A Newly Designed Collagen-Based Bilayered Scaffold for Skin Tissue Regeneration

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Abstract: In this study, a bilayered collagen-based membrane was prepared to mimic skin structure as a potential candidate for wound dressing application. To achieve the desired bilayered structure similar to skin, freeze-drying and electrospinning methods were used consecutively. The macroporous sublayer was prepared by freeze-drying of collagen intended for the absorption of exudates, while the upper layer was electrospun onto the freeze-dried part as an impermeable part to microorganisms. Nanofiber layer was loaded with silver nanoparticles for antibacterial activity. In order to improve biostability, double-layered materials were crosslinked by glutaraldehyde vapor. The morphology of the developed structures was assessed by SEM and the integrity of two layers was confirmed. The water uptake capacity of the scaffolds in physiological conditions was found to be around 738%. Afterwards, silver nanoparticles were sprayed to the upper part in order to obtain an antibacterial layer, and fibrinogen was immobilized to the sublayer for the stimulation of healing process. Agar zone inhibition test showed the antibacterial characteristics of the scaffolds. By providing structural and chemical similarity to natural skin tissue, the designed material can be a potential scaffold for skin tissue regeneration.

Keywords: Antibacterial, electrospinning, freeze-drying, silver nanoparticles, wound dressing.

1. INTRODUCTION

The skin, which acts as a protective barrier between the body and the environment, is the largest organ of our body. Because of its covering function, it is directly exposed to the harmful microbial, mechanical, thermal and chemical factors [1-3]. Skin loss is caused by several conditions such as burn injuries, chronic wounds, surgical procedures, and limited blood circulations. The skin can be able to heal itself; however, some medical assistance should be employed to accelerate the repair and regeneration process in case of a serious damage.

Skin defects can be classified as epidermal, superficial partial-thickness, deep partial-thickness and full-thickness skin wounds [1]. Epidermal, superficial and partial-thickness wounds can regenerate by self-healing [4-6]. But, full-thickness skin wounds in which a destruction of the epithelial regeneration process are more severe. The size of wound has a critical importance for epithelialization. For example, full-thickness wounds with more than 1 cm in diameter need skin grafting to prevent extensive scar formation [5-7]. An ideal graft should be nonimmunogenic, readily available, stimulate the healing process, reduce the pain and cause little or no scar formation.

Autografts, allografts and xenografts have been used for skin damage as natural skin substitutes. However, these naturally derived skin substitutes cannot meet the demand completely due to limited donor sites, risk of infection, slow healing, and association with the formation of scar [8]. Donor graft shortage problem can be solved by using cell-free or allogenic cell-containing skin substitutes. These enhance wound healing process by preventing fluid loss and providing critical dermal matrix components such as growth factors, cytokines to the wound bed. These can be used as temporary coverings until an autograft available. Or, these structures can stay in the wound during healing or longer. However, allografts might still have risk of viral contamination [1,7,8]. According to the reports in the past two decades, great efforts have been made to create substitutes that mimic human skin by the application of tissue engineering approaches. In skin tissue engineering, different biological and synthetic materials are combined with *in vitro*-cultured cells to produce functional tissues [7,8]. Results demonstrate that effective wound healing can be achieved in the presence of the dermis and epidermis layer in the skin substitutes. The most critical issue in skin tissue engineering is the scaffold as a

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E-ISSN: 2311-8717/14

matrix for the direction of the cells and integration of the graft [9, 10].

Many types of skin substitutes consisting of a biological/synthetic scaffold material have been developed by several groups until this date [11-16]. Some of these are commercially available such as Skinethic™ RHE, Episkin™, Epiderm™, Epiderm FT™. These are designed as only an epidermal substitute or a full-thickness skin substitutes. Some of them consist of keratinocyte cells seeded on a natural or synthetic carrier material simulating the human epidermis. Other types involve additionally of a dermal layer of human fibroblasts embedded in different scaffold types.

By mimicking skin structure, as mentioned above, several bilayered skin substitutes have been created and some of them are commercially available. One example of bilayered biosynthetic epidermal substitute is comprised of a silicone membrane (the outer part) and a knitted nylon mesh (Biobrane®) [1,8]. Both layers are covered by a cross-linked collagen that forms a 3D structure to allow adherence and initiate the wound healing process. Integra®, representing the oldest available dermal equivalent, is composed of a 3D porous collagen matrix with about 10–15% chondroitin-6-sulphate and an outer silicone sheet [1,8]. However, these dressing materials have still some drawbacks in practical aspects.

The biomimetic scaffolds in different forms (e.g. sponge, fibrous, or gels) have been produced by using natural and synthetic polymers. For the production, well-known techniques are used such as particle leaching, freeze-drying, gas expansion, phase separation and electrospinning. The freeze-drying method is used for the fabrication of porous scaffolds and the pore size can be controlled by the freezing rate [17, 18]. Electrospinning technique is a simple and inexpensive way for the control of thickness and composition, fiber diameter, and porosity of membranes. Electrospun fibers with diameters ranging from nano to micrometer scale have been produced by using various synthetic polymers such as poly(ε-caprolactone) (PCL), polylactic acid (PLA), polyglycolic acid (PGA), poly(lactide-co-glycolide) (PLGA) and natural polymers such as chitosan, collagen and gelatin. Several ongoing research and clinical trials have been carried out in the field of electrospun nanofibrous scaffolds for skin tissue regeneration [19].

In this present work, as potential wound dressing material, a bilayered collagen-based membrane was prepared by mimicking skin structure. The sublayer has a macroporous structure prepared by freeze-drying of collagen and the upper layer was electrospun onto the freeze-dried part as an impermeable layer to microbial attack. The layers of the scaffolds were incorporated with silver nanoparticles (Ag NPs) and fibrinogen, for antibacterial activity and stimulation of wound healing process, respectively. As fulfilling the all requirements of an ideal dressing material, the designed structures have a great potential as scaffold for skin tissue regeneration.

2. MATERIALS AND METHODS

2.1. Materials

To obtain the bilayered scaffolds, the most abundant extracellular matrix protein of skin tissue, collagen, was isolated from Wistar rat tails according to the method as described elsewhere [20]. Glutaraldehyde (GA), 25% water solution, was purchased from Shanghai Pharm. Co. (China). All other reagents and solvents are of analytical grade and used as received.

2.2. Preparation of Collagen Sponge by Freeze-Drying

Collagen solution was prepared in HAc in 0.5% (w/v). Then it was poured into a small petri dish and frozen at -20^{O}C. In order to obtain more stable structure, petri dishes were immersed in precooled ethanol at -20°C for 5h and then lyophilized for 24 h to obtain a porous collagen sponge.

2.3. Preparation of Bilayered Scaffolds

In this stage, the collagen sponges were coated collagen nanofibers by electrospinning to mimick the epidermal layer of natural skin. This top layer in nanofiber form has also effect as a barrier towards bacterial attack.

Acid soluble, lyophilized collagen was dissolved in 1,1,1,3,3,3 hexafloro-2-propanol (HFIP) in 0.085 gr/ml concentration. Then, this solution was placed into a 1.0 ml syringe mounted in a syringe pump (NE1000-Programmable Single Syringe Pump, USA). The syringe was capped with a 23-gauge blunt end needle. The positive lead from a high voltage supply was attached to the external surface of the syringe needle. The collagen sponge produced in previous section was mounted on a grounded target at a 15 cm distance from the tip. The syringe pump was set to deliver the solution at a rate of 1.5 ml/h and the high voltage (19kV) was applied across the solution.

2.4. Crosslinking of Bilayered Scaffolds

In order to improve the biostability, the bilayered collagen scaffolds were crosslinked with GA vapor. The samples were put inside a desiccator containing aqueous GA solution (25% v/v) for 72 h. To prevent the toxicity that may be caused by unreacted GA, the scaffolds were immersed in 0.02M of glycine solution for 4 h. Finally, they were washed with distilled water excessively and dried before use.

2.5. Characterization of Bilayered Scaffolds

Morphological Characterization

The morphology of the developed structures was observed by a scanning electron microscope (SEM; Leica Cambridge S360). The samples were further examined by SEM to evaluate the influence of crosslinking in the fiber morphology and overall structure.

Water Uptake Rate and Capacity

The double-layered scaffolds were cut into the 0.5x0.5 cm pieces and weighed. Then they were soaked into the phosphate buffer solution for 15 min at 37^{o}C. After that, the excess solution on the wet scaffolds was removed by filter paper and the samples were weighed again. The measurements were repeated in every 15 min until reach the equilibrium. The swelling ratio of the scaffolds was defined as the ratio of weight increase (w-w_0) to the initial weight (w_0). Each value was averaged from three parallel measurements.

2.6. Fibrinogen and Silver Nanoparticle (Ag NP) Loading to the Bilayered Scaffolds

Fibrinogen Immobilization

In order to achieve slower release, fibrinogen protein was covalently immobilized on the scaffolds. In a typical procedure, 2 mg fibrinogen was dissolved in 0.941 ml MES buffer. Then, 0.785 mg NHS and 2.165 mg EDC were added to the solution. For the immobilization, the scaffolds in 0.5x0.5 cm dimensions were soaked in this solution for 2 hours. The samples were then rinsed by PBS and water for 2 hours and left for drying.

The release of fibrinogen from scaffolds was investigated by placing them in a PBS buffer and shaking at 37^{o}C. PBS samples from the medium were taken in every 15 min. Fibrinogen content of the scaffolds was determined by using Bradford reagent. The protein in the scaffolds was dyed with Commassie blue and the measurements were performed by a UV-spectrophotometer (Schimadzu, Japan) at 595 nm.

Ag NP Synthesis and Loading onto the Scaffolds

Ag NPs were synthesized by reducing silver nitrate in the presence of sodium citrate according to the Turkevich's method [21]. The nanoparticles were characterized by UV-spectrophotometer and Zeta Sizer (NanoZS, Malvern Instruments, UK). The nanoparticles were loaded to the upper layer of the scaffold by spraying. The resulted structures were examined by SEM.

2.7. Antibacterial Activity Assay

The antibacterial properties of the bilayered scaffolds were evaluated by zone inhibition test. The Escherichia coli (*E. coli*, ATCC25992) were obtained from the American Type Culture collection (ATCC) (Manassas, VA, USA). The strains were cultured in 2ml nutrient broth overnight at 37°C with a shaking rate of 170rpm. Following day, the cell density was measured by spectrophotometer at 600nm. Several dilutions were applied to reach the defined number of the bacteria ($1.6x10^5$) for the test. Sterilized nutrient agar was dispensed by pouring 15± 2 ml into each standard (90x10mm) flat bottom petri dish to obtain firmly solid agar before inoculating. The bacterial cells were then transferred to the petri dishes using a glass rod. The samples were gently placed onto the agar surface and then incubated at 37^{o}C for 24h. The bilayered scaffolds without Ag NPs and sterile gauze were used as negative controls. To observe the comparative antibacterial activity of Ag NPs to a well-known antibiotic agent, silver sulfadiazine (1% and 10% solution) containing scaffolds were used as positive control. Antibacterial assay was performed in at least triplicate. Antibacterial activity was evaluated by measuring inhibition zone width against the test organism using the following equation:

$$W = (T - D)/2$$

Where W=width of clear zone of inhibition in mm; T=total diameter of test specimen and clear zone in mm; D=diameter of the test specimen in mm

Also, the bacterial zone inhibition was visualized by an imaging system (Vilber-Lourmat, Germany).

3. RESULTS

3.1. Morphology of the Bilayered Scaffolds

Skin tissue is composed of two main layers which differ from each other in the sense of morphological

structure, cell types and biochemical properties. The top layer functions as a bacterial protecting, gas and moisture transportation control membrane, whereas the bottom layer, dermis, is mainly responsible for remodeling of the skin. In the case of extensive wounds such as full-thickness burns, skin tissue can rarely heal spontaneously and thus require temporary or permanent protection to stimulate wound healing. The use of tissue engineering constructs may provide complete and scar free regeneration of the skin tissue. The most crucial point in the design of these constructs is that their ability of resembling to native skin both anatomically and functionally.

In order to obtain the layer that will represent dermis and assist to repair it, collagen porous sponges were prepared by freeze-drying. The SEM image of collagen sponges are given in Figure **1**. It was observed that the pores were aligned in a particular direction and the whole sample present a planar cellular structure (Figure **1**). Since the influence of processing conditions is well investigated for freeze-drying process, the obtained structure herein can be considered as a result of cold ethanol treatment and freezing temperature [22, 23]. In addition, pore size of the collagen sponge was measured to be around 50-200μm which is in the range of the preferred pore size for skin tissue engineering scaffolds (50-500μm) [24-26]. Thus, it can permit the ingrowth of cells, vascularization and regeneration of the tissue.

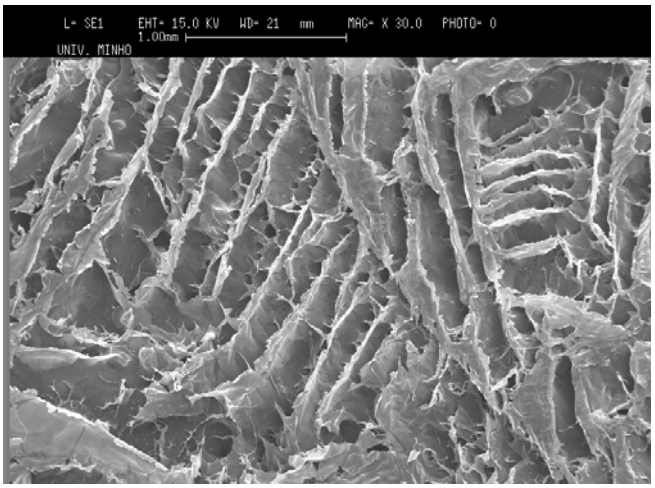

Figure 1: SEM micrograph of the freeze-dried bottom layer of the bilayered scaffold.

Nanofibrous top layer of the scaffolds was formed by electrospinning of collagen onto the sponges. From SEM images as shown in Figure **2**, collagen fibers were smooth, uniform, and fine, with a fiber diameter between 300nm-1.4μm. The nanofibrous structure of the top layer of the scaffold can meet the requirements to mimic the physical function of the natural epidermis. This function is expected to be provided by a small porosity of the electrospun layer that can prevent bacterial impenetration whereas allowing oxygen and water transport. Moreover, electrospun nanofibers have shown to increase cell attachment due to the relatively high specific surface area [27, 28]. Additionally, the smooth and continuous nanofiber formation without any bead was a proof of the utilizing of optimum processing conditions for this particular study.

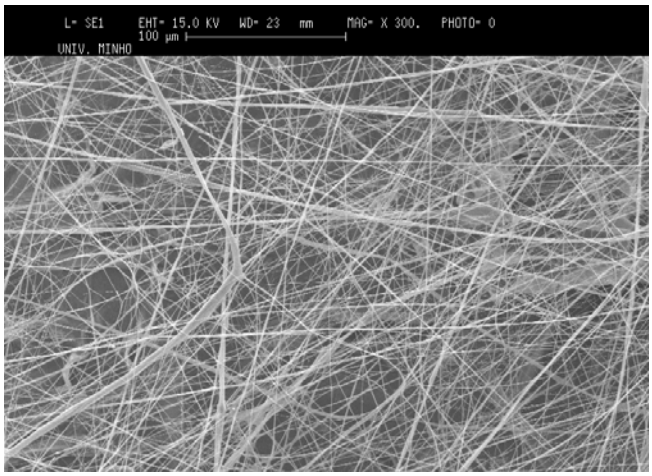

Figure 2: SEM micrograph of electrospun top layer of the bilayered scaffold.

Figure **3a** and **b** show lateral SEM images of the bilayered scaffold prepared by means of two step methodology. A very good adhesion between two layers was observed indicating the total integrity of the scaffolds. This result is mainly due to the use of same material for both layers. The trace amount of solvent residue during electrospinning process has partially dissolved the very top of the spongy layer and this layer act as a glue to combine both sponge and electrospun parts of the scaffolds. Furthermore, the scaffold was able to maintain this integrity after crosslinking with GA vapor. Using glutaraldeyhde in the vapor form would not only allow the crosslinker to penetrate into the deepest part of the samples, but also will minimize the toxic effect of this reaction [29].

3.2. Water Uptake Rate and Capacity

Materials in hydrogel form, like collagen sponges, have a functional capacity to absorb fluids or donate moisture to the wound environment. They are often used to treat second-degree burns and many partial-and-full-thickness wounds [30-33]. Collagen bilayered scaffolds exhibited an ability to absorb large amounts of water. The average swelling ratio was determined from wet and dry samples weights. The scaffolds were

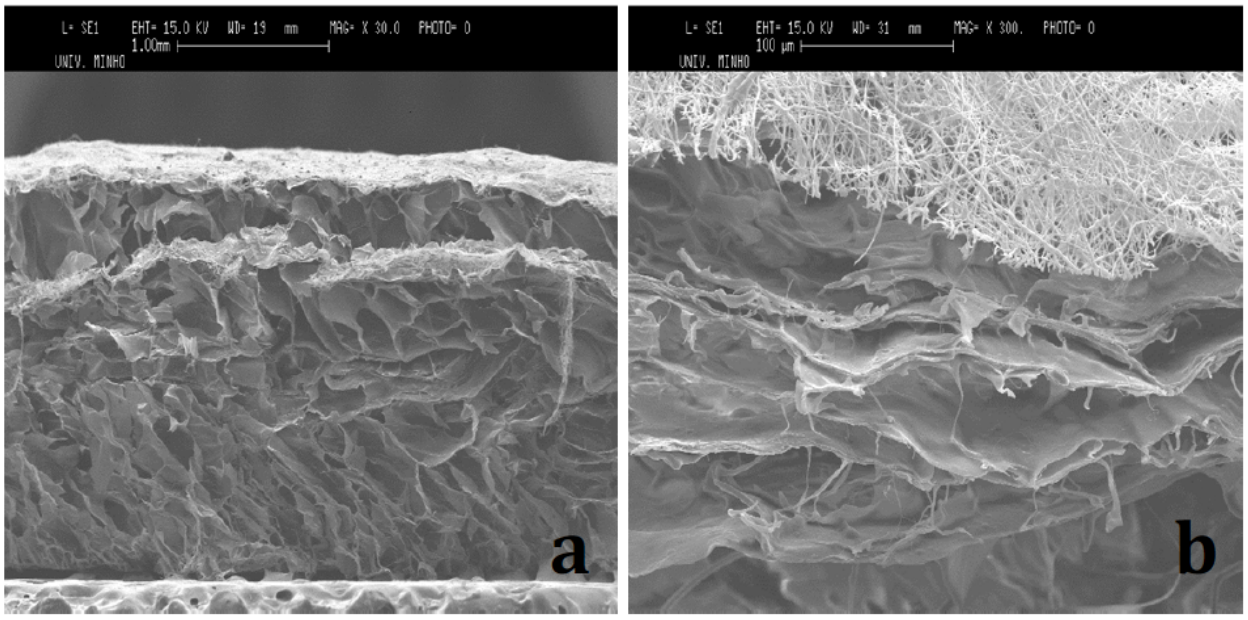

Figure 3: SEM micrograph of bilayered collagen scaffold (Lateral view); **a**) x30, **b**) x300.

rapidly uptake the water in first 30 minutes and reached the equilibrium swelling ratio of 738% after 75 min (Figure **4**). The high water content in prepared sponges was due to the hydrophilic nature of collagen as well as the highly porous structure of the sponges that has ability to keep the water inside those pores.

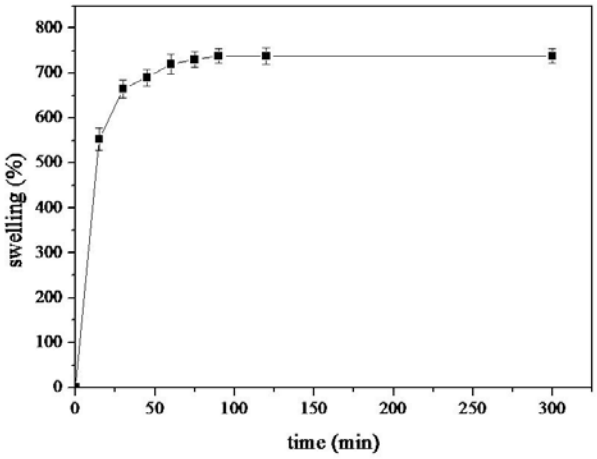

Figure 4: Swelling curve of scaffolds in pH 7.4 PBS at 37°C.

3.3. Fibrinogen Immobilization and Silver Nanoparticle Loading to the Bilayered Scaffolds

Fibrinogen Release from the Scaffolds

Fibrinogen is known as an acute-phase protein and liver is the primary source of plasma fibrinogen. It functions as a bridge to many types of cells and provides a temporary matrix at wound healing site in which cells can proliferate, organize, and acquire specialized functions [34]. In natural healing process, fibroblasts migrate into the wound site after 5 days of injury and the main function of fibrin and fibrinogen starts in this phase. They stimulate fibroblasts to proliferate, and express appropriate integrin receptors and migrate to wound site [35, 36]. Considering all these important function of fibrinogen in wound healing, herein, we immobilized fibrinogen to the bilayered scaffolds. The release characteristic of fibroingen from the scaffolds was given in Figure **5**. Fibrinogen was released with an initial burst phase followed by a slower release (inset graph) and the release was completed within 5 days. The total amount of released protein was 1.095 mg which was about 50% of the initial used fibrinogen amount.

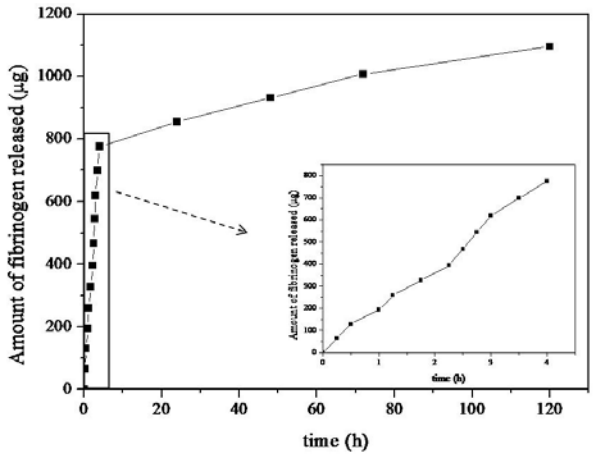

Figure 5: Cumulative release of fibrinogen from bilayered collagen scaffolds.

Characterization of Ag NPs and NP Loaded Scaffolds

Preventing of infection is one of the most crucial issue to be considered during wound healing process.

To bring in this property to the wound healing material, Ag NPs have been widely used in many different studies [37, 38]. In this particular study, Ag NPs synthesized from $AgNO_3$ by Turkevich Method were used to obtain antibacterial epidermal-like top layer. The size of the particles was measured to be around 37nm by Zeta Sizer, which is very similar to other studies used this method in literature [21]. Moreover, their optical properties were also investigated by UV spectrophotometer and max absorbance was observed at 430nm (data not shown).

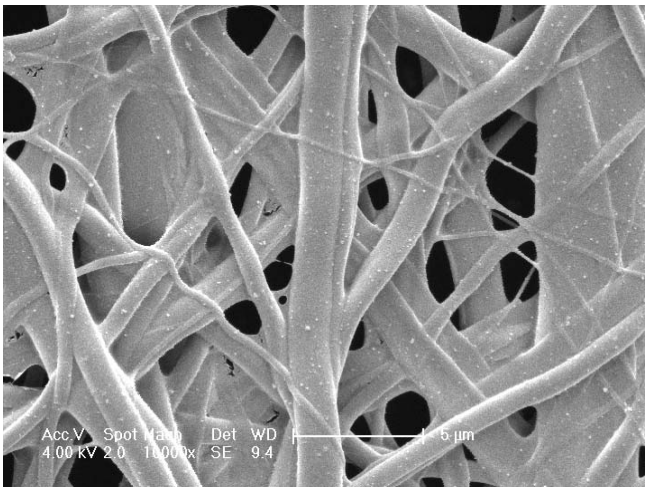

Figure 6: SEM micrograph of Ag NPs incorporated top layer of the bilayered scaffold.

In the last step of the preparation of bilayered and multifunctional scaffolds, Ag NPs were loaded to electrospun top layer of the constructs by a simple spraying methodology. Figure **6** presents the final morphology of the electrospun toplayer of the scaffolds. It was possible to see the homogenous distribution of Ag NPs along the nanofibers. However, some alterations on fiber morphology were also noticed, which might be caused by GA crosslinking process. But, there were no changes in the overall integrity and porous structure of the layer.

3.4. Antibacterial Activity

Our focus was to develop a bilayered scaffold with an antibacterial top layer to protect the newly formed skin tissue from a bacterial attack. *E. coli*, the predominant pathogenic bacteria responsible for severe burn wound infections, was selected as model organism. The images of agar plates were given Figure **7a** and **b**.

Additionally, the antibacterial activities of the tested samples and control samples were expressed in terms of zone inhibition width (W) as shown in Table **1**. The control group, with no Ag NPs, showed no antibacterial activity against *E. coli* after 24h. However, the clear zone around the scaffold with Ag NPs indicates the antibacterial activity of this sample. The bacterial inhibition in the plate was also observed around the scaffolds loaded with different amount of commercial available well-known antibiotic AgSD as expected [37]. Although they have similar effect on reducing bacterium colonization, Ag NPs are more preferable than the silver ion containing antibiotics due to the emergence of antibiotic-resistance bacteria and limitations to use of antibiotics [39].

Thus, all these results suggest that spraying Ag NPs on the electrospun top layer of bilayered scaffolds resulted in the achievement of its antibacterial potency against Gram negative bacterial strain.

4. DISCUSSION

This study reported the preparation of constructs based on collagen by mimicking the physical and

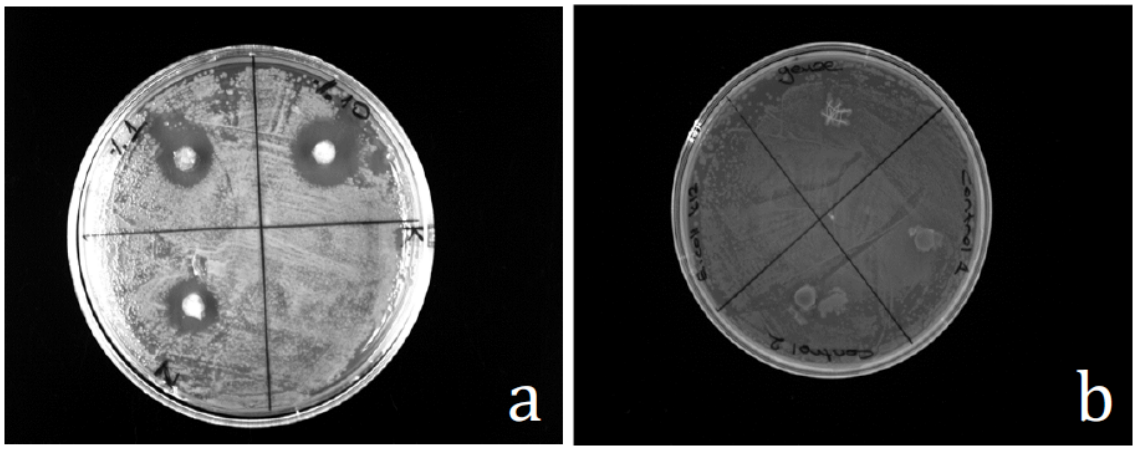

Figure 7: Images of zone inhibition test against *E. coli*: **a**) Ag NPs (N) and AgSD (1% and 10%) incorporated bilayered scaffolds; **b**) Non-loaded bilayered scaffolds (control 1 and 2) and gauze.

Table 1: Antibacterial Activity of the Samples Against *E. coli*

	Scaffolds with Ag NPs	Scaffolds with 1% AgSD	Scaffolds with 10% AgSD	Scaffolds without Ag NPs	Gauze
Zone inhibition width, W (mm)	5	5.5	6.5	-	-
Antibacterial Property	+	+	+	-	-

partially chemical structure of the natural skin to serve as a scaffold for skin tissue regeneration and repair. The different production method, namely freeze-drying and electrospining, were combined to achieve the final desired structures. The prepared bilayered scaffolds were with a good integrity between highly freeze-dried porous bottom layer and less porous electrospun top layer. They were able to maintain this integrity during the immersion in PBS at physiological conditions and showed very high water uptake capacity, which might make them favorable as a skin substitute. Furthermore, silver nanoparticles were produced and incorporated to the top layer of the scaffolds to gain the antimicrobial activity. This activity against common wound pathogen, *E. coli*, was confirmed by a zone inhibition test. Lastly, the bottom layer of the scaffolds was loaded with fibrinogen to stimulate the fibroblast adhesion and growth as it is in a natural skin tissue. The findings indicate that this newly design bilayered collagen scaffolds may be appropriate as a skin tissue engineering scaffold.

ACKNOWLEDGEMENT

This study was supported by Yıldız Technical University, Extensive Research Project Fund, 2011-07-04-KAP05.

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Received on 12-11-2013 Accepted on 18-11-2013 Published on 25-12-2013

DOI: http://dx.doi.org/10.12974/2311-8717.2013.01.01.2