Nanomed. J. 5(3): 180-185, Summer 2018

RESEARCH PAPER

Evaluating the effect of pH on mechanical strength and cell compatibility of nanostructured collagen hydrogel by the plastic compression method

Ayoob Karimizade, Sakine Takallu, Esmaeil mirzaei *

Department of Medical Nanotechnology, School of Advanced Medical Sciences and Technologies, Shiraz University of Medical Sciences, Shiraz, Iran

ABSTRACT

Objective(s): One of the main constraints of collagen hydrogel scaffolds for using in tissue engineering is mechanical weakness. Plastic compression (PC) is a physical method to overcome the mechanical limitation of collagen hydrogel.

Materials and Methods: In this study, the effects of pH on mechanical and biological properties of PC hydrogels were investigated. Collagen hydrogels were fabricated at neutral (pH=7.4) and alkaline pH (pH=8.5), and then underwent plastic compression to prepare final hydrogels. The stability, mechanical properties, morphology and cell compatibility of hydrogels were investigated.

Results: The results illustrated that increasing in polymerization pH was associated with improvement in both tensile strength and elastic modulus of hydrogels. Furthermore, cell viability assay confirmed cell survival in both hydrogels prepared at alkaline and neutral pH.

Conclusion: The results suggest that a slightly basic pH during hydrogel production is an appropriate approach to construct PC collagen hydrogels with an enhanced stability and mechanical properties as well as better handling before PC process.

Keywords: *Collagen, Hydrogel, Mechanical properties, pH, Plastic compression*

How to cite this article

Karimizade A, Takallu S, mirzaei E . Evaluating the effect of pH on mechanical strength and cell compatibility of nanostructured collagen hydrogel by the plastic compression method. Nanomed J. 2018; 5(3): 180-185. DOI: 10.22038/nmj.2018.005.0008

INTRODUCTION

Skin is the exterior and the largest organ in human, where it has an important role to protect the body from potentially harmful events and agents, such as fluid loss, infection, chemicals, or UV irradiation [1]. Therefore, damage to the skin can cause the infectious and chronic lesion to the body. Each year, many patients refer to plastic surgery for the skin transplant due to chronic skin injuries [2]. Using autografts or allografts is the main way to repair damaged skin. However, autograft is often associated with donor site pain and morbidity whilst, the use of allografts may bring about the risk of disease transmission and immunorejection [3–5]. Today biocompatible scaffolds are used commonly to solve this problem. Scaffolds can be fabricated in 3-D structure, be seeded with cells, and later transplanted into the defective skin [6,7].

Various types of biomaterials have been used as a constituent of scaffolds, but because of the high biocompatibility of natural materials, the researchers are interested in using natural materials. Polymeric biomaterials have been illustrated to possess potentially excellent characteristics which make them suitable scaffold candidate for tissue repair [8]. Among various polymeric biomaterials, collagen, which is the main extracellular matrix (ECM) protein in tissues, has been used widely to repair skin. Collagen in the extracellular matrix causes the cell to be attached to the matrix via integrin. Therefore, it can be used as a matrix for cell culture. Collagen also provides a situation for cellular differentiation and, obviously, cellular migration [9,10].

One of the most common scaffolds for skin repair is collagen hydrogel that can mimic extracellular matrix of natural tissues. Collagen hydrogels contain a lot of water and provide

* *Corresponding Author Email: e_mirzaei@sums.ac.ir*

Note. This manuscript was submitted on May 6, 2018; approved on June15, 2018

the possibility for 3D cell culture in the scaffold. Researches show that matrices based on collagen not only do bioactive properties but also stimulate cell migration in scaffolds [11].

Applying collagen hydrogels as tissue engineering scaffold is limited due to its mechanical weakness. There are several ways to enhance mechanical properties of collagen hydrogel. One of them is chemical cross-linking of a collagen hydrogel with chemical reagents such as glutaraldehyde; however, the chemicals are usually cytotoxic and cause brittle hydrogels [12]. Compression is a physical way to increase the mechanical strength of hydrogels without toxicity. Plastic compression (PC) is a method commonly used for this purpose. In this method, collagen hydrogel loses its water by applying pressure and consequently denser collagen hydrogels is formed. The compressed hydrogel is mechanically stronger than the uncompressed one [13].

There are several factors including collagen concentration, collagen source, polymerization temperature, ionic strength and polymerization pH that affect the formation of collagen hydrogels [14]. pH during fabrication of a hydrogel influence the mechanical properties of the collagen hydrogels [15-17]. Some researchers have been investigated the relation between pH changes and hydrogel polymerization. According to Gobeaux et al, collagen showed smaller fiber size and less organization in hydrogels fabricated in higher rates of pH compared to lower pH [15,18].

However, the effects of polymerization pH on mechanical and biological behavior of PC collagen have not been studied yet. pH affects the mechanical strength and handling of pre-compressed collagen hydrogels which are substantial factors in the correct compression. In this study we investigated the effect of pH on mechanical and biological properties of plastic compression hydrogels.

MATERIALS AND METHODS

Cell culture

In vitro tests were carried out using Normal Human Dermal Fibroblast (NHDF) cell line. These cells were chosen owing to the great ability of interacting with biomaterials, non-malignancy, and high reproducibility. Dulbecco's modified Eagle's medium (DMEM)/F12 supplied with 10% FBS and 1% pen/strep (50 U/ml penicillin and 50 µg/ml streptomycin) was the favorable culture medium to the cells. Standard incubation conditions (37 °C temperature and 5% humidity) were facilitated for the cultured cells over the study period. There was also a regular necessity to implementing subculture every 3 days due to high proliferation rate of the NHDF cells. Cell counting routinely performed by trypan blue exclusion method at each subculture time.

The cells were seeded on 25 mm^2 culture flask and subcultured using 0.05% trypsin–EDTA. Cells of the first to sixth passages were used in all experiments.

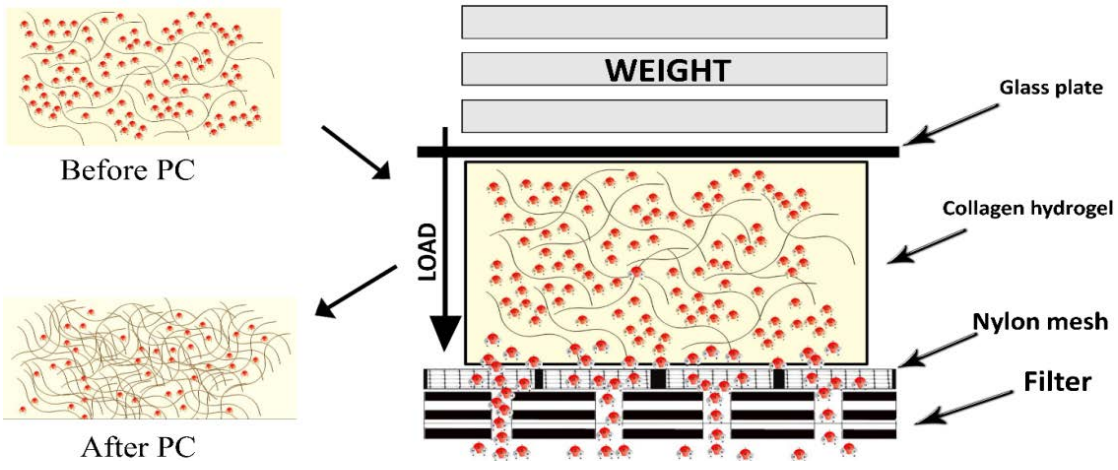

Fig 1. Plastic compression (PC) process. Schematic diagram showing plastic compression of collagen hydrogel including loading and blotting elements, and nylon meshes. Red bullets indicate water molecules

Preparation of compressed collagen hydrogel at two pH

Before preparing the collagen solution, all reagents were chilled on ice to prevent early gel formation. To prepare collagen hydrogel, 15 mg rat tail collagen type I, which was extracted according to the previously reported protocol [19], was dissolved in 4 ml acetic acid (0.02 N). After that, 4 ml phosphate-buffered saline (PBS) (pH= 7.4) was added to collagen solution.

Hydrogel collagen was made in two different pH, 7.4 and 8.5, which was adjusted by dropwise addition of 5N NaOH. The solutions were then incubated at 37º for 1 hour. The prepared hydrogels underwent plastic compression according to the previous reports [20,21]. The hydrogels were transferred to plastic compression device and compressed on the nylon mesh absorbent using a mass of 50 to 100g for 5 to 10 minutes. (Fig1).

Cell culture in hydrogel

For 3-D culturing of fibroblast into collagen hydrogels, culture medium was used instead of PBS. 3.6 ml DMEM (Gibco BRL) without cells was added to 3.6 mg/ml collagen solution. 0.4 ml NHDF cell suspension in DMEM with concentration of 100,000 cells/ml was gently mixed to previous solution. The solution was allowed to be mixed and made homogenous solution. The solutions were incubated in 37 ºC for 1hour. Then hydrogels were transferred to plastic compression device and were compressed on the nylon mesh absorbent using a mass of 50 to 100 g for 5 to 10 minutes. Compressed hydrogels were cultured in DMEM for 7 days in 37 ºC with 5% CO_2. The medium of hydrogels was changed every other day. All of the steps were done under sterilized condition.

Mechanical test

Mechanical properties of compressed hydrogels were measured using a universal testing machine (Zwick/Roell, Z020, Germany) at a crosshead speed of 2 mm. min^{-1} at room temperature. The compressed collagen hydrogels in two groups (pH=7.4 and 8.5) were clamped with two pieces of test grippers, and a stretching force was applied at a rate of 2 mm. min^{-1} until gel rupture, the maximum load and elastic modulus were recorded by the system. Five specimens from each group were tested.

Quantification of gel weight loss

To investigate water retention capability of hydrogels, hydrogel weight loss on absorbent paper was studied. The amount of weight loss after a certain time indicates the stability of hydrogels. The compressed hydrogels were put on the absorbent paper in a humid chamber and were weighted after every 30 minutes. The absorbent paper was changed after each time interval. All weight measurements were performed on five gel replicates per condition. The weight loss was calculated based on the initial weight and the retained weight after each period.

Scanning Electron Microscopy (SEM)

The hydrogels were fixed by immersion in 2.5% glutaraldehyde solution in PBS for 2 hours and then washed thrice in PBS. Then the hydrogels were dehydrated using a graded series of ethanol solutions, followed by further drying using freeze dryer. Specimens were sputter-coated with gold. Pictures were acquired using TESCAN-Vega 3 (Czech Republic) scanning electron microscope.

Cell viability test

The viability of fibroblasts seeded inside compressed collagen hydrogels was surveyed by LIVE/DEAD assay. The compressed hydrogels containing cells, as described previously, were made (in both of pH 7.4 and 8.5), and transferred to the 6-well plate. Hydrogels were cultured in DMEM for 7 days in 37 C with 5% CO_2. The viability of fibroblasts within the compressed collagen hydrogels was evaluated using a Live/Dead Cell Double Staining Kit (Sigma). The kit has two-color fluorescence, the live cells stained green and dead cells were red. The cell survival on compressed hydrogels was evaluated according to the Kit manual after 1 day and 7 days of cell culture. A dead cell positive control was produced by treating fibroblast-containing collagen constructs with 70% methanol before staining with the LIVE/DEAD viability kit. Fluorescence microscopy pictures were taken using Olympus DP73 digital camera connected to a Fluorescence microscopy Olympus IX81equipped with FITC filter (U-MW-IB3). Images were processed with Photoshop 7.0 Adobe Systems Inc.

Statistical analysis

All values were presented as means ± standard deviations (SD). Statistical analysis was performed with Student's t-test and p-value less than 0.05 was considered statistically significant.

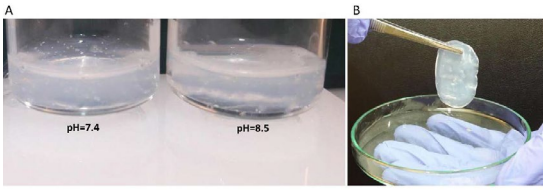

Fig 2. (A) Collagen hydrogels made at pH=7.4 and pH=8.5, both of them had a same appearance.(B) PC hydrogel

RESULTS AND DISCUSSION

Plastic compression hydrogels

Proper mechanical strength is a crucial factor for skin substitutes since it should have an ability to be handled with forceps and also being sutured during surgery. Collagen hydrogels have very low mechanical strength in such a way that they disintegrate during the movement with the forceps. PC method is a physical method to improve the mechanical strength of collagen hydrogel [13,22,23]. Collagen hydrogels have been made in two pH, neutral pH of 7.4 and basic pH of 8.5. Both the hydrogels showed similar appearance as shown in Fig. 2A. The hydrogels were then compressed until reaching a constant thickness. The compressed hydrogels which were prepared in two pH have also a same appearance. Both PC hydrogels were flexible but strong enough to be easily handled with forceps without rupture (Fig 2B).

Table 1. Comparison load and time of comperssion in different pH

Groups of hydrogels	Load to the same final thickness(g)	The time to reach the same thickness at constant pressure (S)
pH= 7.4	53.8 ±1.1	187.8 ± 3.32
pH= 8.5	83.6 ± 1.2	317 ± 6.63

Compression load and time were different in two groups to reach a constant and same thickness. In the group of pH=8.5, more force (83.6 ± 1.2 g) was applied to achieve a constant thickness compared with the group with pH=7.4 (53.8 ±1.1 g). Furthermore, the time to reach the same and constant thickness at constant pressure in the group of pH= 7.4 was 187.8 ± 3.32 seconds and in another group (pH= 8.5) were 317 ± 6.63 seconds. There is a statistical significance in load and time of compression ($p<0.05$) (n=5) (Table 1). It indicates that the scaffolds made in the higher pH had a higher mechanical strength and stability. Also after the compression, the prepared hydrogels could be moved with forceps without being raptured, while this was not possible before the compression (Fig 2B).

Hydrogel weight loss

The stability of plastic compressed hydrogels was surveyed trough weight loss measurement as described in section 2.5. The amount of weight loss after a certain time indicates the stability of hydrogels. Our results revealed that compressed hydrogels, which were made in pH=8.5, had less weight loss in comparison with compressed hydrogels in PH=7.4. (Fig 3). This shows higher stability collagen hydrogels prepared in pH=8.5 than the hydrogels made in neutral pH. Resistance to weight loss is a factor that is used as a criterion in collagen hydrogels for its mechanical stability. On the other hand, the remaining water in the hydrogel acts as a niche for cells and facilitates delivery of cell metabolites and signaling. Therefore, collagen hydrogels that are able to hold more water can provide the proper condition for cell growth. Furthermore, the stability of hydrogels during handling and integrity of collagen network structure is associated with hydrogels resistance in keeping their water. We have investigated the resistance of hydrogels to water loss in hydrogels made in two different pH. Those which were made in a higher pH showed more resistance to water loss. This can be attributed to the effect of pH on collagen fibrillation. A Higher pH makes tighter networks which keep more water inside [24,25].

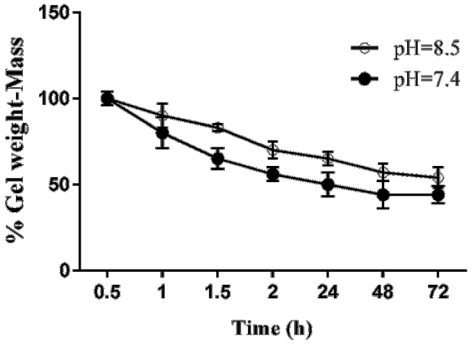

Fig 3. Time course of compressed hydrogels wet weight loss. Error bars indicating standard deviation (n = 5)

Mechanical properties of PC hydrogels

The tensile strength of collagen hydrogels was measured by a mechanical testing device in two different hydrogels groups to assess the effect of pH on the mechanical strength of collagen hydrogels. The maximum load and elastic moduli of hydrogels are shown in Fig 4. The compressed hydrogel made in pH=7.4 showed a maximum load

of 0.38±0.01 N. The maximum load increased to 0.45± 0.01 N for compressed hydrogel made in pH=8.5. In addition, compressed hydrogel made in pH=8.5 showed higher elastic modulus compared to hydrogel made in pH=7.4. The elastic modulus for compressed hydrogel made in pH=7.4 and pH=8.5 was 0.27 ±0.01 MPa and 0.35 ± 0.01 MPa, respectively. The mechanical properties were increased before the hydrogels were put at PC device (Table 1). Therefore, when hydrogels were compressed, it results in hydrogels with higher mechanical strength.

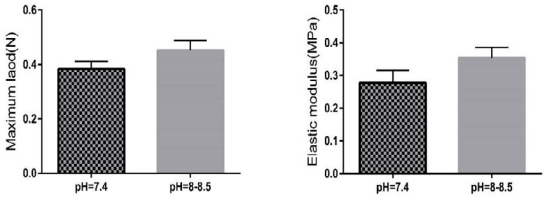

Fig 4. Mechanical properties of compressed hydrogels which were made in pH= 7.4 and pH=8.5. (left) Maximum load; (right) Elastic modulus. There were significant differences in maximum load and elastic modulus between hydrogels were made in pH=7.4 and 8.5 groups (p<0.05) (n=5)

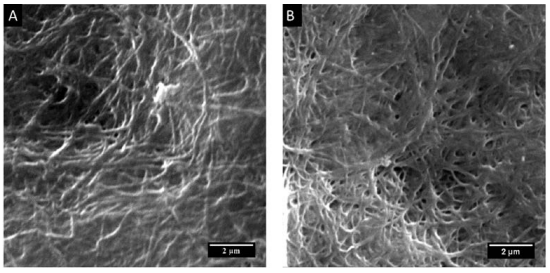

Fig 5. SEM images of collagen hydrogels. Overview of compressed collagen hydrogels made at pH=7.4 (A) and pH=8.5 (B)

Scanning Electron Microscopy (SEM)

The SEM images of PC hydrogels are reported in Fig 5. Fig 5 A-B show the top surface of the collagen hydrogels which were prepared in pH=7.4 and pH=8.5 respectively. Within both of the hydrogels, fibrils of collagen were observed in the range of nanometer with a random orientation. The hydrogels made in higher pH revealed nanofibers with higher diameter. The hydrogels made in basic pH showed mean fiber diameter of 200±30 nm, while the mean fiber diameter for neutral hydrogel was 145±50.

Cell viability

Collagen hydrogels proper candidate for 3-D cell culture because collagen hydrogels provide a good substrate to simulate cell growth [9,10]. Various factors during hydrogel preparation may affect the cell survival in hydrogels. The survival of the cells is affected by PC process. Indeed, a percentage of cell population has died after compression [13]. However, the population of cells can reach a normal level after 7 days post compression [22]. Hydrogel scaffolds were evaluated for cell survival by LIVE/DEAD viability kit. The percentage of the living cells on the first day in the pH=7.4 group was 66±1.8, and in the pH=8.5 group was 65.4±1.9. Furthermore the percentage of the living cells was 93.4±1.5 in pH=7.4 and 94.4±1.1 in pH=8.5 after 7 days. There were no significant differences between the two groups on the first and the seventh day (p<0.05) (n=5) (Fig 6). This indicates that using basic pH (pH=8.5) during formation of collagen hydrogel does not affect cell survival in comparison with a neutral pH.

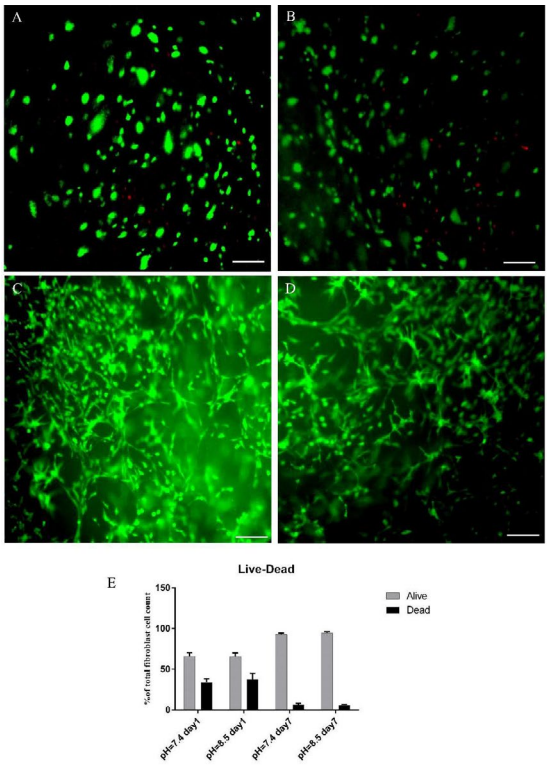

Fig 6. (A-D) Live-Dead stainning of fibroblast in hydrogels at pH=7.4 (A, C) and pH= 8.5 (B,D), top panel after 1 day cell culture, bottom panel after 7 days cell culture.Green color represents alive fibroblasts while red color represents dead cells. (e) The percentage of viable fibroblasts in two groups of pH, in 1 day and 7days after compression. There was no significant deference between two groups at first day and seventh day (p<0.05) (n=5)

Scale bar = 100 µm

CONCLUSION

Mechanical weakness is one of the main constraints of collagen hydrogels that limits its application for many tissue engineering applications such as skin substitutes. PC is a method for mechanical enhancement of hydrogel collagen. We showed that the hydrogels made at a pH higher than the neutral pH have more mechanical properties and handling. In addition, the results showed that the cells at this pH were able to survive. Therefore, slightly basic pH during hydrogel production is an appropriate approach to provide PC collagen hydrogels with enhanced stability and mechanical properties as well as a better handling before PC process.

ACKNOWLEDGMENTS

This project was part of a Master of Science thesis in medical nanotechnology and financially supported by Shiraz University of Medical Sciences (SUMS), Shiraz, Iran. (Grant No. 1396-01-74-15389).

REFERENCES

1. Abou Neel EA, Cheema U, Knowles JC, Brown RA, Nazhat SN. Use of multiple unconfined compression for control of collagen gel scaffold density and mechanical properties. Soft Matter. 2006; 2(11): 986–992.
2. Salgado AJ, Coutinho OP, Reis RL. Bone tissue engineering: State of the art and future trends. Macromol Biosci. 2004; 4(8): 743–765.
3. Laurencin C, Khan Y, El-Amin SF. Bone graft substitutes. Expert Rev Med Devices. 2006; 3(1): 49–57.
4. Laurencin CT, Ambrosio AMA, Borden MD, Cooper Jr. JA. Tissue engineering: Orthopedic applications. Annu Rev Biomed Eng. 1999. p. 19–46.
5. Kneser U, Schaefer DJ, Polykandriotis E, Horch RE. Tissue engineering of bone: The reconstructive surgeon's point of view. J Cell Mol Med. 2006; 10(1): 7–19.
6. Oakes BW. Orthopaedic tissue engineering: From laboratory to the clinic. Med J Aust. 2004; 180(5 SUPPL.): S35–38.
7. Braddock M, Houston P, Campbell C, Ashcroft P. Born again bone: Tissue engineering for bone repair. News Physiol Sci. 2001; 16(5): 208–213.
8. Novikova LN, Novikov LN, Kellerth J-O. Biopolymers and biodegradable smart implants for tissue regeneration after spinal cord injury. Curr Opin Neurol. 2003; 16(6): 711–715.
9. Rosso F, Marino G, Giordano A, Barbarisi M, Parmeggiani D, Barbarisi A. Smart materials as scaffolds for tissue engineering. J Cell Physiol. 2005; 203(3): 465–470.
10. Rosso F, Giordano A, Barbarisi M, Barbarisi A. From Cell-ECM Interactions to Tissue Engineering. J Cell Physiol. 2004; 199(2): 174–180.
11. Serpooshan V, Julien M, Nguyen O, Wang H, Li A, Muja N, Henderson JE, Nazhat SN. Reduced hydraulic permeability of three-dimensional collagen scaffolds attenuates gel contraction and promotes the growth and differentiation of mesenchymal stem cells. Acta Biomater. 2010; 6(10): 3978–3987.
12. Charulatha V, Rajaram A. Influence of different crosslinking treatments on the physical properties of collagen membranes. Vol. 24, Biomaterials. 2003. p. 759–767.
13. Brown RA, Wiseman M, Chuo C-B, Cheema U, Nazhat SN. Ultrarapid Engineering of Biomimetic Materials and Tissues: Fabrication of Nano- and Microstructures by Plastic Compression. Adv Funct Mater. 2005; 15(11): 1762–1770. 14. Antoine EE, Vlachos PP, Rylander MN. Review of collagen i hydrogels for bioengineered tissue microenvironments: Characterization of mechanics, structure, and transport. Vol. 20, Tissue Eng Part B Rev. 2014. p. 683–696.15. Gobeaux F, Mosser G, Anglo A, Panine P, Davidson P, Giraud-Guille M-M, Belamie E. Fibrillogenesis in Dense Collagen Solutions: A Physicochemical Study. J Mol Biol. 2008;376(5):1509–1522.
16. Yang Y-L, Motte S, Kaufman LJ. Pore size variable type I collagen gels and their interaction with glioma cells. Biomaterials. 2010; 31(21): 5678–5688.
17. Raub CB, Unruh J, Suresh V, Krasieva T, Lindmo T, Gratton E, Tromberg BJ, George SC. Image correlation spectroscopy of multiphoton images correlates with collagen mechanical properties. Biophys J. 2008; 94(6): 2361–2373.
18. Sung KE, Su G, Pehlke C, Trier SM, Eliceiri KW, Keely PJ, Friedl A, Beebe DJ. Control of 3-dimensional collagen matrix polymerization for reproducible human mammary fibroblast cell culture in microfluidic devices. Biomaterials. 2009; 30(27): 4833–4841.
19. FITCH SM, HARKNESS MLR, HARKNESS RD. Extraction of Collagen from Tissues. Nature. 1955 Jul 23; 176: 163. 20. Bitar M, Salih V, Brown RA, Nazhat SN. Effect of multiple unconfined compression on cellular dense collagen scaffolds for bone tissue engineering. J Mater Sci Mater Med. 2007;18(2): 237–244.
21. Buxton PG, Bitar M, Gellynck K, Parkar M, Brown RA, Young AM, Knowles JC, Nazhat SN. Dense collagen matrix accelerates osteogenic differentiation and rescues the apoptotic response to MMP inhibition. Bone. 2008; 43(2): 377–385.
22. Levis HJ, Brown RA, Daniels JT. Plastic compressed collagen as a biomimetic substrate for human limbal epithelial cell culture. Biomaterials. 2010; 31(30): 7726–7737. 23. Hu K, Shi H, Zhu J, Deng D, Zhou G, Zhang W, Cao Y, Liu W. Compressed collagen gel as the scaffold for skin engineering. Biomed Microdevices. 2010; 12(4): 627–635.
24. Ní Annaidh A, Bruyère K, Destrade M, Gilchrist MD, Otténio M. Characterization of the anisotropic mechanical properties of excised human skin. J Mech Behav Biomed Mater. 2012; 5(1): 139–148.
25. Varkey M, Ding J, Tredget EE. Advances in skin substitutes-potential of tissue engineered skin for facilitating anti-fibrotic healing. J Funct Biomater. 2015; 6(3): 547–563.