose (final concentration 1, 2, and 3% w/v) was dissolved in deionized water by boiling. 1% and 2% chitosan were prepared by dissolving chitosan in 1% acetic acid solution. Gelatin powder was dissolved in deionized water to make the final concentration 0.5% and 3% w/v. In order to optimize the scaffold properties, different ratios of agarose, chitosan and gelatin were listed in Table 1. The polymer solution

Table 1 The solution mixture of agarose, chitosan and gelatin in different ratios were used to prepare AG-CH-GEL composite scaffolds.

Condition	Agarose	Chitosan	Gelatin
1	1%	1%	0.5%
2	1%	2%	3%
3	2%	1%	0.5%
4	2%	2%	3%
5	3%	1%	0.5%
6	3%	2%	3%

containing agarose, chitosan and gelatin was cross-linked by using 0.5 mL glutaraldehyde solution (0.2%, v/v) and incubated at -80°C for 24 hours. Then, the solution mixture was subsequently frozen dry under vacuum at -100°C for 48 hours (Martin Christ, Germany). The scaffold was dried at room temperature and cut into discs (thickness: 0.5 cm and diameter: 1.5 cm) for further studies.

Water uptake ability

The scaffold was cut into discs (thickness: 0.5 cm and diameter: 1.5 cm) and put into 24 well plate. After that, the scaffold was weighed (W_{dry}) and immersed in 1XPBS (pH 7.4) for 1 hour. Then the scaffolds were removed from 24 well plate and placed on a gauze pad for 5 minutes to absorb the excess water. Finally, the scaffolds were weighed (W_{wet}) to determine water uptake ability as formula below.[27, 33]

$$\textbf{Water uptake ability (\%)} = \frac{W_{wet} - W_{dry}}{W_{dry}} \times 100$$

***In vitro* degradation**

The AG-CH-GEL scaffolds were weighed (W_1) and transferred to 24 well plate filled with sterile 1XPBS solution (pH 7.4) at 37°C for 1 week. After 1 week, triplicated samples were removed and dried at 80°C in a hot air oven and then weighed (W_2). The degree of degradation was determined by dry weight change as formula below.[27, 34]

$$\textbf{Degradation rate (\%)} = \frac{W_1 - W_2}{W_1} \times 100$$

Cell seeding and culturing on scaffolds

Normal human fibroblast cells (Hs68) were grown in an incubator at 37°C with 5% CO_2 in DMEM—HG supplement with 1% penicillin/streptomycin and 10% fetal bovine serum. Culture medium was changed every 3 days, when they reached 80% confluence. The scaffold was cut into disc (thickness: 0.5 cm and diameter: 1.5 cm) and immersed in 2% NaOH to neutralize the residual acid in the scaffold. All scaffolds were washed 5 times with deionized water. Both sides of the scaffold were sterilized using 70% ethanol follow by exposure under ultraviolet irradiation in a laminar flow hood for 30 min. Human fibroblast was counted by tryphan blue exclusion and plated at a density of 1×10^6 cells/20 µL for each scaffold. To allow cell adhere to the scaffold, cell seeding on the scaffold were then cultur in a CO_2 incubator for 2 hours. After

that, the scaffold was covered by the medium for 5 mL. Cellular scaffolds were incubated at 37°C in a CO_2 incubator and culture medium was replaced every three days. The cellular scaffold was harvested at 1 week and 2 weeks for further study.

Cell viability assay

To determine the viability and proliferation of normal human fibroblast in AG-CH-CL scaffold, an MTT assay was carried out. The cellular scaffold was harvested at 1 week and 2 weeks, washed three times in PBS, and then incubated with MTT solution (0.5 mg/mL) for 1 hour After 1 hour, the solution was discarded, then 100 µL of isopropanol was added for solubilized the insoluble formazan before measuring OD. The optical density at 570 nm was then measured using a plate reader spectrophotometer (VersaMax).

Hematoxylin and eosin (H&E stain)

After 1 week and 2 weeks of culture, constructs were washed three times in PBS, and fixed in 4% phosphate-buffered paraformaldehyde for 18-24 hours, dehydrated through a graded series of ethanol, wax-embedded in paraffin, then sectioned in 10 µm thick slices. The tissue sections for histological analysis were soaked in xylene for 5 minutes for removed paraffin, 90%, 80% and 70% ethanol were used as rehydrated reagents. After rehydrated process, the sections were stained with hematoxylin and eosin. The sample was dehydrated again and mounted with coverslip slides using permount. All types of the scaffolds also observed the microstructure by H&E staining.

Statistical Analysis

All experiments were conducted in triplicate, and data were represented as means ± standard deviation. Data were analyzed using GraphPad Prism (v.8.0.2) by one-way ANOVA with Tukey's multiple comparison tests. $p < 0.05$ was regarded as statistically significant.

Results

Microstructure analysis of the AG-CH-GEL scaffolds

The photography image and H&E staining of AG-CH-GEL scaffolds were observed as shown in Fig. 1A-1L. The biocomposite scaffold was fabricated by freeze-drying method, which could generate three dimensional sponge-like structures scaffold. The AG-CH-GEL scaffolds were white to pale yellow in color. We further analyzed the microstructure of biomaterial scaffolds using H&E staining. The entire AG-CH-GEL scaffold showed a similar pattern of non-uniform distribution of pore morphology (Fig. 1B-1L). The polygonal shape and random orientation of pores were observed in the condition of the scaffold 3, 5 and 6 as shown in Fig. 1I, 1K, and 1L, respectively. The pore size diameter of the biomaterial scaffold

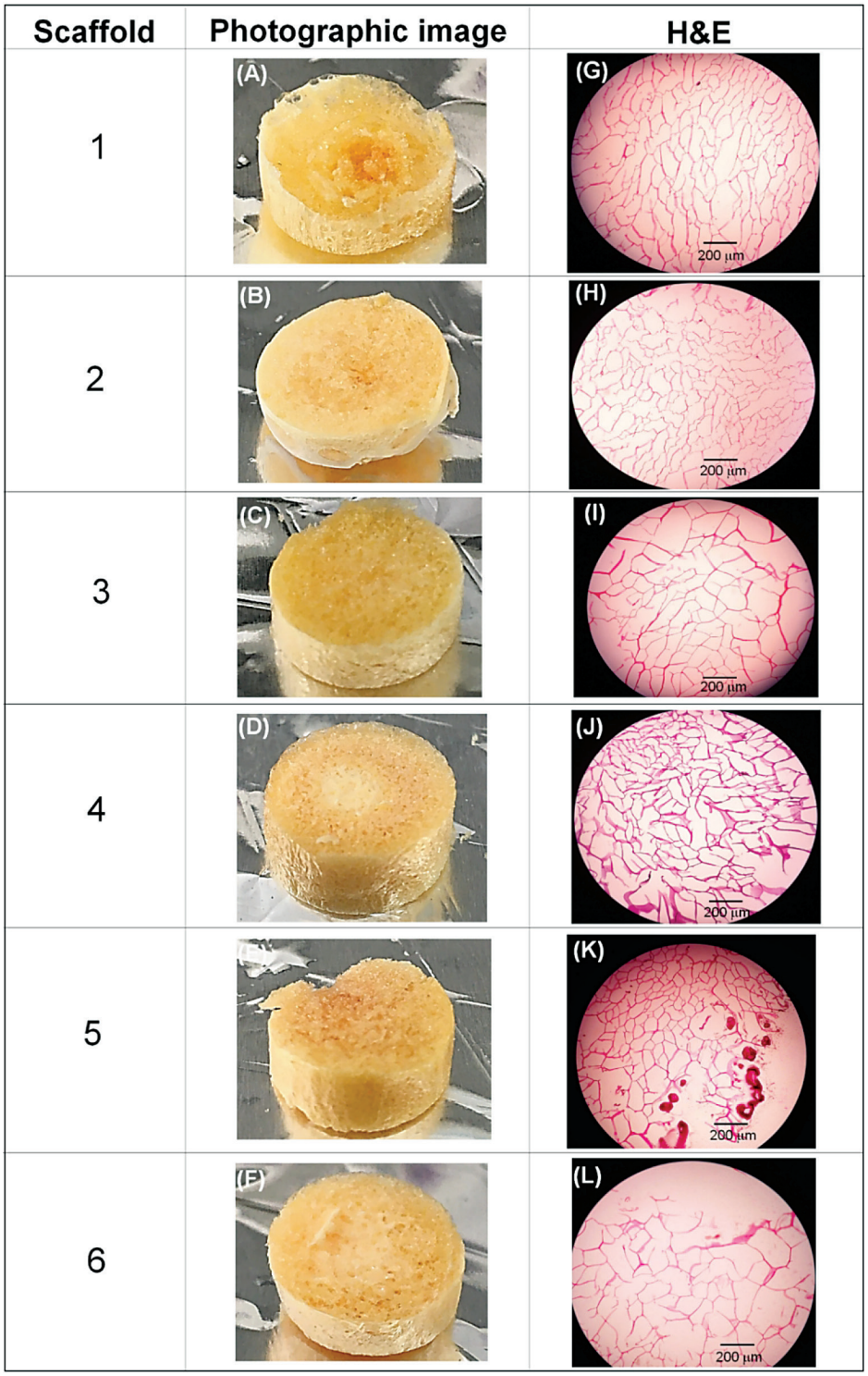


Fig. 1 Photography and H&E staining image of composite scaffold in different conditions. Scale bar = 200 µm

also exhibited a similar range which varies from 151 to 183 μm.

Characterization of the AG-CH-GEL scaffolds

To study the water absorption properties of the composite scaffold, all types of the scaffold were weighed before being soaked in 1X PBS. It was observed that all the scaffolds had water uptake ability up to 80% (Fig. 2). The percentage water uptake ability of scaffolds 1, 2, 3, 4, 5, and 6 were 79.88 ± 1.87, 81.09 ± 1.71, 78.31 ± 2.22, 80.93 ± 2.46,

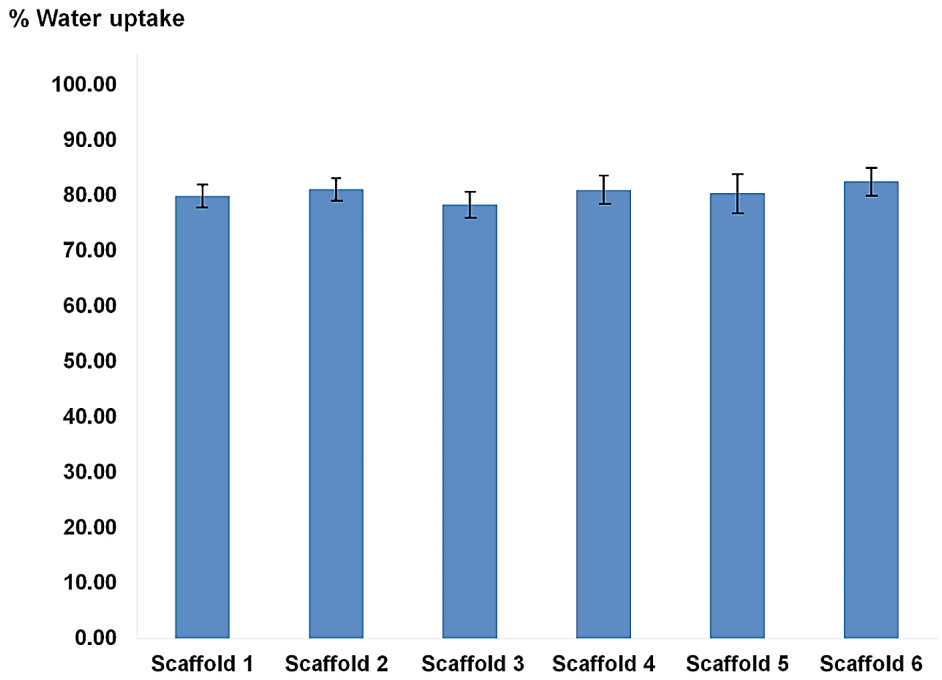


Fig. 2 The water absorption capability of the scaffold composite. Data were shown as mean ± SD. Each error bar represents the standard error of the mean (SD) from triplicate experiments by One-Way ANOVA. Statistically significant differences between untreated control and toxin treatment group are indicated by asterisks (* p value < 0.05).

80.32 ± 3.35 and 82.46 ± 2.40, respectively. However, there was no significant difference between the water uptake ability.

Degradation characteristics of all scaffolds were examined after 1 week of incubation in 1X PBS (pH 7.4). The percentage of degree of degradation of scaffolds 1, 2, 3, 4, 5, 6 was 6.92 ± 1.85, 6.87 ± 1.37, 7.67 ± 2.23, 10.38 ± 2.20, 8.72 ± 1.02 and 11.72 ± 1.55, respectively (Fig. 3). The degree of degradation of the scaffold 6 was found to be highest, while the scaffold 2 exhibited the lowest degradation rate. In addition, the degradation rate of scaffold 6 at 1 week showed

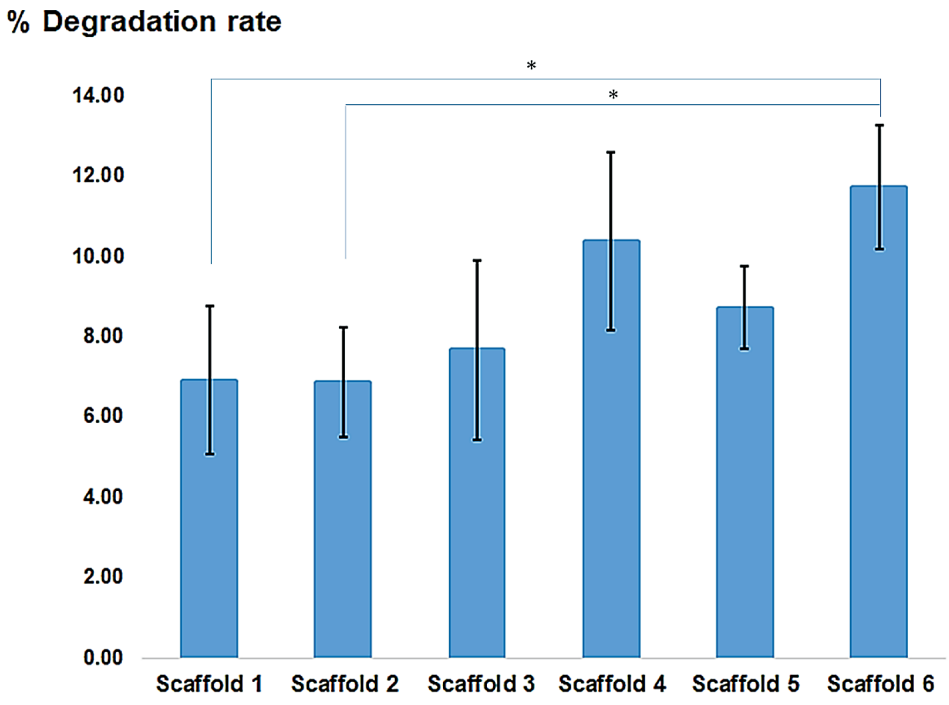


Fig. 3 The degree of degradation of scaffold 1, 2, 3, 4, 5, 6 was evaluated. Data were shown as mean ± SD. Each error bar represents the standard error of the mean (SD) from triplicate experiments by One-Way ANOVA. Statistically significant differences between untreated control and toxin treatment group are indicated by asterisks (* p value < 0.05).

a significant difference from the degradation rates of scaffold 1 and 2.

Viability of human fibroblast within the scaffolds

MTT assay was performed to evaluate the cytotoxicity of the scaffold. This method detects cell growth and proliferation since mitochondria oxidize the MTT solution, giving a typical blue-violet product quantified by spectrophotometry. Fig. 4 shows the value of absorbance when culture fibroblast on scaffolds 4 and 6 at 1 and 2 weeks. The numbers of fibroblast cells culture both on scaffold 4 and scaffold 6 increased significantly at indicated time point. As well as, the number of fibroblast cells culture on scaffold 6 at 2 weeks significantly increased when compared to fibroblast growth on scaffold 4 as demonstrated in Fig. 4. Unfortunately, the value of absorbance at 570 nm could not be detected in the other constructs. We further investigated whether fibroblast cells could adhere to scaffold by using H&E staining. H&E results suggested that human fibroblast proliferation on scaffold 4 and 6 remarkably over time as demonstrated in Fig. 5A-D. The morphology of fibroblast growth on the scaffold exhibited both round cell and long spindle shapes.

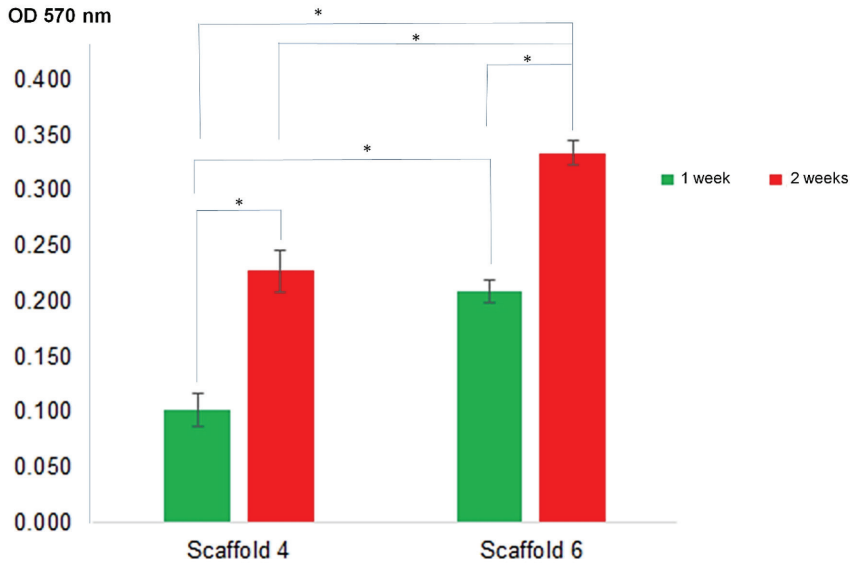


Fig. 4 The cell viability of fibroblast on scaffold 4 and scaffold 6 was evaluated by MTT assay at 1 week and 2 weeks. Data were shown as mean ± SD, n = 3. Each error bar represents the standard error of the mean (SD) from triplicate experiments by One-Way ANOVA. Statistically significant differences between each group of scaffold and the differences between culture period are indicated by asterisks (* p value < 0.05).

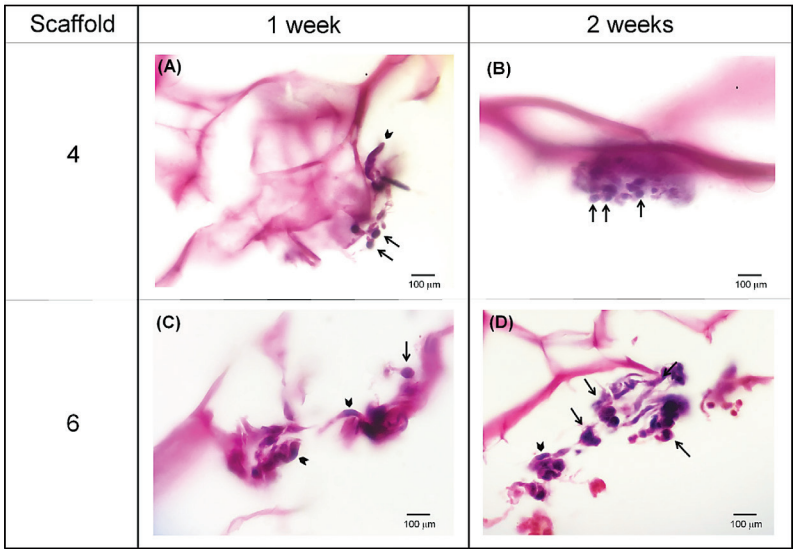


Fig. 5 Histological images of fibroblast cells cultured on the scaffolds 4 after (A) 1 week and (B) 2 week. As well as Figure C and D demonstrated fibroblast cell growth on scaffold 6 at 1 week and 2 weeks, respectively. Black arrows head indicates spindle shape of fibroblast cell growth on the AG-CH-GEL scaffold and arrow indicate round shape of fibroblast cell. Images were captured with 100X magnification. Scale bar = 100 μm

Discussion and Conclusion

In this research, the 3 types of natural biomaterials; agarose, chitosan, and gelatin were designed via a freeze-drying method on the basis of creating three-dimensional microenvironment to promote fibroblast cell growth and proliferation. After the freeze-drying process, the mixtures of material are frozen and subsequently removed solvent under vacuum. The formation of the porous structure as well as pore volume and pore morphology is dependent on many factors such as freeze temperature, solution concentration, nature of solvent and solute, and the control of the freeze direction.[35, 36] Thus, freeze-drying technology could produce three-dimensional porous scaffolds with porosity beyond 90% and a pore diameter range of 20-400 μm.[37] The entire scaffold has three-dimensional porous structure after freeze-drying process. This structure would be beneficial for the growth of cells. Biomaterials scaffold with spongy pores is a mimic microenvironment of native tissue. This network structure includes pores that can be filled with culture medium, water molecules as well as metabolic waste that can transport efficiently throughout the scaffold. The appearances and morphological structures of the scaffold were observed by photography images. However, there were no differences in the gross appearances of the scaffolds. The microstructure of biomaterial scaffolds showed similar pattern of polygonal shape and random orientation of pore. The average pore size diameter of the biomaterial scaffold also exhibited similar range which varies from 151 to 183 μm. The largest pore size was found in scaffold 4 which consisting of 2% agarose, 2% chitosan, and 3% gelatin.

Because of the water absorption ability of the entire scaffold up to 80%, cells can receive the nutrient from culture media. The water absorption capability could increase due to the high porosity and large pore size.[38, 39] These properties of the scaffold, result in cell attachment to the surface of scaffold and growth. The degradation rate of scaffold 6 showed a significant difference from the degradation rates of scaffold 1 and 2 in 1 week. The highest degradation rate was found with higher gelatin concentration in the composite scaffold, scaffold 4 (10.38%) and 6 (11.72%). In comparison to our previous study[28] that fabricated the freeze-drying scaffold consisting of 3% agarose, 2% chitosan and 3% collagen, the degradation rate at 1 week of those scaffolds were only 3% which was lower than the scaffold containing gelatin. This might be due to the fact that gelatin is a denatured form of collagen. Moreover, gelatin is highly susceptible to several proteases, thus the composite scaffold contains high concentration of gelatin might be faster degradation than the others composite scaffolds.

The cell viability and proliferation were performed using MTT assay. At indicated time point, the MTT assay revealed that only scaffold 4 and scaffold 6 could promote cell growth.

Scaffold 4 which consists of 2% agarose, 2% chitosan, and 3% gelatin was observed with a few numbers of fibroblasts in the 1st week. Further analysis revealed that increased culture time correlates with an increase in the cell number on the scaffold 4. Also, scaffold 6 which consisting of 3% agarose, 2% chitosan, and 3% gelatin, found human fibroblast proliferation remarkably over time. This finding suggested that scaffold 4 and 6 could promote cell adhesion and proliferation. The optimal solution mixture of 2-3% agarose, 2% chitosan, and 3% gelatin and subsequent freeze dry method might create the suitable microenvironment for fibroblast. Many biomaterials have been reported their suitable properties for supporting fibroblast adhesion. Collagen and gelatin are widely applied to tissue engineering because of their biocompatibility and biodegradability.[21, 22] Furthermore, collagen and gelatin contain the RGD motif (Arginine-Glycine-Aspartic acid sequences) that are associated with cell adhesion.[40, 41] Silk fibroin is a natural biopolymer with various applications in the human body as a suture for surgery and wound skin treatment. Because of their biocompatibility, biodegradability, elasticity, and mechanical strength, ligament fibroblasts were seen to attach and proliferate well on the silk scaffolds, forming cell aggregates. As well as, fibroblast gradually increased in size and fused with adjacent cell aggregates to cover wide areas of the scaffolds.[42] Chitin and chitosan are a linear carbohydrate biopolymers, their structure are similar to GAGs in the extracellular matrix, which benefits cell adhesion.[3, 7] Moreover, chitin and chitosan have excellent bioactive properties, such as biodegradability, non-toxicity, and antimicrobial activity.[18, 19] Therefore, chitin and chitosan scaffolds are reported to promote rapid fibroblast proliferation and accelerate wound healing.[18, 19, 43]

However, very low cell growth on the scaffold during the 1st week of cell culture was observed when compared to our previous work.[28] This finding implied that the early stages of cell growth on the scaffold required adhesion molecules to bind to integrin binding site on the surface of the scaffold before starting to proliferation.[44] Thus, the initial cell-scaffold interaction is a significant step toward successful tissue engineering. Cell adhesion is the first stage of cell proliferation which affects the final biological performance of the scaffold.[45-47] As well as, cell attachment is influenced by several scaffold properties including biomaterials, mechanical properties, porosity, pore size, and connectivity. The reason could be that the composite scaffold which consists of 2-3% agarose, 2% chitosan, and 3% gelatin might be promoted the initial cell-scaffold interactions. However, we cannot observe any fibroblast cells on the other scaffold. These might be happened before cell starting to proliferate, those cells have to adaptation to new environment and produce an

integrin to bind to integrin binding site on the surface of the scaffold.[46] In addition, some of the cells will move directly through the bottom surface of the culture plate. This result suggest that the scaffold 1-3 and scaffold 5 might not suit for cell culture in term of promoting cell adhesion as well as the structure inside those scaffolds might obstruct resulting in low cells growth and proliferation. Thus, understanding the details of the cell attachment process and the type of cell—material interaction is crucial.

In conclusion, the biomaterial scaffold was prepared by blending the agarose, chitosan, and gelatin solution followed by a freeze-drying method. The three-dimensional sponge-like structures scaffold with a pore size diameter range 151 to 183 μm were synthesized. The composite scaffold which consists of 2-3% agarose, 2% chitosan, and 3% gelatin could be suggested as a promising material to promote fibroblast cell growth in tissue engineering.

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Conflicts of Interest

The authors declare no conflict of interest.

References

1. Jaipaew J, Wangkulangkul P, Meesane J, *et al.* Mimicked cartilage scaffolds of silk fibroin/hyaluronic acid with stem cells for osteoarthritis surgery: Morphological, mechanical, and physical clues. Mater Sci Eng C Mater Biol Appl 2016; 64: 173-82.
2. Somaiah C, Kumar A, Mawrie D, *et al.* Collagen promotes higher adhesion, survival and proliferation of mesenchymal stem cells. PLoS One 2015; 10: e0145068.
3. Manferdini C, Gabusi E. Chitosan-based scaffold counteracts hypertrophic and fibrotic markers in chondrogenic differentiated mesenchymal stromal cells. J Tissue Eng Regen Med 2019; 13: 1896-911.
4. Stokols S, Tuszynski MH. Freeze-dried agarose scaffolds with uniaxial channels stimulate and guide linear axonal growth following spinal cord injury. Biomaterials 2006; 27: 443-51.
5. Ng KW, Wang CC, Mauck RL, *et al.* A layered agarose approach to fabricate depth-dependent inhomogeneity in chondrocyte-seeded constructs. J Orthop Res 2005; 23: 134-41.
6. Buschmann MD, Gluzband YA, Grodzinsky AJ, *et al.* Mechanical compression modulates matrix biosynthesis in chondrocyte/agarose culture. J Cell Sci 1995; 108 : 1497-508.

7. Badhe RV, Bijukumar D, Chejara DR, *et al.* A composite chitosan-gelatin bi-layered, biomimetic macroporous scaffold for blood vessel tissue engineering. Carbohydr Polym 2017; 157: 1215-25.
8. Chua BY, Al Kobaisi M, Zeng W, *et al.* Chitosan microparticles and nanoparticles as biocompatible delivery vehicles for peptide and protein-based immunocontraceptive vaccines. Mol Pharm 2012; 9: 81-90.
9. Liu M, Zheng H, Chen J, *et al.* Chitosan-chitin nanocrystal composite scaffolds for tissue engineering. Carbohydr Polym 2016; 152: 832-40.
10. Ratanavaraporn J, Damrongsakkul S, Sanchavanakit N, *et al.* Comparison of Gelatin and Collagen Scaffolds for Fibroblast Cell Culture. J Met MaterMiner 2006; 16: 31-6.
11. Kathuria N, Tripathi A, Kar KK, *et al.* Synthesis and characterization of elastic and macroporous chitosan-gelatin cryogels for tissue engineering. Acta Biomater 2009; 5: 406-18.
12. Lien SM, Ko LY, Huang TJ. Effect of pore size on ECM secretion and cell growth in gelatin scaffold for articular cartilage tissue engineering. Acta Biomater 2009; 5: 670-9.
13. Zhang S, Chen L, Jiang Y, *et al.* Bi-layer collagen/microporous electrospun nanofiber scaffold improves the osteochondral regeneration. Acta Biomater 2013; 9: 7236-47.
14. Tamaddon M, Burrows M, Ferreira SA, *et al.* Monomeric, porous type II collagen scaffolds promote chondrogenic differentiation of human bone marrow mesenchymal stem cells in vitro. Sci Rep 2017; 7: 43519.
15. Stokols S, Tuszynski MH. The fabrication and characterization of linearly oriented nerve guidance scaffolds for spinal cord injury. Biomaterials 2004; 25: 5839-46.
16. Zhao R, Xu Z, Li B, *et al.* A comparative study on agarose acetate and PDLLA scaffold for rabbit femur defect regeneration. Biomed Mater 2019; 14: 065007.
17. Meinel L, Hofmann S, Karageorgiou V, *et al.* The inflammatory responses to silk films in vitro and in vivo. Biomaterials 2005; 26: 147-55.
18. Saravanan S, Leena RS, Selvamurugan N. Chitosan based biocomposite scaffolds for bone tissue engineering. Int J Biol Macromol 2016; 93: 1354-65.
19. Oryan A, Sahvieh S. Effectiveness of chitosan scaffold in skin, bone and cartilage healing. Int J Biol Macromol 2017; 104: 1003-11.
20. Chang YH, Wu KC, Wang CC, *et al.* Enhanced chondrogenesis of human umbilical cord mesenchymal stem cells in a gelatin honeycomb scaffold. J Biomed Mater Res A 2020; 108: 2069-79.

21. Huang Y, Onyeri S, Siewe M, Moshfeghian A, Madihally SV. In vitro characterization of chitosan-gelatin scaffolds for tissue engineering. Biomaterials 2005; 26: 7616-27.
22. Gentile P, Nandagiri VK, Daly J, *et al.* Localised controlled release of simvastatin from porous chitosan-gelatin scaffolds engrafted with simvastatin loaded PLGA-microparticles for bone tissue engineering application. Mater Sci Eng C Mater Biol Appl 2016; 59: 249-57.
23. Bellich B, Agostino ID, Semeraro S, *et al.* "The Good, the Bad and the Ugly" of Chitosans. Mar Drugs 2016; 14: 99.
24. Su K, Wang C. Recent advances in the use of gelatin in biomedical research. Biotechnol Lett 2015; 37: 2139-45.
25. Song JH, Kim HE, Kim HW. Production of electrospun gelatin nanofiber by water-based co-solvent approach. J Mater Sci Mater Med 2008; 19: 95-102.
26. Bhat S, Tripathi A, Kumar A. Supermacroprous chitosan-agarose-gelatin cryogels: in vitro characterization and in vivo assessment for cartilage tissue engineering. J R Soc Interface 2011; 8: 540-54.
27. Merlin Rajesh Lal LP, Suraishkumar GK, Nair PD. Chitosan-agarose scaffolds supports chondrogenesis of Human Wharton's Jelly mesenchymal stem cells. J Biomed Mater Res A 2017; 105: 1845-55.
28. Chonanant C. Fabrication the biomaterial scaffold for tissue engineering by freeze-drying technique. J Med Tech Phy Ther 2019; 31: 370-81.
29. Baron RD, Perez LL, Salcedo JM, *et al.* Production and characterization of films based on blends of chitosan from blue crab (Callinectes sapidus) waste and pectin from Orange (Citrus sinensis Osbeck) peel. Int J Biol Macromol 2017; 98: 676-83.
30. Manni L, Ghorbel-Bellaaj O, Jellouli K, *et al.* Extraction and characterization of chitin, chitosan, and protein hydrolysates prepared from shrimp waste by treatment with crude protease from Bacillus cereus SV1. Appl Biochem Biotechnol 2010; 162: 345-57.
31. Chonanant C. Fabrication the biomaterial scaffold for tissue engineering by freeze-drying technique. J Med Tech Phy Ther 2019; 31: 370-81.
32. Mao JS, Cui YL, Wang XH, *et al.* Preliminary study on chitosan and gelatin polyelectrolyte complex cytocompatibility by cell cycle and apoptosis analysis. Biomaterials 2004; 25: 3973-81.
33. Yan LP, Wang YJ, Ren L, *et al.* Genipin-cross-linked collagen/chitosan biomimetic scaffolds for articular cartilage tissue engineering applications. J Biomed Mater Res A 2010; 95: 465-75

34. Tripathi A, Melo JS. Preparation of a sponge-like biocomposite agarose-chitosan scaffold with primary hepatocytes for establishing an in vitro 3D liver tissue model. RSC Advances 2015; 5: 30701-10.
35. Brougham CM, Levingstone TJ, Shen N, *et al.* Freeze-Drying as a novel biofabrication method for achieving a controlled microarchitecture within large, complex natural biomaterial scaffolds. Adv Healthc Mater 2017; 6: doi: 10.1002/adhm. 201700598
36. Wang C, Jiang W, Zuo W, *et al.* Effect of heat-transfer capability on micropore structure of freeze-drying alginate scaffold. Mater Sci Eng C Mater Biol Appl 2018; 93: 944-9.
37. O'Brien FJ, Harley BA, Yannas IV, *et al.* Influence of freezing rate on pore structure in freeze-dried collagen-GAG scaffolds. Biomaterials 2004; 25: 1077-86.
38. Tadros T. Polymeric surfactants in disperse systems. Adv Colloid Interface Sci 2009; 147-148: 281-99.
39. Ma P, Choi J. Biodegradable scaffolds with well defined interconnected special pore network. Tissue Eng 2001; 7: 23-33.
40. Kempf M, Miyamura Y, Liu PY, *et al.* A denatured collagen microfiber scaffold seeded with human fibroblasts and keratinocytes for skin grafting. Biomaterials. 2011; 32: 4782-92.
41. Ratanawaraporn J, Danrongsakkul S, Sanchavanakit N, *et al.* Comparison of gelatin and collagen scaffolds for fibroblast cell culture. J Met Mater Min 2006; 16: 31-6.
42. Liu H, Fan H, Toh S *et al.* A comparison of rabbit mesenchymal stem cells and anterior cruciate ligament fibroblasts responses on combined silk scaffolds. Biomaterials. 2008; 29: 1443-53.
43. Barbalinardo M, Biagetti M, Valle F, *et al.* Green Biocompatible Method for the synthesis of collagen/chitin composites to study their composition and assembly influence on fibroblasts growth. Biomacromolecules 2021; 22: 3357-65
44. Röder A, García-Gareta E, Theodoropoulos C, *et al.* An assessment of cell culture plate surface chemistry for in vitro studies of tissue engineering scaffolds. J Funct Biomater 2015; 6: 1054-63.
45. Chen H, Liu Y, Jiang Z, *et al.* Cell-scaffold interaction within engineered tissue. Exp Cell Res 2014; 323: 346-51.
46. Adams JC. Cell-matrix contact structures. Cell Mol Life Sci CMLS 2001; 58: 371-92.
47. Andrews KD, Hunt JA. Upregulation of matrix and adhesion molecules induced by controlled topography. J Mater Sci Mater Med 2008; 19: 1601-8.