Kemik Rejenerasyonunda Kullanım Amaçlı Nanolif Tabakası ile Güçlendirilmiş Hidrojel Geliştirilmesi ve Karakterizasyonu
Development and Characterization of Nanofiber-Reinforced Hydrogel for Bone Regeneration

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Özetçe*— Kemik doku, inorganik ve organik malzemelerden oluşan, deforme edici kuvvetlere sürekli maruz kalan kompozit bir malzemedir. Kemik rejenerasyonu, doku içinde ve dışında çeşitli sinyallerin indüksiyon ve iletimine dayalı bir sistem ile kemik oluşturucu ve kemik deforme edici hücrelerin göçü ve aktivasyonu yoluyla gerçekleşir. Klinik uygulamalarda, bu işlem ya ameliyat yoluyla osteogenez sürecini zorlayarak ya da farklı greft tipleri kullanılarak gerçekleştirilir. Bu malzemelere alternatif olarak hidrojel gibi malzemeler kullanılarak da gerçekleştirilmektedir. Hidrojeller, ECM'ye benzer bir yapı sağlaması, degredasyon kinematiğinin kontrol edilebilir ve biyouyumlu olması sebebi ile kemik rejenerasyonunda tercih sebebidir. Ancak içeriğindeki yüksek su konsantrasyonuna bağlı olarak, mekanik dayanımları özellikle kemik doku mühendisliği uygulamaları açısından yetersiz olabilmektedir. Mekanik mukavemetteki bu kayıp, sistemde bazı sınırlamalara neden olmaktadır. Bu çalışmada, hidrojellerin mekanik dayanımındaki problemlerin üstesinden gelmek amacı ile nanolif tabakalarıyla takviye edilmiş hidrojel yapılarının üretimi gerçekleştirilmiştir. Bu amaçla, PLGA nanolifleri elektroeğirme yöntemiyle üretilmiş ardında Poli (etilen glikol) diakrilat (PEGDA) hidrojel yapıları nanolif tabakaları güçlendirilerek katman-katman üretilmiştir. Sonuçlara göre, fiber takviyeli yapılar içindeki hücre çoğalması 7 günlük süreç boyunca artarak devam etmiş ve sadece hidrojel içeren tabaka yapıya kıyasla daha etkin bir şekilde hücre çoğalmasını desteklemiştir. Ayrıca, nanolif takviyeli hidrojel ve sadece PEGDA içeren yapılar mekanik olarak test edildiğinde, nanolif ile güçlendirilen hidrojelin mekanik dayanıklılığında artış gözlenmiştir. Sonuç olarak, nanofiber takviyeli hidrojel yapılarının kemik rejenerasyonunda kullanımı, gelecekte çalışmalar için umut vadetmektedir.

Anahtar Kelimeler — Kemik, Rejenerasyonu, Hidrojel, Güçlendirme, Mekanik Dayanıklılık

Abstract*— Bone tissue is a type of composite material that is composed of both organic and inorganic materials, constantly expose various deforming forces. Bone regeneration occurs through a complex system of induction and conduction of various signals inside and outside the tissue, migration and activation of bone forming and bone deforming cells. In clinical studies, this process is achieved either forcing the osteogenesis process by surgical operation or using different types of grafts. Also, hydrogels are preferred due to their ability to initiate and support bone regeneration in various ways, such as carrying growth factors, providing a constriction similar to ECM, easy to control their degradation kinematic and the biocompatible nature. However, due to their high water content, mechanical strength decreases and results in a poorly executed bone regeneration process. In this study, hydrogel structures that is reinforced by nanofiber (NF) sheets were produced to overcome mechanical strength related problems. After poly (lactic-co-glycolic acid) (PLGA) NFs were produced with electrospinning technique, PEGDA (Poly(ethylene glycol) diacrylate) hydrogel structures were produced by using layer-by-layer method and reinforced by the NF sheets within the hydrogel layers. Our results indicate that cell viability and cell proliferation within the fiber-reinforced structures increased through 7 days and cell number was significantly higher compared to only hydrogel containing layered structure. Furthermore, NF-reinforced hydrogel structures and structures containing only PEGDA were tested mechanically, the mechanical strength of the NF reinforced hydrogel was higher compared to control group. In conclusion, NF-reinforced hydrogel structures are promising for bone regeneration in future.

Keywords — Bone, Regeneration, Hydrogel, Reinforcement, Mechanical Strength

I. INTRODUCTION

Bone tissue is a type of connective tissue, and a composite material, that contains several types of cells, blood vessels [1, 2] Structural uniqueness of the bone results in a multifunctional tissue that takes place within the skeletal system and provides support, protection, assistance in movement, mineral homeostasis [3]. Bone structure is formed by osteogenesis process with the contribution bone cells which are osteoblasts, osteoclasts and osteocytes[4]. Bone tissue is a multifunctional tissue exposes many forces within the body which results in deformation or fracture and additional support for bone regeneration.

Biomaterials can be used for a variety of time period, and can contribute to the human body for treatment, replacing, or

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helping the any type of organ, tissue or a function [5]. Biomaterials possess various characteristics and properties due to their purpose of usage and how long it should possess these properties in order to assist, help and replace the targeted tissue lacking any rejection [6]. Especially for bone tissue regeneration, biomaterials that possess osteoconductivity, osteoinductivity and osteointegration are preferred. Synthetic polymers are biomaterials that are produced in laboratory conditions [7]. In tissue engineering applications, specific type of bioresorbable synthetic polymers are used[8]. Those polymers are used in various forms in order to provide an environment, promote proliferation, initiate differentiation for cells and replace the defected tissue semi-permanently. Polymers such as PLGA, PGA and PLA are widely used in bone tissue engineering applications [9]. Also, there are polymers who goes through a cross-linking step in order to provide desired tissue structure. A common example is hydrogels which are basically a 3D network that is formed by crosslinked polymeric segments with an ability to absorb a good amount of biological fluids or water [10]. PEGDA is one of those polymers that is usually used in the form of hydrogel.

Hydrogel is a type of biomaterial that has both synthetic and natural origin types and defined as macromolecular structures that formed by cross-linking. Hydrogels have water affinity, which is a result of their chemical or mechanical crosslinking process. Hydrogels can swell when they come into contact with liquids like body fluid. Their swelling depends on the crosslinks which affects the length, density, chemical nature and stability of the hydrogels [11]. PEG based hydrogels are commonly used biomaterials in bone regeneration due to their high functionalization capability and their easy to shape nature. However, their mechanical strength in a great amount of lose due to its ability to interact with large amount of water and their tendency to swell. This problem can be overcome by reinforcing the hydrogel structure with appropriate materials such as polymer NFs [12, 13]. In this study, NF reinforcement method was used by using layer-by-layer approach with PLGA NF and PEGDA hydrogels to enhance mechanical properties of hydrogels and support the hydrogel structure both macro and micro levels.

II. MATERIAL AND METHOD

A. Fabrication and Characterization of PLGA Nanofibers

NFs were produced via electrospinning technique by using PLGA (85:15) at a 3% wt constancy by dissolving in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP; Matrix Scientific; Colombia). The parameters for electrospinning was 15 cm of needle-collector distance, 20 kV of voltage, 1.0 mL/s of injection rate, 1200 rpm to obtain aligned structure. The NF were imaged with a Scanning Electron Microscope (SEM; Carl Zeiss Microscopy, Germany) at 3 kV accelerating voltage after coating with gold (QUORUM; Q150 RES; East Sussex; United Kingdom) at 20 mA for 60 seconds. The scale bars in the images of were obtained from the SEM software and the fiber diameters and distributions were analyzed with IMAGEJ software (National Institutes of Health, Bethesda, MD, USA) to determine the average fiber size.

B. Production of PLGA Nanofiber Reinforced Hydrogel and Characterization

10% (w/v) PEGDA and 0.5% (w/v) (Irgacure 2959) was dissolved in PBS to fabricate hydrogels. 10% (w/v) PEGDA and 0.5% (w/v) (Irgacure 2959) was dissolved in PBS to fabricate hydrogels. The optimized UV crosslinking conditions are exposure duration of 200 seconds, power density of (686 mW/cm^2) and surface to UV source distance of 5.5 cm. The first layer was formed by adding 200 µL PEGDA solution to the mold (diameter: 32 mm) and UV was exposed according to the optimized parameters. 1 cm^2 nanofiber sheet was placed on top of the first layer. Then 200 µL PEGDA solution was added and exposed to UV light. Rheology test was conducted at Izmir Katip Çelebi University Central Research Laboratories Rheological Analysis Laboratory to measure the change in mechanical strength of the NF-reinforced hydrogel structures. Hydrogels were loaded on Peltier Plate (Hybrid Rheometer Discovery HR-2, TA Instruments, New Castle, DE). The gap was 1500 µm and applied shear rate was 0.1-1000 1/s. Angular frequency was applied as 0.1-100 rad/s.

C. Cell Seeding on PLGA Nanofiber Reinforced Hydrogel

Human bone marrow derived mesenchymal stem cells (hMSCs) (HMSC-AD-500, CLS cell lines Service, Lot#102, Eppelheim, Germany) was cultivated in basal medium (DMEM supplemented with 10% FBS, 100 units/mL penicillin, 100 µg/mL streptomycin). Medium of cultures were replaced with fresh medium at 3 and 7 days (d) and cells at passage 3 were used for seeding. Before seeding the cells, NFs were exposed to UV light for 1 hour and rinsed with 70% ethanol for 30 minutes in order to sterilize. NFs were washed with PBS and conditioned in cell medium for 1 hour. hMSCs cells ($5x10^4$ cells/mL) were seeded on NFs. HUVECs (Human Umbilical Vein Endothelial Cells) are encapsulated inside the hydrogel at the density of 10^6 cells/mL. Briefly, hydrogel solution was filtered by using a 0.20 µm and cell platelet was suspended on the solution. 200 µL of solution was transferred to syringe mold and exposed to UV light. After the first layer is formed, BMSC seeded NF was placed on top of hydrogel. 200 µL hydrogel solution was added on NF and exposed to UV light with same parameters. MTT (3- (4,5-dimethyl-2-thiazolyl)-2,5-diphenyl2H-tetrazolium bromide, Vybrant (R), ThermoFisher) assay was used to determine the proliferation and attachment of the cells. Briefly, the medium was removed and 10% of MTT solution were added on the cells at dark. The cells were incubated for 4 hours at 37°C and 5% CO_2 . After incubation, MTT solution was removed and 500µl DMSO was added to each well, waited for 10 minutes. Cell number on each NF was determined by using a UV spectrophotometer (Biotek Epoch 2) , reading the absorbance values of each well at 575 nm. The cell number were calculated by comparing the values obtained with the calibration curve previously created for the same cell line. Cell proliferation of NF-reinforced hydrogel structures was determined by using MTT analysis at 1, 4 and 7. Also, live and dead assay (Biovision, Live/Dead Cell Viability Assay Kit) was

conducted to observe cell viability of the cells within the NF-reinforced structure. Briefly, Solution A and Solution B were prepared at dark. Cell medium was removed and hydrogel was washed with PBS for 3 times. Dye mixture was added to each well and incubated for 30 minutes at 37°C. After incubation, remaining dye solution was removed, and hydrogels were washed with PBS for 3-5 times. Cell images were taken by using fluorescent microscope (Olympus CKX41, Tokyo, Japan).

III. RESULTS AND DISCUSSION

SEM photomicrograph and NF diameter distribution histogram PGLA NF produced by electrospinning is given in Figure 1. Diameter of the resulting NFs used throughout the study ranged from 100 to 600 nm and the mean diameter was calculated to be 328±40 nm.

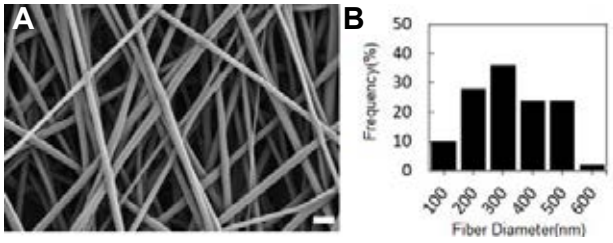

Figure 1. A) SEM images B) Size distribution graph of electrospun NFs. Scale bar represents 2µm

Cell proliferation on PEGDA hydrogel, NF, and NF reinforced hydrogel group was evaluated by MTT assay with respect to incubation time of 1, 4 and 7 days (Figure 2). At Day 1, the ratio of cell number on PEGDA, NF and NF+PEGDA were 1200429 ± 88853, 1065872 ± 112161, and 1876494 ± 191617, respectively. After Day 4, the cell number was the highest on the NF+PEGDA (4905660 ± 445138), followed by NF (3061301 ± 109071) and PEGDA (2166379 ± 195682). Moreover, at Day 7, cell number on NF+PEGDA (7721504 ± 281775; $P < 0.001$) were significantly higher than NF (6740225 ± 338993) and PEGDA (4646392 ± 327529). Cell proliferation in all groups increased for 7d. The results indicated that cells adhere to both the NF and hydrogel,and proliferate. The structure are able to allow penetration of cell medium and provide enough void volume for cells to proliferate to provide cell-cell signaling.

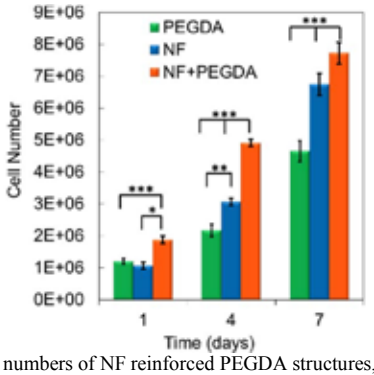

Figure 2. Cell numbers of NF reinforced PEGDA structures, only PEGDA structures and NFs

Cell viability analysis was evaluated with live/dead assay at day 7 (Figure 3). Results showed that NF-reinforced hydrogel structures supports cells viability better compared to other groups. In the literature, similar attempts of using reinforcement of hydrogels increased cell viability [12, 13]. These outcomes supported the idea that NF-reinforced hydrogel structures can be used for bone regeneration process however additional tests like osteocalcin assay and ALP assay needs to be conducted for a better observation of the structures effect on bone regeneration.

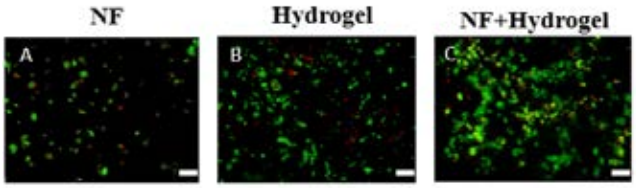

Figure 3. The cell viability on NF layer (A), Hydrogel layer (B) NF reinforced layered hydrogel structure(C) (green: LIVE cells, red: DEAD cells) (Scale bar represents 100 nm).

As shown in Figure 4, rheology analysis indicated that NF-reinforced hydrogel structures has as better mechanical strength when compared to only PEGDA hydrogel containing structures. However, breakage in the structure was observed during the analysis, hydrogel part was deformed but the fiber part was not affected. This outcome should be tested in future studies by using compression test.

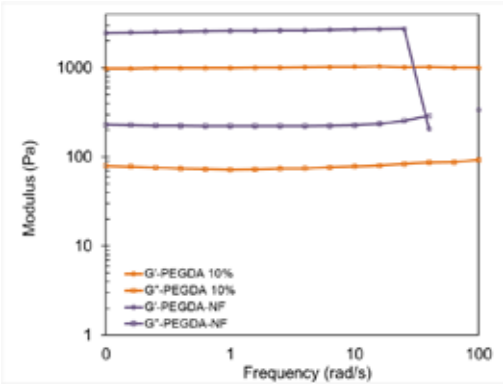

Figure 4. Rheology analysis of PEGDA and PEGDA+NF structures. G' and G" represents storage and loss modulus, respectively.

IV. CONCLUSION

Due to the inadequate mechanical properties of hydrogels, there are only few studies on building bone matrix mimetic scaffold by hydrogels. Therefore, there is still tremendous demand for new approaches and strategies for enhancing mechanical properties of hydrogels. In this project, this limitation were tried to overcome by using aligned electrospun PLGA NFs and reinforcing hydrogels with NFs. The results indicated that mechanical strength of hydrogels was increased and cells proliferated in the structure produced structure for a certain time point. In conclusion, using NF-reinforcement method to increase the mechanical strength of hydrogel

biomaterials has a promising potential for bone regeneration processes. In future study, additional mechanical tests are planned to be conducted and peptide epitopes for osteogenic vasculagenic differentiation will be integrated to this model. After osteogenic differentiation results are obtained, vasculagenic differentiation studies will be performed to develop vascularized bone tissue in this model. This biomimetic approach to develop NF reinforced vascularized bone tissue model in vitro by using peptide conjugated hydrogel could be used for healing of metabolically demanding bone defects.

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