REPUBLIC OF TURKEY

ACIBADEM MEHMET ALİ AYDINLAR UNIVERSITY

INSTITUTE OF NATURAL AND APPLIED SCIENCES

DEVELOPMENT OF HYDROXYAPATITE COATED AND BIOACTIVE ORTHOPEDIC SUTURES

BENGİSU KUBAŞIK

MASTER THESIS

DEPARTMENT OF MOLECULAR AND TRANSLATIONAL BIOMEDICINE

SUPERVISOR

Assist. Prof. Özgül Gök

ISTANBUL 2022

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DECLARATION

I hereby declare that; this thesis has been composed by myself based on the data in line with the scientific rules and ethical principles of responsible conduct of research. All information, data, comments, analyses have been collected and processed through scientific, academic writing style, and literature used have been duly shown by giving reference to the original sources in accordance with the publication ethics. I emphasize that I have not violated any patents and copyrights throughout this study and writing this thesis.

21.09.2022

Bengisu Kubaşık

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I would like to thank my supervisor Assist. Prof. Özgül Gök for her patience and wisdom. Her guidance through my masters help me reach my potential as a proper researcher. I would like to thank GOK Lab members for their support as well.

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I would like to dedicate this thesis to my grandmothers (Rezzan Güngör and Sultan Kubaşık).I hope both of you are proud of me.

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LIST OF ABBREVIATIONS AND SYMBOLS

ACN	Acetonitrile
Arg, R	Arginine
ATR/FT-IR	Attenuated total reflection Fourier transform infrared
Cys, C	Cysteine
DCM	Dichloromethane
dH_2O	Distilled water
DIC	N,N'-Diisopropylcarbodiimide
DMF	Dimethylformamide
Et_3N	Triethylamine
FA	Formic acid
Gel-MAL	Gelatin-maleimide
GMBS	N-γ-maleimidobutyryl-oxysuccinimide ester
Gly, G	Glycine
Ser, S	Serine
SPPS	Solid phase peptide synthesis
Tyr, Y	Tyrosine

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SUMMARY

In this project, in order to accelerate bone tissue fusion and regeneration by making surface modifications to the orthopedic sutures used in the clinic; 10% Hydroxyapatite (HAp) (by weight), which has the potential to facilitate fusion with bone tissue, and 1% (by weight) small peptide sequences that selectively attach osteoblast bone cells were added to the surface of the sutures. As a result of this addition, it was ensured that the sutures contributed to the integration of the bone tissue after implantation. In addition, 0.5% of α-Tocopherol (Vitamin E) (by weight) was encapsulated in this surface coating, resulting in slow and sustained release. In this way, one step of the treatment will be bone tissue regeneration by reducing oxidative stress.

Keywords: Osteo Adhesive Peptide, Bioactive Sutures, Surface Modifications, Hydroxyapatite, Osseointegration

ÖZET

Hidroksiapetit Kaplı Ve Biyoaktif Ortopedik Süturlerin Geliştirilmesi

Bu proje'de klinikte kullanılmakta olan ortopedik sütürlere yüzey modifikasyonu yapılarak kemik doku kaynaşması ve rejenerasyonunu hızlandırmak için; süturların yüzeyine kemik dokusu ile kaynaşmasını kolaylaştıracak potansiyele sahip ağırlıkça %10 Hidroksiapetit (HAp) ile seçici olarak osteoblast kemik hücrelerinin tutunmasını sağlayacak ağırlıkça %1 oranında küçük peptit dizileri eklendi. Bu eklenme sonucunda süturlerin implantasyon sonrası kemik dokusu ile entegrasyonuna katkıda bulunması sağlanması amaçlanmıştır. Ayrıca, ağırlıkça %0,5 oranında α-Tocopherol (Vitamin E) bu yüzey kaplamasına enkapsüle edildi ve yavaş ve sürekli salımı sağlandı. Bu sayede tedavinin bir adımı da oksidatif stresin azaltılarak kemik doku rejenerasyonu olacaktır.

Anahtar Sözcükler: Kemik Hücre Bağlayıcı Peptit, Biyoaktif İp, Yüzey Modifikasyonu, Hidroksiapetit, Kemik İntegrasyonu

1. BACKGROUND AND AIM OF THE STUDY

The stability of the threads (sutures) used during the implantation of orthopedic biomaterials to patients and their healthy integration with the surrounding bone tissue play a very important role in the acceptance of the implant by the body.In order to increase the effectiveness of the sutures used in orthopedic surgeries and to treat possible bone damage in the operation area, it is aimed to create a multifunctional coating that can integrate selectively into the bone tissue. Therefore, the subject of this project is to investigate the effect of sutures on bone tissue regeneration and integration with bone tissue after implantation as a result of coating orthopedic sutures with HAp and adding small peptide sequences that will facilitate the attachment of osteoblast bone cells selectively to the surface. In addition, it is predicted that bone tissue healing and regeneration can be improved thanks to the slow and continuous release of Vitamin E molecules. In the light of the data to be obtained in this project, both a master's thesis will be completed; and an innovative product can be obtained as a result of the development of an existing technology.

2. INTRODUCTION

2.1 Biocompatible Implants

Biomedical materials have been used in medical applications since its rapid development. Orthopedic metal implants area prominent example of biomedical materials. They have outstanding mechanical and biomedical properties. A metal implant is a major aid for stabilization and adjustment of defective bones, it's also beneficial for mechanical unity. However, metal usage in a body as a substitution for a solid tissue comes with its own issues. A metal implants biocompatibility with the implanted area is an issue with utmost importance(1).

To serve safely and retainable for a longer period without rejection, these metals should have a few essential properties such as excellent biocompatibility, high corrosion and wear resistance, suitable mechanical properties, osseointegration, ductility and high hardness. Biocompatibility is defined as the ability of metal implant to be used in close connection with living tissues without causing adverse effects to them. The body parts or tissue of a patient that comes into contact with the metal implants should prevent from any physical irritation and toxicity. The success of the metal implants highly depends on the level of compatibility and acceptance of the implant by the human body. However, the biocompatibility of metal implants extremely depends on their corrosion behaviorthe higher the corrosion of implants, the more of its toxic ions rates are released into the body routinely, and greater risk of adverse effects on the body can occur(2).

As in the case of metal implants, the orthopedic sutures that is used in this study also has biocompatibility issues. Coating the metal implant or orthopedic suture's surface with a biomaterial that is going to enhance metal implants or orthopedic sutures biocompatibility with the implanted area is going to eliminate any biocompatibility issues. This biomaterial must be a specific osseointegration enhancer in the designated area(1). In this study, we are focused on modification of orthopedic suture's surface area to enhance biocompatibility with the implanted

area,by specific enhancement of osteointegration in the implanted area while reducing oxidative stress during bone tissue regeneration.

2.2. Surface Modification Strategies

Surface modificationiskey to obtain a biocompatible and bioactive implant. Corrosion of metal implants from the human body environment and toxic materials coming from metal implant to human body is prevented by modification of metal implant's surface. Although orthopedic suture used in this study does not degrade in the human body environment and doesn't cause toxicity by degradation.Orthopedic sutures surface can be modified to enhance its efficacy on applied area. Researchers have been using various surface modification techniques to enhance osseointegration, osteoinductivity and osteoconductivity of orthopedic suture implants.Coating of the implant with natural or synthetic polymers are an example of strategies for surface modification (3). Surface modification strategies can be categorized into physical modification techniques and chemical modification techniques.

2.2.1. Physical modification techniques

The appropriate surface modification proceduresincreasecharacteristics of titaniumcompounds, such as weariness strength, change of shape and functionality. These procedures also improve explicit surface attributesessentialfor various clinical demands(4). Occurrence of chemical responses is not observed in thermal spraying and physical vapor deposition surface modification techniques. In thermal spraying and physical vapor deposition techniques; modification of titanium and its compounds surface depends on thermal, kinetic, and electrical energy. Surface coating materials are melted into fluid droplets and surface modification is achieved with kinetic energy provided by high-speed in thermal spraying procedure. In order to achieve a surface film with physical vapor deposition; coating material is provided in the shape of atoms, molecules or ions transported onto substrate surface for coating material to condense. Atom, molecule and ion shape of coating materials can

be generated by heating in capacitance, electron beam, laser or electrical discharge in vacuum(5).

In thermal spraying, coating materials are melted with heat to form fluid droplets and these droplets continuously stick and build-up on the surface of coated substrate. This build-up contains layers of fluid droplets, unstiffened material domains and solid particles. Heat, that is used in thermal spraying, is either provided by flame or plasma jet. That's why, spraying methods are divided according to heat source which are flame spraying and plasma spraying. Maximum temperature that can be generated from plasma and flame spraying is the main difference between them (5).

2.2.2. Chemical modification techniques

Chemical conduct, electrochemical conduct (anodic oxidation), sol–gel, chemical vapor deposition, and biochemical modification are examples of chemical modification methods. Chemical, electrochemical or biochemical reactions occur between the border of titanium and the solution. In chemical vapor deposition method, surface of the substrate is coated with chemicals in gas phase. Chemicals in gas phase becomes non-volatile on the substrate. In sol-gel method, chemical reactions occur in the solution rather than substrates surface and solution. Sol is a colloid in which are particles that do not settle and cannot be filtered with centrifuge. Colloids dispersed phase is very small (1–1000 nm) and van der Waals attraction and surface charges are dominant interactions. In sol-gel, sol is the colloid suspension of solid particles in a liquid and gel is the substance that provides a solid backbone structure to sol (5).

2.3. Coating Strategies for Bone Regeneration

2.3.1. Synthetic Polymers

Poly (ε -caprolactone) (PCL), poly lactic acid (PLA), poly glycolic acid (PGA) and their copolymers, e.g., Poly (lactide-*co*-glycolic acid) (PLGA), were used as bone regenerative polymers because of their biocompatibility and their adaptable

biodegradation properties. Conduction of thermally induced phase separation (TIPS) porogen leaching/solvent casting, electrospinning, gas foaming, rapid prototyping, or 3D printing methods to acquire synthetic polymers would result in open porous scaffolds (6).

2.3.2. Natural Polymers

Gelatin is a hydrolyzed version of collagen and favored over collagen. Gelatin's solubility in water and other solvents is higher than collagen. The amino acids in gelatin include an assortment of purposeful groups, as shown in Figure 2.1, amino groups from lysine components that can be used for chemical reactions such as crosslinking. This crosslinking ability of gelatin ensures its usage with peptides and/or bone regenerative chemical components(6,7)

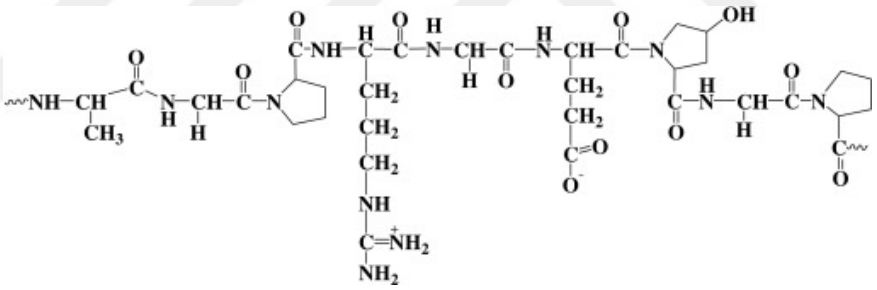


Figure 2.1 Chemical components of gelatin (8).

Chitosan is a polysaccharide with a linear structure and consists of (β 1 → 4)-linked 2-amino-2-deoxy-D-glucopyranose units, as shown in Figure 2.2, which are partially N -acetylated, and takes place in the shells of crabs and shrimps. Chitosan has been known as enhancing cell adhesion, proliferation and osteoblast differentiation by its cationic properties. Chitosan can take a shape of 3D scaffolds with the help of 3D printing and nanotechnology but chitosan does not give satisfying bone repairment results on its own. Chitosan must be blended with different natural or synthetic polymers (3,6,8).

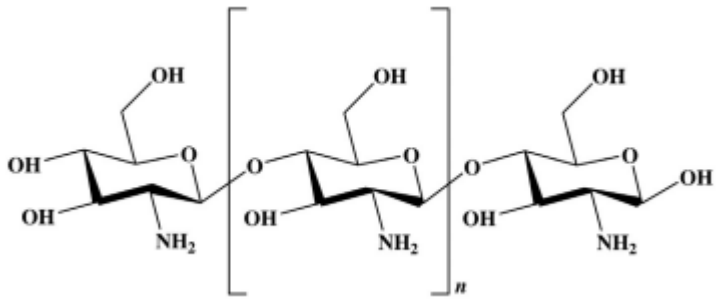


Figure 2.2. Chemical components of chitosan (8).

2.3.3. Hydroxyapatite Coatings

Hydroxyapatite (HAp), $Ca_{10}(PO_4)6(OH)_2$, was first introduced approximately 60 years ago as an implant coating material for its bioactive and osteoconductive properties. Its primary mineral unit compatible with natural bone, as it's shown in Figure 2.3, because HAp accounts for almost 65% of the total bone mass. When HAp used as a coating biomaterial, it boosts the osteoconductivity by encouraging increase and attachment of osteoblast cells on the surface of the implant. Wear resistance of the implant increases with the assistance of coated HAp including osseointegration. HAp also has the ability to delivering other drug molecules to the designated area (3,8,9).

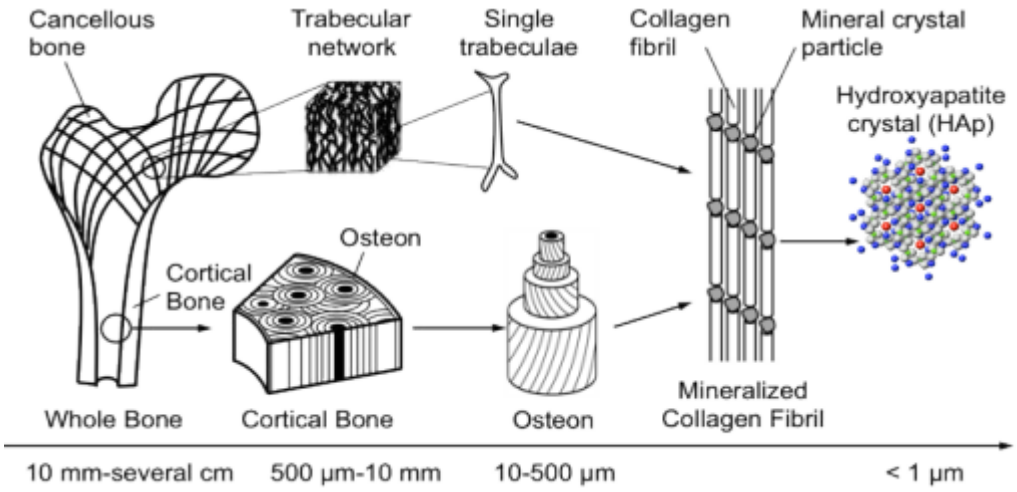


Figure 2.3. Hydroxyapatite is shown to be a component of a bone(11).

2.4. Bioactive Sutures

2.4.1. Drug Eluting Sutures

Vitamin E (α-Tocopherol), shown in Figure 2.4, is known to have a prominent positive impact on maintaining bone structure and regenerating bone cells. Its control over the levels of inflammatory mediators, ROS, growth factors and hormones, ensures Vitamin E a potential regulatory role in bone metabolism(10). It has been proven that vitamin E deficiency increases lipid peroxidation and stimulates osteoclast activation and bone resorption(11). Researchers revealed in a dog model study that vitamin E contributes to earlier healing of the broken bone line, firmer mineralization and remodeling of bone callus(11).

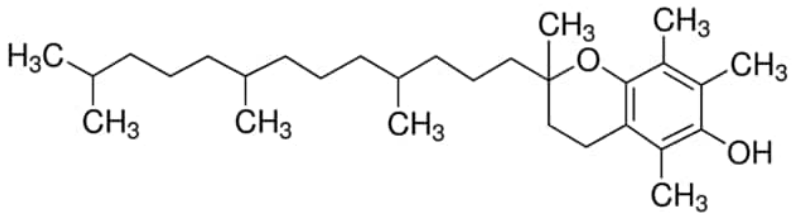


Figure 2.4. Structure of (±)-α-Tocopherol (12).

2.4.2. Peptide-Decorated Sutures

Osteo adhesive peptide (KRSR), (lysine-arginine-serine-arginine), structure shown in Figure 2.5, and KRSRGYC (lysine-arginine-serine-arginine-glycine-tyrosine-cysteine) have been known as peptides that are osteo adhesive. Heparan-sulfate-binding motif of these peptides allows them to bind to cell surface proteoglycan molecules. These peptides also specifically encourage the attachment of osteoblast cells while inhibiting the attachment of fibroblast (9,13). Researchers also shown in molecular simulation that KRSR peptide binds to the surface of an oxidized titanium implant in a balanced position (13).

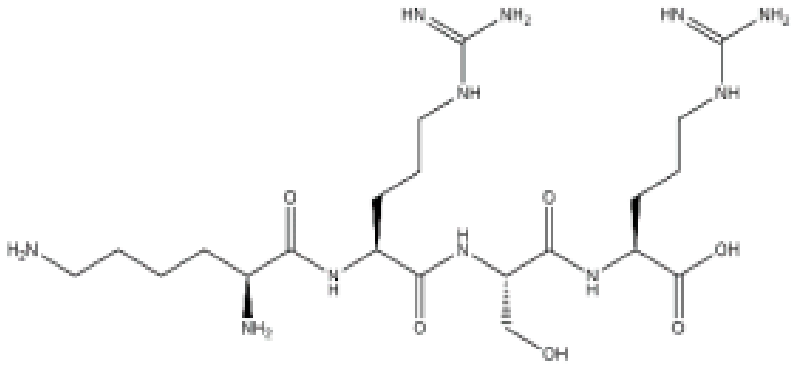


Figure 2.5. Structure of KRSR peptide(14).

2.4.3. Solid Phase Peptide Synthesis

The Solid Phase Peptide Synthesis (SPPS) method creates a peptide chain by a series of deprotection and coupling cycles for each added amino acid. Peptide formation direction is from C-terminal to N-terminal in SPPS. When the desired peptide length is achieved, peptide is cleaved from the solid support which is resin. The first amino acid is connected to resin by the amino acid's carboxyl group. Every other aminoacid are attached to each other through removal of protection group from aminoacid that is going to connect to attached aminoacid (15).

3. MATERIALS AND METHODS

3.1. Materials

Unless it was specified, all chemicals used in this study such as Gelatin (obtained from bovine skin), Hydroxyapatite, (±)-α-Tocopherol (Vitamin E) were purchased from Sigma Aldrich. Amino acids and resin were obtained from CEM.Millipore Direct-Q® 8 UV Water Purification System provided double distilled water used to prepare the solutions. Fibroblast cell strain used in the cytotoxicity assay was *Mus musculus* ATCC CRL-2648. All cell culture commertials were obtained from TPP in sterile condition. FBS and DMEM were received from Gibco.

3.2. Preparation of Coated Sutures

3.2.1. Coating and characterization of sutures with Gelatin polymer

Solutions of gelatin polymer (obtained from bovine skin) at different concentrations (0.5, 1 and 2% by weight) were prepared with pH7.4 10mM phosphate buffer saline PBS (MP Biomedicals) using a heating magnetic stirrer (Velp®Scientifica). The solution was prepared by stirring at 300 rpm for each concentration. Gelatin solutions were prepared as homogeneous solutions using a sonicator (Velp® Scientifica)and then were passed through 0.45 mm filters (TPP®) and stored at +4 °C.

Suture pieces cut in 1 cm lengths as is and coated with these prepared gelatin solutions with two 24-hour incubation processes at both room temperature (RT) and +40 °C, sequentially. Fresh polymer solution was used each time. When the second incubation period was completed, the suture samples removed from the solution were washed twice with double distilled water, dried by lyophilization in Freeze Dryer (Labconco)and stored at +4 °C.

All suture pieces obtained after this coating procedure were characterized in terms of their functional groups, durability and morphological properties.

Functional group analysis: The characteristic bond structures on the surface of the sutures were determined comparatively using the Fourier Transform Infrared Spectroscopy (FT-IR) instrument, in the uncoated state and after coating in dry state.

Stability experiments: Coated sutures were incubated in pH7.4 10mM PBS solutionat both room temperature and body temperature, separately, for 24 hours. They were visualized by Scanning Electron Microscopy (SEM) (Thermo Quattro S) and the presence of polymeric coating layer on suture surface was evaluated.

Morphological analyzes: Coated and uncoated sutures were analyzed using SEM in terms of coating layers on their surfaces and porosity together with the surface smoothness. In addition, step-by-step elemental analyzes of the coatings on the suture surface were made after each procedure with the Energy Dispersive x-ray Spectroscopy detector (EDS)(EDAX) connected to the SEM, and the coating efficiency was calculated.

3.2.2. Coating and characterization of sutures with hydroxyapatite-loaded gelatin solutions

Suture pieces cut in 1 cm lengths and coated with gelatin solution that was determined from multiple analyzes and different HAp concentrations (1,2,4,5,10,20 % by weight). HAp solutions were made by dissolving HAp in pH 5,5 0,1 M Acetate Buffer. Fresh gelatin polymer solution was used each time. These gelatin-HAp coated sutures were treated with a 24 hour incubation processes at the temperature determined from the previous stability experiments. When the incubation period was completed, the suture samples removed from the solution and washed twice with double distilled water, lyophilized in Freeze Dryer(Labconco), dried, and stored at +4 °C. The previous characterization methods and experiments, that were done to gelatin coated sutures, were applied to determine the most desired HAp concentration. Only difference was that HAp-gelatin coated sutures were incubated in pH 5.5 0,1 M Acetate Buffer in stability experiments. The results obtained from characterization experiments were evaluated by comparing the results of sutures without HAp and sutures with non coated suture.

3.2.3. Solid phase peptide synthesis (SPPS) of KRSR

KRSR was synthesized with The Liberty Blue™ Automated Microwave Peptide Synthesizer (CEM). The synthesis scale was set as 0,5 mmole. Prior to peptide synthesis, 0,071 g Rink Amide Protide resin was weighed and become distended in 10 mL N, N-dimethylformamide (DMF) at 30 minutes mark.

Our osteo adhesive peptide consists of Lysine (Lys, K), Arginine (Arg, R) and Serine (Ser, S). All aminoacids used in this peptide synthesis was in L form. 0,19 grams of Lysine was dissolved in 2 mL of DMF. 0,91 grams of Arginine was dissolved in 7 mL of DMF and 0,16 grams of Serine was dissolved in 2 mL of DMF.

Table 3. 1. Consecutive information about synthesized Osteo adhesive peptides.

Osteo adhesive Peptides	**Amino acid Conformation**	**pI**	**Net charge**	**Molecular weight ($gmol^{-1}$)**
KRSR	L form	12,5	+3	544,63
KRSRGYC		10,5	+2,95	868,01

N,N'-Diisopropylcarbodiimide (DIC) was used as activator and Oxyma was used as activator base.0,5 mL of DIC was prepared with 6,5 mL with DMF while 0,568 grams of Oxyma was prepared in 4 mL of DMF. Deprotection cocktail was prepared with 3,8 mL Piperdine in 15,2 mL DMF.

At the end of the peptide synthesize, resin has to be cleaved from the peptide. The device that was used to cleave resin from peptide is called Razor™ Peptide Cleavage System (CEM). The cleavage cocktail that was used to for this device's action consists of 4.75 mL of trifluoroacetic acid (TFA), 125 µL of triisopropylsilane (TIS) and 125 µL of double distilled water (ddH2O). The peptide cleavage was carried out

at 38°C for 30 minutes. The cleaved peptide was precipitated in cold diethyl ether overnight and then centrifuged 6000 rpm for 30 minutes. After centrifugation, diethyl ether was carefully discarded and peptide pellet was dried by vacuum. Dried peptide pellet was put in +4°C.

3.2.4. Purification analysis and characterization of KRSR

KRSR was prepared in 50% acetonitrile (ACN) to have a concentration 0,5 mg/mL and sonicated to be dissolve homogeneously. KRSR was analyzed to check its purification and characterization with 6420 Triple Quad LC/MS(Agilent Technologies) system. Firstly, 10 µL of samples were directly injected to mass detector to be analyzed in scan mod between 50 to 2200 mass (m) range for 1 minute. Mobile phases contain 0.05% formic acid (FA) and samples were injected to mass detector in the 50% ACN. Single (+1) charge of the peptide and positively charged (+4) ion peak were observed 525.7 m/z, and 113.1 m/z, respectively in the spectrum scanned at positive ESI mode.

3.2.5. Preparation of Gelatin-coated sutures containing KRSR and loaded with Hydroxyapatite

In particular, according to the results obtained from the stability experiments, the suture with higher amount of HAp crystals on its surface was determined. KRSR peptide was synthesized as described above. Solutions containing different amounts of KRSR peptide (0,51 and 2% by weight) were prepared with 50% Acetonitrile this time under optimized conditions of gelatin polymer and HAp crystal percentages.

3.2.6. Characterization of vitamin E ((±)-α-Tocopherol)

Vitamin E was prepared in 50% acetonitrile (ACN) to have a concentration 1 mg/mL and sonicated to dissolve homogeneously. Vitamin E was analyzed to check its characterization with 6420 Triple Quad LC/MS (Agilent Technologies) system. Firstly, 10 µL of samples were directly injected to mass detector to be analyzed in

scan mod between 50 to 2200 mass (m) range for 1 minute. Mobile phases contain 0.05% formic acid (FA) and samples were injected to mass detector in the 50% ACN. After scanning, sample was analyzed in single ion monitoring (SIM) mode for the 1 distinct charge (z) molecular ion that was seen in the scan spectrum. 1 SIM mode was performed for this molecular ion peak value as 431.2 m/z ion for the positive scan. By applying a collusion energy of 15 units, the molecule was fragmented and the obtained abundant fragment was found to have a value of 165.1 m/z. After the preparation of multiple reaction monitoring (MRM) method for Vitamin Eby using this information, its different concentrations (from 1000ppb to 0.2442 ppb by serial dilution) were prepared in 50% ACN in H_2O and analyzed with the same MRM method, after which the area under curve values of the obtained drug peaks in each spectrum was related with the concentration to draw the specific calibration curve for Vitamin E molecule.

3.2.7.Preparation of gelatin-coated sutures containing KRSR and loaded with hydroxyapatite and vitamin E

In particular, according to the results obtained from the stability experiments, the suture with higher amount of HAp crystals on its surface was determined. Stability experiments also was applied to all concentration of KRSR peptide and the most stable and efficient concentration was chosen according to coated sutures stability experiments and morphological properties. Vitamin E (0,5, 1, 2 % by weight) dissolved in 96% ethanol was added into the chosen gelatin solution, HAp solution and KRSR solution. Separate suture pieces were coated with these three solutions individually.

3.3.Optimization of Suture Surface Modification

3.3.1. Solid phase peptide synthesis (SPPS) of KRSRGYC

KRSRGYC was synthesized with The Liberty Blue™ Automated Microwave Peptide Synthesizer (CEM). The synthesis scale was set as 0,5 mmole. Prior to

peptide synthesis, 0,071 g Rink Amide Protide resin was weighed and become distended in 10 mL N, N-dimethylformamide (DMF) at 30 minutes mark.

KRSRGYC consists of Lysine (Lys, K), Arginine (Arg, R), Serine (Ser, S), Glycine (Gly, G), Tyrosine (Tyr, Y) and Cysteine (Cys, C). All aminoacids used in this peptide synthesis was in L form as shown in Table 3.1. Both K and Y were weighed to be 0,19 grams and each one of them were dissolved in 2 mL of DMF. 0,78 grams of R was dissolved in 6 mL of DMF. 0,16 grams of S was dissolved in 2 mL of DMF. 0,12 grams of G was dissolved in 2 mL of DMF and 0,24 grams of C was dissolved in 2 mL of DMF.

0,9 mL of DIC was prepared with 10,1 mL with DMF while 0,853 grams of Oxyma was prepared in 6 mL of DMF. Deprotection cocktail was prepared with 6 mL Piperdine in 24 mL DMF.

The procedure for cleavage of KRSRGYC from resin was exactly identical to cleavage of resin from KRSR peptide.

3.3.2. Purification analysis and characterization of KRSRGYC

Synthesized KRSRGYC were prepared to check its purification using 1260 Infinity Quaternary LC (Agilent Technologies) with 18 prep RP-HPLC hydrophobic 6 µm C18 column (Agilent VariTide RPC 250x10 mm ID). 2,5 mg/mL KRSRGYC peptide solution prepared in 50% ACN solution was sonicated to become homogeneous. The column was connected to B solution which was ACN and A solution which was ddH_2O including 0,025% TFA.KRSRGYC solution was first run in between 1-80% B% for 30 minutes to scan at which percentage of B the peptide peak can be seen. Afterwards detecting the B% of the sample peak, between 55-70% B was focused. Relevant pure peaks for peptides were collected with fraction collection. At the end of the purification, ACN was evaporated from KRSRGYC

peptide solution with Rota Evaporator and lyophilized in Freeze Dryer (Labconco), dried and put in +4°C.

3.3.3.Conjugation of gelatin with KRSRGYC peptide

In order to enhance osteo adhesive peptide amount on suture, an idea of conjugating KRSR to gelatin was proposed. To achieve this conjugation KRSR must have thiol group. KRSRGYC was synthesized, as previously described, for possessing cysteine amino acid that has thiol group.

First part of this conjugation was making gelatin-maleimide. As shown in Table 3.2 It was achieved by mixing 100 milligrams of gelatin with 10 mL % 1 PBS. This 1% Gelatin solution was stirred in a magnetic stirrer in 300 rpm without heat while 1 mL of anhydrous Dimethyl sulfoxide (DMSO) was dissolving 4,25 milligrams of N-γ-maleimidobutyryl-oxysuccinimide ester (GMBS) (Thermo Fisher).2,1 µL of Triethylamine (Et_3N) was also added into gelatin solution with GBMS solution. Reaction took place for 2 days.

Table 3.2. Materials used for gelatin-maleimide formation

Name of the chemicals	Equivalent	Molecular Weight (kDa)	n (mmole)	Mass (mg)	Volume
Gelatin	1	131,6 ±4,8	$7{,}57 \times 10^{-4}$	100	
GMBS	20	280,23	0,015	4,25	
Et_3N	20	101,19	0,015	1,53	2,1 µL
1% PBS					10 mL
Anhydrous DMSO					1 mL

Afterwards Gelatin-maleimide solution was dialyzed for a day on heating magnetic stirrer (Velp® Scientifica) at 400 rpm. 1L baker filled with 800 mL ddH_2O. Gelatin-maleimide solution was put into Spectrum™ Spectra/Por Biotech-Grade RC Dialysis

Membrane Tubing (Thermo Fisher) membrane and both ends of the membrane was clasped with dialysis clip. Three times on every hour ddH_2O was replaced with a fresh ddH_2O. At the end of dialysis, gelatin-maleimide (Gel-MAL) that has 137,6 kDa molecular weight was obtained.

Gel-MAL was conjugated with KRSRGYC with chemicals used in the reaction as shown in Table 3.3.

Table 3.3. Materials used for gelatin-KRSRGYC formation.

Name of the chemicals	**Equivalent**	**Molecular Weight (kDa)**	**n (mmole)**	**Mass (mg)**	**Volume**
Gel-MAL	1	137,6	$5,81 \times 10^{-4}$	80 mg	
1% PBS					8 mL
KRSRGYC	20	868,01	0,0116	10,1 mg	
DMSO					1 mL
Et_3N	20	101,19	0,0116	1,18 mg	1,6 µL

80 milligrams of Gel-MAL dissolved in 8 mL % 1 PBS. This 1% Gelatin solution was stirred in a magnetic stirrer in 300 rpm without heat while 1 mL of DMSO was dissolving 10,1 mg KRSRGYC and 1,6 µL of Triethylamine (Et_3N) was also added into Gel-MAL with KRSRGYC peptide solution. Reaction took place for 2 days.

Afterwards Gelatin-KRSRGYC conjugate solution was dialyzed for a day on heating magnetic stirrer (Velp® Scientifica) at 400 rpm without heat. 1L baker filled with 800 mL ddH_2O.

Gelatin-KRSRGYC conjugate solution was put into Spectrum™ Spectra/Por Biotech-Grade RC Dialysis Membrane Tubing (Thermo Fisher) membrane and both ends of the membrane was clasped with dialysis clip. Three times on every hour

ddH_2O was replaced with a fresh ddH_2O. At the end of dialysis, Gelatin- KRSRGYC conjugate that has approximately 155 kDa molecular weight was obtained.

3.3.4.Coating sutures with Gel-KRSRGYC conjugate and Hydroxyapatite

In three different vials 10% HAp (by weight) was mixed with gelatin and KRSRGYC peptide. One suture was coated with 10% HAp (by weight), 1% Gelatin (by weight) and 0,5% KRSRGYC peptide (by weight). Another suture was coated with 10% HAp, 1% Gel-KRSRGYC conjugate. Last suture was coated with 10% HAp, 5% Gel-KRSRGYC conjugate. All sutures had 300 µL total solvent. This experiment set up showed comparison of sutures that had same percentage of gelatin and sutures that had same percentage of KRSRGYC peptide.

3.3.5.Comparison of peptide content for KRSR and KRSRGYC coated sutures concentration

In order to compare KRSR and KRSRGYC coated sutures peptide amount, three sutures that were mentioned above and an additional suture that was coated with 10% HAp, 1% Gelatin and 0,5% KRSR was chosen as samples for concentration measurement. After coating of these sutures, the remaining solution to suture was analyzed in Nanodrop One Microvolume UV-Vis Spectrophotometer (Thermo Scientific) at 214 nm. Standard curve was obtained from Pierce™ Quantitative Fluorometric Peptide Assay (Thermo Scientific™). Concentration of gelatin and osteo adhesive peptides before coating suture was listed below.

Table 3.4. Gelatin and osteo adhesive peptides concentration

Sample ID	Amount of Gelatin	Amount of KRSR	Amount of KRSRGYC	Total Amount of solvent
1	3 mg	1,5 mg		300 µL
2	3 mg		1,5 mg	300 µL
3	2,67 mg		0,33 mg	300 µL
4	13,35 mg		1,65 mg	300 µL

The equation that was used to convert concentration of the samples from µM to µg/mL is shown below;

Concentration (mg/mL) = (Concentration (µM) / standard curve) × (MW of peptide (Da) / 10^6)

(eq1)

3.3.6.Coated sutures osteo adhesive peptide and drug release profiles

Peptide and drug release experiments were carried out for these suture samples. While performing the stability test on these sutures, 100 µL samples were taken from the buffer receptor solutions in which the suture pieces were incubated at different time intervals (0, 1, 2, 4, 6, 8, 12, 24, 48, 72 and 96 hours). These collected samples were analyzed in 6420 Triple Quad LC/MS (Agilent Technologies) for the amount of free peptide and drug molecules in them.

First, two separate methods specific to this peptide and Vitamin E were created in the LC-MSMS device. Then, two different standard curves were created with peptide and Vitamin E solutions prepared at different concentrations (2000, 1000, 500, 200, 100, 50, 25 ppb) using pure peptide molecules and pure Vitamin E molecules, and release samples were analyzed using these curves. Thus, depending on time, how much peptide and what is removed from the suture surface simultaneously.Information about the release profiles related to the release of vitamin E has been obtained.

3.4.*In vitro* Cytotoxicity Assay of Bioactive Materials

Cytotoxicity of polymer coated sutures and its peptide and drug loaded versions were assessed by CCK-8 viability assay against *Mus musculus*fibroblast cell lines (ATCC CRL-2648). Cells (10000 cells/well) were seeded in a 96-well plate as quadruplet in 100 µL appropriate culture medium and incubated at 37 °C for 24 hours for cells to adhere completely. Unmodified and functionalized sutures were sterilized by UV light of biosafety cabinet for almost 30 minutes. Cells were treated with these suture pieces and incubated at 37 °C for 24 hours. After removal of sutures, the left adherent cells at the bottom of the wells were washed with 100 µL PBS twice. The number of viable cells was determined by CCK-8 cell viability assay by adding 10 µL CCK-8 reagent in 100 µL fresh medium onto wells and at the end of 1hour incubation absorbance values at 450 nm were measured via a microplate reader. Relative cytotoxicity values were calculated in an excel document with respect to the value obtained for non-treated cell-containing control well.

4. RESULTS

4.1. Coating of Sutures with bioactive materials

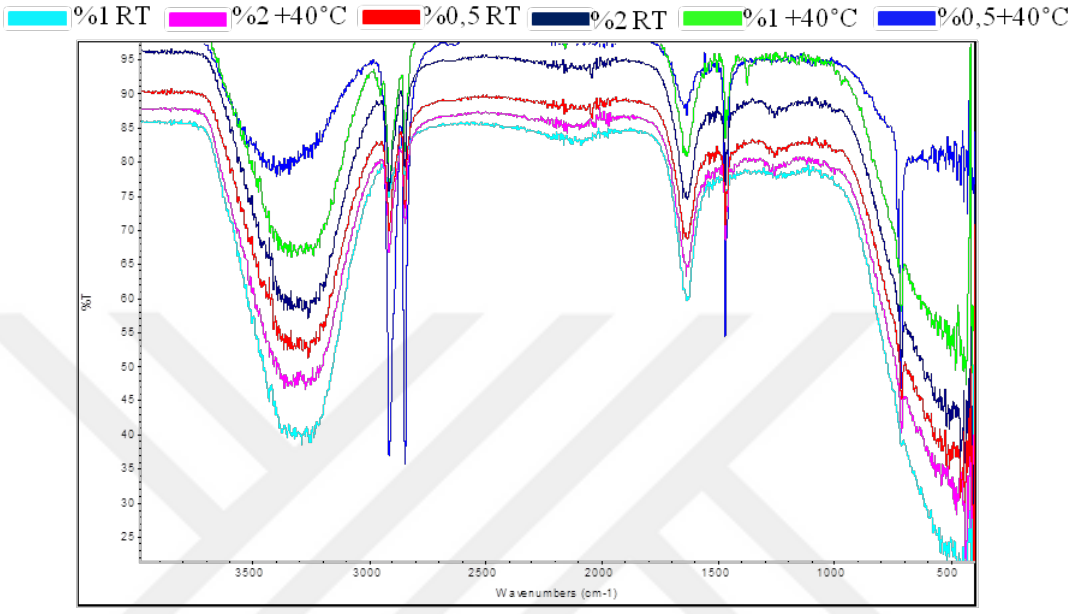


Figure 4. 1. FT-IR Spectroscopy Analysis of Gelatin coated sutures with 0,5%, 1% and 2% polymer concentrationsat RT and +40 °C, separately.

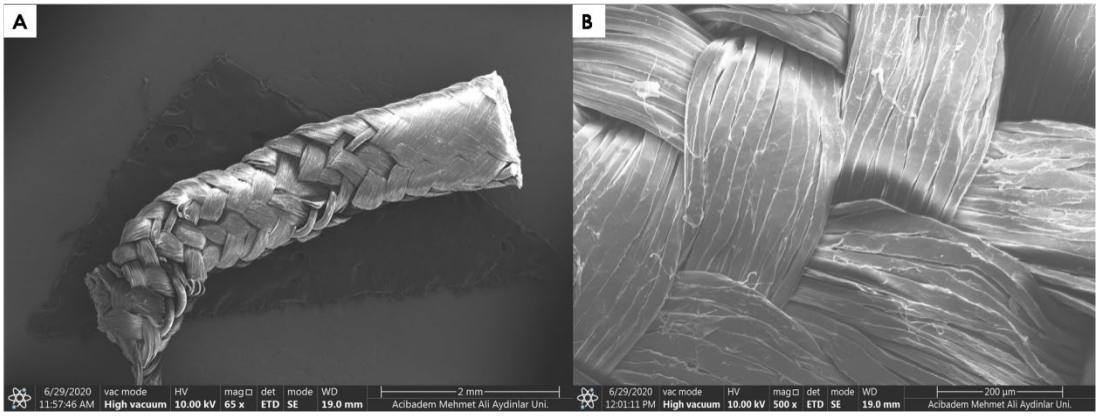


Figure 4. 2. SEM images of 0,5% (w/v) Gelatin coated suture at RT. (A) scale bar:2 µm (B) scale bar: 200 µm.

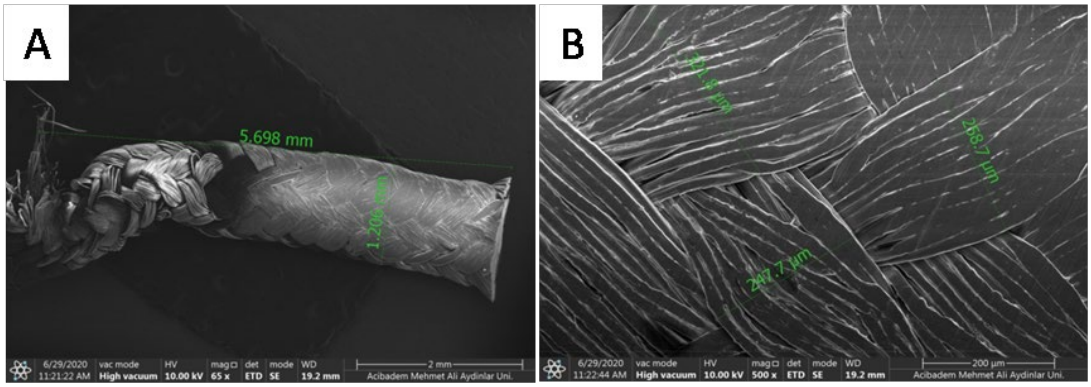


Figure 4. 3. SEM images of 1% (w/v) Gelatin coated suture at RT. (A) scale bar:2 µm (B) scale bar: 200 µm.

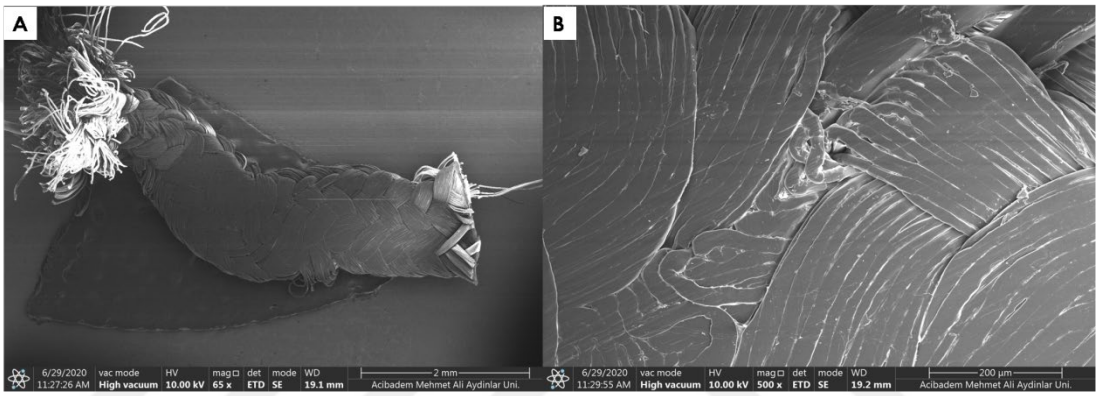


Figure 4. 4. SEM images of 2% (w/v) Gelatin coated suture at RT. (A) scale bar:2 µm (B) scale bar: 200 µm.

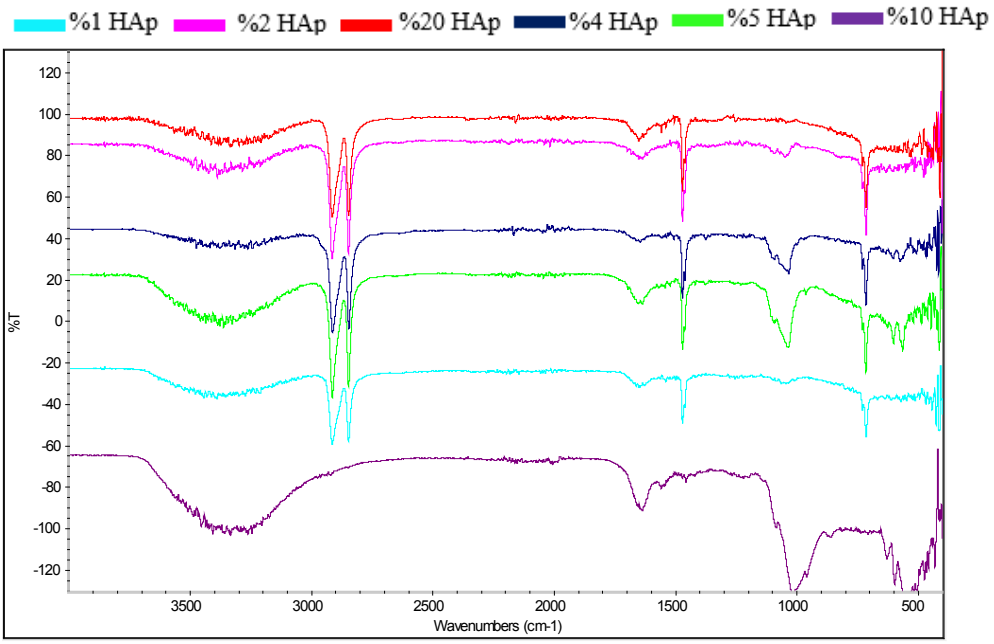


Figure 4. 5. FT-IR Spectroscopy Analysis of HAp coated sutures.(All sutures are covered with 1% Gelatin (w/v) at room temperature)

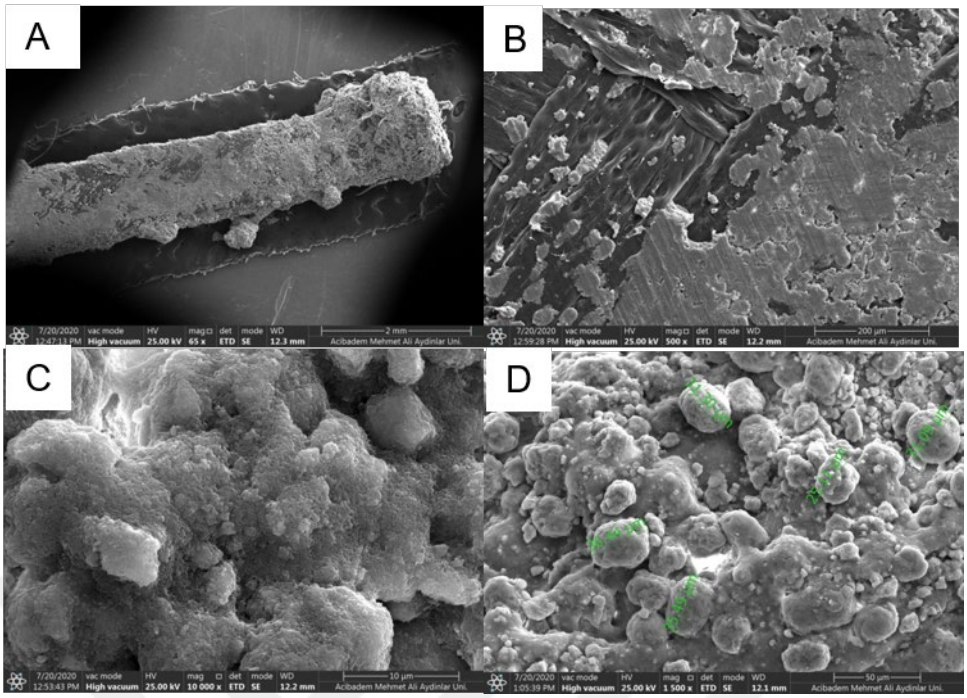


Figure 4.6. SEM images of suturecoated with 10%HAp and %1 gelatin and incubated in RT. (A) scale bar: 2 µm. (B) scale bar: 200 µm (C) scale bar: 10 µm (D) scale bar: 50 µm.

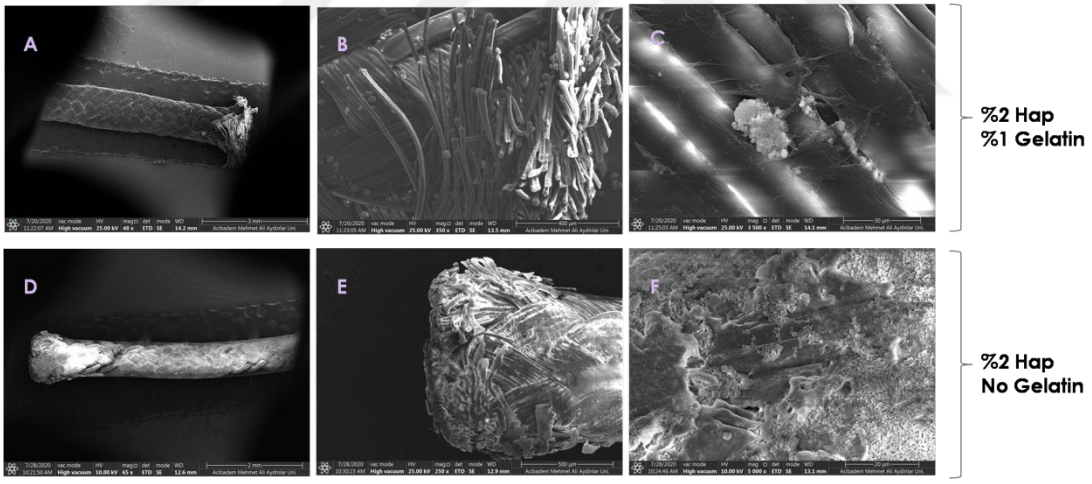


Figure 4.7.SEM images of Hap coated (RT) sutures: with and without Gelatin (control experiment 1).

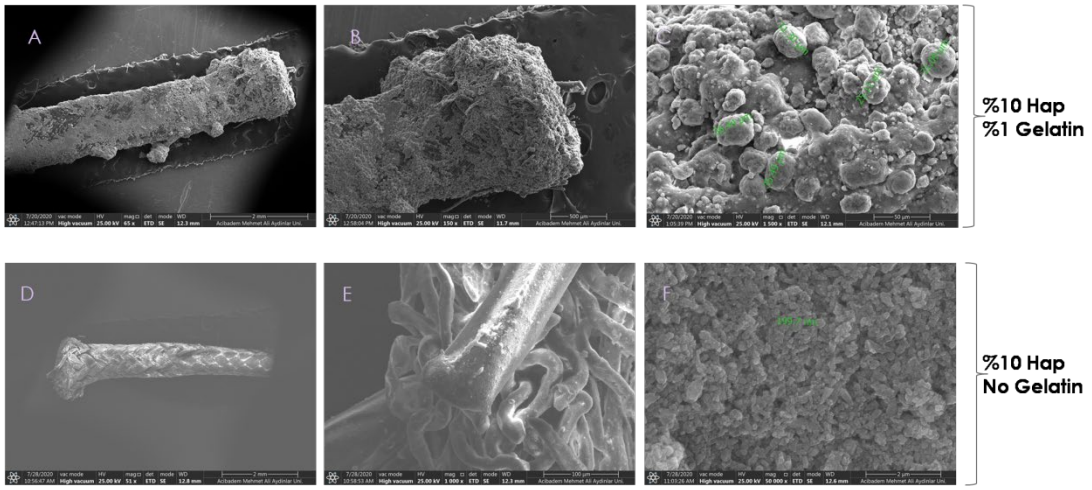


Figure 4.8.SEM images of Hap coated (RT) sutures: with and without Gelatin (control experiment 2).

Figure 4.9. LC-MS/MS analysis for KRSR peptide.

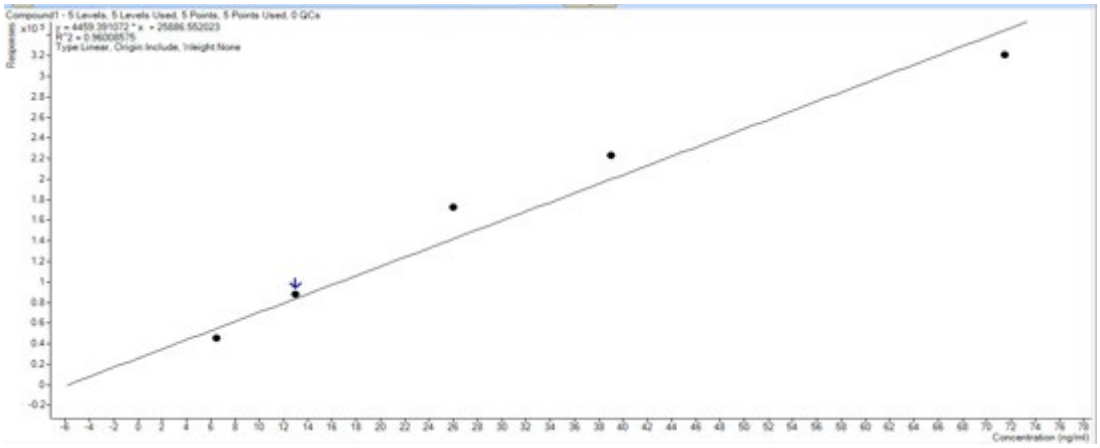


Figure 4.10. Calibration curve of KRSR obtained by LC/MS.

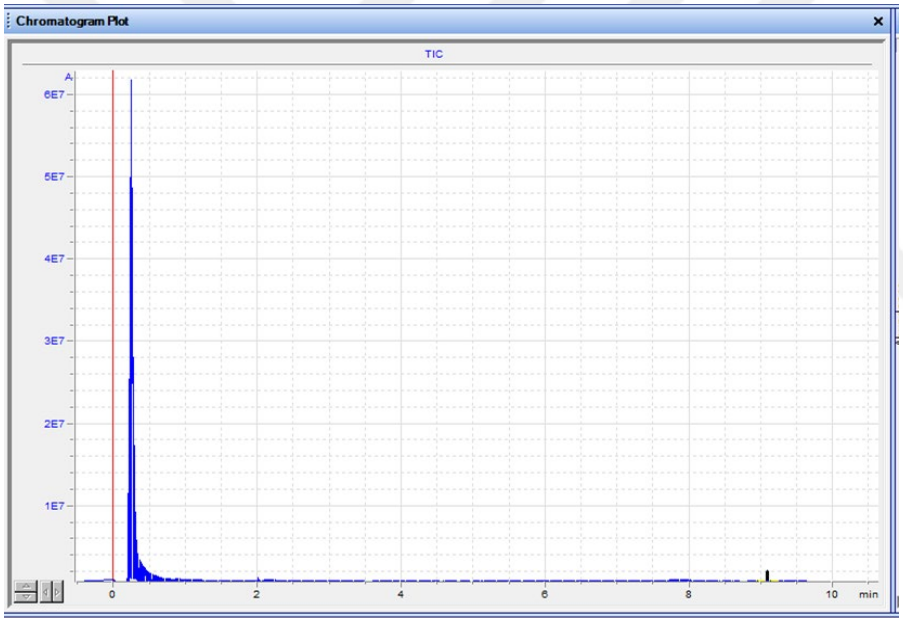


Figure4.11. Purity of KRSR peptide obtained by LC-MS/MS.

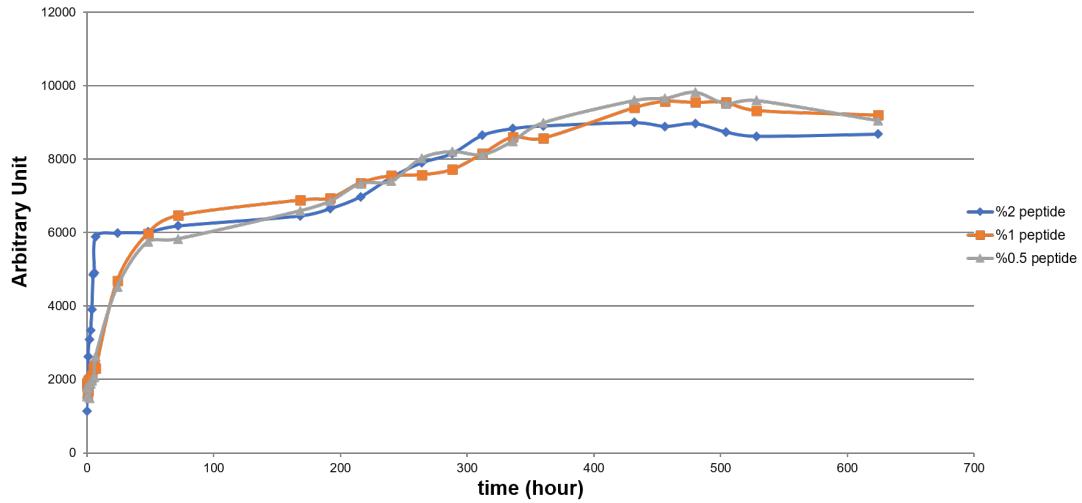


Figure 4.12. Release profile of osteo adhesive peptide KRSR from Gelatin-coated sutures.

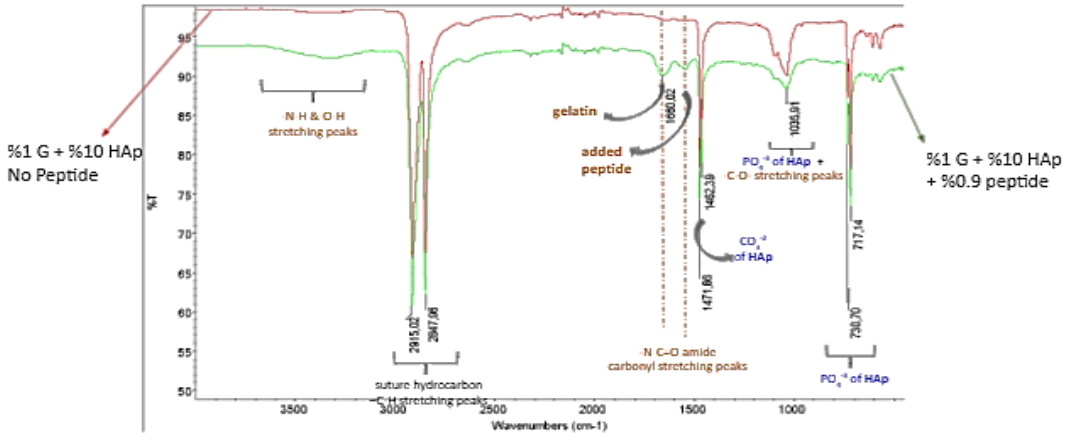


Figure 4.13. FT-IR analysis of % Gelatin + 10 % HAp with or without KRSR peptide.

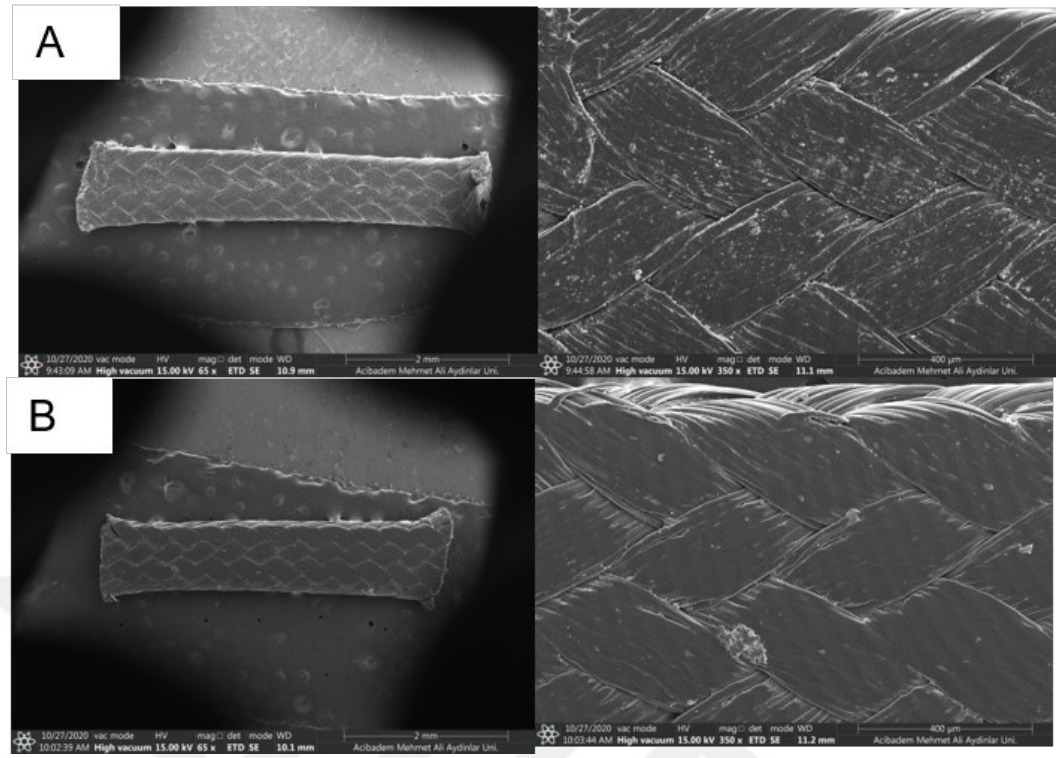


Figure 4.14. Comparison of 1% Gelatin (w/v) + 10 % HAp (w/v) with (A) scale bar: 2 µm and 400µm or (B) scale bar:2 µm and 400µm without KRSR peptide in SEM.

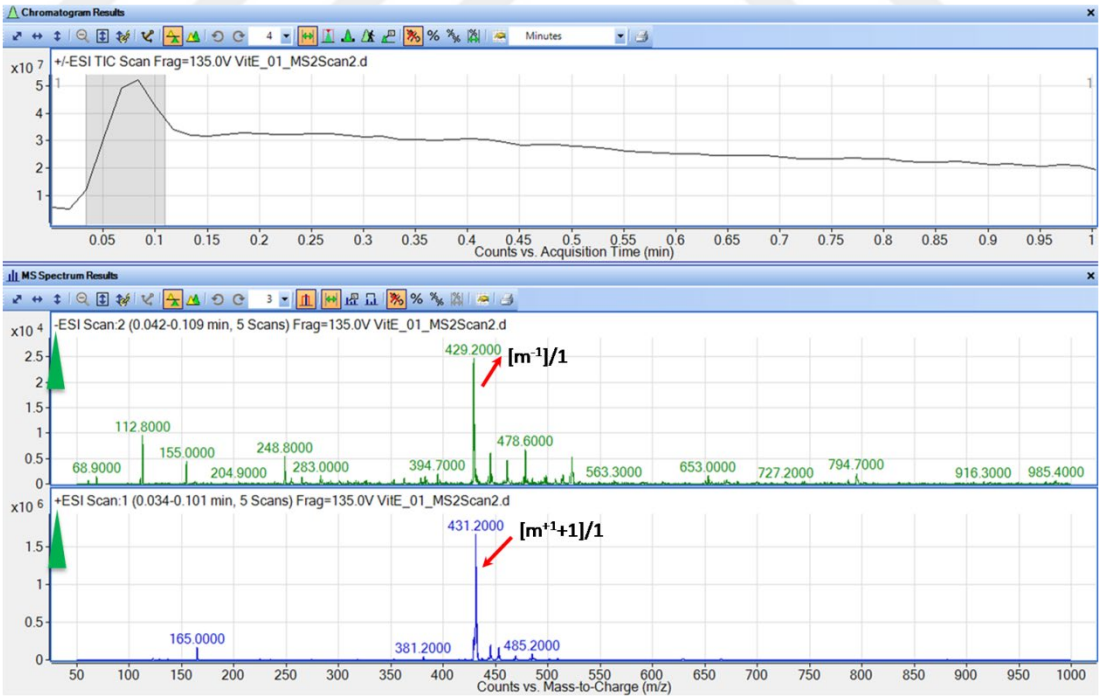


Figure 4.15. LC-MS/MS analysis for Vitamine E.

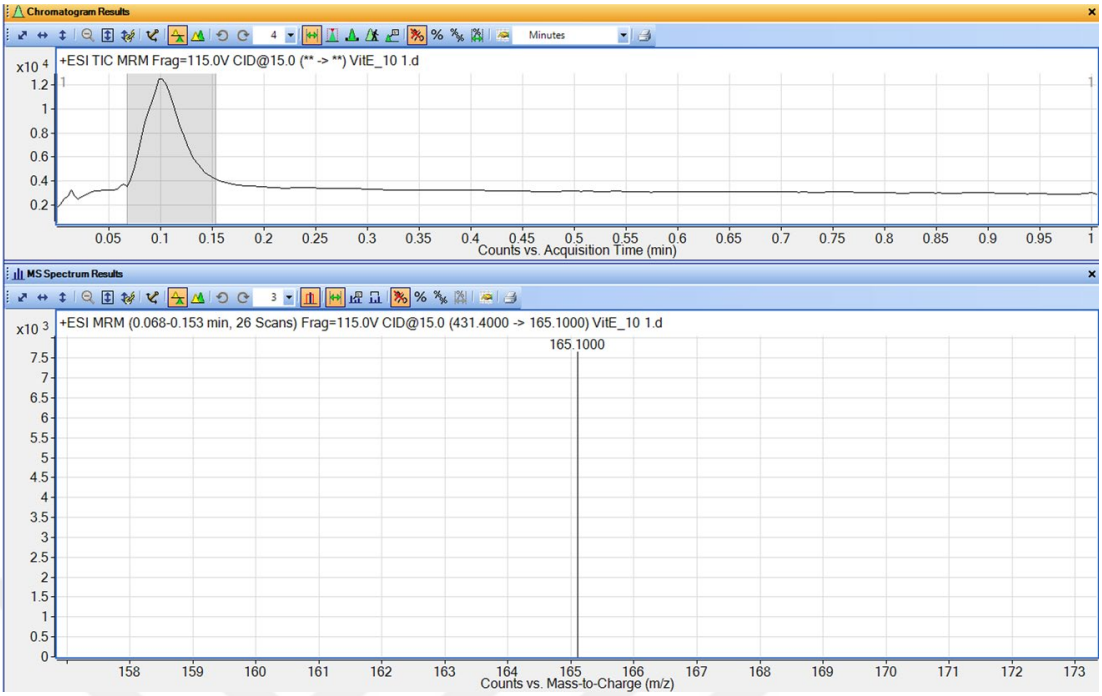


Figure 4.16. MRM method for Vitamin E obtained from LC-MS/MS.

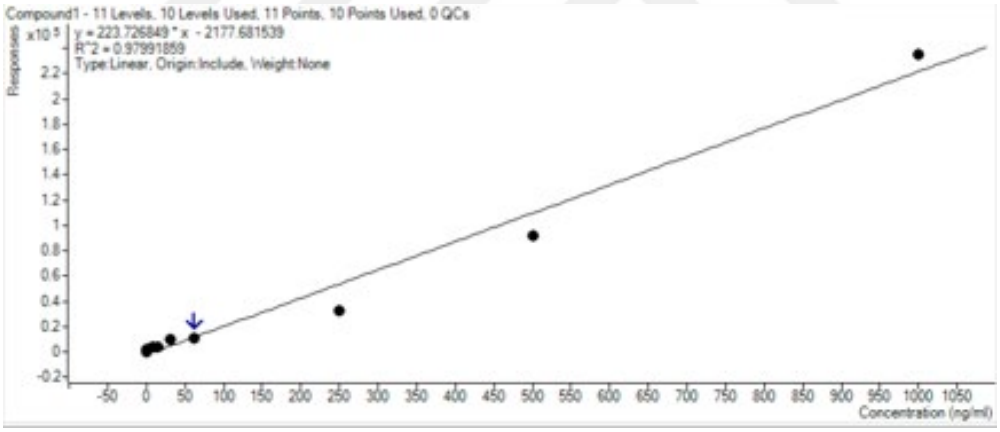


Figure 4.17. Calibration curve of Vitamin E by LC-MS/MS.

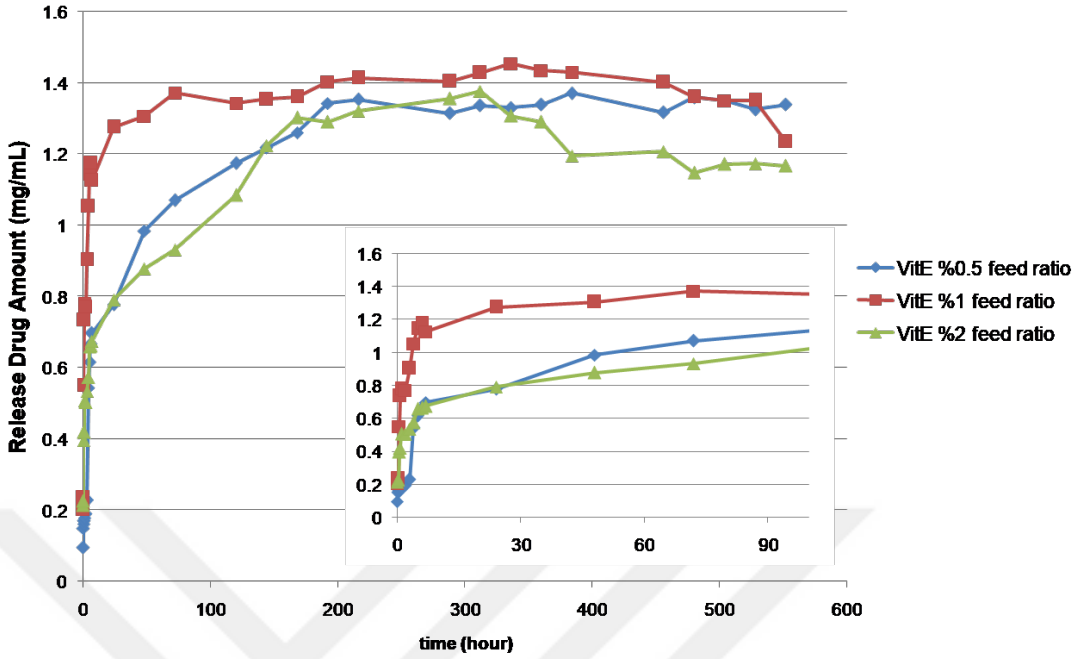


Figure 4.18. Vitamin E release amount for 23 days of period.

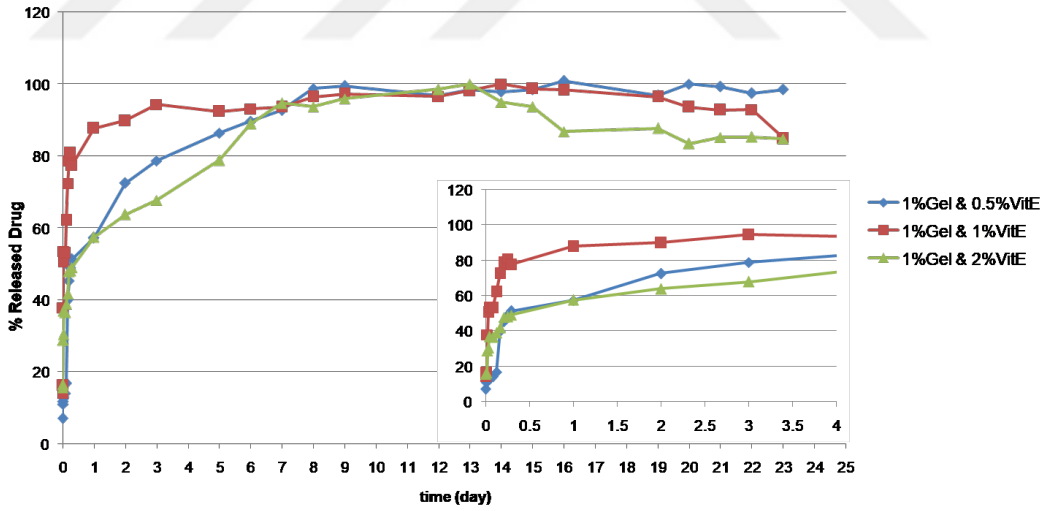


Figure 4.19. Vitamin E release percentages with respect to maximum loaded amount of Vitamin E for 23 days of period.

4.2. Optimization of Suture Surface Modification

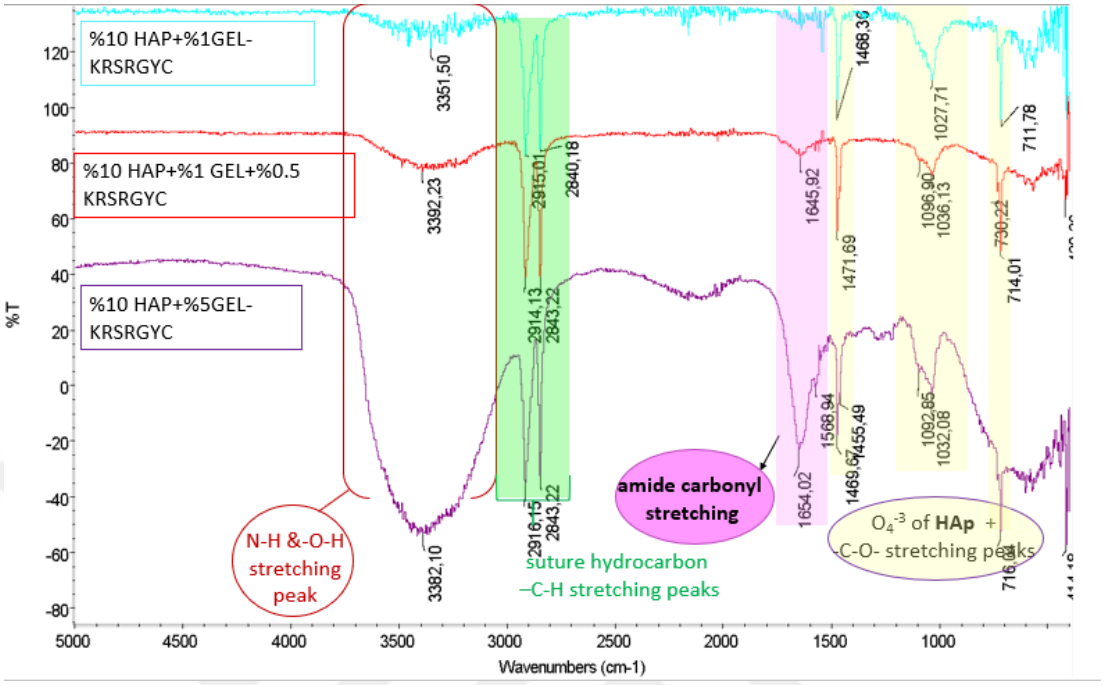


Figure 4.20. FTIR analysis of conjugated 1% and 5% gelatin- KRSRGYC and non-conjugated %1 gelatin and 0,5% KRSRGYC.

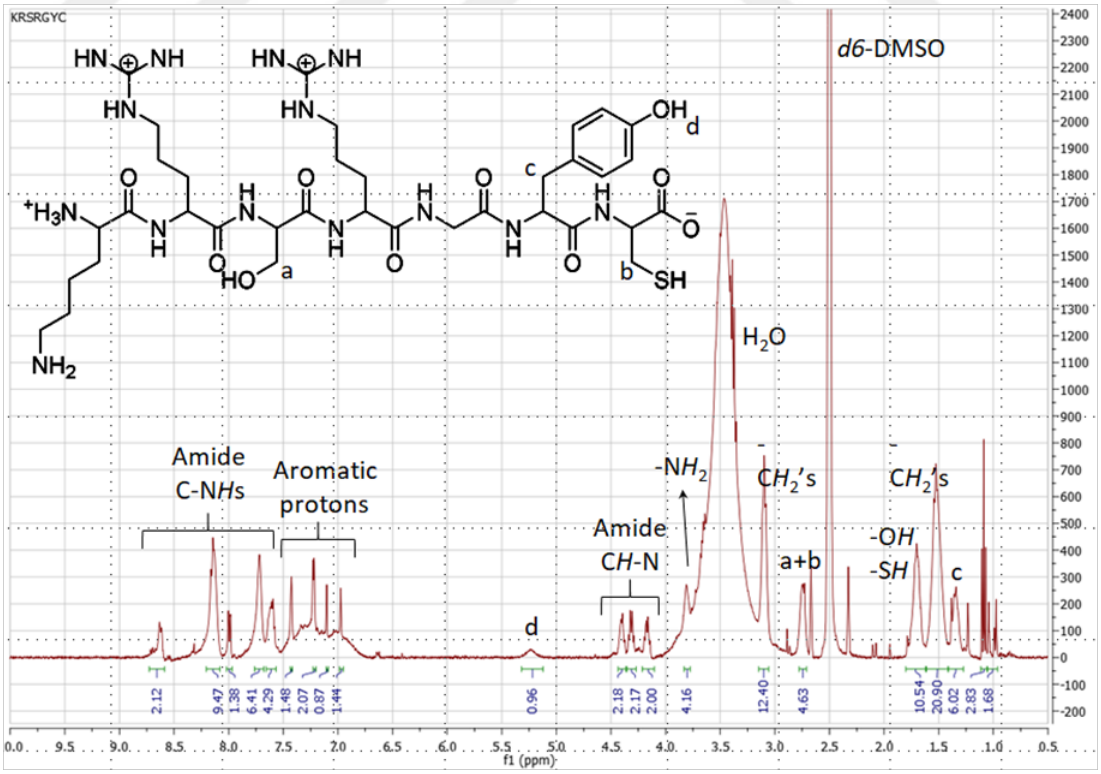


Figure 4.21. ^{1}H NMR spectrum for KRSRGYC peptide taken in d_6-DMSO.

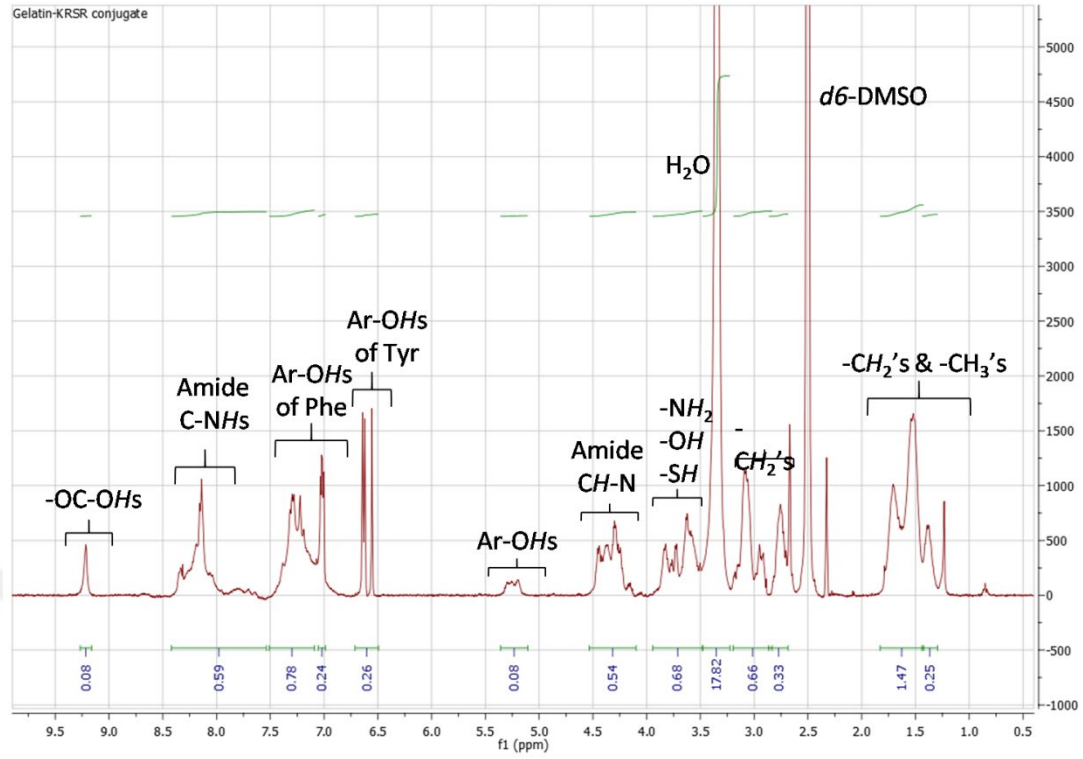


Figure 4.22. ^{1}H NMR spectrum for KRSRGYC peptide taken in d_6-DMSO.

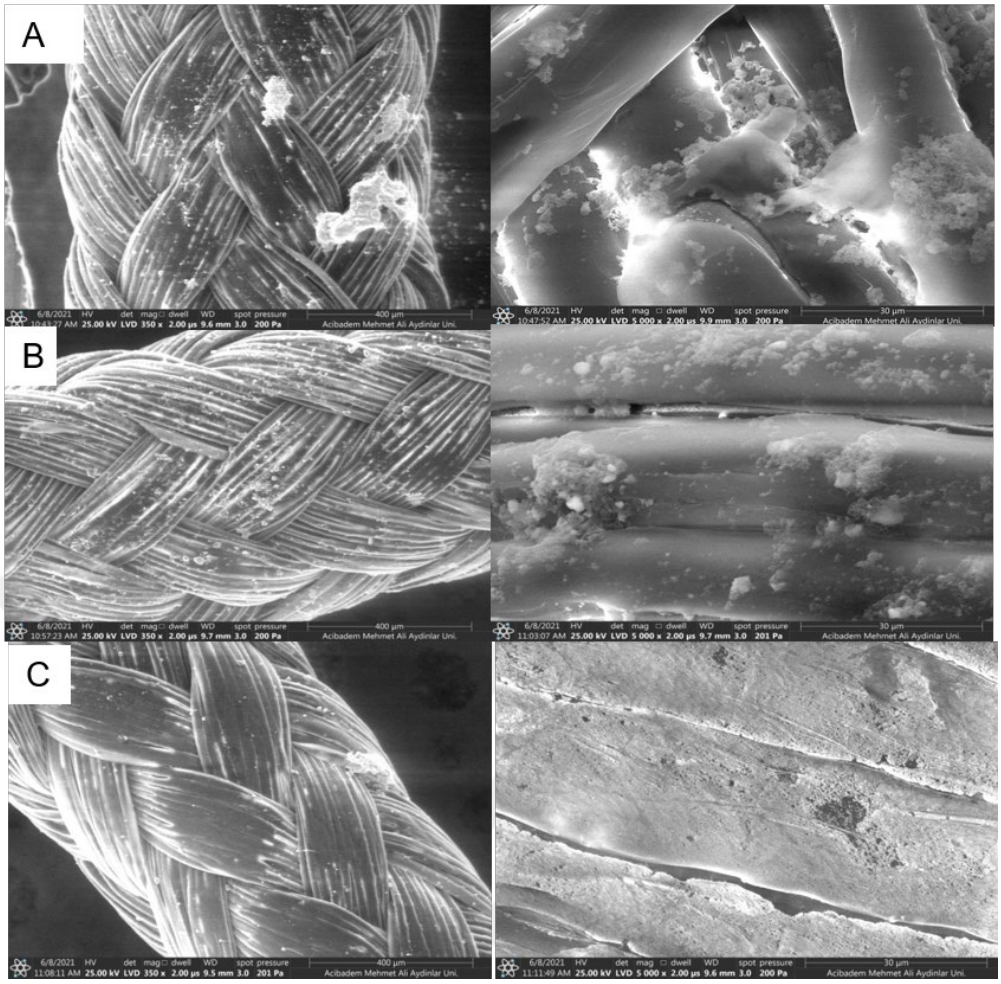


Figure 4.23. SEM images of (A) non-conjugated %1 gelatin and 0,5% KRSRGYC (B)1%gelatin- KRSRGYC and(C) %5 gelatin-KRSRGYC.

4.3.*In vitro* Cytotoxicity Assay of Bioactive Materials

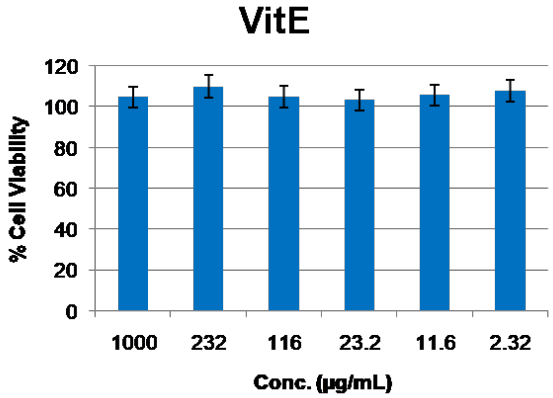


Figure 4.24.*in vitro* cytotoxicity experiment for Vitamin E against L929 mouse fibroblast cell line.

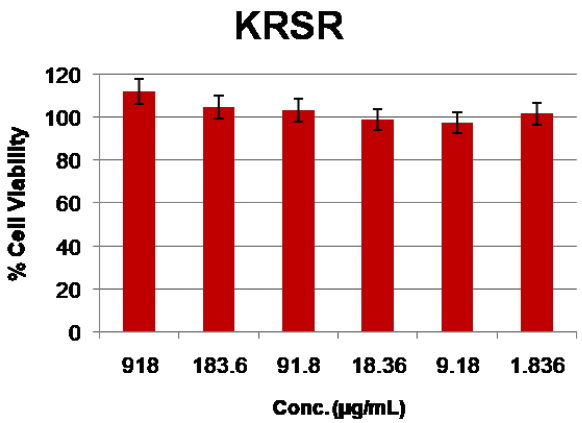


Figure 4.25. *in vitro* cytotoxicity experiment for KRSR peptide against L929 mouse fibroblast cell line.

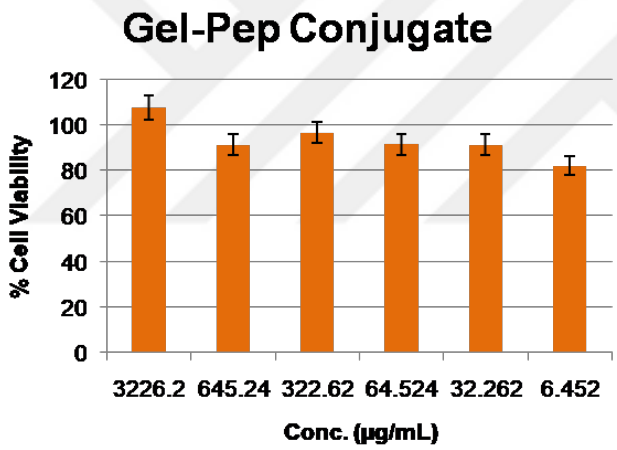
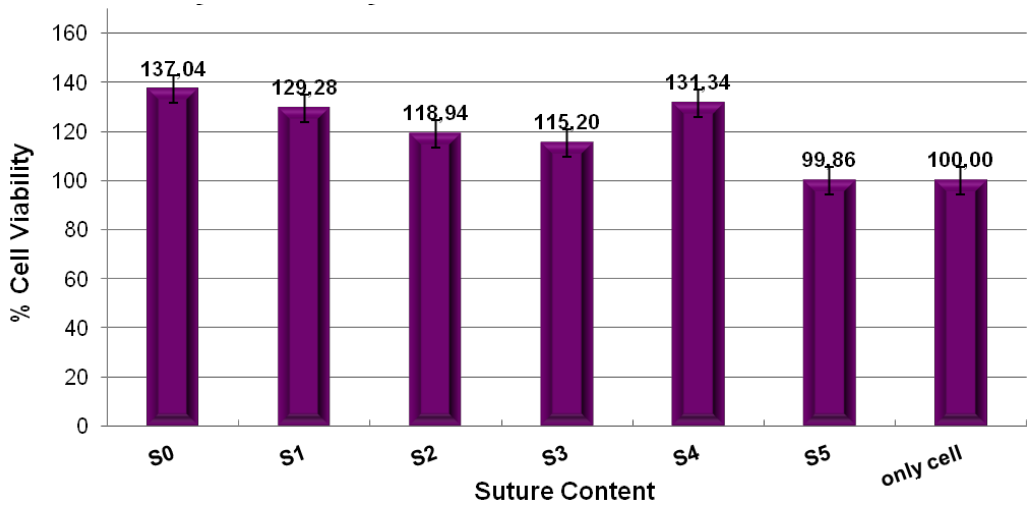


Figure 4.26. *in vitro* cytotoxicity experiment for Gelatin-KRSRGYC peptide conjugate against L929 mouse fibroblast cell line.



S0= only suture
S1= S0 + %1Gel + %10HAp
S2= S0 + %1Gel + %10HAp + %0.5KRSR
S3= S0 + %1Gel + %10HAp + %0.5KRSR + %0.5VitE
S4= S0 + %1Gel + %10HAp + %0.5pep Gel-Pep
S5= S0 + %1Gel + %10HAp + %0.5pep Gel-Pep + %0.5VitE

Figure 4.27. *in vitro* cytotoxicity experiments against L929 mouse fibroblast cell line.

5. DISCUSSION AND CONCLUSION

In this study, a suture used for the fixation of orthopedic sutures was modified to have a more biomimetic surface, which is expected to enhance the adhesion of bone cells at the implantation site after the surgery. For this purpose, a polymer-based coating strategy was applied as following literature procedures with slight modifications. By taking the general composition of the extracellular matrix for bone tissues into account, one can easily apprehend that collagen of different types is the most abundant polymer that keeps the cells together by promoting their adhesion and gives the mechanical properties to the bone tissue necessary for its durability and stiffness. From this knowledge, we would like to use gelatin as the main coating component for modifying the surface of the suture. Gelatin is a polypeptide that is derived from the cleavage of collagen protein, and due to its enhanced water solubility and availability, it is evaluated as one of the primary natural biopolymers for not only tissue engineering but also drug delivery purposes. Hence, it was used as a polymer that could keep other bioactive materials stable on the suture surface in this study. Different concentrations of gelatin solutions (0.5, 1 and 2% by weight) prepared in distilled water were incubated with suture pieces at both room temperature and 40 °C to cover the surface of suture pieces homogeneously. It was seen from SEM images that %1 gelatin was the best to cover the surface of the suture completely, with no detectable difference compared to %2. So for further studies, all coating procedures were conducted by using 1% gelatin solution.

Next, hydroxyapatite (HAp) was included in the system for making the coating layer more similar to natural bone tissue. Although its water solubility is low because of its crystalline structure, a slightly acidic environment was used to dissolve HAp powder, and this way, we could prepare a homogeneous solution of gelatin and HAp together. Each suture is coated with 1% Gelatin at room temperature and covered with Hydroxyapatite in different concentrations. Especially SEM images pointed out that 10% HAp has the best results for having detectable crystals on the surface. Surprisingly, the surface of the suture coated with 20% HAp included solution did not keep more crystals compared to 10%, which might be an indication of the capacity of gelatin polymer used as a glue to keep the HAp crystals on the surface.

This was also proven by control experiments, where HAp solutions were utilized for suture coating without any gelatin polymer, as a result of which almost no crystals were detected on the surface. So it was observed that gelatin increased the amount of hydroxyapatite attached to the suture and allowed hydroxyapatite to spread more evenly to the suture. To take it to a more advantageous level, the surface of this orthopedic suture was decorated with an osteoblast adhesive peptide as a biomolecule providing a more-natural surface for the bone-related cells at the implantation site. Timing is also another important parameter for the acceptance of sutures with surrounding bone tissue, which is why a quicker integration of sutures to the tissue is a topic attracting great attention in recent years that needs to be improved via the incorporation of bioactive molecules. Together with HAp, it is very well-known from previous studies that cell-biomaterial interaction is positively affected by peptide-based biomolecules. That is why; a peptide with a sequence of KRSR was included in this coating layer to make the surface of the suture more biomimetic. A study conducted in 2006 prevailed in the conjugation of this peptide to calcium phosphate-based materials in the form of KRSRGYC, whose thiol group at the end of cysteine amino acid was utilized for its conjugation. By following the same strategy, we first inserted active maleimide groups into the side chains of gelatin polymer with the help of a linker molecule, 6-maleimide hexanoic acid, using an NHS/EDCI coupling reaction to obtain a maleimide functionalized polymer: GEL-MAL. Then, the peptide synthesized by SPPS method was conjugated to this polymer via 1,4 Michael addition reaction using the thiol group of cysteines aminoacid at the end of the peptide. The sutures coated with 1% Gelatin, 10% HAp and 0.5% by weight 1%, and 2% peptide at room temperature were tested 39 for release at 37°C. According to this LC-MS/MS analysis; The most stable (controlled) release for peptide concentration at the time was determined to be 0.5% (wt). This conclusion was derived by evaluating the release profile of this adhesive peptide from the suture surface, conducted at different conditions and the released amount of peptide was analyzed by LC-MS/MS.

Next, we have also considered the possible inflammation at the site of implantation after the surgery, and in order to promote wound healing by eliminating chronic inflammation, we have included Vitamin E as an anti-inflammatory drug

molecule for the therapeutic purpose, of the coating layer of the suture. Although acute inflammation is desired for starting the healing process after the suture application, Vitamin E (Vit E) is known for its stimulating effect on bone healing. So, different concentrations of Vit E (0.5% 1% and 2% by weight) were included in the coating solution of 1% Gelatin, 10% HAp, and 0.5% peptide. Although the loading efficiency is low, VitE containing layer on the suture was subjected to the release studies at various conditions and based on LC-MS/MS results, 0.5% Vitamin E was seen to exhibit a more stable and controlled release than sutures coated with other concentrations. This profile is expected to provide a sustained release of VitE molecules from the surface to promote bone healing over a longer period of time after the surgery.

Overall, in this project, the coating of sutures, which are frequently used in orthopedics, both as HAp and the selective peptide was achieved successfully to increase their orthopedic efficacy, and the commercially available sutures were shown to be utilized as multifunctional bioactive orthopedic sutures that can slowly and continuously release the drug α-Tocopherol (Vitamin E), which induces bone regeneration. Also, osteoblast adhesive peptide which is conjugated to the coating polymer with a non-cleavable linker stays on the suture with the coating layer as a stable component for enhancing the cell adhesion for a longer period of time, unless the polymer itself gets degraded on the suture. There are currently no orthopedic sutures that can provide these two features in the presence of HAp, neither in the literature nor in the market.

Therefore, it is foreseen as a very high probability that this strategy will emerge as a novel procedure to obtain more biomimetic orthopedic products with both therapeutic and regenerative abilities. In addition, it is envisaged that this coating technique can be optimized further to apply to other medical problems which require the use of this kind of suture.

6. REFERENCES

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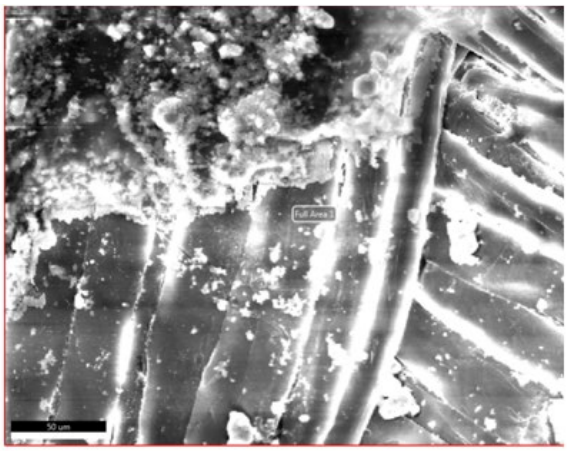
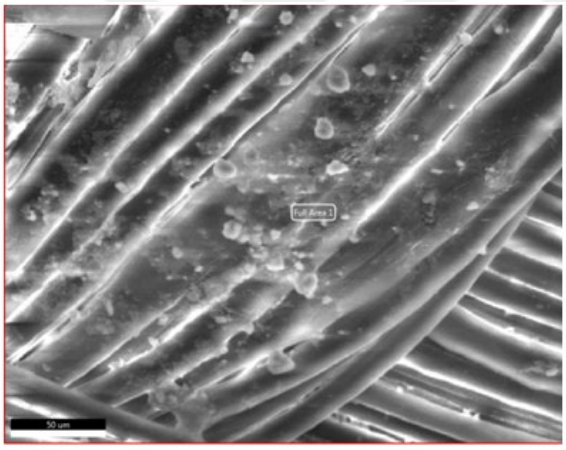
7.APPENDIX



Smart Quant Results

Element	Weight %	Atomic %	Error %
C K	71.52	80.03	5.74
N K	3.2	3.07	49.13
O K	16.17	13.58	14.52
P K	2.66	1.15	3.95
S K	0.05	0.02	68.29
CaK	6.41	2.15	3.1

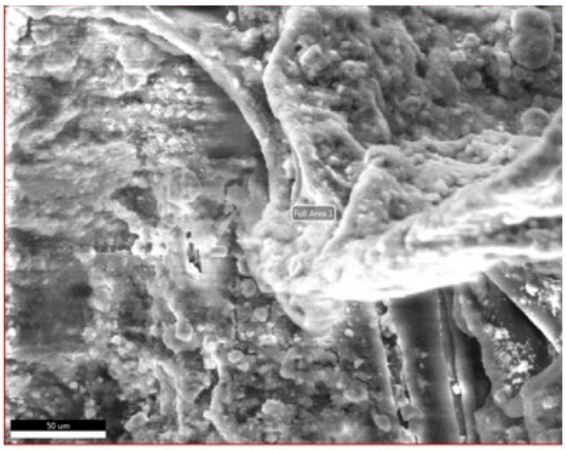
Figure7.1. 10%HAp (w/v), 1% gelatin(w/v) and 0,5% KRSRGYC (w/v) coated suture's EDS result obtained from SEM.



Smart Quant Results

Element	Weight %	Atomic %	Error %
C K	76.14	84.27	5.76
N K	0.84	0.8	99.99
O K	14.07	11.69	15.2
P K	2.62	1.13	4.9
S K	0.09	0.04	63.57
CaK	6.23	2.07	3.91

Figure 7.2.10%HAp (w/v) and conjugate 1% gelatin- KRSRGYC (w/v) coated suture's EDS result obtained from SEM.



Smart Quant Results

Element	Weight %	Atomic %	Error %
C K	14.33	22.8	11.34
N K	4.76	6.49	23.93
O K	42.8	51.12	11.08
P K	10.04	6.19	3.95
S K	0.14	0.08	55.85
CaK	27.93	13.32	1.78

Figure 7.3.10%HAp (w/v) and conjugate 5% gelatin- KRSRGYC (w/v) coated suture's EDS result obtained from SEM.

8. CURRICULUM VITAE