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Effect of Different Number of Glutamic Acid Containing Peptide on Biomineralization and Cell Proliferation

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***Abstract*—Bone organic matrix is formed primarily collagen type I, bone cells and non-collagenous proteins while inorganic matrix is formed by calcium phosphate crystals mainly in the form of hydroxyapatite. Most bone diseases occur due to abnormalities of calcium and phosphate homeostasis and deficiencies during remodeling. Due to the limited osteoinductive capacity of scaffolds, mineralization and osteointegration of these scaffolds are limited. Therefore, bioactive peptides are widely used for scaffold modification to stimulate their influence. In the literature, it is shown that surface modification with glutamic acid templated peptides is effective on nucleation and crystallization of hydroxyapatite. The aim of this study was to evaluate thr effect of surface modification with various number of glutamic acids containing peptides on cell viability, proliferation and mineralization. PLGA (Poly-lactide-co-glycolide) electrospun nanofibers (NFs) were conjugated with peptides including different repetitive glutamic acid and mineralized into SBF (stimulated body fluid). Then, MSCs (Mesencymal Stem Cells) were seeded on NFs. Mineralization amount on NFs were evaluated by Ca Assay and XRD (X-Ray diffraction). It was observed that proliferation and mineralization were significantly higher on the two glutamic acid (GLU) containing peptide conjugated groups compared to other groups.**

Keywords — Bone tissue engineering; surface modification; peptide.

I. INTRODUCTION

Bone matrix is composed of two components, organic and inorganic. The organic matrix of the bone is composed mainly of collagen type-1. Collagen fibers act as a mold for mineralization and maturation of osteoprogenitor cells besides providing elasticity to bone. The inorganic matrix is composed of particularly calcium, phosphate, magnesium, sodium and potassium minerals. Calcium and phosphate (CaP) are found in the collagen fibers mainly in the form of hydroxyapatite (HA). These apatite nanocrystals provide stiffness and gain osteoconductive properties to bone. Non-collagenous ECM proteins such as bone sialoprotein (BSP), osteopontin (OP) and osteocalcin (OC) play major role on CaP deposition on collagenous fibers. These proteins contain glutamic acid (GLU) ranging from 2 to 6 repeated sequences which control nucleation, growth and stabilization of CaP nanocrystals on collagen fibers.

Since random and oriented electrospun nanofibers can mimic collagen fibers in natural bone, electrospinning is mostly used in bone tissue engineering. Electrospinning has wide range of polymer to form scaffold in desired morphology and porosity. Among synthetic polymers PLGA has widely used in tissue engineering because of its high biocompatibility and biodegradability. However, it is shown that PLGA scaffolds by produced electrospinning do not induce cell adhesion and proliferation because of its hydrophobic surface and lack of biological recognition parts. Therefore, additional surface modification of PLGA nanofibers with various technique might be required.

Functionalization of scaffold surface to reduce their limitations is an interesting and promising field in bone tissue engineering [1]. Surface modification with bioactive peptides promote cell attachment, spreading and differentiation. In bone tissue engineering, enhanced mineralization and cell attachment are critical. Studies have demonstrated that GLU sequences in non-collagen proteins initiate nucleation and growth of CaP crystals. However, any comparative study of the effect of different recurrent GLU chains on biomineralization has yet to be studied. The aim of this study is to determine the effects of surface modification of PLGA nanofibers with peptides containing different GLU sequences on biomineralization, cell attachment and viability. PLGA (Poly-lactide-co-glycolide) electrospun nanofibers (NFs) were conjugated with peptides including different repetitive GLU and mineralized into SBF (stimulated body fluid). Then, MSCs (Mesencymal Stem Cells) were seeded on NFs. Mineralization amount on NFs were evaluated by Ca Assay and XRD (X-Ray diffraction).

A. Peptide Synthesis

EEGGGG, EEEEGG, EEEEEE peptide sequences were synthesized manually on 4-Methylbenzhydrylamine (MBHA) (0.67 mmol/g loading capacity).100 mg resin was swelled in 3 ml DMF solution for 30 minutes. F-moc protected groups was removed by using 20% piperidine in DMF for 30 minutes. F-moc protected amino acids (2 eq) was dissolved in DMF and

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added to resin. Then, HBTU (2 eq), HOBt (2eq) and diisopropylethylamine (DIPEA) (4 eq) were added to the resin solution and mixed in orbital shaker for coupling for 4-6 hours. The resin was tested for the presence of unreacted amines using the Kaiser reagents. When the result was positive resin was washed with 3 ml DMF at least 3 times and coupling process was repeated until Kaiser Test was obtained as negative. When Kaiser Test result was negative resin was washed with 3 ml DMF at least 3 times. After that, resin was put into deprotection solution (20 % piperidine in DMF) for 30 min to remove F-moc protecting groups. Then, Kaiser Test was made to ensure F-moc protecting groups were removed. If Kaiser test result was negative deprotection process was repeated until obtain a positive result. When the test result was positive resin washed with 3 ml DMF at least 3 times and filtered. Coupling process was repeated for next amino acid until desired peptide sequence was obtain. Finally, peptide was cleaved from resin with 95% TFA (trifluoroacetic acid), 2.5% TIPS (triisopropylsilane), 2.5% H_2O solution for 2 hours. Solution was poured into cold-diethyl ether and precipitated in -20°C for 24 hours. Suspension was centrifuged, supernatant was removed, and pellet was freeze-dried.

B. Fabrication of Nanofibers

7wt % PLGA (50:50) mixture was dissolved in 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP; Matrix Scientific; Columbia). Polymer mixture was injected into a syringe and syringe pump was used to transfer mixture through needle. Needle was connected positive charged Pt electrode of high voltage supply. The aligned nanofibers were collected by an aluminum rotating wheel. Electrospinning was occurred on 13 mm circular glass coverslips (VWR, Bristol, CT, USA) under conditions of 1 ml/h injection rate, 20 kV electrical potential, 1200 rpm rotation speed and 15 cm distance between needle and collector.

C. Peptide Conjugation on Nanofibers

Cold atmospheric plasma was applied for 45 seconds with 1.5 frequency and 31 kV output voltage. Nanofibers were washed with deionized water then they incubated with 2 mM EDC and 5 mM NHS in 0.1M MES solution for 40 min to produce carboxyl rich surface. After that nanofibers were conjugated with related peptides sequences in 1 mM sterile PBS at 4°C for 24h. Before characterization nanofibers were washed with PBS completely.

D. Biomineralization on PLGA-GLU Nanofibers

PLGA-GLU nanofibers were incubated in 1.5-fold concentrated stimulated body fluid (SBF) at 37°C for 24 hours. The SBF solution was prepared by dissolving sodium chloride, potassium chloride, calcium chloride monohydrate ($CaCl_2.H_2O$), magnesium chloride hexahydrate ($MgCl_2.6H_2O$), sodium bicarbonate ($NaHCO_3$), and monosodium phosphate (NaH_2PO4) (all purchased from Sigma-Aldrich) in deionized water to a final pH of 4.2. Next, 60 mM $NaHCO_3$ solution was added to adjust pH to 7.4. Prepared SBF filtered and centrifuged before incubation. Parafilm was used to prevent CO_2 diffusion. Incubation solution was changed for every 6 hours to refresh calcium and phosphate minerals. After incubation, nanofibers were washed with deionized water and dried.

Surface morphology were determined by Scanning Electron Microscopy (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. After drying, structure of CaP crystal was determined by XRD analysis in Izmir Katip Celebi University. The CaP crystal structure on NFs was determined by a 405S5 wide-angle X-ray diffractometer (XRD, Panalytical Empyrean) with CuKα radiation source at 30 kV. The amount of CaP nucleation on the nanofibers was measured using a QuantiChrom calcium assay (Bioassay Systems, Hayward, CA, USA) according to manufacturer's instructions.

E. Cell Attachment and Proliferation Assay

Mesenchymal stem cells (MSCs) (CLS cell lines, Eppelheim, Germany) were obtained from Tissue Engineering and Regenerative Medicine Laboratory in Izmir Katip Celebi University. Nanofibers were sterilized by leaving under ultraviolet radiation for 1 hour and immersing 70% ethanol for 30 min then fibers were washed three times with sterile PBS. After sterilization, nanofibers were conditioned in basal medium supplemented with 10% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin, for 1 h. After conditioning, basal medium was removed and nanofibers were dried, each sample was seeded with $5x10^4$ cells/cm^2. Nanofibers were incubated in 5% CO_2 at 37°C. Cell proliferation was evaluated by MTT analysis (Vybrant® MTT Cell Proliferation Assay Kit, Invitrogen, Waltham, MA, USA) on 1st, 4th and 7th days. Briefly, MTT solution (5 mg/mL) was added into culture medium (with 10% concentration) and incubated for 2 h at 37°C, 5% CO2. Next, the medium was replaced with DMSO (Sigma Aldrich, St. Louis, MO, USA), and the optical density for each well was measured at 570 nm using a Synergy™ HTX Multi-Mode Microplate Reader (BioTek, Epoch 2). Measured absorbance values were associated to the cell number by using a calibration curve constructed with reference number of cells.

Cell seeded nanofibers were incubated with Alexa Fluor® 594 Phalloidin and DAPI to stain actin filaments and nucleus. Briefly, cultured cells fixed with 4% paraformaldehyde for 15-20 min at room temperature and washed twice with 1x wash buffer. Next, cells were permeabilized with 0.1% Triton X-100 in 1x PBS for 1-5 min then washed twice 1x wash buffer. Blocking solution was applied for 30 min. Each fiber was washed three times with wash buffer. TRITC- conjugated Phalloidin was incubated simultaneously with the secondary antibody for 30-60 min. Nanofibers were washed three times with wash buffer and incubated in DAPI for 1-5 min, followed by washing three times. Stained cells were observed under fluorescent microscopy.

II. RESULTS

Experimental groups shown in the Table 1 were incubated in SBF for 24 hours. Nanofiber morphology and CaP nucleation were examined by SEM Imaging in Figure1. It is observed that all groups have similar morphology. Also CaP nucleation were observed in each group.

TABLO I. *EXPERIMENTAL GROUPS*

	Experimental Groups			
Conjugated Peptide	***EEGGGG***	***EEEEGG***	***EEEEEE***	***GGGGGG***
Group Name	NF1	NF2	NF3	CONTROL

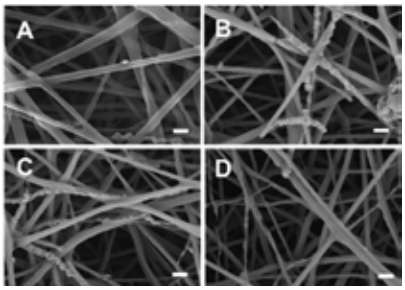

Figure. 1. *SEM Images of Nanofibers (A)PLGA-GGGGGG (B) PLGA-EEGGGG (C)PLGA-EEEEGG (D) PLGA-EEEEEE. The scale bar represents 1μm*

CaP nucleation on NF groups were assessed by XRD and Ca Assay. Comparison of X-ray diffraction spectra of CaP crystals deposited on NF1, NF2, NF3 and Control groups after 24 hours incubation in modified 1.5xSBF was shown in Figure 2A. The highest apatite peak was observed in NF1. Peak centered at 31.8° is one of the characteristic diffraction peaks of apatite crystals. Similarly, calcium assay results in Figure 2B confirmed XRD results by showing the highest Ca amount on NF1 group.

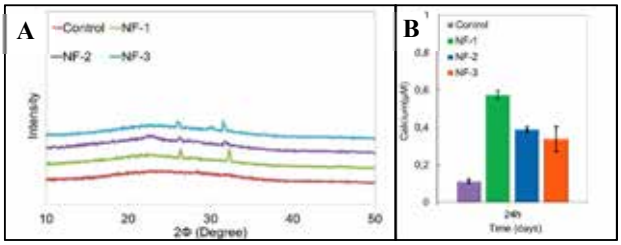

Figure. 2. (*A). X-ray Diffraction Spectra of CaP Crystals Deposited on PLGA NF (B) Amount of CaP nucleation and growth*

Cell viability was evaluated by an MTT test on 1st, 4th and 7th days after cell seeding. As shown in Figure 3, control group had less cell number compared to peptide conjugated nanofibers. Each peptide conjugated nanofibers had higher cell viability on 1st, 4th and 7th days. On 1st 4th and 7th days NF-1 had the most effective results for cell adhesion. However, MTT test results indicate that peptide conjugation does not has any negative effect on cell viability. On the contrary, repetitive amino acid sequences can be influential on cell adhesion and viability. Moreover, cell morphology images at day 7 in Figure 4 was obtained under light microscope. Results were shown that cells adhered on NFs in each experiment group. Furthermore, morphology of cells cultured on peptide-conjugated nanofibers resemble to osteoblasts.

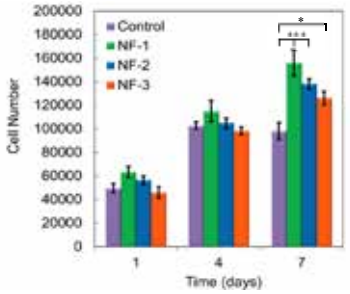

Figure.3. *Results of the MTT assay of MSC cell cultured on NFs after 1,4, and 7 days of incubation.*

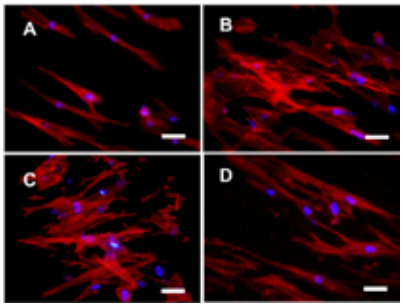

Figure.4. *Cell morphology images of peptide conjugated nanofibers at day 7 (A)PLGA-GGGGGG (B) PLGA-EEGGGG (C)PLGA-EEEEGG (D) PLGA-EEEEEE. The scale bar represents 20μm*

III. CONCLUSION

In summary, biomimetic peptides including different number of GLU conjugated NF have been successfully fabricated via electrospinning. After conjugation of GLU templated peptides, PLGA NF showed accelerated proliferation compared to control group. Moreover, GLU templated peptides increased mineralization compared to control. Karaman *et. al* stated that GLU provides sites for CaP nucleation [2]. In this study, two GLU containing peptide shown better effect on CaP nucleation and MSCs proliferation compared to more GLU containing groups. Therefore, increasing number of GLU negatively effects cell proliferation and mineralization.

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