LOCAL DELIVERY OF BIOACTIVE AGENTS
FOR BONE TISSUE ENGINEERING

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FOR BONE TISSUE ENGINEERING

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Many cases of bone defects require bone grafts addition into the body. Bone tissue substitutes have more advantages over other bone grafts because of low infection risk and abundance of materials. Local delivery of drugs or bioactive agents can help in these diseases' treatments and bone growth overall while avoiding side effects or drug interference. To accelerate local bone regrowth, a research about developing a controlled local release system of bioactive agents to bone cells was conducted in this study using bone tissue substitute as a template for the controlled local release system.

Wet (Monoaxial) electrospinning can produce 3D scaffolds that can either deliver a hydrophilic or a hydrophobic bioactive agent. However, to fabricate a 3D scaffold that can hold and maintain both for local delivery, and has the desired bone graft scaffold characteristics, a coaxial wet electrospinning system was used for the first time in bone tissue engineering. After optimization studies on scaffolds made from different combinations of polymer solutions and various electrospinning systems,

four groups were chosen based on some micro and macro properties of their respective scaffolds.

Additional studies, including release profiles tests and *In situ* and *In vitro* characterizations, were carried out to evaluate their bone tissue engineering related properties and see their potency as local drug delivery system using vitamin K2 and calcitonin as a hydrophobic and a hydrophilic bioactive agent respectively. One of these scaffolds demonstrate better characteristics, but further studies to enhance its mechanical properties and optimize the bioactive agents' dosages are needed.

Keywords: Calcitonin Salmon, Vitamin K2, Bone Scaffold, Controlled Drug Delivery, Dual drug release, Wet Coaxial Electrospinning.

ÖZ

BİYOAKTİF AJANLARIN LOKAL SALIMI İLE
KEMİK DOKU MÜHENDİSLİK

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Kemik hasarların vakalarında birçoğunda, vücuda kemik grefti eklenmesini gerekmektedir. Kemik dokusu yerine geçen malzemeler, bol olması, enfeksiyon ya da hastalık taşıma riskinin düşük olması nedeniyle, diğer kemik greft türlerine göre üstünlük gösteriyor. İlaçların ya da biyoaktif ajanların lokal salımı, bu hastalıkların tedavisine ve genel olarak kemik büyümesine yardım ederken yan etkileri veya ilaç etkileşimleri önlemektedir. Lokal kemik geliştirme sürecini hızlandırmak için, bu araştırmada kemik hücrelerine yönelik biyoaktif ajanların lokal kontrollü salım sistemi kemik doku temelli greftler kalıp olarak kullanılarak yapılmıştır.

Islak (Tek parçalı) elektroeğirme kullanarak hidrofilik ya da hidrofobik biyoaktif ajanı salabilen üç boyutlu iskele üretilebilmektedir. Ancak, ikisinde lokal salımı sürdürebilen ve istenilen kemik iskelesi özelliklerini içeren 3 boyutlu iskele üretmek için, kemik doku mühendisliğinde ilk defa koaksiyel ıslak elektroeğirme sistemi kullanılmıştır. Farklı polimerik solüsyon kombinasyonları ve farklı elektroeğirme sistemleri kullanılarak optimizasyon çalışmaları yapıldıktan sonra, iskelelerin mikro ve makro özelliklerine bağlı olarak dört grup seçilmiştir.

Seçilen bu grupların kemik doku mühendisliğine yönelik özellikleri ve hidrofilik biyoaktif ajan olarak vitamin K2'nin, hidrofobik biyoaktif ajan olarak kalsitoninin lokal salınımına yönelik salım profil testleri ve *In situ* ve *In vitro* karakterizasyonları yapılmıştır. Bu iskelelerin biri daha iyi karakterler göstermiştir ama mekanik özelliklerini geliştirmek ve bioaktif ajanların dozajlarının optimizasyonu için daha fazla çalışmalar gerekmektedir.

Anahtar kelimeler: Kalsitonin Salamon, Vitamin K2, Kemik Hücre Taşıyıcı iskeleti, kontrollü ilaç iletimi, çift ilaç salımı, ıslak çift aksiyal elektro-eğirilmek

To Dignar, may your soul rest in peace

ACKNOWLEDGEMENTS

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ABBREVIEATIONS

ALP: Alkaline Phosphatase

ANOVA: Analysis of variance

Calc: Calcitonin

CE: Coaxial Electrospun by Ethanol Collector

CW: Coaxial Electrospun by Water Collector

DMEM: Dulbecco's modified Eagle's medium

DMSO: Dimethyl Sulphoxide

EDTA: Ethylenediaminetetraacetic acid

ES: Electrospun

FBS: Fetal bovine serum

FDA: Food and Drug Administration

FITC: Fluorescein isothiocyanate

FTIR: Fourier transform infrared spectroscopy

HFIP: Hexafluoro isopropanol

HPLC: High-performance liquid chromatography

ME: Monoaxial Electrospun made by ethanol collector;

MTT: 3-(4,5-dimethylthiazol–2-yl)-2,5-diphenyltetrazolium bromide

MW: Monoaxial Electrospun made by water collector;

P-188: Poloxamer 188

PBS: Phosphate buffered saline

PCL: Polycaprolactone

PI: Propidium iodide

PPM: Part Per Million

PVA: Polyvinyl acetate

Saos-2: "Sarcoma osteogenic"; human osteosarcoma cell line

SEM: Scanning electron microscope

UV: Ultraviolet

VK2: Vitamin K2

Wt/vol: weight/volume

β-LG: Beta Lactoglobulin

CHAPTER 1

INTRODUCTION

1.1. Bone

1.1.1. Bone Structure and Composition

Bone is dynamic hard connective tis sue that is continually regenerating by its ability to restore and redesign itself with no scar left (Sommerfeldt & Rubin, 2001).

Bone is composed of two main tissues, cancellous (spongy) and cortical (dense) bones (Fig. 1-1). Cancellous bone located in the interior side of bone and is characterized as a porous structure. It forms large part of epiphyses (large ends of long bones), scapula (shoulder blade bone) and ribs. It can weaken sudden stresses. Cortical bone on the other hand is in the exterior side of bone and establishes the outer structure of bones, cortical is also much less porous which make it hold much more mechanical stress. It makes up the larger part of long bones of arms and legs. The ratio of cortex to cancellous bone differs from bone to bone according to the load bearing functions. For example in vertebral bones 90 % is cancellous whereas at the femoral neck bone only 25% is cancellous (Britannica, 2015a, 2015b; Brydone, Meek, & Maclaine, n.d.; Wehrli, n.d.).

Bone is a heterogeneous structure composed of minerals, mainly hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_{2)}$ with fraction of carbonate, magnesium and acid phosphate, and an organic matrix consisting of small amounts of lipids, high amount of proteins; 85-90% being collagen , the rest being noncollagenous proteins.(Boskey, 2007; Clarke, 2008; Young, 2003). The main types of cells in bone tissue are osteocytes, osteoblasts, osteoclasts and bone lining cells. In mature bone tissue

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osteocytes represent over 90% of all cells, they are distributed across the mineralized matrix and form a comprehensive network together with osteoblasts and lining cells, similar to the sensory network in morphology and function in that they can communicate mechanical stress.

Osteoblasts are responsible for bone tissue formation and releasing bone matrix protein. Osteoblasts forms and release collagen and noncollagenous proteins on an evenly molar basis. Osteoclasts are responsible for resorption of bone tissue. Bone lining cells cover inactive bone surfaces and work as osteogenic precursor.(Clarke, 2008; Long, 2011; Marks & Popoff, 1988; Miller, de Saint-Georges, Bowman, & Jee, 1989; Rachner, Khosla, & Hofbauer, 2011).

The formation and resorption of bones are correlated with each other by yet unknown process, when formation or resorption increase, the other will increase too and vice versa. However, resorption happens much faster than formation leading to decrease in bone mass when there is an increase in resorption even though it is correlated with formation.(Harada & Rodan, 2003).
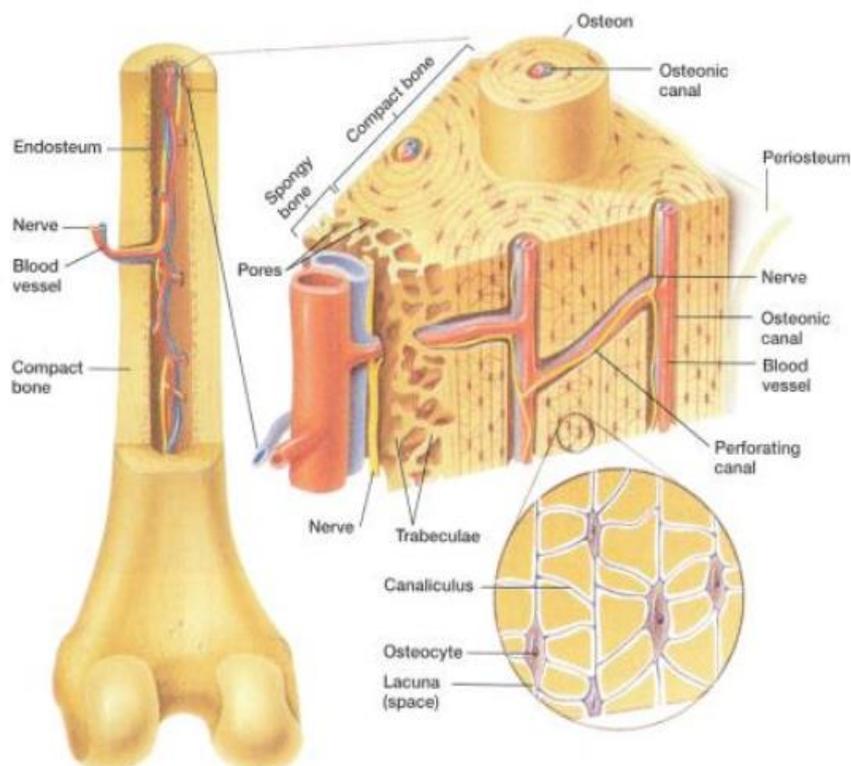
Fig. 1- 1 Structure of human bone showing bone, cancellous bone and compact bone (Shier et al., 1996)

1.1.2. Bone Remodelling

Bone remodelling is the process in which bone is regenerated for sustaining bone strength and homeostasis. Remodelling means that distinct parts of old or damaged bone are continually removed and replaced with new mechanically stronger and healthier bone by adding proteinaceous mineralized matrix. Remodelling involves four consecutive stages (Fig. 1- 2); starts with activation and resorption which is followed by reversal and finishes with bone formation. Remodelling happens at sites that need repair and at healthy sites in a randomized manner (Clarke, 2008).

Bone remodelling occurs according to physiological conditions through osteoblast and osteoclast cells. The balance in formation and resorption in healthy adults keeps maintenance of bone mass and bone turnover (Boyle, Simonet, & Lacey, 2003; Harada & Rodan, 2003).
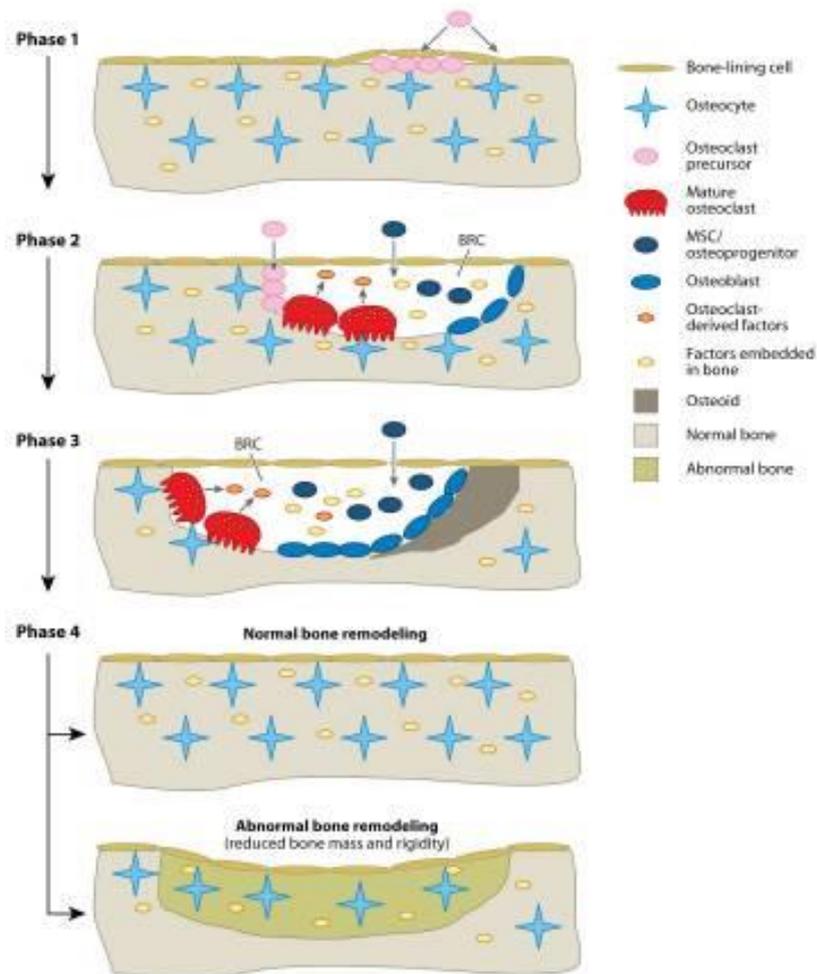
Fig. 1- 2 Phases of Bone remodeling process (Peel, 2012)

Remodelling has several functions such as restoration of old bones containing microdamages to maintain bone mechanical strength and to avoid the reduction of bone function while it gets old. This process is also important for homeostasis of calcium and phosphate in the body as bones act as storages sites for these essential elements (Burr, 2002; Harada & Rodan, 2003; Parfitt, 2002).

Bone formation is performed by osteoblasts as they produce a distinctive mixture of extracellular proteins involving mainly collagen type I, osteocalcin and alkaline phosphatase. Extracellular matrix is affluent in collagen type I and then it is mineralized by calcium phosphate's accumulation in the matrix as hydroxyapatite(Hall, 2005). Some osteoblasts differentiate to osteocytes when entombed inside bone matrix while the rest change to be bone lining cells or go through apoptosis (Bonewald, 2011). Osteoclasts are the main cells of bone resorption, which is crucial for bone remodelling. Osteoclasts stimulate osteoclastogenesis by expressing receptor for activation of nuclear factor kappa B (NF-κB), (RANK), and its ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) (Teitelbaum, 2000).

1.1.3. Bone Function

Bone offers structural support by acting as levers during muscle mediated movement of the body and its parts. It is also important in shielding the internal organs like brain, heart and lungs from trauma besides it role in load bearing especially through cortical bone structure. Bone acts in hematopoiesis since it covers and protects bone marrows and also have a critical role in homeostasis in the body by the mineral matrix which works as a mineral reservoir (Clarke, 2008; Kartsogiannis & Ng, 2004; Nyman et al., 2006; Parfitt, 2002; Rodan, 1992).

1.1.4.   Bone Diseases and Defects

1.1.4.1.   Osteoporosis

Osteoporosis is a bone disease characterized by decreasing the bone density. The disease occurs when bone resorption surpasses bone formation which results in alteration in the micro-architecture of the bone (Fig. 1- 3). This change in bone metabolism occurs by decreasing activity of osteoblasts and/or by increasing activity of osteoclasts. Osteoporosis occur in around 40% of Caucasian postmenopausal women, furthermore fractures from osteoporosis can increase mortality rate by 20% for the next 12 months (Rachner et al., 2011; Titorencu, Pruna, Jinga, & Simionescu, 2014).
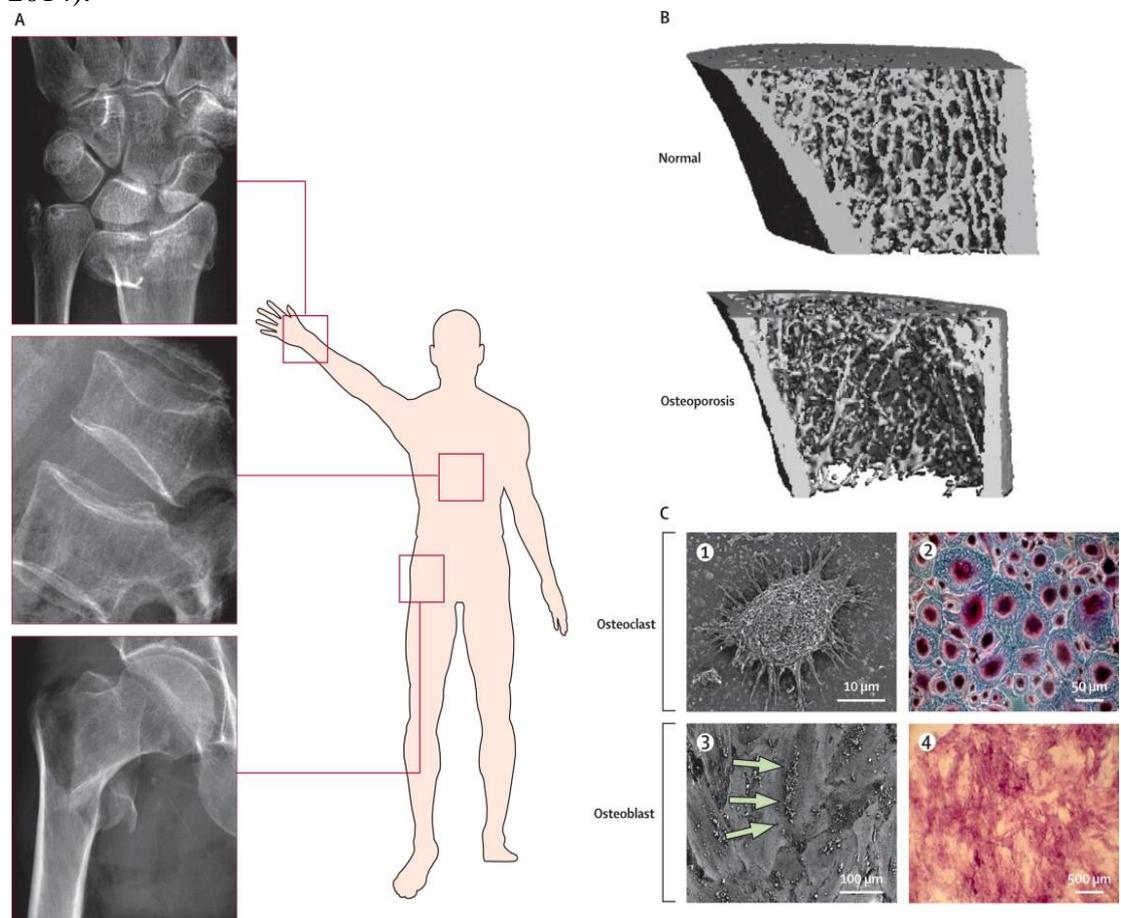
Fig. 1- 3 Osteoporosis effects shown A) on the wrist, spine and hip X-ray images B) Trabecular bone thinning shown on a microcomputed tomography. C) 1,2 osteoclasts, 3,4 osteoblasts cells shown by microscope (Rachner, Khosla, & Hofbauer, 2011)

1.1.4.2.   Paget's Disease

Unlike osteoporosis that takes place systematically, Paget's disease is localized. It is defined as a disease caused by the existence of huge osteoclasts which causes an enormous bone resorption. In bones where Paget's disease goes through, first a lytic phase and then an increase in formation of woven bone occurs in compensation of bone. which makes irregular bone causing mechanically weaker bone. Surgery could be in need to recover from Paget's disorder(Peel, 2012)

1.1.4.3.   Osteosarcoma

Osteosarcoma is the most widely speared and known malignant tumour affecting bones of different age groups. It tends to occur in long bones at the metaphysis (Fig. 1- 4). It is characterized as bone swelling and harsh pain. The therapy include chemotherapy at first, seconded by a surgery to remove the tumour, and followed by chemotherapy again (Isakoff, Bielack, Meltzer, & Gorlick, 2015).
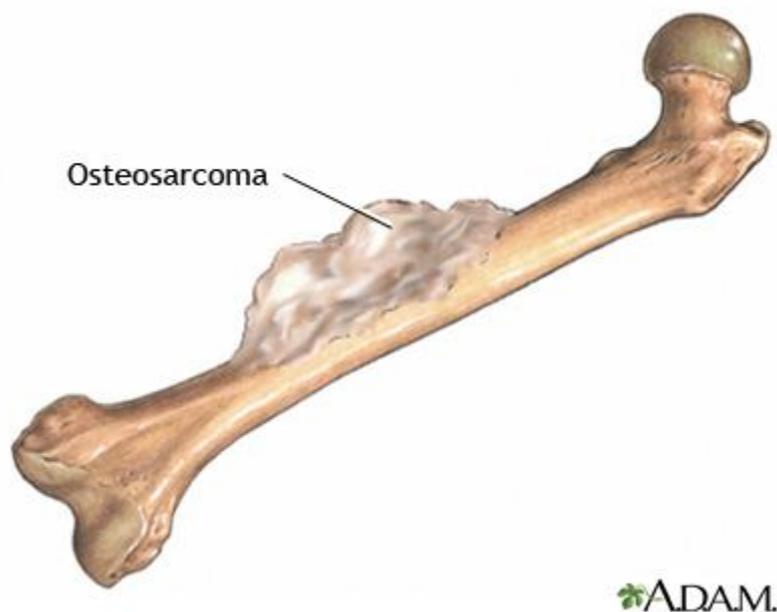
Fig. 1- 4 Osteosarcoma on long bone ("Bone tumor," 2016)

1.1.4.4.   Bone Metastases

Cancer which had spread from the primary tumour site to other sites causes the most mortality between cancer patients. Bone metastases is known to be silent linked to serious, usually intractable, pain. It is caused by tumour cells that invades the bone and then either osteoblasts or osteoclasts are affected making irregularity in bone structure. Bone metastases create osteolytic, osteoclast activation, and osteoblastic, osteoblastic activation, events by releasing factors to activate both (Mundy, 2002).

1.1.5. Current Treatments

Non-pharmacological management is a crucial and very wide concept that should be taken into consideration for prevention of any fracture. Nutrients are important in that aspect especially intake of calcium and vitamin D3 (also with sun exposure) while avoiding smoking and excess alcohol or caffeine consumption.

Weight-bearing exercises, muscle strengthening exercises and physical therapy enhance posture, balance, strength and agility. These activities cause reduction in the risks of falling and fractures which may lead to morbidity and mortality. Fall prevention strategies should be in consideration especially for the elderly (Body et al., 2011; Tella & Gallagher, 2014).

The use of pharmacological drugs should be taken into account for many of these bone disorders (Fig. 1-5). For example, Bisphosphonates, calcitonin, estrogens, parathyroid hormone and denosumab are FDA approved drugs for osteoporosis (Cosman et al., 2014), while some of these, such as calcitonin and bisphosphonates, are used for Paget's disease (Michou & Brown, 2011).

It should be noted that combination therapy of different classes of drugs can be beneficial for the treatment as it was shown to cause higher fractures reduction than the use of single drug. Combination therapies includes, bisphosphonate with denosumab, bisphosphonate with raloxifene, and estrogen and testosterone have all shown to provide better results than each alone (Ivanova et al., 2015).
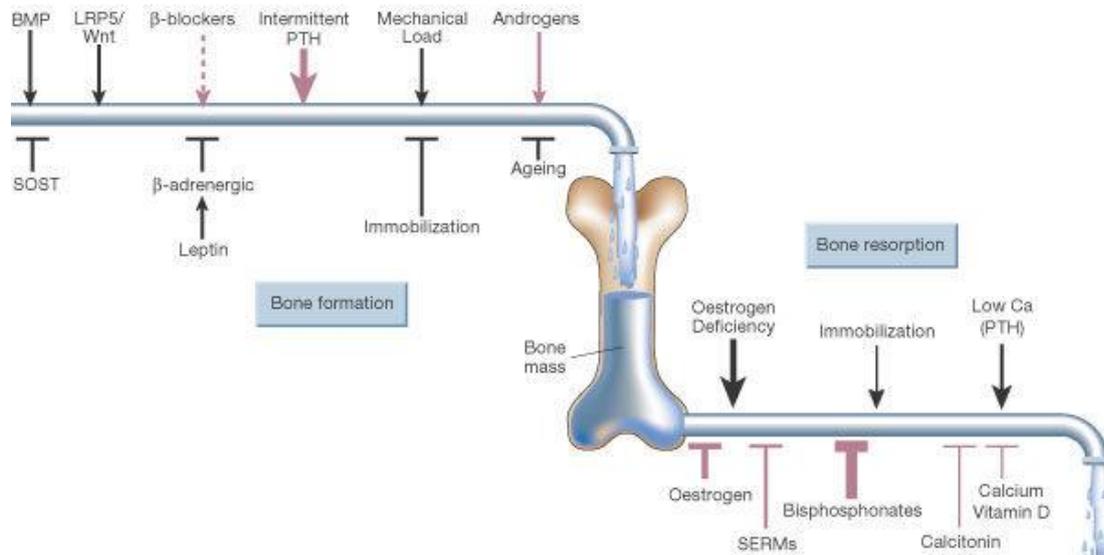
Fig. 1-5 Servo system sustaining bone mass levels. Physiological (black) and pharmacological (pink) stimulators (arrows) and inhibitors (T shaped symbols) of bone formation and resorption. (Harada & Rodan, 2003)

1.2. Bone Substitutes and Tissue Engineering Approaches

The need for surgery because of bone defects is evidenced by the fact that there is roughly four million bone grafting operations or bone substitutes performance every year around the world (Reid, 1968).

The bone graft is applied to stimulate and augment the regeneration of new bone especially in cases like healing of bone fractures or replacing lost bone caused by trauma or disease. Bone grafts have three major types which are autografts, allografts and bone tissue substitute (Polo-Corrales, Latorre-Esteves, & Ramirez-Vick, 2014). Autografts are bone grafts taken from patients' another bone site. It is the most widely used graft but it has problems including pain or donor site related risks and not having enough graft material at the donor site.

Allografts are bone grafts taken from another body. They don't have problems such as limited supply or donor site morbidity but have their own problems such as infection risk and low osteogenic capability (Polo-Corrales et al., 2014). Bone substitutes are biomaterials made of natural or synthetic materials that imitate the composition and/or microstructure of bones.

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Bone tissue engineering which is in focus of most recent biomaterial studies aims to improve osteogenicity, osteoconductivity and osteoinductivity by adding growth factors and progenitor cells to bone substitutes in the form of scaffolds. It has lower risk of contamination and infection, immunogenicity and no risk of disease transmission. Bone tissue engineering studies has been done and still going on with plentiful of building materials. (Polo-Corrales et al., 2014; Porter, Ruckh, & Popat, 2009).

Osteoconductivity is the property of a scaffold that induces bone cells to adhere, develop and live on it. Osteoinductivity is the ability to increase bone regeneration by biomolecular signalling and to let undifferentiated cells to develop into osteoblasts and chondrocytes (Di Martino et al., 2011; Olszta et al., 2007; Polo-Corrales et al., 2014). Osteogenicity is the ability for bone cells to create bone tissue.

Xenografts are bone grafts taken from animals. It is considerably unsuitable because of high risk of disease transmission, infection and immunogenicity (Porter et al., 2009).

To create an optimum scaffold four points must be considered while producing the scaffold:

1-Biocompability: It is the ability of a scaffold to maintain regular cellular activities, with no toxic affects either locally or systemically.

2-Mechanical characteristics: The mechanical characteristics and properties of bone scaffold should be matching to those of native bones which in turn vary for cortical or cancellous bone. However, it is generally hard to obtain because of the significant variation in morphology and mechanical properties such as load bearing stress even for same type of bone at different sites (Williams, 2008) .

3-Pore size: Interconnected porosity is essential for oxygen and nutrients diffusion across cells and for cell migration throughout the scaffold (Rouwkema, Rivron, & van Blitterswijk, 2008). Pore sizes between 10-400 μm are considered good enough (Porter et al., 2009), when there are both microscale and macroscale pores, scaffold acts better so it is preferred to have both scales of porosity on bone scaffolds (Woodard et al., 2007).

4- Biodegradation: It is a critical element for making bone scaffolds, since scaffolds should be degraded in a controlled rate to create space for more cells to migrate or develop(Lichte, Pape, Pufe, Kobbe, & Fischer, 2011; Olszta et al., 2007; Williams, 2008).

Scaffolds should maintain three properties for an optimum bone scaffold, osteoconductivity, osteoinductivity and angiogenesis, the ability of blood vessels to develop.(Olszta et al., 2007; Williams, 2008)

1.3. Scaffold Fabrication Methods

1.3.1. Electrospinning

Electrospinning is a simple and flexible method to develop fibrous structures (Yang, Yang, Wang, Both, & Jansen, 2013). Electrospinning is a method for achieving fibres with micro and nanoscale diameters using various. Basically, polymer solutions are injected through a syringe and then a high electric field is created between the positively charged polymer solution at the syringe tip and negatively charged collector (or ground connected collector). The electrospun polymer produced by this method contain high surface area and that is why it is considered to be suitable for tissue engineering in general (Huang, Zhang, Kotaki, & Ramakrishna, 2003; Liao, Chan, & Ramakrishna, 2010).

It can be used for creating nanofibrous scaffolds that support interaction between cells and matrix (Liao et al., 2010). Through electrospinning a fibrous scaffold can be produced with fibres of various diameters. Thus, the scaffold can imitate the bone matrix in terms of similarity to collagen fibres' diameters and topography (Flemming, Murphy, Abrams, Goodman, & Nealey, 1999; Friess, 1998; Green, Jansen, van der Waerden, & Von Recum, 1994).

Electrospinning setup (Fig. 1-6) includes different components to work, a polymer solution reservoir with a syringe , a syringe pump to control the flow rate of the injected solution, a collector for the aim of collection of the produced scaffold or mat, a power supply to produce an electric field between the reservoir and the collector (Di Martino et al., 2011).
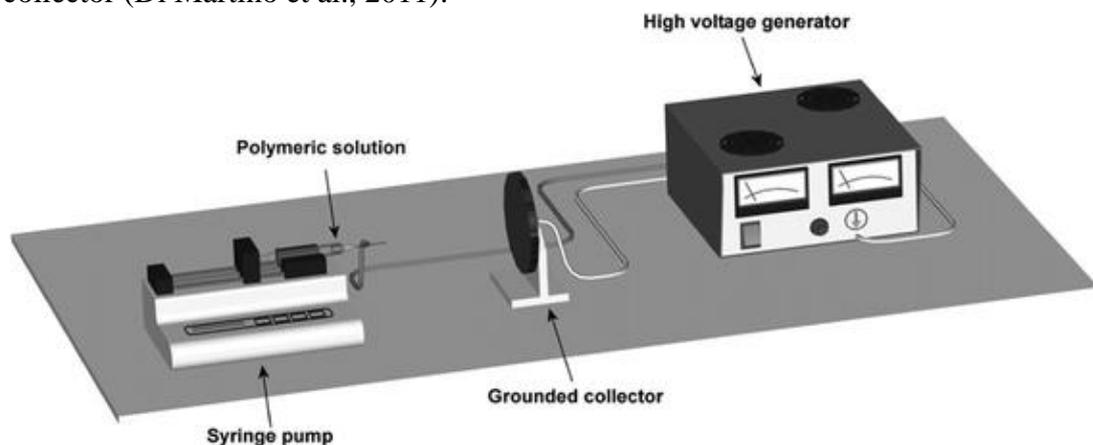
Fig. 1- 6 Schematic representation of an electrospinning setup (Di Martino et al., 2011)

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Electrospinning parameters are categorized into three; processing parameters, solution properties and ambient parameters. Processing parameters include collector type, needle diameter, distance between collector and syringe tip, solution feed rate, nozzle's configuration and applied voltage. Solution properties such as polarity, volatility and dielectric constant of the solvent besides solution viscosity and concentration. (Bhardwaj & Kundu, 2010; Ramakrishna, Fujihara, Teo, Lim, & Ma, 2005; Sill & von Recum, 2008).Ambient parameters include temperature, humidity etc.

Electrospinning has versatile options when it comes to material choice for drug delivery applications. Electrospinning can enable entrapment of various bioactive agents (antibiotics, anti-inflammatory agents, anticancer agents, peptides/proteins, vitamins, etc) by dissolving or dispersing the agent inside the polymer solution. The release of the entrapped agent will be mostly based on the diffusion and/or dissolution of the bioactive agent through the polymer fibers.

The biodegradation rate of the polymer would affect the release rate drug to be through diffusion entirely or by enhanced diffusion event with erosion of polymer matrix. In general, a fast release profile is observed with fibrous delivery systems because of high surface area. (Agarwal, Wendorff, & Greiner, 2008).

1.3.2. Wet Electrospinning

Conventional electrospinning create electrospun scaffolds consisting of compact/dense fiber network which makes it hard for cells to penetrate into the center of the scaffold (Yang et al., 2013). Several different methods by electrospinning were reported that can create 3-D scaffolds including changing the collector to a micro-patterned model, eliminating electricity from sprayed fibre by chemical blowing agents, and taking out microparticles placed between nanoparticles. Nevertheless, all of them issued problems on the fibre density management or for scaling up for the industry (Yokoyama et al., 2009).

To obtain bigger pores and higher porosity, a new electrospinning method was developed by making the collector a solvent such as methanol and letting the nano/micro fibers drop directly into the solvent (Fig. 1- 7). This type of electrospinning

is called wet electrospinning and it can create 3D scaffolds that are more appropriate for tissue engineering applications than conventional electrospinning method (Ki et al., 2007).
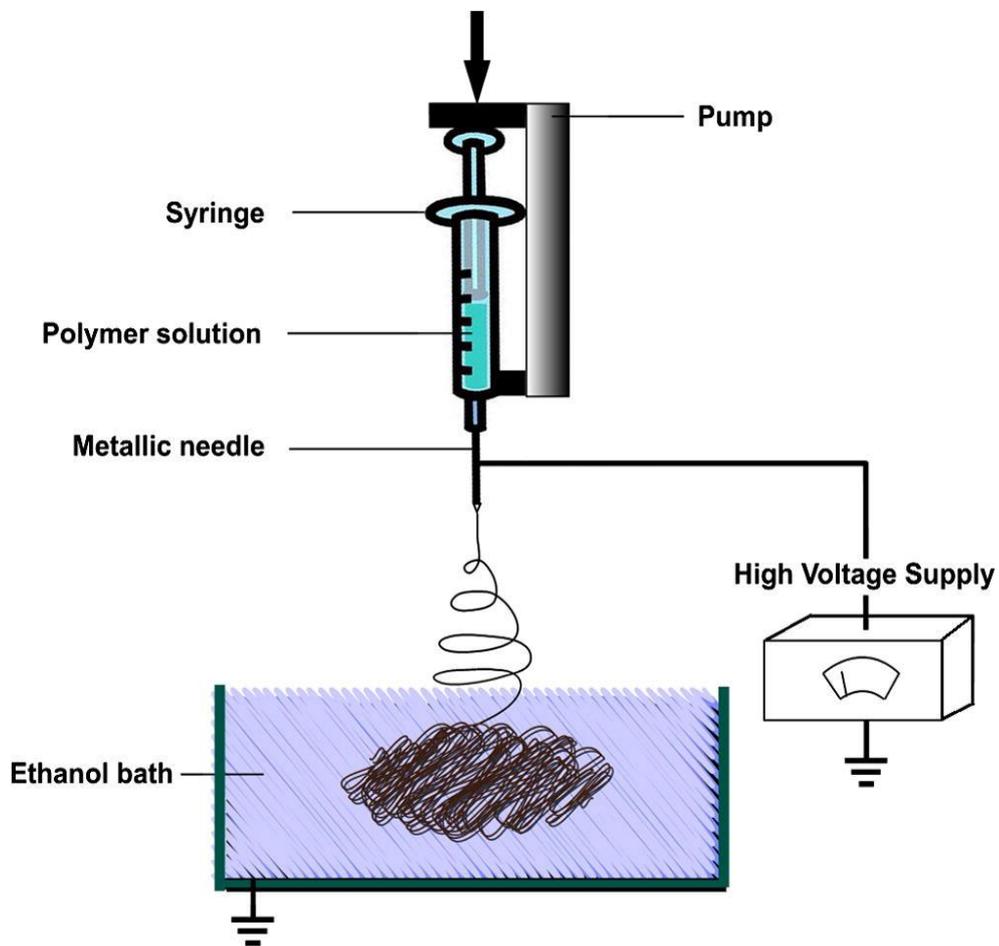
Fig. 1- 7 Scheme for wet electrospinning representing how the scaffold is in bulk formation (3-D) instead of a mate or a 2D membrane form obtained in conventional Electrospinning) (Yang et al., 2013)

1.3.3. Coaxial Electrospinning

Coaxial electrospinning differentiates from conventional electrospinning in that it creates core-shell fibres by using two different capillaries, one inside the other, where each is fed through a different polymer solution (Fig. 1- 8). When coaxial electrospinning starts, the high voltage will ease the evaporation of the solvents of two polymer solutions of both layers, thus forming core-sheath composite fibers (Díaz, Barrero, Márquez, & Loscertales, 2006).

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Possible usages for coaxial electrospinning include conserving a biological agent from environmental factors, providing a delivery method for a bioactive agent to be released in a fixed/controlled rate, and modifying the sheath layer of the fibers for surface properties without affecting the core (Braghirolli, Steffens, & Pranke, 2014).
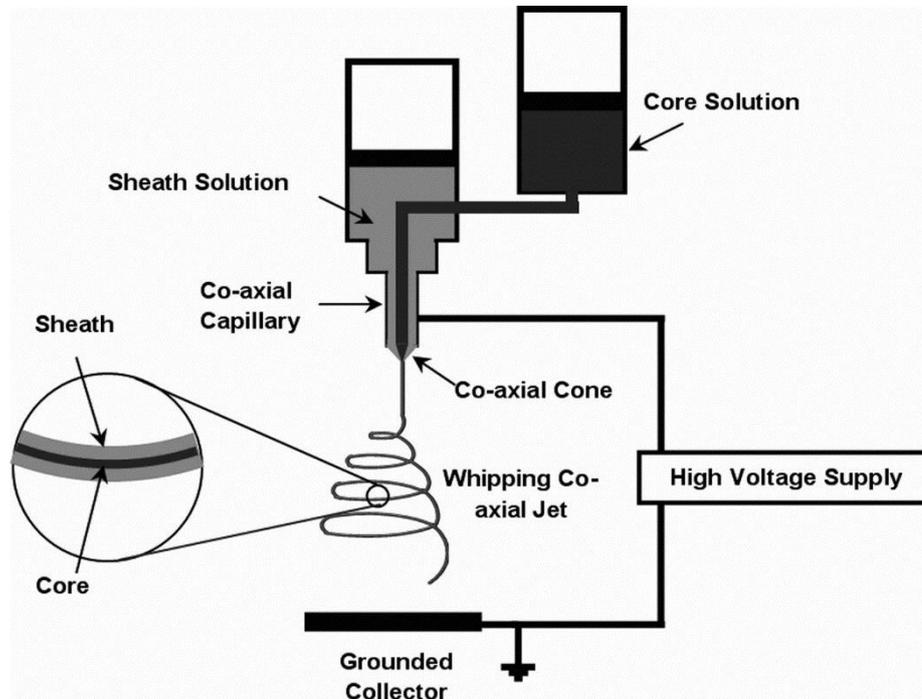
Fig. 1- 8 Schematic showing the coaxial electrospinning setup (Moghe & Gupta, 2008)

1.4. Controlled Release Systems

1.4.1. Controlled Release and Local Delivery of Drugs

Controlled release systems are created to change the pharmacokinetics of the bioactive agents, by acting as a reservoir and barrier for the agent(s) they are loaded with (Lee, Glendenning, & Inderjeeth, 2011). The administration frequency of a bioactive agents in conventional drug formulations is very important considering its route of administration and side effects. By developing a controlled release system, most of the problems of conventional systems can be optimized to achieve better drug pharmacokinetics (Allen & Cullis, 2004) with less systemic effects and more therapeutic efficacy (Maleki, Latifi, Amani-Tehran, & Mathur, 2013). Besides these discontinuation of drug by patients' due to difficulty of drug intake and following schedule, and side effects can also be solved by a controlled release drug delivery system (Lee et al., 2011).

Making a controlled release system for local bioactive agent delivery rather than systemic delivery can accelerate the local performance and even totally avoid potential systemic effects of while minimizing the risk of overdose and increasing bioavailability of the agent at the desired concentration at the main site (Mouriño et al., 2013; Rambhia & Ma, 2015).

Bone scaffolds have the potential to be local controlled release systems to provide localized treatment with more optimum doses for a longer time and at the same time they can provide surface and regenerative agents for cells to accelerate bone regeneration (Mouriño et al., 2013; Rambhia & Ma, 2015)

1.4.2. Bioactive agents used in this study

1.4.2.1.   Vitamin K2 (Menaquinone)

Vitamin K is a lipid soluble vitamin having an important role in the liver as it is involved in synthesis of active forms of coagulation factors. Vitamin K has the main chemical formula (2-methyl-1,4-naphthoquinone) and it is classified into three subsides Vitamin K1 aka phylloquinone, Vitamin K2 aka menaquinone and Vitamin K3 menadione (Plaza & Lamson, 2005; Strople, Lovell, & Heubi, 2009)

Vitamin K2 shows an influential effect on bone regeneration and reducing bone loss, particularly in Osteoporosis. It is also known to be one of the most prescribed osteoporosis therapy agents in Japan (Cockayne et al., 2006; Plaza & Lamson, 2005).

VK2 slows down osteoclastogenesis and bone resorption; and it acts in the posttranslational modifications of several proteins like osteocalcin which is engaged in calcium uptake and mineralization of bone (Koshihara, Hoshi, Okawara, Ishibashi, & Yamamoto, 2003; Plaza & Lamson, 2005). Menaquinone 4 (MK-4) (Fig. 1- 9) is known to be the prevailing kind of Vitamin K2 in animals generated by the conversion of exogenous and bacterial naphthoquinones (Plaza & Lamson, 2005).

However, 1 mg of Vitamin K2 is enough to interfere with anticoagulant drugs and antagonize their effect. (Crowther, Donovan, Harrison, McGinnis, & Ginsberg, 1998; Pharmaceutical Society of Great Britain, 2017) .This may lead to decrease the consumption of vk2 in which means avoiding its benefits.
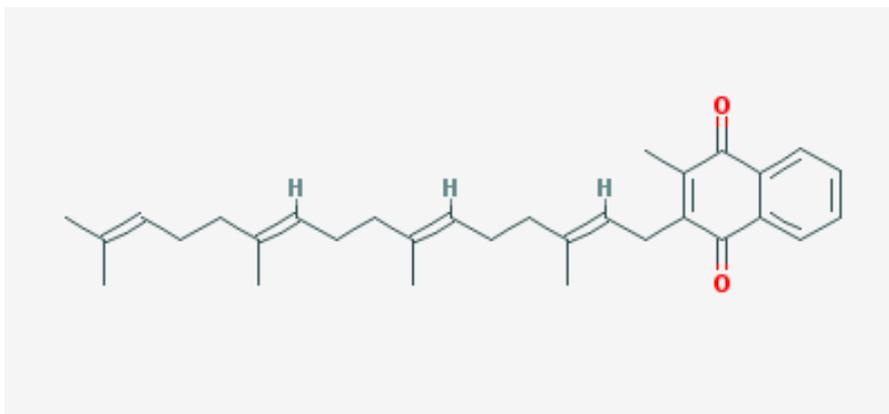
Fig. 1- 9 Chemical formula for Vitamin K2 Menaquinone 4 ("menatetrenone | C31H40O2 - PubChem,)

1.4.2.2.   Calcitonin

Calcitonin is a peptide composed of 32 amino acids having a disulphide bond between its residues at 1 and 7, and an amidated carboxy-terminal (Fig. 1- 10 &

Fig. 1- 11) (Breimer, MacIntyre, & Zaidi, 1988). Calcitonin can powerfully bind to osteoclasts and can decrease significantly their resorptive activity; especially the salmon calcitonin is 50 times more potent than human calcitonin (CHAMBERS & MOORE, 1983; Zaidi, Moonga, & Abe, 2002)

By inhibition of bone resorption, calcitonin can cause rapid decrease in calcium levels in blood. Calcitonin is used especially in high turnover of bone metabolism, and calcitonin has the unique effect which cause retraction of osteoclasts by changing the intracellular calcium levels (Naot & Cornish, 2008).

Calcitonin has side effects including abdominal pain, headache, malignancy, hypertension and polyuria (increasing urine output) (Pharmaceutical Society of Great Britain, 2017).
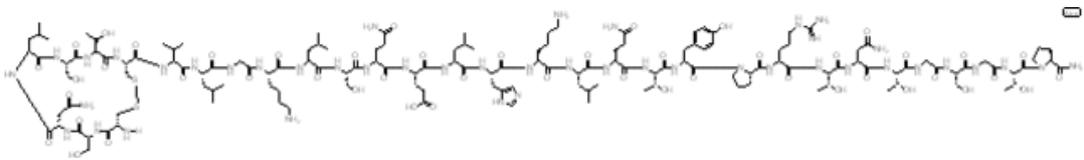
Fig. 1- 10 Calcitonin Salmon chemical formulation (Wishart,Knox, A. Geo et al)
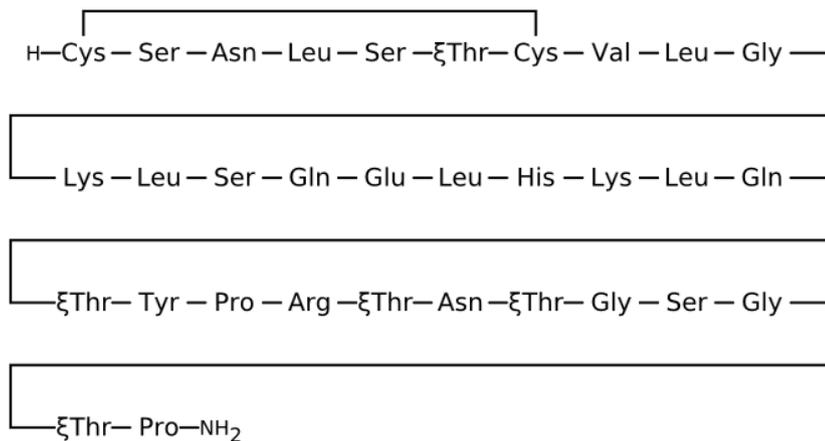
Fig. 1- 11 Aminoacid sequences of Salmon Calcitonin (National Center for Biotechnology Information)

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1.5. Aim of the Study

Bone defects can occur because of many different reasons ranging from trauma to osteosarcoma. Many of them need surgical operations, and adding a bone graft/substitute is necessary for returning to the healthy state. In the field of developing bone substitutes, new biomaterials that can also enhance regeneration capacity of the bone and deliver various bioactive agents/molecules is the focus for researchers. Meanwhile local and controlled release systems represent a challenge for researchers because of their benefits on avoiding side effects and interactions, and their potency for more effective treatment. Scaffolds make a perfect sense as local delivery systems because they are applied exactly at the site of defect. Besides that, they are aimed to direct tissue growth at the site while delivering the required agents for healing and regeneration. Wet electrospinning has the advantages of producing scaffolds with high porosity, 3D shape and high surface area making it an ideal bone scaffold that can also act as a local drug delivery system. Coaxial electrospinning has the ability to provide a core-shell fibrous structure in which delicate agents like proteins can be protected at the core from the outside environment. Also, it has the ability to make a system of dual release where the core and the shell will have different drug/bioactive agents and will have different release profiles owing to their locations within fibers.

In this study, developing a dual agent delivery system for a hydrophilic agent and a hydrophobic agent that will also act as bone scaffold was aimed. For that, designing a novel electrospinning method by applying coaxial electrospinning with wet electrospinning to produce a bone scaffold that has the benefits of both types was used. In addition, vitamin K2 and calcitonin salmon were chosen for their different therapeutic effects to be used for a dual agent release. Moreover, the scaffold was made to be releasing both vitamin k2 and calcitonin salmon as a novel drug delivery system for their different therapeutic effects which can be used in combination therapy.

Fig. 1- 12 Schematic structure for the hypothesis of this method

CHAPTER 2

MATERIALS AND METHODS

2.1.1. Materials

DMEM High Glucose with stable glutamate and with sodium pyruvate, Fetal Bovine Serum (FBS), Penicillin: Streptomycin solution, were purchased from Biowest (France).

Trypsin was obtained from Bibco (UK).

Fluorescein isothiocyanate (FITC)-phalloidin was bought from Thermo-Fisher Scientific (USA).

Protease Inhibitor Cocktail (EDTA-Free), ALP and BCA Detection Kit were obtained from ABCAM (UK).

P-Nitrophenyl phosphate Liquid substrate system, β-lactoglobulin from bovine milk (β-LG), Poly (vinyl Alcohol) (PVA), β-Glycerophosphate Disodium Sulfhydrate, Triton X-1, and Dimethyl Sulfoxide (DMSO)were all acquired from Sigma-Aldrich (USA).

Thiazolyl Blue Tetra Zolium Bromide, Vitamin K2 Menaquinone (K2), Gelatin from porcine skin, Pluronic F-68 (C3H6o.C2H4O) (Poloxamer 188), Kolliphor 188 (Poloxamer 188), Cupric Sulphate Pentahydrate, L-Ascorbic Acid 99%, Dimethyl Sulfoxide >= 99.5% (GC), Trypan Blue Solution (0.4%), Methanol (HPLC grade), Ethanol (HPLC grade) were purchased from SIGMA (Germany).

Poly [Ethylene Glycol] (PEG) (Mn:20000) and PCL (Poly CaproLactone) (6-caprolactone polymer) (mn: 70,000 – 90,000) were bought from ALDRICH (USA). 1,1,1,3,3,3 Hexafluoro-2-propanolol (HFIP), Magnesium Chloride - Hexahydrate,Iron (III) Chloride Hexahydrate, Triton X-100, Methanol (HPLC Grade), Acetic Acid were acquired from Merck (Germany).

Alamar Blue, MTT reagent (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) and Propidium iodide (PI) were purchased from Invitrogen (Germany).

Albumin Standard solution, Coomassie Plus Protein Assay Reagent and Fluorescein isothiocyanate (FITC)-phalloidin were bought from Thermo scientific (USA).

Calcitonin, Salmon was purchased from Calbiochem (USA).

2.2. Methods
2.2.1. Quantification Studies
2.2.1.1. Quantification of Vitamin K2

To establish protocols for quantifying Vk2 in release samples, Spectrophotometry method was applied.

2.2.1.1.1. Spectrophotometric Method

Ethanol was added to aliquots of release samples at ratio; 1:1, (Ethanol:PBS ; (0.1 M, pH 7.4)). The optical densities were measured at 247 nm. Pure ethanol:PBS 1:1 was used as Blank and the concentration of Vk2 was determined using previously constructed Vk2 calibration curve in ethanol:PBS 1:1 (range: 2.5 - 100 μg/ml) (Appendix A).

2.2.2. Quantification of β-Lactoglobulin

To establish protocols for quantifying β-LG in release samples, several methods were applied like spectrophotometric and Coomassie Plus (Bradford) Assay, but they failed to obtain qualified results because of interference with scaffolds components. To obtain such results, HPLC method was applied.

2.2.2.1.1. HPLC Method

Beta Lactoglobulin released from scaffolds into PBS (0.1 M pH 7.2) at 37°C was measured by HPLC method. A Shimadzu HPLC equipment and BioSep SEC-s3000 column (5qm x 300mm x 7.8 mm) was used with PBS (0.1 M pH 7.2) as mobile phase at a flow rate of 1 ml/min. Detection was performed at 214 nm at 37°C. The amount of B-Lactoglobulin was then calculated using a previously constructed calibration curve in PBS (Appendix A).

2.2.3. Preparations of Polymer Solutions for Electrospinning

PCL, Gelatin, Poloxamar-188, PEG, $MgCl_2$ and PVA solutions at different concentrations and combinations were prepared with various solvent systems using HFIP, Chloroform, Acetic acid. After complete dissolution, they were used in conventional wet electrospinning or co-axial wet electrospinning systems for preparing scaffolds.

Scaffolds loaded with bioactive agents were prepared by mixing these bioactive agents with the polymer solution before electrospinning.

2.2.4. Preparation of Scaffolds via Different Electrospinning Methods
2.2.4.1. Conventional Wet Electrospinning

The set-up (Fig. 2- 1) for electrospinning tool was made to be in vertical position so jet formation is enhanced by gravitational forces, consisted of NE-1000 syringe pump (New Era Pump Systems Inc, New York, USA) placed on the plexiglass cabinet made up of PMMA (Kesit Pleksi, Ankara, Turkey), rotational collector stand (Gözeler Elektronik, Ankara, Turkey) was inside the cabinet , a glass dish was placed above it, High Voltage Power Supply (Inovenso Inc, Istanbul, Turkey) was connected

to syringe's needle to make electric voltage (5 – 30 kV) between needle tip and the stand which is connected to ground.

The distance between the tip and collector was in range of 6 – 16 cm. The glass dish was filled with either distilled water or ethanol for different groups. After the electrospun was made, it was removed from the glass dish into a plate and freezed at -80°C in an ultra-low freezer (Nuaire, Playmouth USA), and then it was lyophilized via freeze-drier (Labconco Corporation, Kansas City USA).
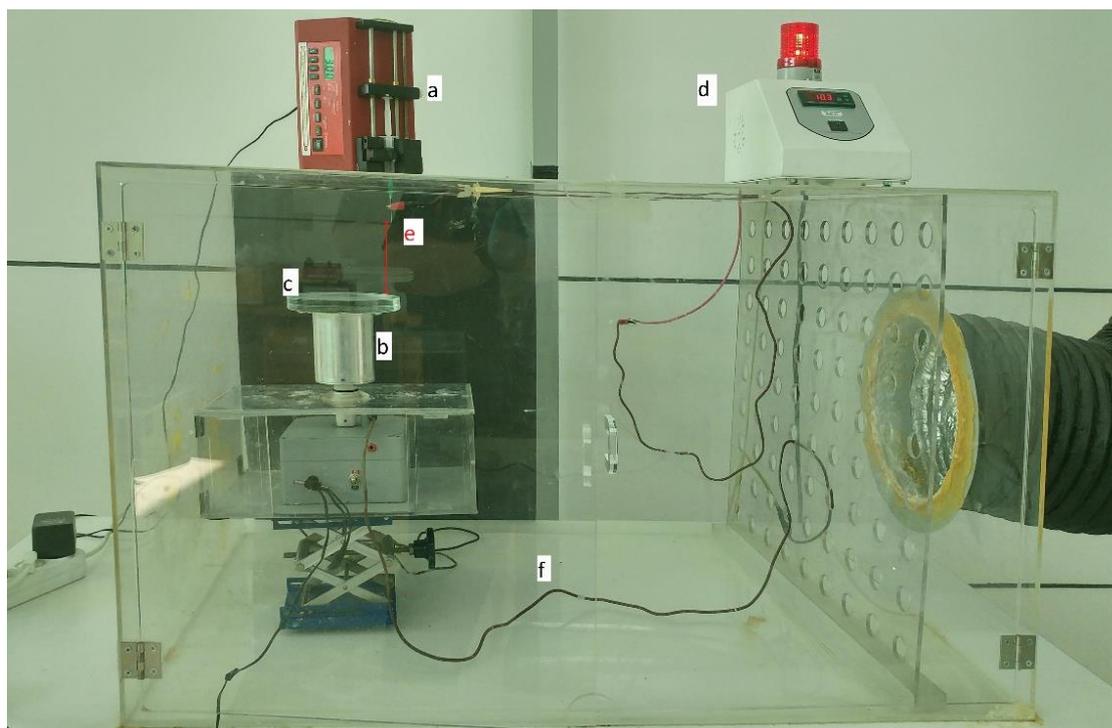
Fig. 2- 1 The setup for electrospinning a) NE-1000 syringe pump b) Rotational collector stand c) Glass dish contain the collector solvent d) High voltage source e) Distance between needle tip and collector f) cable connecting the stand to the ground

2.2.4.2.   Wet Co-Axial Electrospinning

The set-up for electrospinning tool was made to be in vertical position so jet formation is enhanced by gravitational forces. The system consisted of two syringe pumps, both are connected by tubes to a specially made needle for co-axial electrospinning (Fig. 2- 2) and a glass dish was placed at the bottom. All set up was inside a cabinet made of glass and aluminium.

The glass dish was filled with either distilled water or ethanol. After the electrospinning process, produced scaffolds was removed from the glass dish into a plate and cooled down to -80°C inside a deep freezer, and then it was lyophilized via freeze-drier (Labconco Corporation, Kansas City USA).
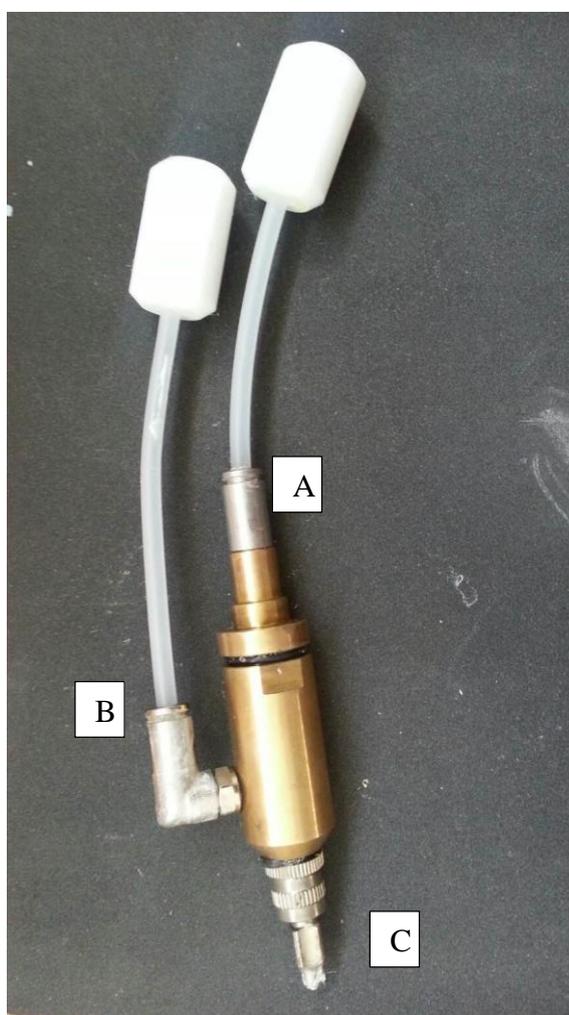
Fig. 2- 2 Coaxial needle a) Core solvent input b) Shell solvent input c) Core-Shell Output

2.2.5. Characterization of Electrospun Scaffolds
2.2.5.1.   Macro Scale Morphology Observation

The gross morphology of scaffolds was examined with the naked eye and handling. They were characterized as; i) had 2D or 3D structures. ii) having large or small volume, iii) formed a round, flat or amorphous shapes, and iv) has a tough or soft structure when evaluated physically.

2.2.5.2.   SEM Analysis

Scanning electron microscopy was used for microscale morphology analysis of electrospun scaffolds including fiber morphologies, diameters and average pore sizes. To prepare samples for SEM analysis, they were coated with gold and examined by SEM devices Quanta 400F Field Emission SEM device (FEI, USA) in Central Laboratory at METU (Ankara, Turkey).

2.2.5.3.   FTIR

To determine the presence of any chemical interactions between scaffold components and/or bioactive agent, FTIR analyses were made by a spectrometer (HYPERION 1000, Bruker Inc., UK). Each sample was crushed into powder and then mixed with Potassium Bromide, after that each sample had 50 scans within the wavenumber range; 400-4000 cm^{-1} with 4 cm^{-1} resolution. Correction of the spectra for background and atmosphere inside the FTIR was done for all samples. Spectragryph software (version 1.2.4; Menges 2017) was used to demonstrate the results.

2.2.5.4.   Degradation Study

Electrospun scaffolds were cut into cylindrical shaped samples, each with weight ~5 mg. Then, each piece was immersed in 4 ml PBS solution (0.1 M, pH 7.2) and incubated at 37°C inside water bath device (Nüve Bath NB 5, Turkey) for 10 days. PBS was periodically changed in every 2 days. After drying, scaffolds were weighed again and the weight loss percentages were calculated.

$$\text{Weight Loss (\%)} = \frac{\text{initial dry weight} - \text{final dry weight}}{\text{initial dry weight}} \times 100$$

2.2.5.5. Porosity Measurements

Porosity percentage of scaffolds and their pore size distribution were measured using Coremaster 60 Mercury Porosimeter (Qunatochrome Coorporation, Florida, USA) under low pressure (0-50 psi for 200 - 4 µm diameter) and Helium Ultrapycnometer 1000 (Quantachrome Corporation, Florida, USA) in Central Laboratory at METU (Ankara, Turkey). In mercury porosimeter analysis, Washburn equation was used to measure pore diameter in relation to the applied pressure. In helium pycnometer analysis, Archimedes' principle for fluid displacement and Boyle's gas law were used to measure volume and true density.

2.2.5.6. Swelling Test

Electrospun scaffolds Samples from selected groups of were cut into similar shaped and weight, they were immersed in 4 ml PBS solution (0.1 M, pH 7.4) and incubated at 37°C inside water bath (Nüve Bath NB 5, Turkey) for 10 days. PBS was periodically changed in every 3 days. Scaffolds were taken out and put over filter paper to get rid of excess water and then weighted, at different time points

$$\text{Swelling (\%)} = \frac{\text{Swollen weight} - \text{initial weight}}{\text{initial weight}} \times 100$$

Swollen weight is the weight of scaffolds at different times after submission in water. Initial weight is the weight of scaffold before being immersed in water.

2.2.5.7. Mechanical Tests on Scaffolds

Cylindrical shaped scaffold samples with a base diameter of 10 mm and 8 mm height were prepared. Compression test was applied to them by a mechanical tester (Univert Mechanical Test System, CellScale, Canada) by a 1 N load cell at a 5 mm/min crosshead speed tear. Young's modulus and 50% strain values were determined from stress-strain curves obtained from force displacement graphs.

2.2.6. Studies on Agents' Loading into Electrospun Scaffolds
2.2.6.1.　　Release Study
2.2.6.1.1.　　Vitamin K2

Scaffolds were cut into disc shaped samples and weighed. They were then put inside dialysis tubing's and immersed in 3 ml PBS solution (0.1 M, pH 7.2). Release studies were carried out at 37°C in a shaking water bath (Nüve Bath NB 5, Turkey) for 7 days, aliquots (1 ml) from the PBS around tubing's were collected at intervals and replaced with fresh PBS. Calculations of released amounts were done as described above for different bioactive agents. Dialysis tubing membrane acts like a sieve preventing molecules over 12,000 Daltons from passing. Therefore, it is expected to restrict the gelatin component of the scaffolds from being collected with aliquots. Thus, interference of Gelatin on release sample readings with spectrophotometry was not considered.

2.2.6.1.2.　　Beta-Lactoglobulin

Scaffolds were immersed in 3 ml PBS solution (0.1 M, pH 7.2) and incubated at 37°C inside water bath device (Nüve Bath NB 5, Turkey) for 7 days, aliquots from the PBS solvent were collected at intervals and replaced with PBS as described in previous part.

2.2.7. Cell Culture Studies
2.2.7.1.　　Cell Culture Conditions

Human osteogenic sarcoma cell line (Saos-2 cells) were cultured in a medium composed of DMEM - High Glucose with stable glutamine supplemented with FBS (10% V/V) and Penicillin: Streptomycin (100 U/ml) at 37°C under humidified atmosphere of 5% CO_2 – 95% air in incubator (Panasonic MCO-5AC-UV-PE IncuSafe, Japan). Every 3 days the medium was refreshed. Cells were passaged when they reach over 80% confluency by trypsinization with Tryspin-EDTA (0.1% v/v).

Saos-2 cells were seeded onto sterilized cell culture plates for monolayer cell studies. Saos-2 cells were seeded onto sterilized electrospun scaffolds for scaffold studies. Scaffolds were sterilized by UV exposure for 2 hours and then submerged in DMEM with 10% FBS, 1% Penicillin:Streptomycin and incubated at 37 °C under humidified atmosphere of 5% CO_2 – 95% air in incubator for 24 hours to verify the sterilization process .

2.2.7.2. Cell Viability Study

Effects of Vk2 and calcitonin on cell viability was determined through MTT assay (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide) in 96 well plate. In this assay, a monolayer of Saos-2 cells seeded as 15000 cells on each well were incubated in a medium composed of DMEM - High Glucose with stable glutamine supplemented with FBS (10% v/v) and Penicillin: Streptomycin (10 Unit/ml). The cell culture medium was used as blank control and as media for analysing the effects of samples for different incubation periods. In sample groups Vk2 was added to wells at different concentrations (0.028, 0.056, 0.112 Molar) and Calcitonin was added to different wells at different concentrations (0.0036, 0.0072, 0.0145 Molar). The control groups of cells grown in same cell culture media without addition of bioactive agents was used for each incubation period.

MTT solvent (5 mg MTT per 1 ml of the same medium) was added to each well, incubated for 4 hours at dark at 37°C under humidified atmosphere of 5% CO_2 – 95% air in incubator (Panasonic MCO-5AC-UV-PE IncuSafe, Japan). After that solution was replaced with DMSO and waited for 30 minutes by continuous shaking. Finally, the color change was measured by a well plate reader (GMI Biotech 3550, USA) at 540 nm wavelength. The test was considered for 3 different time intervals (24 hours, 48 hours and 72 hours) with 3 replicates for each group (n=3).

Scaffolds were trimmed into equal discs of 2 mm in diameter and set inside a 48 well microplate. Then, scaffolds were seeded with Saos-2 cells at density of 20,000/disc and incubated for 3 days.

For cell viability and attachment, Alamar Blue assay was done on 1st, 3rd, 7th days of incubation. Alamar Blue assay was done by first removing the medium and washing the discs with PBS (0.1 M, pH 7.4), then a solution made of 10% Alamar Blue and 90% DMEM with phenol red was added to each disc and incubated for 4 hours at dark. Optical density was measured at 570nm and 600nm for the added solutions on cell seeded scaffolds (samples), for the added solutions on cell free scaffolds (control of scaffold samples) for the added solutions on monolayered cells without scaffolds (control group 1), and for media without cells and scaffolds (control group 2).

2.2.7.3.   ALP Test

Increase in ALP enzyme activity is known as late differentiation marker for osteoblasts as mentioned by Barragan-Adjemian et al.   To study the effects of bioactive agents on differentiation to osteoblast cells Alkaline phosphatase assay (ALP) was applied to cells after certain treatment periods with the free solution and scaffolds loaded forms.

A growth medium consisting of DMEM - High Glucose with stable glutamine supplemented with FBS (10% v/v), Penicillin: Streptomycin (500 U/ml), dexamethasone (0.001 mg/ml), Beta-glycerophosphate (216.04mg/100ml), Ascorbic Acid (5 mg/100 ml) was prepared. Cells were seeded to a 48 well-plate for free solution or onto scaffolds at a density of 25000 cells/well and 25000 cells/scaffold, within the aforementioned growth medium and ALP activities were studied for 7th and 14th days.

Briefly, A lysis buffer was prepared by adding protease inhibitor cocktail (1% v/v) into an aqueous solution containing triton X-100 (1% v/v), tris (hydroxymethyl) aminomethane (50 mM) and NaCl (150 mM).
For ALP activity analysis, the growth medium was removed from well-plates and they were washed twice with PBS (0.1 M pH 7.2). After adding lysis buffer (75 μl) plates were incubated for 1 hour at 37°C by continues shaking. Lysates were collected from well-plates and transferred into another plate, lysis buffer containing $MgCl_2.6H_2O$ (10 mM) were added. Then, they were incubated for 1 hour at 37∘C by continues shaking in the presence of p-nitrophenylphenol solution. Finally, the optical densities were measured at 405 nm.

2.2.7.4.   Cell Morphology Anal-yses

Cells' morphologies were on various scaffolds were studied by SEM analysis. Cells seeded and incubated on electrospun scaffolds were fixed with 4% paraformaldehyde in PBS (0.1 M pH 7.2) at different time points. Scaffolds were first washed with PBS and then they were dehydrated with incubation in a series of ethanol solution with increasing ethanol amount from 20% to 100%. Following this, hexamethyldisilazane is applied for 20 minutes to dry samples before storage in desiccator. Finally, SEM analysis was done as described in section 2.2.5.2.

2.2.7.5.   Confocal Laser Scanning Microscopy

Saos-2 cells were seeded into scaffolds and were cultured for a week, then these cells were fixed as mentioned in section 2.2.7.4, after that these cells were stained with Fluorescein isothiocyanate (FITC)-phalloidin for actin and stained with Propidium iodide (PI) for nuclei (Mandal & Kundu, 2009). A confocal laser scanning microscope (Zeiss LSM 510, Germany) was used to obtain fluorescence images from stained samples. 70 μm z-stack was taken.

2.2.8. Statistical Analysis

All data were expressed as mean ± standard deviation. SPSS software was used for the One-Way Analysis of Variance (ANOVA), with Tukey's multiple comparison test for post-hoc analysis. Differences were considered as significant for $p < 0.05$.

CHAPTER 3

RESULTS AND DISCUSSIONS

3.1. Results of Electrospinning Experiments

Choosing the proper polymeric solution to produce a desired scaffold depends on the properties of solute and solvent, and also the concentrations. Different combinations of polymers and solvents were tested at different concentrations for obtaining a suitable scaffold via electrospinning (Table 3-1, Table 3-2). Produced materials were examined by macroscale morphological observation such as shape (Fig. 3-3) and volume, and by microscale SEM analysis (Fig.3-1, 3-2, 3-4, 3-5, 3-6) to find out the most proper combinations and concentrations.

At first, PCL was used alone using HFIP solvent. PCL was selected because of its well-known properties; PCL is a biocompatible, biodegradable polymer with quite good mechanical characteristics. It is an FDA approved polymer for use in biomedical applications (Ren, Wang, Sun, Yue, & Zhang, 2017). PCL facilitate cellular maturation and is mostly used in bone scaffolds (Groppo et al., 2017). Gelatin is a hydrophilic, biocompatible, and biodegradable biopolymer (Ghasemi-Mobarake et al). Gelatin was added to PCL to improve biomimetic properties of the scaffolds as it is a derivative of collagen, the most abundant protein in bone matrix.

During initial trials, a 2D shape/structure was resulted of both dry and wet Electrospinning due to the hydrophobicity of PCL. However, this situation was avoided and 3D structure is mostly obtained when gelatin was mixed with PCL. To be assured about the best mixture ratios of PCL and gelatin, three mixtures were tested; i) PCL is more than gelatin, ii) gelatin is more than PCL, and iii) PCL and gelatin mixed equally. When gelatin was more than PCL, it gave a 2D scaffold which was not membranous but was very sticky to the collector. The other two combinations gave

large, 3D shaped scaffolds that are smooth, but a little fluffy, and soft. It was also not in a good fibrous structure as aimed with ES process. The scaffold which was prepared with more PCL was bigger in size. Microscale SEM analyses showed that the equal amounts of PCL and gelatin resulted in a more porous structure which is preferable. Adding vk2 to the mixture didn't change the structural outcome of the scaffolds as seen in (Fig. 3-1, Fig. 3-2 and Table 3-1), but ethanol present in scaffold collection media had the risk of dissolving some of the added vk2 which might cause losing drug/bioactive agent from the scaffolds. The reason behind this issue is that vk2 is a hydrophobic compound that dissolves in ethanol.

To overcome this problem, water was used in collection environment of ES process, but the resulted scaffold was a membrane that was not in 3D structure. The main reason behind that result is thought to be the difference of surface tension and/or density between polymer phase and collection media water and ethanol.

Considering this situation, three approaches were applied. The first was making the polymer solution denser by adding metals to it. $MgCl_2$ and $FeCl_3$ were added instead of gelatin and a 3D scaffold was yielded but it didn't achieve good results in microscale analysis (Fig. 3- 2 8a, 8b). Fibers were not homogenous; Some had diameters around 0.1 μm while others around 0.7μm. Pores were barely appeared because of closeness and condensed formation of fibers.

The second approach was adding surfactant to the water collector, which should decrease the surface tension of the water leading to creation of a 3D scaffold, but no observable effect was seen and again a membrane was obtained.

The third approach was to add surfactant to the polymer mixture, which resulted in a good 3D structure scaffolds. The reason why adding a surfactant to the collector media didn't help creating a 3D scaffold but adding a surfactant to the polymer mixture did, can be predicted to be the concentration of surfactant in the collector is much less than in the polymer solution, and when the surfactant is in the polymeric solution and also by the difference in formed polymer fibers when surfactant is added to polymer solution. Two surfactants were tested, each with different strategy and several ratios inside the polymeric solution.
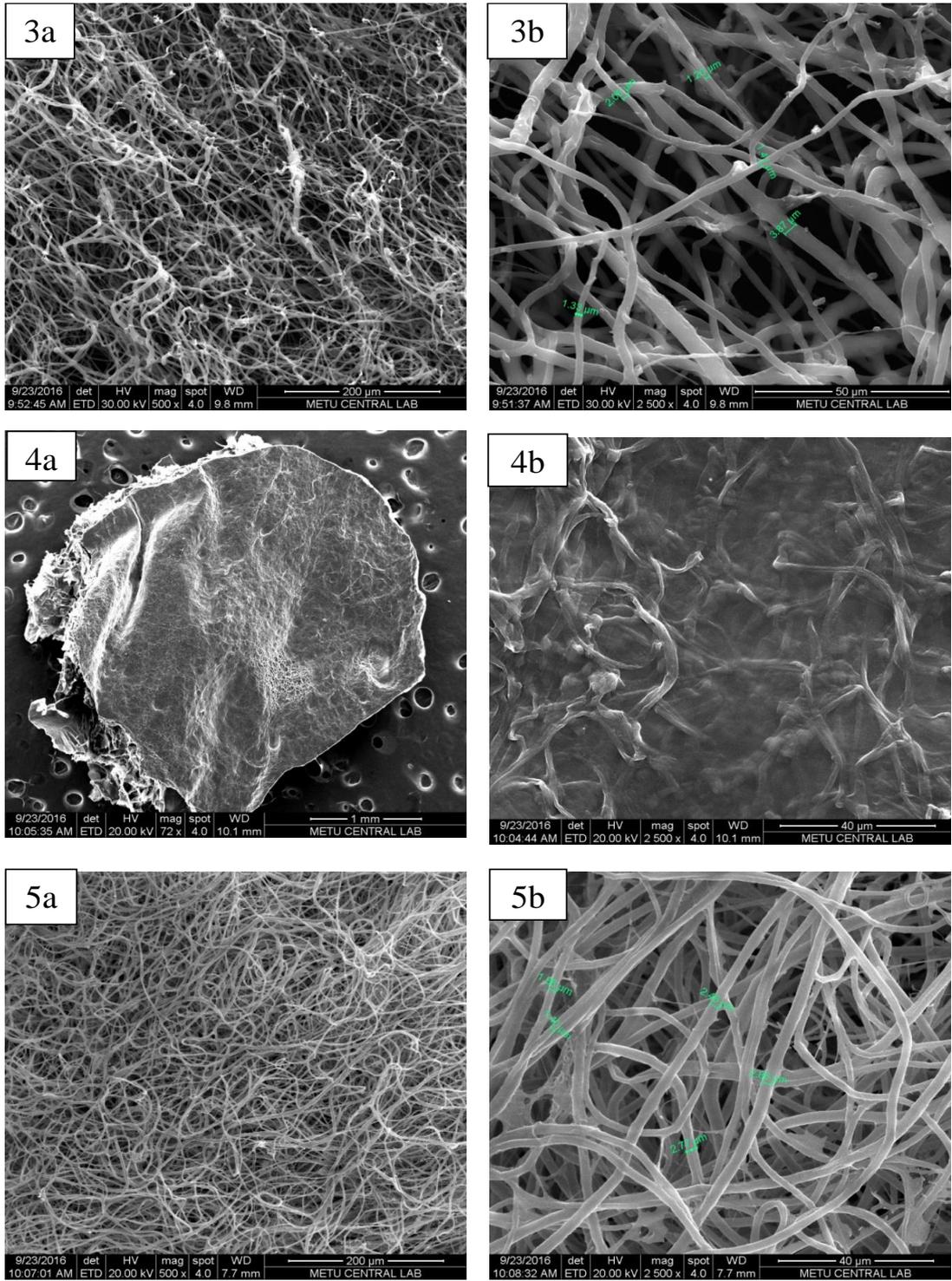
Fig. 3- 1 SEM images of electrospun scaffolds, Number codes on figures are corresponding to those given in Table 3-1, a) 500x, b) 2500x magnification 3) 5%PCL + 5% Gelatin, ethanol – 4) 2.5% PCL + 7.5% Gelatin, Ethanol – 5) 7.5%PCL + 2.5% Gelatin, ethanol

PVA (Poly Vinyl Alcohol) is a hydrophilic, biocompatible and biodegradable polymer that is commonly used as surfactant (Zhuang 2012). While adding PVA, the polymer composition was kept around 10% in total. One of the polymer composition had equal amounts of PCL, Gelatin and PVA, and resulted in a 3D shaped scaffold that is round, having medium toughness and size. Pore sizes were good enough for cells to penetrate them, and fiber diameters were around 1-2μm. However, the scaffold was a bit melted which closed some pores and increased some fiber diameters significantly and that resulted in making the scaffold less homogenous in structure (Fig3-2 9a, 9b).

The other two scaffolds that included PVA were prepared by keeping its amount around 10% wt/vol of polymeric solution. Instead of preparing scaffolds with equal amounts of PCL and gelatin, higher PCL/gelatin ratio applied. Here, while one of the groups had more Gelatin than PVA, the other had a more PVA than gelatin. Both of these trials have resulted in a membrane shaped scaffold. Studies showed that adding more PCL than gelatin in a polymer mixture resulted in a more round and tougher scaffold. However, using more PCL than gelatin with PVA addition resulted in a membrane because of PCL hydrophobicity which made it more repel to water in turn making a membrane.

The other surfactant used in this study was P-188 (Poloxamer 188, Pluronic® F68, Kolliphor® P 188). P-188 is an FDA approved non-ionic surfactant which is a biocompatible and biodegradable polymer (Bueno 2014, Shelke 2007, Taslo 2013)

Instead of making the whole polymer solution concentration around 10% wt/vol, since equal amount of PCL and Gelatin created a 3D scaffold with PVA and not the 10% ratio, here the original ratio of 5% wt/vol gelatin mixed with 5% wt/vol PCL was kept and P-188 was added to it in 3 different ratios; 1%, 5% and 10%. All these added ratios resulted in a 3D scaffold. However, they had different physical characteristics, with increasing the ratio of P-188 the resulted scaffold becomes less flat and more round in shape, tougher and bigger in size. Looking at these three scaffolds on a microscale (Fig 3-4) reveals that with 1% wt/vol P-188, the scaffold has no apparent fibers or pores, it seems to be melted or fused. While the scaffold with 5% wt/vol P-188 ratio had visible fibers and pores, the melting effect was still observable.

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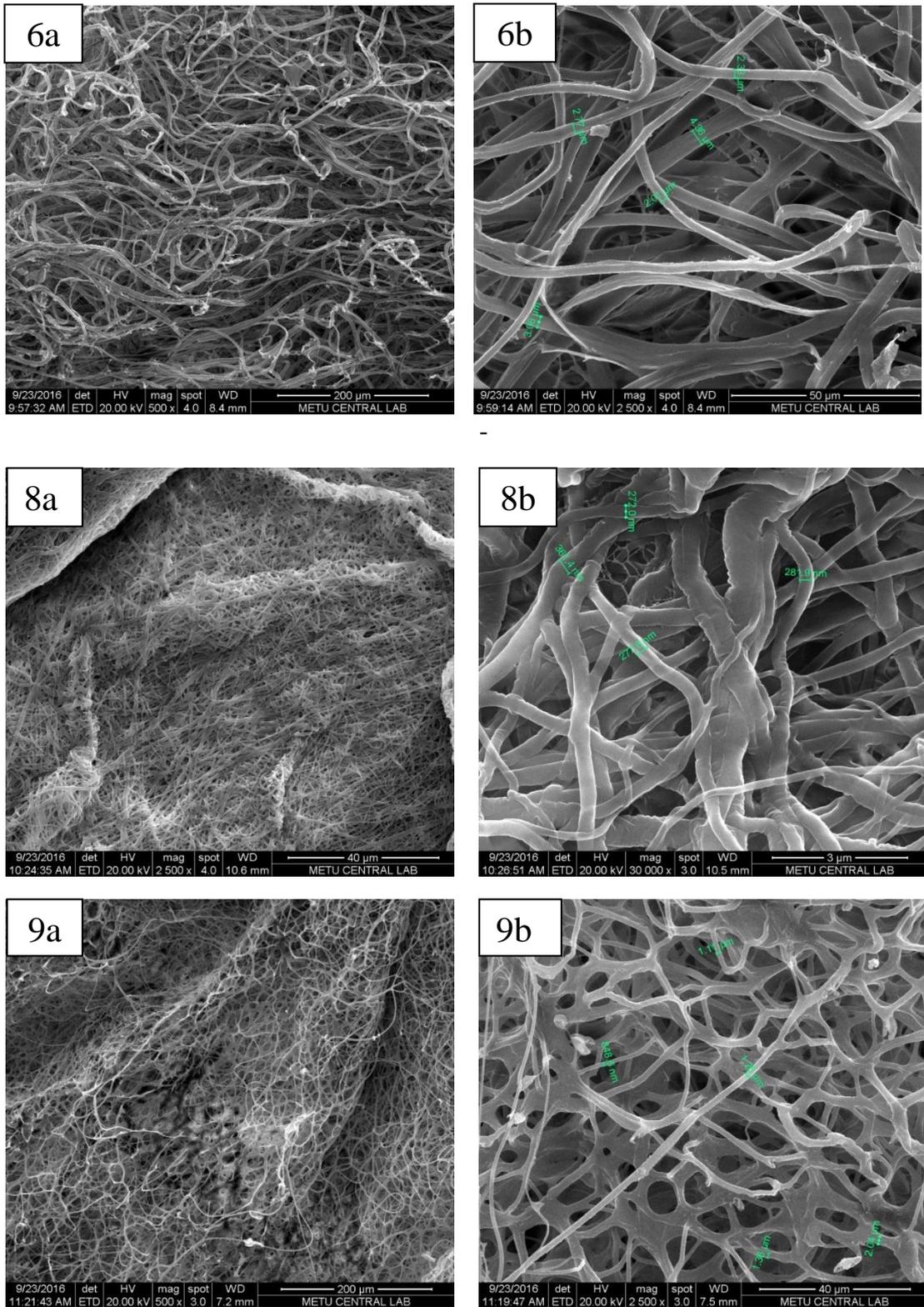
Fig. 3- 2 SEM images of electrospun scaffolds. Number codes on figures are corresponding to those given in Table 3-1, a) 500x, b) 2500x magnification 6) 5% PCL + 5% Gelatin, ethanol – 8) 10% PCL + 10% MgCl2+ FeCl3, ethanol – 9) 3%PCL + 3% Gelatin + 3% PVA, ethanol

However, in terms of tissue engineering they were unfavorable as bone cells wouldn't be able to penetrate into both scaffolds.

In the meantime, the scaffold with 10% wt/vol P-188 didn't result in any melting effect. They had clearly seen fibrous structure with fibers in the range of 0.5 2µm and having micropores between 10-50 µm range.

Since adding metals did make the scaffold in 3D shape even without gelatin, PCL was mixed with P-188 but without gelatin to be sure that gelatin has an effect on 3D scaffold along with P-188. Two polymer mixtures were tested, one with 1% P-188 ratio and the other with 5% ratio, and both included PCL at 10% ratio. Both ended to become a membrane like scaffold insuring that gelatin is indeed has an effect on making the scaffold in 3D shape.

Since the good quality of the aforementioned scaffold that is made with 10% wt/vol P-188, it was also tested again but with ethanol in collector, instead of water, in order to compare it to the scaffolds made from 5% wt/vol PCL and 5% wt/vol gelatin in ethanol based collector. The resulted scaffold was also big and round like the one used water as a collector, but it was soft and its fibers were much larger, some of which being above 10 µm (Fig 3-4).

The aforementioned scaffolds can be used for loading with either a hydrophobic bioactive agent or a hydrophilic one, in theory they should also hold more than one hydrophobic bioactive agent or more than one hydrophilic agent. However, loading different agents into same matrix-scaffold environment would affect both loading ratios and release profiles of the two agents depending on various parameters (Surfactant presence, solvents used, solubility of the agents etc.) Therefore, in this study, a single bioactive agent for loading into such (monoaxial electrospun) scaffolds was tested on selected groups of scaffolds.

However, to prepare a scaffold containing both a hydrophobic and a hydrophilic bioactive agent loaded into different sections of the scaffold, coaxial electrospinning can be applied. In such systems, the shell contains the bioactive agent that won't be dissolved in the collector and the core contains the bioactive agent which would be dissolved in the collector if it weren't shielded by the shell. In this type of scaffold, dual loading of Vk2 and a model protein was investigated as they are expected to not affect each other's loading and might have less effect on release profiles by being compartmentalized. From Fig. 3-7, it can be clearly seen that coaxial electrospinning successfully produced coaxial fibers with one layer shielding the other.

For that, two strategies were tested, the first is when ethanol was used as a collector and the core-shell structure would be able to contain hydrophobic (in core) – hydrophilic (in shell) bioactive agents. The second is when water was used as a collector and the core-shell structure would be able to contain hydrophilic-hydrophobic bioactive agents respectively.
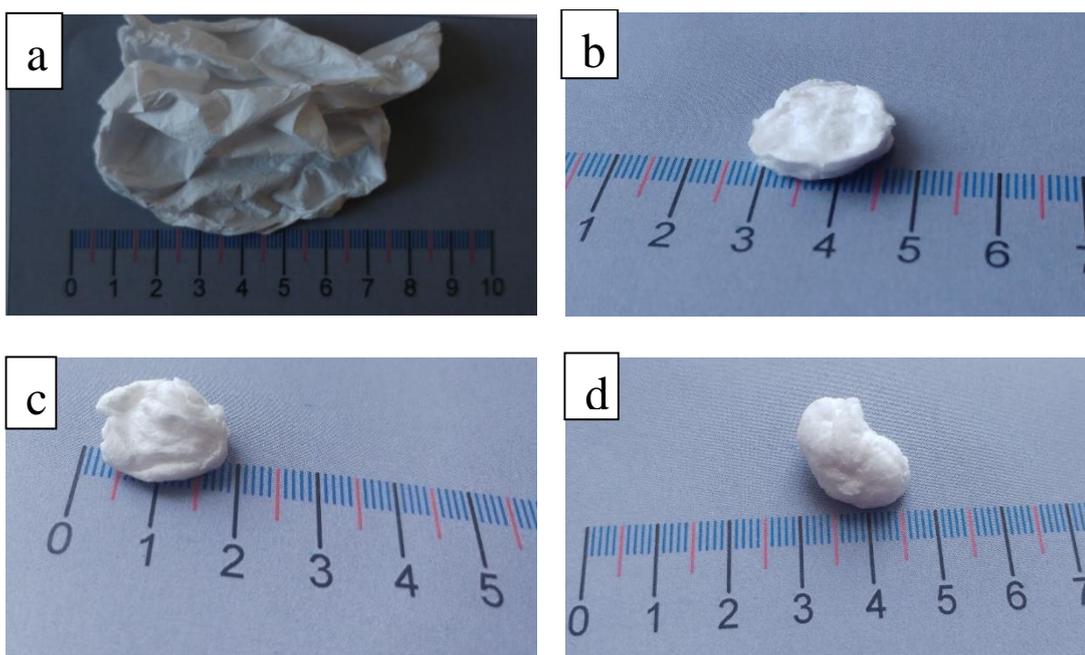
Fig. 3- 3 representative figures for main shapes of scaffold a) membrane b) flat c) amorphous d) round

Table 3- 1 Polymeric solvents concentrations, collector, and macroscale and microscale characteristics of electrospuns made by Monoaxial wet electrospinning, all polymeric solutions had HFIP as a solvent, % ratios represents concentration weight/volume

| #	PCL	Gelatin	P-188	PVA	VK2	MgCl$_2$ + FeCl$_3$	Collector	Macroscopic characteristic	Microscopic Characteristics
1	10%						Ethanol	Membrane	
2	10%						Water	Membrane	
3	5%	5%					Ethanol	3D Soft, large, flat	Good Fiber and pore diameters
4	2.5%	7.5%					Ethanol	2D shape	
5	7.5%	2.5%					Ethanol	3D Soft, Large, Round	
6	5%	5%			+		Ethanol	3D Soft, large, flat	Good Fiber and pore diameters
7	10%				+		Ethanol	Membrane	
8	10%					10%	Water	3D Soft, medium size, Flat	Good Fiber small pore diameters
9	3%	3%		3%			Water	3D Medium toughness large, round	Melting issue occurred
10	6%	3%		1%			Water	Membrane	
11	6%	1%		3%			Water	Membrane	
12	5%	5%	1%				Water	3D Soft, Medium Size, Flat	Extreme melting
13	5%	5%	5%				Water	3D Tough Large Amorphous	Good Fiber and pore diameter but condensed fiberss
14	5%	5%	10%				Water	3D Tough, Large, Round	Good Fiber and pore diameters
15	5%	5%	10%				Ethanol	3D Soft, Large, flat	Good and Smooth Fibers with good pore diameters
16	10%		1%				Water	Membrane	
17	10%		5%				Water	Membrane	
18	5%	5%					Water	Membrane	

Fig. 3- 4 SEM images of Eelectrospun scaffolds. Number codes on figures are corresponding to those given in Table 3-1, a) 500x, b) 2500x magnification   12) 5%PCL + 5%Gelatin + 1% P-188, ethanol - 13) 5%PCL + 5%Gelatin + 5% P-188, ethanol - 14) 5%PCL + 5%Gelatin + 10% P-188, water – 15) 14) 5%PCL + 5%Gelatin + 10% P-188, ethanol

A series of optimization experiments (Table 3-2) were needed for coaxial electrospinning studies of the scaffolds:

When ethanol is used in collector media, at first, similar content of monoaxial electrospun, 5% wt/vol gelatin with 5% wt/vol PCL, was applied. However, the electrospinning failed to operate at these concentrations. Then, the concentrations were increased to 10% wt/vol PCL and 10% wt/vol Gelatin as shell and core polymer solutions respectively and vice versa for aqueous collectors.

As long as PCL was used in the shell, the resulted scaffold was tough, big, flat and showed good fiber diameter distribution. On the other hand, pores were very scattered and hard to notice as much fibers are oriented in one way but not in different directions (Fig 3-5). But when PCL was used in the core, the resulted scaffold was soft, big, flat and had both good fiber diameters and pore sizes (Fig 3-5). Since PCL is a hydrophobic compound, then adding a hydrophobic bioactive agent to the PCL solution would provide its homogenous distribution, controlled loading and release.

Instead of HFIP, chloroform was also tested as solvent of core polymer based on the idea that different solvents of core and shell would make a better separation during electrospinning. The resulted scaffold was small, tough and round, and while it had good shape and porous structures, the scattered not homogenous fibers was considered as the scaffold was not very suitable for drug delivery (Fig.3-5).

P-188 was added to see its effect on these scaffolds and it was in the shell part with gelatin, the resulted scaffold was soft and round but its fibers were melted such that pores were closed (Fig. 3-5). On the other hand, when P-188 was added to both the core and shell in similar amounts as 5% wt/vol PCL and 5% wt/vol P-188 in core and 5% wt/vol gelatin and 5% wt/vol P-188 in shell, the resulted scaffold was soft, large and flat, with good fiber diameters and good pore sizes (Fig.3-5)

Since adding more PCL did lead to a membrane like scaffold in the initial single phase (monoaxial) electrospinning studies, another concentration was considered for the coaxial study by decreasing PCL ratio to 2.5% wt/vol in the core, and the resulted scaffold was a membrane, which proves that the equal amounts of

PCL and gelatin are needed for a better 3D scaffold when it comes to using water as a collector.

When using water as a collector, similar to the content of its monoaxial electrospun, 5% wt/vol gelatin, 5% wt/vol PCL and 10% wt/vol P-188 were used; here gelatin solution phase was the core and so a hydrophilic bioactive agent (model protein) which should be in the core would be loaded into this phase with more ease. P-188 needed to be both in the core and shell together in equal ratios (as in the case of above mentioned, second type of coaxial ES with PCL core), otherwise co-axial electrospinning was not successful if ratios of core and shell weren't equal to each other. The resulted scaffold was a soft, large and amorphous scaffold with a good fiber diameter range; 1-3μm and even though some pores were closed due to some melting, overall pore sizes were good enough for cells to move inside the scaffold (Fig. 3-6).

When core was gelatin based, water was used as a core polymeric solvent based on the idea that it would cause better separation, however the co-axial electrospinning failed to proceed.
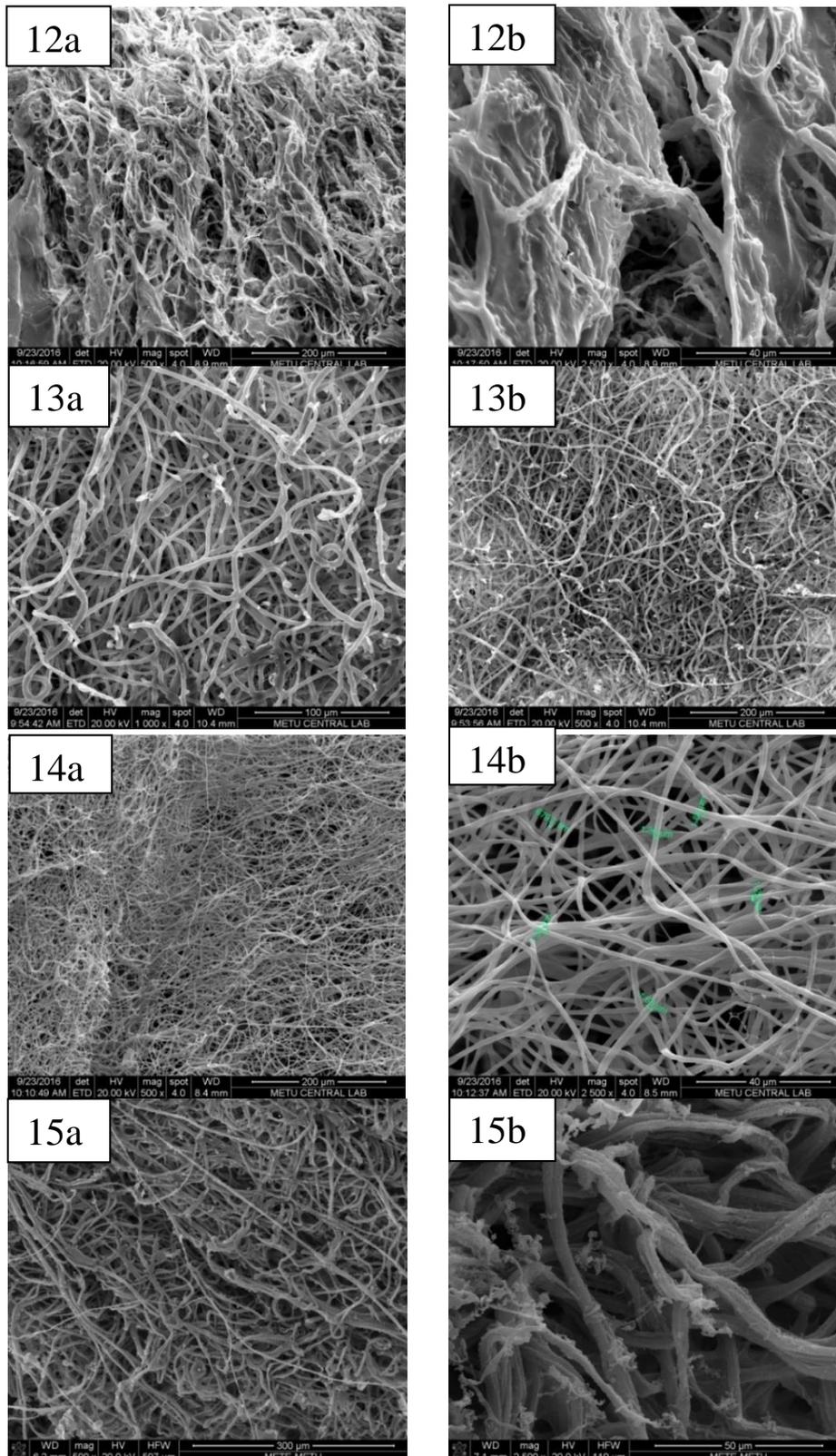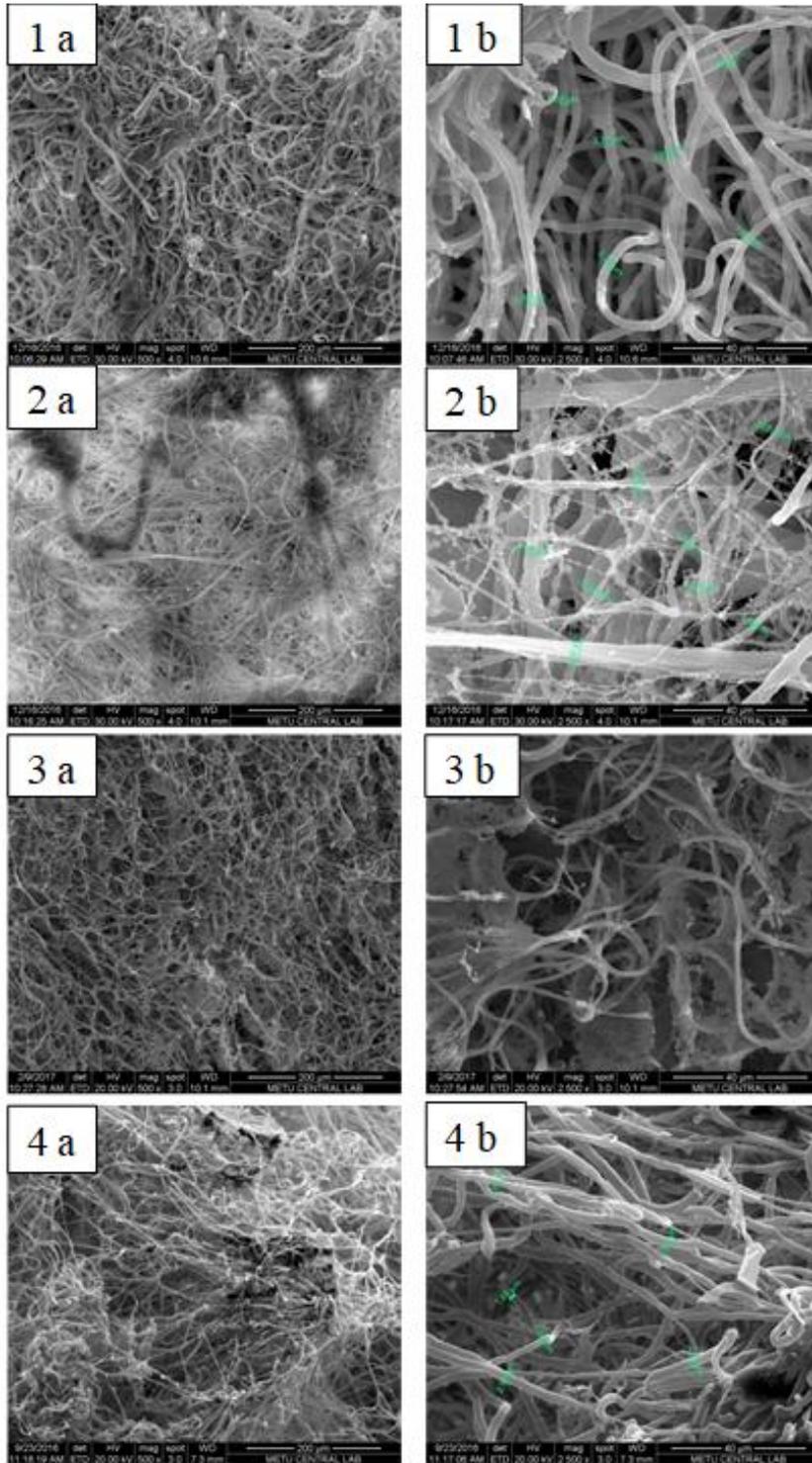
Fig. 3- 5 SEM images of electrospun scaffolds. Number codes on figures are corresponding to those given in Table3-2, a) 500x, b) 2500x magnification 1) Core: 5%PCL+: 5% P-188, Shell: 5%Gelatin +: 5% P-188, ethanol – 2) Core: 10% PCL, Shell: 10% Gelatin, ethanol;   3)Core: 10% PCL, Shell: 9%Gelatin +1% P-188.,ethanol 4) Core: 10% Gelatin, Shell: 10% PCL, ethanol
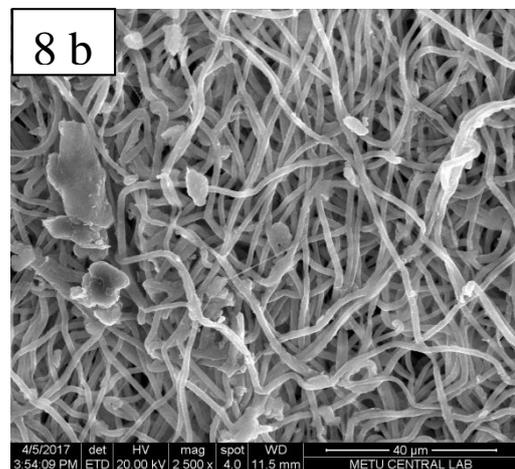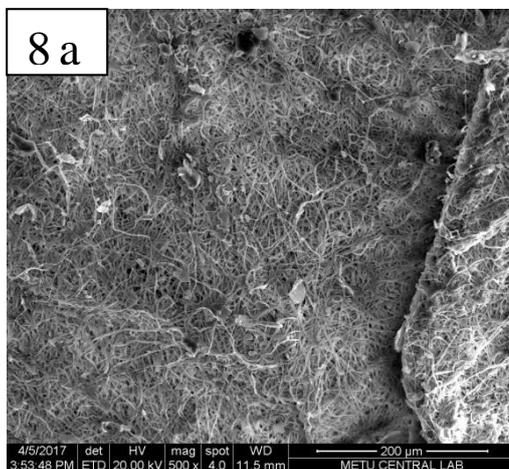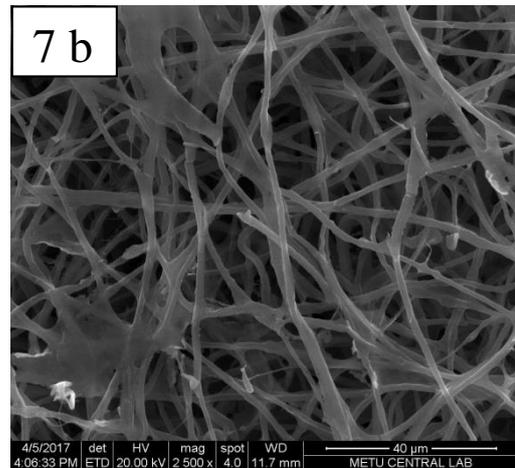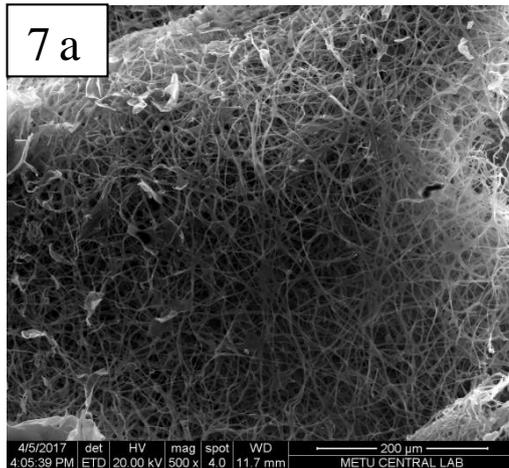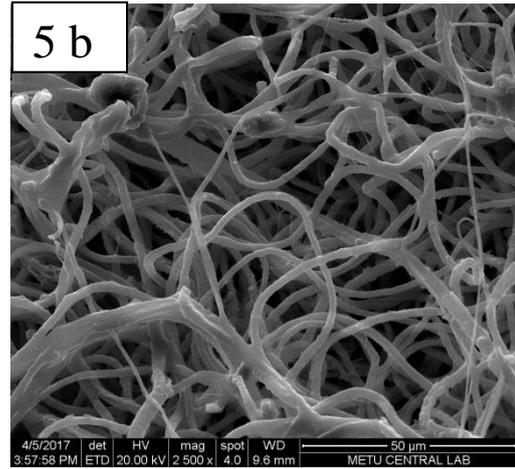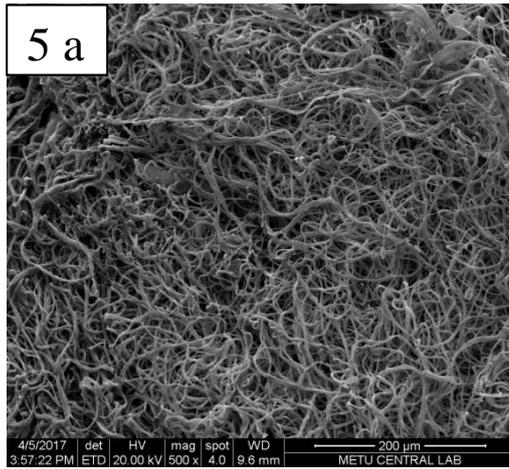
Fig. 3- 6 SEM images of electrospun scaffolds, Number codes on figures are corresponding to those given in Table3-2, a) 500x, b) 2500x magnification   5) Core: 10% PCL, Shell: 10% Gelatin, ethanol 7) Core: 5%Gelatin+: 5% P-188, Shell: 5%PCL +: 5% P-188, water8) Core: 10% PCL, Shell: 5% Gelatin + 5% P-188, water.
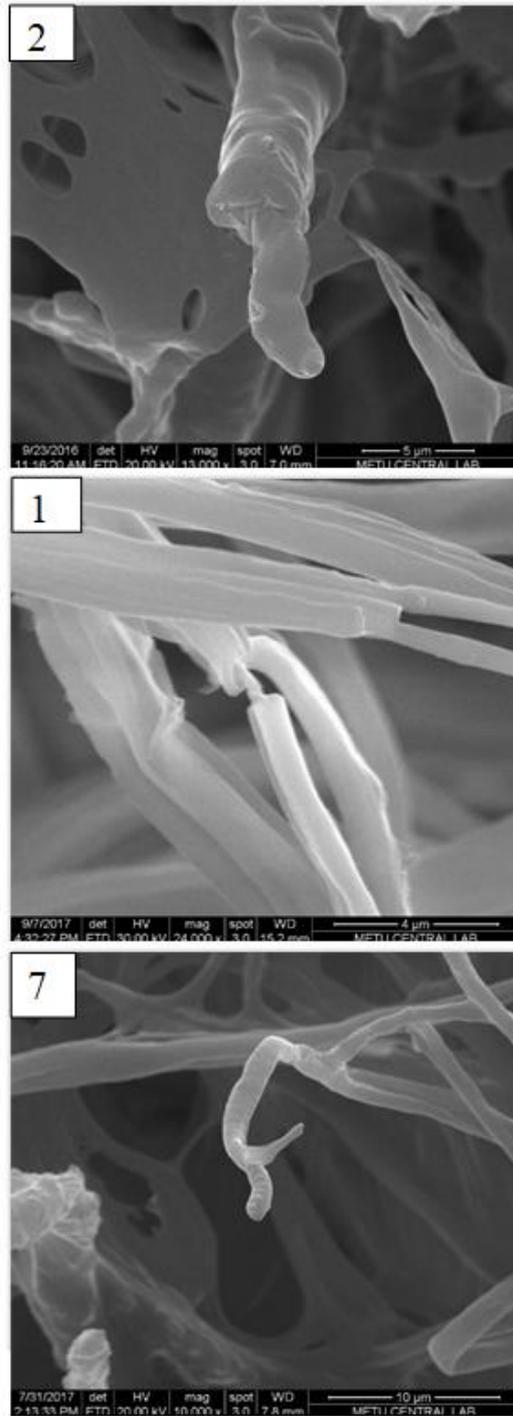
Fig. 3- 7 Structure of coaxial electrospun nanofibers taken by SEM of samples exposed by freeze fracturing with liquid nitrogen showing both the core and shell of electrospun fiber. Number codes on figures are corresponding to those given in Table 3-2.

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Table 3- 2 Polymeric solvents concentrations, collector, and macroscale and microscale characteristics of electrospuns made by Coaxial Wet Electrospinning, all polymeric solutions had HFIP as a solvent except for number 2 where the core had chloroform as a solvent. % ratios represent concentrations as weight/volume

#	PCL	Gelatin	P-188	Collector	Macroscale Characteristics	Microscale characteristics
1°	Core: 5%	Shell 5%	Core: 5% Shell: 5%	Ethanol	Soft, Large, Flat	Good fiber and pore diameters
2°	Core: 10% (Chloroform)	Shell 10%		Ethanol	Tough, Large, Flat	Not good fibers with good pore diameters
3°	Core: 10%	Shell: 9%	Shell:1%	Ethanol	Soft , Medium size, Round	Melting problem
4°	Shell: 10%	Core: 10%		Ethanol	Tough, Large, Round	Good fibers but pores are hard to distinguish
5°	Core: 10%	Shell: 10%		Ethanol	Soft, Large, Flat	Good fiber and pore diameters
6°	Shell: 2.5%	Core: 5%	Core: 5% Shell: 7.5%	Water	Membrane	
7°	Shell: 5%	Core: 5%	Core: 5% Shell: 5%	Water	Soft, Medium size, Morpheus	Good fiber and pore diameters
8°	Core: 10%	Shell: 5%	Shell: 5%	Water	Soft, Large, Flat	No pores due to condensed fibers

In this study, further evaluation of scaffolds was done by selecting certain groups considering all of the electrospinning studies.

1) If the bioactive agent is a hydrophilic compound then it can be mixed with a polymer solution made from 5% wt/vol PCL and 5% wt/vol Gelatin to be electrospinned using ethanol as a collector. A 3D scaffold that has good fiber diameter and good pores sizes, large volume but soft and flat structure would be obtained. this type of scaffolds will be labelled as (ME; *Monolithic*/monoaxial electrospun by *Ethanol* collector)

2) If the bioactive agent is a hydrophobic compound then it can be mixed with a polymeric solution made from 5% wt/vol -PCL 5% wt/vol Gelatin and 10% wt/volP-188 to be electrospinned using water as a collector. A 3D scaffold that has good fiber diameter and good pores sizes, large volume with tough and round structure can be achieved. In this study, this type of scaffolds will be labelled as (MW; *Monolithic*/monoaxial electrospun by *Water* collector)

3) If two bioactive agents, one hydrophobic and the other hydrophilic, would be used in an electrospun scaffold such that hydrophobic one will be in the core, then an electrospun was made by coaxial wet electrospinning using ethanol as a collector, where 5% wt/vol PCL and 5% wt/volP-188 as a core mixed with the hydrophobic bioactive agents, and the shell would contain 5% wt/vol gelatin and 5% wt/volP-188 and with the hydrophilic bioactive agent. In this study, this type of scaffolds will be labelled as (CE; *Coaxial* electrospun by *Ethanol* collector)

4) If two bioactive agents, one hydrophobic and the other hydrophilic, would be used in an electrospun scaffold such that hydrophilic one will be in the core, then an electrospun was made by coaxial wet electrospinning using water as a collector, where 5% wt/vol gelatin and 5% wt/volP-188 as a core mixed with the hydrophilic bioactive agents, and the shell would contain 5% wt/vol PCL and 5% wt/volP-188 and with the hydrophobic bioactive agent. In this study, this type of scaffolds will be labelled as (CW; *Coaxial* electrospun by *Water* collector).

3.2. Characterization of Selected Electrospuns
3.2.1. Degradation

Degradation experiments reveal that after 10 days of incubation, scaffolds lose between twenty to sixty percent of their weights (Fig. 3-8), core/shell electrospuns have higher degradation than monoaxial electrospuns, and electrospuns made with ethanol collector lose much more of their weight than their corresponding electrospuns prepared with water collector in both monoaxial and coaxial systems. That cause CE to have statistically significant degradation comparing to MW and CW. The higher percentage of weight loss for CE (ethanol collector) than ME (ethanol collector) could be due to more gelatin being removed from scaffolds because it is on the shell of CE and is more presented to outer environment than in scaffolds of type ME and MW, which both have gelatine-PCL mixed in a single phase during fiber formation. Besides that, more loss of weight in both types of ES systems when ethanol collector is used might be due to loss of gelatin into water phase (dissolving the superficial gelatines) during preparation of scaffold, thus leaving less gelatin on the surface. This shows importance of core shell structure and collector type for degradation result.
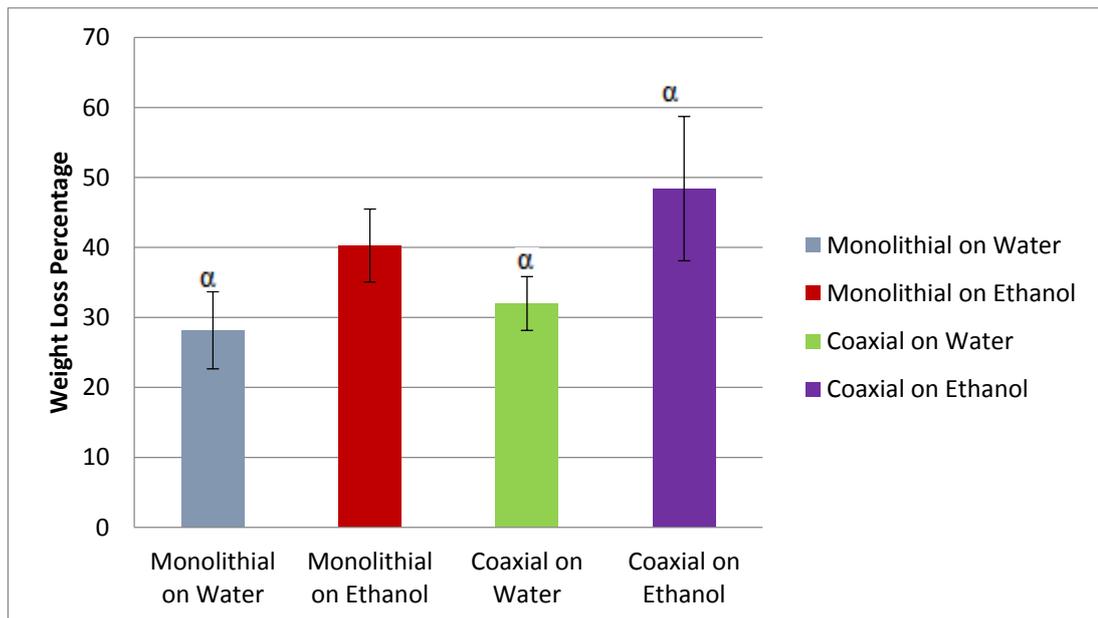
Fig. 3- 8- Degradation percentage of scaffolds after being submitted in PBS (0.1 M, pH 7.4) inside a water bath at 37°C for 10 days (n=3). α: statistically significant difference between degradation of CE and degradation of MW or CW

3.2.2. Water Retention

Water retention test was applied to the four selected groups of scaffolds (Fig. 3- 9). All scaffolds were swollen in the first two days, following that swelling amounts sharply decreased at the fourth day. After that swelling of ME and CE are increased and decreased slightly to be settled around 150% weight gain, while MW continued to decrease slowly till around 125% weight gain, and interestingly after fourth day CW continued to increase until it is around its original weight, that is because it didn't gain much weight through first two days but decreased sharply in the next two days losing around 22% of its original weight which should be the effect of degradation.

Comparing these results with degradation studies on section 3.2.1, weight decreases of these scaffolds between the second and fourth day can be explained as a result of degradation especially since CW loses weight to less than its original weight and less than the degradation loss after ten days of incubation.

ME, which increases for more than 2.5 folds, can be considered the less suitable scaffold for bone tissue substitute while MW and CE are considered much better alternatives because they are much more stable and were constantly around 150% weight gain.

The immediate decrease of weight gain for scaffolds produced by CW method causes it to have the least swelling scaffold property and make it great to be preferable as bone tissue substitute. This group has least swelling ratio probably due to more hydrophobic character of the fiber surfaces as they have PCL in the shell layer of fibers that were produced with coaxial ES method.

To decrease swelling ratio, crosslinking, which wasn't applied in this study, can be applied as it would stabilize the scaffolds' structure and slow down degradation process. However, due to risks of cross-linking the loaded protein (as it is incorporated into fibers during formation) during any crosslinking protocol, it was aimed to develop and characterize the developed bone scaffolds without such modifications.
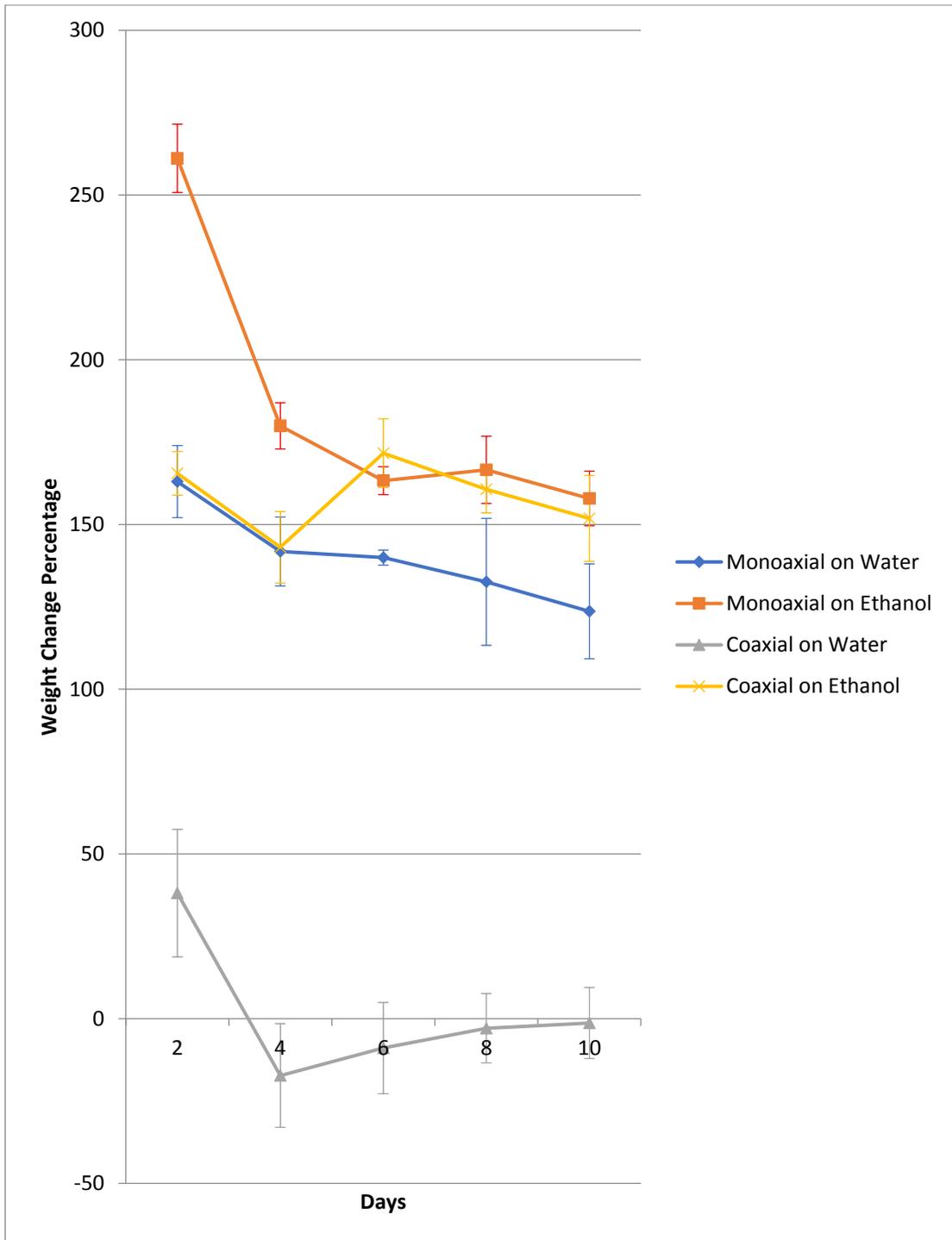
Fig. 3- 9 Swelling Percentage for scaffolds after being incubated in PBS (0.1 M, pH 7.4) inside water bath at 37°C for 10 days, weight of the scaffolds was measured on 2nd 4th,6th ,8th and 10th days (n=3).

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3.2.3. Porosity Measurements

Porosity measurements revealed that CE type of scaffold has the highest porosity, 67.63%, while MW and ME have similar porosities, 40.20% and 48.28% respectively, and CW has the lowest porosity value 15.10%. This would suggest that when gelatin is in the shell and PCL is in the core of the polymer then the porosity of the scaffold would increase and if gelatin is in the core and PCL is in the shell of the polymer then the porosity will decrease. Although CW has the lowest porosity, its degradation is expected to cause an increase in the porosity with time, making it also suitable for cell attachment and migration.

ME and MW are expected to be more suitable than CW, but less than CE when it comes to porosity characterization. CE in the other hand has high porosity, making it the most suitable for bone tissue engineering among the four scaffold groups; with high porosity, cells will have more ease to move towards inside and they can survive better due to more diffusion of substances, and gases through pores (Ruixin et al., 2017).

Pore size distributions of different scaffolds are shown in figures 3.10 – 3.13. Pore sizes of ME and CE are distributed uniformly between different diameters (4-240μm), while MW pores are concentrated between 20-200 μm diameters and have much less pores below 20 μm, and CW has more uniform distribution than MW but still has less pores below 20 μm comparing to above 20 μm. This may indicate that when water is used as a collector, then small pores, less than 20 μm would mostly
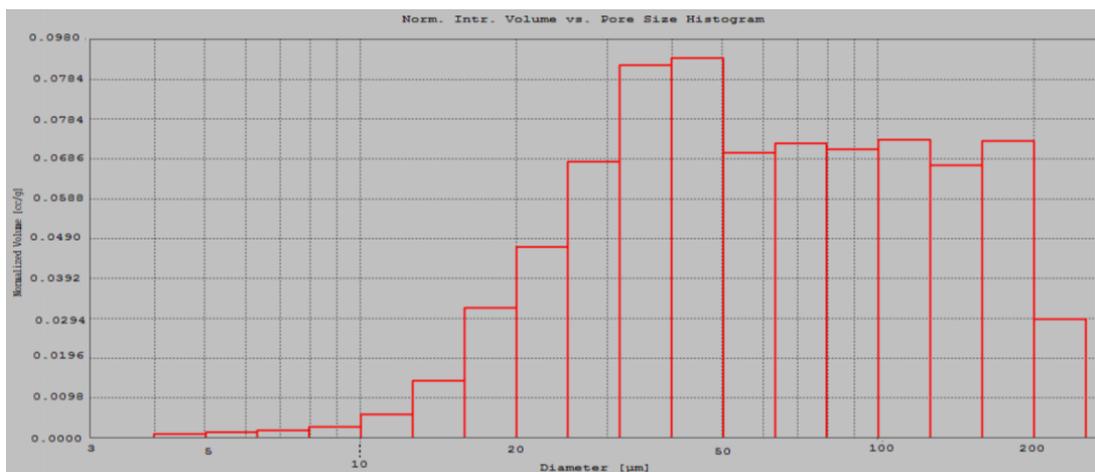
Fig. 3- 10 Pore size distribution of MW scaffolds

close. All four scaffolds have good pore size distribution for bone tissue engineering. That is because to bone scaffolds are suggested to be better if they were distributed between 10-400 μm as mentioned earlier (Porter et al., 2009).
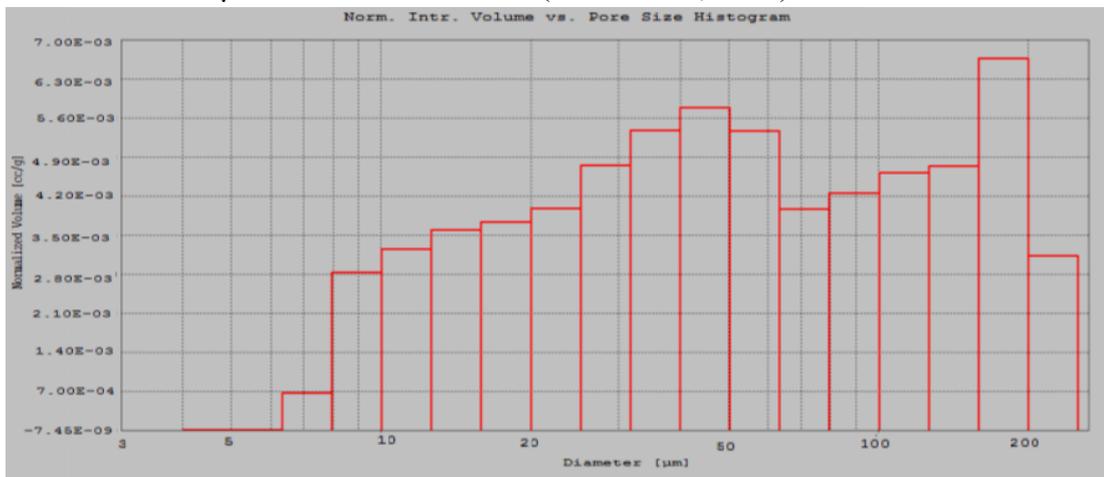
Fig. 3- 11 Pore size distribution of CW scaffolds

Fig. 3- 12 Pore size distribution of CW scaffolds
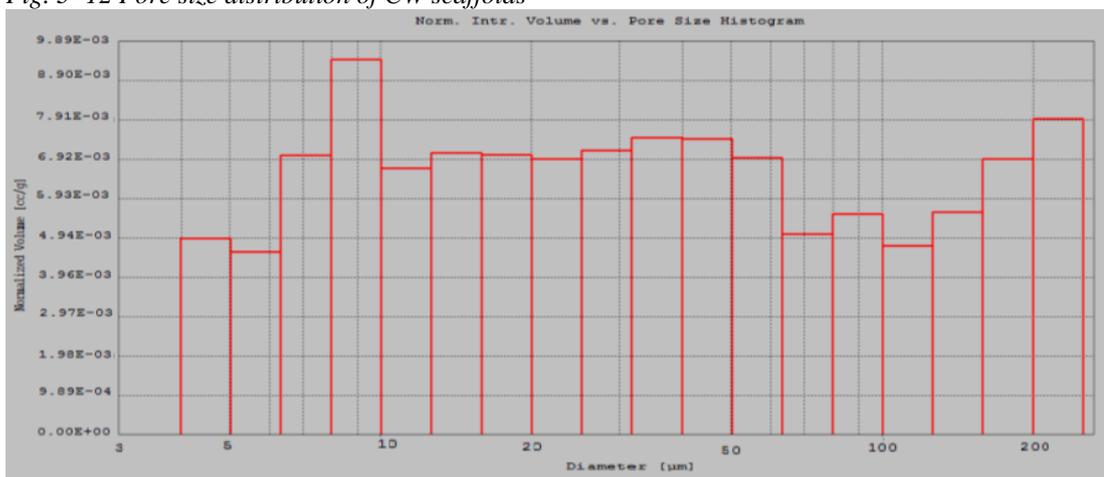
Fig. 3- 13 Pore size distribution of CE scaffolds

3.2.4. FTIR

FTIR studies were performed to the four selected groups of scaffolds as loaded with bioactive agents and as free from bioactive agents to see if there is any clear difference between them and to see if bioactive agents would cause any recognizable change in chemical structure like new bond formation between polymers and bioactive agents, also to see if any gelatin, PCL or P-188 stayed in the scaffold by comparison to other references (APPENDIX E). The first notable difference between scaffolds is that stretches and bends of scaffolds that were produced in water collector were different from those of scaffolds that used ethanol as collector (Fig. 3-14)

The broadband between 3600-3200 cm^{-1} indicates a hydroxy group (-OH) bond, and it is more intense in ME and CE than MW and CW. Similar increase in intensity is observed around bands;1650 cm^{-1} and 1550 cm^{-1}, which indicate for an amide bond between (C=O) and (N-H) and a secondary amine bond respectively. All these suggest that part of gelatin was lost with usage of water collector.

MW and CW have higher intensities between 1750 cm^{-1} and 1650 cm^{-1}, also between 1300 cm^{-1} and 1000 cm^{-1}, both of which indicate (C=O) and (C-O) respectively and combination of these point toward the availability of PCL and P-188 is higher in MW and CW than CE and ME.

From these differences, it appears that there is partial loss of materials with wet electrospinning. And the loss of polymeric material is determined by the solvent used as a collector during the electrospinning procedure.

When comparing between scaffolds loaded with bioactive agents and scaffolds free from bioactive agents, it appears that scaffolds loaded with vk2 increase their intensities at absorption bands1720 cm^{-1} and 1170 cm^{-1} which shows presence of much more (C=O) and (C-O) groups with the decrease around 1650 cm^{-1} indicating a quinone groups which is found in molecular structure of vk2.
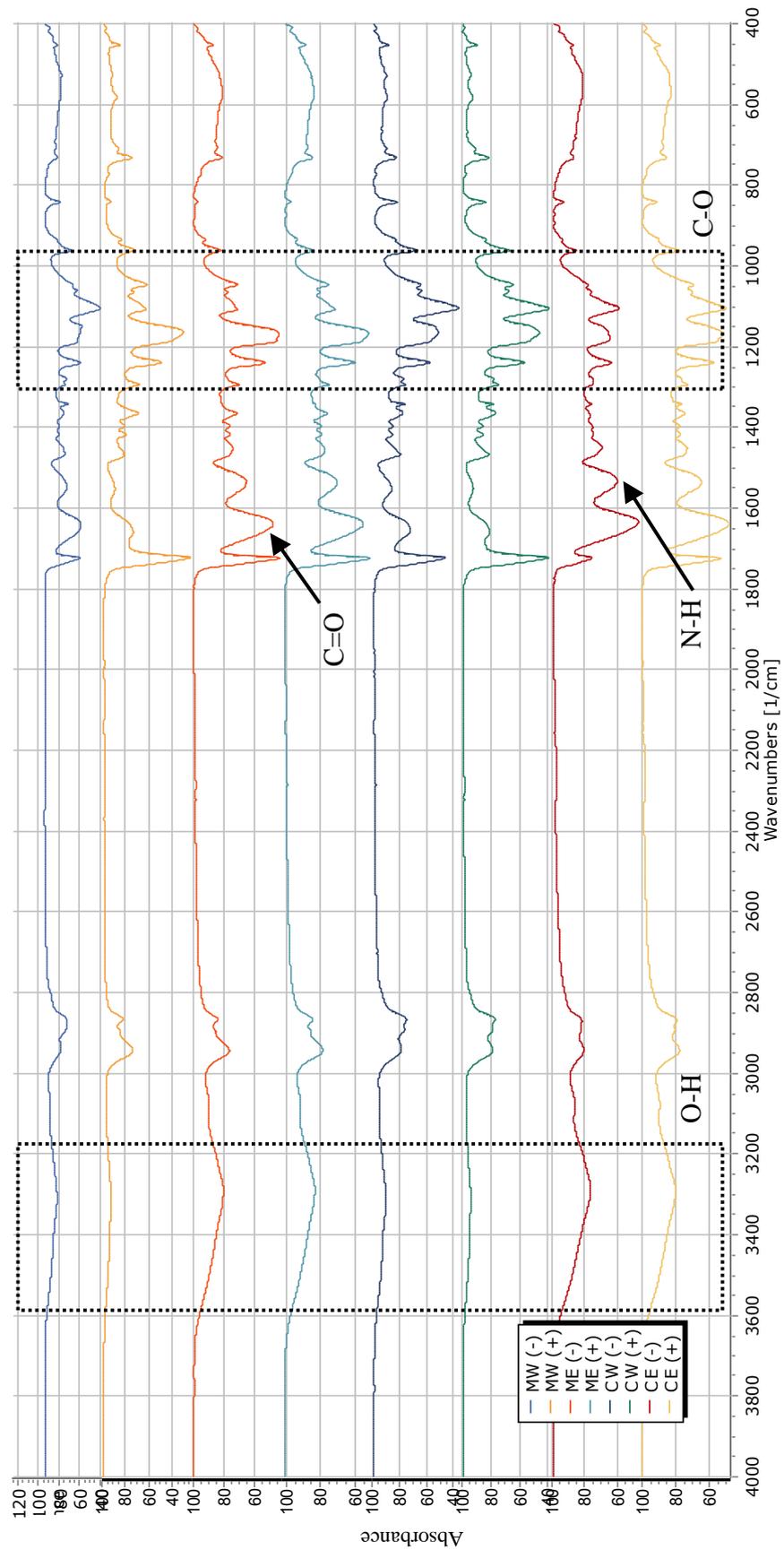
Fig. 3- 14 FTIR spectra of MW, ME, CW and CE scaffolds, (+) is for loaded scaffolds, (-) is for agents' free scaffolds

3.2.5. Mechanical Tests

In order to evaluate mechanical properties of the selected groups of scaffolds compression tests were applied to scaffolds. All scaffolds showed over 50% strain deformation upon stress and Stress-Strain curves were drawn for each of them. Young's modulus, stiffness and 50% stress point were obtained from the curves and presented in Table 3-3 and Figures 3-15 – 3- 18.

Scaffolds didn't show high values of strength; therefore, they were thought to be more suitable for non-load bearing bone tissues like skull. In a work done by Hamlekhan et.al., they were able to produce a scaffold composed of PCL, gelatin , and hydroxyapatite that has the following properties; ultimate tensile strenght is around 3.73 MPa , stifness is around 131 MPa and young's modulus is around 23.5 MPa .(Hamlekhan, Mozafari, Nezafati, Azami, & Hadipour, 2010). However, Ultimate compressive(tensile) strength (UTS) couldn't be determined because scaffolds didn't break and stress was less than 1 MPa, even though strain get to over 50%.

Instead of UTS, 50% deformation point was used to evaluate these scaffolds, similar approach was used by Hejazi et.al. as they used 50% deformation point for their mechanical study (Hejazi & Mirzadeh, 2016).

In order to make the scaffolds more suitable for load bearing bone sites other modifications as cross-linking or adding bioglass or hydroxyapatite like minerals might be suggested. There is no clear difference between coaxial and monoaxial scaffolds but scaffolds that used ethanol as a collector were tougher than scaffolds produced in water collector. This might be more preservation of contents with no/less solubility of both gelatin and PCL in ethanol collector compared to water. Besides that, FTIR results also indicated some strong bond formations (amide bonds) within scaffolds which might have caused the increased strength.

Table 3- 3 Compressive mechanical parameters obtained from stress-strain curves of different scaffolds (n=3).

Scaffold	Young Modulus (kPa)	Stress (kPa) at 50% strain
MW	5.9 ± 0.6	2.6 ± 0.5
ME	25.6 ± 2.9	19.3 ± 6.7
CW	9.97 ± 0.6	4.8 ± 3.6
CE	58.9 ± 5.4	38.4 ± 6.5
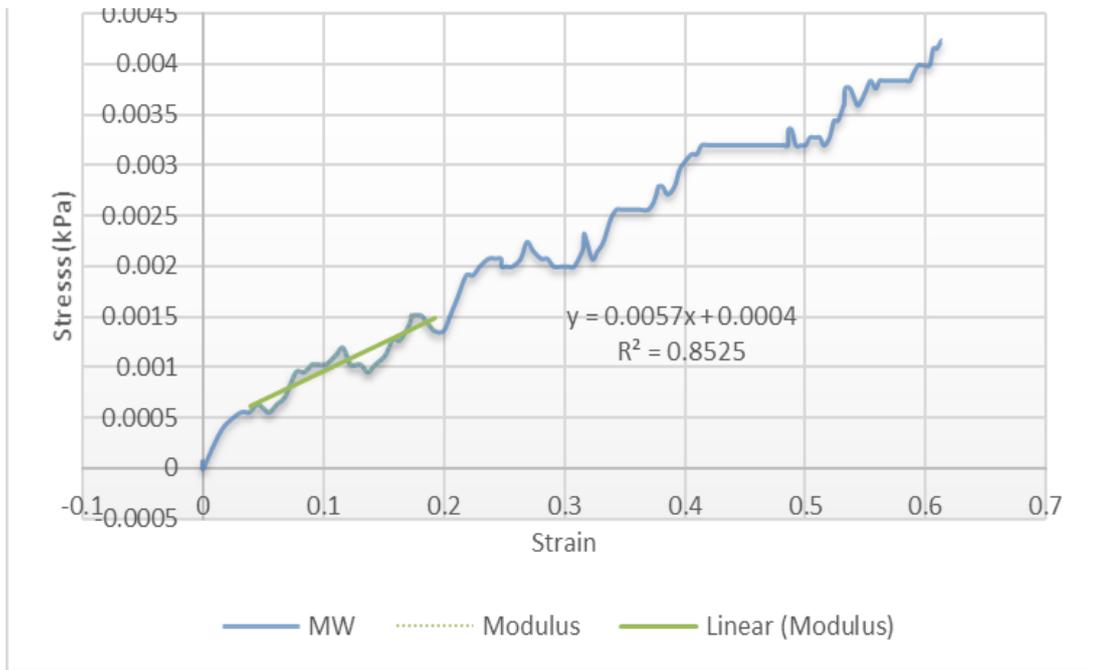
Fig. 3- 15 Stress-Strain Curve of representative for MW scaffold
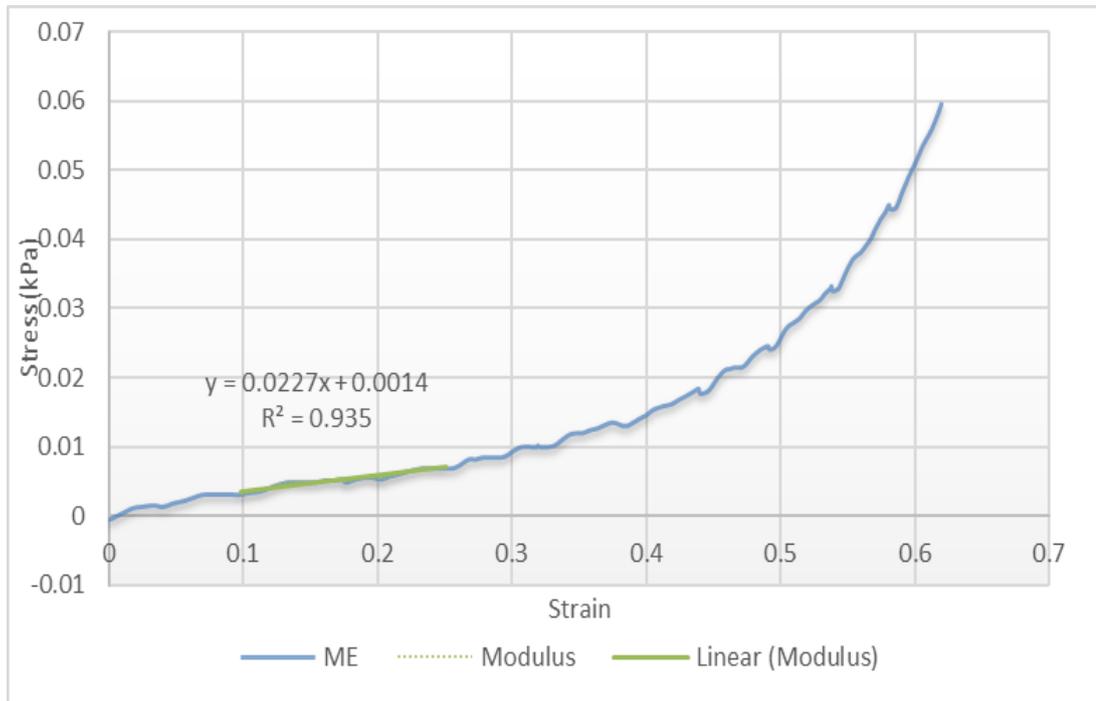
Fig. 3- 16 Stress-Strain Curve of representative for ME scaffold
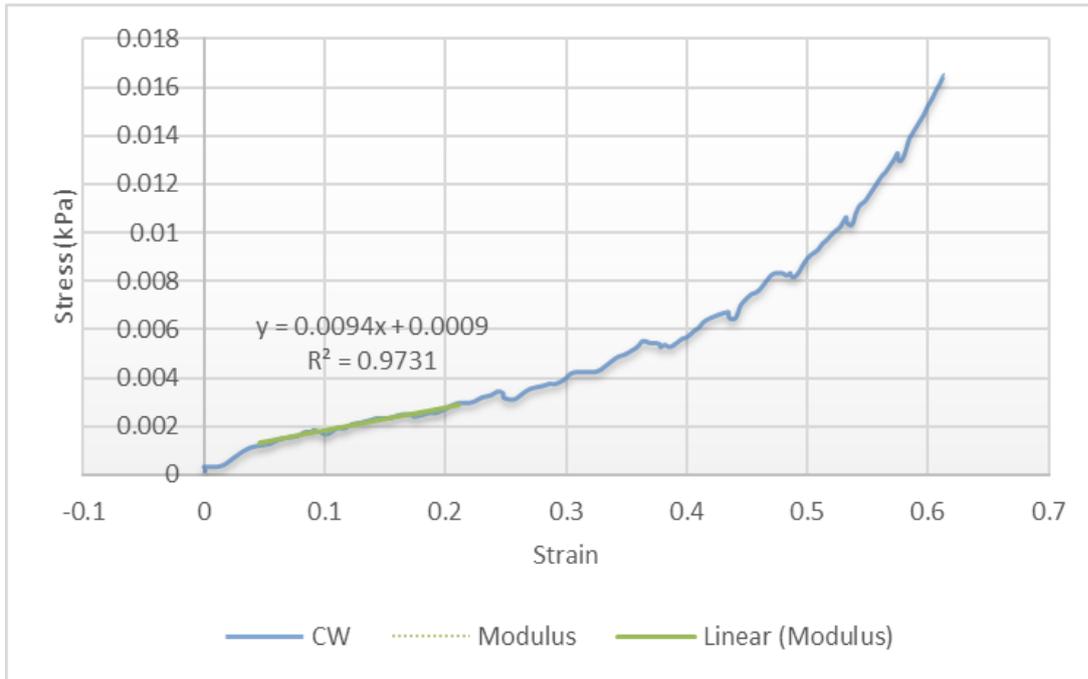
Fig. 3- 17 Stress-Strain Curve of representative for CW scaffold
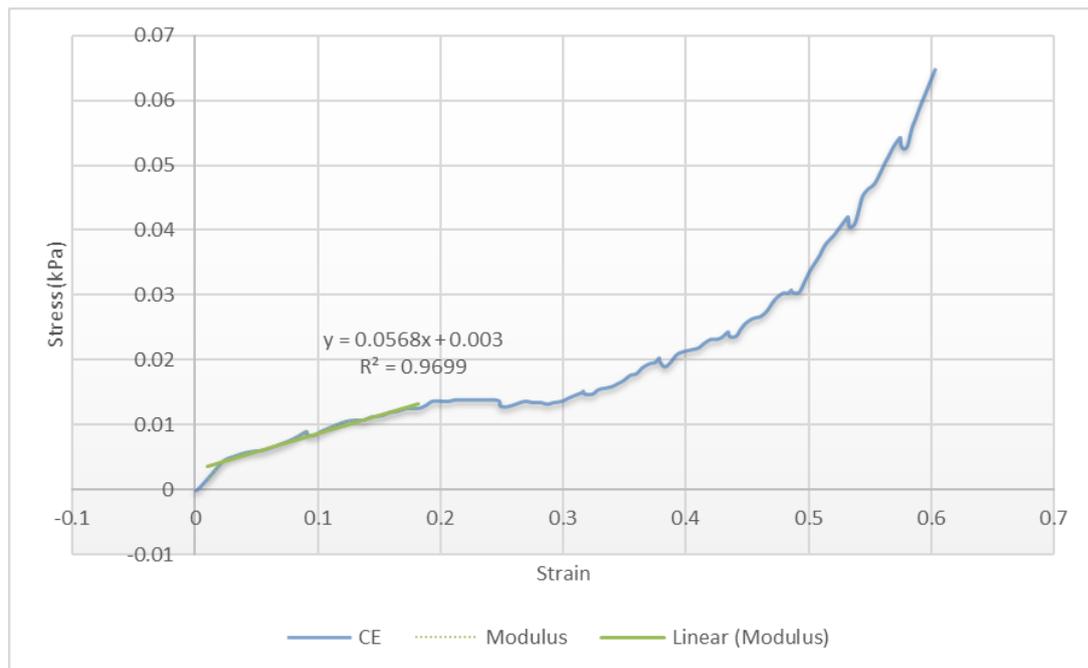
Fig. 3- 18 Stress-Strain Curve of representative for CE scaffold

3.3. Release Profiles of bioactive agents

Electrospun scaffolds were evaluated for in vitro release profiles by using HPLC and UV-Spectrophotometry. While MW and ME were loaded with only vk2 or β-LG respectively, CW and CE were loaded with both of them.

From Fig 3-19 and Fig. 3-20, it is apparent that all the releases had burst effect phenomena. Then release occurs with slightly decreasing and small amounts overtime.

As expected, CW had the lowest burst release of all groups since β-LG is loaded into the core of the electrospun scaffold and covered by hydrophobic PCL shell. Nevertheless, CE and CW had similar release amounts in the forward days. ME had more release in the burst and in the following days which could be caused by the higher porosity of the scaffold.

In contrary to β-LG release behaviour, burst effect with vk2, was much lower, probably owing to the hydrophobicity and thus less aqueous solubility of vk2. The release of both β-LG and vk2 can be considered slow and stable in the following days of burst effect. Vk2 release from CW was less than that of CE, in which could mean that porosity is more effective on modifying release of this agent than its localization in core or shell of the fibers, and that this could also be the case of releasing β-LG.

Much like β-LG, Vk2 h0ad higher release profile in the monoaxial electrospun.
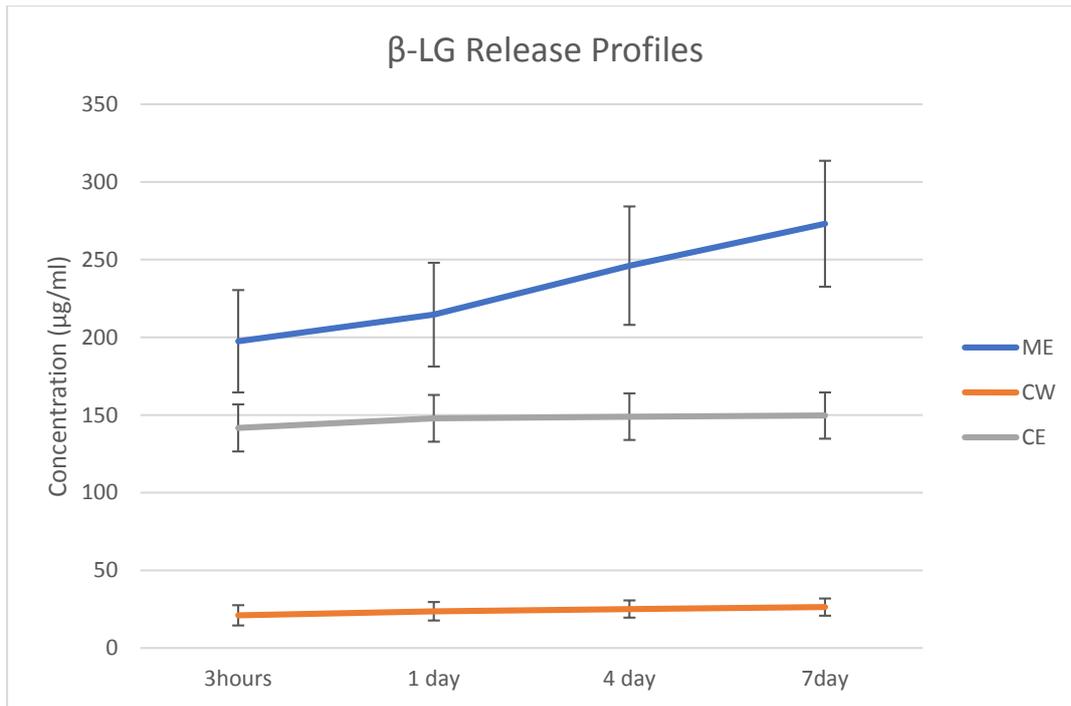
Fig. 3- 19 β-LG cumulative release profile of ME, CW and CE scaffolds (n=3).
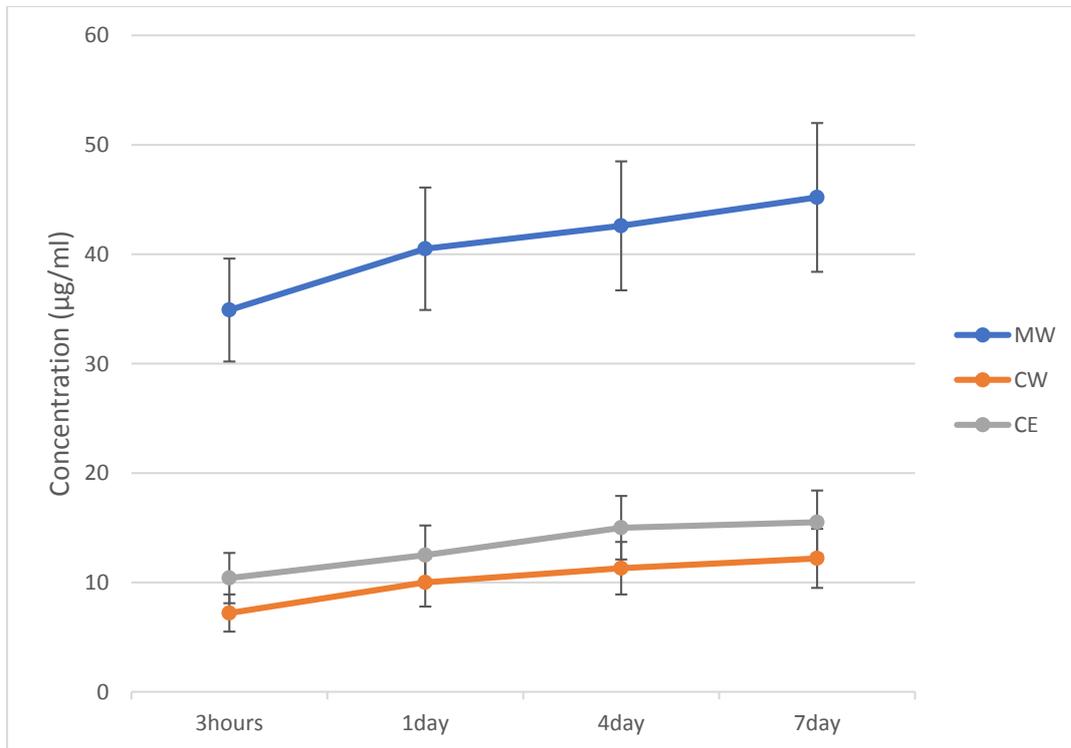
Fig. 3- 20 Vk2 cumulative release profile of MW, CW and CE scaffolds (n=3).

3.4. Cell and Bioactive Agent/Scaffold Interaction Studies

3.4.1. Monolayer/2-D cell culture studies

3.4.1.1.   Cell Viability Studies for Bioactive Agents

To examine the effect of bioactive agents on a specific cell line of bone forming cells (osteoblast), (Saos-2), and to find out the suitable amount of a bioactive agent that should be released from scaffolds, MTT cell viability assay was done on Saos-2 Cells with different concentrations of bioactive agents at different periods of times and compared to control group viability; Saos-2 Cells incubated without any bioactive agent.

In these studies, considering the ultimate aim of the designed scaffold calcitonin, the peptide hormone that was selected for its effects on bone tissue, and Vk2 were investigated for their effects or potencies on bone cells in cell culture studies.

It is apparent from the results (Fig.3-21) that calcitonin acts best at concentration of 25 ppm; it increases cell viability especially on the first two days. However, concentrations of 12.5 and 50 ppm don't have an increasing effect of cell viability, on the contrary, they do have a cytotoxic effect by reducing the cell viability.

Contrariwise, VK2 has a cytotoxic effect on the first day which increases with increased concentrations, but a clearly positive effect on cell viability owing to VK2 was seen on the second and third days; as cell viability increases more than control at all concentrations. When considered for the 3 incubation periods, VK2 has good effects on cell viabilities at 12.5 and 25 ppm. concentrations.

3.4.1.2.   ALP Activity

Upon addition of VK2, ALP activity of Saos-2 cells did not change considerably for two weeks period; in the first week, there was a slight decrease in the activity for 12.5 and 50 ppm VK2 treated cells, but in the second week all of the concentrations showed an increase in ALP activity with 12.5 and 50 ppm VK2 groups being higher than with 25 ppm (Fig.3-22). Interestingly, when cells where treated with calcitonin, ALP activity of Saos-2 cells increased in the first week but decreased in the second week (Fig - 23).
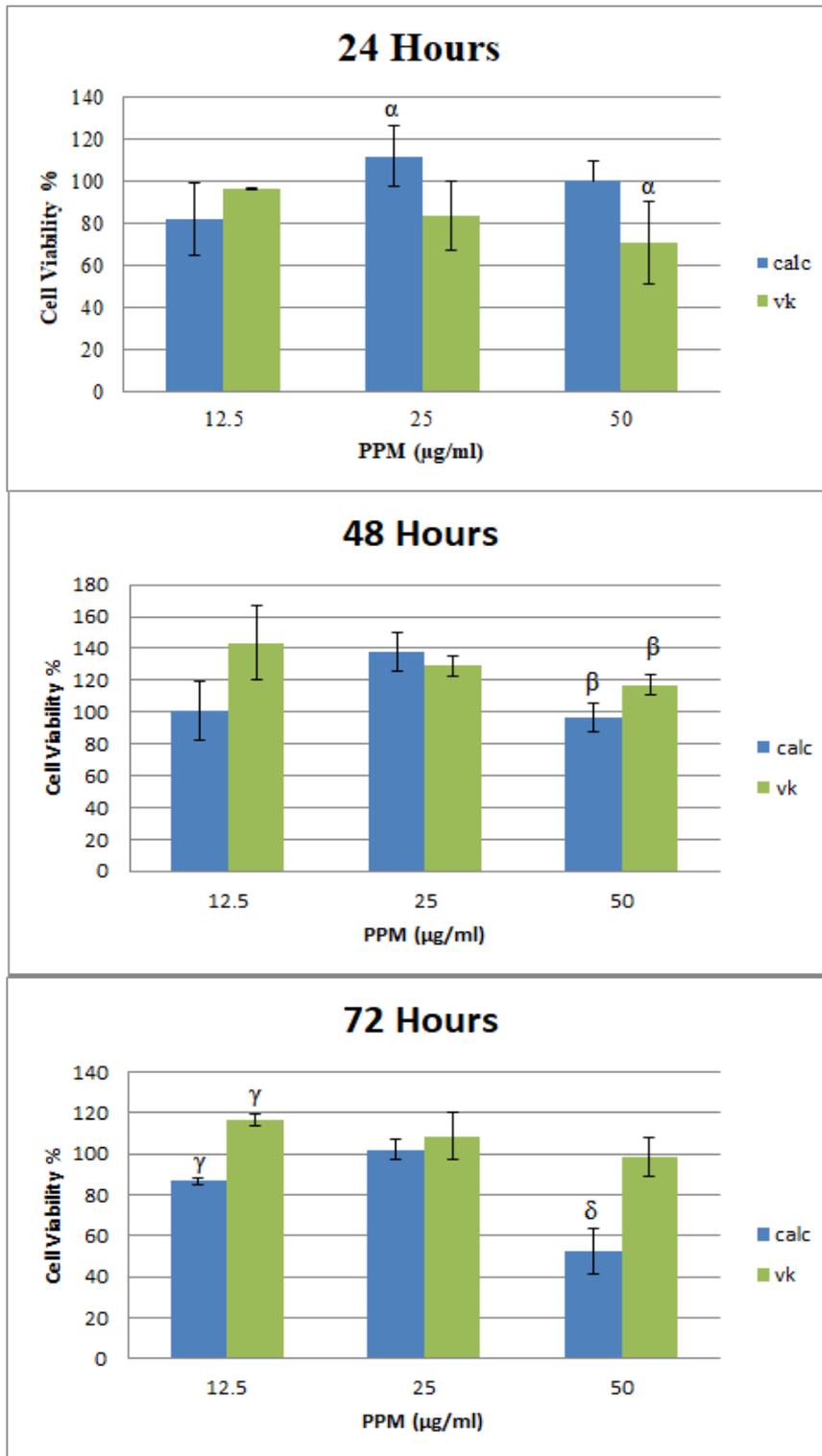
Fig. 3- 21 MTT assay on 2D culture of Saos-2 cells showing the viability% of cells after adding calcitonin and vitamin k2 for 3 days comparing to a control group without any additive (n=3). α: statistically significant difference between 25 µg/ml calc and50 µg/ml vk2, β: statistically significant difference between 50 µg/ml calc. and 50 µg/ml vk2, γ: statistically significant difference between 12.5 µg/ml calc. and 12.5 µg/ml, δ: statistically significant difference between 50 µg/ml calc and other groups.

When considering ALP activity as an indication of osteogenic differentiation, for the short time period calcitonin is a better candidate, but when considering a longer time then VK2 has proven to be better for increasing this activity. The reason why there is much less ALP activity on cells when treated with vk2 than cells treated with calc. might be related with DMSO used in dissolving vk2. Vk2 can't dissolve easily in the cell culture medium, then adding that cells was the necessary way in which was also applied to the control group.
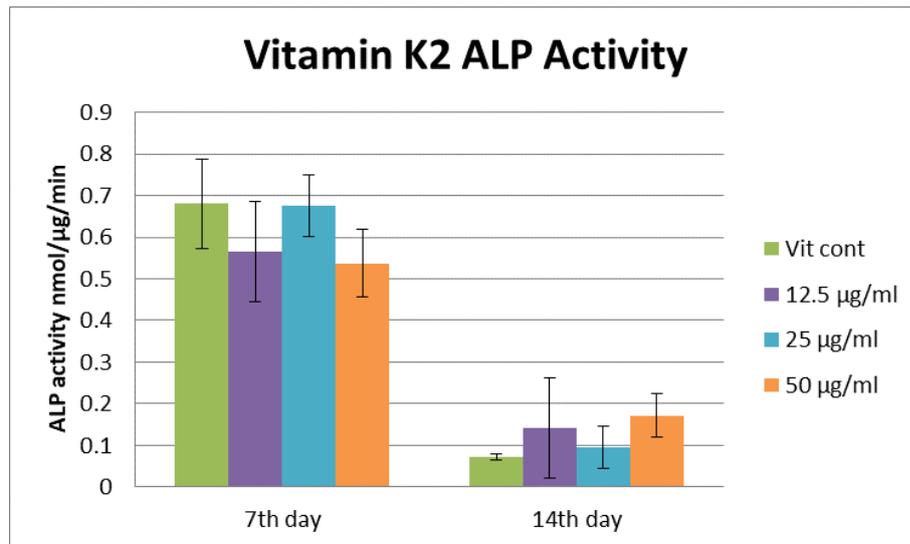
Fig. 3- 22 ALP activity of VK2 on Saos-2 cells, DMSO was added to all groups to dissolve vitamin k2 (n=3).
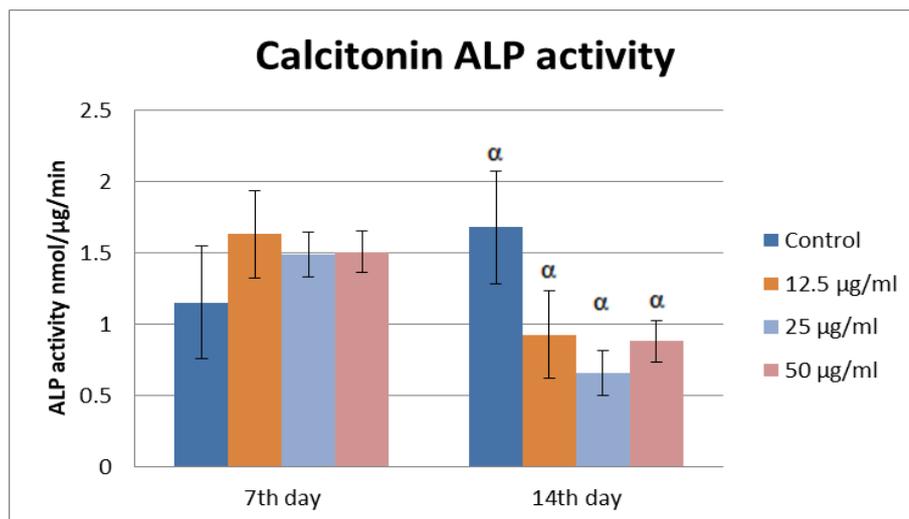
Fig. 3- 23 ALP activity of calc. on Saos-2 cells (n=3), none of the groups contained DMSO. α: statistically significant difference between control group on 14th day and other groups on 14th day

3.4.2. Cell culture studies on 3D Scaffolds

3.4.2.1. Cell Viability Assays for Scaffolds

Alamar blue assay was accomplished in different time frames (1,4,7 and 14 days) over scaffolds either loaded with bioactive agents or free from bioactive agents (Fig. 3-24)

Through the first week, cell viability wasn't constant and most of scaffolds loaded with bioactive agents did not cause statistically significant change than scaffolds free from bioactive agents, except for seventh day when a couple of statistically significant decrease and increase for Coaxial scaffolds happened. Comparing ES types to each other in their effect on cell viability, there is no clear result since they increase and decrease throughout the week and there is no statistically significant result showing one group of them to have higher or lower value than the other type (considering each type is tested on both loaded and not loaded with bioactive agents)

After 14 days, there were different results, as MW (without bioactive agent) and CW (loaded with vk2 and calc) scaffolds proved to be better than other scaffolds when it comes to cell viability because of the statistically significant higher values comparing to all scaffolds that used ethanol collector. And since CE scaffolds prompted statistically significantly less cell viability than other scaffolds after 14 days, and through the first week CE didn't cause any increase over others but showed some statistically significant decrease to other scaffolds, all these imply that the usage of ethanol collector isn't much suitable for cell viability.

ME was the only scaffold through 2 weeks where it had higher cell viability when it was loaded with bioactive agents, meanwhile MW was the opposite in which` it resulted in a negative effect when loaded with vk2, although these changes are mostly not statistically significant, they suggests that vk2 may have caused a negative effect on scaffolds similar to negative effect with its increased concentration as shown with monolayer cell viability tests on section 3.4.1.1, which in turns means that further studies with lower vk2 concentrations should be considered.
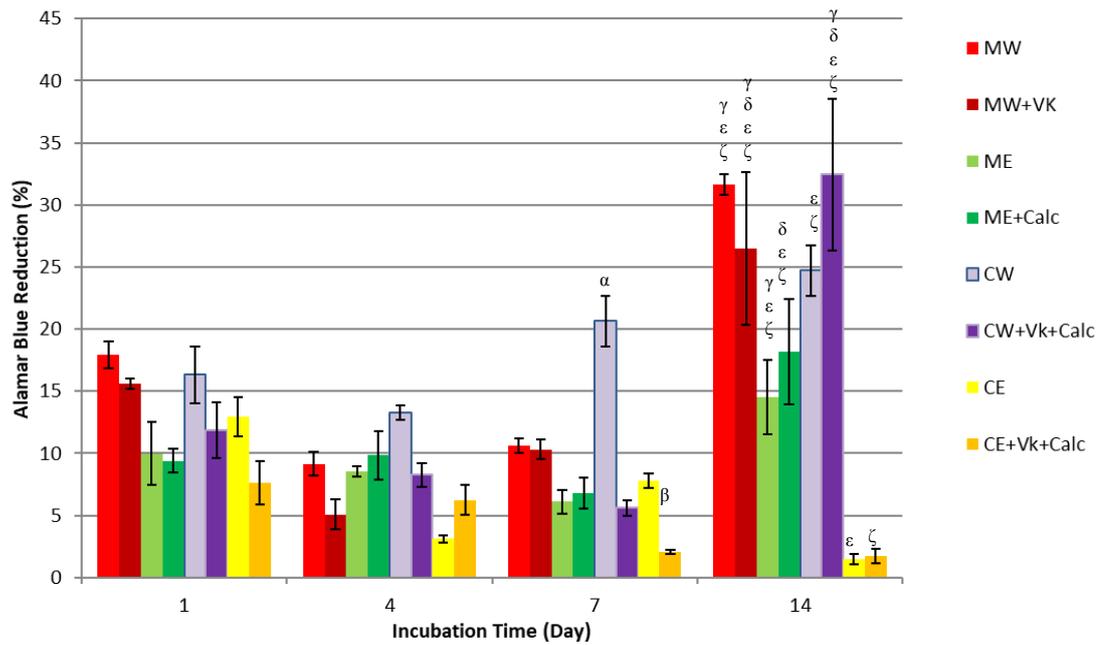
Fig. 3-24 Alamar blue assay results on Saos-2 cells through a week on MW, ME, CW and CE scaffolds (n=3).
α: statistically significant difference between (CW) and all other groups in 7th day, β: statistically significant difference between (CE+vk2+calc) and other groups in 7th day, γ: statistically significant difference between (ME) and other groups in 14th day, δ: statistically significant difference between (ME+calc) and other groups in 14th day, ε: statistically significant difference between (CE) and other groups in 14th day, ζ: statistically significant difference between (CE+calc+vk2) and other groups in 14th day

3.4.2.2. ALP Activity

ALP assay was done on scaffolds loaded with bioactive agents and for scaffolds free from bioactive agents to see the effect of scaffolds and the effect of released bioactive agents on the differentiation of cells with bone forming characteristics. From Fig. 3-25 and Fig 3-26, bioactive agents also increase the activity over ME in the second week. However, coaxial electrospun scaffolds had a decrease in ALP when both vk2 and calc were added to them in the first week for CW, but no statistically significant change in the second week for CW or in the two weeks for CE. That means when vk2 and calc were mixed together they may have a negative effect although they had a positive effect on monolayer cells and scaffold seeded cells, when each of them is given alone. Further studies should repeat the ALP assay but with different

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concentrations for combined bioactive agents seeded. cells, when each of them is given alone. Further studies should repeat the ALP assay but with different concentrations for combined bioactive agents.
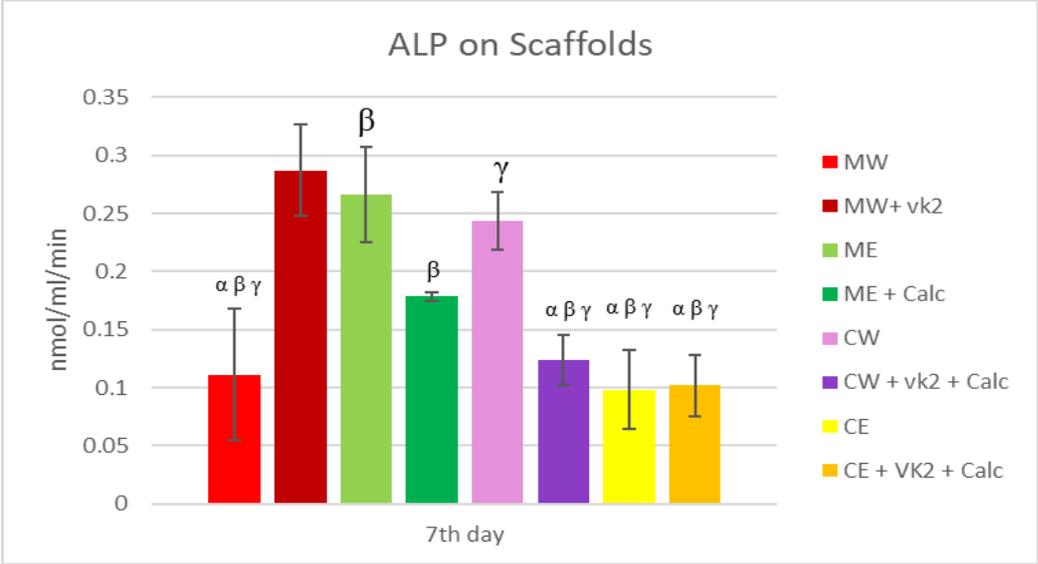
Fig. 3- 25 ALP activity on Saos-2 cells in after a week on MW, ME, CW and CE scaffolds (n=3). α: statistically significant difference between (MW + vk2) and other groups, β: statistically significant difference between ME and other groups, γ: statistically significant difference between CW and other groups.
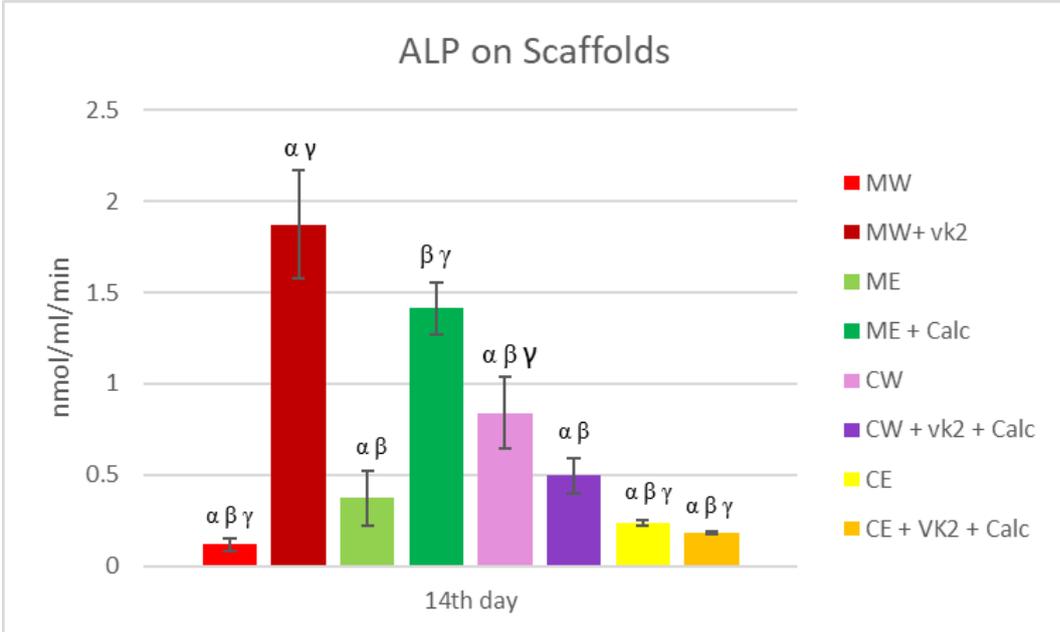
Fig. 3- 26 ALP activity on Saos-2 cells after 2 weeks on MW, ME, CW and CE scaffolds (n=3), α: statistically significant difference between (MW+vk2) and other groups, β: statistically significant difference between (ME+calc) and other groups, γ: statistically significant difference between CW and other groups

3.4.2.3.   Cell Morphology Analysis

SEM analysis was performed on scaffolds that cells were cultured and then fixed to them after 1 day and 7 days. Figures 3-27 – 3-30 shows fixed cells over different scaffolds that had loaded with bioactive agents and scaffolds without any bioactive agents.

On the first day, there were more cells on MW, ME and CW scaffolds and they attached more to scaffolds that release bioactive agents but CE showed the opposite. On the 7th day, scaffolds loaded with bioactive agents had many cells attached to them comparing to the 1st day and many of them were proliferated. Unfortunately, on the seventh day, CE scaffold which wasn't loaded with bioactive agents was full of bacteria which avoided evaluation of Saos-2 cell proliferation. Cells couldn't be found at CW scaffold free from bioactive agents These can be interpreted as cells attaches well in the scaffolds and even more in scaffolds loaded with bioactive agents.

MW 1st day X2500			
MW+ Vk2 1st day X2500			
MW 7th day X2500		MW 7th day X20000	
MW+ Vk2 7thday X2500		MW+ Vk2 7th day X10000	
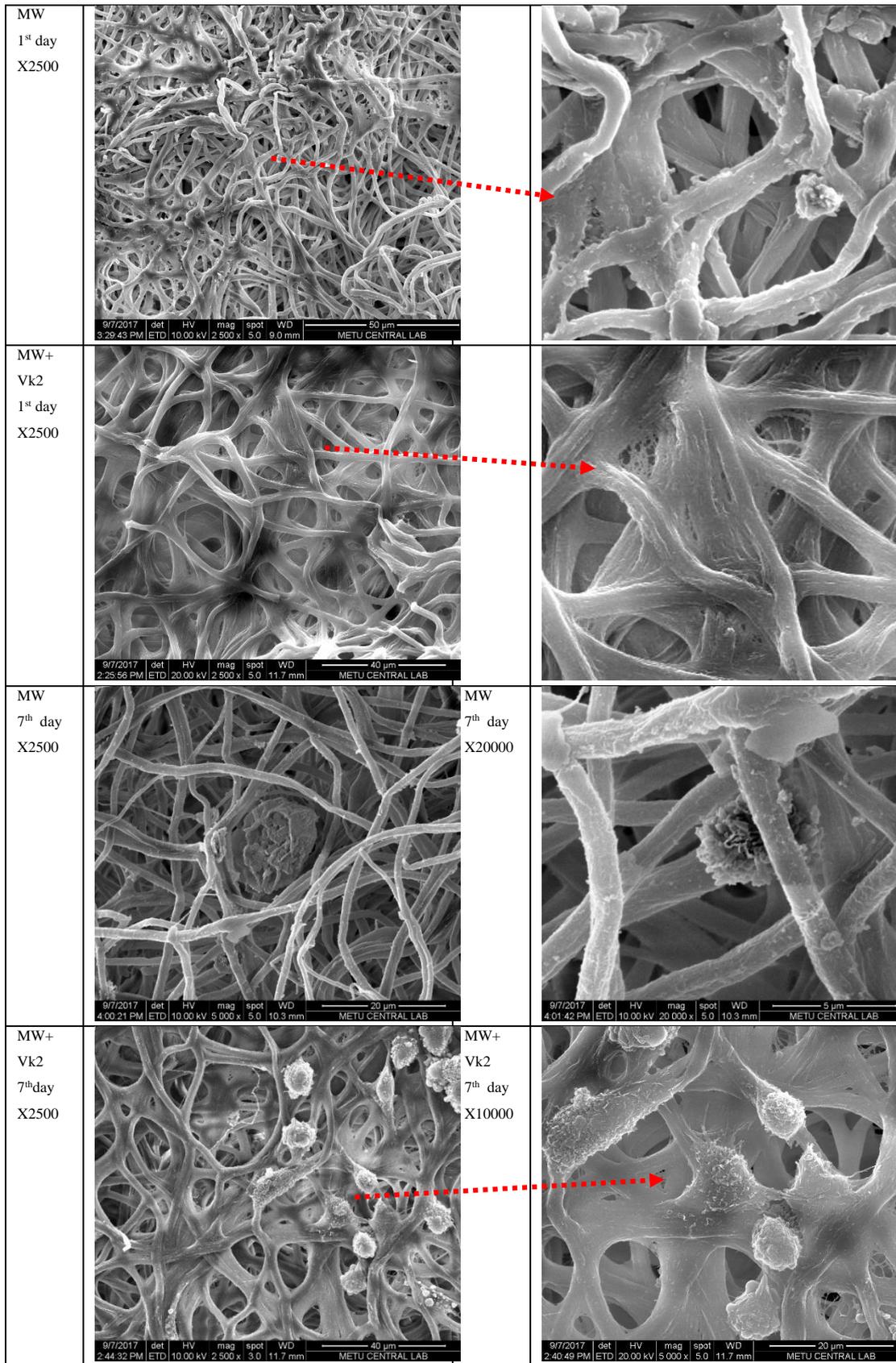
Fig.3-27 Cell attachment on MW scaffold at 1st and 7th day of culturing by SEM

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ME 1st day X2500		ME 1st day X10000	
ME+ Calc 1st day X2500		ME+ Calc 1st day X10000	
ME- 7th day X2500		ME 7th day X10000	
ME+ calc 7th day X2500		ME+ calc 7th day	
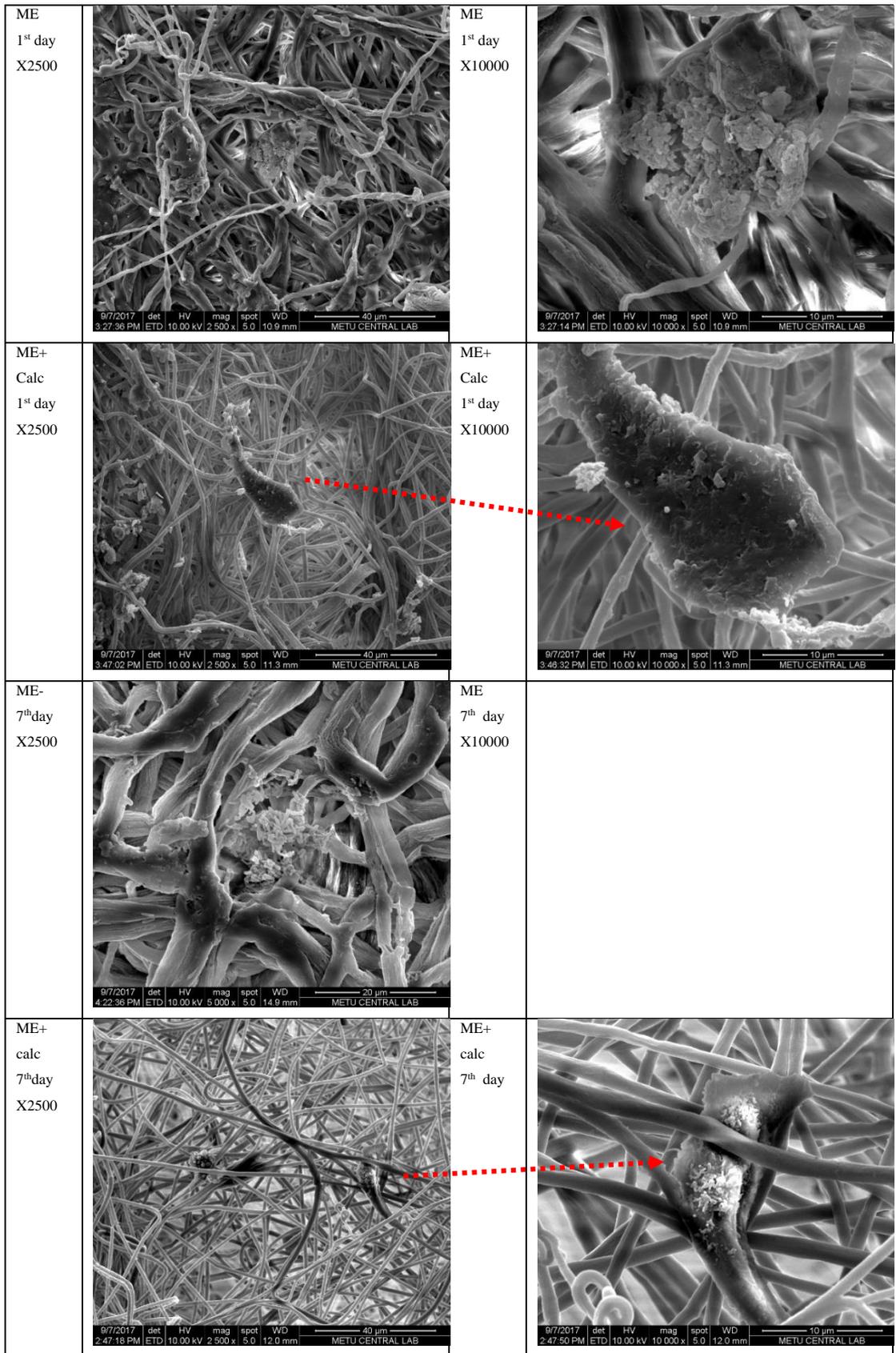
Fig.3-28 Cell attachment on ME scaffold at 1st and 7th day of culturing by SEM

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CW 1st day X2500		CW 1st day X5000	
CW+ vk2+ calc 1st day X2500		CW+ vk2+ calc 1st day X10000	
CW 7th day X2500			
CW+ vk2+ calc 7thday X5000		CW+ vk2+ calc 7th day X10000	
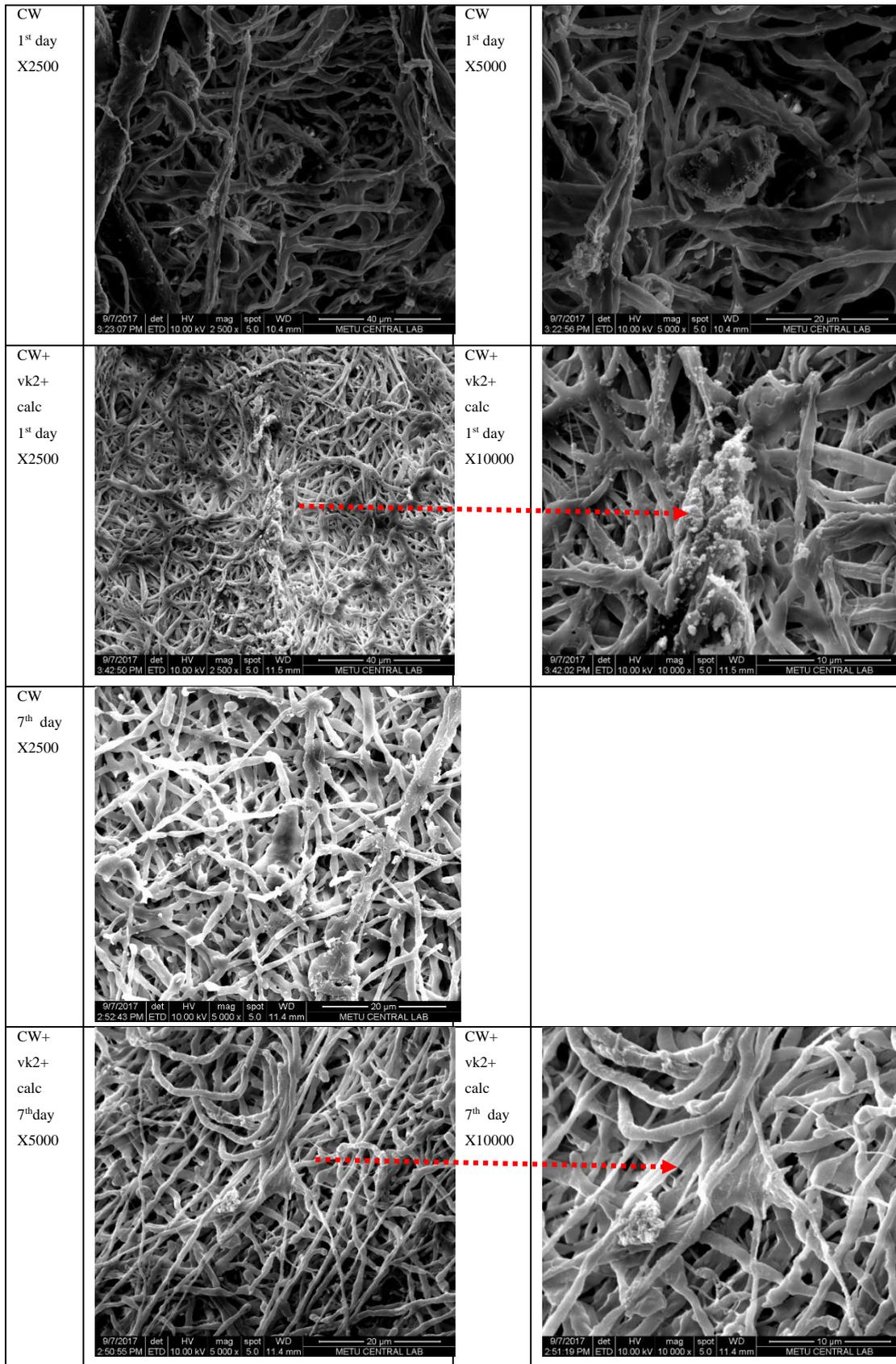
Fig.3-29 Cell attachment on CW scaffold at 1st and 7th day of culturing by SEM

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CE 1st day X2500		CE 1st day X10000	
CE+ vk2+ calc 1st day X2500		CE+ vk2+ calc 1st day X20000	
CE 7th day X5000		CE 7th day X20000	
CE+ vk2+ calc 7thday X2500			
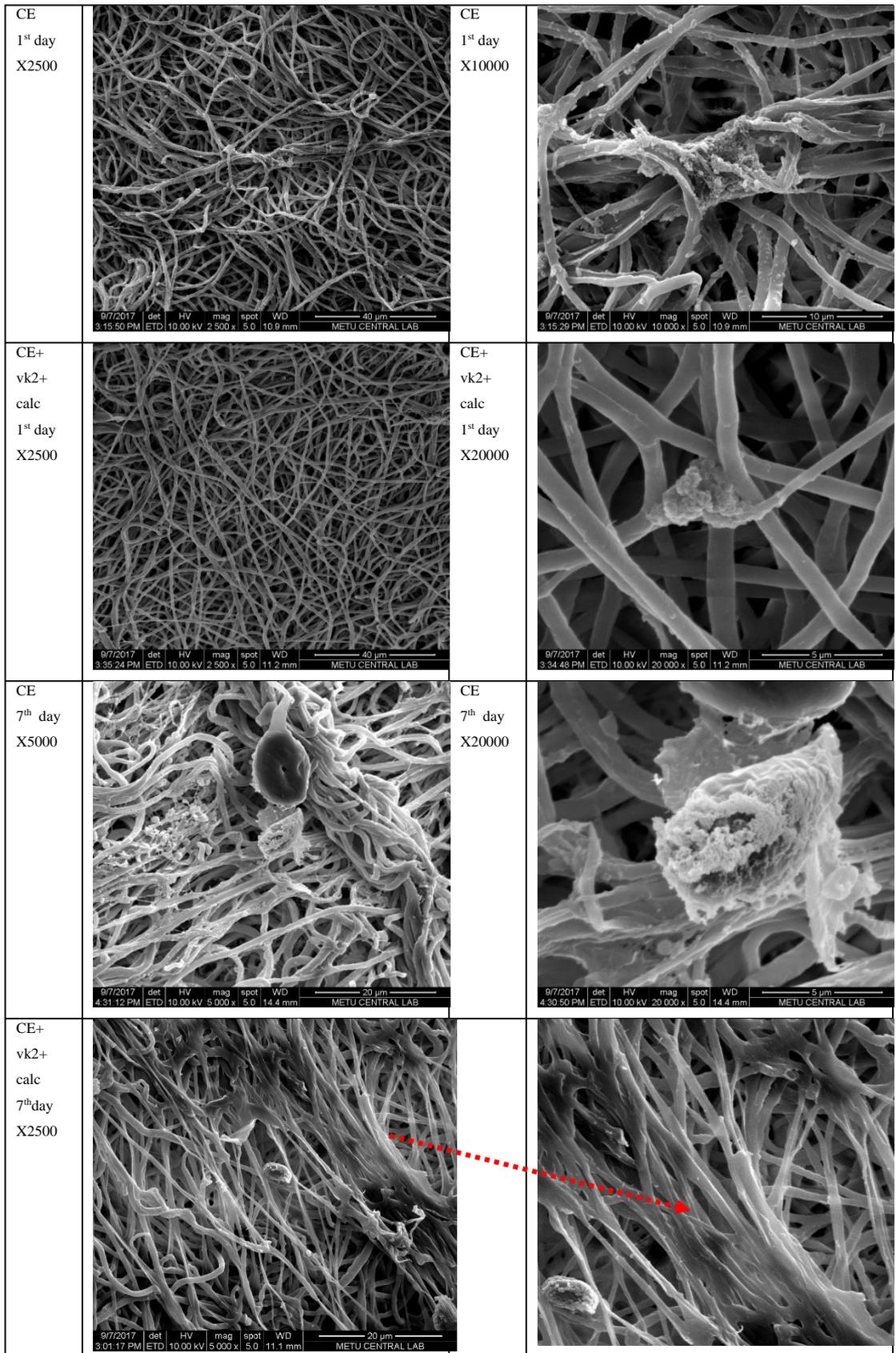
Fig.3-30 Cell attachment on CE scaffold at 1st and 7th day of culturing by SEM

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3.4.2.4. Confocal Laser Scanning Microscopy

To further evaluate these scaffolds in terms of Cell adhesive properties and cell migration into scaffolds, confocal laser scanning microscopy was used. Cells were seeded into scaffolds and cultured for a week and then they were fixed and stained with FITC-phalloidin for actin cytoskeleton of cells and PI for cell nuclei. Confocal micrographs showed that FITC-phalloidin stained scaffold fibers in green instead of actin cytoskeleton yet the cells can be distinguished by the nuclei which was stained in red by PI (Fig.3-32). Cells were able to adhere onto scaffolds's surface and penetrate inside the scaffold to more than 30 μm (Fig.3-31, Fig. 3-32)
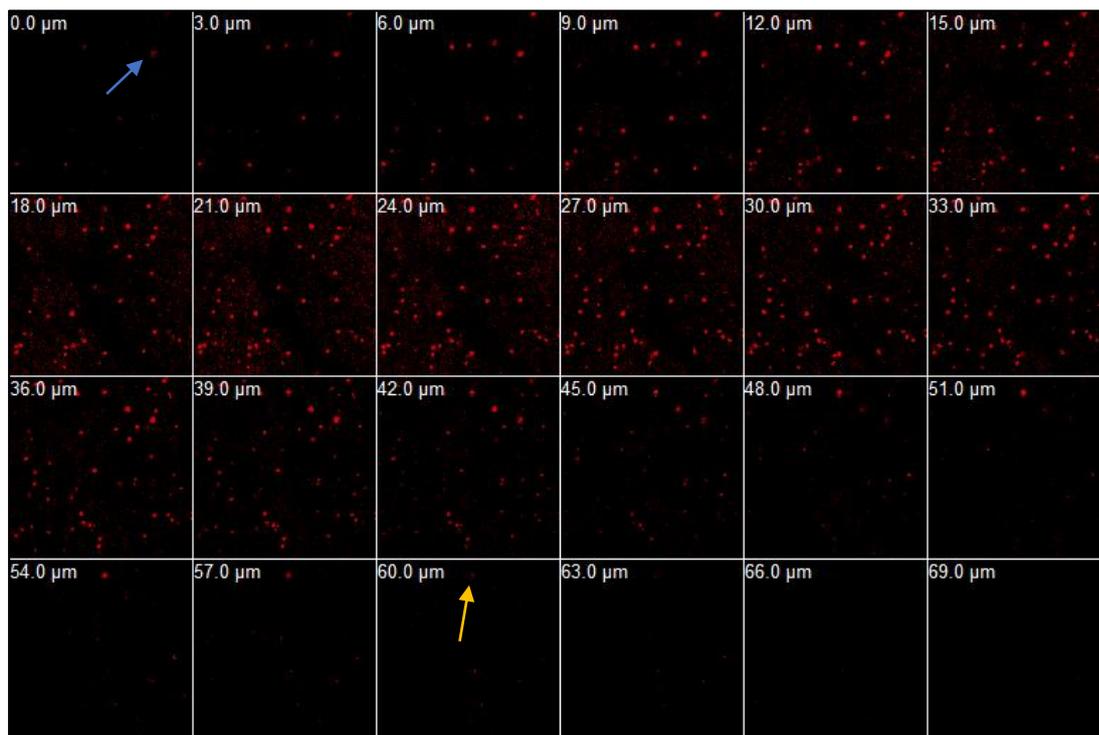
Fig. 3- 31 Representative of confocal microscope z-stack images of Saos-2 cells seeded scaffolds showing different planes from surface to bottom with 3 μm plane thickness each. Cells' nuclei were stained with PI (Red). Blue arrow point toward a cell in the surface while yellow arrow point toward a cell migrated inside the scaffold.
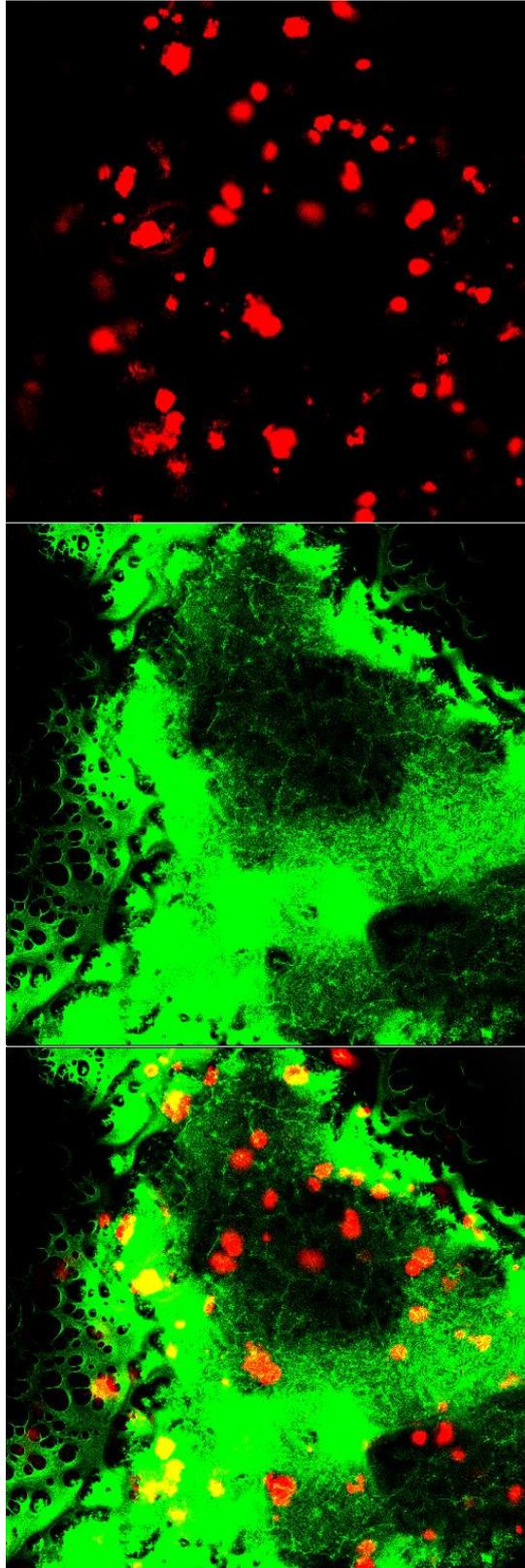
Fig. 3- 32 Representative of confocal microscope images of Saos-2 cells seeded scaffolds. Cells' nuclei were stained with PI (Red) while scaffold fibers were stained with FITC- phalloidin (Green).

CHAPTER 4

CONCLUSION

Tissue engineering is still a challenge and many researches are working on its development. This study was focusing on enhancing the development through local delivery of bioactive agents directly to bone cells by adding them into the building materials of bone substitute grafts.

In this study, 3D scaffolds were made with wet electrospinning and they were designed to hold either a hydrophilic (calcitonin salmon) or a hydrophobic (vitamin k2) bioactive agent or both. Scaffolds prepared with (monoaxial) wet electrospinning system were not suitable for loading both type of agents because there would be a significant loss of one of these bioactive agents depending on the type of collector solvent. Thus, a novel method was applied by combining a second type of electrospinning system, co-axial electrospinning, with wet electrospinning to produce 3D scaffolds that can have two layers, one shielding the other from the loss of bioactive agent caused by the collector.

In order to evaluate the resulting scaffolds, four scaffolds where chosen by microscale SEM analysis and macroscale observation to further study them and see whether- coaxial electrospuns has better *In situ*, *In vitro* and agent release profile, and also to determine which electrospinning collector is more advisable to use.

In this study, a coaxial electrospun scaffold produced by 5% PCL, 5% P-188 in the core and 5% gelatin ,5% P-188 in the shell, with water used as an electrospinning collector was considered to be better than the rest of the scaffolds evaluated in this study, because it had good degradation ratio, swelling ratio and pore size distribution.

Not to mention, it was better in releasing agent profile and proliferation rate, yet it had low mechanical strength that should be improved.

This study shows that by using wet coaxial electrospinning method, both a hydrophilic and a hydrophilic bioactive agent/drugs can be released locally in a controlled manner for combination therapy through incorporation with bone substitute grafts.

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APPENDIX A

CALIBRATION CURVES FOR VK2 AND β-LG
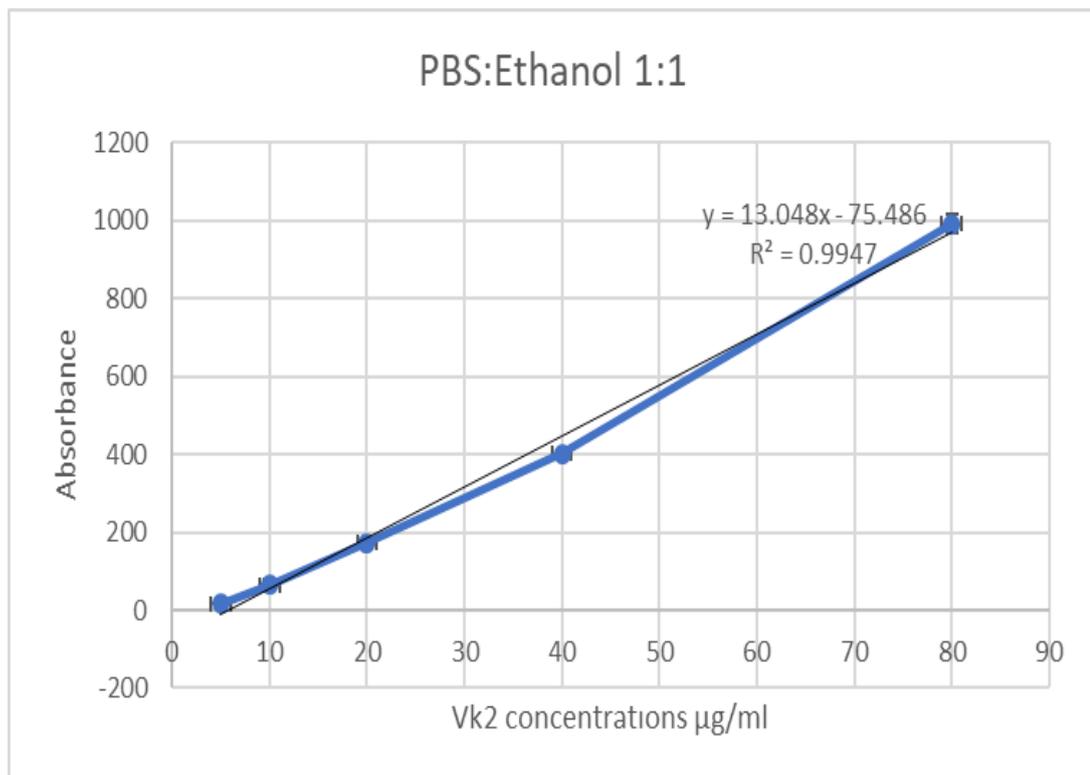
Fig. A1 Calibration curve of VK2 dissolved in (PBS:Ethanol 1:1 PBS (0.1 M pH 7.4)) solvent at different concentrations
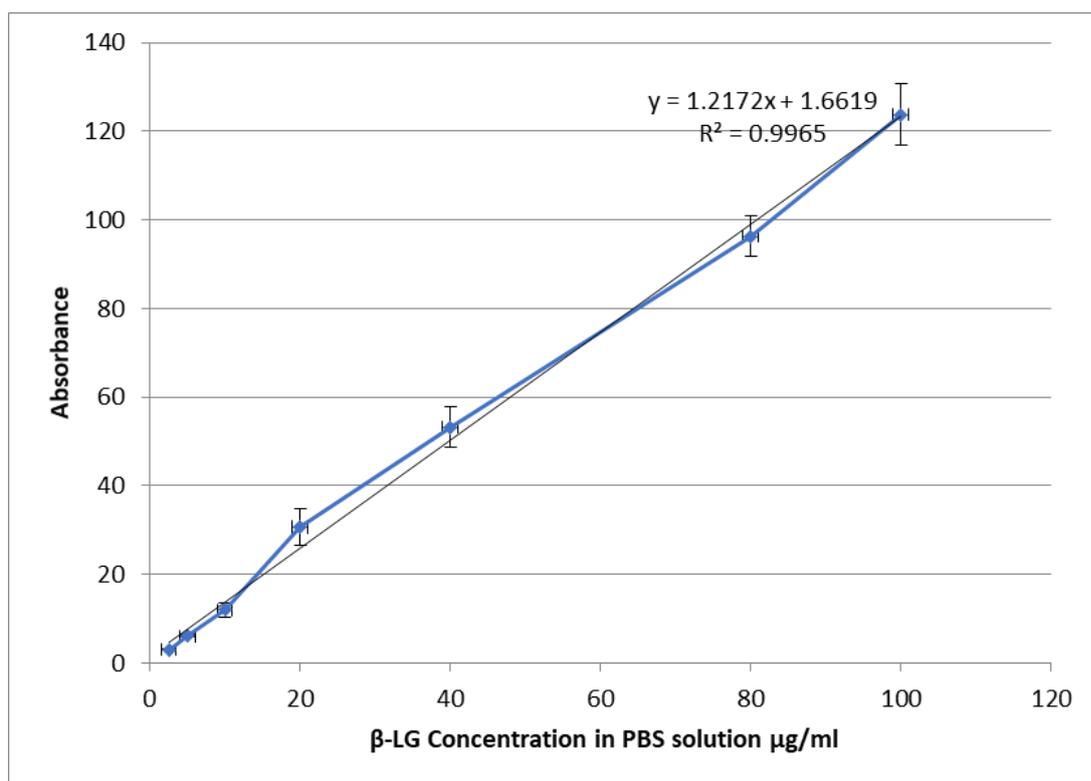
Fig. A2 Calibration curve of β-LG dissolved in PBS (0.1 M pH 7.4) solvent at different concentrations
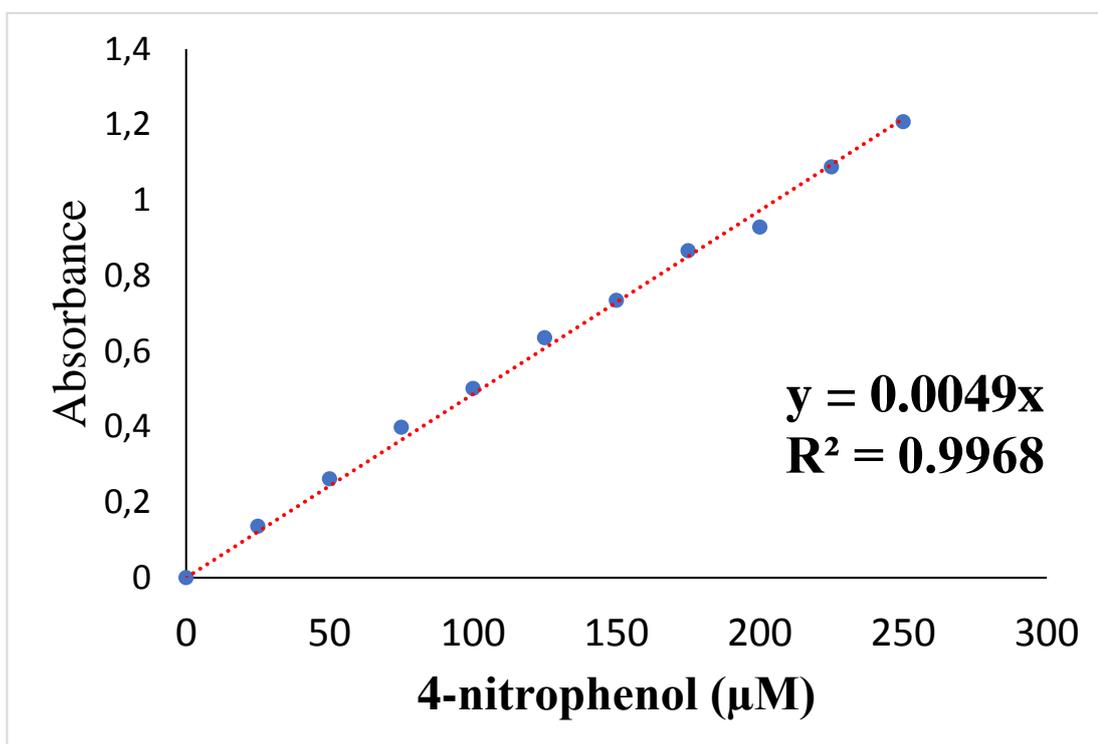
CALIBRATION CURVE FOR ALP

$$y = 0.0049x$$
$$R^2 = 0.9968$$

Fig. B Calibration Curve for ALP activity assay. 4-nitrophenol was used as a standard.
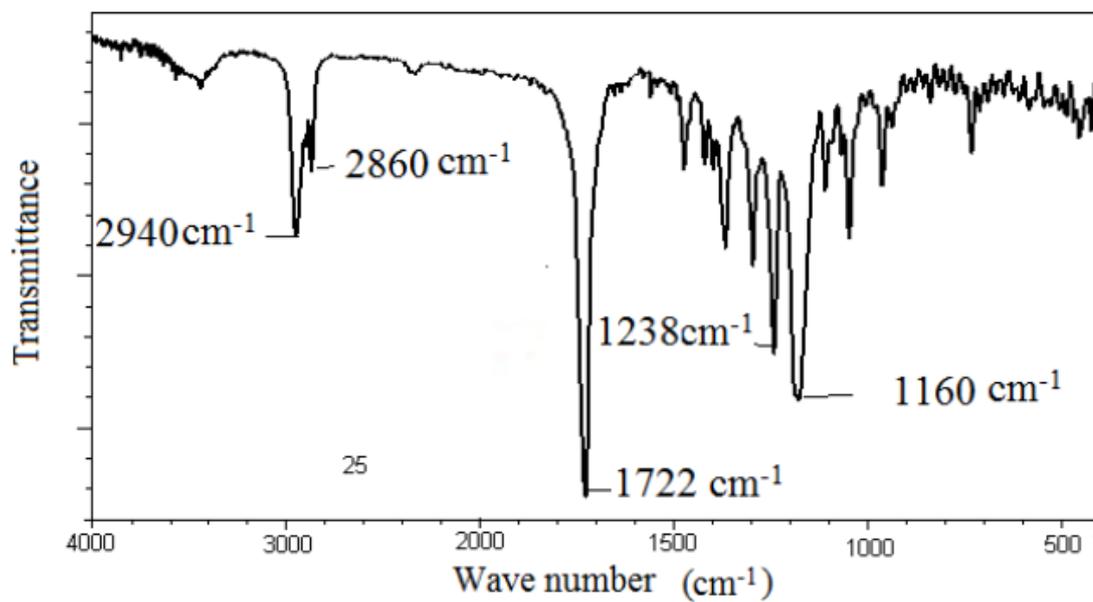
APPENDIX C

FTIR OF POLYMERIC COMPUNDS

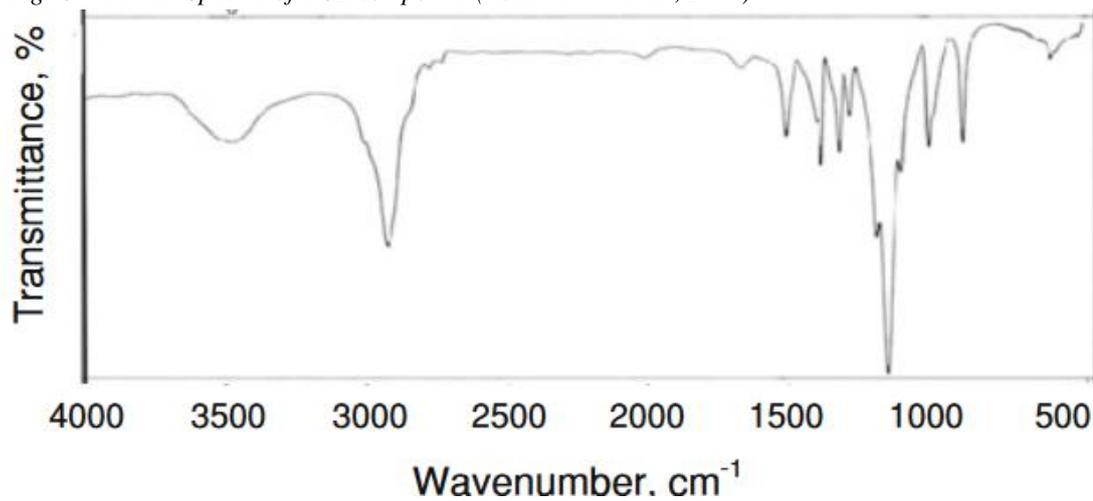*Fig. C – 1 FTIR Spectra of PCL compound (Abderrahim et al., 2015)*
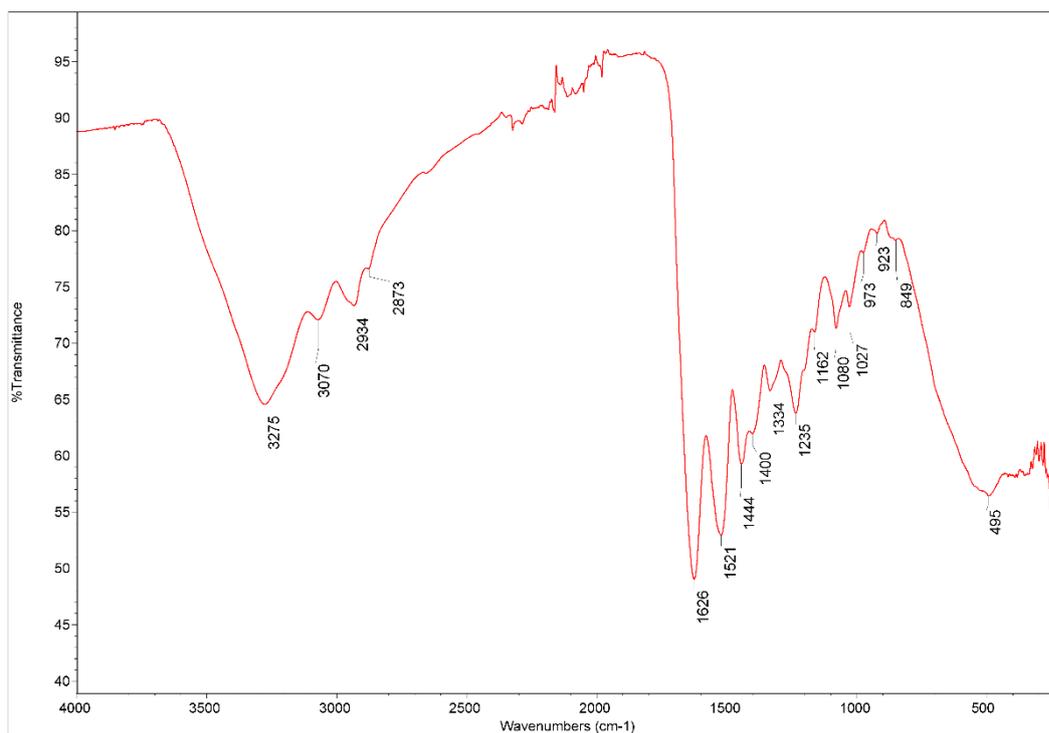

Fig. C – 2 FTIR Spectra of P-188 compound (Ha et al., 2012)

Fig. C – 3 FTIR Spectra of Gelatin compound (Institute of Chemistry University of Tartu, 2015).