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# Synergistic effect of selenium and genipin triggers viability of 3T3 cells on PVA/Gelatin scaffolds

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*Purpose*: The aim of this study was to reveal the first time synergistic effect of GP and selenium (Se) on 3T3 cells seeded on natural and non-cytotoxic porous scaffolds with poly(vinyl alcohol) (PVA) and gelatin (GE). *Methods*: Electrospinning scaffolds were produced as PVA/GE/GA crosslinked with glutaraldehyde (GA) and freeze/dried scaffolds crosslinked with genipin (GP) were divided into two groups as PVA/GE/GP5 and PVA/GE/GP8. The scaffolds were investigated in terms of pore morphology, swell ratio, biodegradation, and biocompatibility. The biocompatibility of the material was tested *in vitro* by MTT assay on 1, 2, and 3 days to test the cell viability of 3T3 cells. *Results*: It was observed that Se triggered the excellent cell growth and proliferation on electrospinning and freeze drying PVA/GE scaffolds. *Conclusions*: Selenium with PVA/GE scaffolds can be a promising candidate for wound healing application, as it significantly increases cell viability on scaffolds. It is thought that the synergistic effect of selenium with genipin may be an important step in tissue engineering applications. The preliminary study can be supported by *in vivo* studies in the future.

*Key words*: *gelatin, genipin, 3T3 cells, scaffold, selenium*

## 1. Introduction

Tissue engineering, which was used as the common application of medicine and engineering, has aroused interest among scientists to improve and functionalize its structural functions in tissue/organ damage or loss [15]. Different than the other organs, skin is known as a barrier to protect the internal environment from external influences when it faces injurious bacteria and effects of physical and chemical. When the skin is exposed to some harmful microbial, thermal, mechanical and chemical effects daily, it may not heal spontaneously, which can cause scarring [6]. Over the past few years, attempts have been made to produce skin mimicking substitutes using advanced tissue engineering approaches. Thus, clinical applications were made to support the healing of both acute and chronic wounds. Skin tissue engineering applications target to repair the damaged skin using three-dimensional polymeric porous scaffolds. The appropriate roughness and hydrophilicity of the surface support suitable situations for colonization by cells through better adhesion and, as a result, preferable integration of the forming tissue with the porous scaffolds. Although there are many studies about the skin tissue scaffolds from past to present, they still have disadvantages due to their bio incompatibility [14], [39]. Hence, the scaffolds should have optimal features such as biocompatibility, biodegradability, mechanical strength and interrelated controlled porosity [37]. The polymer used in scaffold creation, poly(vinyl alcohol) (PVA), is water-soluble, economic, biocompatible and biodegradable synthetic hydrophilic, semi-crystalline polymer with good chemical and thermal stability [17]. Gelatin (GE), a type of collagen derivatives obtained from the skin and bones, is often preferred in tissue engineering applications due to the features of its biocompatibility

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and biodegradability. Furthermore, GE enables more cells to adhere to scaffolds due to the containing arginine-glycine-aspartic acid (RGD), glycine, proline, and 4-hydroxyproline structures that increase the cohesion of cells [7], [8]. The promising new generation engineering applications aim to produce scaffolds with an informational function, for instance, using materials that facilitate cell attachment, proliferation, and differentiation that are much better than polymers [17]. In this study, we used selenium (Se), which is known to have a positive effect on proliferation. Se, an important trace element, has been used for the proliferation of different cell lines in cell culture media for many years and plays an important role in biological functions [7], [19], [27], [38]. Se was proven to prevent apoptosis at lower concentrations [8], [10], [25]. Different Se concentrations have different effects on the cells. However, the functions of these effects have not yet been explained [35]. It has been proven that the risk of skin cancer is very high in people with less selenium amount compared to individuals with normal level [32]. Se contains the enzymes glutathione peroxidase (GSH-Px) and thioredoxin, which protect the skin from injuries caused by UV, which has the skin cancer risk [22], [31]. Electrospinning and freeze/drying methods are the most preferred methods due to their simple, and inexpensive properties. The electrospinning technique has an easy structure, high-yield, cost--effective and specific orientation nanofibers to produce polymeric nanofibers compared to other nanofiber manufacturing techniques. This method can produce ultrafine fibers with diameters between micrometers and nanometers using natural and synthetic biopolymers with interrelated pore structure, high porosity, large surface area, superior tensile strength, and many other unique properties. Thus, electrospinning nanofiber scaffolds are widely used in wound dressings due to these outstanding features [1], [26], [29], [44]. The scaffolds obtained by the freeze/drying method used to produce polymeric tissue scaffolds are vacuumed in a lyophilizer to remove residual water. Porous scaffolds of various sizes are obtained in this manner, facilitating the feeding of the cells and also providing an advantage in the disposal of the residues produced, helps to feed the cells, and also provides an advantage in the disposal of produced residues [13]. In this project, the porous scaffolds were prepared with the combination of equal amounts of the PVA, and GE (1:1 w/w) polymers using the electrospinning and freeze/drying methods. Since PVA/GE scaffolds are not stable in the biological environment and easily swell and lose their fibrous structure when in contact with water, they are cross-linked to increase their structural strength [9], [20], [23]. The electrospinning and freeze/dried scaffolds were cross-linked with glutaraldehyde (GA) and genipin (GP), respectively. Although GA is known to be toxic, it is widely used. Two different ratios of genipin cross-linker were used for observing whether effects on freeze/drying scaffolds. GP is a non-toxic, natural cross-linking agent isolated from the fruit of the *Gardenia jasminoides* plant [23]. Morphological analysis, biodegradability and biocompatibility well ratio, cytotoxicity of scaffolds, and also attachment of 3T3 fibroblast cells supplemented with selenium were investigated on PVA/GE scaffolds crosslinked with GA and GP.

# 2. Materials and methods

## 2.1. Materials

Bovine gelatin (GE) was purchased from Kervan, poly(vinyl) alcohol (99% hydrolyzed, $M_w$ 98.000 g/mole, Inner Mongolia Shuangxin), glutaraldehyde (GA) were purchased from Sigma–Aldrich, genipin (GP) was purchased from Lesen Phytochem & Herbs Extracts Solutions, 3T3 cells were used from (Istanbul University--Biophysics), DMEM-F12 (Dulbecconun Modified Eagle Medium F-12), Tyripsin (25% EDTA), FBS (Fetal Bovine Serum), penicillin-streptomycin solution. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Gibco), dimethyl sulfoxide (DMSO) were purchased from Gibco.

## 2.2. Preparation of electrospinning of PVA/GE/GA

8% w/v PVA was dissolved in distilled water at 80 °C for 3 hours. 10% w/v GE was dissolved in distilled water: acetic acid (7:3 v/v) for 30 minutes at room temperature with slow stirring. The PVA aqueous solution was added with GE solution as PVA/GE (1:1 w/w) and the solution was magnetically stirred for 30 minutes. The mixture was taken to a plastic syringe fitted with a needle (G16, diameter = 0.16 mm) and were put up in the electrospinnig device. The syringe was directed by a syringe infusion pump. An electric field was formed at the room temperature using a high voltage (24 kV) between syringe needle, and metal collector. The distance between the collector and needle was optimized to 15 cm, and the flow rate of the solution from the needle was adjusted

to 25 µl/min. The scaffolds were cross-linked with 2% glutaraladehyde for 12 hours after the electrospinning was ended (PVA/GE/GA). Morphology of the electrospun PVA/GE/GA fibers was examined by SEM. The proposed cross-linking reaction of PVA and glutaraldehyde is in Fig. 1a.

## 2.3. Preparation of freeze/drying PVA/GE/GP

8% w/v PVA was dissolved in distilled water at 80 °C for 3 hours. 10% w/v GE was dissolved in distilled water at 60 °C for 1 hour. The PVA aqueous solution was added with gelatin solution as PVA/GE (1:1 w/w) and the solution was magnetically stirred for 30 minutes. 0.75% GP was added by dissolving in distilled water for cross-linking this freeze/drying PVA/GE mixture. Two mixtures were obtained by different GP ratios. 5 ml of GP was added to 10 ml of PVA/GE mixture to form the first concentration (PVA/GE/GP5). The second concentration was obtained by adding 8 ml GP to a 10 ml PVA/GE mixture (PVA/GE/GP8). The mixtures were taken into 5 ml syringes kept at room temperature for 24 hours, and stored (at –20 °C for 24 hours). The end portions of the injectors were cut and the materials removed. Frozen mixtures were transferred to the lyophilizer for 24 hours. At the end of this period, the polymers were cut with a scalpel into pieces of 1 mm in thickness. It was washed with PBS after standing at 0.75% glycine (in distilled water) for 8 hours. The proposed cross-linking reaction of gelatin and genipin is in Fig. 1b.

## 2.4. Morphology

Adhesion and morphological images of the cells on PVA/GE scaffolds were observed by SEM after 24 and 48 hours of culturing. The cell-seeded samples

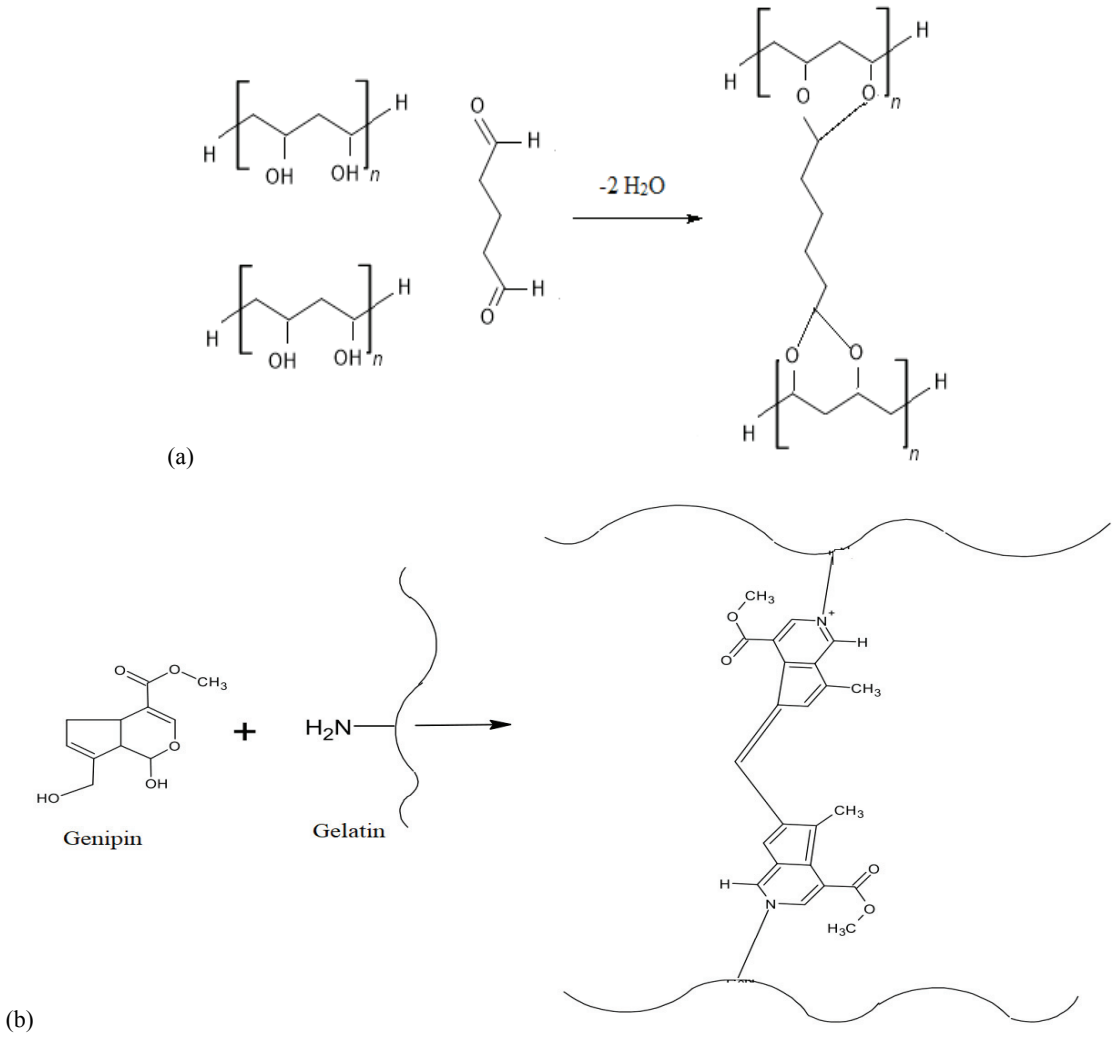


Fig. 1. The proposed cross-linking reaction of (a) PVA and glutaraldehyde (b) gelatin and genipin

were washed with PBS and fixed with 2.5% by weight GA (in PBS) for 30 minutes at room temperature. The samples were dehydrated with different proportions of ethanol (30, 50, 70, 90 and 100%) and washed with hexamethyldisilazane (HMDS) for 5 minutes. It was placed on an SEM sample holder and analyzed using SEM (Zeis) after each sample was coated with platinum.

## 2.5. Fourier transform infrared (FTIR)

The structural information of PVA/GE scaffolds was determined using Agilent Cary 630 Fourier transform infrared spectroscopy (FTIR). FTIR spectrum of PVA/GE was examined in the wavelength range of 4000–1000 $cm^{-1}$.

## 2.6. Swell ratio

Samples were immersed in 25 mL PBS (37 °C, pH 7.4) to determine the percentage of water absorption of PVA/GE scaffolds. First, the dry weights of the scaffolds were calculated as $W_d$. Then, the samples were weighed at specific time intervals (10, 20, 30, 40 and 60 minutes) to determine their wet weight after taking from PBS solution ($W_w$). Swelling ratios of the scaffolds were calculated according to the following definition:

Swelling Ratio = $[(W_w - W_d)/W_d]$ where $W_d$ and $W_w$ are dry and wet weights of the scaffolds. The results were reported as an average of three measurements.

## 2.7. *In vitro* degradation of scaffolds

*In vitro* degradation studies were applied to determine the weight loss of scaffold samples. The dry weight of samples was recorded as $W_i$, and then, they were immersed in PBS for 7 weeks at the same temperature environment at 37 °C. The PBS was not altered throughout the experiment. Samples were removed and the water collected on the samples was taken using filter paper and dried at 100 °C for 2 hours, and their final wet weights were taken as $W_f$. Degradation rates of the samples were observed with the following formula. The results were reported as an average of three measurements.

$$\% \text{ Degradation} = [(W_f - W_i)/W_f] \times 100\%.$$

## 2.8. Cell culture

3T3 (mouse fibroblast) cells were cultured in DMEM F-12 supplemented with 10% FBS, and 1% antibiotic (100 μg/ml streptomycin, and 100 U/ml penicillin), and were incubated (37 °C, 5% $CO_2$). The *in vitro* cell culture applications were evaluated for each scaffold. Each sample was sterilized with ultraviolet light radiation, and with 70% alcohol, and then was taken in 24-well plates.

## 2.9. MTT analyses

### 2.9.1. Determination of Selenium cytotoxicity

3T3 cells were seeded in 96-well cell plates in triplicate at a density of 15 × 103 cells/well. After 24 hours, cells were treated with Se (50 nM–1 μM) for 24, 48, and 72 hours (37 °C, 5% $CO_2$). After Se exposure, 4 μl MTT 3-(4,5-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dissolved in 5 mg/ml PBS was given into each well and incubated for 3 hours. At the end of the incubation, the medium was removed and replaced with the same amount of DMSO (Dimethyl Sulfoxide), followed by waiting in a dark for 30 minutes. Absorbance values were measured at 570 nm with the Gen5 program according to formazan crystal density in the wells.

### 2.9.2. Determination of the cytotoxicity of the cell-seeded scaffolds

$5 \times 10^4$ cells/well were seeded on the placed scaffolds in 24-well plates in 1 ml DMEM and they were incubated (37 °C, 5% $CO_2$) for 24 hours. After this period, 200 nM Se was added to the cell-seeded scaffolds and was incubated for 24 hours (37 °C, 5% $CO_2$). Then, 50 μl MTT (5 mg/ml in PBS) was added to the culture wells and incubated for 4 hours (37 °C, 5% $CO_2$). After the incubation, 1 ml DMSO was added instead of the MTT reaction medium. The absorbance values were determined using the Gen5 program at wavelengths of 570 nm [13].

## 2.10. Statistical analysis

Data are shown as means with standard deviation. At least 3 replicates were done for each experimental setup. Statistical significance between the control condition and each of the exposure samples was calculated with Student's *t*-test ($p < 0.05$).

# 3. Results

## 3.1. Morphology of PVA/GE electrospun and freeze/drying scaffolds

PVA/GE scaffolds were shown in Fig. 2. The color of the electrospun nanofiber scaffolds was white and opaque without being crosslinked by GA (Fig. 2a). The freeze/dried scaffolds that were structured with added GP were green and spongy (Fig. 2b).

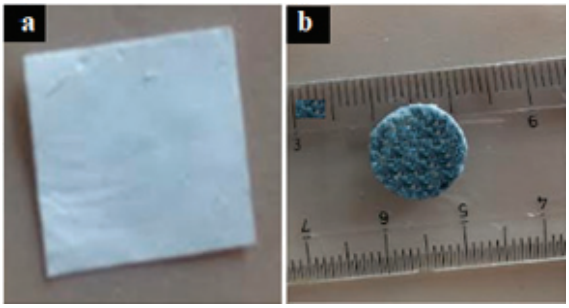


Fig. 2. (a) PVA/GE/GA electrospun nanofiber scaffolds are white and opaque, (b) the freeze/dried scaffolds are green, spongy structures

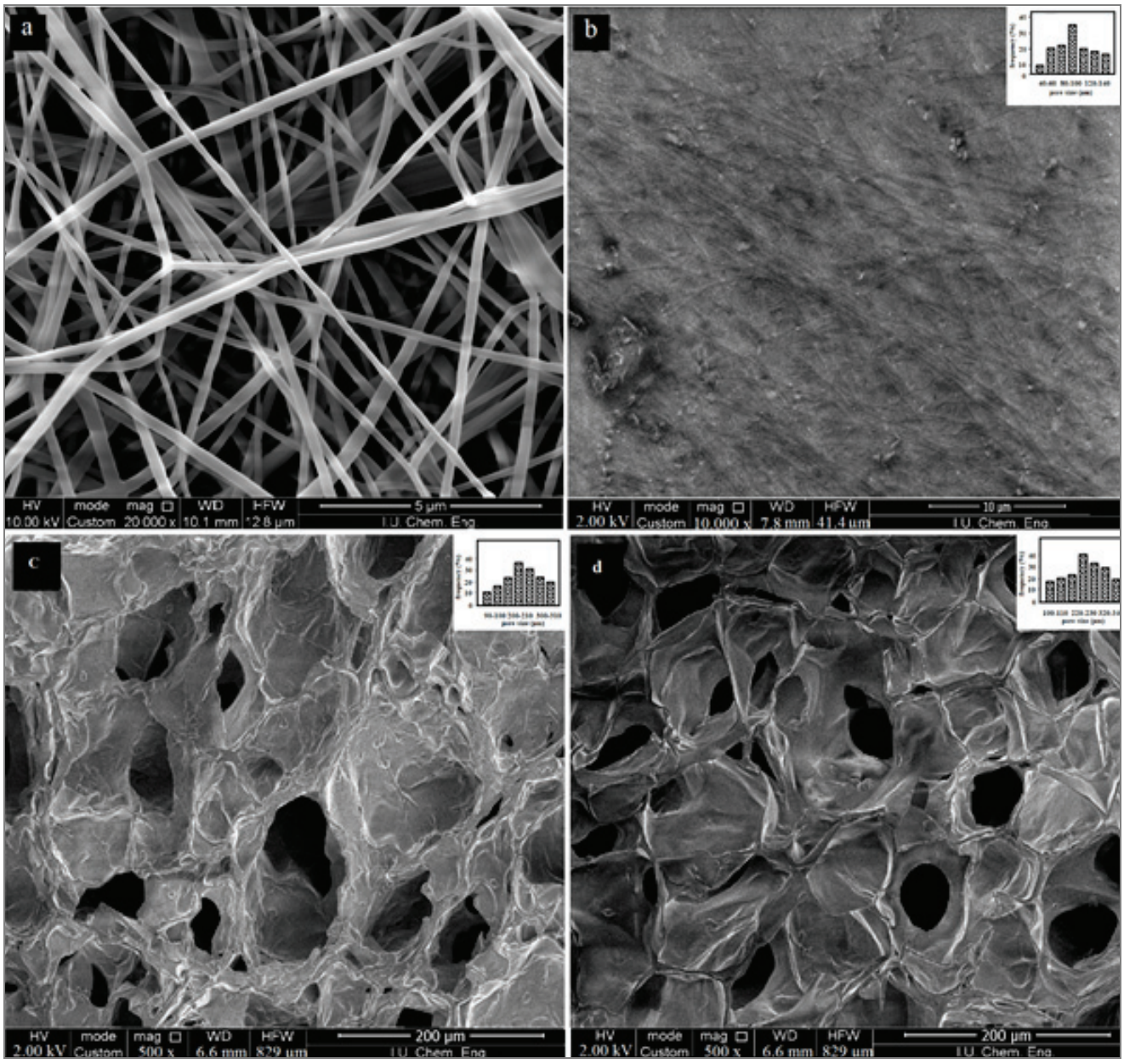


Fig. 3. Cross-sectional of SEM image of scaffolds prepared by electrospinning technique with different magnification. Average pore size of scaffold: (a) without GA (× 20 k), (b) with GA (× 10 k). SEM images of freeze/dried scaffolds containing the different ratio of genipin (× 0.5 k), (c) PVA/GE/GP5, and (d) PVA/GE/GP8

In Figure 2, SEM images at the cross-section of PVA/GE/GA electrospun and PVA/GE/GP5-PVA/GE/GP8 freeze/dried scaffolds with their pore size distribution plotting obtained by Image J Software are demonstrated. High flow rates are generally avoided in spinning because droplet occurs. In the present experiment, the appropriate ranges of flow rate and voltage were determined as 20–25 ml/min and 20–25 kV to obtain a smooth and beadless PVA/GE/GA electrospun scaffold. The obtained electrospun scaffolds were crosslinked with glutaraldehyde for binding the free amino groups of gelatin and were stabilized. While the average pore size was 7 µm un-cross-linking, it increased with cross-link to 90 µm (Fig. 3a, b).

Freeze/dried PVA/GE scaffolds were formed with two different volumes of genipin solution with a concentration of 0.75% (PVA/GE/GP5 and PVA/GE/GP8). The average pore size of the PVA/GE/GP5 scaffolds is 210 µm, whereas the PVA/GE/GP8 scaffolds are 230 µm in Figs. 3c and d.

## 3.2. Fourier transform infrared (FTIR)

Determined spectra for PVA/GE scaffolds in wavenumber between 4000–1000 $cm^{-1}$ were demonstrated in Fig. 4. The FTIR spectra of all the scaffolds demonstrated broadband in the range of 3000–3500 $cm^{-1}$, and the distinct absorption hill at 2975 $cm^{-1}$ was related to the O-H group. C-H stretching vibration which was symbolized at 2835 $cm^{-1}$, was combined in 2975 $cm^{-1}$ peak. The peak at 1720 $cm^{-1}$ was correlated with the C=O vibration group of gelatin polymer. Gelatin spectrums indicated distinctive peaks between 3000 $cm^{-1}$ and 3150 $cm^{-1}$ because of N-H stretching secondary amide group. C-H group at 2975 $cm^{-1}$, and C=O group around 1720 $cm^{-1}$.

## 3.3. Swelling ratio

PVA/GE/GA electrospun scaffolds showed the highest swell ratio, the sample swelled up to 25 g/g in 40 min and reached equilibrium in 60 min (Fig. 5a). While PVA/GE/GP5 reached its maximum swelling rate (30 g/g) in 40 minutes, the PVA/GE/GP8 reached its (25 g/g) in 30 minutes. The swelling rate of PVA/GE/GP5 reached an equilibrium between 30 and 60 minutes, while that of PVA/GE/GP8 was recorded between 40 and 60 minutes (Fig. 5b).

## 3.4. *In vitro* degradation

The degradation percentages of PVA/GE scaffolds were observed in PBS solution for seven weeks at 37 °C. Percentage degradation of the scaffolds was plotted. The PVA/GE/GA electrospinning scaffold showed a maximum rate of 70% at week seven (Fig. 5c). PVA/GE/GP5 with low genipin concentration showed a faster degradation with a maximum of 75%, while

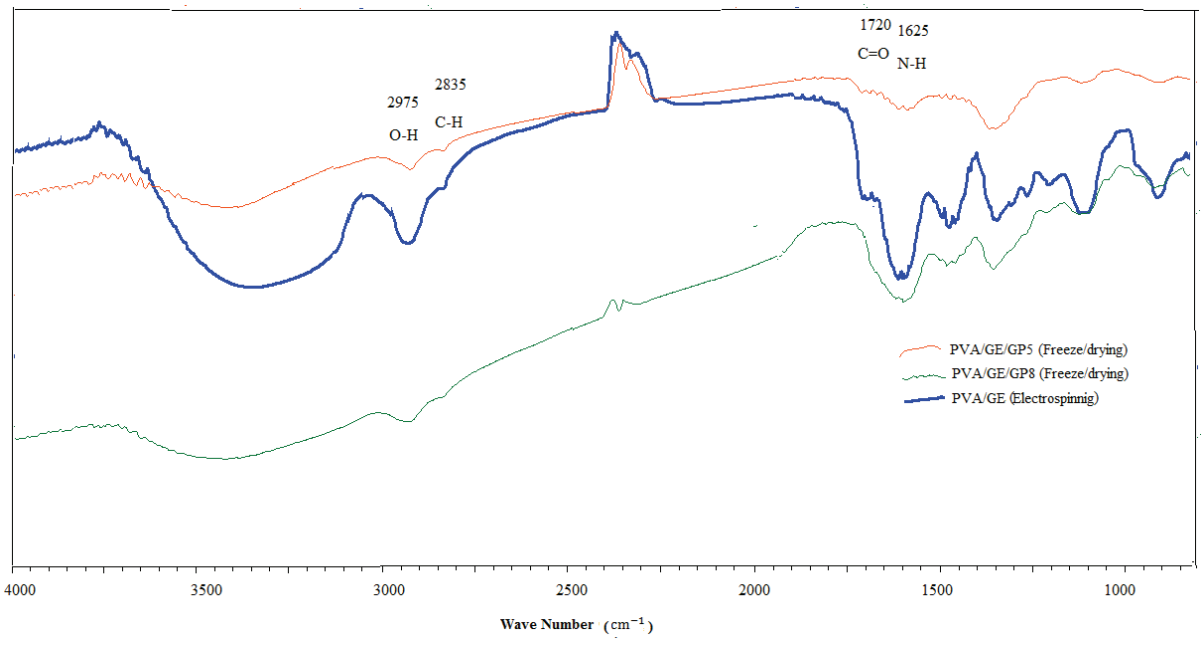


Fig. 4. The FTIR analysis of PVA/GE electrospun and freeze/drying scaffolds without cross-linked (4000–1000 $cm^{-1}$)

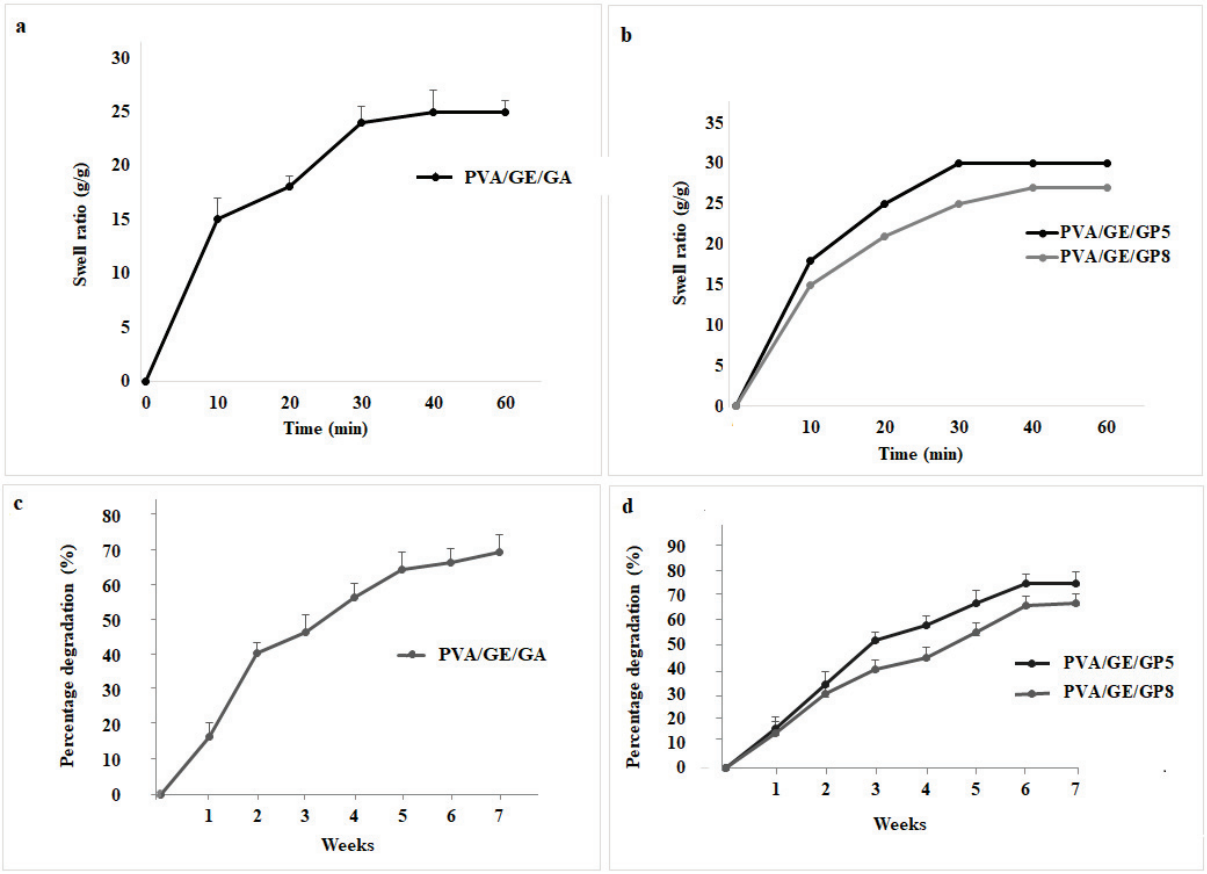


Fig. 5. Swelling and degradation rates of scaffolds: (a) PVA/GE/GA, (b) PVA/GE/GP5-PVA/GE/GP8, (c) PVA/GE/GA, (d) PVA/GE/GP5-PVA/GE/GP8

PVA/GE/GP8 with a higher genipin concentration showed a maximum degradation of 67% from its original weight at week seven (Fig. 5d).

## 3.5. Cell-seeding on the scaffolds

3T3 fibroblasts cultured on GA cross-linked PVA/GE/GA electrospinning and GP cross-linked PVA/GE/GP5-PVA/GE/GP8 freeze/dried scaffolds showed adhesion after 48 hours in the scanning electron micrograph images (Figs. 6a–c). This work has good biocompatibility and is nontoxic to the growth of cells.

## 3.6. Cell viability

### 3.6.1. Se cytotoxicity

3T3 cells were treated with increasing concentrations of selenium (50 nM, 100 nM, 200 nM, 500 nM, 1 µM, 10 µM, 100 µM, 1 mM), and incubated for 24,

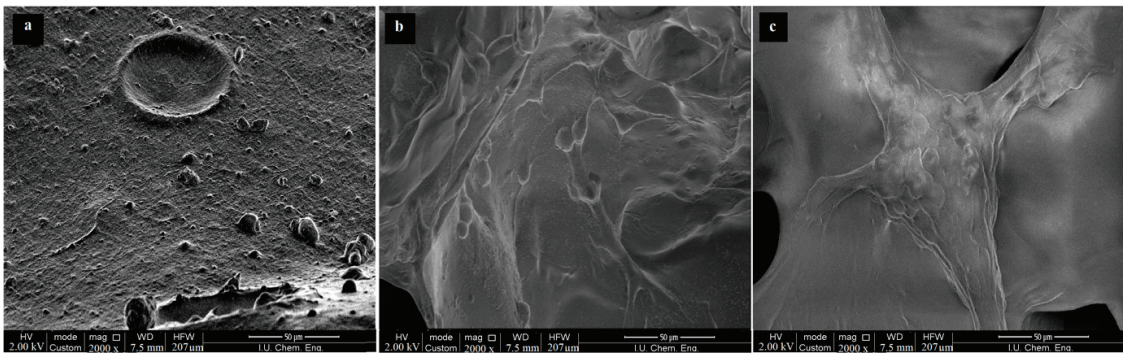


Fig. 6. SEM images of 3T3 fibroblast cells on crosslinked scaffold after 48 hours of culturing (× 2 k): (a) PVA/GE/GA, (b) PVA/GE/GP5, (c) PVA/GE/GP8

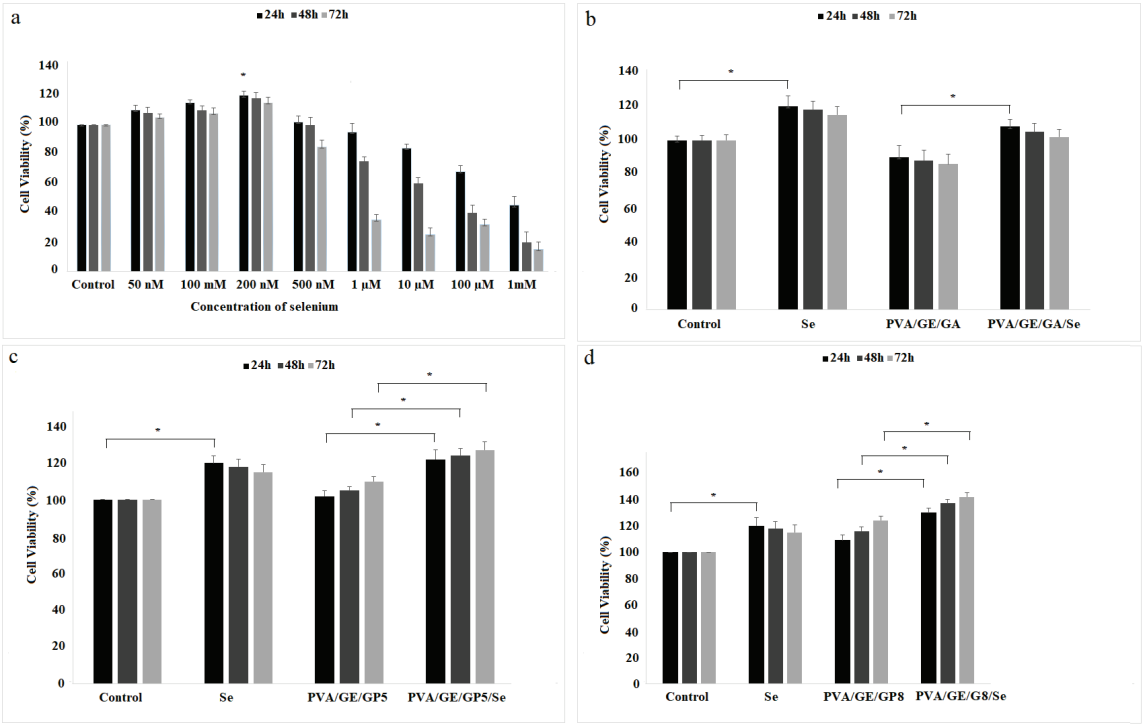


Fig. 7. (a) MTT analysis in 3T3 fibroblast cells treated with increasing selenium concentrations for 24, 48, 72 hours; (b) Viability of 3T3 fibroblast cells-seeded PVA/GE/GA electrospun scaffold with and without selenium for 24, 48, 72 hours. Viability of 3T3 fibroblast cells-seeded freze/dried scaffolds with and without selenium for 24, 48, 72 hours; (c) PVA/GE/GP5 (d) PVA/GE/GP8

48 and 72 hours for MTT analysis in Fig. 7(a). The optimum Se dose was determined to be 200 nM with approximately 120% viability in 24 hours. Statistical analysis revealed significant viability in 200 nM ($p < 0.05$). Experiments were repeated three times.

### 3.6.2. Se cytotoxicity and viability of 3T3 cells on PVA/GE/GA and PVA/GE/GP5-PVA/GE/GP8 scaffolds

Four groups were established: the control, Se, PVA/GE/GA, PVA/GE/GA/Se scaffolds, respectively for electrospinning (Fig. 7(b), Se, PVA/GE/GP5, PVA/GE/GP5/Se and control, Se, PVA/GE/GP8, PVA/GE/GP8/Se scaffolds, respectively, for freze/drying methods (Figs. 7c and d). The control which is the first group includes only the cells. The second group contains cells and Se. The third group contains cells and the scaffolds. The last group has the cell, selenium, and scaffold.

In the PVA/GE/GA electrospinning scaffold, when it was compared with the control, the viability decreased by 10% from 100% to 90% (24 h). It decreased by 12% to 88% (48 h), by 14% to 86% (72 h). When selenium was supplemented to these scaffolds, the viability increased from 90% to 108% (24 h), from 88% to 105% at (48 h), from 86% to 102% (72 h).

Compared to the control the viability on the PVA/GE/GP5 freeze/dried scaffolds was recorded as 105% (24 h), 112% (48 h) and 120% (72 h), before the selenium treatment. On the other hand, compared to the control the viability on the PVA/GE/GP8 freeze/dried scaffolds was recorded as 109% (24 h), 116% (48 h), and 124% (72 h), without selenium treatment. After the addition of selenium in PVA/GE/GP5 freeze/dried scaffolds, the vitality increased from 105% to 126% (24 h), from 112% to 132% (48 h), from 120% to 138% (72 h). On the other hand, the vitality in PVA/GE/GP8 freeze/dried scaffolds rised from 109% to 130% (24 h), from 116% to 137% (48 h), from 124% to 142% (72 h). Compared to the control, genipin and selenium together increased cell viability by 26% and 30% in PVA/GE/GP5 and PVA/GE/GP8, respectively (24 h). In the controls examined, the viability increased by 32% (48 h), by 38% (72 h) for PVA/GE/GP5 samples and by 37% (48 h), by 42% (72 h) for PVA/GE/GP8 samples.

## 4. Discussion

In the present study, we demonstrated a synergistic effect of the genipin and selenium for the proliferation of cells using PVA/GE scaffolds. 3T3 fibroblasts cells

were then cultured on the obtained cross-linked PVA/GE scaffolds to examine the *in vitro* biocompatibility of the PVA/GE scaffolds. Widely used electrospun nanofiber and freeze/dried scaffolds in wound dressings were preferred [40].

SEM images demonstrated at the cross-section of PVA/GE/GA electrospun and PVA/GE/GP5-PVA/GE/GP8 freeze/dried scaffolds with their pore size distribution plotting obtained by Image J Software. We chose electrospinning because it has an important role in controlling the pore sizes of scaffolds [1]. We used glutaraldehyde for electrospinning as it proved to have the ability to form suitable porosities in tissue engineering applications [20]. However, although the GA showed a significant effect on the porosity, inter-pore connectivity and mechanical properties of the scaffolds, it showed a cytotoxic effect on the cells in our study. Because electrospinning PVA/GE scaffolds dissolved in GP solution, we had to use GA instead of GP for cross-linked of these scaffolds.

Scaffold colors differed due to cross-linking. Samples cross-linked with GA were white and opaque, while those cross-linked with GP were green. This research is coherent with the results explained and proved the formation of a blue pigment is the consequence of oxygen-radical-induced polymerization of genipin [3]. Although the surface of both types of scaffolds was smooth, the shape of the electrospun and freeze/dried scaffolds are like thin-film and radial patterns, respectively. In this study, we preferred GE in enabling the epithelialization and granulation of tissues which are the most crucial required step for wound healing and also owing to the RGD motifs of GE which increases the adhesion, proliferation, migration of fibroblast cells [11], [45]. The mixture of PVA with GE solution was used for providing the mechanical strength to the GE (PVA/GE, 1:1 ratio) due to the low mechanical strength of GE [41]. In the freeze/drying method, porous scaffolds which have effective pores were obtained by sublimation of ice crystals.

SEM demonstrated that pores inside the scaffolds were interconnected in irregular designs, and the pore size was related to the genipin concentration. Samples containing low genipin had higher pore numbers and lower pore diameters compared to higher genipin concentrations, in this study. Although studies are supporting these observations [9], [20], [23], there are also opinions that the porosity decreases as the cross-link concentration increases [5], [35]. The cause of this dilemma is unknown. However, we hypothesize that the increase in the pore diameter with the increase of the genipin is due to the ring-opening polymerization of the genipin and the feature of long-range cross-linking, which has been shown by the group of Mu et al. [21]. It has been known that the appropriate porosity size supports the proliferation of fibroblast cells and is, therefore, important in skin tissue engineering studies [42].

To observe the chemical structure of PVA/GE electrospun and freeze/dried scaffolds without cross-linking was used FTIR spectroscopy technique. Bending of N-H group at 1625 $cm^{-1}$. Yang and et al. [35] demonstrated that when gelatin and PVA combine, the form of hydrogen bond is cut, thus the ranges are more narrow and sharp. So, in this study, FTIR results can give a piece of information about that mixture of PVA and gelatin has good compatibility for the scaffold.

The swell is known to be significantly needed at the porosity, inter-pore connectivity, and mechanical properties of the scaffolds [18]. Two items are important for the swell of porous scaffolds: 1) nature of formation polymers, and its hydrophilic feature; 2) the relation of the volume of pores and total volume [16]. In our study, the swelling ratio was high and compatible because the scaffolds contain PVA and have a high cross-linking effect. It is known that PVA has a significant role in the high swell and it can hold very many water molecules since all free hydroxyl groups of PVA prevent chemical cross-link [2], [43]. Cross-linked scaffolds with a high genipin ratio showed lower swelling values than scaffolds with a lower genipin ratio. The rising in swelling depends on cross-linking time and could be referred to as the oligomerization of genipin with the time that can have enhanced porosity of the fibers and let more swelling occur [18]. The scaffolds applied in tissue engineering are necessary to have high water absorption capacity for the arrest of the loss of body fluid, especially water absorption capacity in skin tissue engineering plays an important role in keeping the wound moist, which prevents the wound from stretching [14], [28]. Our results demonstrated that water permeability reduced and the capability of water-absorbing increased due to the increased crosslinking ratio which outcomes in more water molecules being trapped within the network.

One of the most crucial properties of the ideal scaffold should be the degradability to enable the cells to generate their extracellular matrix in skin tissue engineering applications [16], [32], [33]. Structures with high cross-connections have higher mechanical strength compared to others [23]. In our study, scaffolds with higher genipin concentrations were less fragmented than scaffolds with lower genipin concentrations.

Cell adhesion to the scaffold is an important parameter for tissue applications [4], [35], [43]. In our study, the morphology of cells attaching to the surface of the scaffolds demonstrated that they had cytocompatibility.

Selenium cytotoxic experiments are based on some studies. Higher concentrations of Se lead to apoptosis, low concentrations of selenium provide survival [34], [38]. In our study, as a result of cytotoxic experiments, selenium doses higher than 200 nM decreased cell viability (statistical analysis revealed significant viability in 200 nM).

Nanofibrous tissue scaffolds obtained by electrospinning methods are known to have an important role in skin tissue engineering by enabling cellular adhesion [12], [32], [33]. In our study, Se significantly increased the vitality compared to each control group within 24, 48, 72 hours.

The lower cell viability can be thought to be due to the GA cross-linker with high cytotoxicity according to the genipin [20]. It has been observed that selenium increases viability in both scaffold and non-scaffold conditions. Our results confirmed a significant increase in vitality when Se was added to the samples compared to the control without Se. Se is an important trace element that triggers apoptosis when it is given at high doses in cancerous cells. Low dose of Se has been shown to increase the vitality and proliferation of cells on healthy cells [8], [10], [25], [34].

Cell viability was significantly increased in genipin cross-linked scaffolds compared to control in freeze/drying methods. The increased cell viability can be explained due to the GE, because genipin is a natural non-toxic cross-linker, we can think that genipin could be used frequently in wound healing and skin tissue engineering applications where it can increase cell proliferation and growth, as shown in [4], [18].

The MTT assay was evaluated to calculate the number of living cells on the cross-linked scaffold for 24, 48, and 72 h. These analyzes were performed as certain groups in the presence, and absence of Se and scaffolds. The lower cell viability can be thought to be due to the GA cross-linker with high cytotoxicity according to the genipin [20]. Genipin and selenium were analyzed in separate studies on vitality [15], [25], [34], [38], but no study have been found that researched them together. Although a study shows that genipin increases the viability of various cells in cross-linked scaffolds, there is no clear study on 3T3 cells [24]. In our research, synergistic effects of both genipin and selenium increased vitality significantly together.

# 5. Conclusions

In this research, bead-free, tough and smooth scaffolds containing PVA/GE were successfully fabricated via electrospinning and freeze/drying methods. In result of analyzes, tissue scaffolds had biocompatibility, appropriate swelling capacity, good degradation rate and high pores for the adhesion of cells. The synergistic effects of GP and Se together were examined on viability of cells on scaffolds. Compared to the control group, Se did not have cytotoxicity in cell seed scaffolds. It showed that Se exhibits strong biosafety. Due to the fact that Se triggered the excellent cell growth and proliferation on electrospinning and freeze drying PVA/GE scaffolds, it can be suggested that it has potential to be used as wound dressing. So far, the effect of Se on cell viability of different cross-linked and cell-seeded scaffolds obtained using two different methods was not investigated. It has been determined that Se increases cell viability on cell seeded scaffolds (PVA/GE/GA, PVA/GE/GP5, and PVA/GE/GP8), and also that Se and GP together contribute to vitality. The synergistic effects of Se and GP showed significantly of the originality of our article. Although there are many studies on wound healing, the studies are quite limited on acceleration of wound healing. It can be thought that this limit will be eliminated due to the synergistic effects of Se and GP together. When considered these interactions of Se and GP, it can be aimed to observe the effects on skin tissue engineering in the future and to conduct *in vivo* studies to accelerate wound healing.

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## References

[1] ABRIGO M., MCARTHUR S.L., KINGSHOTT P., *Electrospun nanofibers as dressings for chronic wound care: Advances, challenges, and future prospects*, Macromolecular Bioscience, 2014, 14, 772–792.

[2] ARAMWIT P., SIRITIENTONG T., KANOKPANONT S. et al., *Formulation and characterization of silk sericin-PVA scaffold crosslinked with genipin*, Int. J. Biol. Macromol., 2010, 47, 668–675.

[3] BUTLER M.F., NG Y.F., PUDNEY P.D.A., *Mechanism and kinetics of the crosslinking reaction between biopolymers containing primary amine groups and genipin*, J. Polym. Sci. Part A Polym. Chem., 2003, 41, 3941–3953.

[4] CHANG W.H., CHANG Y., LAI P.H. et al., *A genipin--crosslinked gelatin membrane as wound-dressing material: In vitro and in vivo studies*, J. Biomater. Sci. Polym. Ed., 2003, 14, 481–495.

[5] CHEN K.Y., LIAO W.J., KUO S.M., TSAI F.J., CHEN Y.S., HUANG C.Y. et al., *Asymmetric chitosan membrane containing collagen I nanospheres for skin tissue engineering*, Biomacromolecules, 2009, 10, 1642–1649.

[6] CHOI S.M., SINGH D., KUMAR A. et al., *Porous three-dimensional PVA/gelatin sponge for skin tissue engineering*, Int. J. Polym. Mater. Polym. Biomate., 2013, 62, 384–389.

[7] GANTHER H.E., HAFEMAN D.G., LAWRENCE R.A. et al., *Selenium and Glutathione Peroxidase in Health and Disease*, Essential and Toxic Element, 1976, 71, 952–958.

[8] GOPEE N.V., JOHNSON V.J., SHARMA R.P., *Sodium selenite--induced apoptosis in murine B-lymphoma cells is associated with inhibition of protein kinase C-δ, nuclear factor κB, and inhibitor of apoptosis protein*, Toxicol. Sci., 2004, 78, 204–214.

[9] GORCZYCA G., TYLINGO R., SZWEDA P., AUGUSTIN E., SADOWSKA M., MILEWSKI S., *Preparation and characterization of genipin cross-linked porous chitosan-collagen-gelatin scaffolds using chitosan-$CO_2$ solution*, Carbohydr. Polym., 2014, 901–911.

[10] GUAN L., HAN B., LI Z. et al., *Sodium selenite induces apoptosis by ROS-mediated endoplasmic reticulum stress and mitochondrial dysfunction in human acute promyelocytic leukemia NB4 cells*, Apoptosis, 2009, 14, 218–225.

[11] GUO S., DIPIETRO L.A., *Critical review in oral biology and medicine: Factors affecting wound healing*, J. Dent. Res., 2010, 89, 219–229.

[12] HAYASHI Y., FURUE M.K., OKAMOTO T. et al., *Integrins Regulate Mouse Embryonic Stem Cell Self-Renewal*, Stem Cells, 2007, 25, 3005–3015.

[13] HUANG C.Y., HU K.H., WEI Z.H., *Comparison of cell behavior on pva/pva-gelatin electrospun nanofibers with random and aligned configuration*, Sci. Rep., 2016, 6, 37960.

[14] KOBIELARZ M., TOMANIK M., MROCZKOWSKA K., SZUSTAKIEWICZ K., ORYSZCZAK M., MAZUR A., ANTOŃCZAK A., FILIPIAK J., *Laser-modified PLGA for implants: in vitro degradation and mechanical properties*, Acta of Bioengineering and Biomechanics, 2020, Vol. 22, No. 1.

[15] LANGER R., VACANTI J.P., *Tissue engineering*, Science (80-), 1993, 260, 920–926.

[16] MAHNAMA H., DADBIN S., FROUNCHI M. et al., *Preparation of biodegradable gelatin/PVA porous scaffolds for skin regeneration, Artif Cells*, Nanomedicine Biotechnol., 2017, 45, 928–935.

[17] MALAFAYA P.B., SILVA G.A., REIS R.L., *Natural-origin polymers as carriers and scaffolds for biomolecules and cell delivery in tissue engineering applications*, Adv. Drug. Deliv. Rev., 2007, 59, 207–233.

[18] MEKHAIL M., WONG K.K.H., PADAVAN D.T. et al., *Genipin-cross-linked electrospun collagen fibers*, J. Biomater. Sci. Polym. Ed., 2011, 22, 2241–2259.

[19] MCKEEHAN W.L., HAMILTON W.G., HAM R.G., *Selenium is an essential trace nutrient for growth of WI-38 diploid human fibroblasts*, Proc. Natl. Acad. Sci. USA, 1976, 73, 2023–2027.

[20] MIRZAEI E., FARIDI-MAJIDI R., SHOKRGOZAR M.A. et al., *Genipin cross-linked electrospun chitosan-based nanofibrous mat as tissue engineering scaffold Genipin cross-linked chitosan nanofibrous scaffold*, Nanomed. J., 2014, 1, 37–146.

[21] MU C., ZHANG K., LIN W., LI D., *Ring-opening polymerization of genipin and its long-range crosslinking effect on collagen hydrogel*, J. Biomed. Mater. Res. – Part A, 2013, 101, 385–393.

[22] NATHAN C., XIE Q.W., *Regulation of biosynthesis of nitric oxide*, Journal of Biological Chemistry, 1994, 13725–13728.

[23] NGUYEN T.H., VENTURA R., MIN Y.K. et al., *Genipin Cross--Linked Polyvinyl Alcohol-Gelatin Hydrogel for Bone Regeneration*, J. Biomedical Science and Engineering, 2016, 9, 419–429, 28.

[24] NYAMBAT B., MANGA Y.B., CHEN C.H., GANKHUYAG U., ANDI PRATOMO W.P., SATAPATHY M.K. et al., *New insight into natural extracellular matrix: Genipin cross-linked adipose derived stem cell extracellular matrix gel for tissue engineering*, Int. J. Mol. Sci., 2020, 21, 4864.

[25] PAPP L.V., LU J., HOLMGREN A. et al., *From selenium to selenoproteins: Synthesis, identity, and their role in human health*, Antioxidants and Redox Signaling, 2007, 775–806.

[26] PILEHVAR-SOLTANAHMADI Y., DADASHPOUR M., MOHAJERI A. et al., *An Overview on Application of Natural Substances Incorporated with Electrospun Nanofibrous Scaffolds to Development of Innovative Wound Dressings Mini-Reviews*, Med. Chem., 2018, 18, 414–427.

[27] RAYMAN M.P., *The argument for increasing selenium intake*, Proc. Nutr. Soc., 2002, 61, 203–215.

[28] REVERCHON E., CARDEA S., RAPUANO C., *A new supercritical fluid-based process to produce scaffolds for tissue replacement*, J. Supercrit. Fluids, 2008, 5, 365–373.

[29] RIEGER K.A., BIRCH N.P., SCHIFFMAN J.D., *Designing electrospun nanofiber mats to promote wound healing-a review*, Journal of Materials Chemistry B, 2013, 1, 4531–4541.

[30] SARAL Y., UYAR B., AYAR A. et al., *Protective effects of topical alpha-tocopherol acetate on UVB irradiation in guinea pigs: Importance of free radicals*, Physiol. Res., 2002, 51, 285–290.

[31] SONG H., KIM J., LEE H.K. et al., *Selenium inhibits migration of murine melanoma cells via down-modulation of IL-18 expression*, Int. Immunopharmacol., 2011, 9, 236–242.

[32] SUBRAMANIAN A., KRISHNAN U.M., SETHURAMAN S., *Fabrication, characterization and in vitro evaluation of aligned PLGA-PCL nanofibers for neural regeneration*, Ann. Biomed. Eng., 2012, 40, 2098–2110.

[33] SUNDARAMURTHI D., KRISHNAN U.M., SETHURAMAN S., *Electrospun nanofibers as scaffolds for skin tissue engineering*, Polymer Reviews, 2014, 54, 348–376.

[34] U□UZ A.C., NAZIRO□LU M., ESPINO J. et al., *Selenium modulates oxidative stress-induced cell apoptosis in human myeloid HL-60 cells through regulation of calcium release and caspase-3 and -9 activities*, J. Membr. Biol., 2009, 232, 15–23.

[35] YANG C., WU X., ZHAO Y. et al., *Nanofibrous scaffold prepared by electrospinning of poly(vinyl alcohol)/gelatin aqueous solutions*, J. Appl. Polym. Sci., 2011, 121, 3047–3055.

[36] YAN L.P., WANG Y.J., REN L., WU G., CARIDADE S.G., FAN J.B. et al., *Genipin-cross-linked collagen/chitosan biomimetic scaffolds for articular cartilage tissue engineering applications*, J. Biomed. Mater. Res. – Part A, 2010, 95, 465–475.

[37] YANG S.F., LEONG K.F., DU H. et al., *The design of scaffolds for use in tissue engineering*. Part II. *Rapid prototyping techniques*, Tissue Eng., 2002, 1, 1–11.

[38] YÜKSEL E., NAZIROĞLU M., ŞAHIN M., ÇIĞ B., *Involvement of TRPM2 and TRPV1 channels on hyperalgesia, apoptosis and*

*oxidative stress in rat fibromyalgia model: Protective role of selenium*, Scientific Reports OPEN, 2017, 7–17543.

[39] ZHANG B., CU Y., YIN G. et al., *Synthesis and swelling properties of hydrolyzed cottonseed protein composite superabsorbent hydrogel*, Int. J. Polym. Mater. Polym. Biomater., 2010, 59, 1018–1032.

[40] ZHAOA X., SUN X., YILDIRIMERA L., LANGA Q., YUAN Z.L., ZHENGA R., ZHANGH Y., CUIA W., ANNABIA N., KHADEMHOSSEINIA A., *Cell infiltrative hydrogel fibrous scaffolds for accelerated wound healing*, Acta Biomater., 2017, 49, 66–77.

[41] ZHONG S.P., ZHANG Y.Z., LIM C.T., *Tissue scaffolds for skin wound healing and dermal reconstruction*, Wiley Interdisciplinary Reviews, Nanomedicine and Nanobiotechnology, 2010, 2, 510–525.

[42] ZHANG Y.Z., VENUGOPAL J., HUANG Z.M., LIM C.T., RAMAKRISHNA S., *Crosslinking of the electrospun gelatin nanofibers*, Polymer (Guildf), 2006, 47, 2911–2917.

[43] ZHOU Y., YANG D., CHEN X. et al., *Electrospun water-soluble carboxyethyl chitosan/poly(vinyl alcohol) nanofibrous membrane as potential wound dressing for skin regeneration*, Biomacromolecules, 2008, 1, 349–354.

[44] XIAO J., DUAN H., LIU Z. et al., *Construction of the recellularized corneal stroma using porous acellular corneal scaffold*, Biomaterials, 2011, 32:6962–71.

[45] WALDECK H., CHUNG A.S., KAO W.J., *Interpenetrating polymer networks containing gelatin modified with PEGylated RGD and soluble KGF: Synthesis, characterization, and application in in vivo critical dermal wound*, J. Biomed. Mater. Res. – Part A, 2007, 82, 861–871.