TIPTEKNO'18
TIP TEKNOLOJİLERİ KONGRESİ
8-10 Kasım 2018 / Gazi Magosa / KKTC

Comparison the Effect of RGD Peptide Conjugation on Titanium Discs with Different Methods on Cell Adhesion and Proliferation

Günnur ONAK, Belgin YURTSEVEN
Biomedical Engineering
İzmir Katip Çelebi University
İzmir, TURKEY
gunnur.onak@ikc.edu.tr, belgin.yurtseven@windowslive.com

Oğuzhan GÖKMEN, Ozan KARAMAN*
Biomedical Engineering
İzmir Katip Çelebi University
İzmir, TURKEY
gokmennoguzhann@gmail.com, ozan.karaman@ikc.edu.tr
*Corresponding author.

***Abstract*— Metallic biomaterials have high fracture toughness and tensile strength compared to other implant materials such as ceramics and polymers. Accordingly, metallic implants are widely used in orthopedic. Titanium (Ti) is a frequently preferred biomaterial due to the properties such as resistance to corrosion, non-toxicity to the human body and mechanical strength. However, when Ti implants cannot be sufficiently osteo-integrated in the host bone, bone tissue formation fails and limits the use of these implants. Today, Ti surfaces can potentially be functionalized to provide useful additional features such as overcome above corrosion resistance, increasing bioactivity and osteo-integration properties. The ability of cells to attach to Ti implants and secrete extracellular matrix (ECM) molecules improve new functional tissue formation. Integrins which present in the cell structure, play an active role in cell adhesion and interact with short amino acid sequences in extracellular matrix molecules. Especially, the RGD (Arg-Gly-Asp) sequence, has been described as mediating cells of various plasma and ECM proteins, including fibronectin, vitronectin, type 1 collagen, osteopontin and bone sialoprotein. Bioactive peptide constructs that are conjugated to the surface of implants have the potential to increase implant cell interaction, inhibit fibrous tissue formation, and provide direct osteointegration of the implant. The most effective way of achieving peptide conjugation to the implant surface, which is an important step at this point, can be considered as a parameter directly affecting osteointegration. In this study, we aim to evaluate the efficacy of commonly used Ti-peptide conjugation methods on cell attachment and proliferation. Moreover, competitive conjugation success was interpreted by fluorescence imaging technique, contact angle measurement and AFM (Atomic Force Microscopy).**

Keywords — Bone tissue engineering; surface modification; peptide.

I. INTRODUCTION

Metals are commonly used biomaterials in orthopedic disorders due to their bone-like properties. These metallic biomaterials such as titanium and titanium alloys, stainless steel and Co-Cr alloys are commonly used in implant applications. However, due to the inadequate osseointegration, which causes micromotion of the implant, these implants might fail at the early stages of integration. Titanium (Ti), particularly Ti6Al4 V, is widely used with its osseointegration ability, which supports proper bone formation and mechanical properties [1]. Furthermore, modulus and hardness properties of Ti are close to cortical bone tissue and has suitable mechanical properties (bending, fatigue strength and maximum load) in terms of support of the load carrier [2].

Osseointegration of Ti implant involves formation of new bone tissue in direct contact between implant surface and bone tissue. Implant surface modification is required to achieve high bioactivity with optimal biological responses of surrounding osteogenic cell environment. different surface modification techniques have been applied to titanium implants. The aim of surface modifications is to produce bioactive osteoinductive surface of implants to enhance the bone tissue formation.

Short and bioactive amino acid sequences can function as a bioactive unit between integrin receptors of cells and material surface. The RGD (arginine–glycine–aspartic acid) acts as a binding unit for alpha/beta integrin receptors during osteoprogenitor cells attachment and proliferation. Although enhanced cellular adhesion on RGD peptide coated with different reaction on Ti implants were studied, the comparison of these reaction on peptide conjugation efficacy has yet to be studied. In this study, we aim to compare the effect of commonly used titanium peptide conjugation methods on cell attachment and proliferation. Moreover, competitive conjugation success was interpreted by fluorescence imaging technique, contact angle measurement and AFM (Atomic Force Microscopy).

II. MATERIALS AND METHODS

A. Peptide Synthesis

All chemicals used for peptide synthesis were purchased from AAPPTEC (Louisville, KY, USA). Gly-Arg-Gly-Asp-Ser (GRGDS) peptide was synthesized using on 4-Methylbenzhydrylamine (MBHA) resin. After, 200 mg of resin was swollen in 4 ml of DMF for 30 minutes, 20% piperidine in DMF was added to resins for 15 minutes and the Fmoc protecting group is removed. 2 equivalents (according to the resin substitution) of Fmoc-protected aminoacids dissolved in DMF and was added to resin. HBTU (2 eq), hydroxybenzotriazole (2 eq, HOBT) and N, N-diisopropylethylamine (4 eq, DIEA) were added to the mixture. Amino acid couplings were performed for 3 hours in an orbital mixer. The results of binding and deprotection reactions were checked by ninhydrin test. For cleavage reaction, the peptide

978-1-5090-2386-8/16/$31.00 ©2018 IEEE

was separated from the resin with the solution containing trifluoroacetic acid (TFA): triisopropylsilane (TPS): water (H_2O) in the ratio 95: 2.5: 2.5. For the GRGDS peptide FITC conjugation, Lysine amino acid containing two amino groups and one carboxyl group was added at the end of the sequence. The Fmoc protecting group was separated by 60 mL of a solution containing 2.85 mL of TFA, 0.075 mL of distilled water, 0.075 mL of triisopropylsilane and 57 mL of DCM. The resins were shaken 6 times for 5 minutes in 10 mL of solution and then washed in DCM until the yellow color disappeared. Finally, the resins were washed with 5% (v/v) DIEA in DCM. 389.4 mg FITC and 256.8 µL DIEA were prepared in 3 mL DMF and added to the resins. The reaction chamber was covered with aluminum foil. Peptides coupled with and without FITC were TFA evaporated under high vacuum and the peptide washed 3 times with cold diethyl ether. After the treatment, the remaining pellets were cooled and dried in a lyophilizer device.

B. *Peptide Conjugation*

For conjugation of Titanium (Ti) Discs by Silanization Method, titanium disks were treated in concentrated sulfuric acid at room temperature for 15 minutes and washed extensively with water. After being treated in boiling water for 10 minutes, it was washed 5 times with water, rinsed with acetone and dried under vacuum for 12 hours. The silanization was carried out by incubating 30 ml of dry toluene samples containing 0.5 ml (2.15 mmol) of 3-aminopropyl triethoxysilane (APTES) for 3 hours at 120°C. After the reaction, the substrate was ultrasonically washed with chloroform (5 times), acetone (2 times), methanol (5 times) and concentrated water. Only the silanized titanium substrate surface was placed in a small vial with 400 µl of N, N-dimethylformamide (DMF) containing 3mg (28mM) N-succinimidyl-3-maleimidopropionate (SMP) in contact with the solution, after incubation for 1 h at room temperature (with 2 min sonication every 10 min) the substrate was washed with DMF (5 times) and with water (10 times). Substrates were immediately subjected to the next step to prevent hydrolysis. The substrate was incubated in 300µL of purified water containing 1.35 mg of GRGDC (10 mM) for 2 hours at room temperature (with 1 sonication every 10 minutes). The GRGDC grafted substrates were thoroughly washed with water, dried with nitrogen and stored in argon.

For, EDC/NHS reaction, the titanium samples were rinsed with deionized water and incubated in 2mM EDC and 5mM NHS in 0.1M MES solution for 40 min. For peptide conjugation (1 mM in sterile PBS), incubated for 24 hours at 4°C in the refrigerator and washed before characterization.

For another conjugation method, titanium samples were stored at 60°C for 24 hours in a 30% H_2O_2 (Hydrogen Peroxide) solution. The hold samples were washed several times with acetone. It was then washed three times with dry acetone. The samples were released in the CDI solution (20 mg/ml) in dry acetone for 30 minutes. The samples were washed five times with dry acetone. The GRGDS peptide was dissolved in a concentration of 1 mg/ml in 100 mM filtered $NaHC0_3$ (Sodium Bicarbonate). It was shaken in the mixer at room temperature for 36 hours. The samples were washed 5 times with phosphate buffered saline (PBS).

C. *Surface Analysis of Titanium Disc*

To determine peptide surface coverage on NF, fluorescein isothiocyanate (FITC) attached peptide was conjugated to titanium discs as. FITC labelled peptide conjugated titanium disc groups, shown in the Table 1, were imaged with an inverted fluorescent microscope (Olympus CKX41, Tokyo, Japan).

TABLO I. *EXPERIMENTAL GROUPS*

	Experimental Groups		
Crosslinkers	***APTES***	***EDC/NHS***	***CDI***
GROUP NAME	GROUP1	GROUP2	GROUP3

Effect of peptide conjugation on titanium disks on surface hydrophilicity was evaluated by water contact angle measurements using KSV Attension Theta goniometer (Biolin Scientific, Stockholm, Sweden). Briefly, 10 µl deionized water was dropped to the nanofiber surface, photographed and the contact angle (θ) was calculated.

Images were recorded by a Multimode atomic force microscopy (AFM) equipped with a Nanoscope V (Veeco Inc., CA, USA) operating in tapping mode employing a silicon cantilever probe (Bruker Nano Inc., CA, USA with a force constant of 3 N m^{-1} and resonance frequency of 75–92 kHz). Fibers were directly deposited on mica substrate and scanned in dry conditions.

D. *Cell Attachment and Proliferation Assay*

Mouse fibroblast cells L929 (CLS cell lines, Epithelium, Germany) were cultivated. Cells were plated in primary culture media containing DMEM medium, 10 % Fetal Bovine Serum (FBS), 100 U/ml penicillin and 100 mg / ml streptomycin and incubated in 5% CO_2 at 37°C. Culture media was changed every two days and cultures that reached 80% density were passaged at the appropriate passage rate using 0.25% Trypsin / EDTA solution. To ensure adequate storage in each passage, the cells were frozen in freezing mediums in a controlled manner and stored in a liquid nitrogen tank at 196°C. Titanium discs prepared for cell culture were sterilized using 70% ethanol and UV. For cell viability analyzes, $4x10^4$ cells / cm^2 were seeded on the prepared titanium disc specimens. MTT(3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-etrazolium bromide, Vybrant®, ThermoFisher) was used. Briefly, MTT solution was (5 mg/mL) added into culture medium (with 10% concentration) and incubated for 2 h at 37°C. Next, the medium was replaced with 500 lL DMSO (Sigma Aldrich, St. Louis, MO, USA), and the optical density for each well was measured at 540 nm using a SynergyTM HTX Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA).

III. RESULTS

The efficacy of CAP treatment on peptide conjugation was also characterized via observing FITC intensity by using fluorescence microscope. FITC labeled RGD peptides were used to show the titanium surface coverage with peptides. Briefly, FITC labelled RGD peptides were conjugated with titanium discs. The images were then compared to all groups. As shown in Figure1, the highest fluorescence intensity was

observed in Group 1. Only titanium discs were used as negative control and as expected no fluorescence was observed, which confirms that the fluorescence observed in the other two images came from the label FITC. The lowest fluorescence intensity was observed on Group 4.

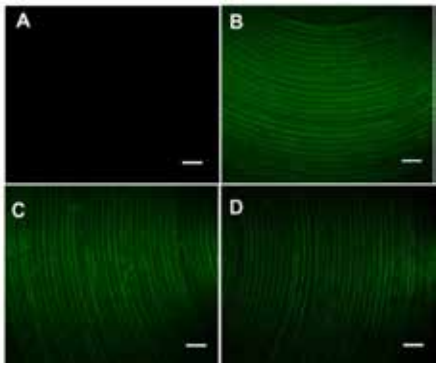

Figure. 1. *Fluorescent microscopy images of (A)Control (B)Group 1 (C)Group 2 (D) Group 3 fluorescein isothiocyanate (FITC) labeled RGD peptide conjugated (Scale bar represents 200μm).*

The impact of CAP treatment for titanium discs on hydrophilicity was also demonstrated using water contact angle (θ) measurement. The water contact angles were determined based on the images taken right after 10 µL of deionized water was applied to the surface and they were compared to that of non-CAP treated NF. It can be easily seen in Figure2 that the contact angle (θ) dropped from 83.73 ± 2.23º down to, 45.22 ± 5.21º, 30.21 ± 2.34º and 76.12 ± 1.21º in Group1, Group2 and Group3, respectively.

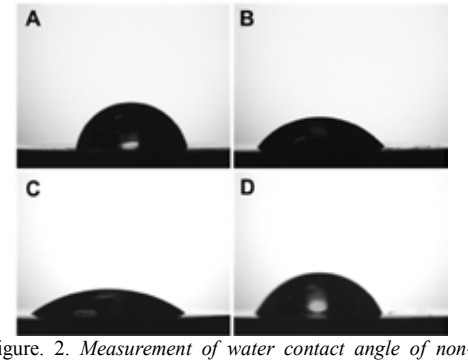

Figure. 2. *Measurement of water contact angle of non-CAP (A)Control (B)Group 1 (C)Group 2 (D) Group 3,, 83.73 ± 2.23º, 45.22 ± 5.21º, 30.21 ± 2.34ºand 76.12 ± 1.21º, respectively.*

Surface roughness of titanium discs was evaluated by AFM and results were shown in Figure 3. Surface area measurements of control, Group1, Group2 and Group3, 34,554nm, 378,00 nm, 288,467nm and 42,238 nm, respectively.

Cell proliferation on surface modified Ti discs was assessed by MTT assay. As shown in Figure 4, cell adhesion, which can be correlated with day 1 MTT results, was higher on Group2 compared to control group. After day 4 and day 7, cell number was the highest on Group2 followed by Group1, Group3 and control group.

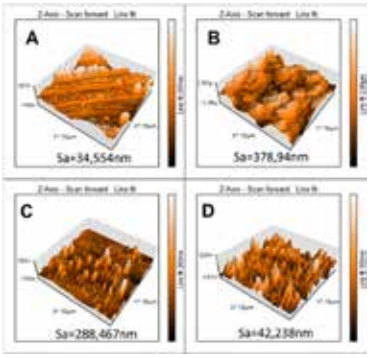

Figure. 3. *Representative 3D topographical view of titanium discs (A)Control (B)Group 1 (C)Group 2 (D) Group 3*

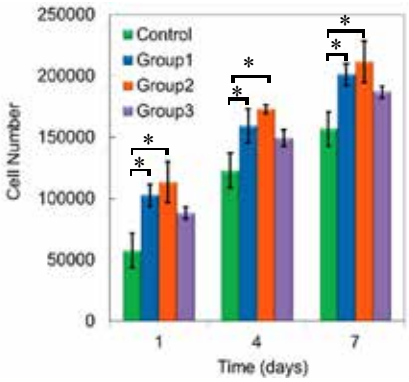

Figure. 4. *Results of the MTT assay of L929 cell cultured on control RGD conjugated Ti surfaces after 1,4, and 7 days of incubation.*

IV. Conclusion

In this study, peptides were successfully conjugated on Ti discs with all defined methods. When fluorescent microscope images were compared, it was depicted that peptide conjugation was achieved in Group1with higher efficacy where CDI used, followed by Group2 and Group3. Nevertheless, it was observed that cell number in Group2 for 7day were higher compared to other groups. It is assumed that cold plasma application during conjugation increased -OH and -COOH groups. Therefore, cell attachment was successfully achieved, even though RGD peptide conjugation efficacy was less than Group1. Moreover, it is indicated that peptide conjugation decreases Sa value in AFM results, increased surface roughness, and leads better cell attachment.

References

[1] A. Civantos, E. Martinez-Campos, V. Ramos, C. Elvira, A. Gallardo, and A. Abarrategi, "Titanium coatings and surface modifications: Toward clinically useful bioactive implants," *ACS Biomaterials Science & Engineering,* vol. 3, pp. 1245-1261, 2017.

[2] R. Bosco, E. R. U. Edreira, J. G. Wolke, S. C. Leeuwenburgh, J. J. van den Beucken, and J. A. Jansen, "Instructive coatings for biological guidance of bone implants," *Surface and Coatings Technology,* vol. 233, pp. 91-98, 2013.