ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL

PREPARATION AND CHARACTERIZATION OF PCL/LIGNIN SPONGES FOR BONE TISSUE ENGINEERING APPLICATIONS

M.Sc. THESIS

Gizem ELMALI

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

KEMİK DOKU MÜHENDİSLİĞİ UYGULAMALARI İÇİN PCL/LİGNİN SÜNGERLERİNİN HAZIRLANMASI VE KARAKTERİZASYONU

YÜKSEK LİSANS TEZİ

Gizem ELMALI
(521181108)

Moleküler Biyoloji-Genetik ve Biyoteknoloji Anabilim Dalı

Moleküler Biyoloji-Genetik ve Biyoteknoloji Programı

Tez Danışmanı: Prof. Dr. Fatma Neşe KÖK

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Gizem Elmalı, a M.Sc. student of ITU Graduate School student ID 521181108, successfully defended the thesis entitled "PREPARATION AND CHARACTERIZATION OF PCL/LIGNIN SPONGES FOR BONE TISSUE ENGINEERING APPLICATIONS", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

Thesis Advisor : **Prof. Dr. Fatma Neşe KÖK** .....
Istanbul Technical University

Jury Members : **Prof. Dr. Candan ERBİL** .....
Istanbul Technical University

Prof. Dr. Halime KENAR .....
Acıbadem University

Date of Submission : 25 March 2022
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To my family,

FOREWORD

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May 2022 Gizem ELMALI

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ABBREVIATIONS

DMEM : Dulbecco's Modified Eagle Medium
EDX : Energy Dispersive X-ray Spectroscopy
FBS : Fetal Bovine Serum
FTIR : Fourier Transform Infrared Spectroscopy
HAp : Hydroxyapatite
PBS : Phosphate-Buffered Saline
rpm : Revolutions per minute
SEM : Scanning Electron Microscopy

SYMBOLS

%	**:** percent
cm^{-1}	**:** wavenumber
g	**:** gram
h	**:** hour
mbar	**:** millibar
mg	**:** milligram
ml	**:** milliliter
mm	**:** millimeter
mM	**:** millimolar
nm	**:** nanometer
^{o}C	**:** degrees celsius
pH	**:** potential of hydrogen
U	**:** unit
v	**:** volume
w	**:** weight
µm	**:** micrometer

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PREPARATION AND CHARACTERIZATION OF PCL/LIGNIN SPONGES FOR BONE TISSUE ENGINEERING APPLICATIONS

SUMMARY

Organs and tissues may be damaged by accident, congenital anomalies, diseases and similar reasons. Although living tissues have the capacity to heal these damages, large defects or severe tissue losses can only be repaired to a certain extent. Tissue engineering techniques have been developed to trigger, assist and accelerate the healing process, or directly replace the targeted tissue. In tissue engineering, the most suitable three-dimensional scaffold structure is produced to match the characteristics of the targeted tissue and provide the initial structural integrity and organizational backbone for cells to replace the damaged tissues and organs. Especially bone and its related diseases can cause a significant impact on a person's health and quality of life if they are not treated and healed properly. Millions of bone injuries have been occurring each year and they are not only affecting people's life but also cause to a serious burden on country's economies. Bone tissue engineering was presented as a solution to address these problems.

In this thesis, a synthetic and natural polymer blend was used to create a scaffold that is suitable for bone tissue engineering. Polycaprolactone (PCL) was used as synthetic polymer, while alkaline lignin was used as natural polymer. Solvent casting, particulate leaching and freeze-drying methods were used to create porous sponge-like scaffolds. PCL and lignin samples were prepared at different lignin concentrations (0, 10, 15, and 20 mg/ml) while keeping the PCL concentration constant at 200 mg/ml, PCL200, PCL200/Lignin10, PCL200/Lignin15 and PCL200/Lignin20, respectively. A sample with PCL concentration of 100 mg/ml and lignin concentration of 20 mg/ml was also prepared to see the effect of PCL concentration (PCL100/Lignin20). For all scaffolds, except for the control sample, NaCl crystals were used as porogen.

Lignin containing scaffolds were appeared more brownish than pure PCL scaffolds. Since the structural integrity of PCL100/Lignin20 sample was very poor, and PCL200/Lignin20 without the salt addition cannot be removed from the mold surface, they were not used in further experiments.

Even though, mechanical properties of the scaffolds have not been studied in this thesis, based on preliminary visual and manual analysis rest of the PCL/Lignin type scaffolds were observed to be more flexible than PCL constructs.

Water uptake analysis showed that there is no significant difference between the samples with different lignin concentrations. The water uptake behavior of the scaffold, however, was dramatically increased with the lignin addition. While the water uptake for PCL200 was 44%, it was 380-400% for the samples with lignin after 24 h. In Fourier-Transform Infrared Spectroscopy (FTIR) analysis, typical peaks for PCL are identified for all scaffold types. Even though special peaks for

lignin could not be observed, a reduction in the intensity of a peak, which represent –OH groups, was determined in all PCL/Lignin scaffolds compare to the PCL200. This can be evidence of reactions and new bonds between PCL and lignin polymers. Since water uptake and FTIR analysis showed that there is no significant difference between different lignin concentrations, it was decided to continue with only one sample type, PCL200/Lignin10, for further experiments.

The porosities of pure PCL200 and PCL200/Lignin10 determined as 73% and 76%, respectively using liquid displacement technique. Pore sizes were investigated with ImageJ software on SEM images. For PCL200 scaffold, 31% and 58% of the pores on the surface were in the range of 100-300 μm and 300-600 μm, respectively. For PCL200/Lignin10, 30% and 43% of the pores were in the range of 100-300 μm and 300-600 μm, respectively. In addition, many pores that are smaller than 20 μm were observed for both sponges. The melting temperature of PCL200/Lignin10 scaffold was %5.4 higher than PCL200 scaffold as determined by Differential Scanning Calorimetry. The increased melting point might be the result of a new bond between PCL and lignin.

Hydrolytic degradation was not seen within 7 days for both samples, but enzymatic degradation in lipase presence was observed for both sponges. After 7 days, only 43% weight loss was recorded for PCL200/Lignin10, while PCL200 sponge was completely degraded after the fifth day. Lignin's natural resistance to enzymatic degradation or the modifications to form an alkaline lignin might be the reason of this unexpected decrease in degradation.

Biomineralization process was triggered by soaking scaffolds to modified-simulated body fluid solution. The mineralization rate was analyzed by SEM. SEM micrographs revealed significant mineral accumulations for both scaffold types at the end of the 7th day. When the 7th day samples were compared, it was seen that the hydroxyapatite-like structures were mostly deposited on top of each other in the PCL200 sample, while the aggregations were more evenly spread over the surface on the PCL200/Lignin10 sample. Furthermore, even though an intense accumulation was observed on the scaffold surface of the PCL200, the inside of the pores as appeared to be smooth. Lignin containing samples, on the other hand, had more mineral deposition within the pores. The elemental analysis of the formed hydroxyapatite-like structures was analyzed by EDX. It was observed that the Ca/P ratio of the accumulations on the PCL200/Lignin10 samples at all determined times was closer to the ideal hydroxyapatite Ca/P ratio (1.67).

Finally, cell proliferation assay was performed with hFOB cells to examine the effects of lignin addition on cell adherence and proliferation on the surface. The proliferation of hFOB was significantly higher on PCL200/Lignin10 scaffolds compared to PCL200. The proliferation on PCL200/Lignin10 sponge was found to be 212% and 50% higher after day 3 and 7, respectively.

Further studies are necessary in order to evaluate the usability of PCL200/Lignin10 sponge scaffolds in bone tissue engineering. However, the findings obtained within this thesis suggest that this blend is promising candidate for bone tissue engineering.

KEMİK DOKU MÜHENDİSLİĞİ UYGULAMALARI İÇİN PCL/LİGNİN SÜNGERLERİNİN HAZIRLANMASI VE KARAKTERİZASYONU

ÖZET

Doku ve organlar, kaza, doğumsal anomaliler, hastalıklar ve benzeri nedenlerle zarar görebilir. İnsan vücudunun bu hasarları iyileştirme kapasitesi, özellikle büyük travmalarda, oldukça sınırlıdır. Bu sebeple, iyileşme sürecini tetiklemek, hızlandırmak ve bu sürece yardımcı olmak veya doğrudan hedeflenen dokunun yerini alabilecek bir yapı geliştirmek için doku mühendisliği teknikleri geliştirilmiştir.

Doku mühendisliğinde, hedeflenen dokunun özellikleri için en uygun üç boyutlu iskele yapısı oluşturulur. Bu yapı hücrelerin hasarlı doku ve organları yeniden yapılandırması, yerini alması veya onarması için ilk yapısal bütünlüğü ve omurgayı sağlar. Özellikle kemik ve ilişkili hastalıklar, uygun şekilde tedavi edilmediği ve iyileştirilmediği takdirde kişinin sağlığı ve yaşam kalitesi üzerinde ciddi olumsuz etkilere neden olabilir. Her yıl milyonlarca kemik yaralanması meydana gelmekte ve bunlar hasta rahatı açısından olumsuz etkileri yanında ülke ekonomileri için de ciddi bir yük oluşturmaktadır. Bu nedenle, kemik doku mühendisliği bu sorunlara bir çözüm olarak düşünülmüştür.

Bu tezde, kemik doku mühendisliğinde kullanıma uygun bir yapı iskelesi oluşturmak için sentetik ve doğal bir polimer karışımı kullanılmıştır. Sentetik polimer olarak polikaprolakton (PCL), doğal polimer olarak alkali lignin tercih edilmiştir.

PCL fiziksel ve biyolojik özellikleri nedeniyle doku mühendisliği iskelelerinde sıklıkla tercih edilen bir alifatik poliesterdir. Biyouyumlu ve biyobozunur yapısı ve düşük erime ve camsı geçiş sıcaklığı tercih edilme sebeplerindendir. Ancak bozunurluğu, benzeri sentetik polimerlere kıyasla daha yavaş olduğundan, sert ve yük taşıyıcı dokuların iskeleleri için kullanımı daha uygundur. Aynı zamanda birçok sentetik polimerden daha ucuz olması ve yüksek miktarlarda üretilebilmesi de avantajlarındandır. Buna karşın, diğer birçok sentetik polimer gibi organizmada enflamasyonu ve oksidatif stresi tetikler ve hidrofobik özelliğinden dolayı hücre adezyonu ve çoğalması için uygun bir platform oluşturmaz. Bu dezavantajlara rağmen bu polimerin sıklıkla tercih ediliyor olmasının nedenlerinden biri, diğer polimerlerle kolayca kopolimer veya karışım oluşturabilmesidir. Böylece avantajlarının ön plana çıkarılacağı ve dezavantajlarının elimine edilebileceği yapılar oluşturulabilir.

Lignin doğada en bol bulunan ikinci polimerdir ve çoğunlukla damarlı bitkilerin hücre duvarında bulunur. Yılda 70 milyon tondan fazla lignin kağıt endüstrisi yan ürünü olarak üretilmektedir ve bu miktarın yalnızca %2'si farklı sektörlerde kullanılarak ticarileştirilmektedir. Ligninin yapısı, bitkiler arasında önemli ölçüde farklılık gösterebilir. Ligninin kimyasal yapısı oldukça karmaşıktır ve bu nedenle standart bir yapıdan söz edilemez. Lignin birçok farklı fonksiyonel gruptan oluşabilir ve özellikleri bu fonksiyonel grupların kimyasal olarak modifiye edilmesiyle değiştirilebilir. Kırılgan ve karmaşık yapısına karşın biyouyumlu oluşu, termal

kararlılığı ve antibakteriyel özellikleri nedeniyle ilgi konusu olmuştur. Aynı zamanda hücreleri oksidatif strese karşı koruduğu gözlemlenmiştir. Buna rağmen ligninin medikal araştırmalarda kullanımı oldukça nadirdir ve bu alanda ciddi bir açık bulunmaktadır.

Bir doku mühendisliği iskelesi oluşturulurken kullanılacak polimerler kadar, bu polimerlerden maksimum verimin alınabilmesi için iskele yapımında doğru tekniğin kullanılması da önemlidir. Fonksiyonel doku ve organların oluşturulabilmesinde üç boyutlu hücre kültürlerinin kurulabilmesi için gözenekli sünger yapılı iskelelerin kullanımı yaygındır. Gözenekli yapılar, yüzey alanı genişlikleriyle yeni doku oluşumu için verimli bir alan ve hücrelerin madde alışverişi yapabileceği bir mikro çevre oluşturur. Özellikle büyük gözenekli (100-600 μm) iskelelerin konak kemik dokusuyla daha iyi bir entegrasyona, damarlanmaya ve kemik hücrelerinin yayılımına olanak sağladığı, geçirgenliğin gözenek boyutu arttıkça artmasıyla kemik büyümesinin desteklendiği görülmüştür. Ancak gözenek boyutu ve miktarı ile mekanik güç arasında ters bir orantı bulunmaktadır. Bu sebeple mekanik anlamda güçlü sentetik polimerler ile yüksek gözeneklilik sağlayan ve hücre çoğalımı için çok daha elverişli bir ortam sunabilen doğal polimerler birlikte kullanılarak bu problemin önüne geçilebilir.

Bu tezde farklı avantaj ve dezavantajlara sahip PCL ve lignin polimerleri bir karışım olarak kullanılarak ve bu karışımdan sünger yapılı gözenekli yapılar oluşturularak uygun bir kemik doku mühendisliği iskelesi eldesi hedeflenmiştir.

Gözenekli sünger benzeri yapı iskeleleri oluşturmak için çözücü dökümü, parçacık uzaklaştırma ve dondurarak kurutma yöntemleri kullanılmıştır. Farklı konsantrasyonlarda PCL ve lignin içeren iskele yapıları oluşturmak için farklı miktarlardaki polimerler kloroform/DMF (2:1, v/v) içerisinde çözdürülmüştür. PCL için 200 mg/ml konsantrasyonu sabit tutularak, 0, 10, 15 ve 20 mg/ml lignin içeren örnekler hazırlanmış ve sırasıyla; PCL200, PCL200/Lignin10, PCL200/Lignin15, PCL200/Lignin20 olarak adlandırılmıştır. Ayrıca PCL konsantrasyonun etkisinin araştırılabilmesi için 100 mg/ml PCL ve 20 mg/ml lignin içeren bir örnek de hazırlanmıştır (PCL100/Lignin20). Tüm bu örnekler için porojen olarak tuz (NaCl) partikülleri kullanılmıştır. Dondurma ve kurutma işlemlerinden sonra gözenekli yapı oluşturmak için seri yıkamalar ile tuz uzaklaştırılmıştır. Ek olarak porojen (tuz) kullanılmadan bir örnek hazırlanarak porojen kullanılan örneklerden farkının gözlemlenmesi amaçlanmıştır (PCL200/Lignin20/nS).

Tüm PCL/Lignin iskelelerinin görsel olarak saf PCL iskelelerinden daha kahverengi olduğu gözlemlenmiştir. PCL konsantrasyonunun etkisinin gözlemlenmesi amacıyla oluşturulan PCL100/Lignin20 yapısında, örneğin sonraki deneylerde kullanımına imkan sağlayacak kadar bir yapısal bütünlük elde edilememiştir. PCL200/Lignin20/nS numunesinin dondurarak kurutma sonrasında kalıp yüzeyine yapıyı bozmadan çıkarılamayacak kadar yapıştığı gözlemlenmiş, organik çözücülerin kullanılması nedeniyle, örneğin kalıptan çıkmasını kolaylaştıracak başka bir kalıp tipi veya kalıp ile polimer çözeltisi arasında bir ara yüz kullanılması mümkün olmamıştır. Her iki yapı da başka deneylerde kullanılmaya uygun bulunmayarak çalışmadan çıkarılmıştır.

Bu tezde iskelelerin mekanik özellikleri incelenmemiş olsa da, ön görsel ve manuel analizlere dayalı olarak PCL/Lignin iskelelerinin PCL yapılarından daha esnek olduğu gözlemlenmiştir.

Su alma analizi sonucu, farklı lignin konsantrasyonları ile hazırlanmış örnekler arasında anlamlı bir fark gözlemlenmemiştir. Ancak saf PCL örneği ile kıyasladığında örneklerin su alma davranışı lignin ilavesiyle çarpıcı biçimde artmıştır. Bir gün sonunda PCL200 için su alımı %44 iken lignin eklenen örneklerde %380-400 aralığında kaydedilmiştir. Doku mühendisliği iskelelerinin içindeki kimyasal bağlarının incelenebilmesi için Fourier Dönüşümlü Kızılötesi Spektroskopisi (FTIR) yapılmıştır. PCL için tipik pik noktaları, her iskele tipi için de gözlemlenmiştir. Lignin için özel pik noktaları elde edilememiş olsa da, tüm PCL/Lignin tipi iskelelerde, PCL'ye kıyasla, –OH gruplarını temsil eden pikin yoğunluğunda bir azalma tespit edilmiştir. Bunun, PCL ve lignin polimerleri arasında gerçekleşen reaksiyonlar ve yeni bağlar için bir kanıt olabileceği düşünülmüştür. İki analiz için de saf PCL numunesi ile ligninli numuneler arasında fark gözlemlenmesine karşın, farklı lignin konsantrasyonlarına sahip numuneler arasında kayda değer bir fark elde edilmemiştir. Bu nedenle, daha sonraki deneyler için lignin oranının en düşük olduğu örnek olan PCL200/Lignin10 ile devam edilmesine karar verilmiştir.

PCL200 ve PCL200/Lignin10'un gözeneklilikleri, sıvı yer değiştirme yöntemiyle, sırasıyla %73 ve %76 olarak hesaplanmıştır. SEM görüntüleri üzerinde ImageJ yazılımı kullanılarak gözenek boyutları incelenmiştir. PCL200 iskelesinin yüzeyindeki gözeneklerin %31'i ve %58'i, PCL200/Lignin10 iskele yüzeyinin %30'u ve %43'ü, sırasıyla 100-300 µm ve 300-600 µm aralığında olarak bulunmuştur. Ayrıca 20 µm'den küçük birçok gözenek tespit edilmiştir. Diferansiyel Taramalı Kalorimetre ile yapılan analizde PCL200/Lignin10 iskelesinin erime sıcaklığı PCL200 iskelesinden %5.4 daha yüksek elde edilmiştir. FTIR sonuçlarını destekler nitelikte artan erime sıcaklığının, PCL ve lignin arasında oluşmuş olabilecek yeni bağların sonucu olabileceği tahmin edilmektedir.

Her iki numune için 7 günün sonunda hidrolitik bozunma gözlemlenmemiş olmasına karşın iki sünger için de lipaz varlığında enzimatik bozunma gözlemlenmiştir. Lignin eklentisi bulunan sünger örneğinin enzimatik bozunma hızının saf PCL'e kıyasla çok daha yavaş olduğu gözlemlenmiştir. PCL200/Lignin10 için 7 gün sonunda sadece %43 kütle kaybı kaydedilirken, PCL200 süngeri beşinci günden sonra tamamen bozunmaya uğramıştır. Ligninin enzimatik bozunmaya karşı olan doğal direncinin veya alkali lignin oluşturmaya yönelik yapılan modifikasyonların, bozunmadaki bu beklenmedik düşüşün nedeni olabileceği düşünülmektedir.

İskelelerin modifiye-yapay vücut sıvısı (m-SBF) çözeltisine batırılmasıyla biyomineralizasyon süreci tetiklenmiştir. Süreci hızlandırmak için üç kat daha yoğun m-SBF kullanılmıştır. Süreç sonucunda oluşan hidroksiapatit-benzeri yapıların yoğunluğu SEM cihazı ile analiz edilmiştir. SEM görüntüleri, her iki örnek tipinde de 7. günün sonunda yüzeyde hidroksiapatit-benzeri yapıların yoğun bir şekilde biriktiğini göstermiştir. Örneklerin 7. gün sonuçları karşılaştırıldığında PCL200 örneğinde mineral yapılarının çoğunlukla birbiri üzerine biriktiği gözlemlenirken, PCL200/Lignin10 örneğinde bu birikmelerin yüzeye daha eşit bir şekilde yayılarak gerçekleştiği gözlemlenmiştir. Ek olarak, PCL200 örneğinin yüzeyinde gözlemlenen yoğun birikime rağmen porların iç yüzeylerinin birikme olmaksızın pürüzsüz bir yapıda olduğu tespit edilmiştir. Lignin içeren örneklerin por iç yüzeylerinde ise yüzeyde olduğu şekilde hidroksiapatit-benzeri yapıların birikimi gözlemlenmiştir. Oluşan hidroksiapatit benzeri yapıların element analizi EDX ile gerçekleştirilmiştir. Belirlenen tüm zamanlarda, PCL200/Lignin10 numuneleri üzerindeki birikimlerin

Ca/P oranlarının ideal hidroksiapatit Ca/P oranına (1,67) daha yakın olduğu tespit edilmiştir.

Son olarak, lignin ilavesinin iskele yapıları üzerinde çoğalabilecek hücre sayısı üzerindeki etkisinin incelenebilmesi için hFOB hücre hattıyla deneyler gerçekleştirilmiştir. hFOB hücre sayısı, PCL200'ye kıyasla PCL200/Lignin10 iskelelerinde önemli ölçüde daha yüksek olarak kaydedilmiştir. PCL200/Lignin10 süngeri üzerindeki proliferasyonun 3. ve 7. günlerde sırasıyla %212 ve %50 daha yüksek olduğu gözlemlenmiştir.

PCL200/Lignin10 sünger yapı iskelelerinin kemik doku mühendisliğinde kullanılabilirliğini değerlendirmek için daha ileri çalışmalara ihtiyaç vardır. Ancak bu tez kapsamında elde edilen bulgular, elde edilen yapının umut vadettiğini göstermektedir.

1. INTRODUCTION

1.1 Purpose of Thesis

Humans have a limited capacity to regenerate most of their tissues and organs that have been severely damaged by diseases, traumas, or developmental anomalies, and current remedies are insufficient. Tissue engineering has emerged as a promising solution to address this medical requirement. Scaffolds are used as 3D matrix in tissue engineering to provide the initial structural integrity and organizational backbone for cells to reconstruct, replace, or repair damaged tissues and organs. Scaffolds are constructed with biomaterials and fabrication techniques have been developed to tailor the architecture for specific purpose.

In this thesis, a blend composed of synthetic and natural polymers is used to produce a scaffold that is suitable to use in bone tissue engineering. Polycaprolactone (PCL) was chosen as synthetic polymer due to its good mechanical properties and biodegradable nature. Moreover, PCL has high processivity and easily blend with other polymers, cheap and can be found in large quantities. Lignin, which is used as natural polymer, was selected because of its high durability, thermal stability, antibacterial properties, good biocompatibility. Lignin also has high capacity to protect cells from the effects of oxidative stress. By blending these two polymers, it is aimed to form biocompatible, biodegradable scaffolds that trigger the formation of hydroxyapatite for bone formation and provide a suitable growth environment for bone tissue cells.

1.2 Tissue Engineering

Diseases, injuries, and developmental defects are serious problems that causes severely damaged tissues and organs. Because the human kind has a very limited regeneration ability, tissue and organ failures have become major economical and healthcare concerns (Gurtner et al., 2008; Persidis, 1999). Although it is tried to find solutions to these problems with organ donations, these are not permanent and

efficient solutions due to the low number of donors, the low probability of donor-recipient match, limited time for transplantation, increasing transplant waiting list and the increase in the elderly population (Chandra et al., 2020).

Tissue engineering (TE) has emerged as a viable approach for meeting this medical requirement. TE is a multidisciplinary discipline that brings together subjects including biology, chemistry, engineering, medicine, pharmaceuticals, and material sciences to create structures that imitate the extracellular matrix (ECM) of natural tissues to heal or replace damaged tissues and organs (Ogueri et al., 2019; Theocharis et al., 2016). As a result, advances in TE have occurred in parallel with advances in fields such as novel biomaterials (Stratakis, 2018), three-dimensional (3D) bioprinting technologies (Murphy and Atala, 2014), nanotechnology integration, stem-cell technologies (Hasan et al., 2018), and gene editing technologies (Hasan et al., 2018). (Pulgarin et al., 2017).

Tissue engineering may be divided into three categories: cells, materials, and scaffolds and their manufacturing techniques. All of these aspects may be programmed to achieve a certain goal (Chandra et al., 2020).

1.2.1 Cells

There are several cell sources for TE, each with its own set of advantages and disadvantages. Ex vivo growth of multipotential cell populations, such as autologous, allogeneic, progenitors, adult unipotent or multipotent stem cells, and induced pluripotent stem cells (iPSCs) (Figure 1.1), and after that their transplantation into damaged areas, is the current technique for TE (Chandra et et al., 2020;Griffith and Naughton, 2002). Autologous cells are used in TE to avoid host immune response. Even so, these type of cells are hard to collect and time-consuming to produce, which makes this approach hard to apply (Petreaca and Martins-Green, 2020).

Because of their unique regeneration potential and immunomodulatory features, adult stem cells or iPSC might be preferable (Hanson et al., 2014). They have the ability to develop into the desired cell types or into stem cell-derived transplant cells (Clarke et al., 2018). Adult stem cells, like mesenchymal stem cells, usually do not cause an immunological response after being transplanted. Despite the fact that, the sorts of cells they may differentiate into are limited, tissue repairing and regeneration can be induced (Wu et al., 2018). iPSCs differentiate from adult stem cells by their

potential to develop into any kind of cell and giving opportunity to change and repair any mutations that might be exist in these cells by using gene editing methods (Wertheim and Leventhal, 2015). In principle, iPSCs should not trigger any immunological reactions because they directly produced from patient's own cells (Clarke et al., 2018). However, it has been reported that after transplantation and differentiation of the iPSCs, some immune reactions observed in in vivo studies (Zhao et al., 2011). Even though it is not fully understood, the mechanism behind the responses to autologous cells might be because of ex vivo differentiations of the cells in the implantation preparation process (Wertheim and Leventhal, 2015).

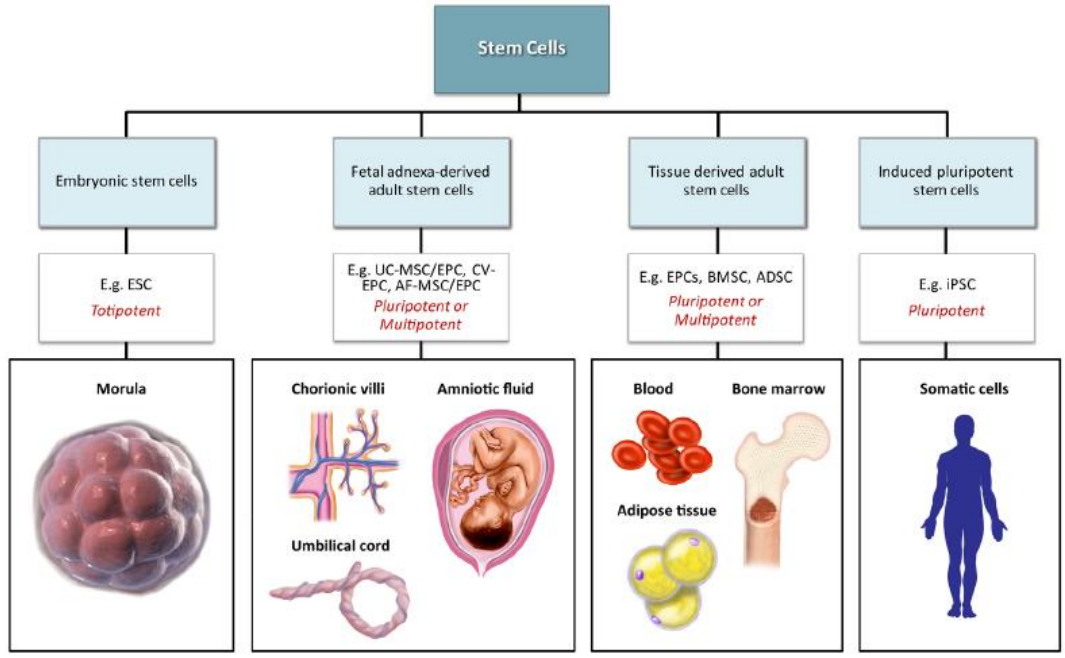

Figure 1.1 : Different sources of cells for tissue engineering, (Al-Himdani et al., 2017).

1.2.2 Biomaterials

In 1976, first Consensus Conference of the European Society for Biomaterials defined biomaterials as "a nonviable substance used in a medical device, designed to interact with biological systems." However, the concept of biomaterials has developed in a way that the definition changed to "material designed to interface with biological systems to evaluate, treat, enhance, or replace any tissue, organ, or function of the body" (O'Brien, 2011).

The purpose of the first generation biomaterials in TE scaffolds were to perform in a way that can replace the needed tissue without any toxical effect to the host. In the

second generation the bioactive and biodegradable biomaterials are preferred to increase transplantation efficiencies. For the third generation, in addition to second generation biomaterials with great properties and performances, the addition of instructive substances are proposed to initiate special useful biological reactions in the host (Qu et al., 2019).

Natural polymers, synthetic polymers, and ceramics are the three main types of biomaterials, each having its own set of benefits and drawbacks.

Ceramics like hydroxyapatite (HAp) and tri-calcium phosphate are commonly used in hard-tissue engineering, such as bone regeneration. They are very similar to bone in chemical and structural aspects (Hench, 1998). These properties make them highly biocompatible and it has been shown that they can improve differentiation and proliferation of osteoblasts (Ambrosio et al. 2001). Unfortunately, their brittleness, hard to-shape structure and inability to sustain the mechanical loading required for remodeling limit their uses (Wang, 2003).

Natural polymers are derived from plants, animals and microorganisms, which can be summarized as renewable resources, hence, they are widely abundant in nature. Natural polymers offer a wide range of applications, including plasters, absorbent materials, cosmetics, drug delivery, and TE scaffolds (Malafaya et al. 2007, Shanmugam et al. 2005). In the field of tissue engineering, natural polymers have a number of advantages and disadvantages. They promote great cell adhesion and development, are biocompatible and biodegradable, have high water binding behaviors, cytocompatibility, and low possibility of provoking an immune response. Also their degradation products can be used in cellular metabolism (Asghari et al., 2017; O'Brien, 2011; Abbasian et al., 2019; Gomes et al., 2013). However, it is very challenging to create a homogeneous and reproducible scaffold by using natural materials (O'Brien, 2011). On the contrary, their main disadvantages such as possibility of microbial contamination, lower tunability, uncontrollable hydrolysis rate and low mechanical strength, limit their practice for hard tissue engineering (Reddy et al., 2021). Polysaccharides (lignin, chitosan, alginate etc.) and proteins (collagen, gelatin etc.) are natural polymers that are available and preferred in applications (Leach et al., 2003).

In the quest to create scaffolds, a variety of synthetic polymers have been studied. Synthetic polymers have a number of advantages, including customizable features like strength, degradation rate, and microstructure, they are reproducible in large scales (Liu and Ma, 2004), and able to facilitate the regeneration of damaged or diseased tissue structure and function (Reddy et al., 2021). As a result, synthetic polymers have been approved for use in tissue engineering scaffolds. While these materials have had a lot of success, they do have certain disadvantages, such as the absence of cell attachment sites and the need for chemical changes to improve cell adherence (Reddy et al., 2021). The most commonly investigated synthetic materials are polycaprolactone (PCL), polyglycolic acid (PGA), polylactic acid (PLA), and their copolymers (Kluge and Mauck, 2011). Despite the fact that several synthetic polymers have varying amounts of good biodegradability, biocompatibility, and mechanical qualities, no polymer owns all of these key features at optimal levels by itself (Ciardelli et al., 2005).

As mentioned above, biologically derived polymers provide important advantages over synthetic materials, including lower toxicity and improved bioactivity. However, the insufficient engineering features of bio-based polymers typically limit their use (Chesterman et al. 2020). On the other hand, synthetic polymers have demonstrated several benefits over natural polymers, such as more controllable chemical compositions to create different scaffolds with properties that fit the varying application (Reddy et al., 2021). So, it is suggested that no single material can meet all of the requirements for tissue replacement by its own. Instead, a blend tissue scaffold produced from both natural and synthetic biopolymers can be used to meet all medical criteria (Sheikholeslam et al., 2018). They have been combined to take advantage of their favorable properties to overcome the disadvantages of each particular type of material (Tran et al., 2018). The natural and synthetic polymer blends that carries inherent biocompatibility of natural polymers and good physicochemical qualities of synthetic polymers, has been investigated by many researchers to create a good tissue engineering scaffold (Reddy et al., 2021).

In this project, PCL synthetic polymer and Lignin natural polymer are used to create a composite scaffold and formation of hydroxyapatite inorganic component on scaffold was studied along with the other properties that are mentioned later.

1.2.2.1 Hydroxyapatite

Hydroxyapatite (HAp) is an inorganic component. They mostly found in human hard tissues like teeth and bones (Arora et al., 2013). Because of its resemblance in composition to the inorganic component of natural bone, HAp has been frequently used to build different bone regenerating scaffolds as one of the significant bone filler materials (Si et al., 2016). HAp coatings provide scaffold surfaces with antibacterial properties. HAp-based coating techniques have been shown to provide accelerated bone tissue growth and can be used for a variety of orthopedic scaffolds providing effective and long-lasting tissue regeneration, wound recovery and joint repair (Shah et al., 2012).HAp can be used in the process of fabrication of scaffolds by adding HAp nanoparticles into a polymer solution directly, or biomineralization and HAp coating can be triggered by a variety of methods, including dipping the scaffold in simulated body fluid (SBF), immersing in calcium and phosphate solutions, urea-mediated heat treatment, and enzymatic processes (Murphy and Mooney, 2002). Immersing in SBF is one of the most used methods for HAp coating. SBF is a fluid that created to have an ion concentration similar to human blood plasma, so that biomineralization abilities of artificial materials can be studied in vitro (Kim et al., 1997; Cai et al., 2011). However the apatites that are formed by conventional SBF, which is presented by Kokubo et al. in 1990, could not achieve the desired similarity to human bone apatite. Oyane et al. (2003) presented a modified-SBF (m-SBF), which is more optimal for in vitro biomineralization studies with an ion concentration closer to human blood plasma than conventional SBF. Therefore it can produce apatite with a quality and structure closer to human bone apatite.

1.2.2.2 Lignin

Lignin is the second most abundant polymer in nature and is mostly found in the cell wall of vascular plants (Norgren and Edlund, 2014). The structure of lignin can differ substantially across the plants, as well as between extraction methods. Because of that, lignin's chemical structure is highly complicated and a regular structure cannot be mentioned. (Lu et al., 2017). Lignin is mostly consists of three basic monomers called, paracoumaryl, coniferyl and sinapyl alcohol (Figure 1.2). These three monomers forms cross-linked phenylpropanoid units, and different repeating patterns

of these units create different types of lignin (Figueiredo et al., 2018; Thulluri et al., 2016). Lignin can be consist of varying functional groups such as phenolic, methoxy, and an aliphatic hydroxyl groups and characteristics of lignin depends on these functional groups (Bertella and Luterbache, 2020), and by chemically modifiying these groups, any properties of lignin can be altered (Wu et al., 2012; Konduri and Fatehi, 2015).

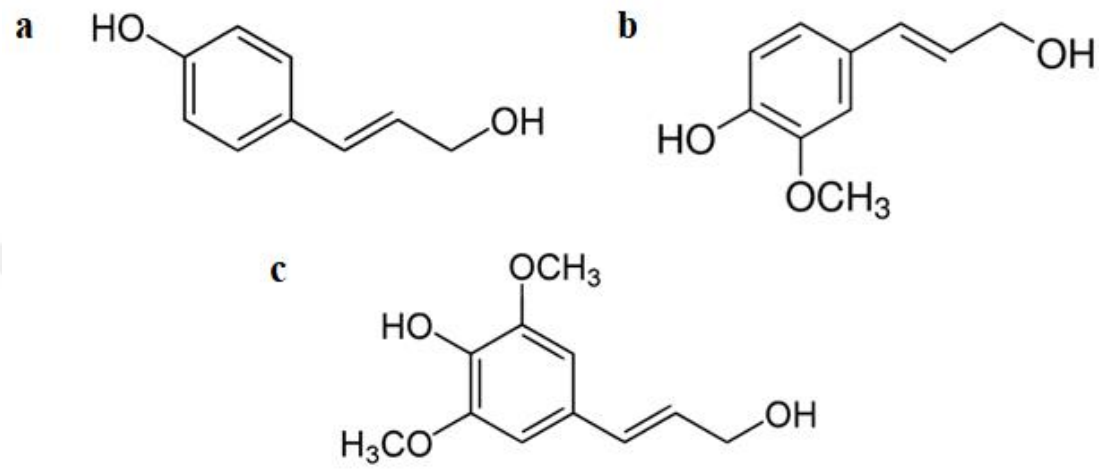

Figure 1.2 : Three basic monomers of lignin; a) paracoumaryl alcohol, b) coniferyl alcohol, c) sinapyl alcohol.

Each year, more than 70 million tons of lignin are generated in the paper sector alone as a by-product, and only 2% of this amount is commercialized using different industries (Naseem et al., 2016; Chatterjee and Saito, 2015). In many studies conducted in recent years, despite the fragile and complex three-dimensional structure of lignin, it has been observed that it has high durability, thermal stability, antibacterial properties, good biocompatibility, and it has been discovered that it has a high capacity to protect cells from the effects of oxidative stress (Sen et al., 2015; Salami et al., 2017). Despite its existing advantages, when looking at the patents and scientific studies published on lignin, it is seen that they are concentrated in the concrete, dispensant and adhesive sectors. There are only a few studies on the use of lignin in biomedical applications compared to other natural polymers, particularly in tissue engineering scaffolding (Table 1.1) (Witzler et al., 2018).

There have been some research on the use of lignin in TE blends with other polymers. Quraishi et al (2015) has reported an enhanced water uptake behavior and better biocompatibility for alginate aerogel when it was used with lignin as copolymers. Techato et al. (2018) reported that agarose-lignin composite hydrogels

had improved on mechanical characteristics. Wang et al. (2019) demonstrated increased HAp formation and efficient adhesion and proliferation of osteoblast cells for lignin/PCL nanofibers.

Table 1.1 : Number of scientific publications on lignin, chitosan and gelatin polymers according to Web of Science, searched on 02 March 2022 (search is refined with "tissue engineering").

Publication Year	lignin	chitosan	gelatin
2021	53	1016	936
2020	52	979	865
2019	52	925	813
2018	39	805	639
2017	32	801	655

1.2.2.3 PCL

PCL is an aliphatic polyester that has undergone extensive research as a biomaterial and is commonly used in 3D scaffolding studies (Chesterman et al., 2020). Because of its physical and biological characteristics, PCL is an ideal polymer to fabricate biomedical materials (Abedalwafa et al., 2012). It is a biocompatible, bioadsorbable, and biodegradable polymer (Asghari et al., 2017). When compared to PGA or PLA, PCL degrades at a much slower rate (Pitt, 1990). Since it takes longer time to degrade than other synthetic polymers that used for the same purpose, it is advantageous to use PCL for hard and load-bearing tissue regenerations (Dwivedi et al., 2019). Another reason why this polymer is preferred is that it is cheaper and available in large quantities compared to other synthetic polymers (Park et al., 2014). Another unusual property is its high thermal stability It has a very low melting temperature and a very low glass transition temperature (Engelberg and Kohn, 1991). However, contrary to these advantages it triggers inflammatory response and oxidative stress in the organism, like many other synthetic polymers, and does not constitute a suitable platform for cell adhesion and proliferation due to its hydrophobic properties (Wang et al., 2018; Intranuovo et al., 2014).

Neat PCL's mechanical characteristics, biodegradability, and biocompatibility are insufficient for some tissue engineering applications, such as bone tissue engineering (Abedalwafa et al., 2012). Despite these disadvantages, a useful property of PCL is it

can easily form copolymers with different polymers with its easily formable structure, thus maintaining its advantage over many other synthetic polymers (Woodruff and Hutmacher, 2010). PCL; thanks to its easy-to-mix structure, it was mixed with many natural and synthetic materials. Good physical properties and enhanced cellular response for PCL-chitosan composite hydrogels and enhanced cell adhesion and extracellular matrix interactions for PCL-alginate fibers were reported (Zhong et al., 2011; Hu et al., 2019). Increased biocompatibility and good cell adhesion and proliferation for fibroblast cells were demonstrated PCL and PLGA blends (Nguyen and Lee, 2010). For PCL and PLA blend nanofibers high mechanical properties and increased cell proliferation and osteogenic differentiation were revealed (Yao et al., 2017)

1.2.3 Scaffolds in tissue engineering

In tissue engineering, scaffold acts as a backbone for cells while in the process of reconstruction, replacement and repairing of the damaged tissue and organs (Figure 1.3) (Langer and Vacanti, 1993). It is critical to create a suitable scaffold to cells. An engineered scaffold cannot always exhibit the exact necessities of the target tissue but there are several features that are considered as crucial for a good tissue construction (Asadian et al., 2020): First of all, the material should be biocompatible to eliminate host immune response problem (Ma and Langer, 1999). It should have proper chemical characteristics to enhance cell attachment, proliferation and differentiation (Mandal and Kundu, 2009a; 2009b). A scaffolds biodegradation rate should match with the growth of the target tissue cells to provide a support as long as it needed. So controllability of the biodegradation is really important (Cima et al., 1991; Kweona et al., 2003). Porosity, pore sizes and interconnectivity between pores are also vital features of a scaffold. They should support cell infiltration and vascularization (Hutmacher 2001; Karageorgiou and Kaplan, 2005). Scaffolds should have mechanical strength enough to maintain the structure during the whole repairing and replacement process of the tissue after transplantation (Karande et al., 2014; Kim et al., 2000; Lee et al., 2001). They should be able to replicate the structure and biological function of the extracellular matrix to encourage cellular processes and deliver bimolecular signals to the cells (Ma, 2008; Li and Shi, 2007; Ito et al., 2003).

The necessity of combining all of these aspects defines the current challenges in the development of a scaffold for TE applications (Mader et al., 2018).

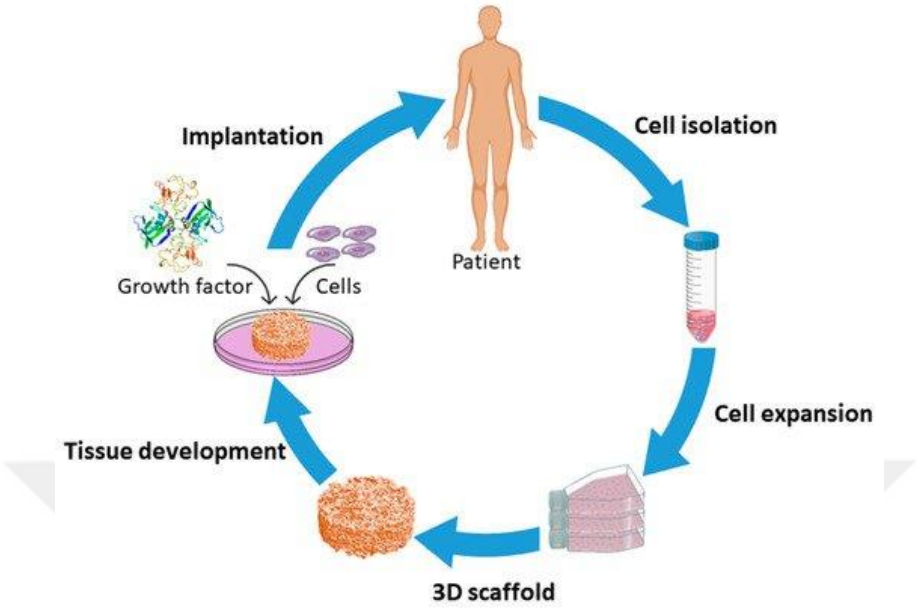

Figure 1.3 : Schematic of the scaffold-based tissue engineering approach, (Asadian et al., 2020).

1.2.4 Scaffold fabrication techniques

Over the years, a variety of methods for producing biodegradable polymers and fabricating various types of scaffolds have been presented. The final product qualities should fit the demands of specific tissues to be regenerated, and the assessment of scaffold construction processes should consider both possible advantages and downsides of each methodology. Solvent casting, particulate leaching, melt molding, gas foaming and freeze-drying are some of the most commonly used conventional techniques. There are also some advanced techniques to produce TE scaffold from both natural and synthetic polymers, such as electrospinning, stereolithography, selective laser sintering, fused deposition modelling, 3D printing, and 3D bioprinting (Reddy et al., 2021).

1.2.4.1 Solvent casting-particulate leaching

One of the most commonly used method to produc scaffold is the solvent casting/particulate leaching method (Abedalwafa et al., 2012). In this technique, scaffolds are made by pouring a polymer solution, which has been solved in the

appropriate solvent, into the mold, along with a suitable porogen. Then solvent and porogen should be removed from the polymer (Figure 1.4) (Prasad et al., 2017).

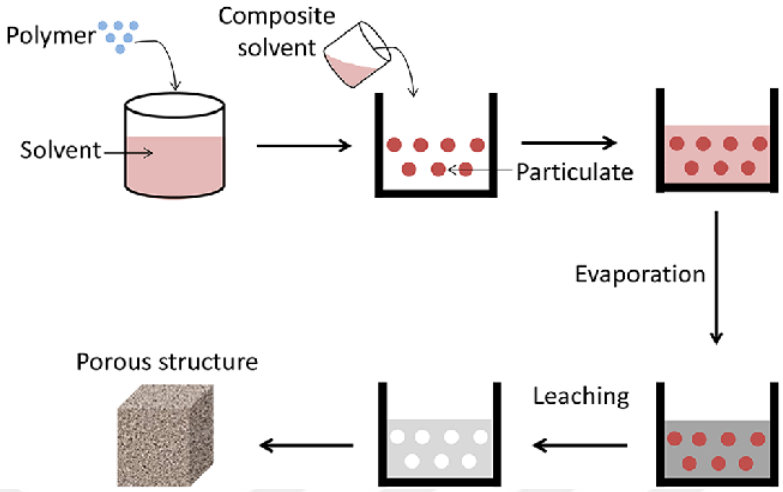

Figure 1.4 : Schematic of the solvent casting-particulate leaching (Kačarević et al., 2019).

Some of the advantages of this technique are; porosity, pore size and crystallinity can be easily controlled, created pores can form interconnections, and it is a simple and reproducible technique. However, it has drawbacks such as residual solvent and porogen material issues, as well as a relatively longer processing time (Sola et al., 2019).

1.2.4.2 Freeze-Drying

In tissue engineering, the freezing method is widely used to build porous structures. The solvent crystals build until the sample is entirely frozen during the freezing process, but the dissolved polymers are not integrated into the frozen solvent. As a result, a porous structure remains after the solvent is evaporated by executing the freeze-drying process (Figure 1.5). The pore structure of the material is affected by a variety of freezing conditions, including freezing temperature, solvent concentration, and solvent type. Keeping the freezing temperature too low (for example, -196 ^{o}C) induces quick production, thus forms small structured ice crystals. Keeping the temperature reasonably high (for example, -20 ^{o}C) allows the formation of huge crystal structures by decelerating the process. It's also crucial to develop a network of interconnecting channels and pores (Qian and Zhang, 2011; Zhang and Cooper, 2007; Gutierrez et al., 2008).

This process has several advantages, including the elimination of separate leaching and the production of highly porous materials. However, porosity is frequently inconsistent, it takes a long time to process, and it is an energy-consuming and costly approach (Fereshteh, 2018).

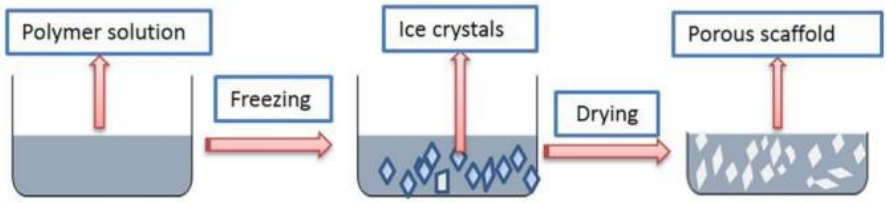

Figure 1.5 : Schematic illustration of freeze drying technique (Ceylan and Bölgen, 2016).

1.3 Bone Tissue Engineering

Half of the chronic diseases seen in people over 50 are bone and related diseases. Therefore, such diseases remain as major clinical problem (Brinker and O'Connor, 2004). Even though human bones are capable of healing themselves in some level, large bone injuries caused by aging, accidents, fracture nonunion, bone tumors, and so on, are beyond their healing capacity and can have a significant impact on a person's health and quality of life (Laurencin et al., 2006; Wang et al., 2016b). Even only bone fractures put a serious burden on the European and American economies €17 billion and $20 billion each year, respectively. In the United States, over 8 million bone injuries occur each year, with around 5% to 10% of these fractures caused by delayed healing or nonunion. Furthermore, a dramatic increase is expected on the number of hip fractures till the 2050, highlighting the significance of improved information of skeletal reparative techniques for bone healing (Kanczler et al., 2020). Bone tissue engineering is presented as a solution to these problems (Yu et al., 2015). Functional bone grafts may be obtained by gathering a biocompatible scaffold, suitable cells, and proper morphogenetic signaling molecules in a 3D complex system. These grafts can then be used to treat a variety of bone disorders and injuries (Laurencin et al., 1999; Langer and Vacanti, 1993).

It is not possible to create a proper scaffold without knowing the morphologies and components of the natural bone. The human skeleton is made up of over 206 distinct bones that change in sizes and shapes over the body; such as large bones (e.g.,

limbs), small bones (e.g., wrist, ankle), flat bones (e.g., sternum, skull), and irregular bones (e.g., pelvis, vertebrae). Bone tissues can be organized in either in a compact or a trabecular structure (Ackerman et al., 1976). Bones have complex hierarchical structures based on the length and width scale (Figure 1.6). This structures include; macrostructures, such as cancellous (trabecular) and cortical (compact) bone; microstructure; such as haversian canals and osteons; submicrostructure, such as lamellae; nanostructure, such as collagen fibers; and sub-nanostructure, such as mineral, collagen, organic proteins (Barth et al., 2011; Rho et al., 1998). The components are arranged and oriented in an irregular, yet optimal, manner in this hierarchically ordered system.

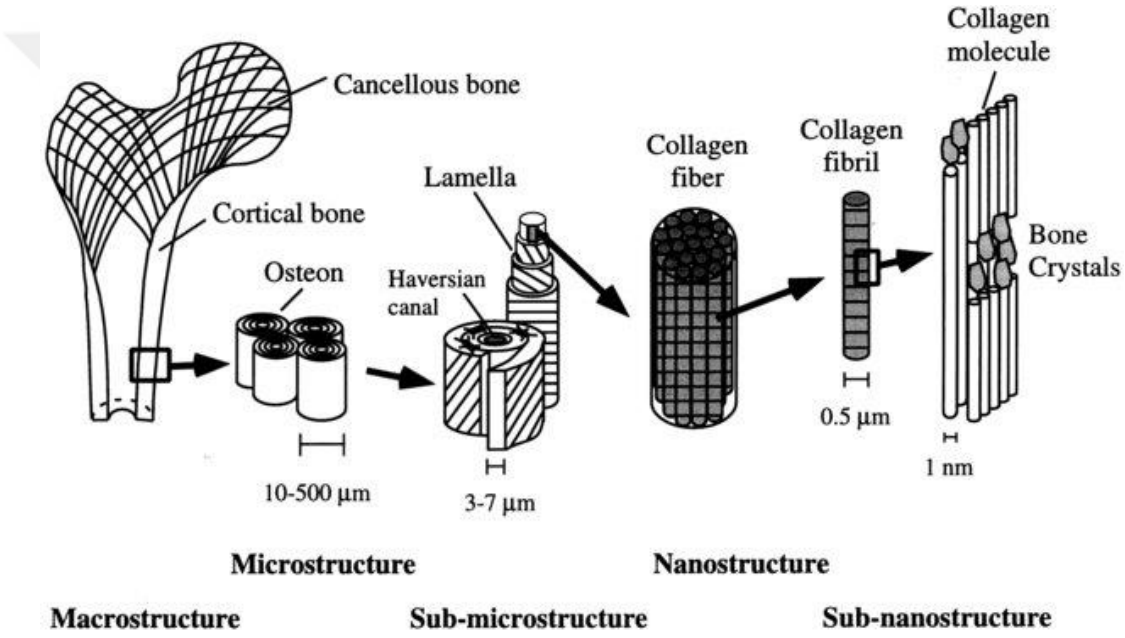

Figure 1.6 : Hierarchical structure of natural bone, (Rho et al., 1998).

It is not possible to select a scaffold material without knowing and understanding the natural bone components. Under dry conditions, collagen and HAp together make for ~95% of natural bone (Ma et al., 2018). Furthermore, more than 200 different noncollagenous matrix proteins contribute to the quantity of signals in the near extracellular environment (Stevens, 2008). The nanocomposite structure is critical for bone's compressive strength and fracture toughness (Stevens and George, 2005).

In this study, PCL and lignin polymers were used to manufacture a sponge-like porous scaffold to be used in bone tissue engineering.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

The list of chemicals that were used in this study and their suppliers were given in Appendix A.

2.1.2 Solutions

The solutions that were used in this study and their compositions were given in Appendix B, and Appendix C.

2.1.3 Laboratory equipment

The laboratory equipment that were used in this study can be found in Appendix D.

2.2 Methods

2.2.1. Fabrication of scaffolds

In this study, PCL with 80 kDa molecular weight and chemically-processing lignin were used. In order to construct sponge type scaffolds, the solvent casting and salt particulate leaching techniques were used as mentioned in Thadavirul et al. (2014). Differently, freeze-drying was applied for the evaporation of the solvents instead of ventilation hood. Chloroform and dimethylformamide (DMF) were used as PCL and lignin solvents, respectively. Lignin and PCL were dissolved in chloroform/DMF (2:1 v/v) together to reach five different final concentrations (Table 2.1). Additionally, a scaffold without the porogen (NaCl crystals) was prepared as control.

The solutions were mixed on magnetic stirrer at room temperature for 3 h. Next, NaCl particles were added as porogen and the mixtures were poured into petri dishes which were used as molds. The molds were placed in -80^{o}C overnight before the freeze-drying process (Alpha 1-2 / LD-plus, Christ, Germany). The ice was sublimed under a vacuum of 0.22 mbar at a temperature of -35^{o}C, maintained for 24 h.

Table 2.1 : Composition of PCL/Lignin sponges.

	PCL Concentration (mg/ml)	Lignin Concentration (mg/ml)	Salt Addition*
PCL200	200	0	+
PCL200/Lignin10	200	10	+
PCL200/Lignin15	200	15	+
PCL200/Lignin20	200	20	+
PCL100/Lignin20	100	20	+
PCL200/Lignin20/nS	200	20	-

* +: salt added; -: no salt added

After evaporation, to leach out salt particles and loosely attached lignin polymers, the sponges were dipped in distilled water for 12 h while water was renewed every 2 h. The scaffolds were air-dried for 24 h, before being placed in a vacuum desiccator until they were needed for further analysis.

2.2.2. Determination of morphological and chemical features

The pore sizes of the samples were examined with ImageJ software program on Scanning Electron Microscopy (SEM) micrographs. The sputter coating with platinum was applied on the surfaces of scaffolds to increase electrical conductivity before examination.

The porosity of scaffolds was measured via the liquid displacement method as mentioned in Arefpour, et al. (2020). Beakers containing 10 ml of ethanol (99.99%, Merck) were prepared and this initial volume was recorded as V1. The scaffolds were immersed in beakers containing ethanol. After 5 min, the increased ethanol volume was recorded as V_2. The leftover volume (V3) was measured after the ethanol-soaked scaffolds were removed. Equation 2.1 was used to calculate the porosity of the scaffolds:

$$\text{Porosity} = (V_1 - V_3) / (V_2 - V_3) \quad (2.1)$$

Fourier transform infrared spectroscopy (FTIR) was used for chemical analysis of PCL sponges with and without lignin. The FTIR spectrum in the 400-4000 cm^{-1} range was scanned.

Effect of lignin addition on the thermal characteristics of the scaffolds was investigated using differential scanning calorimetry (DSC). The thermograms of the samples were recorded by using SETARAM DSC 131 (Setaram, France). Each sample was weighed (~25-30 mg) and placed into 120 µl aluminum pans, and all the measurements were performed between 30 and 600 ^{o}C at a heating ramp rate 10 ^{o}C/min in the nitrogen atmosphere (Bektas et al., 2021).

2.2.3 Water uptake

The scaffolds were cut as triangle-shaped samples with 15 mm in edges and 3 mm in height. In their dry form, each sample was weighed and recorded as W_d. The water uptake properties of the scaffolds were studied by immersing these samples in 10 mL 1X phosphate-buffered saline (PBS; pH 7.4) at 37 °C and 150 rpm shaking speed. Specimens were taken from the solution, weighed (W_w), and reintroduced to the solution. This process was repeated at predefined intervals until equilibrium was established and water uptake was determined using Equation 2.2.

$$\%\text{Water Uptake} = [(W_w - W_d)/W_d] \times 100 \quad (2.2)$$

2.2.4. Enzymatic degradation

Each sample (dimensions around 5x5 mm) with a known dry weight was sterilized with 70% ethanol prior to the degradation test. This also served as a pre-wetting treatment, allowing enzyme solution to permeate into all pores of the sponges (Yoshioka et al., 2010). After washing with sterile PBS, the sponges were immersed in 5 ml of a PBS (pH 7.4) containing 0.1% (w/v) sodium azide to avoid microbial contamination, and in the presence of 0.5 mg (0.1 mg/ml) lipase from *Pseudomonas cepacia* (EC 3.1.1.3, 40 units/mg) to observe the effect of enzymatic degradation. Furthermore, enzyme-free control samples were prepared to observe the presence of hydrolytic degradation. The process was carried out at 37^{o}C with gentle vertical shaking. To maintain enzymatic activity, the buffered enzyme solution was changed every 24 hours. At specified time intervals, replicates were removed from the solution, rinsed with distilled water, and dried under vacuum at room temperature till

constant weight. The degradation rate was determined by the weight loss which is defined as:

$$\%\text{Weight Loss} = [(W_0 - W_d)/W_0] \times 100 \quad (2.3)$$

where W_0 is the initial weight and W_d the weight after enzymatic degradation treatment.

2.2.5. Biomineralization

For the observation of HAp formation, each sample was cut in triangle shapes with 10 mm in edges and 3 mm in height. Each piece was soaked in 25 ml 3X modified simulated body fluid (m-SBF, pH 7.4) and incubated at 37 °C in a shaking incubator for a predetermined time intervals (1, 3, 7 days). To provide appropriate ion concentrations for mineral development, the 3X m-SBF solution was changed every other day. Before characterization, the samples were washed with deionized water and air-dried. Eventually, SEM imaging was used to observe mineralization. In addition, Energy-Dispersive X-ray (EDX) analysis was performed to identify the elements in each sponge, especially for the determination of Ca/P ratios of the mineralized particles.

2.2.6. hFOB cell culture and cell proliferation assay

Cell culturing of the human fetal osteoblast cells (hFOB; ATCC CRL-11372) was carried out as described in Prabhakaran et al. (2009). hFOB were cultured in DMEM medium (1:1) containing 10% FBS and 1% penicillin/streptomycin (v/v; 1:1) and maintained in 5% CO_2 atmosphere at 37 ^oC for 3 days.

Sterilization of the scaffolds was carried out by adapting the method mentioned in Ghasemi-Mobarakeh et al. (2008). Before cell cultivation, first the scaffold constructs were sterilized under UV for 20 minutes, and then incubated in 70% ethanol for 2 hours. After washing 3 times in sterile PBS, they were incubated in fresh PBS for 2 hours. Finally, they were soaked in culture medium overnight.

Prior to the cell seeding on scaffolds, adherent cells were removed from the plate surface (at > 90% confluency after 3 days of incubation) as described in Shimizu et al. (2006). First, the medium was removed and plates were washed with PBS to remove unattached and dead cells, then trypsin/EDTA (0.25% / 0.02% in PBS) was

added on the cells. After 5 minutes of incubation at 37°C, fresh medium was added to stop trypsin activity. The removed cells were collected by centrifugation at 1000 rpm for 5 min and suspended in fresh medium before cell counting. Cell number was determined on Thoma cell counting chamber with trypan blue dying and diluted as much as needed before seeding (Shimizu et al., 2006).

Sterile PCL/Lignin and PCL scaffolds were placed in 24-well plates and hFOB cells were seeded at a number of $10x10^3$ per scaffold. After plates were incubated at 37 ^oC for half an hour to allow cell attachment, the wells were filled with fresh medium. Culture medium was changed after every 3 days.

Cell proliferation on scaffolds was monitored after 1, 3, and 7 days by Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc.) as described in manufacturer's manual. The kit includes a highly soluble form of tetrazolium salt which can be reduced in living cells by dehydrogenase process producing yellow-colored formazan dye. This dye is completely soluble in culture medium and color intensity produced by cell activity can be detected at 450 nm using a spectrophotometer. It is accepted that the amount of produced product is directly related to the number of living cells.

All of the cell proliferation assay process was carried out under dark conditions to minimize the effect on the light-sensitive CCK-8 solution. Scaffolds were transferred to new wells after their incubation times. CCK-8 solution was mixed with DMEM medium in 1:10 ratio and added on the scaffolds till it covers whole structure and incubated at 37^oC for 3 hours. The scaffolds were then washed with 1% SDS to ensure the removal of colored CCK-8 solution product from the scaffolds and cells, as suggested in Phipps et al. (2011). Absorbance of the solutions was measured using a microplate reader at 450 nm. Calculations were made by considering dilution factors.

3. RESULTS AND DISCUSSION

3.1 Characterization of Scaffolds

3.1.1 Morphology of scaffolds

Round-shaped sponge scaffolds were prepared as described in Section 2.2.1. PCL100/Lignin20 samples were obtained with very low mechanical properties, i.e. too fragile to be used in the studies (Figure 3.1). PCL200/Lignin20/nS, on the other hand, strongly adhered to the glass surface and cannot be removed without damaging the structure. Due to the use of organic solvents, a different mold or interface between mold and polymer solution could not be used to facilitate the removal of the sample from the mold (Figure 3.1). For these reasons, these samples were not used in further experiments.

Figure 3.1 : PCL100/Lignin20 (Left) and PCL200/Lignin20/nS (Right) samples.

Rests of the constructed scaffolds were measured as 1.2 cm in diameter and 0.8 mm in height. Although, after the freeze-drying step scaffolds appeared more brownish with the increase of lignin concentration, the colors of the final structures appeared to be the same with the removal of loosely attached lignin in the salt leaching stage (Figure 3.2; A, B, C, D). Overall, all PCL/Lignin blends appeared slightly more brownish than pure PCL scaffolds even after the salt leaching step (Figure 3.2; D and E).

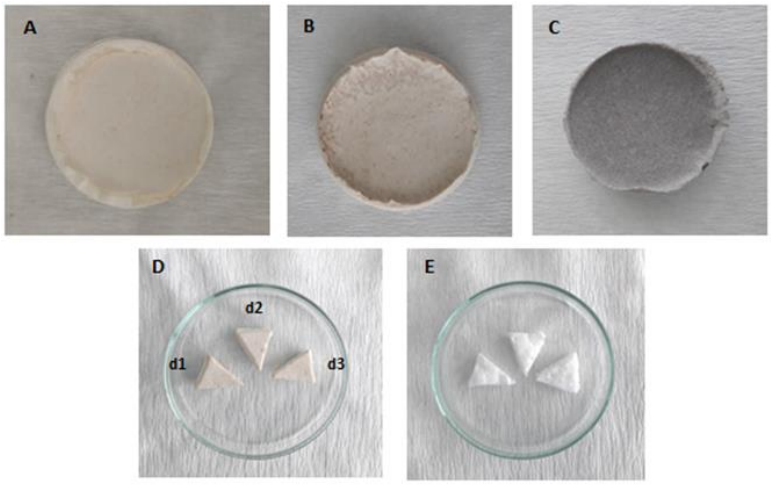

Figure 3.2 : Sponge constructs before salt leaching step: A) PCL200/Lignin10, B) PCL200/Lignin15, C) PCL200/Lignin20; and after salt leaching step: D) d1: PCL200/Lignin10, d2: PCL200/Lignin15, d3: PCL200/Lignin20; E) PCL200.

FTIR and Water Uptake experiments, which are discussed later in Section 3.1.2.1 and Section 3.2, respectively, were performed before pore size and porosity experiments. As discussed in these sections, different PCL/Lignin ratios resulted in similar behaviors only a single blend sample, PCL200/Lignin10, was chosen for further studies.

Even though, mechanical properties of the scaffolds were not studied in this thesis, based on preliminary visual and manual analysis, PCL200/Lignin10 scaffolds were more flexible than PCL200 constructs (Figure 3.3; A3 and B3).

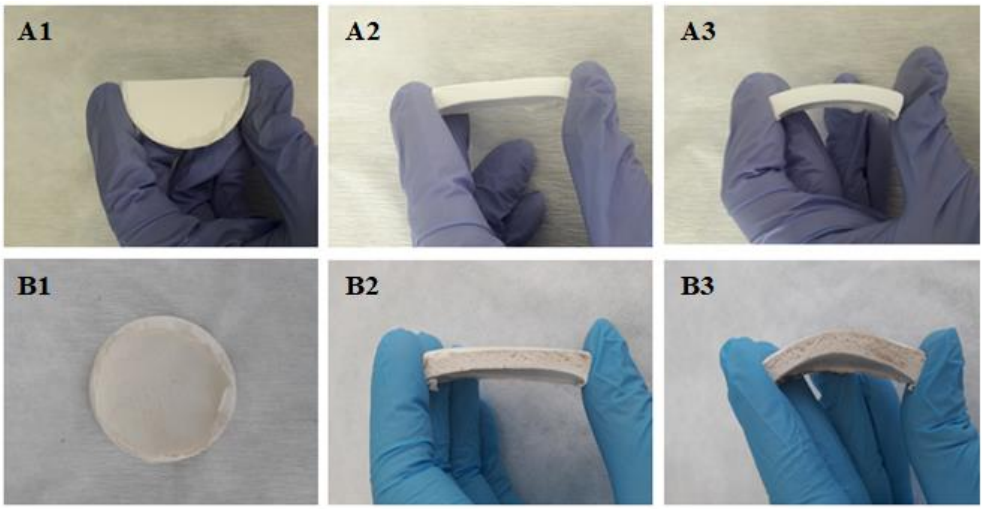

Figure 3.3 : Scaffolds that produced with solvent casting/particulate leaching and freeze-drying methods; A1-3) PCL200; B1-3) PCL200/Lignin10.

For this study, the porosity of the scaffolds was determined as 73% and 76% for PCL200 and PCL200/Lignin10, respectively by liquid displacement analysis. The

pore size analysis, on the other hand, was performed on the 2-dimensional surface images obtained from SEM using ImageJ software (Figure 3.4). In order to exclude pores that are too small to be measured by the program, only the pores with surface areas in the range of 20 – 600 μm were scanned. For PCL200 scaffold, 31% and 58% of the pores were in the range of 100-300 μm and 300-600 μm, respectively. For PCL200/Lignin10, 30% and 43% of the pores were in the range of 100-300 μm and 300-600 μm, respectively. It should be noted that pores smaller than 20 μm also exist in high quantities but the quantification cannot be done via image analysis.

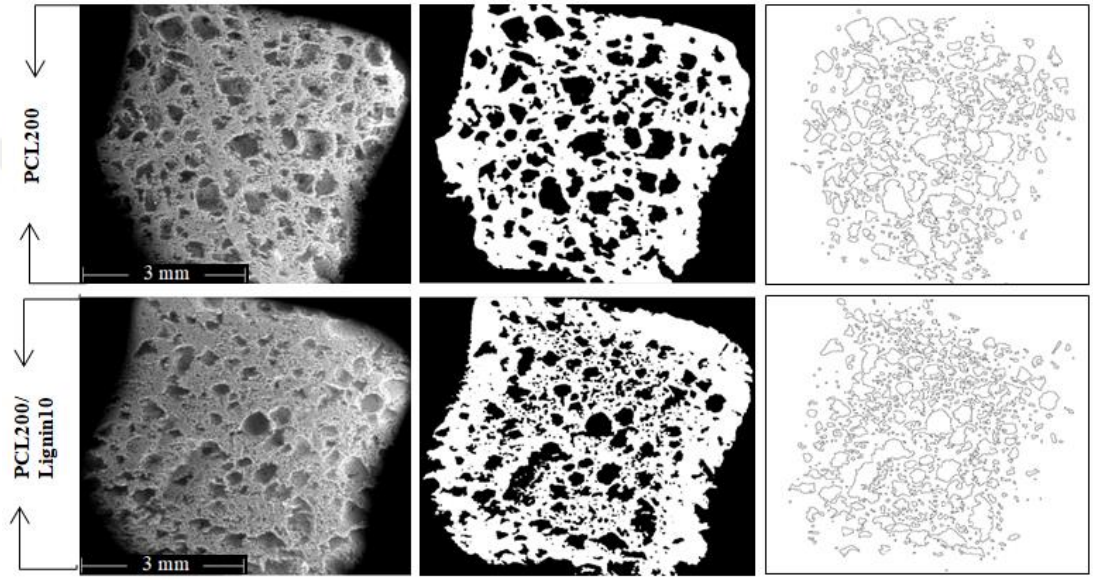

Figure 3.4 : Pore size analysis with ImageJ software on 50X SEM Images.

Scaffold porosity plays a significant role in TE. Not only pore quantity, size and form, but also interconnectivity, and orientation may affect the performance. The usage of porous sponge-structured scaffolds is common to establish 3-D cell cultures for the formation of functional living tissues and organs. Porosity generally induce proliferation and migration of cells, provides an environment for the transmission of nutrients to the structures near the scaffolds. Porous structure of the scaffolds helps cell growth, uniform distribution of cells, and vascularization (Prasadh and Wong, 2018; Yadav et al., 2021; Polo—Corrales et al., 2014). It was also shown that porosity promotes biomineralization which is an essential process for bone tissue engineering. Barrere et al. (2003) reported in their study with porous tantalum that mineralization took place intensively in the porous interior of the structure, but almost no mineralization was found on the flat upper surface; Kuboki et al. (1998) examined while there was no formation on the smooth solid surface of

hydroxyapatite particles, osteogenesis was triggered in the porous structure. The optimum porosity and pore size for bone tissue engineering scaffolds have long been controversial due to different results obtained in different articles (Murphy et al., 2010). In general, the average porosity of tissue engineering scaffolds should be between 60 – 90 % to favor cell growth, but scaffolds with porosity higher than 80% are not recommended for load-bearing bone tissue applications (Chong et al, 2007; Abbasi et al., 2020). Pore sizes also highly important for scaffold performance. Even though large porous (100-600 μm) scaffolds allow better integration with the host bone tissue, bone ingrowth, vascularization and bone distribution, smaller pore sizes are also necessary for different purposes. While pores that are smaller than 1 μm allows protein interactions and enhance bioactivity, pores between 1-20 μm are important for cell developments and cell orientation (Abbasi et al. 2020; S´anchez-Salcedo et al. 2008).

In the light of this information, it can be said that the both of the scaffold structures were in ideal range in terms of porosity and pore sizes.

3.1.2 Chemical structure of scaffolds

3.1.2.1 Fourier-transform infrared spectroscopy (FTIR)

The sponges were characterized using FTIR to identify the effect of lignin presence in surface chemistry (Figure 3.5) by determining specific chemical bond peaks. Typical peaks for PCL were identified for all sponges; The broad peak at 3442 cm^{-1} was considered as the terminal hydroxyl group of PCL (Sharifi et al., 2011). Carbonyl (C=O) stretching vibration was observed at around 1722 cm^{-1} and, the peaks at 2867 cm^{-1} and 2944 cm^{-1} corresponds to C-H groups (Safaeijavan et al., 2014). Asymmetric and symmetric C-O-C streching vibrations gave peaks around 1238 cm^{-1}, 1167 cm^{-1} and 1107 cm^{-1} (Ghorbani et al., 2020; Sharifi et al., 2011). No new peak appeared that identify lignin in any blend of PCL/Lignin, but the reduction of the intensity of –OH groups at 3442 cm^{-1} may indicate new bond formation between these two polymers as mentioned by Sen et al. (2015) and Wang et al. (2017). In the blend sponges, no changes in the peaks were observed with increasing lignin concentrations.

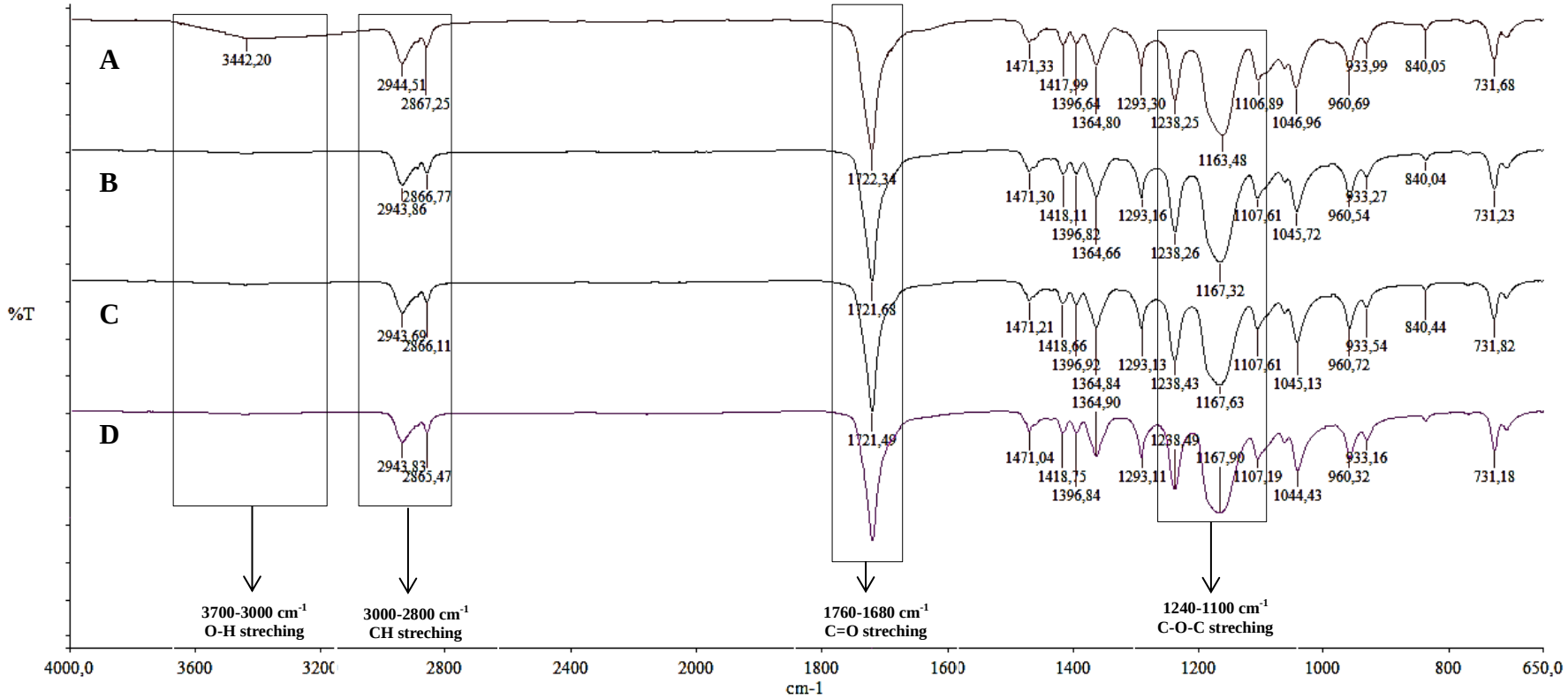

Figure 3.5 : Comperative FTIR spectra of sponge scaffolds: A) PCL200; B) PCL200/Lignin10; C) PCL200/Lignin15; D) PCL200/Lignin20.

3.1.2.2 Differential scanning calorimetry (DSC)

The thermal transition properties of polymeric materials, such as glass transition, crystallization and melting temperatures, can be examined by differential scanning calorimetry (DSC) (Gill et al., 2010). Glass transition and the melting temperatures of PCL are reported around -62°C and 64°C, respectively (Gunatillake and Adhikari, 2003). Because lignin polymer has a complex structure, it has been a challenge to measure its glass transmission temperature. In the literature, the glass transmission temperatures for lignin vary from 90 to 180°C (Li and McDonald, 2014). Its melting temperature is around 170°C (Patel, and Parsania, 2018).

In this study, the melting temperatures of PCL200/Lignin10 sponges were studied using DSC (Figure 3.6). In general, lignin blending increased the melting temperature in comparison to the pure PCL. While melting temperature of PCL200 sponge was determined as 66.3 ^{o}C, PCL200/Lignin10 scaffold showed 5.4% increase with a 69.9 ^{o}C melting temperature. This increase might be the result of a new bond between PCL and lignin, which is also observed in FTIR results. These DSC results confirm the coexistence of lignin with PCL within the sponge structure.

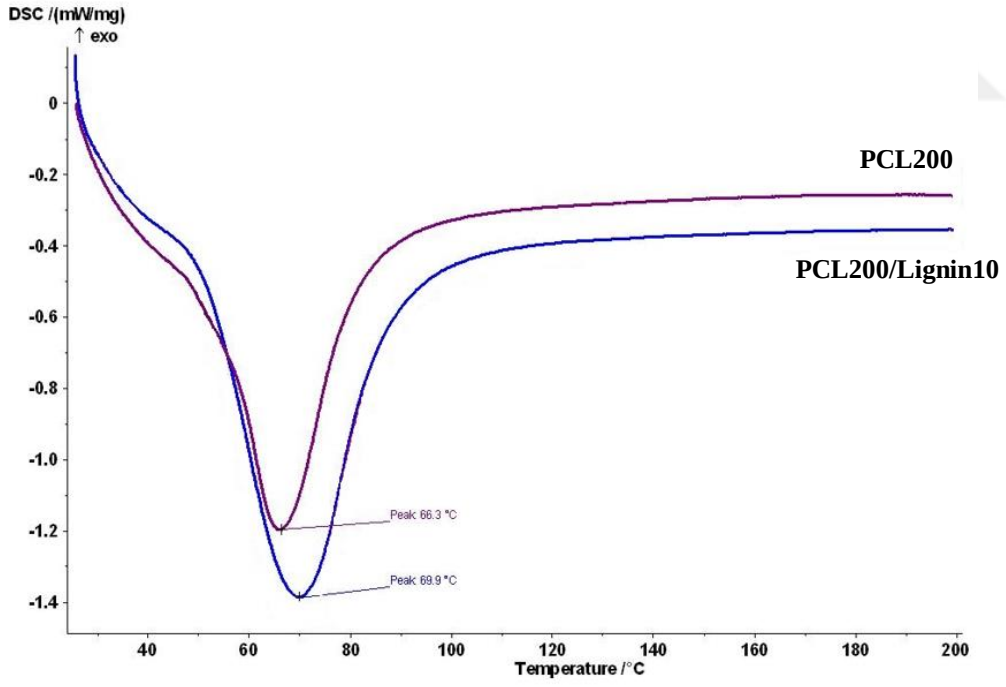

Figure 3.6 : DSC results for melting temperatures of PCL200 and PCL200/Lignin10 sponges.

3.2 Water Uptake

Water uptake capacity of the tissue engineering scaffolds has great importance due to its effects on enzymatic degradation rate, biomineralization capacity, cell viability etc. (Ghorbani et al., 2020). Water uptake can occur in two different ways; “absorbed water” which depends on hydrophilicity of the material, and water that “drawn in” through pores or capillaries of the material. Moreover, it is considered that the water uptake capacity is related to the porosity, since porous material can uptake and store more water in its pores than the nonporous material, which can only store a limited amount of water (Sultana and Khan, 2013).

Water uptake of sponges was measured from the increase in their wet weight (Figure 3.7). The overall water uptake of all PCL/Lignin blends were considerably higher than the pure PCL sponge. PCL is a hydrophobic polymer (Mondal et al., 2016) and even though lignin is a complex polymer with hydrophobic rings, it has hydrophilic groups that attached to those rings (Fatehi, 2017). The alkaline lignin used in this study is prepared through the chemical modifications such as partial desulfonation, oxidation, hydrolysis and demethylation as as mentioned in company website (TCI, Product No. L0082). Even though the effect of these modifications to hydrophilic properties of lignin has not been reported, the significant increase in water uptake capacity of PCL/Lignin sponges compare to PCL, is an evidence for the increased hydrophilic characteristics of this scaffold. Increasing lignin concentration, however, did not cause a significant water uptake difference between the blend samples.

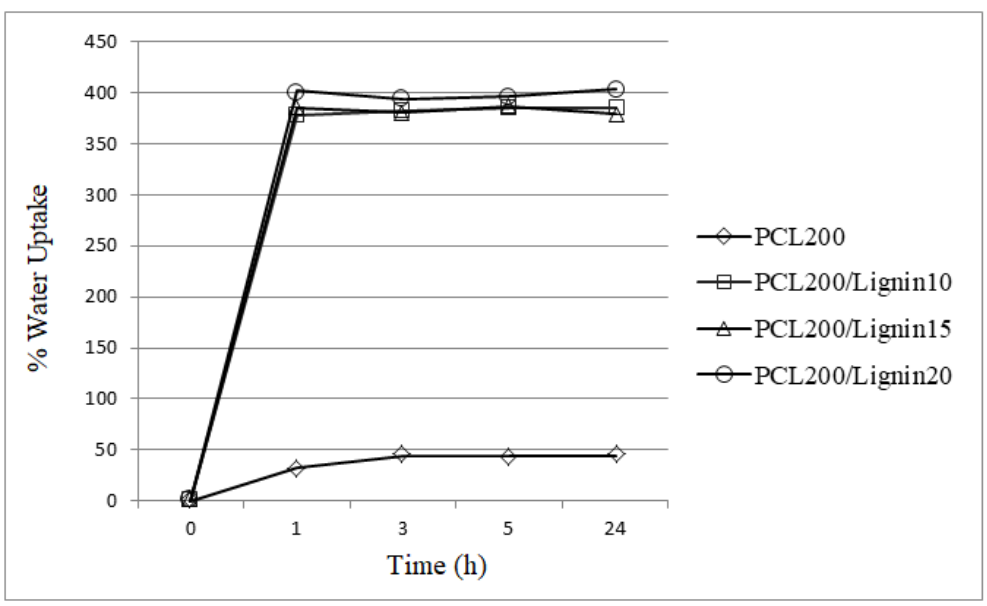

Figure 3.7 : Water uptake of the scaffolds.

When FTIR and water uptake experiments are evaluated together, it is thought that there is a limit on the amount of lignin that can be blended with this PCL concentration. It looks like all scaffolds have similar final lignin concentration after removing loosely attached lignin in the salt leaching stage. Consequently, subsequent experiments were continued with one type of blend sponge, namely PCL200/Lignin10.

3.3 Enzymatic Degradation

The most important role of the use of scaffold in vivo is to preserve its integrity and mechanical properties for sufficient time to allow the formation of new tissue, while also simultaneously degrading and being replaced by that tissue (Vert, 2005). For this reason, the enzymatic and hydrolytic degradation behaviors of a tissue engineering scaffolds has crucial importance.

In this study, enzymatic degradation of PCL200/Lignin10 sponges was studied in the presence of *Pseudomonas cepacia* lipase enzyme. As it can be seen in Figure 3.8 that while PCL200 sponge reached 100% degradation after 5th day, PCL200/Lignin10 scaffold maintained 55% of its initial weight after 7 days of incubation in the presence of the enzyme. Complex structure of lignin makes its enzymatic hydrolysis difficult and this behavior affected the degradation behavior of the scaffolds dramatically. For both samples, no mass loss, hence hydrolytic degradation, was observed in the enzyme-free solution at the end of the 7th day.

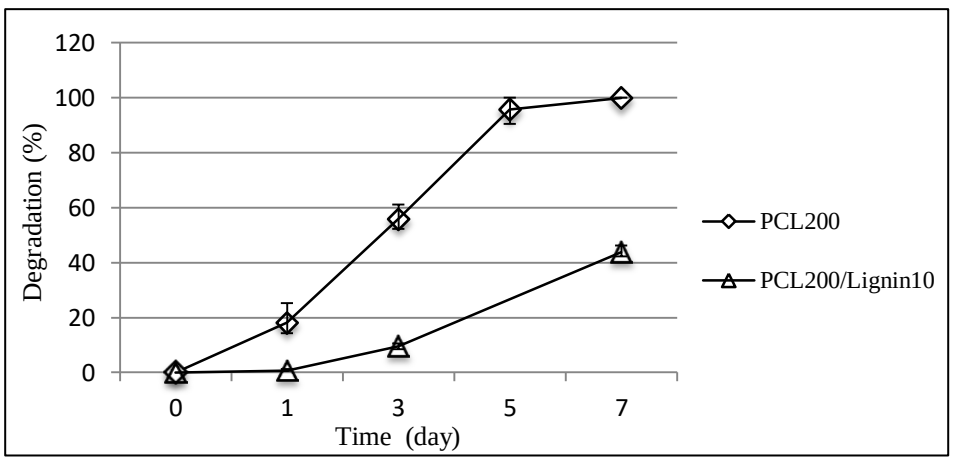

Figure 3.8 : Enzymatic Degradation behavior of PCL200 (diamond-shaped) and PCL200/Lignin10 (triangle-shaped) sponges.

Many studies have been carried out on enzymatic and non-enzymatic degradation of PCL. PCL's hydrophobicity and crystallinity affect its hydrolytic degradation

negatively and slow down the rate of its degradation to such an extent that it can last years (Castilla-Cortázar et al., 2012), which makes this polymer useful for long term applications. To improve biodegradability of PCL and tailor it for specific applications, its usage in a blend or as a copolymer has been proposed (Cha and Pitt, 1990; Buntner et al., 1998; Koleske et al., 1978). Tokiwa and Suzuki (1977) has been reported the first study on activity of lipases on synthetic polyesters and many other studies have shown *Pseudomonas* lipases are highly effective on PCL (Gan et al., 1997; Liu et al., 2000; Castilla-Cortázar et al., 2012). Since enzymatic degradation by hydrolysis mostly occurs on the surface of the scaffold, porous structures are more favorable for higher enzymatic degradation rates. Vidaurre et al. (2008) has reported enzymatic degradation behaviors of different forms of PCL scaffold with *Pseudomonas fluorescens* lipase. Highly porous PCL sponge degraded completely in 100 h, while PCL films, with low porosity, reached %100 degradation rate at around 260 h.

One of the functions of lignin in plants is to protect the organism against biological stresses by inhibiting enzymatic hydrolysis (Boerjan et al., 2003). So naturally its degradation has proven to be challenging as it is naturally resistant to enzymatic degradation (Liu et al., 2020) which might be one of the reasons of low degradation rate of PCL200/Lignin10 sponge compared to PCL200, in this study. Several studies can be found on the degradation behaviors of lignin in the nature by bacteria and fungi (Breen and Singleton, 1999; Bugg et al., 2011; Yiamsawas et al., 2014; Kumar and Chandra, 2020). Due to their slow degradability, lignin and other lignocellulosic containing industrial wastes are major pollutant for environment (Sadh et al., 2018). But it appears that there is a lack of study on biodegradability of lignin in the context of biomedical applications (Wang et al., 2016a), as its usage in this area is already limited as mentioned before.

In most studies, it has been observed that an enhancement in water uptake capacity also causes an increase in enzymatic degradation, since the surface area that can be affected by enzyme is also increased. In addition, natural polymers are mostly used as blends with synthetic polymers to increase both of these properties (Diba et al. 2011; Lei et al., 2012; Pawar and Srivastava, 2019). But in this study, even though lignin addition increased the water uptake capacity compare to the pure PCL scaffold, the enzymatic degradation decreased significantly. Similar results had been reported by Thuaksuban et al. (2011), in a study with PCL and chitosan blends. Their

results also revealed that more chitosan content increased water uptake of the scaffolds, and in contrast, decreased their degradation rate. One of the reasons that can explain this phenomenon suggested by this group is, a permanent bond between the hydroxyl group of chitosan and oxygen atoms of water, which is so called gelatinization, might have been occurred in the filaments of the scaffold and protect the structure against enzyme degradation.

PCL scaffolds were already favorable for replacement of hard and load-bearing tissues because of their low degradation rate (Dwivedi et al., 2019). PCL200/Lignin10 scaffold's longer degradation time might also make it popular for this kind of applications, especially for bone regenerations of elderly people, whom have slower cell regenerations and increased rates of delayed healing (Clark et al., 2017; Liu et al., 2018).

3.4 Biomineralization

One of the essential necessities for a biological scaffold for bone tissue engineering is production of a bone-like apatite on its surface to evaluate the bioactivity and the ability of inducing new bone tissue cells (Mao et al., 2018).

The mineralization of the scaffolds was achieved with 3x m-SBF as mentioned in Section 2.2.5. Three-times saturated m-SBF was used to accelerate the process. Before SEM and EDX analysis, the weight change of the samples, after incubation in 3X m-SBF, was observed (Figure 3.9). In general, more weight gain observed in the lignin added samples. This suggested that biomineralization may be more pronounced in this sample.

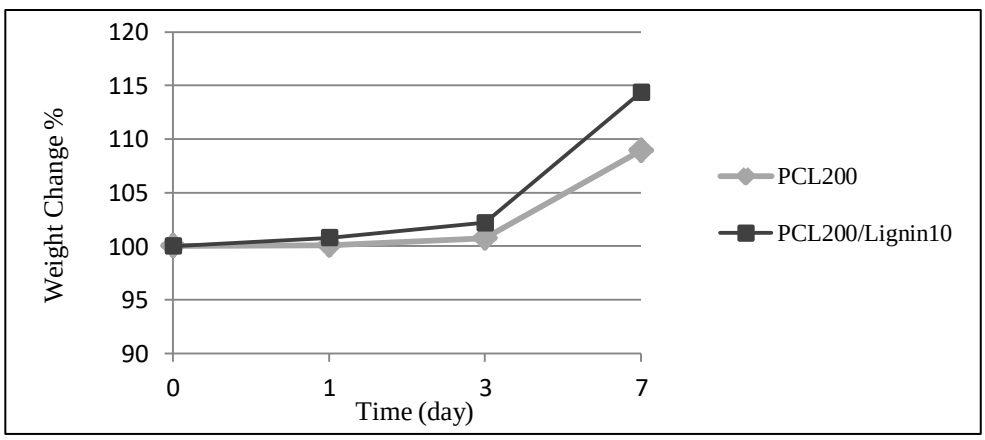

Figure 3.9 : Weight change of scaffolds in 3X m-SBF.

In order to investigate the HAp formations on the surface of scaffolds, SEM and EDX analyses were performed. The 1kx images for both scaffolds can be found in Figure 3.10 and Figure 3.11. Rest of the SEM micrographs with different magnifications can be found in Appendix E and Appendix F.

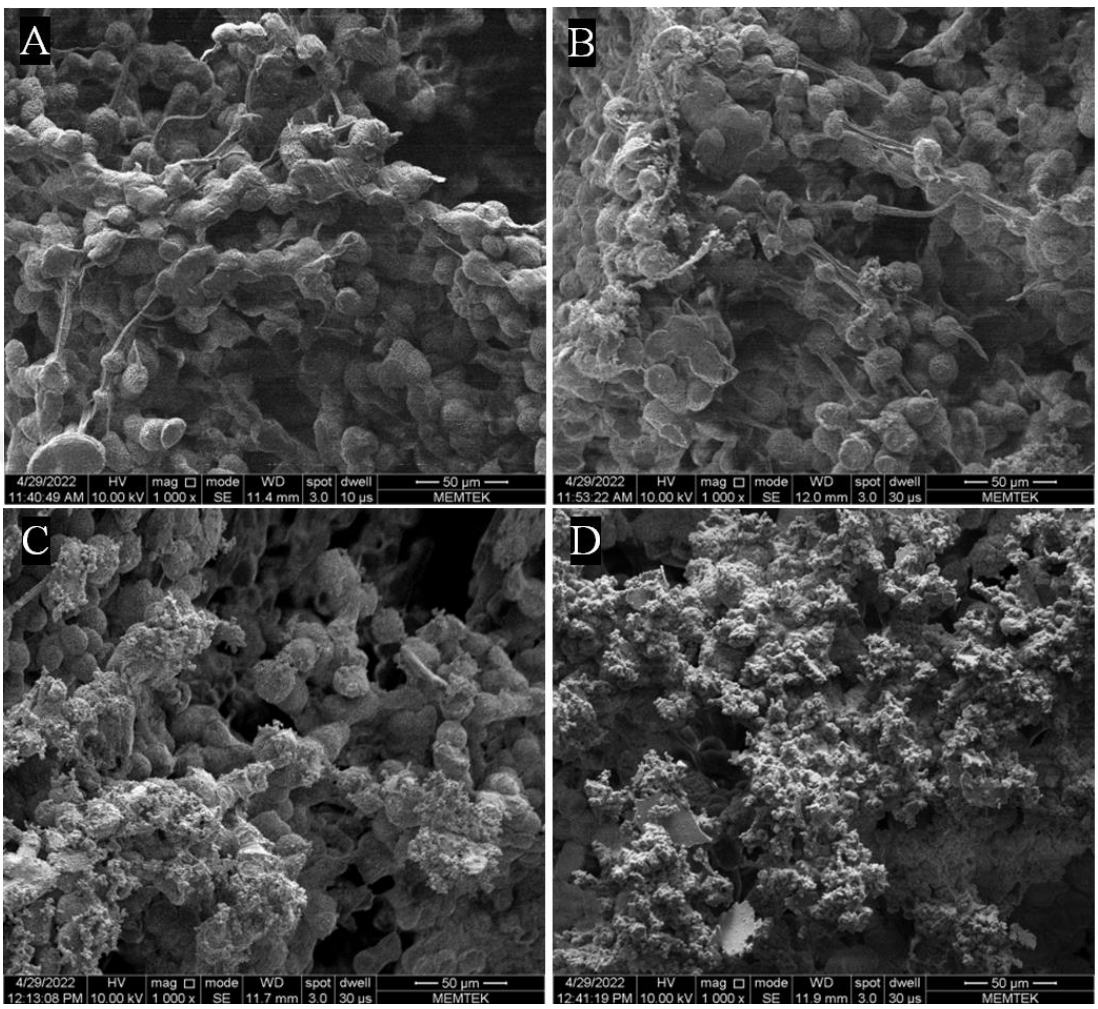

Figure 3.10 : SEM images of PCL200 before (A), and after incubation in 3X m-SBF; day 1 (B), 3 (C) and 7 (D).

An increase in the accumulation of HAp-like structures over time was observed in both samples. SEM micrographs revealed significant mineral accumulations for both scaffold types at the end of the 7th day. When the 7th day samples were compared, it was seen that the HAp-like structures were mostly deposited on top of each other in the PCL200 sample, while the aggregations were more evenly spread over the surface on the PCL200/Lignin10 sample.

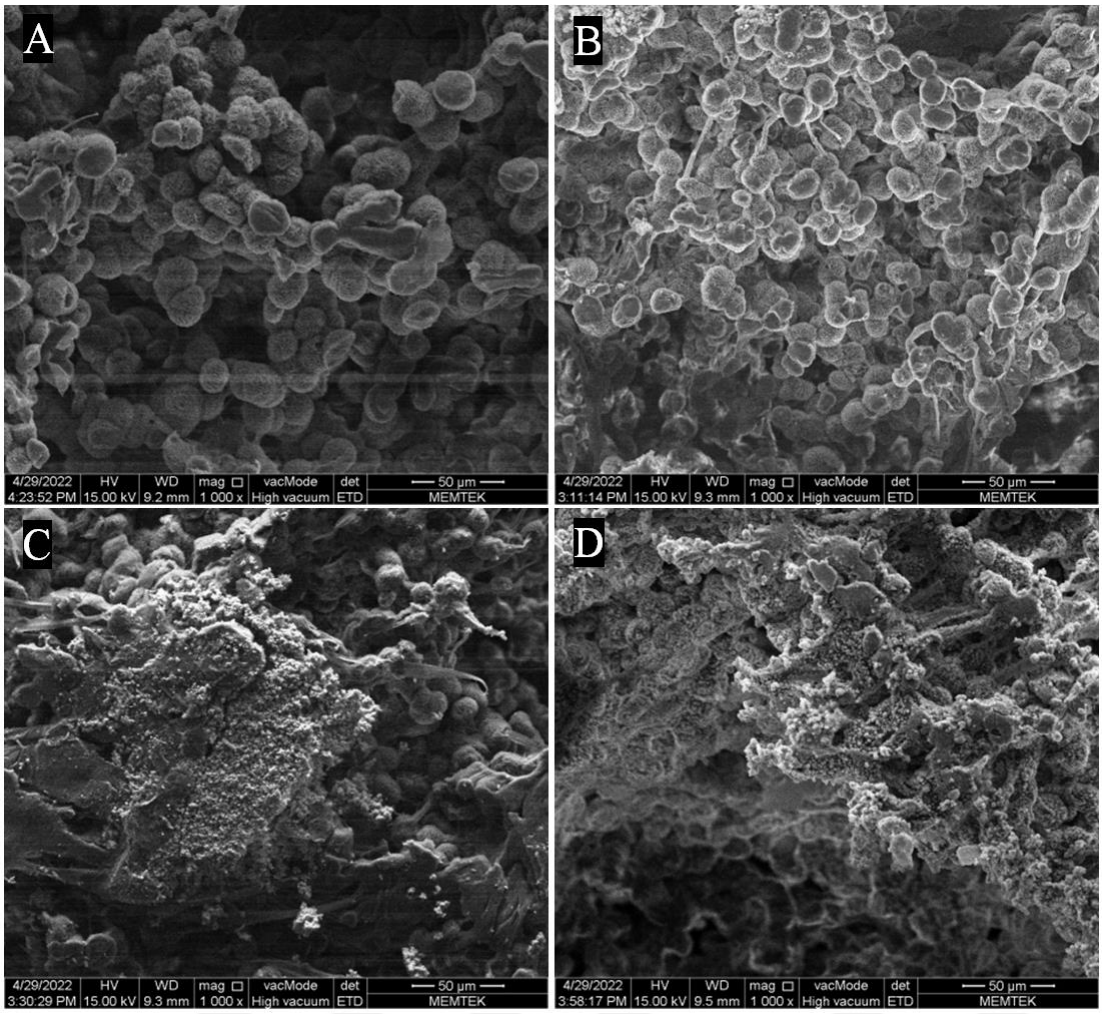

Figure 3.11 : SEM images of PCL200/Lignin10 before (A), and after incubation in 3X m-SBF; day 1 (B), 3 (C) and 7 (D).

For the PCL200 sample, after 7 days of incubation, even though an intense accumulation was observed on the scaffold surface, the inside of the pores as appeared to be smooth, similar to Day 0 sample. Lignin containing samples, on the other hand, had more mineral deposition within the pores. This can be originated from the hydrophobic nature of the PCL sample, causing reduced interaction of m-SBF with the inner surfaces of the pores. Considering the difference between the water uptake characteristics of the samples, m-SBF can be absorbed better by lignin added samples which encourage the deposition of HAp-like structures into the scaffold. The mass change analysis results of the structures strengthen this idea.

Ca/P ratios were calculated by EDX analysis at day 0, 1, 3 and 7, as 0, 0.33, 0.17, 1.50 for PCL200, and 0, 1.26, 1.32, 1.56 for PCL200/Lignin10 sample, respectively. It was observed that the Ca/P ratio of the accumulations on the PCL200/Lignin10

samples at all determined times was closer to the ideal hydroxyapatite Ca/P ratio which is 1.67.

3.5 Cell Proliferation Assay

Good model cell lines are vitally important for studies of human osteoblast function, proliferation, differentiation etc. For this reason, a lot of different osteoblast model systems obtained from primary cultures of human bone fragments and osteosarcoma derived cell lines from human bone tumors (Subramaniam et al., 2002). But these osteoblast cultures showed some limitations due to their slow and/or abnormal growth properties, heterogeneity of phenotype and differentiation, exhibition of different responses to hormones.

Because of these properties, their use for in-vitro biomimetic studies for normal osteoblast has become questionable (Clover and Gowen, 1994). That is why conditionally immortalizing human fetal osteoblasts, hFOB cell line, developed by Harris et al. (1995). These hFOB cells contain a mutation in the SV40 large T-antigen gene which makes them temperature-sensitive mutants. The mutation on this gene allows cells to be active and rapidly divide only at the permissive temperature. Their return to a non-immortalized state can be trigged by changing the incubation temperature of the cells to a restrictive temperature and the cells differentiation and indication the phenotype of mature osteoblasts can be obtained.

hFOB cell proliferations on the scaffolds were determined after 1, 3 and 7 days of cell seeding. Unattached cells were washed with fresh medium first and the scaffolds were transferred to new wells. Previous wells were also observed under optical microscope to determine cell detachment from scaffolds. No notable number of cells was found on the old medium, which indicates good cell adhesion for all scaffold types.

Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Inc.) was used to determine the effect of lignin presence on the proliferation of the hFOB cells. Cell proliferation continually increased with time for all time points and all scaffold types (Figure 3.12). The proliferation of hFOB was significantly higher on PCL200/Lignin10 scaffolds compared to PCL200. The proliferation on PCL200/Lignin10 sponge was found to be 212% and 50% higher after day 3 and 7, respectively. Based on this assay, hFOB cells proliferated faster on

PCL200/Lignin10 sponges than on PCL200 sponges, therefore PCL200/Lignin10 scaffold was considered to be more favorable for osteoblast cell proliferation.

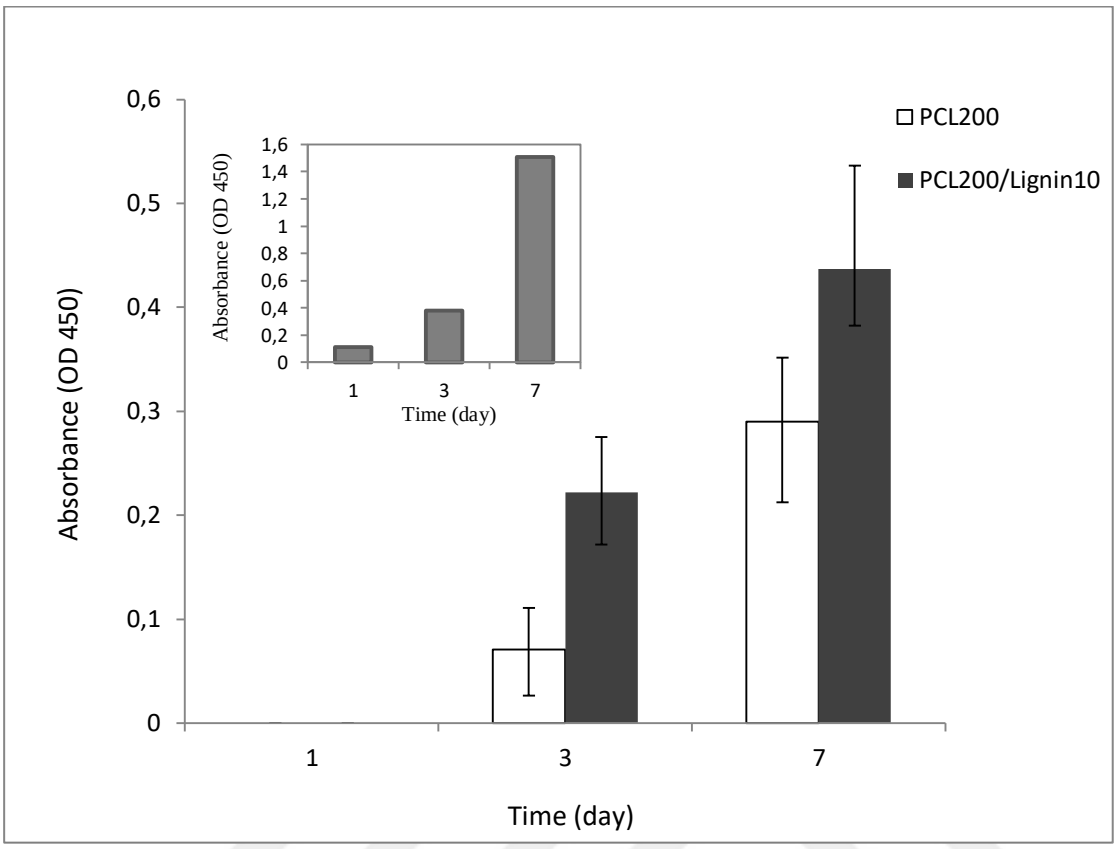

Figure 3.12 : The cell proliferation assay with hFOB cells on PCL200 and PCL200/Lignin10 sponges (the inset shows the growth control).

4. CONCLUSION

This study aims producing a bone tissue engineering scaffold with improved characteristics. In this regard, lignin was added to already known PCL polymer to promote cell attachment and mineralization. Two polymers were solved in appropriate solvents, mixed and poured in molds full of salt particles. After freezing the mixture, and lyophilization of the solvents, salt particles were removed by serial washings. Thus, porous scaffolds of PCL and lignin blends were obtained.

The lignin used in this study is a chemically-processing lignin with increased water solubility. For this reason, although samples with different concentrations of lignin were prepared, the salt leaching stage caused the loss some of the lignin and as far as it was observed, in each time, structures with similar amounts of final lignin concentration were obtained. After it was noticed that weakly bound lignin was removed in the salt leaching step and no difference was observed in terms of water uptake and FTIR analysis between the samples prepared with different lignin concentrations, the experiments were continued with a single PCL/lignin ratio, i.e. PCL200/Lignin10. The porosities of pure PCL and blend scaffolds were very similar, with 73% and 76% porosity, respectively. When the pores in the range of 20-600 μm were examined, it was observed that the lignin addition did not cause a significant difference in the percentage of the 100-300 μm sized pores. However, the percentage of macropores in the range of 300-600 μm was observed to be higher in the PCL200 sample than PCL200/Lignin. Also many, probably hundreds of, pores that are smaller than 20 μm were detected. A reduction in the peak that represent –OH bonds in FTIR analysis and a higher melting temperature for PCL200/Lignin10 sponges were recorded, both of which may indicate the existing of newly formed bonds between the polymers. The water uptake assay showed, even though existence of lignin increases the water uptake characteristics, the polymer may has acted as a shield against enzymatic degradation, since the weight loss for PCL200/Lignin10 sponges were much less than PCL200 sponges. No hydrolytic degradation was observed for both of the sponge structures after 7 days. Although biomineralization results showed significant amount of accumulation of HAp-like structures on both of the scaffolds at the end of 7 days incubation, PCL200/Lignin10 was observed with a

more covered surface than PCL200. Cell proliferation assay demonstrated living cell numbers were higher in PCL200/Lignin10 structures compared to PCL200, which serves as an evidence about this blend is more favorable for osteoblasts.

These results were promising enough to suggest that further investigation may be performed, like analyzing mechanical properties of the PCL200/Lignin10 scaffolds. Also further cell analysis can be performed, such as Alkaline Phosphatase Activity Assay to determine osteogenic differentiation on the scaffolds. The effects of different concentrations and different types and forms of lignin can also be investigated.

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APPENDICES

APPENDIX A: Chemicals and materials

APPENDIX B: Preparation of 1X phosphate buffered saline (PBS) buffer

APPENDIX C: Preparation of 3X modified-simulated body fluid (m-SBF)

APPENDIX D: Laboratory equipment

APPENDIX E: SEM micrographs of PCL200 after biomineralization for day 0, 1, 3, 7 at 50x, 200x, 500x magnifications

APPENDIX F: SEM micrographs of PCL200/Lignin10 after biomineralization for day 0, 1, 3, 7 at 50x, 200x, 500x magnifications

APPENDIX A

Table A.1 : List of chemicals, materials used in this thesis and their suppliers

Chemical/Material	Supplier
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, HEPES	Biomatik
Calcium chloride, $CaCl_2$	Sigma-Aldrich
Cell Counting Kit-8	Dojindo Mol. Tech.
Chloroform	Merck Millipore
Dimethylformamide	Merck Millipore
Di-potassium hydrogen phosphate trihydrate, $K_2HPO_4.3H_2O$	Sigma-Aldrich
Dulbecco's Modified Eagle Medium, DMEM (Cat.No. P04-04510)	Pan Biotech
Ethanol, C_2H_6O	Sigma-Aldrich
Fetal Bovine Serum	Sigma-Aldrich
Hydrochloride, HCl	Sigma-Aldrich
Lignin (Alkaline)	Tokyo Chemical Ind.
Lipase from *Pseudomonas cepacia*	Sigma-Aldrich
Magnesium chloride hexahydrate, $MgCl_2.6H_2O$	Sigma-Aldrich
Penicillin Streptomycin Solution	Sigma-Aldrich
Polycaprolactone (PCL, 80 kDa)	Sigma-Aldrich
Potassium chloride, KCl	Sigma-Aldrich
Potassium phosphate monobasic,KH_2PO_4	Sigma-Aldrich
Sodium azide, NaN_3	Merck Millipore
Sodium bicarbonate, $NaHCO_3$	Sigma-Aldrich
Sodium carbonate, Na_2CO_3	Sigma-Aldrich
Sodium chloride, NaCl	Merck Millipore
Sodium hydroxide, NaOH	Sigma-Aldrich
Sodium phosphate dibasic, Na_2HPO_4	Sigma-Aldrich
Sodium sulphate, Na_2SO_4	Sigma-Aldrich
Trypsin/EDTA	Pan Biotech
Trypan Blue	Sigma-Aldrich

APPENDIX B

1X PBS contains; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4. For this reason;

- 8 g of sodium chloride (NaCl)
- 0.2 g of potassium chloride (KCl)
- 1.44 g of sodium phosphate dibasic (Na_2HPO_4)
- 0.24 g of potassium phosphate monobasic (KH_2PO_4)

were dissolved in 800 ml distillated water. pH of the solution was arranged to 7.4 with hydrochloric acid. The total volume was adjusted to 1 liter. Sterilization of the solution performed with 0.22 μm filter.

APPENDIX C

Ultrapure water was used to prepare m-SBF. m-SBF solution must be prepared in a specific ion concentration. 1X of the solution should contain; 142 mM Na^+, 5 mM K^+, 1.5 mM Mg^{2+}, 2.5 mM Ca^{2+}, 103 mM Cl^-, 10 mM HCO_3^-, 1 mM HPO_4^{2-}, 0.5 mM SO_4^{2-}. For preparing m-SBF in 3X concentration the reagents listed below should be solved in 700 ml ultrapure water according to the order and each should be added after the previous one is completely dissolved. The HEPES was previously dissolved in 100 mL of aqueous 0.2 M NaOH.

- NaCl : 16.209 g
- $NaHCO_3$: 1.512 g
- Na_2CO_3 : 1.278 g
- KCl : 0.225 g
- $K_2HPO_4.3H_2O$: 0.690 g
- $MgCl_2.6H_2O$: 0.933 g
- HEPES : 53.676 g
- $CaCl_2$: 0.879 g
- Na_2SO_4 : 0.216 g
- NaOH (1 M) : 45 ml

The pH was adjusted to 7.4 at room temperature by titrating aqueous 1.0 M of NaOH into the fluid. The total volume of the fluid was adjusted to 1 liter by adding ultrapure water. The freshly prepared m-SBF solution was sterilized by using 0.22 μm pore size filters.

APPENDIX D

Table D.1: List of equipment used in this thesis and their suppliers

Equipment	Supplier
Beakers	ISOLAB
Fourier transform infrared spectroscopy, FTIR	Perkin Elmer
Freeze-dryer	Martin Christ
Freezer, -20 ^{o}C	Arçelik
Freezer, -80 ^{o}C	New Brunswick Scientific
Fume hood	Unbranded
Magnetic stirrer	Cole-Parmer
Mini Sputter Coater	SC7620 Polaron Ranger
Orbital Shaker	Thermo Fisher Scientific
pH metre	WTW inoLab
Pipettes	Eppendorf
Refrigerator, +4 ^{o}C	Vestel
Rocking platform shaker	Heidolph Instruments
Scale	Ohaus
Scanning electron microscope, SEM	JEOL JSM-5410
Syringe and needle	BD Medical
Tissue Culture Plates	TPP

APPENDIX E

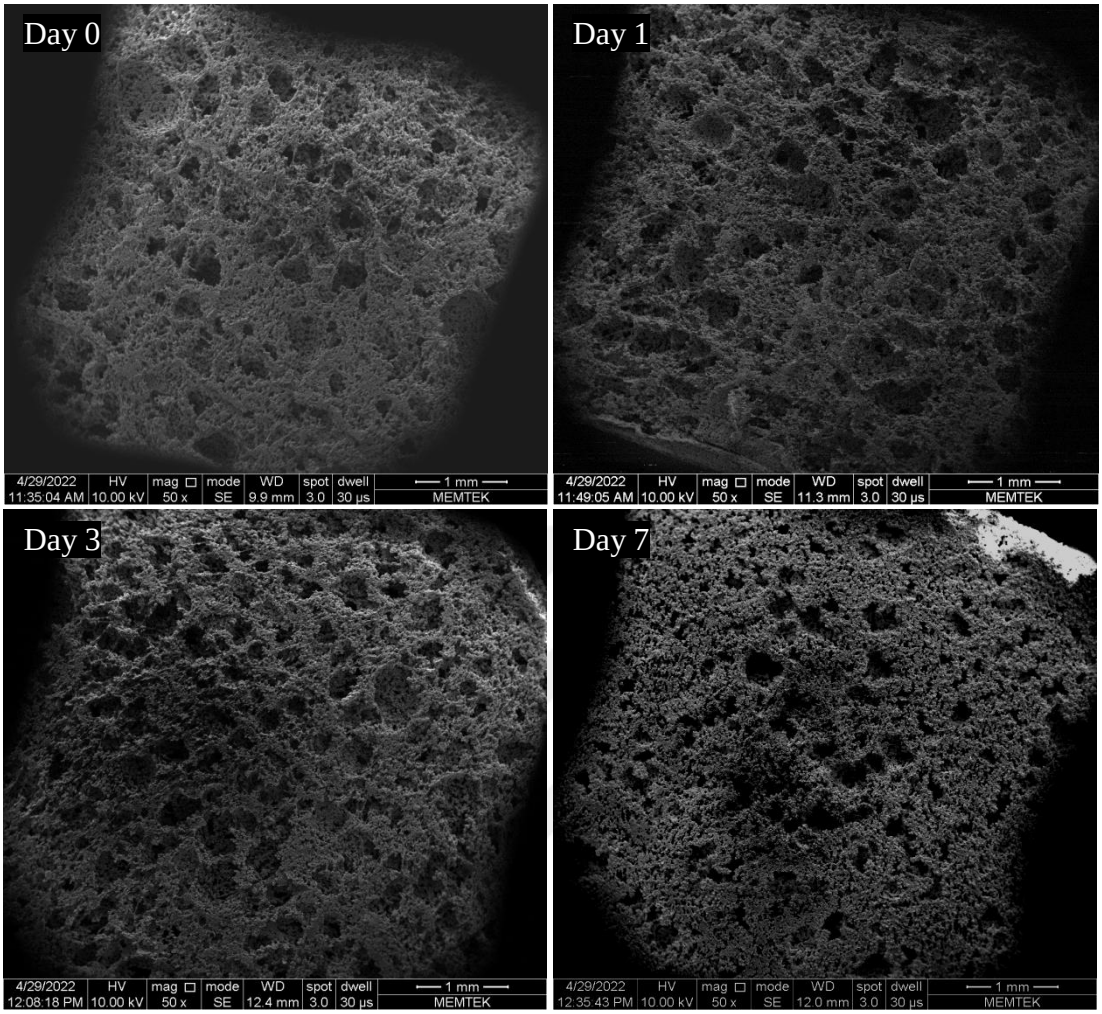


Figure E.1 : SEM images of PCL200 sample at 50X magnification at day 0, 1, 3 and 7.

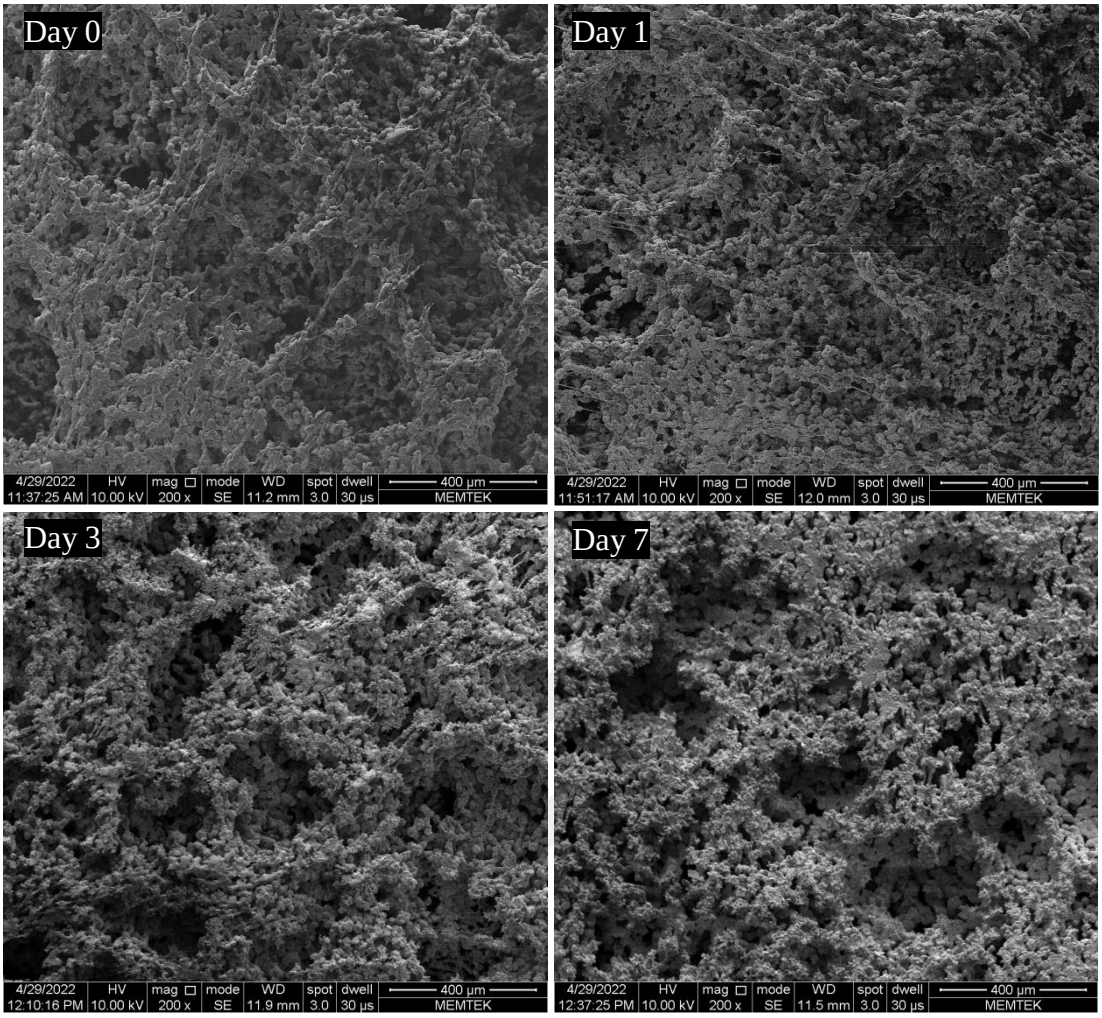


Figure E.2 : SEM images of PCL200 sample at 200X magnification at day 0, 1, 3 and 7.

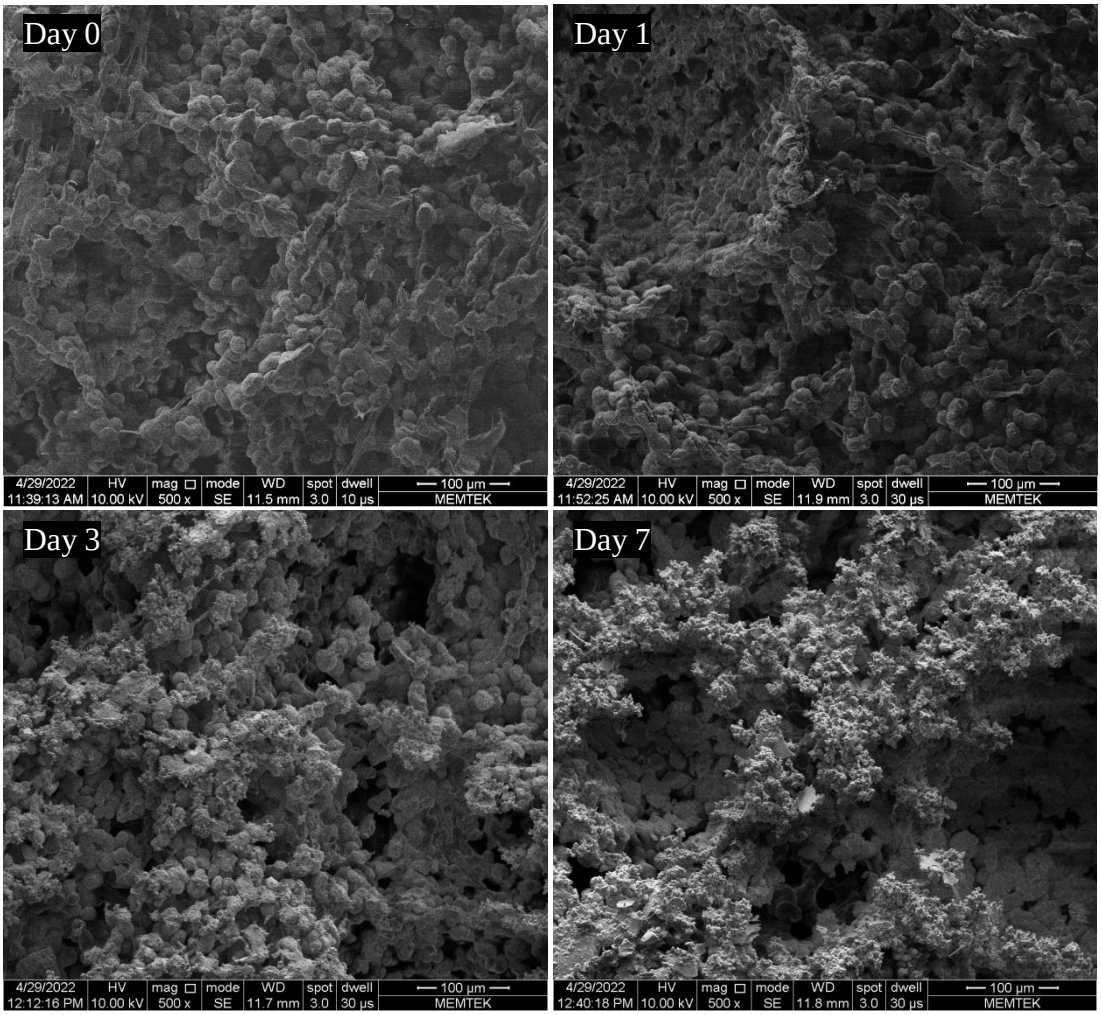


Figure E.3 : SEM images of PCL200 sample at 500X magnification at day 0, 1, 3 and 7.

APPENDIX F

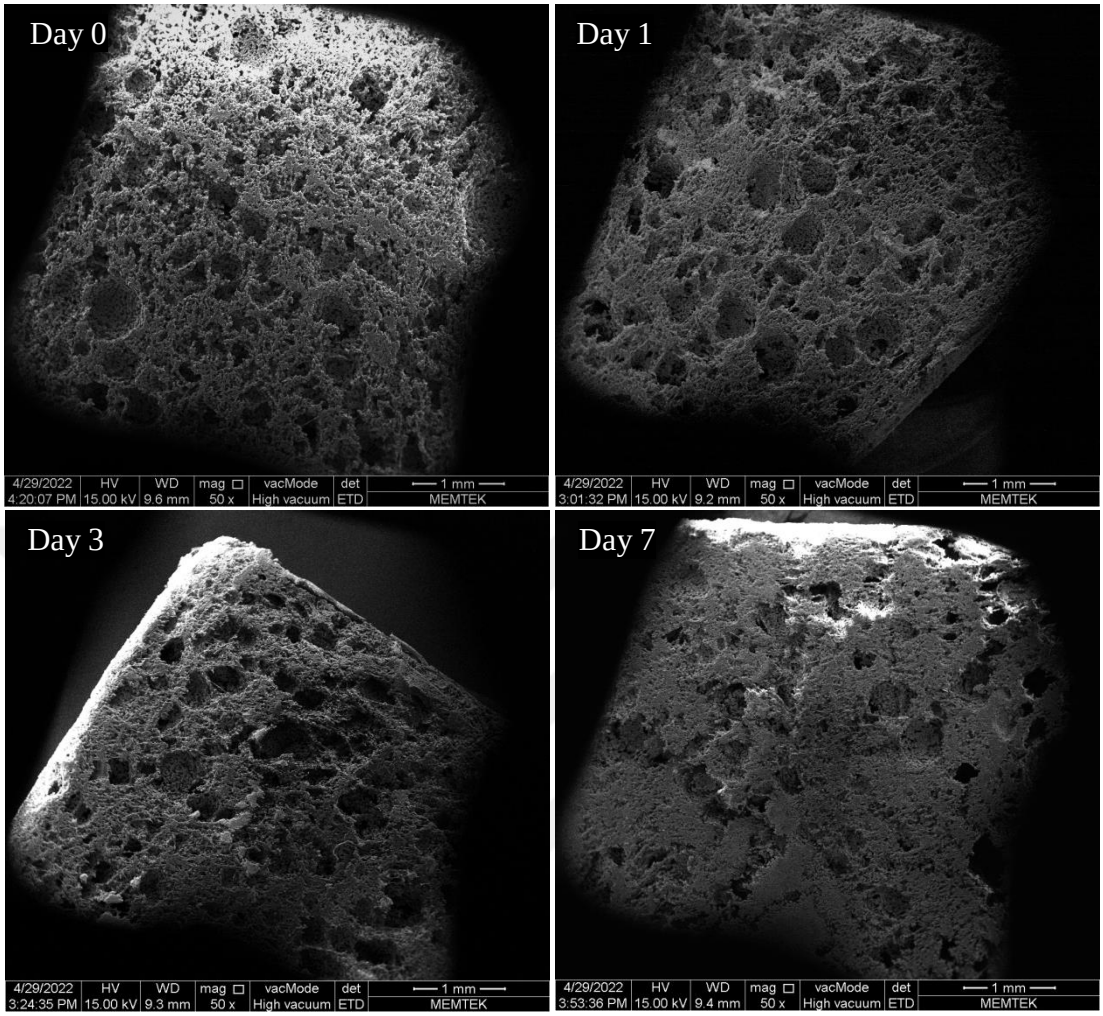


Figure F.1 : SEM images of PCL200/Lignin10 sample at 50X magnification at day 0, 1, 3 and 7.

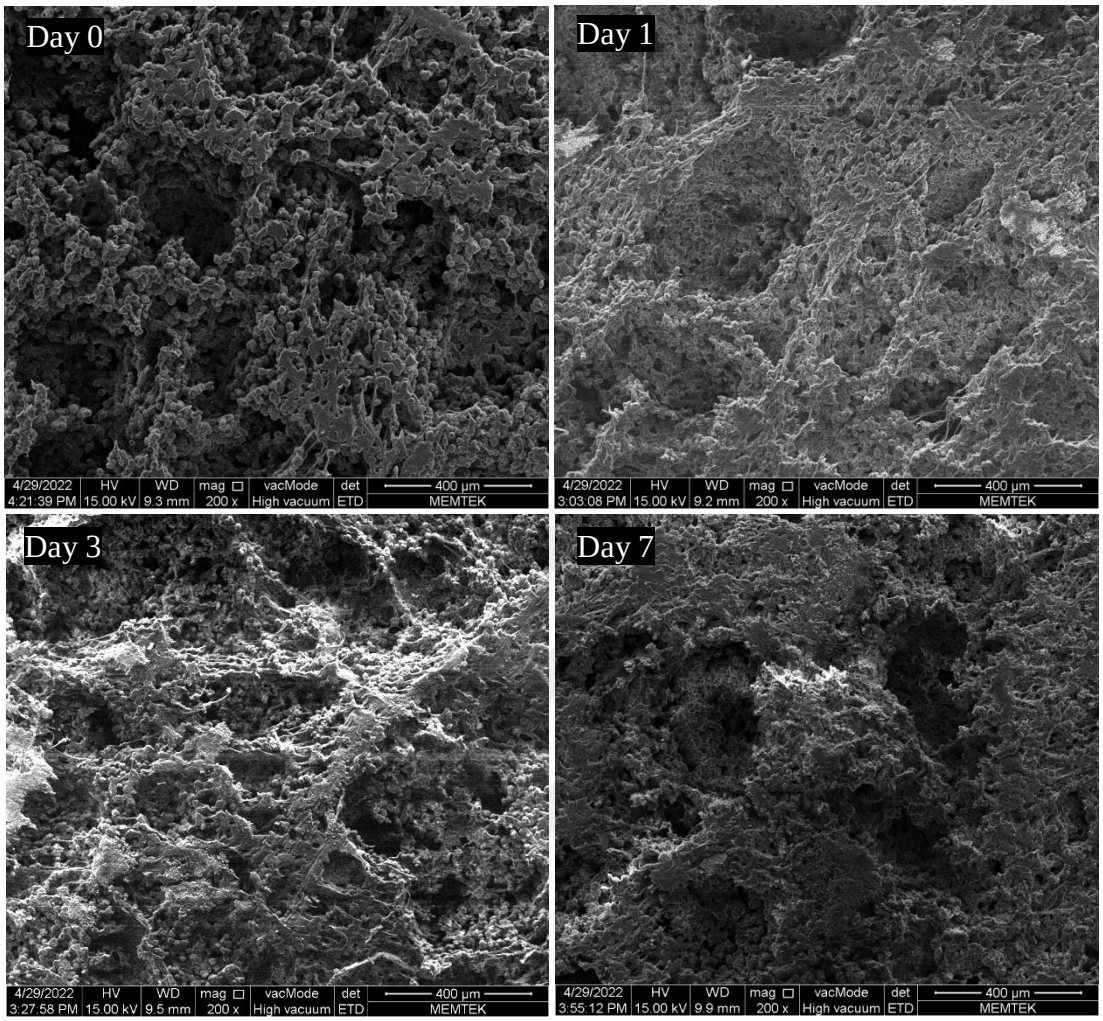


Figure F.2 : SEM images of PCL200/Lignin10 sample at 200X magnification at day 0, 1, 3 and 7.

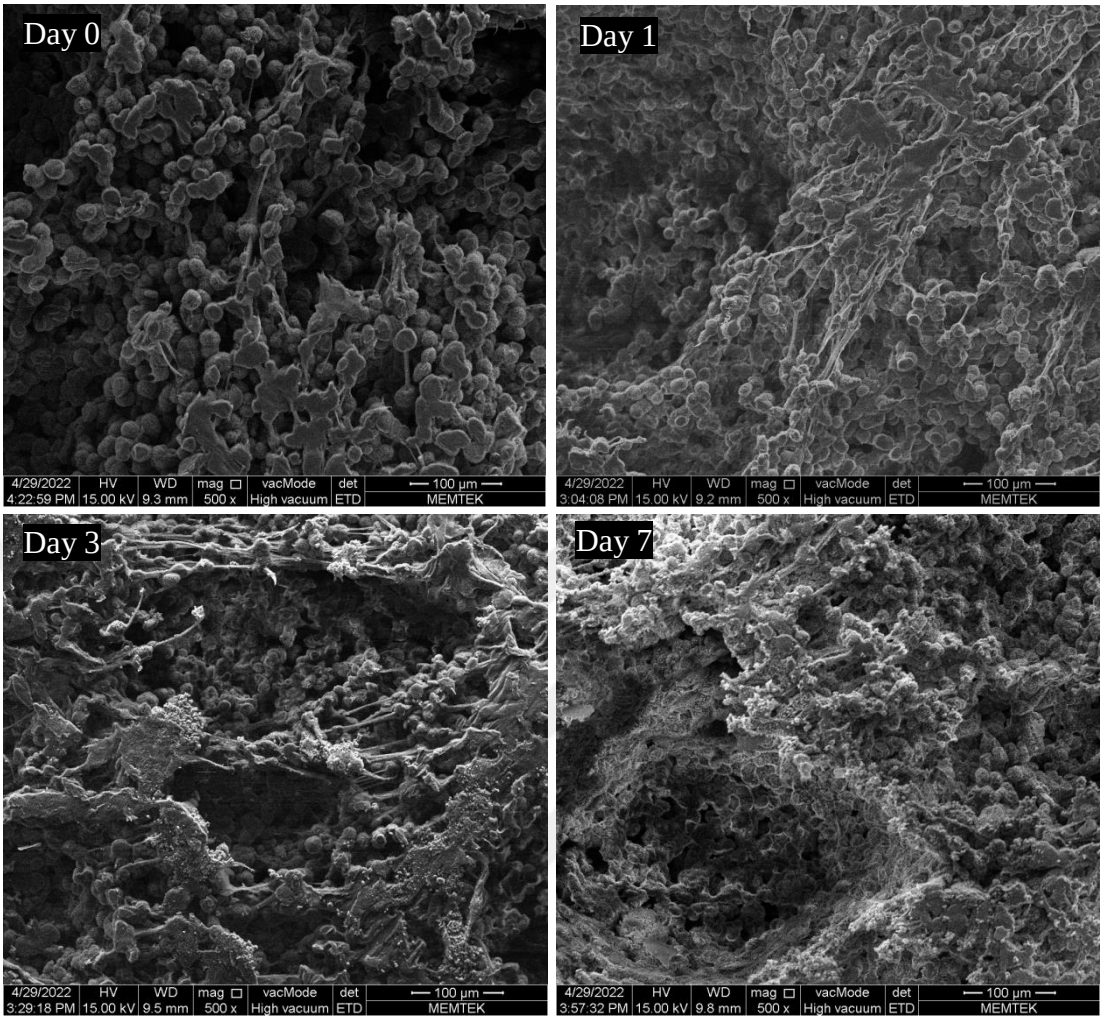
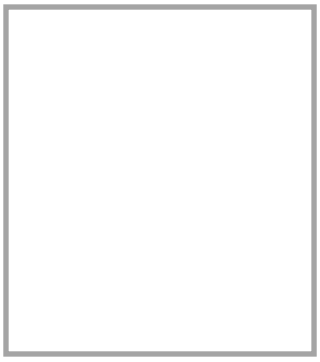


Figure F.3 : SEM images of PCL200/Lignin10 sample at 500X magnification at day 0, 1, 3 and 7.

CURRICULUM VITAE



Name Surname **:** Gizem Elmalı

EDUCATION

- **B. Sc** **:** 2018, Istanbul University, Science Faculty, Molecular Biology and Genetics

PROFESSIONAL EXPERIENCE AND REWARDS

- R&D Personnel in Geneon Biotechnology R&D, Education and Consultancy Co. Ltd.
  - February 2020 – November 2021
- Advisor of Saint Joseph Private French High-School in iGEM 2021 Competition
  - Silver Medal winner

PUBLICATIONS, PRESENTATIONS AND PATENTS

- Participation with Oral Presentation to II. International Green Biotechnology Congress (2019) with "Molecular Analysis of Horizontal Gene Transfer From Transgenic Soybean to *Acinetobacter baylyi* ADP1 Bacteria Strain" project (financially supported by Scientific Research Projects Coordination Unit Of Istanbul University)