PHYSICS AND CHEMISTRY OF SOLID STATE
V. 22, No. 4 (2021) pp. 767-774
Section: Technology

DOI: 10.15330/pcss.22.4.767-774

Vasyl Stefanyk Precarpathian National University

ФІЗИКА І ХІМІЯ ТВЕРДОГО ТІЛА
Т. 22, № 4 (2021) С. 767-774
Технічні науки

PACS: 87.85.G; 62.20.F ISSN 1729-4428

Akram Jassim Jawad[1,2]

Investigation of the Effect of Agarose Gel Concentration and Culture Period on Bio and Mechanical Properties of Chondrocyte Tissue Engineering

[1]*University of Babylon, College of Materials Engineering, Department of Polymer and Petrochemicals Industries Engineering, Al Hillah, Iraq.*
[2]*Queen Mary University of London, School of Engineering and Materials Science, London, UK.,akrammaterials4@gmail.com*

As a gel scaffold for chondrocyte tissue engineering, agarose concentration plays a significant role in the relationship between porosity and nutrition. In this work, the effect of concentration and period cultured on Glycosaminoglycan (GAG) and mechanical properties were studied. A bovine chondrocytes were isolated and seeded in different agarose gel scoffed concentrations, about 4% and 6%, for different period cultured, 0 and 7 days. The Mechanical Test Machine (MTS) and Spectrophotometric with calibration curve method were used to measure mechanical properties, and GAG concentration of the prepared samples, respectively. The results of mechanical tests and GAG contents have shown that there are a wide range of dispersion in the most of the samples, which attribute to different factors. For mechanical properties, these factors could be attributed to anisotropic of the produced chondrocyte with agarose scaffolds, insufficient cells' dispersion within the gel scaffold during seeding and cultured time, and some test procedure condition, such as Earle's Balanced Salt Solution (EBSS) hydration. While for GAG results, those factors could be the differences of the cell growth environment between in-vitro and in vivo media. However, the average maximum stress value increased in 6% agarose from 0.01331 N/mm^2 at 0 days to 0.01678 N/mm^2 at 7 days, as increasing the GAG concentration that indicates increasing chondrocyte growth. Generally, also the GAG concentration increase from 3.7 to 70 μg/ml at 4% and from 6.4 to 55.4 at 6% agarose for 0 and 7 days period cultured, respectively. The recommended way to solve these differences is using a bioreactor, which could introduce more matching between in-vitro and in vivo media.

Keywords: Chondrocyte, Tissue Engineering, Agarose, Biomaterials, Glycosaminoglycan.

Received 27 August 2021; Accepted 16 December 2021.

Introduction

There are many factors that have an effect on mechanical and regenerative properties to regenerate articular cartilage, especially scaffold design and cultured period [1, 2, 3]. Gel based scaffold has been evaluated and applied for its high ability in cartilage tissue engineering, especially agarose based scaffold [4, 5]. Additionally, the important criteria in the design of gel scaffold materials are mechanical, physicochemical properties and 3D structure of the host scaffold, which includes also size, number and dispersions of pores. For example, El-Sherbiny and Yacoub (2013) mentioned that the interconnectivity of pores is a significant factor to make all the cells, which are within 200 μm range from the blood source supply to permit the mass transfer of nutrients materials and oxygen pass [2]. Moreover, Huber and et al. (2000) pointed that the period of cell cultured and nutrition and oxygen supplies play an important role in the regenerative ability of tissue engineering of chondrocytes, which are the main content to survive the extracellular matrix (ECM) [6]. Also, Lee and Bader (1997) clarified that production of the right proteoglycan can be helpful for more chondrocytes healthy, which can be investigated by the

glycosaminoglycan (GAG) content in the system [7]. Buckwalter and Mankin (1998) showed that GAG concentration has an impact on the mechanical properties of the produced tissue of chondrocytes, which means it is a crucial tool to monitor the regenerative ability [8].

In this work, the effect of the agarose gel concentration and culture period on Glycosaminoglycan (GAG) synthesis and stiffness of the chondrocyte/agarose constructs were investigated. The agarose gel concentrations were 4% and 6%, while the period culture were 0 and 7 days. The MTS machine and Spectrophotometric with calibration curve method were used to measure mechanical properties, and GAGs concentration of the prepared samples, respectively.

I. Experimental

Firstly, an articular cartilage were removed from the joint of bovine metacarpal-phalangeal. Then, the chondrocytes were isolated from the extracellular matrix part (ECM) by using pronase and collagenase. Then, the cells were washed and placed again in 10 ml solution of culture medium, which is Dulbecco's Minimal Essential Medium supplemented plus 20% Fetal Calf Serum (DMEM+20%FCS). Preparation 4% and 6% of agarose specimens were done by using a suspension of ultra-low gelling temperature agarose in Earls Balanced Salt Solution (EBSS), which are autoclaved and cooled to 37C for 5 minutes. Then, the isolated cells were mixed into agarose, and molded them into 5 mm height and 5 mm diameter mold. The samples were cultured for 7 days, with changed the culture medium for every 2-3 days.

The MTS machine were used for mechanical characterization, where the core was hydrated with EBSS, with a speed of 0.0167 mm/second to a compression force of 20 % strain, with 10 minutes holding time. The compression and relaxation phases were sited up to be 10 Hz and 1 Hz, respectively, and the same procedure was repeated for 5 samples. Spectrophotometric methods were used to calculate Glycosaminoglycan (GAG) concentration, where the metachromatic shitting of the dye complexes of sulphated GAGs in the absorbance wavelength range from 600 nm to 535 nm were used as an indicator for calibration curve.

II. Results and discussion

Figure 1 shows stress relaxation results of cells/gel scaffold at different gel agarose concentrations (4% and 6%), and different periods cultured (0 days and 7 days), where the test was repeated for five samples, while figure 2 shows the average values curve of that. In figure 3, the stress and strain relationship results were presented, while figure 2 shows the average values curve of that. In

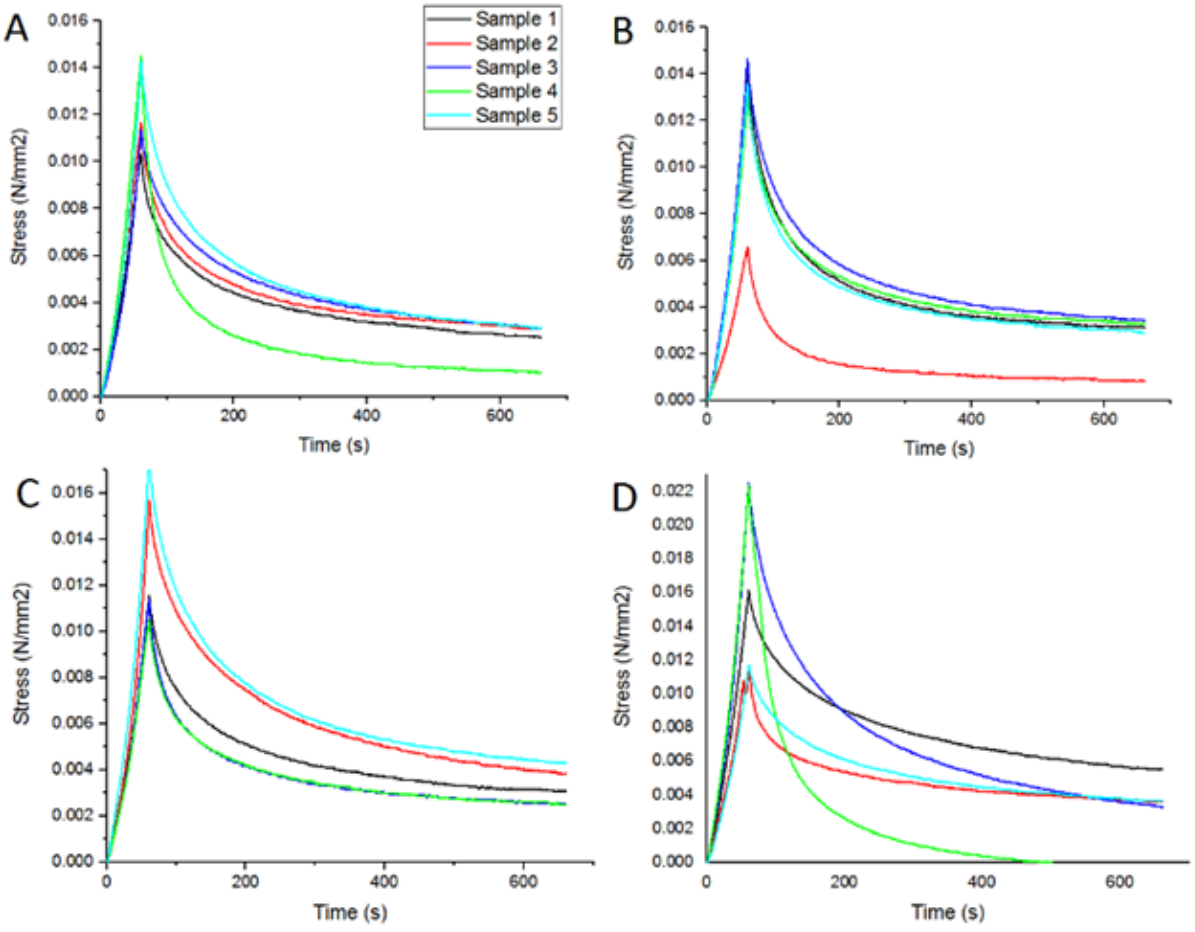


Fig. 1. Stress relaxation results of cells/gel scaffold at different gel agarose concentrations (4% and 6%), and different periods cultured (0 days and 7 days), (A) 4% and 0 days, (B) 4% and 7 days (C) 6% and 0 days (D) 6% and 7 days, five samples for each case.

figure 5 (A) and (B), the individual samples and the mean values of the tangent Young's modulus that were calculated from figure 4 at 15% strain were plotted as a histogram, respectively. After that, the tangent Young's modulus values at 15% strain were analyzed to calculate the mean, maximum, minimum and standard deviation values, which were illustrated in table 1.

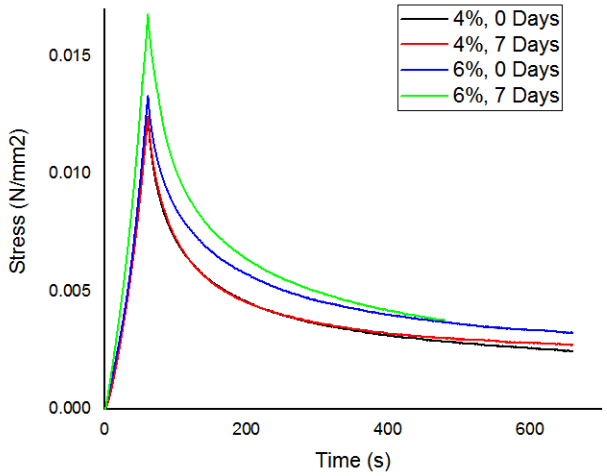


Fig. 2. The average curve of stress relaxation results of cells/gel scaffold at different gel agarose concentrations (4% and 6%), and different periods cultured (0 days and 7 days).

In figure 6 (A) and (B), the five individual and mean values of the total GAG of cells/gel scaffolds were plotted in a histogram form, respectively. The mean, maximum, minimum and standard deviation analysis values of total GAG were calculated and presented in table 2. A comparison between the mean values of total GAG and the tangent Young's modulus were plotted in figure 7. Analyses of pair samples T-test and one way ANOVA data statistics of GAG concentration and the tangent Young's modulus of cells/gel scaffolds were shown in table 3 and 4, respectively.

The relaxation test results shows different range of dispersion, for example there are high fitting and matching values of stress-time curves for all cells/gel scaffold samples at 4 % in both 0 and 7 days cultured periods, except sample number 4 at 0 days and 2 at 7 days, respectively. While, there are a wide and a broad distribution of data for 6 % in both 0 and 7 days cultured periods, as it is clear in figure 1. The average values of these data show that there is no change in the maximum stress at 4% in both 0 and 7 days, while it increases at 6% from 0.01331 N/mm^2 at 0 days to 0.01678 N/mm^2 at 7 days, as it was shown in figure 2.

Stress and strain relationship behaves in the same way in which the relaxation stress curves have behaved, as it was shown in figure 3. The general tendency indicates that there are obvious increasing in the maximum stress point of 6% agarose from 0.0113 N/mm^2 to 0.0150 N/mm^2 at 0 and 7 days, respectively, while it was not a significant change at 4%, as it is clear in figure 4. Similarly, the tangent young

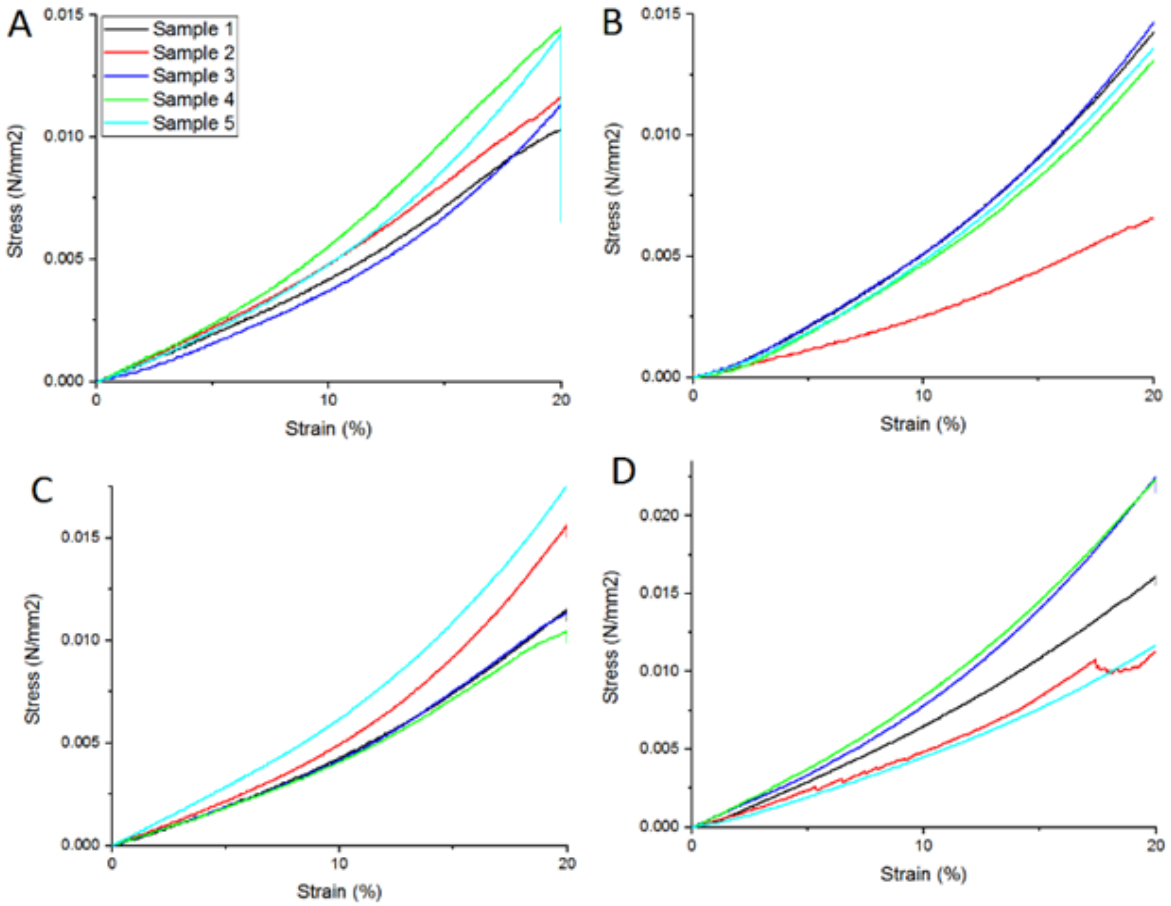


Fig. 3. Stress and strain relationship results of cells/gel scaffold at different gel agarose concentrations (4% and 6%), and different periods cultured (0 days and 7 days), (A) 4% and 0 days, (B) 4% and 7 days (C) 6% and 0 days (D) 6% and 7 days, five samples for each case.

modulus values behaviours were illustrated in figure 5, which illustrates the 6% with 7 days period cultured have more values. This increasing in the mechanical properties with increasing the content of agarose could be attributed to high mechanical properties of agarose, and decreasing in the pores sizes of the scaffold, as the relationships were shown in figure 8 A and B [9, 10, 11]. Furthermore, the optimum pore size of scaffold for chondrocyte cells was found in the literature about 250 to 500 µm [12].

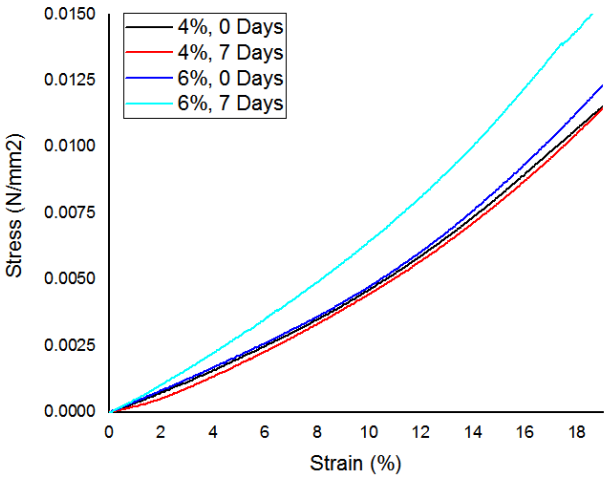


Fig. 4. Average five samples curve of Stress and strain relationship results of cells/gel scaffold at different gel agarose concentrations (4% and 6%), and different periods cultured (0 days and 7 days).

From table 1, there are high diversities and inaccuracy, which could be because one or more of procedure errors. For example, this could be as a result of anisotropic of the produced chondrocyte with agarose scaffolds, which leads different mechanical properties with different loading directions. Another factor might be insufficient cells' dispersion within the gel scaffold during seeding and cultured time as a result of seeding conditions and gravity. Also, it could be some test conditions, such as the differences in EBSS hydration of samples while doing the mechanical characterization, where the short period cultured have higher ability of hydration, which could solve it by immersion of samples for a specific time inside EBSS.

From figure 6 and standard deviation in table 2, the GAG concentration measurements show high non-homogeneity values over all samples. Generally, the GAG concentration values increase from 3.7 to 70 µg/ml at 4% and from 6.4 to 55.4 µg/ml at 6% agarose for 0 and 7 days period cultured, respectively, as it was shown in table 2. That means the 4% agarose concentration show higher content of GAG, which may attribute to high diffusion ability of nutrient and metabolite products inside the cells in lower concentration of agarose [6, 7, 13].

In figure 7, the comparison between GAG concentration and the tangent Young's modulus values shows non-understandable relationship. However, the tangent modulus tend to increase as GAG concentration increasing with changing the period cultured for a specific agarose concentration. This is because after 7 days period cultured the chondrocytes created higher content of the GAG in extracellular matrices (ECM),

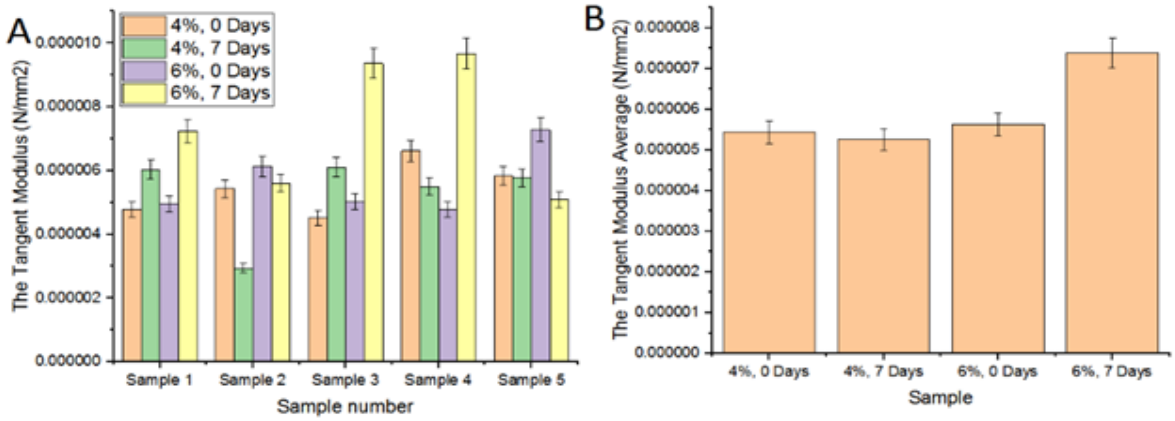


Fig. 5. (A) The values of the tangent Young's modulus at 15% strain of cells/gel scaffold at different gel agarose concentrations (4% and 6%), and different periods cultured (0 days and 7 days), five samples for each case, (B) The mean values of the tangent Young's modulus at 15% strain of cells/gel scaffold at different gel agarose concentrations (4% and 6%), and different periods cultured (0 days and 7 days).

Table 1.

Mean, maximum, minimum and standard deviation analysis values of the tangent Young's modulus at 15% strain of cells/gel scaffold at different gel agarose concentrations (4% and 6%), and different periods cultured (0 days and 7 days).

Sample	Mean	Standard Deviation	Minimum	Maximum
4%, 0 Days	5.44E-06	8.41E-07	4.51E-06	6.62E-06
4%, 7 Days	5.27E-06	1.33E-06	2.94E-06	6.11E-06
6%, 0 Days	5.63E-06	1.06E-06	4.79E-06	7.28E-06
6%, 7 Days	7.39E-06	2.10E-06	5.09E-06	9.67E-06

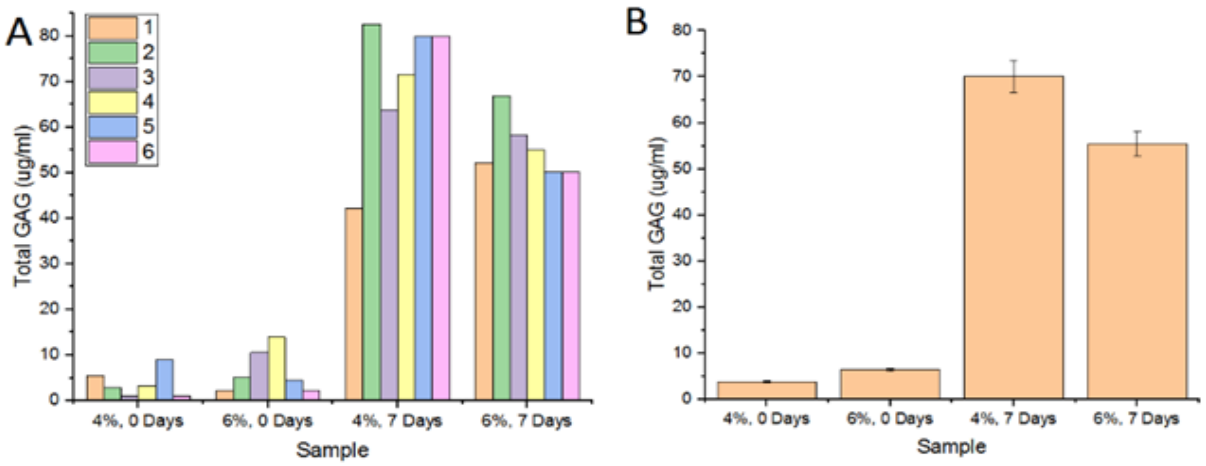


Fig. 6. (A) The values of total GAG of cells/gel scaffold at different gel agarose concentrations (4% and 6%), and different periods cultured (0 days and 7 days), five samples for each case, (B) The mean values of total GAG of cells/gel scaffold at different gel agarose concentrations (4% and 6%), and different periods cultured (0 days and 7 days).

Table 2.

Mean, maximum, minimum and standard deviation analysis values of total GAG of cells/gel scaffold at different gel agarose concentrations (4% and 6%), and different periods cultured (0 days and 7 days).

Sample	Mean	Standard Deviation	Minimum	Maximum
4%, 0 Days	3.77234	3.07055	1.03	9.06635
6%, 0 Days	6.47122	4.78408	2.25	13.99719
4%, 7 Days	70.08477	15.33286	42.21516	82.7
6%, 7 Days	55.48564	6.40141	50.2	66.92647

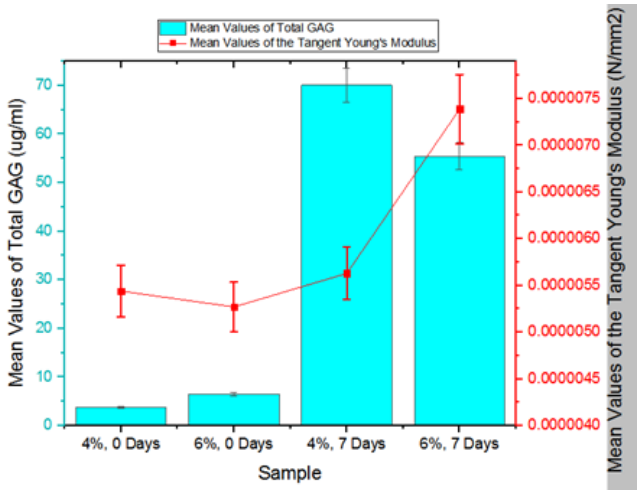


Fig. 7. Comparison between the mean values of total GAG and the tangent Young's modulus of cells/gel scaffold at different gel agarose concentrations (4% and 6%), and different periods cultured (0 days and 7 days).

Table 3.

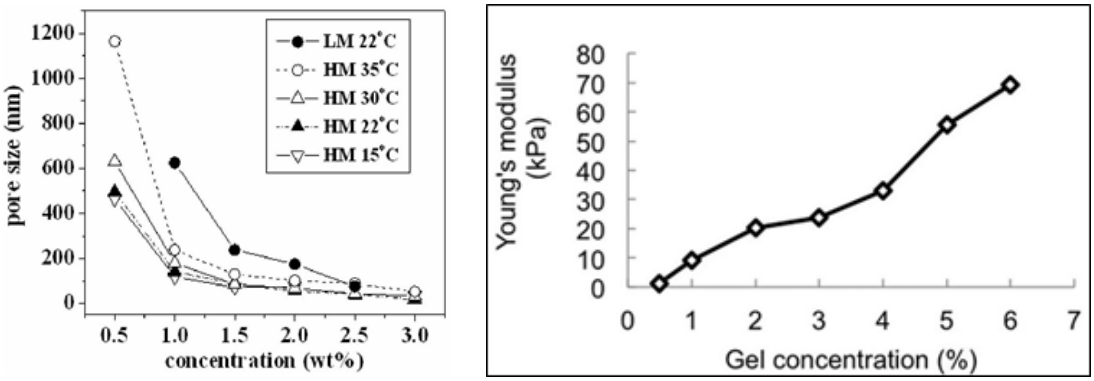
Pair samples T-test data statistics analysis of GAG concentration and the tangent Young's modulus of cells/gel scaffold at different gel agarose concentrations (4% and 6%), and different periods cultured (0 days and 7 days).

Probability>|t|	Total GAG	The Tangent Modulus
Agarose concentration	**Between 0 and 7 period cultured**	
4%	0.000152533	0.8333
6%	0.000011905	0.26873
Period cultured	**Between 4% and 6% agarose concentration**	
0	0.34467	0.73784
7	0.06588	0.06701

Table 4.

One way ANOVA data statistics analysis of GAG concentration and the tangent Young's modulus of cells/gel scaffold at different gel agarose concentrations (4% and 6%), and different periods cultured (0 days and 7 days).

	Total GAG		The Tangent Modulus	
	F Value	**Probability >F**	**F Value**	**Probability >F**
Agarose concentration	**Between 0 and 7 period cultured**			
4%	107.89912	0.00000112	0.05844	0.81506
6%	225.70071	0.0000000344	2.79181	0.13329
Period cultured	**Between 4% and 6% agarose concentration**			
0	1.3524	0.27186	0.10561	0.75353
7	4.63211	0.05685	3.66099	0.09205



a) **b)**

Fig. 8. (a) The relationship between the concentration of high melting (HM) and low melting (LM) agarose, and pore size for different heating temperatures [9], (b) The relationship between the Young's modulus and agarose concentration [10].

which are provided the ability of pressure resistance in tissues [6]. Conversely, the tangent modulus tend to decrease as GAG concentration increasing with changing agarose concentration for a specific period cultured. One of the main issues for the mentioned non homogenates is that there are a wide differences of the cell growth environment between in-vitro and in vivo media, which including stress environment such as shear and compressive forces that has a huge effect on nutrient and GAG producing and dispersion [14, 15]. One suggested way to solve these differences is to use a bioreactor, which could introduce more matching between them [14].

The high distribution and non-homogeneities of data appears again in the analysis of T-test and ANOVA data statistics of GAG concentration and the tangent Young's modulus of cells/gel scaffold. Which has shown low probability values that produce low values of confidence, less than 95%, except that of total GAG analysis between 0 and 7 period cultured at 4% and 6% agarose concentrations, as it was shown in table 3 and 4. In addition to that, the high F values of that exceptions which were shown in table 4 have provided an evidence that there are high variation between 0 and 7 days period cultured of GAG concentration and the tangent Young's modulus because of the regenerative growth of chondrocyte inside the scaffold.

Conclusions

In summary, chondrocyte growth has increased because the average maximum stress value has increased in 6% agarose from 0.01331 N/mm^2 at 0 days to 0.01678 N/mm^2 at 7 days, with increasing the GAG content, which is responsible on chondrocyte growth. Also the GAG concentration has increased from 3.7 to 70 μg/ml at 4% and from 6.4 to 55.4 μg/ml at 6% agarose for 0 and 7 days period cultured, respectively. Additionally, the tangent Young's modulus increased with GAG concentration increasing with changing the period cultured for a specific concentration of agarose. However, the mechanical tests and GAG contents results shown that there are a wide range of homogeneity, differences and dispersion of them in the most of the samples, which attribute to different factors. For mechanical properties, these parameters including anisotropic of the produced chondrocyte with agarose scaffolds, insufficient cells' dispersion within the gel scaffold during seeding and cultured time, and some test procedure condition, such as EBSS hydration. While for GAG results, those factors could be the differences of the cell growth environment between in-vitro and in vivo media. Designing and using a bioreactor could solve these issues by introducing more fitting and matching between in-vitro and in vivo media to produce high level of chondrocyte growth.

Akram Jassim Jawad **–** Researcher University of Babylon Hillah.

[1] L. Kock, C. van Donkelaar, K. Ito, Tissue engineering of functional articular cartilage: the current status, Cell and tissue research 347(3), 613-627 (2012); https:// doi: 10.1007/s00441-011-1243-1.
[2] I. El-Sherbiny, M. Yacoub, Hydrogel scaffolds for tissue engineering: Progress and challenges, Global Cardiology Science and Practice 1(3), 38 (2013); https://doi:10.5339/gcsp.2013.38.
[3] M. Farokhi, F. Jonidi Shariatzadeh, A. Solouk, H. Mirzadeh, Alginate based scaffolds for cartilage tissue engineering: a review, International Journal of Polymeric Materials and Polymeric Biomaterials 69(4), 230-47 (2020); https://doi.org/10.1080/00914037.2018.1562924.
[4] P. Abdollahiyan, F. Oroojalian, A. Mokhtarzadeh, M. de la Guardia, Hydrogel-Based 3D Bioprinting for Bone and Cartilage Tissue Engineering, Biotechnology journal 15(12), 2000095 (2020); https://doi/abs/10.1002/biot.202000095.
[5] M. Salati,. J. Khazai, A. Tahmuri, A. Samadi,.A. Taghizadeh, M. Taghizadeh, P. Zarrintaj, J. Ramsey, S. Habibzadeh, F. Seidi, M. Saeb, Agarose-based biomaterials: opportunities and challenges in cartilage tissue engineering, Polymers 12(5), 1150 (2020); https://doi:10.3390/polym12051150.
[6] M. Huber, S. Trattnig, F. Lintner, Anatomy, biochemistry, and physiology of articular cartilage, Investigative radiology 35(10), 573-80 (2000); https://doi:10.1097/00004424-200010000-00003.
[7] D. Lee, D. Bader, Compressive strains at physiological frequencies influence the metabolism of chondrocytes seeded in agarose, Journal of orthopaedic research 15(2), 181-8 (1997); https://doi/abs/10.1002/jor.1100150205.
[8] J. Buckwalter, H. Mankin, Articular cartilage: tissue design and chondrocyte-matrix interactions, Instructional course lectures 47, 477-86 (1998).
[9] J. Narayanan, J. Xiong, X. Liu, Determination of agarose gel pore size: Absorbance measurements vis a vis other techniques, InJournal of Physics: Conference Series 28(1), 017 2006; https://doi:10.1088/1742-6596/28/1/017.
[10] G. Kazi, K. Rahman, M. Farahat, T. Matsumoto, Fabrication of single gel with different mechanical stiffness using three-dimensional mold, Journal of Biomedical Materials Research Part A 107(1), 6-11 (2019); https://doi/abs/10.1002/jbm.a.36455.
[11] H. Tabani, S. Asadi, S. Nojavan, M. Parsa, Introduction of agarose gel as a green membrane in electromembrane extraction: an efficient procedure for the extraction of basic drugs with a wide range of polarities, Journal of Chromatography A 1497, 47-55, (2017); https://doi:10.1016/j.chroma.2017.03.075. Epub 2017 Mar 29.
[12] S. Lien, L. Ko, T. Huang, Effect of pore size on ECM secretion and cell growth in gelatin scaffold for articular cartilage tissue engineering, Acta biomaterialia, 5(2):670-9 (2009); https://doi:10.1016/j.actbio.2008.09.020.
[13] T. Chowdhury, D. Bader, J. Shelton, D. Lee, Temporal regulation of chondrocyte metabolism in agarose constructs subjected to dynamic compression, Archives of biochemistry and biophysics, 417(1), 105-11, (2003); https://doi:10.1016/s0003-9861(03)00340-0.
[14] E. Darling, K. Athanasiou, Articular cartilage bioreactors and bioprocesses, Tissue engineering 9(1), 9-26 (2003); https://doi:10.1089/107632703762687492.
[15] Y. Kim, R. Sah, A. Grodzinsky, A. Plaas, J. Sandy, Mechanical regulation of cartilage biosynthetic behavior: physical stimuli, Archives of biochemistry and biophysics 311(1), 1-2 (1994); https://doi:10.1006/abbi.1994.1201.

А. Дж. Джавад[1,2]

Дослідження впливу концентрації агарозного гелю та періоду культивування на біо- та механічні властивості інженерії тканин хондроцитів

[1]*Університет Вавилону, коледж інженерії матеріалів, факультет полімерів та інженерії нафтохімічної промисловості, Аль-Хілла, Ірак.*
[2] *Лондонський університет королеви Марії, Школа інженерії та матеріалознавства, Лондон, Великобританія,* akrammaterials4@gmail.com

Як гелевий каркас для інженерії тканин хондроцитів, концентрація агарози відіграє значну роль у взаємозв'язку між пористістю та живленням. У цій роботі досліджено вплив концентрації та періоду культивування на глікозаміноглікан (GAG) та механічні властивості. Хондроцити великої рогатої худоби виділялися в різних концентраціях агарозного гелю, приблизно 4% і 6%, протягом різного періоду культивування від 0 до 7 днів. Для вимірювання механічних властивостей і концентрації GAG у підготовлених зразках використовували метод механічного випробування (MTS) та спектрофотометричний метод з калібрувальною кривою. Результати механічних випробувань та вміст GAG показали, що у більшості зразків є широкий діапазон дисперсності, що пов'язано з різними факторами. Щодо механічних властивостей, ці фактори можна пояснити анізотропністю виробленого хондроцита з агарозними каркасами, недостатньою дисперсністю клітин у каркасі гелю під час висіву та культивування та деякими умовами процедури тестування, такими як гідратація збалансованого сольового розчину Ерла (EBSS). Хоча для результатів GAG цими факторами можуть бути відмінності середовища росту клітин між середовищами in vitro та in vivo. Однак, середнє значення максимального стресу зросло в 6% агарозі з 0,01331 Н/мм^2 на 0 день до 0,01678 Н/мм^2 на 7 день, оскільки збільшення концентрації GAG вказує на збільшення росту хондроцитів. Як правило, концентрація GAG також збільшується з 3,7 до 70 мкг/мл при 4% і з 6,4 до 55,4 при 6% агарозі протягом 0 і 7 днів культивування, відповідно. Рекомендований спосіб вирішити ці відмінності – використовувати біореактор, який може забезпечити більше відповідності між середовищами in vitro та in vivo.

Ключові слова: Хондроцит, тканинна інженерія, агароза, біоматеріали, глікозаміноглікан.