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# Human Hair Keratin Protein, Hair Fibers and Hydroxyapatite (HA) Composite Scaffold for Bone Tissue Regeneration

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**HUMAN HAIR KERATIN PROTEIN, HAIR FIBERS AND HYDROXYAPATITE  
(HA) COMPOSITE SCAFFOLD FOR BONE TISSUE REGENERATION**

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Bachelors of Science in Chemical Engineering

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May 2012

Submitted in partial fulfillment of requirements for the degree of

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**CLEVELAND STATE UNIVERSITY**

November 2014

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Student's Date of Defense: 2014

This thesis is dedicated to my parents Siyum Gebretsadik and Eyerusalem Kebede  
and my wife Tsebaot Kebede

## **ACKNOWLEDGEMENT**

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Last but not least, I would like to thank my wife and my family for all the help and support they showed me.

# **HUMAN HAIR KERATIN PROTEIN, HAIR FIBERS AND HYDROXYAPATITE (HA) COMPOSITE SCAFFOLD FOR BONE TISSUE REGENERATION**

**Samuel Siyum**

## **ABSTRACT**

The field of tissue engineering aims at promoting the regeneration of tissues or replacement of failing or malfunctioning tissue by means of combining a scaffold material, adequate cells and bioactive molecules. Different materials have been proposed for use as three-dimensional porous scaffolds for bone tissue engineering procedures. Among them, polymers of natural origin are one of the most attractive options mainly due to their similarities with the extracellular matrix (ECM), chemical versatility as well as typically good biological performance. In this study, two biocompatible composite scaffolds were developed from natural polymer by tissue engineering approach and tested in vitro. The first Scaffold (SCAF-1) that was developed was composed of human hair keratin protein and human hair fibers (cuticle-cortex). The second scaffold (SCAF-2) was composed of human hair keratin protein, human hair fibers (cuticle-cortex) and hydroxyapatite (HA) particles. SEM and EDX were used to analyze the three dimensional structure, surface chemistry and pore size of the scaffolds. Both scaffolds showed a three-dimensional structure with a pore size ranging from 40-500 $\mu$ m and porosity greater than 50%. Compressive tests were carried out under dry as well as wet conditions for both scaffolds. SCAF-1 showed compressive modulus of 0.009 MPa in wet condition and 0.90 MPa in a dry condition. Likewise, SCAF-2 had compressive modulus of 0.09

MPa in wet condition and 2.7 MPa in dry condition. Cell culture experiments with bone marrow stromal cells demonstrate that the composite scaffolds support cell attachment and proliferation.

Overall, human hair keratin scaffolds have been shown to have a porous three-dimensional structure that induces proliferation of GFP- stromal cells for bone tissue regeneration. These preliminary results suggest that human hair keratin, cuticle-cortex fibers and HA composite scaffolds appear to be an interesting structure for potential studies in bone tissue engineering.

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## **CHAPTER I**

### **INTRODUCTION**

Thousands of surgeries are performed every day to replace or restore bone that has been damaged through illness or trauma. Treatment is typically focused on transplanting bone tissue from one site to another. There are different forms of tissue transplant. In some cases, the bone is transplanted from one part of the body to another on the same patient, which is referred to as autograft. In other cases, the bone is transplanted from one person to another and is referred to as allograft. While these procedures are used to effectively treat the underlying problem, they often have major side effects and shortcomings. One of these limitations is that getting autograft can be very costly. The procedure is also painful and requires a long recovery time [22, 7]. Similarly, allograft also has some side effects; the main one being that it is hard to find the right amount of bone tissue and the immune

system of the patient might reject the donor's bone tissue. These are the type of problems that the growing field of tissue engineering is trying to address. In tissue engineering, a tissue regeneration technique where cells from the body are combined with porous scaffold biomaterial made outside of the body is used to help in the growth of the tissue. A brief illustration of the idea of tissue engineering is shown in the figure 1.

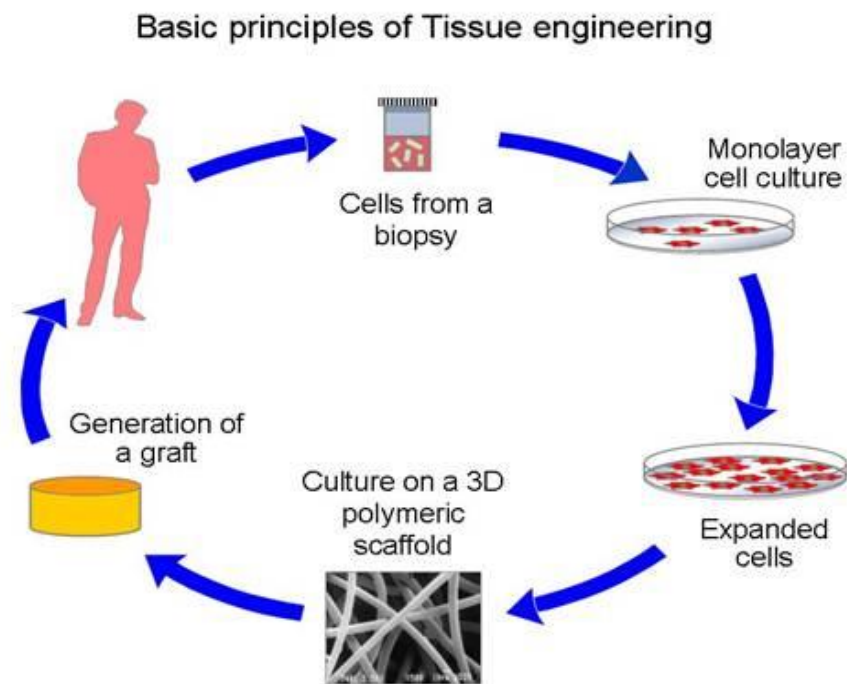


Figure 1: The concept of tissue engineering [9]

Many experiments have been done to investigate materials that can be used in the production of bone scaffold. This research is specifically focused on evaluating the potential of human hair proteins, cuticle fragment and hydroxyapatite composite to be used as a scaffold for bone repair and regeneration. Human hairs are present in a large scale as unused protein rich waste.

Approximately 300,000 tons of hairs per year worldwide are dumped as waste [40]. Large percent of human hair contains keratin protein (KP) and keratin associated proteins (KAP). Keratins are a group of cysteine rich fibrous proteins that show high mechanical strength due to a large number of disulfide bonds. In addition, the keratin proteins from human hair exhibit a cell adhesion motif, leucine -asparatic-acid-valine (LDV), which is recognized by the integrin family of protein [40, 50, 54]. The characteristics mentioned above make human hair protein an ideal molecule to be used in the production of a bone scaffold.

The biomedical use of keratin protein has been investigated intensively in the past ten years. Keratinous materials with some chemical modification or combination have been fabricated for biomedical purposes including films, hydrogels and scaffolds [40, 47, 50]. However, most of the investigations are primarily focused on a successful extraction and physical and chemical properties of the human hair keratin [54].

In this study, human hair protein, cuticle fragment and hydroxyapatite were used to develop a biocompatible bone scaffold. First, Keratin protein was extracted from the human hair using Shindai method [33]. Second, a three-dimensional structured scaffold was created by mixing the keratin protein with cuticle-cortex fiber fragments and coated with hydroxyapatite. Finally the three dimensional structures; biocompatibility, microstructure and mechanical properties were studied to see if hair protein and cuticle-cortex fiber fragments can be used to create a bone scaffold.



## **Chapter II**

### **BACKGROUND**

#### **2.1 Bone**

##### *2.1.1 Bone anatomy*

Bone is a living growing tissue composed of cells, connective tissues, and calcium compounds such as hydroxyapatite and neuron tissues. Bone is primarily made up of bone matrix, which is composed of organic and inorganic material [5, 37, 53]. The organic material primarily consists of type one collagen and proteoglycans. The inorganic material primarily consists of calcium phosphate crystal called hydroxyapatite. The adult human skeleton approximately contains 213 bones that makeup eighteen percent of the human body weight [4]. The bones of a Skeleton

have several functions. They provide protection to internal organs, support structure and movement of the body, serve as a reservoir of growth factors and minerals and balance the pH of blood by releasing alkaline salt [5, 37, 53].

Based on their shape, human bones are classified as long bones, short bones, flat bones and irregular bones. The long bones include: the clavicles, humeri, radii, ulnae, metacarpals, femurs, tibiae, fibulae, metatarsals and phalanges. Short bones consist of the carpal, tarsal, patellae and sesamoid bones. Flat bones consist of the skull, mandible, scapulae, sternum, and ribs. Irregular bones consist of the vertebrae, sacrum, coccyx, and hyoid bone [5]

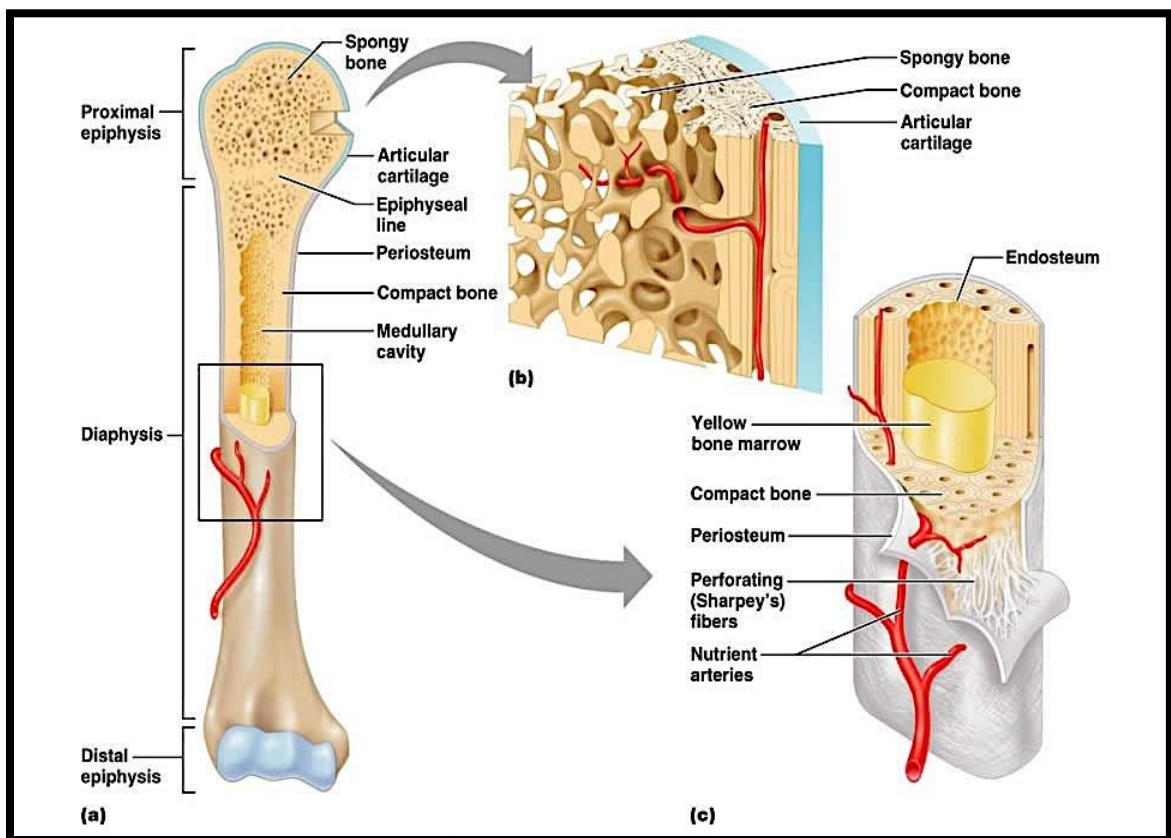


Figure 2: The structure of human bone [4]

The bone tissue can be separated into two main types, the cortical bone and the trabecular bone (Figure 2). The trabecular (spongy) bone accounts for 20% of the total bone mass while the cortical bone accounts for 80%[5]. The trabecular bone is less dense and more elastic bone forming the interior scaffolding and maintaining shape when compressive force is applied. The cortical (compact) bone stands for 80% of the skeleton mass and protects the external shell around every bone in the body.

Bone tissue is classified as either woven or lamellar, according to the arrangement of the collagens in the bone matrix. In a woven bone, the collagens fibers are randomly oriented in many directions. Woven bone forms in early stage of fracture bone repairing. In addition, they have more osteocyte cell per unit volume. In a bone remodeling stage the osteoclast cells remove the woven bone and osteoblast cells will form a lamellar bone. Lamellar bone is a mature bone with formal arrangement of collagen fibers. They are stronger and less flexible than woven bone.

### *2.1.2 Bone Growth and Remodeling*

Bone undergoes growth and continuous remodeling throughout its lifetime. The modeling involves a process by which bones change their whole shape in reaction to physiologic impacts or mechanical force resulting in gradual modification of the skeleton.

The continual adding and removing of bone has several physiological functions. First, bones are able to adjust their strength in proportion to the degree of stress. Second, the shapes of the bone reorganize for proper support of mechanical forces.

There are four main cells responsible for the removal and replacement of bones: they are osteogenic cells, osteoblast cells, osteocyte cells, and osteoclast cells. Osteogenic cells are located along the inner portion of periosteum, in the endosteum, within the blood vessels. These cells are derived from mesenchyme, the tissue from which all connective tissues are formed. Osteoblast cells, also known as bone-forming cells produce collagen and proteoglycans. These cells are also responsible for the mineralization of the bone matrix. The formation of bone by osteoblast is called ossification. Ossification occurs in two ways: first, when bone is made on top of primitive connective tissues, second when cartilage is used as a template [44]. Once osteoblast cells are surrounded by bone matrix they become osteocyte. Osteocytes cells work in order to maintain the bone matrix. On the other hand, osteoclasts cells are responsible for the reabsorption of the bone, they breakdown the mineralized bone matrix by releasing a protein digestive enzyme. The osteoclasts perform their duty effectively when they are in contact with the mineralized bone matrix.

As shown schematically in Figure 3 Bone remodeling cycle includes three complex series of sequential phases that are greatly controlled. The first phase is the resorption during which the old bone will be digested. The second phase is the reversal during which mononuclear cells will be present in the surface of the bone and they will complete the resorption and produce signal that initiates formation of new bone. The final stage is the formation during which the osteoblast cells will lay down new bone matrix until the old bone is removed.

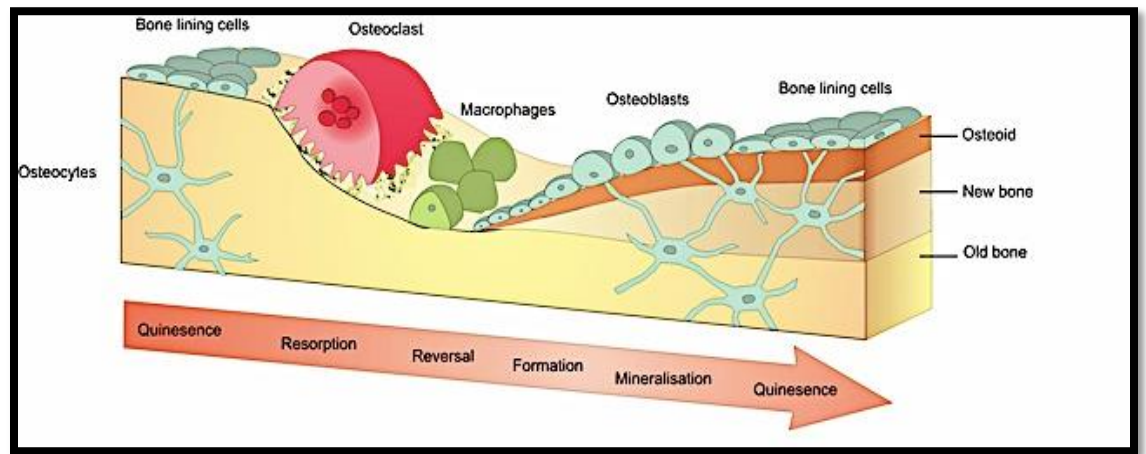


Figure 3: Bone remodeling process [2]

## 2.2 Scaffolds for tissue engineering

The scaffold for tissue engineering needs to have the following characteristics. First, it needs to have three-dimensional porous architecture made up of interconnected pores having varying dimension, from micrometer to millimeter to allow for cell ingress and growth and transportation of nutrients and waste [14].

Second, it needs to be biocompatible. Third it should be capable of degrading and being resorbed as the bone tissue grows after scaffold being implanted. Fourth, it has to have optimum strength and stiffness to retain its structure while the new bone grows into the scaffold. Fifth, it needs to have the right surface chemistry, and surface nano-texture to promote cell attachment and proliferation.

A variety of materials have been used for production of scaffold to replace and repair damaged or traumatized bone tissue. These materials include metals, ceramic, and polymers and their composites. Porous scaffolds made of metallic materials, such as titanium [1, 42], cobalt-chromium [1, 42], have two major disadvantages. First, they are not bio-absorbable and stay within the body for years causing its associated complications, such as bone loss due to stress shielding [1] or medical complications due to wear-debris getting into the blood stream [1,15]. Second, their processibility is very limited. In contrast, polymers and some ceramics have more flexibility because their composition can be tailored to a specific need. Also they can be designed to degrade when in contact with biological fluids. As a result, researchers are studying natural-polymers, synthetic polymers and composite of ceramic and polymers for the bone tissue engineering application [57,41,32,23].

### *2.2.1 Biodegradable scaffolds*

#### *2.2.1.1 Ceramic*

Ceramic is widely being used in the production of bone scaffold because of its similarity with the mineral phase of bone. The most commonly studied ceramics are hydroxyapatite (HA) [22,26] and tri-calcium phosphate [22]. These materials show good biocompatibility with the local bone due to their similar chemical and structural arrangement. They have excellent osteoconductivity [22,3]. In addition, a highly porous HA scaffold mimics the bone tissue structure and provides space and high surface area for osteoblast to deposit bone matrix [22]. However, using HA by itself for the production of scaffold has its own problems. It degrades very slowly compared to other natural-polymers and synthetic-polymers. It also has a high young modulus and low elasticity, it is relatively hard to fabricate to achieve the shape of a local bone.

#### *2.2.1.2 Synthetic Polymer scaffolds*

Different types of synthetic polymers are being used as scaffold because of two main reasons. First, they are relatively easy to use in creating a tailor-made scaffold designed specific to patient needs. Second, chemistry can be designed to be biodegradable when in contact with internal body fluid [30]. Their degradation rate can be controlled by changing their hydrophobicity and crystallinity. Poly-l-lactic

acid (PLLA), poly-glycolic acid (PGA) and poly lactic-glycolic acid (PLGA) are the most often utilized synthetic polymers for three-dimensional scaffold in tissue engineering [46,30,28]. Studies have shown that these materials have the most optimum mechanical strength, biocompatibility, and biodegradability among the polymers [46,28]. Although different polymeric materials have been studied, no single polymer fulfills all the properties that are required to fabricate bone scaffold. For instance, when PLLA, degrades in bulk; it releases lactic acid [28,51]. If the concentration of the lactic acid builds up fast, it will cause the local pH to decrease; which intern may accelerate the degradation rate and cause an inflammatory reaction [51,28]. Likewise, when PGA degrades by hydrolysis reaction, it increases the concentration of carbon dioxide, which lowers the pH and causes death of cells and tissue [51,28].

### *2.2.1.3 Natural polymers*

Natural polymers like silk [26,32,24], collagen [22,28], soy protein [27,35], alginic acid [27], keratin from wool [48,55,48], starch [27,20], cellulose [27,10], agar [27], and chitosan [27,57] have been widely studied for the production of three dimensional structured scaffold for bone and cartilage tissue regeneration applications. Natural polymers provide the advantage of possessing specific biological macromolecules, which the biological environment can recognize. In addition, to their similarity with the extracellular matrix, natural polymers are also likely to minimize the onset of chronic inflammation or immunological reaction and



toxicity, which is often a problem with synthetic polymers. A study conducted by V.Verma *et al.* [49] on the fabrication of three dimensional scaffold from keratin protein for tissue engineering shows that the extracted keratin protein displays an amino acid sequence leucine-aspratic acid-valine (LDV) that is recognized by the integrin family of protein  $\alpha4\beta1$  [5,39,4], which will promote cells to attach to the surface of the scaffold. In a similar manner, J.F.Mano *et al.* [28] reported that type I-collagen scaffolds show good biocompatibility, low antigenicity and ability to be cross-linked which enables tailoring of their structure, degradation and water uptake capacity. Moreover, Mandal *et al.* [26] investigated how to use a  $\beta$ -sheet protein that was extracted form Bombyx mori silk worm to create a high strength scaffold for bone application. They used alkaline hydrolysis [26, 24, 22] method to break down the protein into small fibers. Then, the small protein fibers were mixed with hexafluoroisopropanol solution to facilitate cross linkage between the extracted proteins. This was followed by lyophilization to remove the water and chemical solutions and create the 3-D microporous structure. The microporous scaffold exhibited good mechanical property with a compressive strength of 13Mpa [26]. Furthermore, silk microfiber protein scaffold shows strong similarity with real bone mechanical features such as matrix stiffness and strength [26, 24, 22].

#### *2.2.1.4 Composite Materials*

As discussed above, one single material will not fulfill all the desirable characteristics of biological scaffolds. Henceforth, scholars are investigating

scaffolds that are made up of composite materials for bone tissue regeneration. A material that results when two or more materials are combined physically to produce new material whose properties are greater in a particular application to those of the original material is called composite material. Composite materials are used in bone scaffold production for better biocompatibility, biodegradability, mechanical structure and osteoconductivity. ZrO<sub>2</sub> coated with hydroxyapatite (HA)[55], poly ( $\epsilon$ -caprolactone) (PCL) with hydroxyapatite ceramic powder [17], nano-hydroxyapatite, collagen and PLA [52], silk and hydroxyapatite, nano-hydroxyapatite crystals and polyamide [22], wool-keratin and hydroxyapatite [48,11], chitosan and hydroxyapatite [39] are some of the composite materials that are being currently investigated. For instance, H.-W.Kim *et al.*'s [18] study shows when zirconia bone scaffold is coated with hydroxyapatite, it increases the strength of the scaffold to be greater than a pure hydroxyapatite scaffold [18]. The strength of the zirconia scaffold ranged from 1.6 to 35 MPa with high porosity [18]. Furthermore, in an in-vitro study osteosarcoma cells were found to attach and spread on the surface of the HA coated zirconia (ZrO<sub>2</sub>) scaffold very well [18]. However, Zirconia-HA scaffold shows low degradation rate, which will have a direct impact on the growing tissue by causing inflammatory reaction. Similarly, H.J.Kim *et.al.* [19] studied a silk fiber scaffold coated with HA. "Silk fibroin scaffolds were prepared with the addition of polyaspartic acid during processing, followed by the controlled deposition of calcium phosphate by exposure to CaCl<sub>2</sub> and Na<sub>2</sub>HPO<sub>4</sub>. [19]" The scaffold exhibited good mechanical and osteoconductive property compared to uncoated silk scaffold [19]. Recent studies showed that when natural polymers are

combined with both synthetic polymers and ceramic they improve the desirable characteristics of a scaffold. For instance, Liao *et al.* [22], investigated the potential of nano-hydroxyapatite, collagen and PLA composite scaffold for bone tissue regeneration. This 3-D scaffold mimics the structure of spongy bone [22]. Furthermore, result showed that the n-HA, collagen and PLA scaffold and the natural bone show similarity both in main composition and in microstructure. This scaffold was implanted in to an adult rabbit to study the biocompatibility and the biodegradability property of the scaffold in vivo. The implantation result showed that the scaffold was bioactive. Osteoblast cells proliferated, spread, and adhered through the pores of the scaffold material within a week [22].

### **2.3 Hair Morphology**

Hair is the most sophisticated biological composite material that grows from follicles found in the dermis. Hair has two main parts; the hair root and the hair fiber shaft that extends above the skin surface.

The hair fiber shaft is a multi-cellular tissue with many different structural components and specific chemical composition. The fiber geometry is roughly cylindrical, with a diameter ranging from 90-130  $\mu\text{m}$  [36,12,13]. The hair fibers primarily contain protein, lipids, nucleic acid, carbohydrates and inorganic compounds. The elemental analysis shows human hair fiber consists of approximately 50wt% carbon, 22wt% oxygen, 16wt% nitrogen, 7wt% hydrogen and 5wt% sulfur [36]. The hair fiber is divided into three main sections: the cuticle

(the outer layer), the cortex (the core shaft) and the medulla as shown in Figure 4 [36].

The medulla is an air-filled cavity made up of special types of cells. It is located in the innermost part of the hair shaft. Mostly, only thicker coarse hair shafts contain a medulla.

The cuticle is thin outer layer of the hair fiber that is made up of flattened, oval-shaped or elongated cells. The cuticle mainly contains highly crystallized cysteine rich keratin proteins that protect the hair fiber from physical and chemical damage and provide strength for the hair to maintain its shape. Cuticle consists of four layers (Figure 4) with different concentration of cysteine: the epicuticle outer layer, which is nearly 5nm thin hydrophobic membrane, the  $\alpha$ -layer, the exocuticle and the endocuticle. The cell membrane complex binds the four layers together.

The cortex is composed of tightly bonded spindle shaped cells that contain keratin filaments. Cortex accounts for 70-90 percent of the total weight of hair. It gives the hair its strength, flexibility, elasticity and color. It is made up of ortho-cortex, para-cortex and meso-cortex cells [36,12,13,45]. A study conducted by C.Popescu *et. al.* and his group [36] on the structure of hair shaft shows that these cells differ in terms of their  $\alpha$ -helix and cysteine contents. Ortho-cortex cells contain less matrix material between intermediate filaments and lower sulfur content. On the other hand, para-cortical cells have higher sulfur content.

The cortical cells contain macrofibrils and intermacrofibrillar matrix. The macrofibrils have an approximate diameter of 0.1-0.4  $\mu\text{m}$  and they are composed of microfibrils and intermacrofibrillar matrix [36,13]. Macrofibrils are responsible for the strength of the hair fiber. Intermacrofibrillar matrix is an amorphous structure made from keratin-associated protein (KAP). Keratin-associated protein (KAP) has a molecular weight of 10-25kDa and it contains high concentration of disulfide bonds. Rogers *et al.* [43] state that KAP can be classified into three groups based on its amino acid composition. The high sulfur KAP that contains about 20% cysteine, the ultra high sulfur KAP that contains 30% or more cysteine and 20% serine and the KAP, which, is rich in tyrosine and glycine [43].

The alpha keratins assemble together to form protofilament and protofilament further assembles together to form intermediate filament (microfibrils). The alpha keratin protein has low sulfur content and its molecular weight ranges from 40-60KDa [45,12]. Cytoplasmic and nuclear remnants of the keratinocytes make up the intermacrofibrillar matrix.

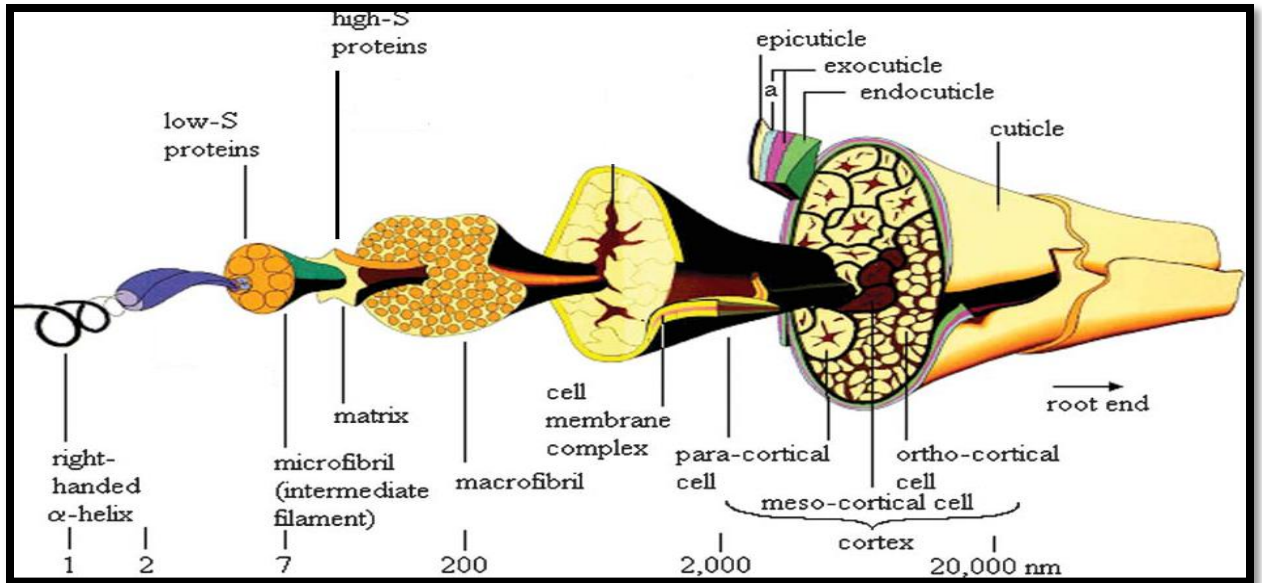


Figure 4: Structure of the alpha-keratin fiber under various magnifications [36]

## 2.4 Keratin based biomaterials for biomedical application

Keratins are cysteine rich insoluble fibrous structural proteins that exhibit high mechanical strength due to a large number of disulfide bonds. These protein are found in hard or filamentous structures such as hair, wool, nail, horns etc [12,13,45,43]. There has been increased investigation on wool and hair keratin based biomaterial in the past ten years because of their biological compatibility and abundance. A. Tachibana *et al.* [49] studied using wool keratin scaffolds for long-term cell cultivation for the first time in 2001. The scaffolds were fabricated by

lyophilization of an aqueous wool keratin solution after controlled freezing, which resulted in a homogeneously porous microstructure with an average pore size of 100 $\mu$ m. Additionally, a long-term cell cultivation study showed that the scaffolds supported the attachment and preosteoblast proliferation of cells [49]. Similarly, Katoh *et al.* [16] investigated the potential of wool keratin for production of scaffold for biomedical application. He used a compression molding and particulate-leaching method to develop S-sulfo keratin sponges with controlled pore size and porosity [16]. The scaffold had more than 90% porosity with pore size ranging from 100-300 $\mu$ m [16].

Recently, the cytocompatibility, in vivo biodegradation and wound healing properties of a keratin biomaterial were investigated by Xu *et al.* [54]. Keratin proteins extracted from human hair were fabricated into three-dimensional porous scaffold by lyophilizing frozen keratin protein solution. Characterization of scaffold was performed using porosity experiments and morphological assessment was made by scanning microscopy, which showed that the scaffold had 90% porosity and highly porous structure with 120 to 220  $\mu$ m pores [54]. Furthermore, in-vivo and in-vitro study showed that the scaffold exhibits good fibroblasts cell compatibility and biodegradability [54].

## **CHAPTER III**

### **MATERIALS AND METHODS**

In this chapter, first, the process of extraction of keratin protein from hair using Shindai method [33] is explained in detail. Second, fabrication of keratin composite scaffolds procedure is discussed. This procedure was developed originally. Third, scaffold swelling, water up taking capacity and porosity measuring procedure is discussed. Fourth, the scaffold's architecture and surface chemistry studying procedure by using SEM is discussed. Fifth, the scaffold's mechanical strength measuring procedure and material is discussed. And lastly, cell proliferation study methods are discussed in detail.



### **3.1 Keratin protein extraction from human hair**

#### *3.1.1 Cleaning and Delipitizing*

Human hair (of a Caucasian woman) collected from a local barbershop, was washed with ethanol and water several times in order to remove any dirt from the surface of the hair. The clean hair was put in a chloroform (Sigma-Aldrich) and methanol solution (2:1) v/v for 24hrs in order to remove any fat from the surface of the hair. The delipidized hair was washed with RO-water and kept in open air overnight to evaporate chloroform and methanol.

#### *3.1.2 Preparation of Buffer solution*

The keratin protein extraction buffer solution was prepared by adding 3.025g of Tris (hydroxymethyl) aminomethane or Tris-base (Fisher), which used as a buffer solution, 197.9g of thiourea (Sigma-Aldrich), 300g of urea (Fisher) and 50ml of 2-mercaptoethanol (Sigma-Aldrich) in 1000ml of deionized water. The pH was then adjusted with 8M HCl (Sigma-Aldrich) to 8.5.

The Tris-HCl was used to keep the keratin extraction buffer solution at a pH of 8.5. Thiourea and urea were used to break down the non-covalent bonds found between polypeptide chains of amino acids. Additionally, 2-Mercaptoethanol was used in order to reduce the disulfide bond found between cysteine.

### 3.1.3 Protein extraction

A delipidized hair (60g) was cut in to smaller pieces with average length around 1mm, followed by mixing it with the 1L buffer solution described above . The solution was juddered by hand for 3min, and kept inside a preheated oven at 50°C for 3 days; the solution was juddered by hand every twelve hours.

A 2 $\mu$ m size filter paper (Whatman) was used to separate the solution containing protein from the cuticle-cortex residue. Then, filtrate was centrifuged at 15,000 $\times$ g (TOMY MX-305) for 20min at room temperature using 50ml centrifuging vial (Flacon), in order to remove small fragments of hair residues. The obtained supernatant was dialyzed against deionized water using SnakeSkin dialysis tubing (Thermo scientific, with a molecular weight cutoff of about 3.5kDa and diameter 16mm). During dialysis the outer water was replaced with deionized water twice a day for four days. Furthermore, the solution that contains proteins started to change its color to milkish because proteins start to aggregate and polymerize. Then dialyzed protein solution having a milkish color was kept in -80°C refrigerator (Thermo Scientific, Revco UXF) for 48hrs. Finally, the frozen protein solution was kept in a lyophilizer (LABCONCO, 4.5 FreeZone) at pressure (3.5pa) and temperature (-48°C) operating conditions for 48hrs. Lastly, 17g of keratin protein powder was recovered.

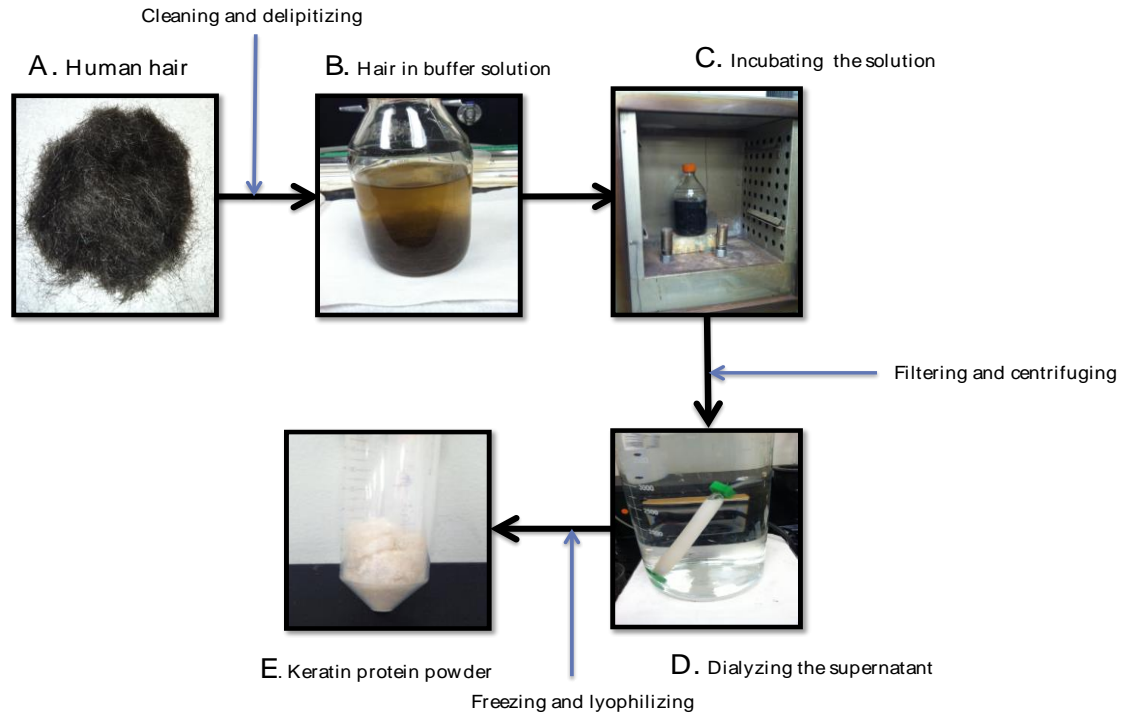


Figure 5: Process of keratin protein extraction from human hair.

### 3.1.4 Sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE)

Protein molecular weight confirmation was performed using gel electrophoresis with 4-20% gradient Tris-HEPES-SDS (Pierce) gel. Samples were prepared in loading buffer containing 1%SDS and heated to 90°C for 5 minutes. Then samples were loaded onto the gels and ran at 90V on a Bio Rad Mini Protean 3 gel box until completion. Staining was performed with gelcode blue reagent (Pierce) for 2 hours then de-stained overnight with RO water.

## **3.2 Fabrication of Keratin scaffolds**

### *3.2.1 Breaking down the hair shaft into cuticle-cortex fragments*

The delipidized hair (2.55g) was cut into smaller pieces of average length approximately 1mm. Then the small pieces of hair were mixed with 400ml of 0.1M NaOH at pH 11.0. Sodium hydroxide was used to make the hair shaft soft by breaking the ionic bond found between polypeptide chains of amino acids. The ionic bond between polypeptides chains of amino acids accounts for 30% of hair shaft strength. Then, the solution was incubated in a preheated oven at 60°C for 24hrs. This was followed by fragmentation of the softened hair shaft into cuticle-cortex fragments by ultrasonic probe (Fisher scientific, Sonic Dismembrator: probe diameter 2.7mm and output power 10watt). Next, the solution was filtered by 112µm size metal sieve to separate the cuticle and cortex residue from the basic solution. Afterward, the cuticle-cortex residue was washed with water for several minutes until the pH was neutral and the recovered cortex and cuticle fragments were stored in 4°C refrigerator.

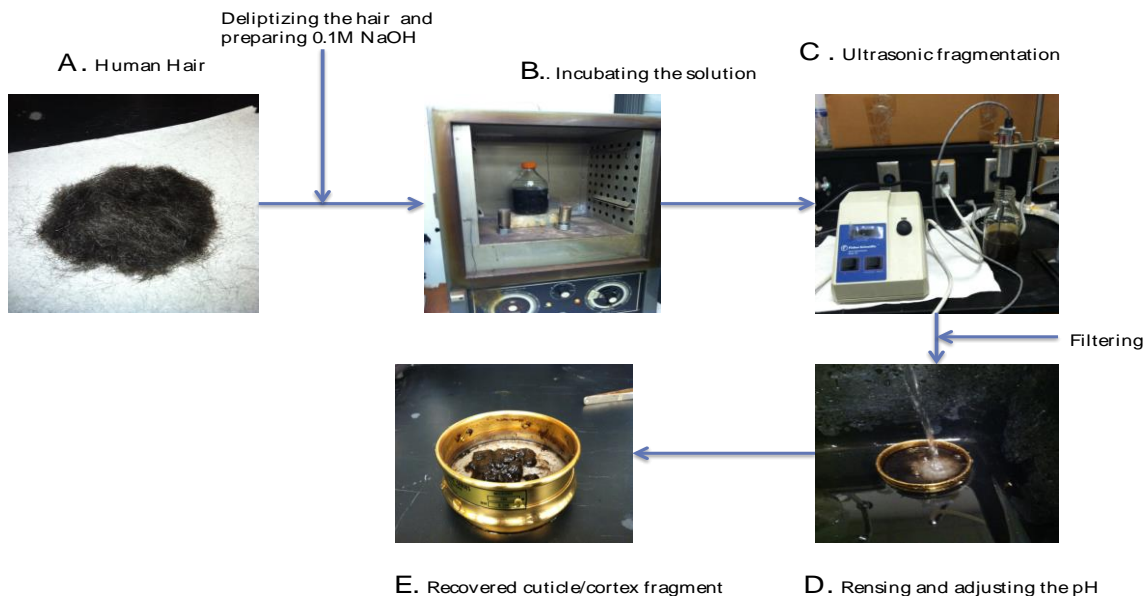


Figure 6: Fragmentation of hair shaft into cuticle-cortex fibers

### 3.2.2 Making the scaffold

A 5%(w/v) keratin solution was prepared by adding 50mg keratin powder in 1ml of keratin extraction buffer solution. Then, 50mg of cuticle-cortex fibers were measured and placed in glass vials. Next, 0.1ml of the 5%(w/v) keratin solution was put into the vials that contain the cuticle-cortex fibers. The mixture was mixed well with a spatula for five minutes. Afterward, 0.1ml of pre-cooled acetone (Sigma-Aldrich) was added into the solution drop by drop, and immediately thereafter the solution was incubated at -20°C refrigerator for one hour and for 30min in 4°C refrigerator. Acetone was used in order to aggregate the protein. A disk shaped cross-linked 3D- structure was recovered.

The recovered disc-shaped structure was washed several times with RO-water and was left overnight in open air. Then, the scaffold was dialyzed against RO-water using snakeskin dialysis tubing (Thermo scientific, with a molecular weight cutoff of about 3.5kDa and diameter 16mm) for five days; the outer water was replaced every day in order to remove acetone and all the chemicals found on the protein extraction buffer. Finally, the dialyzed scaffolds were air-dried and stored under laminar flow hood.

### *3.2.3 Coating the scaffold with Hydroxyapatite (HA)*

A 1%(w/v) HA solution was prepared by adding 0.20g of HA powder (Sigma-Aldrich) in 20ml of RO-water. The solution was then mixed with ultrasonic probe (Fisher scientific, Sonic Dismembrator: probe diameter 2.7mm and output power 10watt) for 10min to form colloids. Then, the three dimensional keratin and cuticle-cortex fibers composite scaffold was placed in a metal sieve with pore size of 115 $\mu$ m and the 20ml solution was poured in to the scaffold. Afterwards, the scaffold was left overnight in an open air.

### **3.3 Swelling and water uptake capability measurement**

The water uptake capacity was measured for both HA, cuticle-cortex and keratin protein (KP) composite scaffold and for cuticle-cortex and KP composite

scaffold. First, the dry weight of the scaffolds was measured on an electronic balance and then the scaffold was placed in PBS of pH 7.4 and kept in a pre-heated oven temperature of 37°C. Every 10min interval for 100min each swollen scaffold was taken out and gently the excess liquid was dried with paper towel and immediately the weight was measured using electronic balance. The experiment was conducted several times using the same conditions. The percentage swelling of the scaffolds at equilibrium was calculated using the following equation:

$$\text{Percentage swelling } (\%) = \frac{(W_{wet} - W_d)}{W_d} \times 100 \dots \dots \dots (1)$$

Where the  $W_d$  is the weight of the dry scaffold and  $W_{wet}$  is the weight of the swollen scaffold.

### **3.4 Porosity measurement**

The porosities of the scaffolds were measured by a liquid displacement method with absolute ethanol (Sigma-Aldrich) as the displacement liquid. First the geometrical volumes of the disc shape scaffolds were calculated by measuring the diameter and height. Second, the scaffold's dry weight was measured. Third, the scaffold was placed in vacuum Erlenmeyer flask and 30ml of absolute ethanol was added and the flask was closed and vacuum was applied for 5 min to remove air bubbles from the pores of the scaffold. Forth, the samples were taken out and

weighed immediately. The porosity of the scaffold was calculated according to the following equation:

$$V_p = \frac{(W_e - W_d)}{\rho_e} \dots \dots \dots (2)$$

$$\phi = \frac{V_p}{V_s} \times 100 \dots \dots \dots (3)$$

where  $w_e$  weight of the scaffold after it is immersed in absolute ethanol,  $W_d$  is the dry weight of the scaffold,  $\rho_e$  is the density of the ethanol,  $V_p$  is the volume of the pores and  $V_s$  is geometrical volume of the scaffold and  $\phi$  is porosity.

### **3.5 Scaffold architecture, surface property and elemental analysis examination**

The microstructure and surface property of the scaffolds were examined by scanning electron microscopy. To prepare samples for SEM images, scaffolds were prepared as above (3.2.2). All the sample scaffolds were mounted on carbon tabbed stubs and air gun was used to blow any dust from the sample then samples were placed in the SEM. The samples were imaged in the SEM under vacuum at a voltage of 10kve and dwell time of 3 $\mu$ s.

The elemental analysis on the surface of the scaffold was performed by Energy-dispersive X-ray spectroscopy (EDX).



### 3.6 Mechanical Tests

Mechanical characterizations of compression property of the scaffolds were performed on an Instron testing frame equipped with a 0.1kN load cell (At College of Polymer Science and Polymer Engineering, University of Akron. Dr. Mark Holman, holtman@uakron.edu) Compression tests were carried out on several scaffolds with approximately 9.7 mm diameters and 4.5 mm heights (figure 7) at room temperature. The scaffolds were tested in dry and wet conditions. Wet scaffolds were prepared by submerging in phosphate buffer saline solution for 48 hrs. Slope of the stress versus the strain curve, which represents the compressive Young's modulus, was calculated for all samples. Compression tests were also performed to varying strains. Samples were compressed 10%, 20% and 30% from the original height.

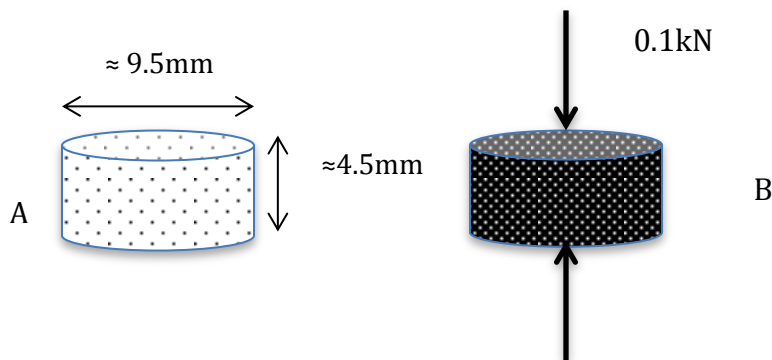


Figure 7: Scaffolds dimensions (A). Compression test on scaffolds using a 0.1kN (B).

### 3.7 Coating tissue culture plate with KP and HA

Human hair keratin protein coated tissue cultures were prepared by adding 16 mg of lyophilized protein powder in to wells of 48-well tissue culture plate

followed by adding 0.3 ml of autoclaved RO-water. The formed mixtures were stirred for several minutes to ensure uniform suspension. Then, the plates were kept at room temperature for 96hrs to form a thick Keratin protein layer. Similarly, tissue culture plates were coated with hydroxyapatite and keratin protein. 8 mg of keratin protein and 8 mg of HA were mixed with 0.3 ml of autoclaved RO- water inside wells of 48-well tissue culture plate. The formed solution was mixed with spatula for several minutes and placed at room temperature for 96 hrs. Finally, the coated plates were washed several times with absolute ethanol and autoclaved water and sterilized using UV radiation for overnight by keeping them in laminar flow hood.

### **3.8 Cell culture**

#### *3.8.1 Growth Media Preparation*

Growth media is a mixture of different solutions that consist of all the important ingredients needed for cell survival and growth. In this research, the media was tailor made for the type of cell line that was used in the experiment. GFP-stromal cells from bone marrow of rat were used for all the experiments.

The media was prepared by adding together 10ml 1 molar HEPES at pH 7, 50ml of Fetal bovine serum (FBS), 5 ml of non-essential amino acid, 5 ml of L-

glutamine and 5ml pen-strep into 430 ml of minimum essential media alpha mix (α-MEM) without ribonucleosides and deoxyribonucleosides solution.

HEPES is zwitterionic organic chemical buffering agent that helps to maintain the physiological pH in the media in spite of changes in the concentration of the carbon dioxide produced by cellular respiration. FBS is the most widely used growth serum supplement for the eukaryotic cell culturing in vitro. It contains nutrients, low level of antibodies, higher growth and attachment factor and protects cells from oxidative damage and apoptosis. Non-essential amino helps to increase cell growth and viability. Penicillin-Streptomycin is an antibiotic used to prevent and inhibit bacterial growth in cell cultures. L-glutamine is used as an amino acid supplement for cell culture media. It improves cell viability and growth by increasing media stability, minimizing toxic amino acid buildup, and maximizing cell performance.

### *3.8.2 Thawing cells*

A cryovial, which contains low passaged frozen GFP cells and 2ml of 10% DMSO and 90% FBS solution, was retrieved from a nitrogen tank. Immediately the cryovial was suspended in warm water bath until the solution was liquefied. Then the solution was instantly transferred into a centrifuge tube that contains 10ml of growth media and centrifuged for 10 min at 800 RPM in a swinging bucket rotor. Supernatant was discarded and the recovered cell pellet was broken by gently raking the centrifuge tube over Styrofoam rake. Afterward, cells were re-suspended

in 5ml growth media and placed in a T75 tissue culture flask and additional 10ml of media was added and incubated in a humid environment at 37°C, 5% CO<sub>2</sub>.

### *3.8.3 Passaging GFP cells*

Cell attachments, shape and contamination were observed under microscope. When 70-80% of cells were attached to the bottom surface of the flask; media was aspirated and flask was washed with 10 ml hank's balanced salt solution (HBSS) to wash any remaining media. This was followed by trypsinization of the Cells by adding 10 ml of 1x trypsin and incubating it for 5 minutes in the incubator. Then the flask was removed and taped on the side and placed back in the incubator for additional 4 minutes. Flask was observed in inverted microscope to make sure cells were detached. Immediately cells were transferred into 50 ml centrifuge tube, which contains 10ml of media and centrifuged at 800 rpm for 10 minutes. Supernatant was discarded and cell pellet was broken by gently raking the centrifuge tube over Styrofoam rake. Afterward, cells were re-suspended in 5 ml growth media. Finally, a sample of cell suspension was diluted with a filtered 0.4% trypan-blue solution and pipetted onto the hemcytometer in order to perform viable and non-viable cells count. The non-viable cells were blue and the viable cells were unstained. Three samples were counted and averaged to determine the cell density in 5ml of cell suspension.

### 3.9 Cell Proliferation Study

#### Summary

Cell proliferation was studied on the two composite scaffolds, which are keratin protein, and cuticle-cortex fibers composite scaffolds (SCAF-1) and keratin protein, cuticle-cortex fibers and hydroxyapatite composite scaffolds (SCAF-2). And on keratin protein coted and hydroxyapatite and keratin protein coated 48-well tissue culture plate. The experiment was set up for 10 days along with the control group. Samples were examined for cellular activity using WST-1 assay kit at a selected culture time interval (2, 6, 10 days). WST-1 assay is a colorimetric assay used for assessing cell viability and cell proliferation. WST-1 cell proliferation assay kit works based on the enzymatic cleavage of the tetrazolium salt WST-1 to formazan by cellular mitochondrial dehydrogenase present in viable cells (figure 6)[23]. During the dehydrogenase process the salt will change its color from pale yellow to dark yellow. The dark yellow formazan absorbance is measured at 450-480 nm and it is directly correlated to cell number.

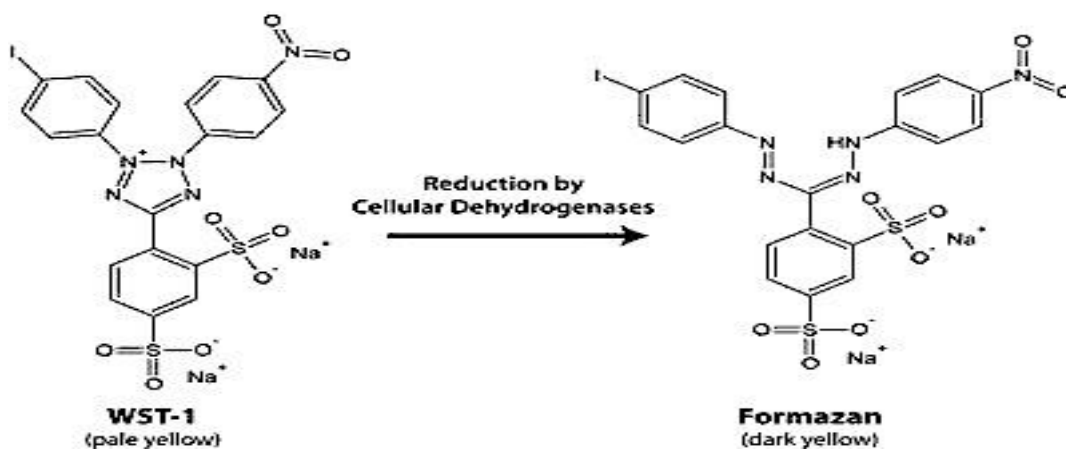


Figure 8: Cleavage of the tetrazolium salt WST-1 to formazan [23].

### *3.9.1 Sample preparation and Sterilization*

Three keratin protein and cuticle composite scaffolds and three keratin protein and cuticle composite scaffolds coated with hydroxyapatite discs were prepared with approximate diameter of 9.5 mm and thickness of 4.5 mm following the protocol mentioned in section 3.2 and 3.2.3. Then, the disks were sterilized with 99.99%(v/v) ethanol and rinsed several times with autoclaved PBS and autoclaved RO-water. Two scaffolds, one with HA the other without, were placed in one 48-well tissue culture plate. In the same manner the rest of the scaffolds were placed in two 48-well tissue culture plates. Finally, they were kept under UV light for 12 hours before they were used for the experiment.

Similarly, two wells on opposite corners of one 48-well tissue culture plate were coated; one with keratin protein and the other with keratin and HA following the protocol mentioned in section 3.7. Following this similar protocol additional two plates were prepared. The wells were washed with absolute ethanol and rinsed with autoclaved RO-water and autoclaved PBS solution in order to be sterilized. Lastly, they were kept under UV light uncovered for 12 hours before they were used for the experiment.

### 3.9.2 Seeding the scaffolds and the coated plates with cells

GFP-bone marrow stromal cells in culture medium were seeded on scaffolds (10,000 cells/scaffold) in 48-well tissue culture plate. Briefly, low passage GFP stromal cells were harvested from T-75 tissue culture flask by following the protocol mentioned on section 3.8.3. Based on the cell number calculation, the suspension volume amount for 10,000 cells was determined to be 38 $\mu$ l. In order to achieve a uniform distribution of cells on the scaffolds and on the coated surfaces during seeding the cell suspension volume was mixed with 262  $\mu$ l of cell culture media in microcentrifuge tubes. The tubes were gently shaken with hand to insure a uniform distribution of cells. Then, the suspension was pipetted into the scaffolds. This was followed by the addition of 200  $\mu$ l media in each which were then incubated in a humidified incubator at 37°C and 5% CO<sub>2</sub>. Furthermore, the media was changed every two days. With the same protocol mentioned above coated plates and control groups also were seeded.

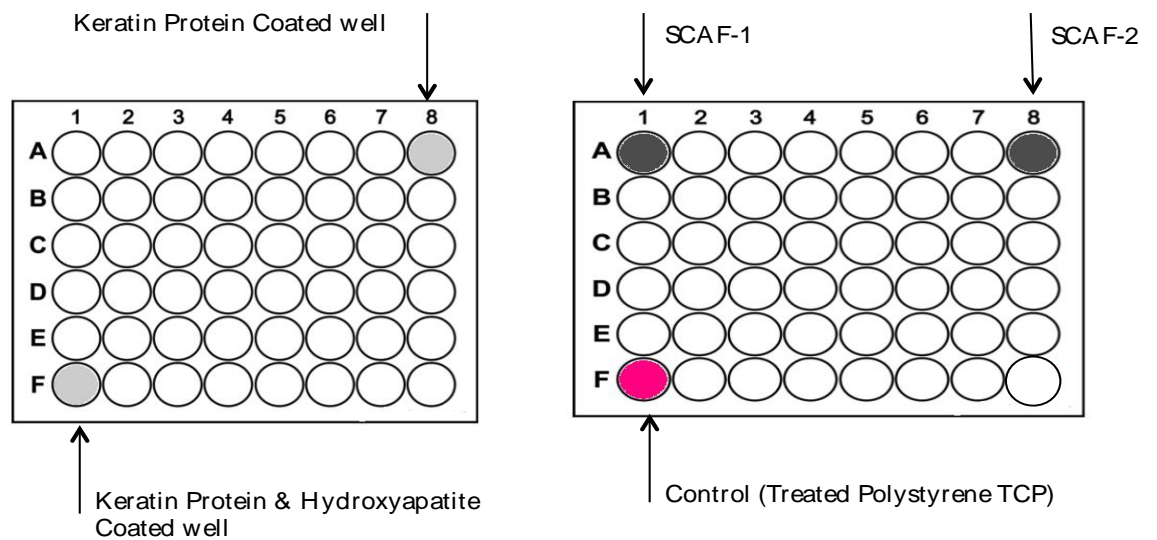


Figure 9: Experiment setup in two 48 well plates. (Three plates samples)

### *3.9.3 Cell proliferation assay*

WST-1, assay was performed to observe viable cells on the scaffolds and coated plates. For this purpose, seeded scaffolds and coated plates in 48 well tissue culture plate were taken out in 96 hours interval. Then 50 $\mu$ l of wst-1 solution was injected into each well and the plate was incubated for 4 hours (37 °C air 5 % CO<sub>2</sub>). However, every hour the plate was gently shaken by hand for few seconds and placed back in the incubator. Finally, 200  $\mu$ l supernatant solution was removed and transferred into 96-well tissue culture plate and the absorbance was recorded at 480nm on a Synergy H1 reader (BioTek).

### *3.9.4 Preparing calibration curve for WST-1 assay*

Calibration curve was prepared to determine the correlation between absorbance reading and the cell number. Different cell numbers ranging from 12,00 cells per well to 40,000 cells per well were seeded in a 48 tissue culture plate and media was added with a final volume of 500  $\mu$ l and cultured for 24 hrs. Then 50  $\mu$ l of wst-1 solution was added to each well and the plate was further incubated for 4hrs. Finally, 200  $\mu$ l supernatant solution was removed and transferred into 96-well tissue culture plate and the absorbance was recorded at 480nm on a Synergy H1 reader (BioTek). The experiment was repeated three times and the average value was used to create the calibration curve.



## **Chapter IV**

### **Results and Discussion**

#### **4.1 Results**

##### *4.1.1 SEM Image of hair shaft*

Scanning Electron Microscope (SEM) image of hair shaft before extraction and after is shown in figure 10 (A and B). The images were taken at high magnification and 5KV. The images portray that the hair shaft loses its organized structural strength after the protein is extracted.

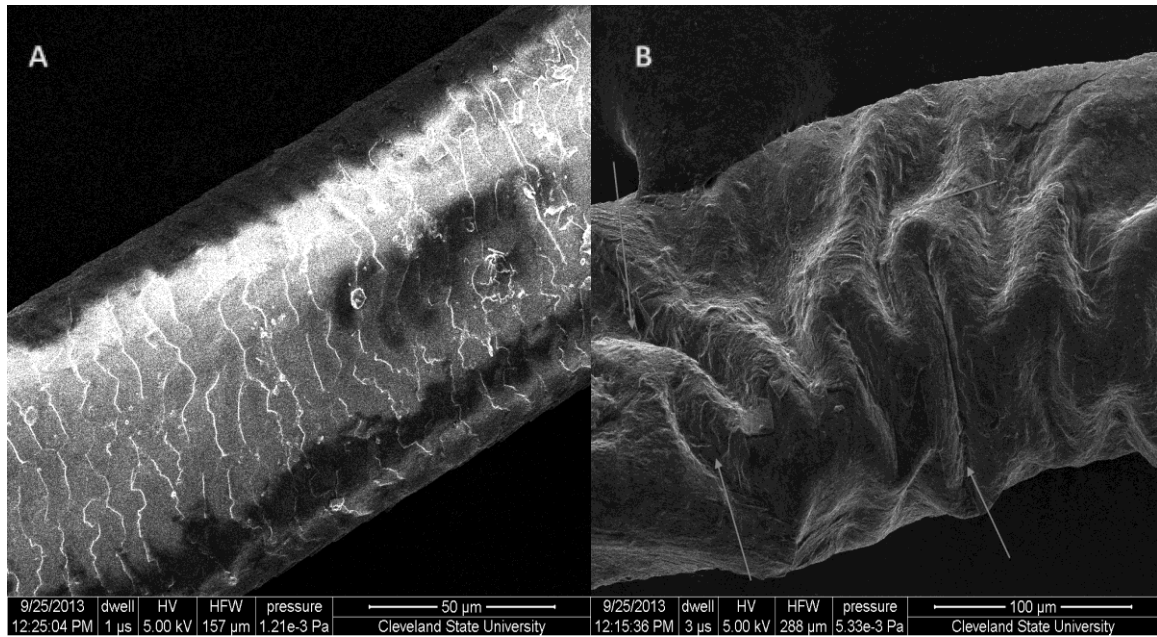


Figure 10: SEM image of human hair shaft before extraction (A) and after extraction (B)

#### 4.1.2 Identification of extracted keratin

The obtained proteins were analyzed by SDS-PAGE. The SDS-PAGE result shows the extracted protein samples have molecular weight range between 38 kDa to 52 kDa as shown on fig (11). Lane 1 on Fig 4.1 shows a molecular weight ladder and lane 2 and 3 are the extracted protein samples both showing the same molecular weight distribution.

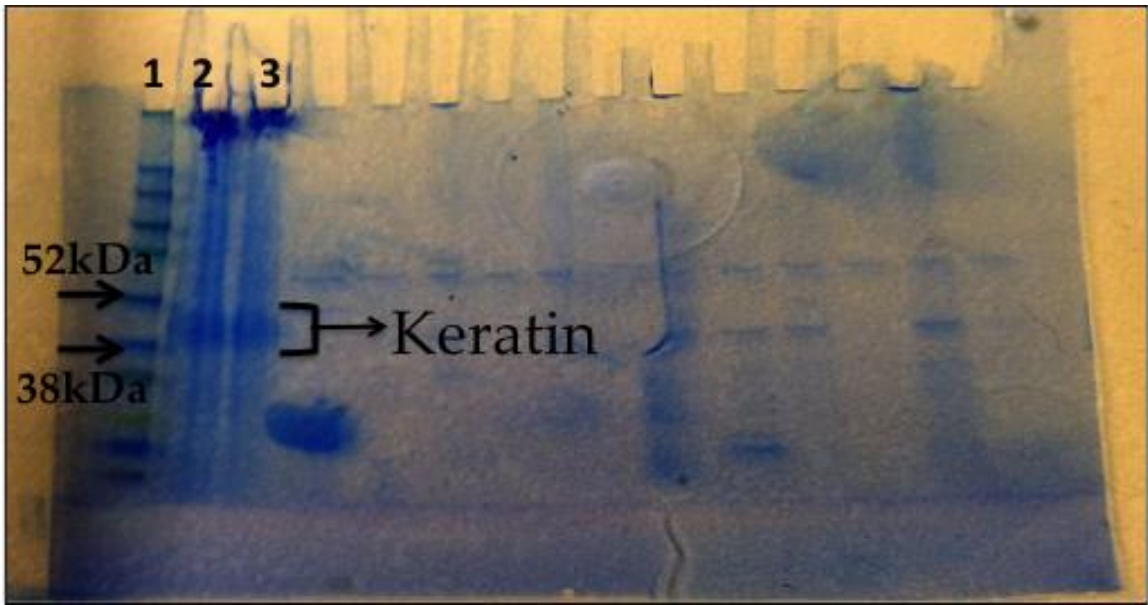


Figure 11: Identification of extracted human hair keratins. SDS-PAGE: LANE 1- molecular weight marker, lane 2 and 3 - Human hair keratin.

#### 4.1.3 Scaffold Fabrication

Scaffolds were prepared following the protocol mentioned in section 3.2.2.

Two composite scaffolds were prepared from keratin protein, cuticle-cortex fiber and HA. Table 1.1 shows the composition of these scaffolds.

<b>SCAF-1</b>	30 mg cuticle-cortex fragment + 0.1 ml of 5%(w/v) keratin protein
<b>SCAF-2</b>	30 mg cuticle-cortex fragment + 0.1 ml of 5%(w/v) keratin protein + 1% (w/v) Hydroxyapatite (HA) solution

Table 4.1 Composition of prepared scaffolds

#### *4.1.4 Scanning Electron Microscopy and Elemental analysis*

##### *4.1.4.1 SEM image of keratin protein and cuticle-cortex fibers scaffold (SCAF-1)*

SEM image of the scaffold was taken in different magnification at low voltage. Figure 12 represents SEM image of keratin protein (KP) and cuticle scaffold (SCAF-1) at different magnification. Figure 12 (A and B) shows a crossed linked three-dimensional pore structure of the scaffold at lower magnification with a pore size range from 50- 500  $\mu\text{m}$ , where the KP appears to have successfully glued the SCAF fibers. Figure 12 (C and D), Illustrates the same scaffold with higher magnification on the cross-linked cuticle-cortex fibers. The fibers are cross-linked by keratin protein that was extracted from the human hair. Figure 12 (E and F), shows the surface of the cuticle-cortex fibers at higher magnification.

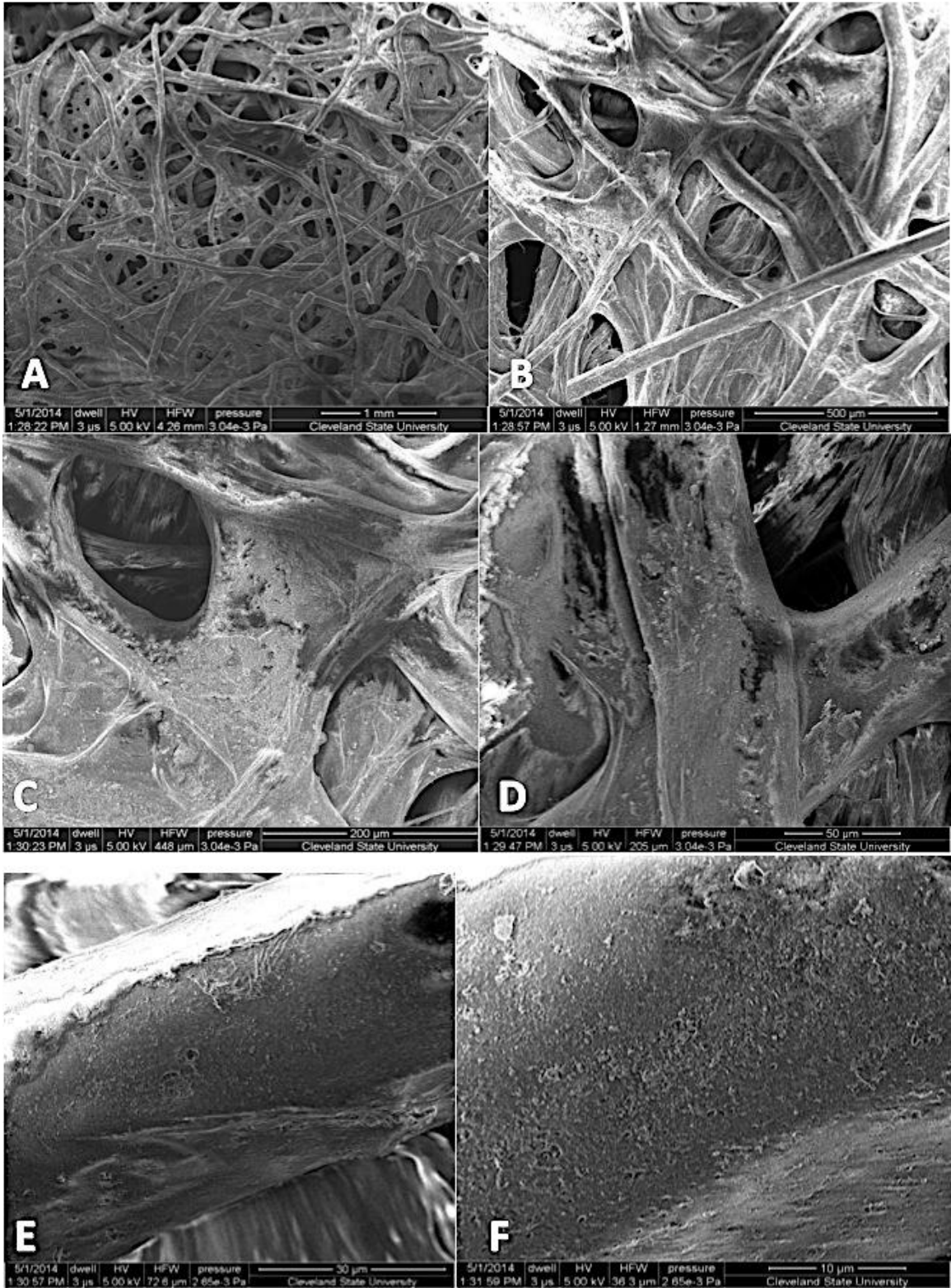


Figure 12: SEM image of keratin protein and cuticle-cortex fiber composite scaffold (SCAF-1) at different magnification.

#### *4.1.4.2 Elemental analyses on the surface of keratin and cuticle-cortex fibers composite scaffold (SCAF-1)*

Elemental analysis was performed on the surface of the scaffold by using energy-dispersive X-ray spectroscopy (EDX). Figure 13 (A) shows the SEM image of the scaffold where the elemental analysis was performed. The elemental distributions are displayed on figure 13(B, C and D). Figure 13(B) shows the distribution of carbon, Figure 13(C) shows the distribution of nitrogen and figure 13(D) shows the distribution of oxygen. As it was expected the elements that were detected are the main building blocks of keratin protein and cuticle-cortex fibers. In addition, the elemental mapping shows the elements are uniformly scattered through out the scaffold and they are both located on the same place. The weight percentage composition of the elements is represented on figure 14. It shows that carbon has the highest weight percent of 54(%wt.) followed by Oxygen 27.2(%wt.) and Nitrogen 18.6(% wt.).

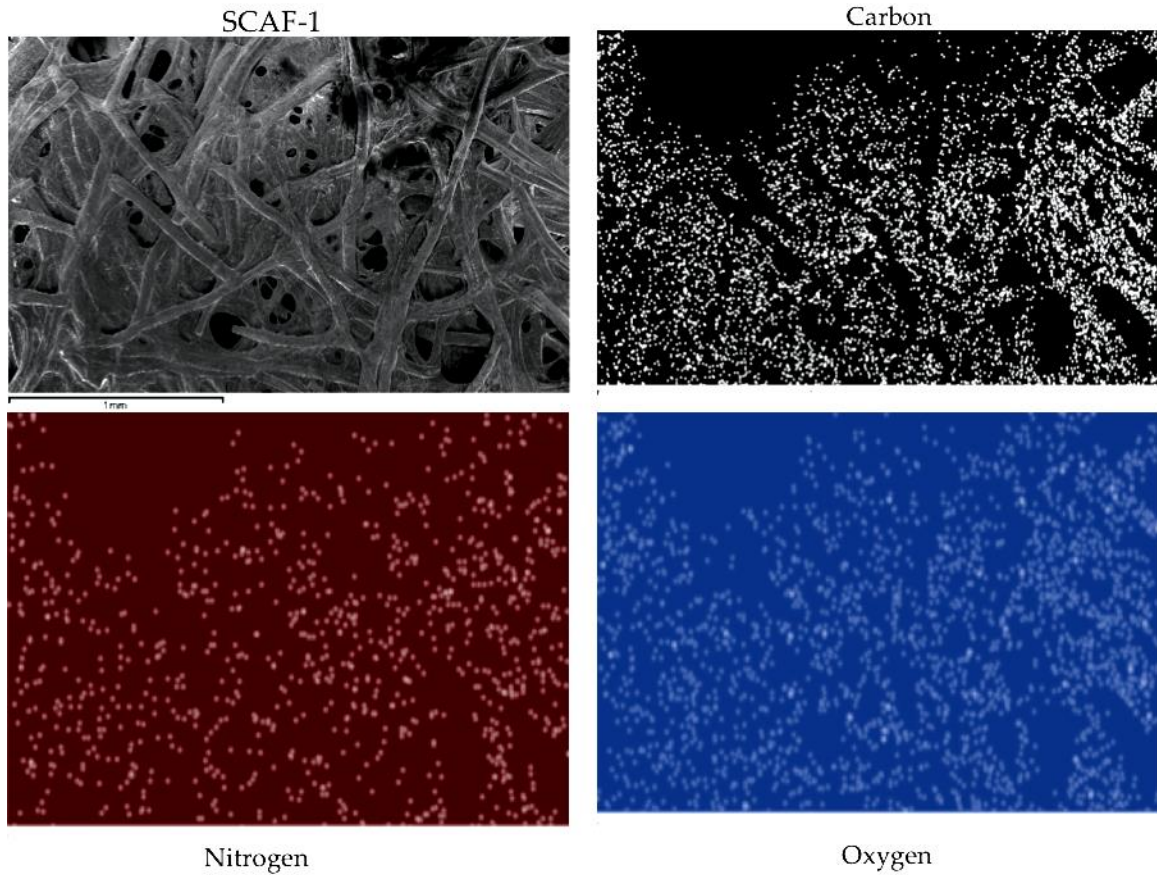


Figure 13: SEM image of SCAF-1 and image of elemental distribution

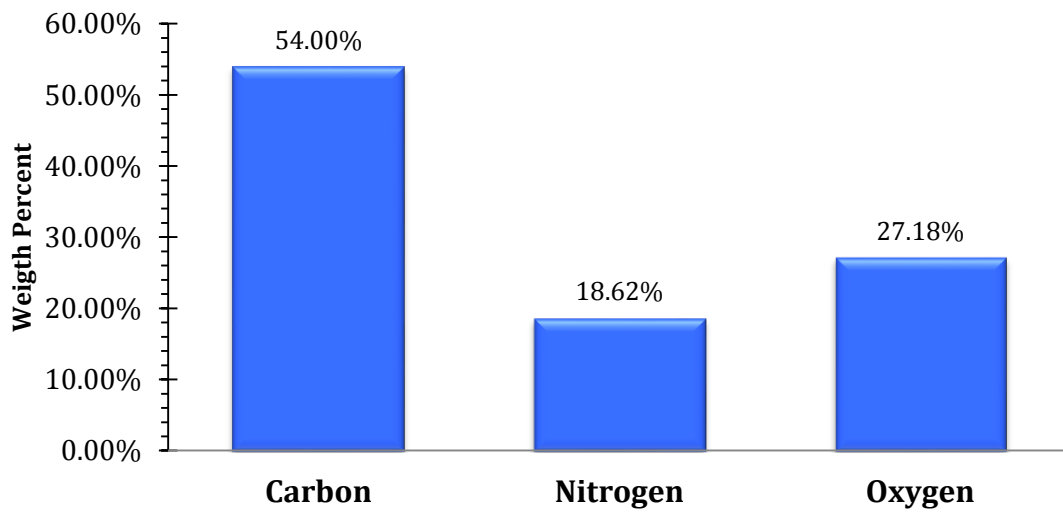


Figure 14: Weight percent distribution of the elements on the surface of SCAF-1

#### *4.1.4.3 SEM image of keratin protein (KP), cuticle-cortex fibers and hydroxyapatite (HA) scaffold (SCAF-2)*

SEM image of the scaffold was taken at different magnification and at 5kilo-volt. Figure (15) shows SEM image of keratin protein (KP), cuticle-cortex fiber and HA composite scaffold (SCAF-2) at different magnifications. Figure 15(A and B) show a crossed linked three-dimensional pore structure of the scaffold at lower magnification with a pore size range of 40- 400  $\mu\text{m}$ . Figure 15 (C and D), show that the hydroxyapatite forms a layer on top of the cross-linked cuticle-cortex fibers. Further magnified images on the surface of the fibers are shown in figure 15 (E and F). The image displays the three-dimensional morphology of HA coating particles on the fibers.



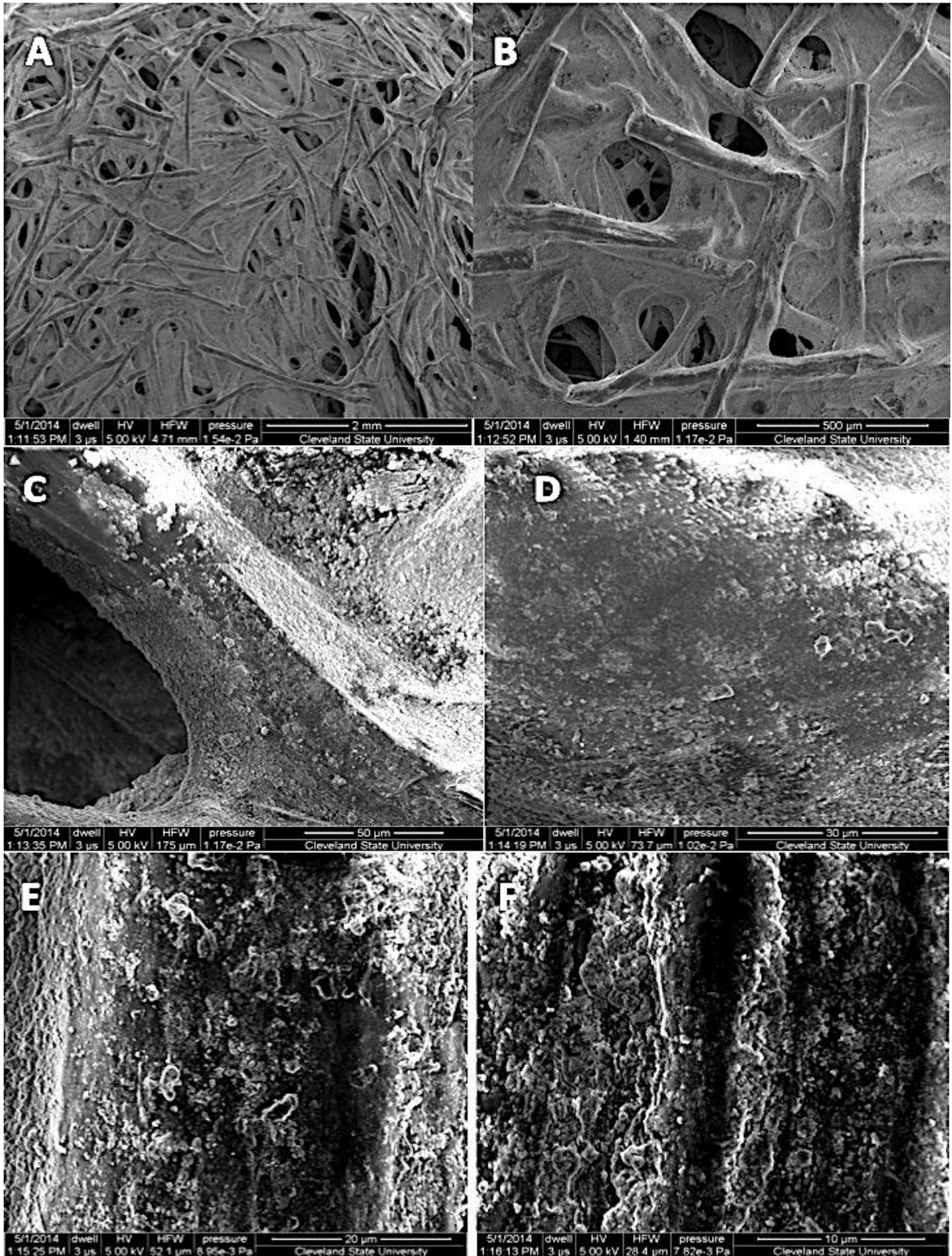


Figure 15: SEM image of keratin protein (KP), cuticle-cortex fibers and hydroxyapatite (HA) composite scaffold (SCAF-2).

#### *4.1.4.4 Elemental analyses on the surface of keratin and cuticle-cortex fibers composite scaffold (SCAF-2)*

##### **I. Before PBS**

Elemental analysis was performed on the surface of the scaffold by using energy-dispersive X-ray spectroscopy (EDX). The elemental distributions are displayed on figure 16 (B, C, D, E, F, and G). Weight percent compositions of the elements are represented on figure 17. Figure 16 (A) shows the SEM image of the scaffold where the elemental analysis was performed. Results from the elemental analysis show that the surface of the scaffold is uniformly coated with hydroxyapatite. Since the elemental compositions for HA were phosphorus and calcium, we were able to detect the presence of these elements throughout the scaffold surface. The weight percent ratio between calcium to phosphorus (Ca: P) was found to be 1.5:1. Furthermore, the elemental mapping shows all the elements are uniformly scattered throughout the scaffold. Calcium and phosphorus are co-located on the same area of the scaffold.

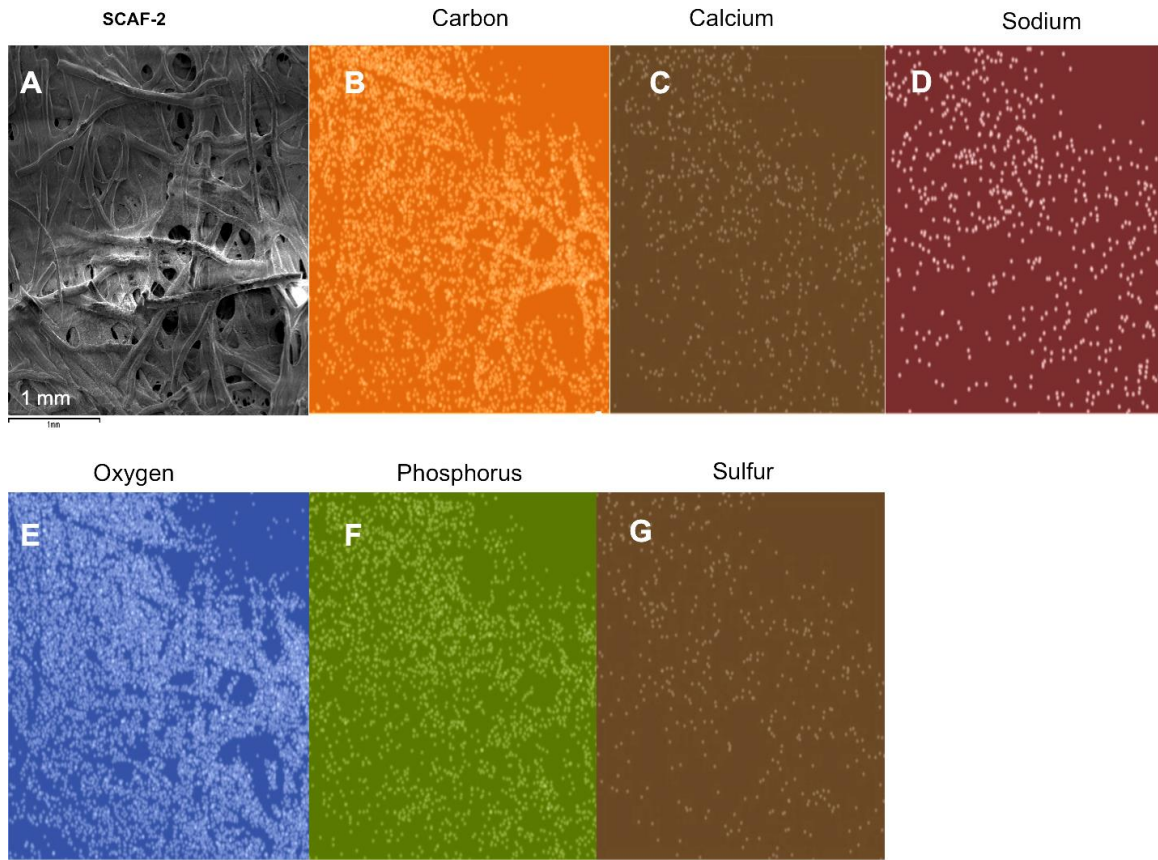


Figure 16: SEM image of SCAF-2 and image of elemental distribution.

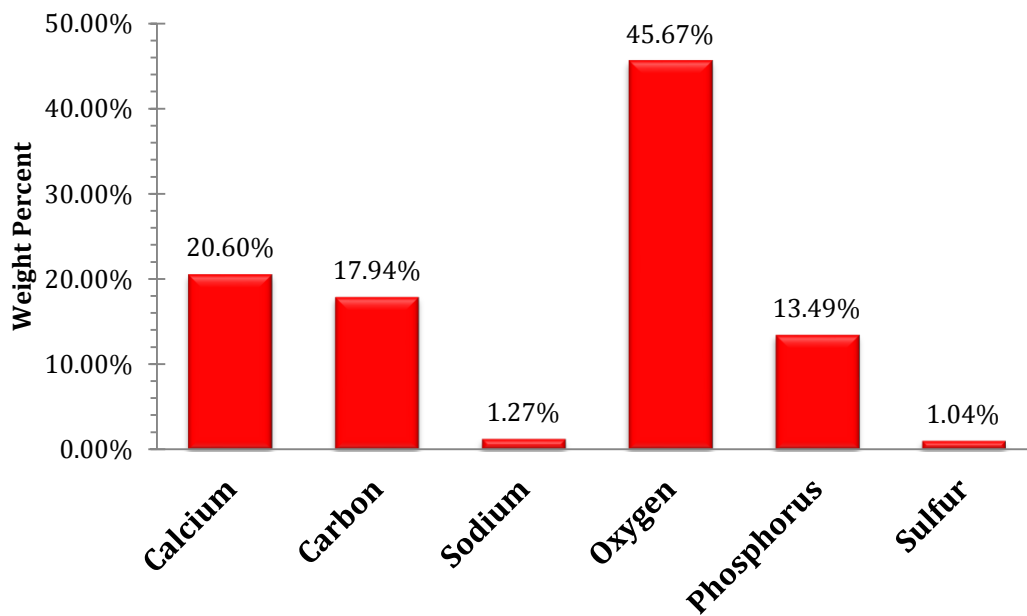


Figure 17: Weight percent distribution of the elements on the surface of SCAF-2.

## II. After PBS

In order to analyze whether HA will stay on the surface of the scaffold when the scaffold is in contact with biological fluids, scaffold coated with HA was kept in PBS for 72hrs at 37<sup>0</sup>c. After 72 hrs, the scaffold was removed from the solution and kept in an open-air overnight to dry. SEM image at higher magnification and elemental analysis was taken in order to see if there was any surface morphology change. The result from the elemental analysis is shown on figure (18 and 19). Figure 18 (A, B, C, D, E, F, G and H) shows the elemental distribution and figure 19 shows the weight percent distribution. The elemental analysis result shows that HA did not wash-out; calcium and phosphorus are still present on the surface of the scaffold. Moreover, the scaffold didn't lose its three-dimensional micro porous structure and the elements were distributed uniformly on the surface of the scaffold. However, small trace of sodium chloride was detected on the surface of the scaffold, which comes from the buffer solution.

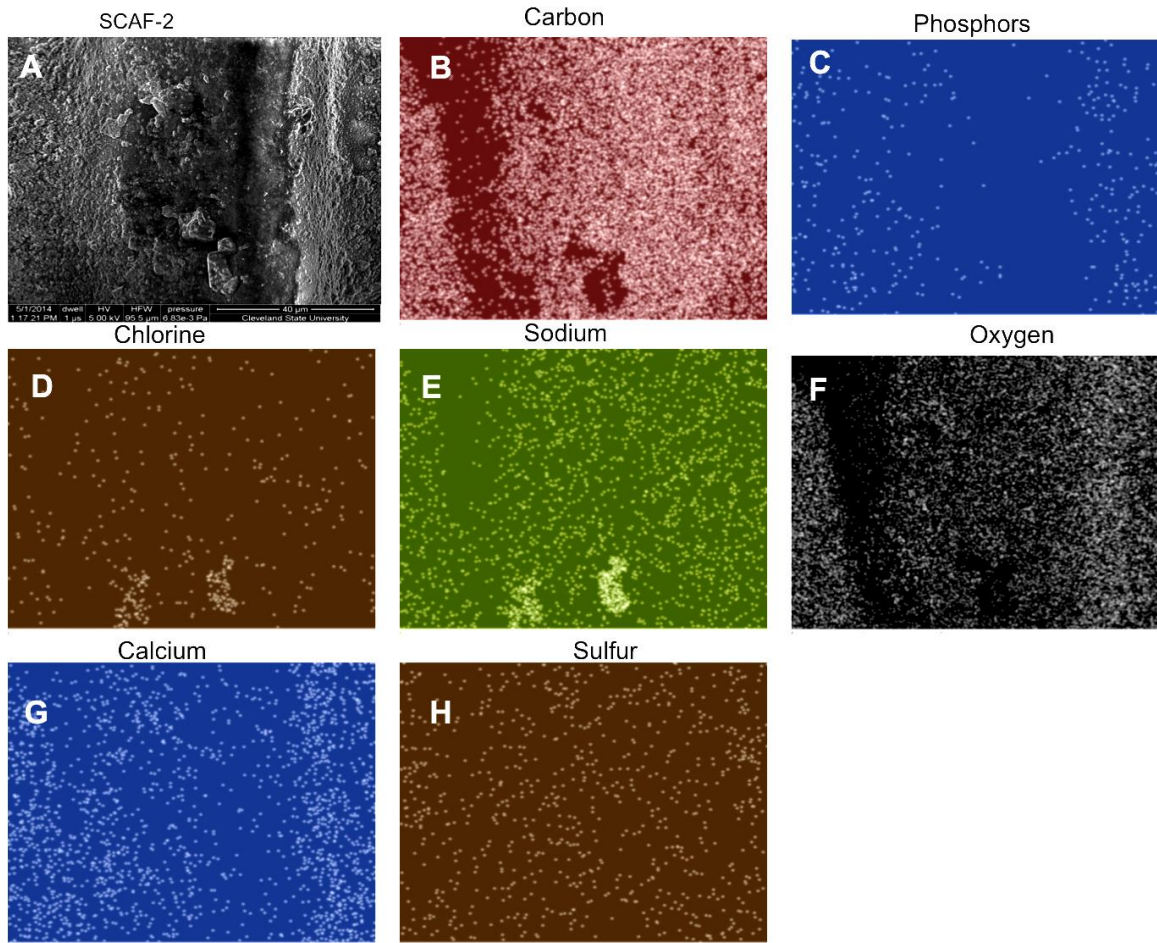


Figure 18: SEM image of SCAF-2 and image of elemental distribution

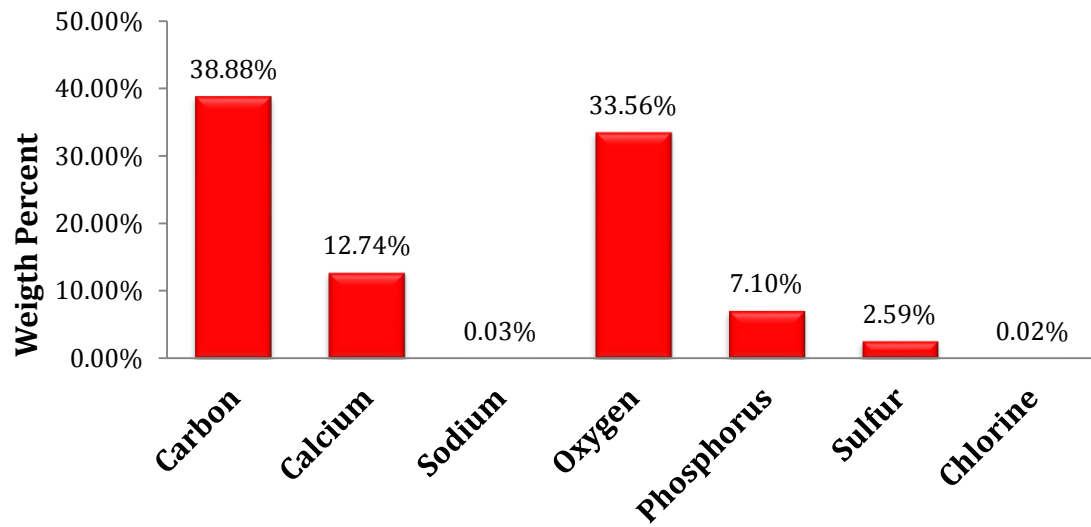


Figure 19: Weight percent distribution of the elements on the surface of SCAF-2.

#### 4.1.5 Porosity

The porosities of the scaffolds were measured by a liquid displacement method with absolute ethanol as the displacement liquid. Results of porosity measurements illustrated in figure 20 are the average values obtained from three replicates. As expected the scaffold composed of hydroxyapatite; keratin protein and cuticle-cortex fibers (SCAF-2) have lower porosity than a scaffold composed of keratin protein and cuticle-cortex fibers (SCAF-1). It is because the pore's volume of SCAF-2 was reduced when the scaffold was coated with hydroxyapatite. The average porosity percentage of SCAF-1 and SCAF-2 is  $70 \pm 10\%$  and  $51 \pm 3\%$ , respectively.

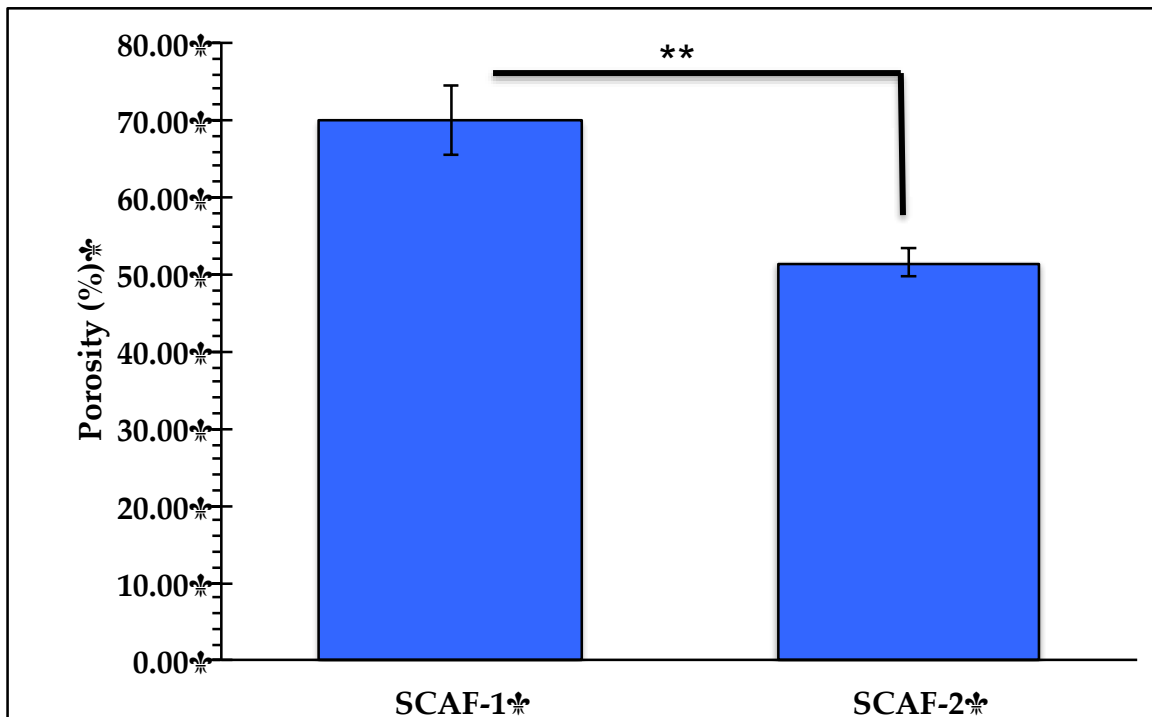


Figure 20: Porosity of the scaffolds. Data are presented as the average  $\pm$  standard deviation (n=3, \*\*p< 0.05)



#### 4.1.6 Swelling and Water uptake capability

Figure 21 represents the swelling behaviors of both SCAF-1 and SCAF-2 in pH 7.4 PBS at 37°C. A rapid swelling occurred during the early soaking of 10 minutes, but after 40 minutes the swelling tendency evidently attenuated for both scaffolds. No significant difference was observed between the two scaffolds. The water uptake percentage of SCAF-1 and SCAF-2 at 90 min is  $152\pm 7\%$  and  $155\pm 10\%$ , respectively.

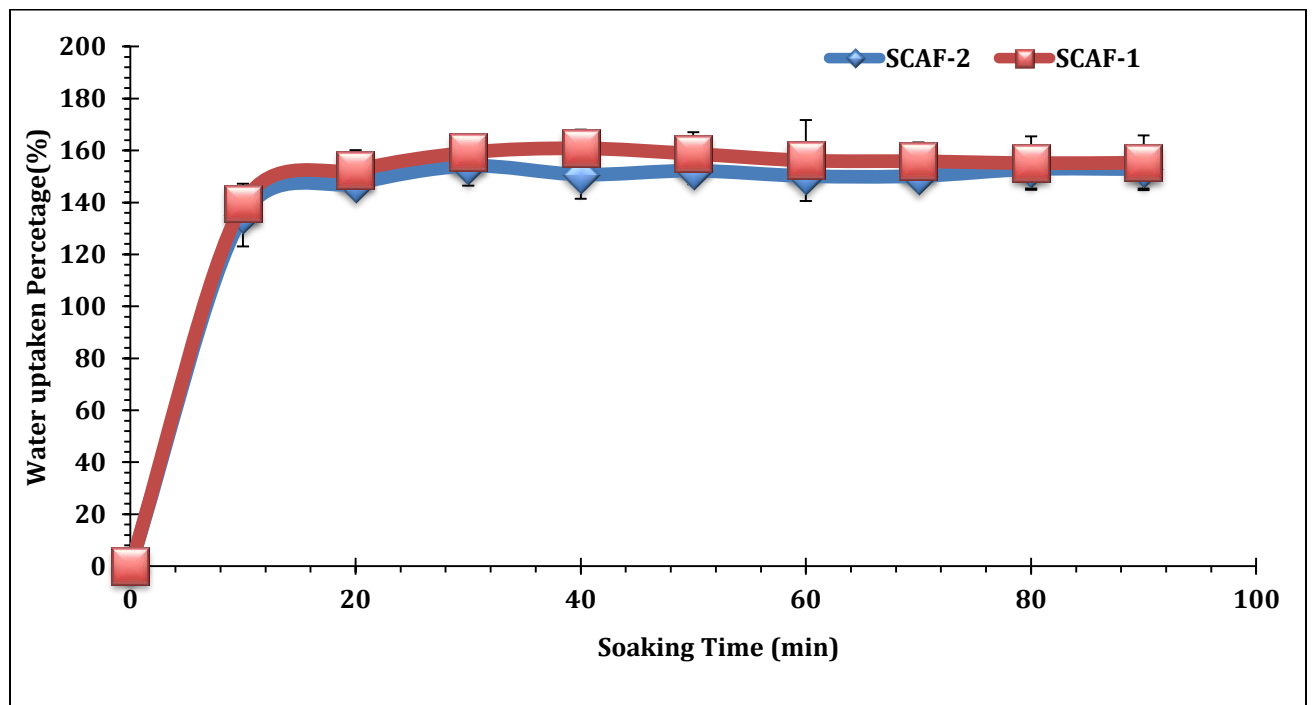
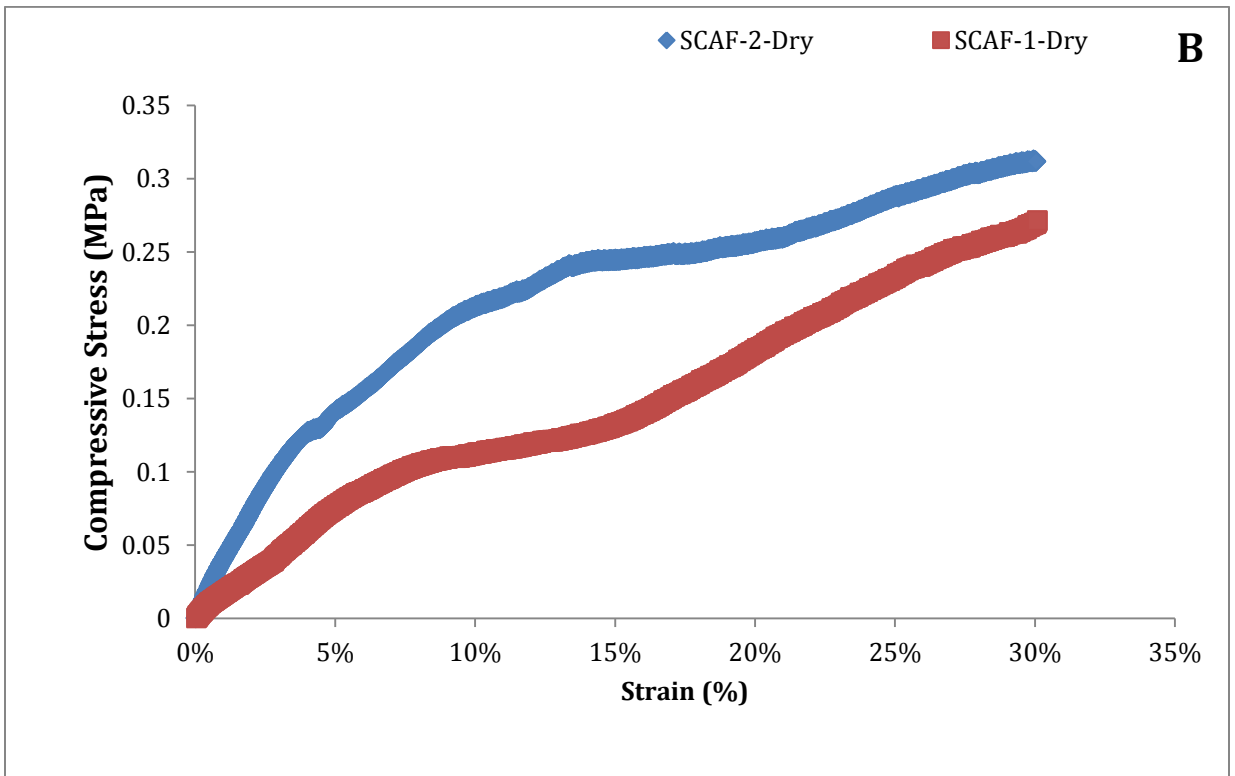
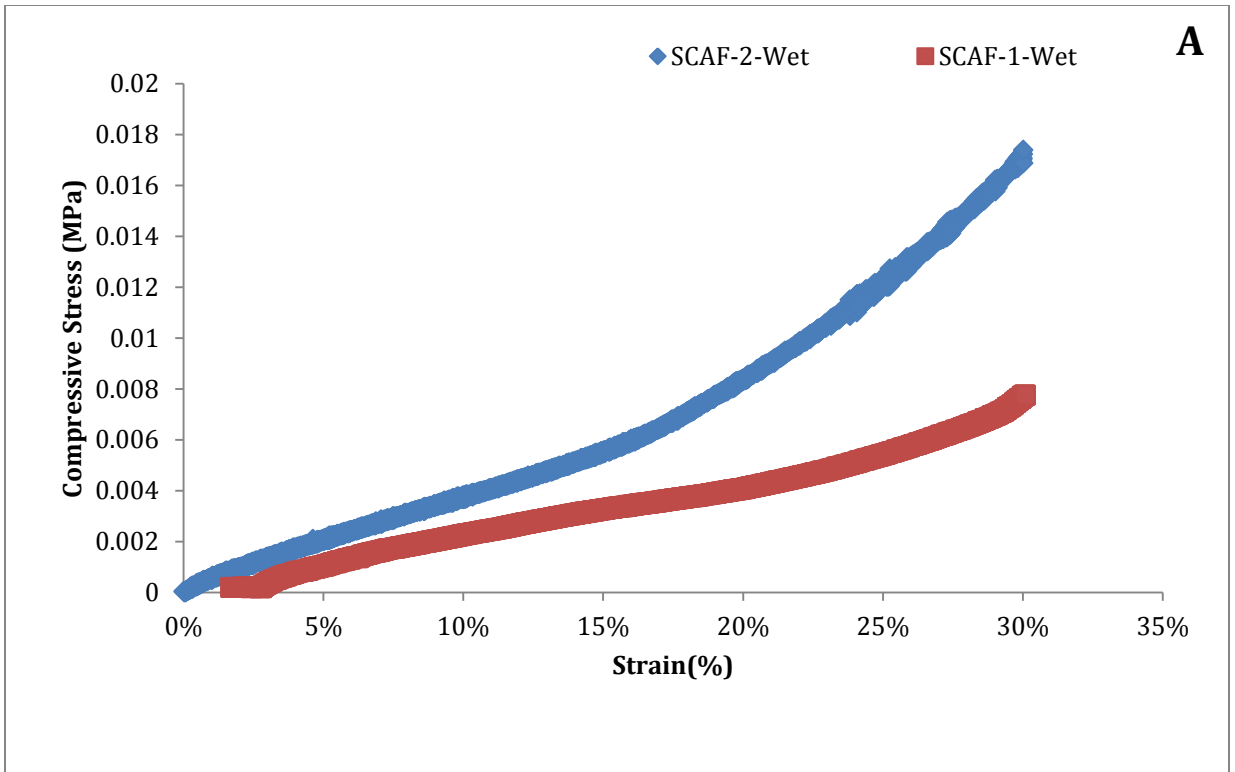


Figure 21: Swelling curves of SCAF-1 and SCAF-2 in pH 7.4 PBS at 37°C. Data from 3 sample replicates are shown (n=3, mean  $\pm$  SD, were  $P \geq 0.05$ )

#### *4.1.7 Mechanical strength*

Mechanical properties of all composition scaffolds were tested under compression in dry and wet condition. The compressive strength of scaffolds in two conditions is shown on figure 22. Wet-SCAF-1 and Wet-SCAF-2 have compressive stress of 0.008 MPa and 0.012 MPa consecutively at thirty percent strain. On the other hand Dry-SCAF-1 and Dry-SCAF-2 have compressive strength of 0.27MPa and 0.31MPa at thirty percent strain. Compressive moduli were calculated at the initial linear slope of stress-strain graph for each sample. The compressive moduli obtained from static tests of both SCAF-1 and SCAF-2 are summarized in Figure. SCAF-1 has compressive moduli of 0.009 MPa in wet condition and 0.90MPa in dry condition. Likewise, SCAF-2 has compressive moduli of 0.09 MPa at wet condition and 2.7MPa at dry condition.





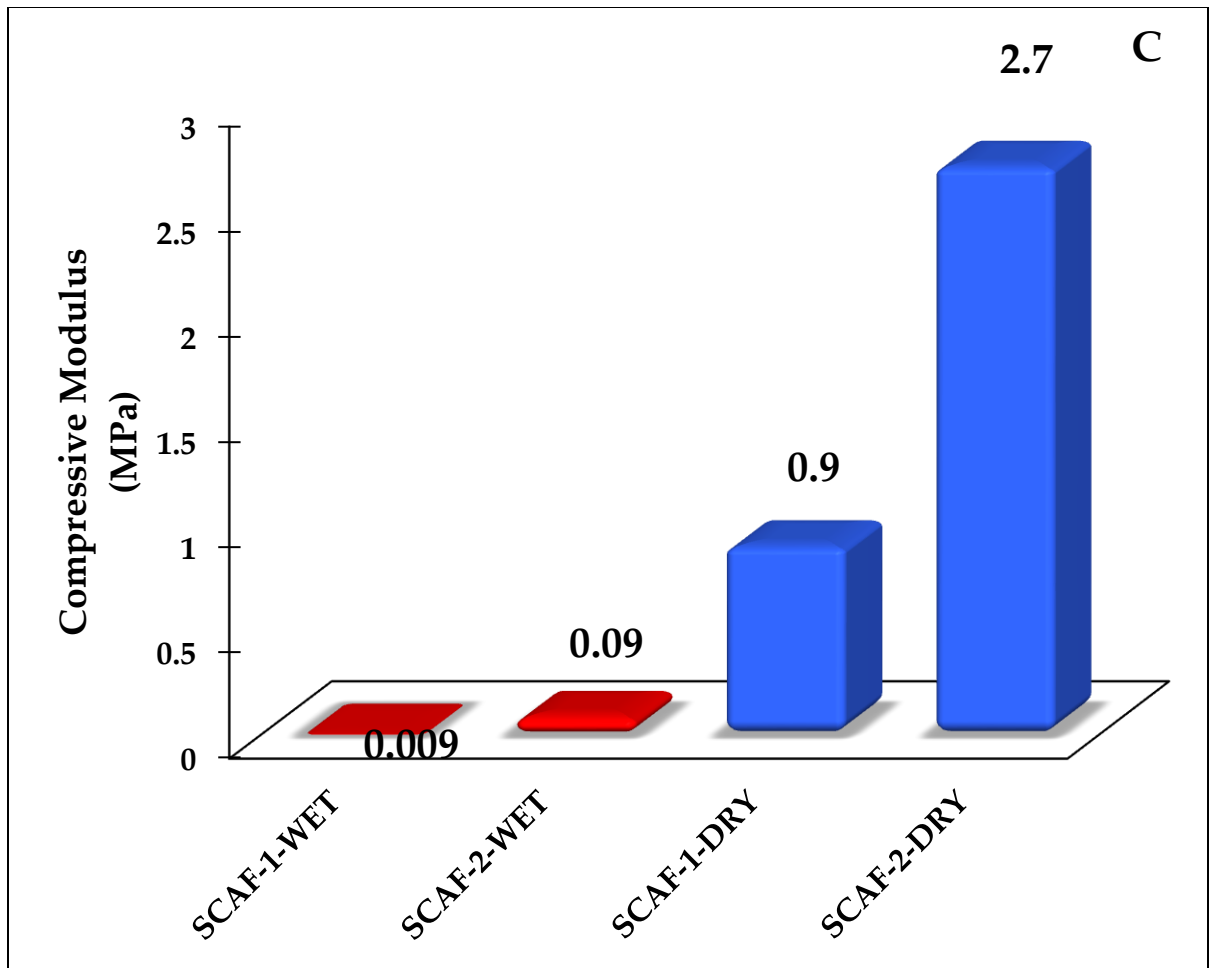


Figure 22: Compressive stress – strain graph of SCAF-and SCAF-2 at wet and dry condition (22.A and 22.B) and scaffolds compressive modulus in both conditions (22.C)

#### 4.1.8 Cell proliferation

##### I. Calibration curve

Calibration curve was prepared to determine the correlation between absorbance reading and the cell numbers. Average result of three different experiments on the relationships between absorbance and number of cells is illustrated on figure 24.

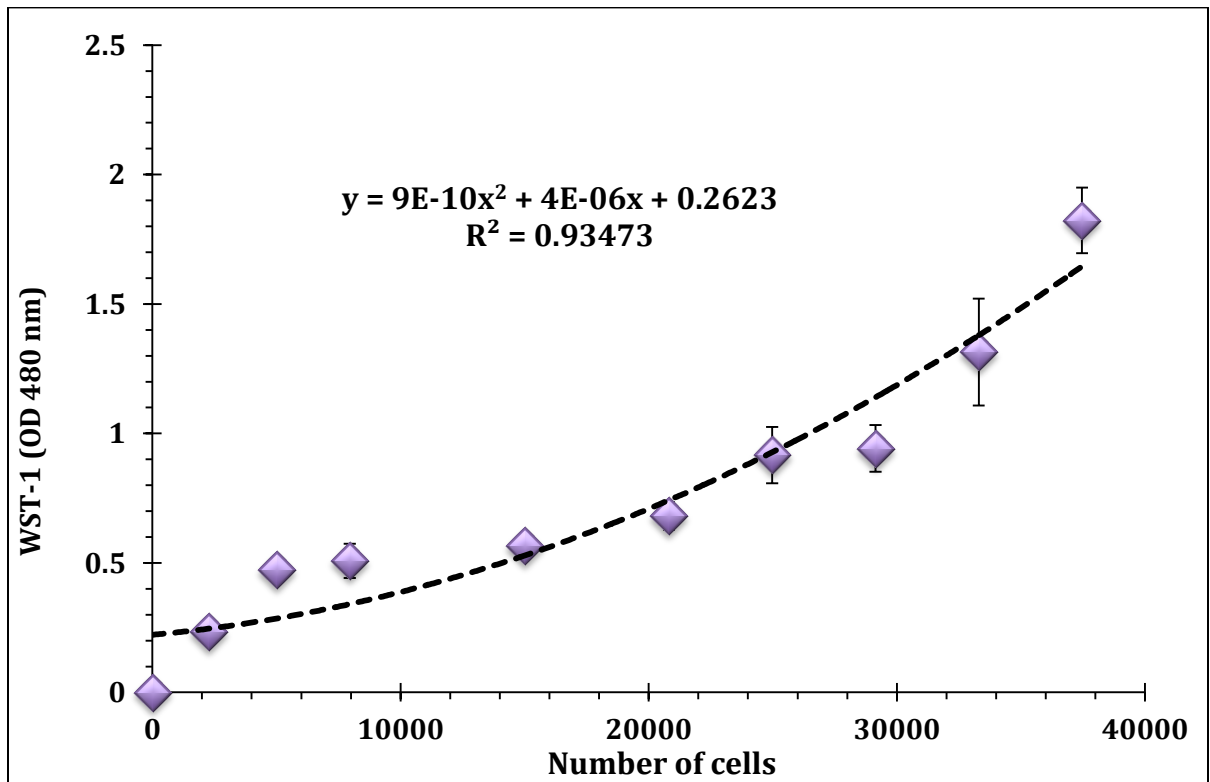
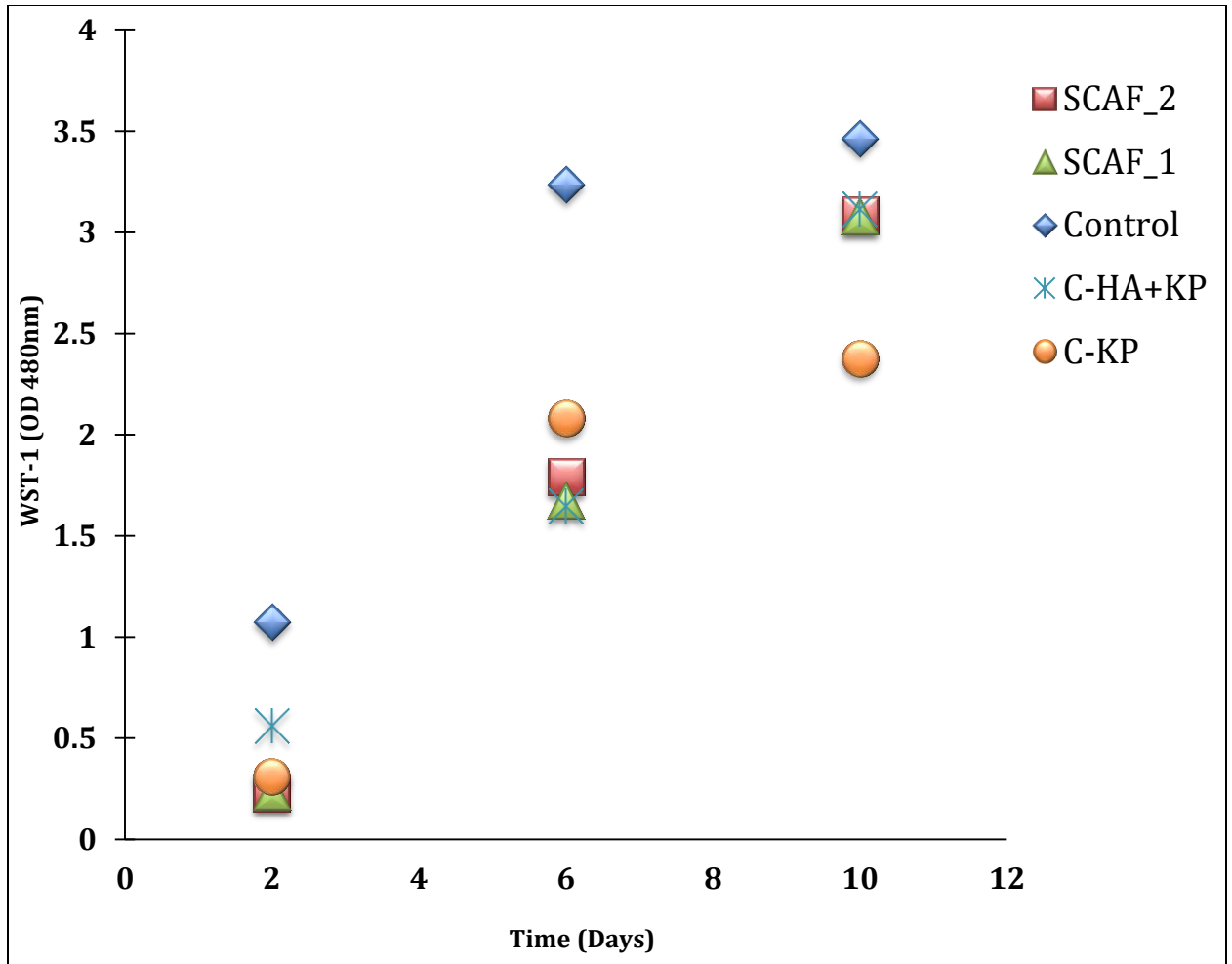


Figure 23: Cell number standard curve for GFP-cell line using the WST-1 cell assay. Different cell number ranging from 1200 per well to 40,000 cells per well were seeded in a 48 tissue culture plate and media was added with a final volume of 500- $\mu$ l and incubated overnight at 37C<sup>0</sup> in a CO<sub>2</sub>. Then 50  $\mu$ l of WST-1 solution was added to each well and the plate was further incubated for 4 hours. Finally, 200  $\mu$ l supernatant solution was removed and transferred into 96-well tissue culture plate and the absorbance was recorded at 480nm. Data from 3 sample replicates are shown (n=3, mean  $\pm$  SD)

## II. WST-1 Assay

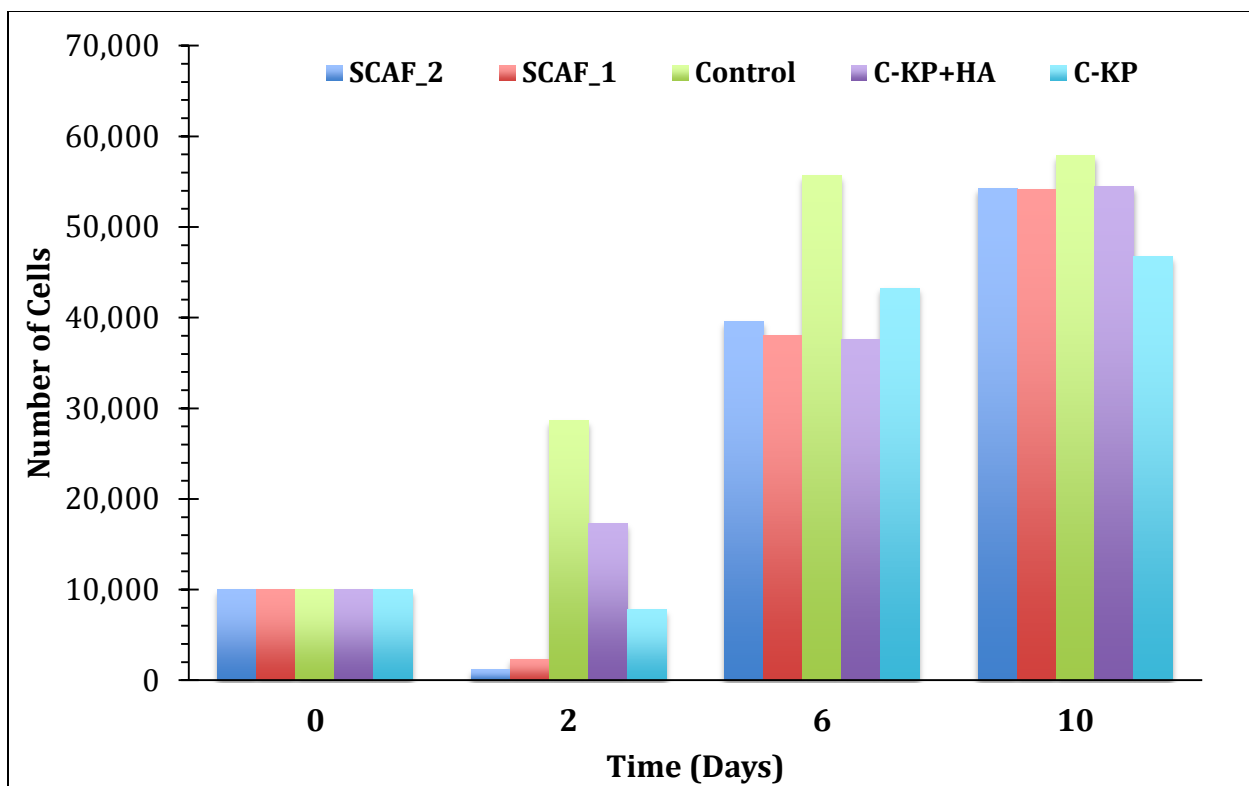
WST-1 data, Figure 25, and cell proliferation data, figure 26, show that cells proliferated both on the keratin scaffolds and on coted tissue culture plates. In the first 2 days, less number of cells were detected on scaffold-1, scaffold-2 and keratin coated tissue culture plate than what was originally seeded (10,000/cells). On the contrary, the cells started to proliferate on the HA and keratin protein coated tissue culture plate and on the control (surface treated polystyrene tissue culture plate) after the first 2 days. After six days cell number increased significantly on both scaffolds and coated tissue culture plates. The cells on scaffold one and two showed a significant growth even more than the cells on the keratin coated plate and HA and keratin coted tissue culture plate that were proliferating during the first two days. As expected, cells on the control group showed significant growth after the first two days as well as six days. Following the tenth day, the cells in scaffold 1 and 2 both continued to proliferate with the same rate showing that there was no distinct difference between the two environments in terms of sustaining the cells. Cells in the control group had also proliferated but at a much slower rate than what had been in previous days. While cells in the HA and keratin coated tissue culture plate and keratin coated tissue culture plate were also showing proliferation, the cells in HA and keratin coated culture plate had a higher rate of proliferation than that of keratin coated plate.



Key for the graph

- **C-HA+KP** - Tissue culture plate coated with keratin protein and HA
- **C-KP** - Tissue culture plate coated with only Keratin protein
- **SCAF-1** - Scaffold composed of cuticle-cortex and keratin protein
- **SCAF-2**- Scaffold composed of cuticle-cortex, keratin protein and HA
- **Control** - Polystyrene tissue culture plates

Figure 24: WST-1 data of cells cultured on the scaffolds and coated surfaces for up to 10 days.



Key for the graph

- **C-HA+KP** - Tissue culture plate coated with keratin protein and HA
- **C-KP** - Tissue culture plate coated with only Keratin protein
- **SCAF-1** - Scaffold composed of cuticle-cortex and keratin protein
- **SCAF-2**- Scaffold composed of cuticle-cortex, keratin protein and HA
- **Control** - Polystyrene tissue culture plates

Figure 25: Proliferation curves of GFP-stromal cells on the scaffolds and coated plates.

	Cell Numbers			
	Day 0	Day 2	Day 6	Day10
SCAF-1	10,000	2,345	38,000	54,135
SCAF-2	10,000	1,234	39,603	54,214
Control	10,000	28,601	55,667	57,840
C-KP	10,000	7,765	43,364	46,756
C-KP+HA	10,000	17,292	37,569	54,447

Table 4.2: Cell numbers on scaffolds and coted plates obtained from WST-1 assay on day 0, day 2, day 6 and day 10. Using equation from figure 23.

## 4.2 Discussion

This project examines the construction, characterization and cytocompatibility of human hair protein scaffold as a potential bone implant option. Keratin proteins extracted from hairs, wools, nails and horns have long been considered as a new type of promising biomaterials [12,45]. Human hair proteins have been chosen as an ideal material due to several advantages. Human hair is the only material of human origin that is abundantly available and can be obtained easily without a daunting process. The proteins extracted from human hair are biodegradable, fulfilling an important criterion of biomaterial selection for constructing scaffolds [50].

Following shindig method of extraction keratin proteins were extracted from the human hair [32]. The extracted human hair in the present study mainly consisted of keratins protein with molecular weight range 38-52 kDa (figure 12), which is in agreement with the reported data [32].

Both groups of scaffolds, SCAF-1 and SCAF-2 (Table 1.1) have multi-porous structure with an average pore size ranging from 40 to 300 $\mu$ m for SCAF-1 and 50 to 300 $\mu$ m for SCAF-2 (figure 12.15). These pore sizes correspond with the optimal porosity for cell adhesion, proliferation and differentiation reported literature [16, 26, 1]. These pore sizes are also analogous to the pore size range found by Vema *et al* [50] and Xu *et al* [47] for keratin protein scaffolds.

Figure 27 shows a comparison between the surface structures of the two scaffolds. The elemental analysis for both scaffolds shows that the elements present on the surface of the scaffolds are the same as the building blocks of bone protein [5, 37, 36]. SCAF-2 elemental analysis shows that hydroxyapatite is uniformly distributed throughout the scaffold creating a rough three-dimensional nano-microstructure on the surfaces of the scaffold. On the contrary, the surface of SCAF-1 is relatively smooth. It is generally believed that surface roughness plays important role in human bone marrow cell adhesion, proliferation and differentiation and detachment strength [8]. A study conducted by Li *et al* showed that rougher HA surfaces are better at enhancing cell attachments and proliferation, than smother surfaces [8].

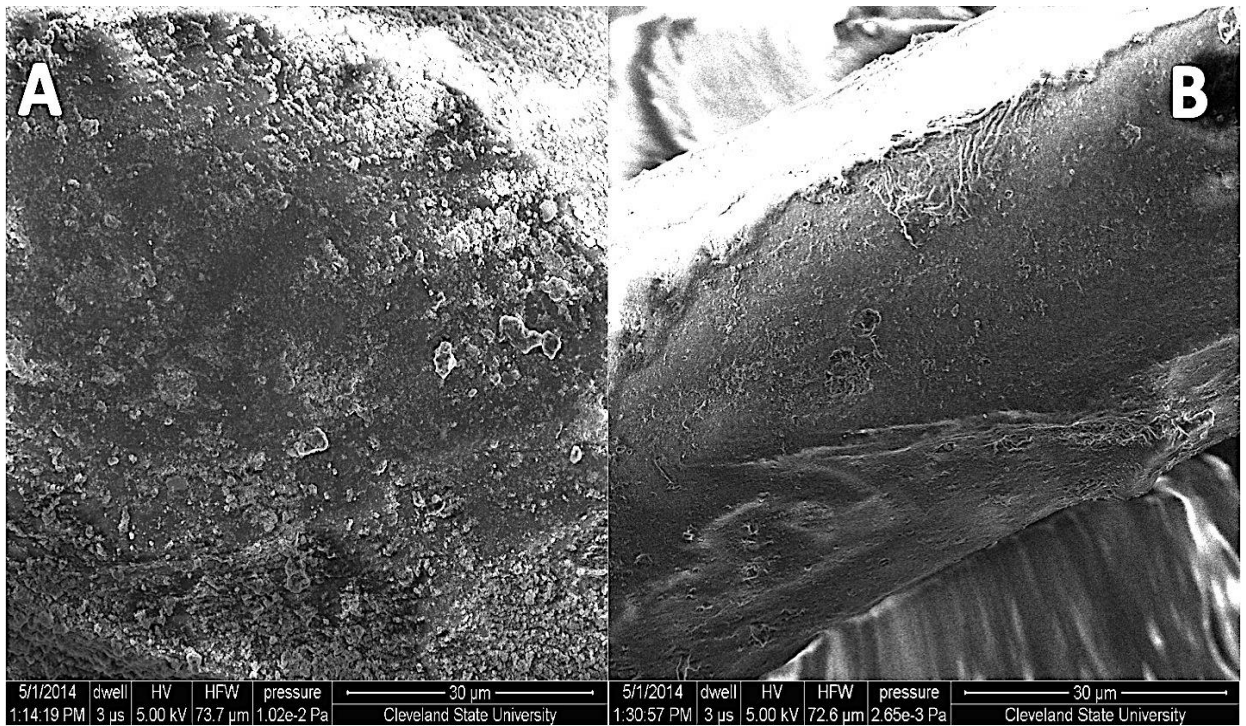


Figure 26: SEM image comparison of SCAF-2 (A) and SCAF-1 (B)



The scaffolds swelled approximately 150% within a period of 90 min and both group of scaffolds had high porosities above 50%. The swelling may have contributed to increase in the pore size, causing an increase in the surface area, making the movement of cells and nutrients easier within the scaffolds and improving the adhesion [50]. Similarly, having highly porous scaffold is desirable, since it can support the growth of tissue via the necessary nutrient transport [21]. Porosity also plays an important role in regulating cell adhesion and migration. High porosity provides a high surface area for cell-matrix interactions, sufficient space for extra cellular matrix regeneration and uniform and efficient cell seeding [21]. Higher porosity could also lead to increased cell adhesion [21].

The mechanical strength of SCAF-2 was higher than SCAF-1 due to HA layer formed on the surface of the scaffold. Compressive testing in hydrated state shows that SCAF-2 had a compressive modulus that was ten times more than SCAF-1 (figure 22.C). As expected, compressive modulus of the scaffolds in dry condition was one to two orders of magnitude higher than the wet. When comparing the compressive modulus of dry scaffold, a similar trend to that of a wet scaffold was observed where SCAF-2 has three times higher modulus than SCAF-1. These results indicate that the presence of HA on the surface of the scaffold also increases the scaffold stiffness.

Cell culture experiments with GFP stromal cells showed that the keratin composite scaffolds are good carrier for these cells, which is essential for bone regeneration and repair. GFP-stromal cell culturing experiments confirmed good

cytocompatibility of the prepared keratin composite scaffolds, as indicated by WST-1 results which recorded the continuous proliferation after seeding and culturing for as long as 10 days (figures 25 and 26). A higher proliferation rate was achieved on the keratin and HA coated tissue culture plate followed by SCAF-2. The presence of HA made the scaffold become more cytocompatible.

According to Fergal *et al.* [33] scaffolds for bone tissue engineering should provide the following essential properties: biocompatibility and biodegradability, open and interconnected porosity, a proper pore size, appropriate chemical and topographical surface properties, stimulation of osteogenic differentiation and a sufficient mechanical strength. Compact bone is known to have a compressive strength measuring anywhere from 150-250 MPa due to variability in bone density [37,1]. Although both composite scaffolds showed low compressive strength as compared to other bio-based scaffolds and natural compact bones, they still present a promising substrate for cell growth and bone regeneration by fulfilling most of the essential properties mentioned above. Since it is not fully understood to what extent the biodegradable scaffolds need to mimic natural bone mechanical properties, because as the scaffold degrades the new bone are expected to grow into the degrading scaffold and provide the load bearing ability. Perhaps this is the reason why ranges of materials with compressive strengths ranging from 2-9 MPa are being examined for bone scaffold application [24,28,3].

## **Chapter VI**

### **CONCLUSION AND RECOMMENDATION**

#### **5.1 Conclusion**

Through past research it has been discovered that bio-based materials are more viable and biocompatible alternative to synthetic materials fabricated for tissue engineering applications. Although many bio-based materials have been used, human hair keratin protein has yet to be researched as a possible material to create three-dimensional structure to promote osteoblast adhesion, proliferation and differentiation.

In this study human hair keratin protein and cuticle-cortex composite scaffold (SCAF-1) and human hair keratin protein, cuticle-cortex and HA composite scaffold (SCAF-2) were studied for bone tissue regeneration. After successful fabrication of the scaffolds, the morphology was determined using SEM allowing for scaffold cross sectional area and top porous structure to be viewed. Both scaffolds showed a three-dimensional structure with a pore size ranging from 40-500 $\mu$ m and porosity greater than 50%. Also, water up taking capacity of the scaffolds were measured and found to be 150% for SCAF-1 and 155% for SCAF-2. Average pore size, scaffold porosity and water up taking capacity results were comparative to porous scaffold used previously in researches that promote cellular adhesion, proliferation and differentiation for tissue engineering application.

In addition, compressive tests were carried out under dry as well as wet conditions for both scaffolds. SCAF-1 showed compressive modulus of 0.015 MPa in wet condition and 0.90 MPa in a dry condition. Likewise, SCAF-2 had compressive modulus of 0.05 MPa in wet condition and 2.7 MPa in dry condition. However, the average compressive strength of SCAF-2 was lower than bone. The ideal compressive strength required for scaffolds is not known and therefore both scaffolds cannot be ruled out as a possible substrate for cell growth and differentiation. In fact GFP-stromal cells showed a significant population increase and proliferation in both scaffolds. Thus, these scaffolds are potential resorbable scaffold for bone implant applications, especially where a cage made of other stiff materials can be made to support the human hair protein based scaffolds during the initial implant stage while the new bone in-growth takes place into the scaffold.

## 5.2 Recommendation

The following are recommendations for potential further studies that can be performed on the scaffolds to find better cell proliferation and differentiation, scaffold morphology and mechanical strength:

- Conduct further study to investigate the effect of different concentration of keratin protein, cuticle-cortex fibers and HA to improve the mechanical structure of the scaffolds without affecting the 3D porous microstructure.
- Study different scaffold fabrication methods in order to control the pore sizes.
- Since the cell numbers were drastically lower on day two, perform live/dead cell assay on day two to confirm cell death.
- Introduce coupling agent like zirconate, titanate and silane between the cuticle-cortex fibers and HA particles in order to improve the mechanical property of the composite scaffolds and to insure HA won't get washed out.
- Since the cell number passes the calibration curve boundary limits after six days, a new calibration curve needs to be used with higher number of initial cell density.
- Perform longer cell culture experiments in order to study formation of extra cellular matrix and mineralization.

- Introduce growth factors like; bone morphogenic proteins (BMPs), which induce the differentiation of mesenchymal cells into osteoblasts to the scaffolds and study the effects.
- Introducing multi-adhesive glycoproteins such as; sialoprotein I and II, which mediate cell attachment and initiate calcium phosphate formation during mineralization process to the scaffolds and study the effects.

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## **APPENDENCES**



## **APPENDIX A**

### **A. Failed Experiments**

#### **I. Scaffold Production and Protein Extraction**

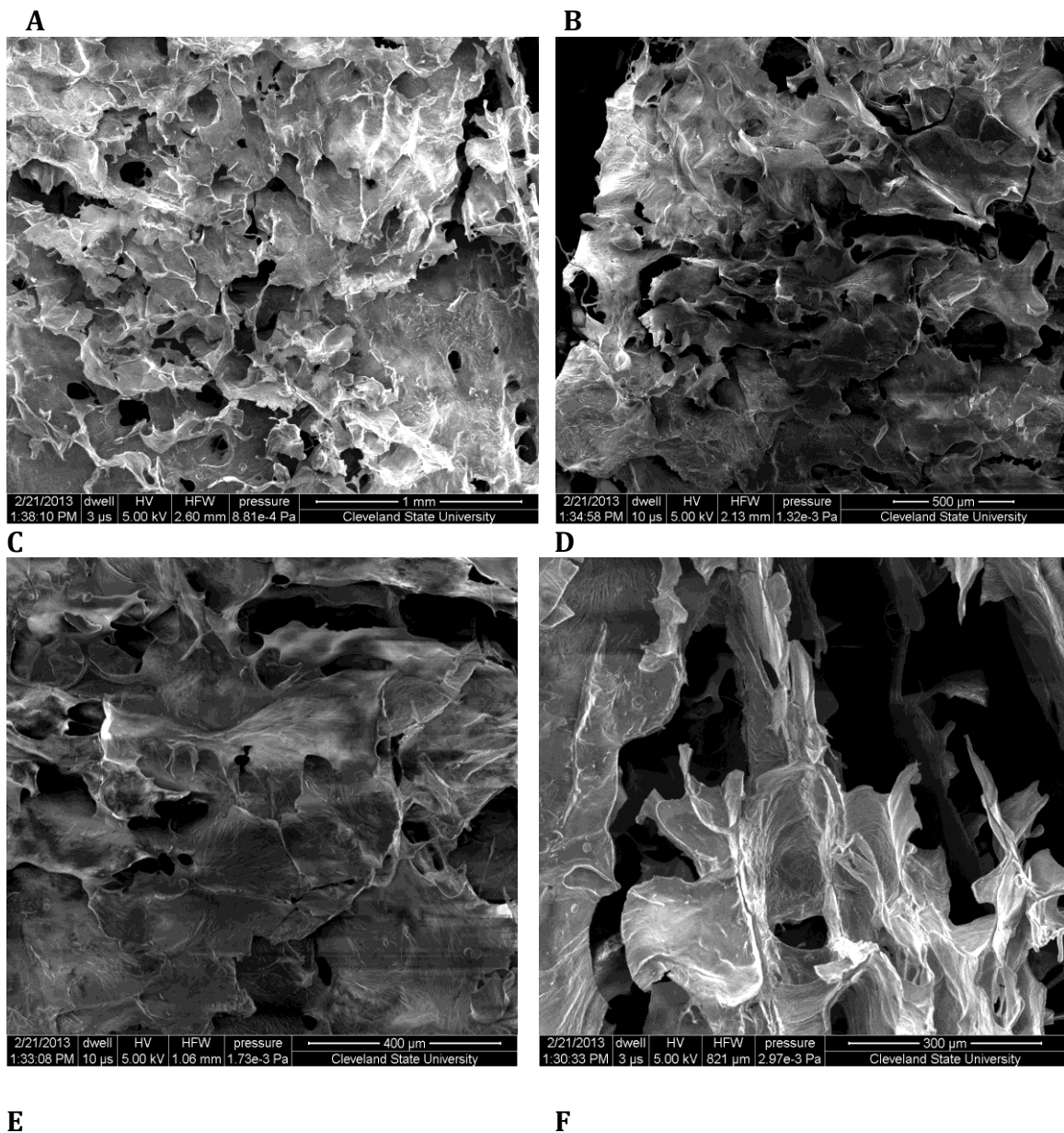
Alkaline hydrolysis of protein procedure was used to extract the protein and to form the scaffold [19]. The procedure and the result are explained in detail bellow.

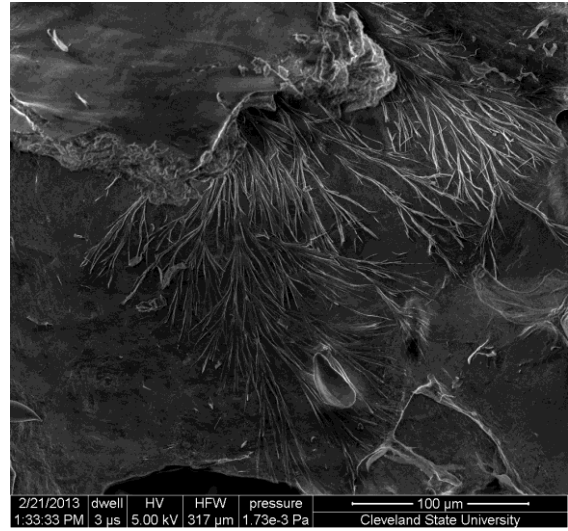
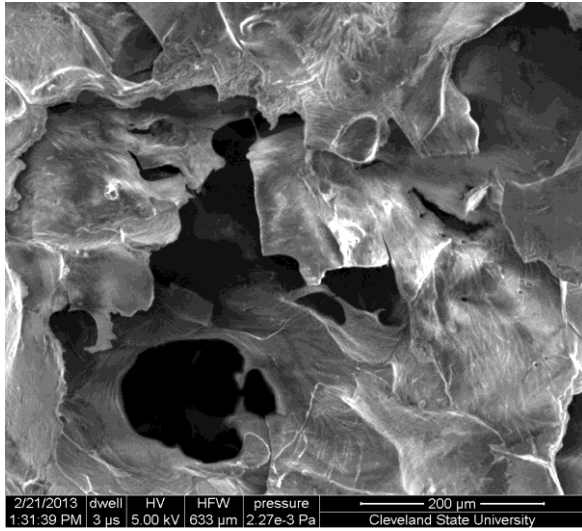
##### **Method**

Human hair collected from a local barbershop, was washed with ethanol and water several times in order to remove any dirt from the surface of the hair and was cut into smaller pieces. Afterward, 0.3 mg of small hairpieces were placed in a 50ml centrifuge tubes and 10 ml of 8M NaOH was poured on them and shaken on a shaker plate at 400 rpm for 10 min. Then the reaction was stopped by adding 40 ml of RO-water. Afterward, the mixture was centrifuged at 2000 rpm for four minutes and the supernatant was discarded and 30ml of RO-water was added to the tube and centrifuged again. The process was repeated several times (eight times) until the pH of the solution was neutral. A pellet was recovered and placed on a dialysis tube and dialyzed against RO-water for four days and water was changed once a day. Then the dialyzed sample was kept in -80 °C freezers for 48 hours and following that the sample was placed in a lyophilizer for 48 hours. Finally, the three-dimensional structure that was recovered was examined by SEM.

## Result

The SEM image of the scaffolds is shown on figure (E1). The image shows that the scaffolds had a porous three-dimensional structure. However, when the scaffold was tested for stability in PBS, it lost its three-dimensional structure and dissolved completely.





**G**

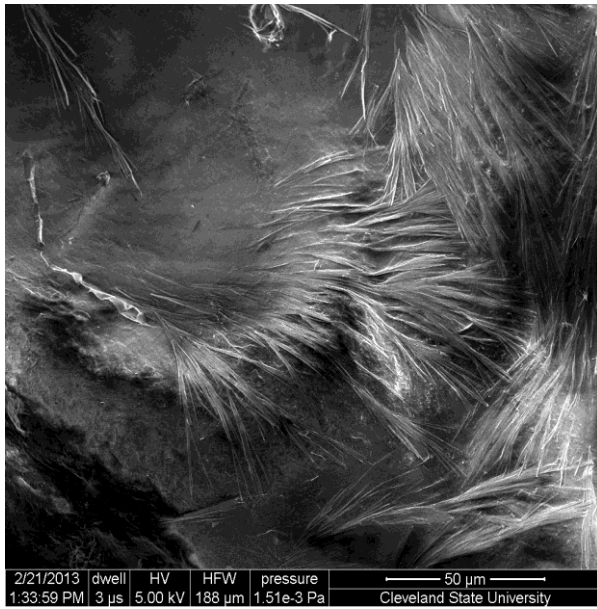


Figure E1: SEM image of three-dimensional structure at different magnification (lower (A) - higher (G))

## II. Coating the scaffold with HA

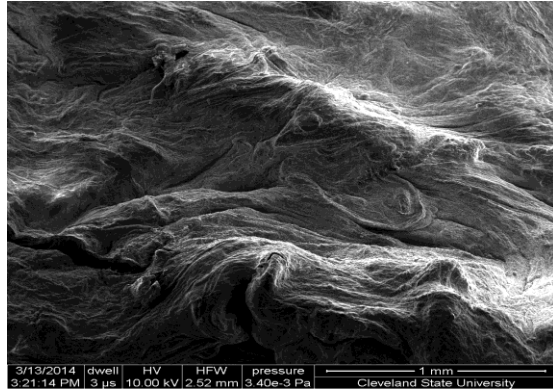
In order to form a composite scaffold we used two methods to deposit HA particles on the surface of the scaffold. The first method was to blend HA particles with the keratin powder and form scaffold following the scaffold making procedure mentioned on section 3.2.2. The Second method was to prepare a HA slurry and to pour it on top of a scaffold that was already created. The second method is explained in detail in section 3.2.3 since it was a successful approach. Here, I will explain why the first approach didn't work.

HA particles measured at different consecration were mixed with keratin powder. Then, keratin powder was dissolved by buffer solution creating a dissolved keratin protein and HA particle mixture. Then the mixture was poured on to 50 mg of cuticle-cortex fragments. The mixture was mixed well with a spatula for five minutes. Afterward, 0.1 ml of pre-cooled acetone (Sigma- Aldrich) was added into the solution drop by drop, and immediately thereafter the solution was incubated at -20 °C refrigerator for one hour and 30 min in 4 °C refrigerator. Following that, a disk shaped cross-linked 3D- structure was recovered.

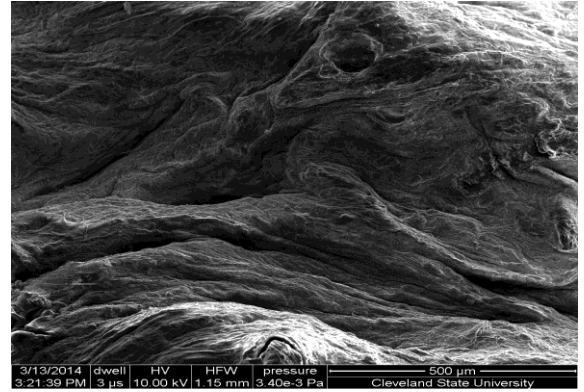
The recovered disc-shaped structure was washed several times with RO-water and left overnight in an open air. Then, the scaffold was dialyzed against RO-water using snakeskin dialysis tubing (thermo scientific, with a molecular weight cutoff of about 3.5 kDa and diameter 16 mm) for five days and during that time the outer water was replaced every day.

## Result

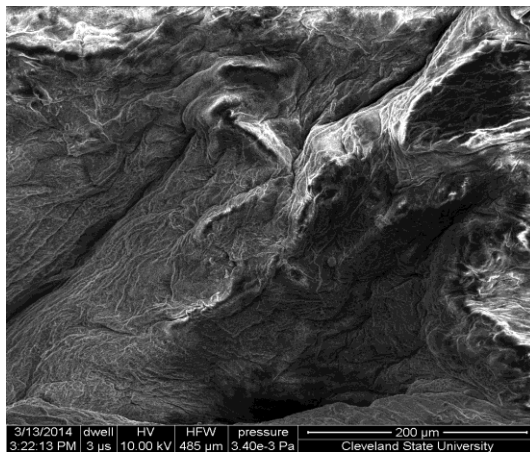
SEM image shows figure (E2) a scaffold that was prepared with HA particles premixed with keratin protein. This method was considered a failure because the HA particles got imbedded into the protein and were generally not seen on the scaffold surface in addition the scaffold didn't have a much of a porous structure. As discussed in the previous chapters since porosity is one of the main important factors that affect cell growth and proliferation, it was decided not to follow this method to form the composite scaffold



A.



B.



C.

Figure E2: SEM image of scaffold that was made from HA particles and keratin protein

### **III. Cell study**

Cell study was conducted on both composite scaffolds using live cell imaging process. However, because the cells were invisible due to the three-dimensional structure of scaffold it became very hard to focus in order to take proper image. Keratin protein and cuticle /cortex fibers also fluoresced which makes it very difficult to distinguish between cells and the fibers especially once the cells start attaching.

Briefly, five thousands cells were seeded to both composite scaffolds (SCAF-1 and SCAF-2) and incubated in a humidified incubator. The study was conducted for four days and images were taken every twelve hours. The images of both scaffolds and cells are shown on figure E3 and E4.

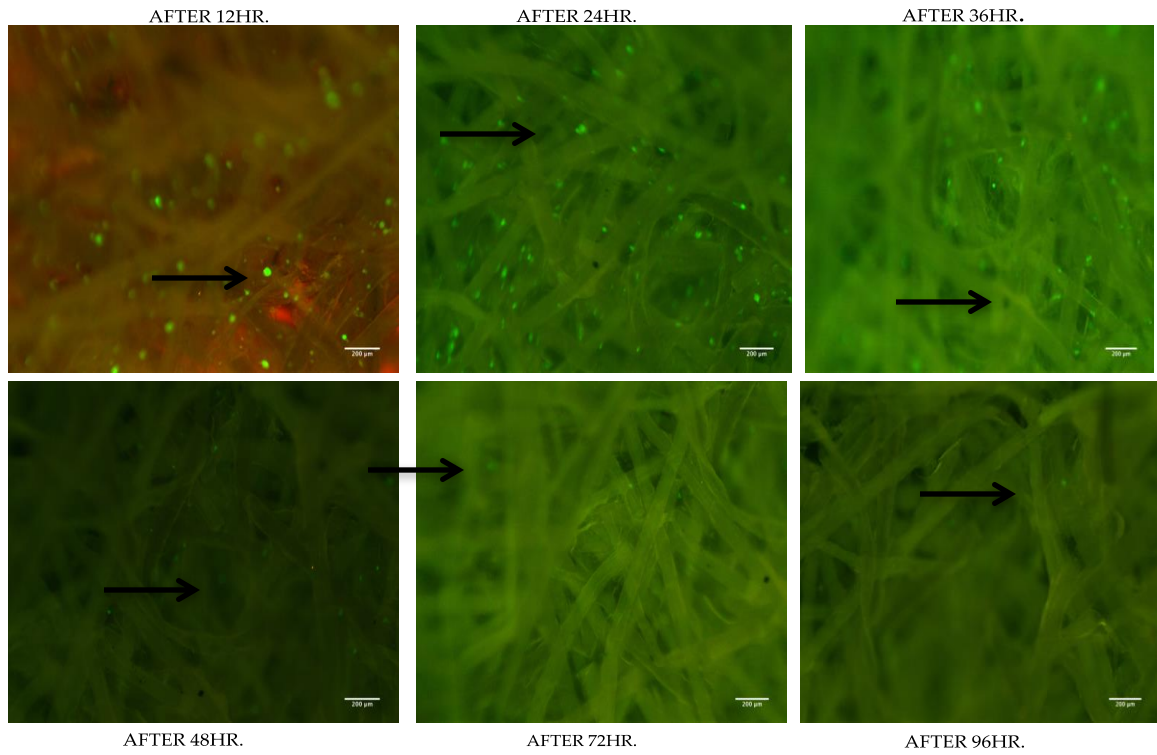


Figure E3: Cell proliferation images on SCAF-2

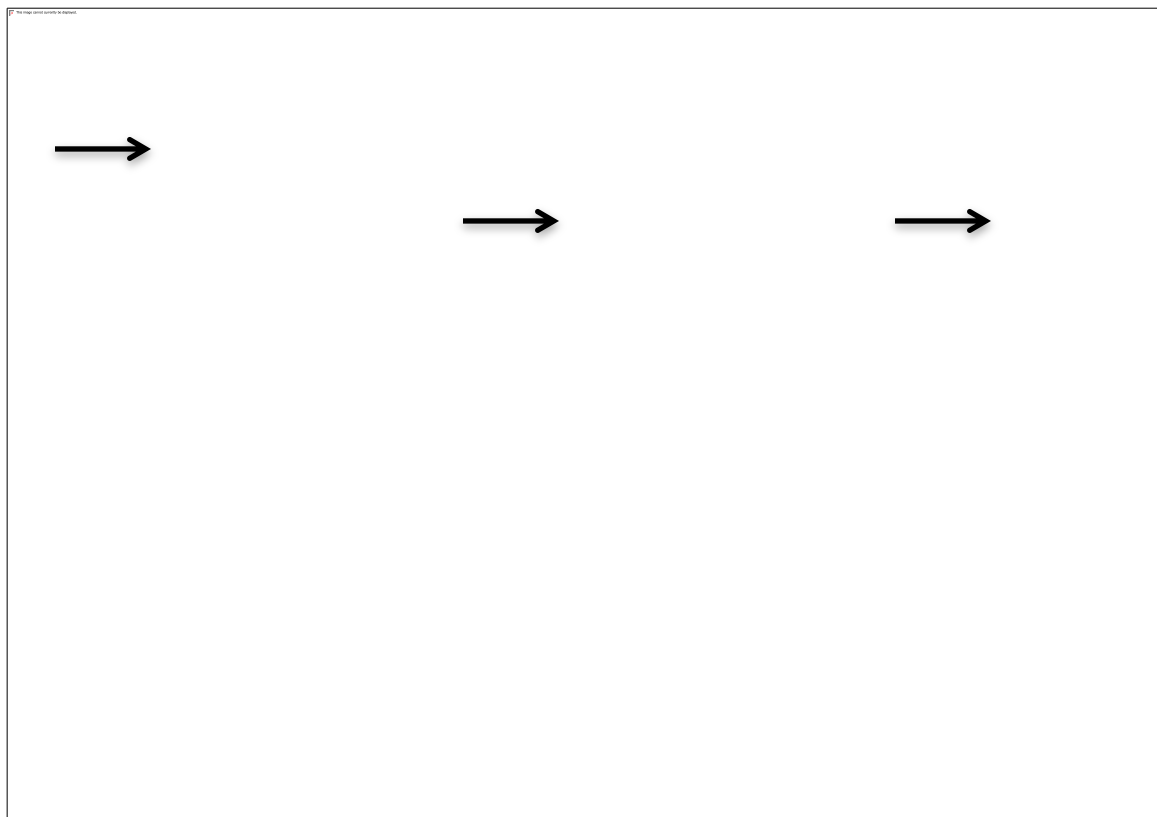


Figure E4: Cell proliferation images on SCAF-1

## APPENDIX-B

### I. Hair keratin protein extraction

1. Wash the hair with ethanol and water
2. Incubate the hair in mixture of chloroform/methanol (2:1,v/v) solution to remove lipids
3. Mix the delipidized hair with a buffer solution containing 25 mM Tris-HCl, pH 8.6, 2.6 M thiourea, 5M urea and 5% 2-mercaptoethano. Hair mass (mg) to buffer (ml) ration is approximately 66.
4. Incubate the mixture at 50°C for three days
5. Filter the mixture and centrifuge at 15,000 X g fro 20min at room temperature
6. Dialyzed the obtained supernatant against deionized water using cellophane tubing (molecular weight cutoff of about 10 kDa) and replace the outer water twice a day.
7. Place the dialyzed solution in -80°C refrigerator for 24hours
8. Lyophilize the frozen solution and recover the protein powder



## APPENDIX-C

### Cell Culturing

#### I. Protocol: Measuring Cell Proliferation using WST-1

1. Culture cells in flat-bottomed 48-well plates in a final volume of 100-250 $\mu$ l/well culture medium in a humidified atmosphere (37°C, 5% CO<sub>2</sub>). Reserve one well as a background control (culture medium without cells).

**Note:** The incubation period and cell density of the culture depend on the particular experimental conditions and on the cell line used. For these experiment setups, a cell density was 10,000 cells/well.

2. Add 20  $\mu$ l of Premixed WST-1 Cell Proliferation Reagent to each well (1:10 final dilution).

**Note:** Premixed WST-1 Cell Proliferation Reagent should be used at a final dilution of 1:10. If cells are cultured in 200 $\mu$ l culture medium, add 20 $\mu$ l Premixed WST-1 Cell Proliferation Reagent per well.

3. Incubate the plate for 0.5 to 4 hr at 37°C in a humidified atmosphere maintained at 5% CO<sub>2</sub>.
4. Shake thoroughly for 1 minute on a shaker.
5. Measure the absorbance at 420–480 nm, using a multiwell plate reader. The reference wavelength should be greater than 600 nm.

**Note:** The absorbance level of the background control well (containing culture medium plus WST-1 Cell Proliferation Reagent, without cells) will depend on the culture medium, incubation time, and exposure to light. Typical background absorbance after 2 hr is between 0.1–0.2 absorbance units.