ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

EFFECT OF SILANIZATION AGENT ON THE DEVELOPMENT OF NANOHYBRID SYSTEMS

M.Sc. THESIS

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Department of Molecular Biology-Genetics and Biotechnology

Molecular Biology-Genetics and Biotechnology Programme

Thesis Advisor: Prof. Dr. Yüksel GÜVENİLİR

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ISTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

NANOHİBRİT SİSTEMLERİNİN GELİŞTİRİLMESİNDE SİLANLAMA AJANLARININ ETKİSİ

YÜKSEK LİSANS TEZİ

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Moleküler Biyoloji-Genetik ve Biyoteknoloji Anabilim Dalı

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NİSAN 2019

Meltem Akkulak, a **M.Sc.** student of ITU **Graduate School of Science Engineering and Technology** student ID 521161117, successfully defended the **thesis** entitled "**EFFECT OF SILANIZATION AGENT ON THE DEVELOPMENT OF NANOHYBRID SYSTEMS**", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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Date of Submission : 20 March 2019
Date of Defense : 29 April 2019

To my family,

FOREWORD

First of all, I would like to express my gratitude to my supervisor Prof. Dr. Yüksel Güvenilir for her guidance, support and understanding throughout my thesis study. I would also like to thank to Msc. Chem. Eng. Yasemin Kaptan and Msc. Chem. Eng. Cansu Ülker for their help and guiding me in laboratory.

I am also thankful to my friends for their friendship and support. Finally, I would like to present my thankfulness to all my family and my parents for their encouragement, great support, understanding and patience during my thesis period and all my life.

March 2019

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ABBREVIATIONS

3-APTES : 3-aminopropyl triethoxysilane
3-APTMS : 3-aminopropyl trimethoxysilane
3-GPTMS : 3-glycidyloxypropyl trimethoxysilane
CALB : *Candida antarctica* lipase B
CALB L : Free form of *Candida antarctica* lipase B
DSC : Differential scanning calorimetry
HNMR : Proton nuclear magnetic resonance
PCL : Poly(ε-caprolactone)
RHA : Rice husk ash
ROP : Ring opening polymerization
SEM : Scanning electron microscopy
TGA : Thermal gravimetric analysis
TMSPDA : Trimethoxycylyl propyl ethylenediamine
v/v : Volume per volume ratio
w/w : Weight per weight ratio
XRD : X-ray diffraction

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EFFECT OF SILANIZATION AGENT ON THE DEVELOPMENT OF NANOHYBRID SYSTEMS

SUMMARY

Biodegradable polymers are important materials with their biocompatible, non-toxic and environmentally friendly properties. Nanohybrids are formed by filling polymer matrices with inorganic nanofillers. Development of nanohybrid systems become an attractive issue because of increasing thermal, mechanical properties and usability of biodegradable polymers. Nanohybrids are used in many applications especially biomedical field like tissue engineering or controlled drug delivery. Polycaprolactone which is an aliphatic biopolyester can be obtained by ring opening polymerization of ε-caprolactone with using chemical catalysts or enzymes. Enzymatic synthesis is generally preferred because it has lots of advantages over chemical synthesis. The main advantage of enzymatic synthesis is that enzymes are non-toxic catalysts whereas chemical ones are toxic. The aim of this study is to synthesize polycaprolactone enzymatically and prepare nanohybrids based on PCL.

Lipase enzyme (EC 3. 1. 1. 3) that found in the group of hydrolases mainly catalyze triglycerides to glycerol and fatty acids. Moreover, lipases mostly used in ester synthesis. Recently with increasing production in industries, enzymes and their immobilization become more important. Immobilization provides many improved properties to enzymes such as improving their activity, stability and reusability. There are several immobilization methods like physical adsorption, covalent binding, cross-linking or entrapment. Physical adsorption is mostly used immobilization method. In physical adsorption enzymes are immobilized on support materials with weak interactions such as van der Waals forces or hydrogen bonding. It is an easy and economic method but weak interaction may cause a leakage problem. Enzymes can be immobilized by covalent bonds or with the help of a spacer arm like gluteraldehyde in cross-linking method. Moreover, enzymes can be immobilized in a cage or polymer matrix in entrapment method.

Enzymes can be immobilized on surface activated support materials. These support materials may be polymers, organic or inorganic materials. Inorganic supports especially contains silica. Rice husk ash obtained from burning of rice husks is a suitable support material with its high silica content. For surface modification of rice husk ashes, many different silanization agents like 3-Aminopropyl trimethoxysilane (3-APTMS), 3-Glycidyloxypropyl trimethoxysilane (3-GPTMS), 3- Aminopropyl triethoxysilane (APTES) and Trimethoxycylyl propyl ethylenediamine (TMSPDA) may be used.

In this study, nanocomposite systems that include PCL and PCL grafted on rice husk ash (RHA) nanohybrids are synthesized by immobilized *Candida antarctica* lipase B (CALB). Moreover, nanohybrids are also synthesized by Novozyme 435.

CALB enzyme immobilized by both physical adsorption and cross-linking methods. RHA was used as support material; 3-APTMS, 3-GPTMS, APTES and TMSPDA were used for surface modification of RHA respectively. Immobilized CALB enzyme was used in the ring opening polymerization (ROP) of ε-caprolactone and formation of PCL/RHA nanohybrids.

First of all, rice husks that obtained from a rice production company were burned at 600 °C for 6 h to obtain RHA. Before immobilization, surface modification of RHA was achieved by mixing RHA with four silanization agents separately in acetone and incubated in water bath. Then, surface-activated RHA was filtered and dried at 60 °C. For TMSPDA activated RHA, glutaraldehyde treatment was also applied. After surface modification, lipase enzyme immobilized on RHA samples. ROP of ε-caprolactone and development of nanohybrid systems were performed by using these immobilized enzymes and Novozyme 435 with enzyme to monomer ratio of 20% (w/w) separately. Reaction conditions (temperature and time) were different for these enzymes. At the end of the reactions it is aimed to obtain polycaprolactone (PCL) chains grafted onto RHA particles, thus polymer composites consisting of PCL and PCL/RHA nanohybrids. After the reaction, final mixture was separated into two fractions as RHA-rich and polymer-rich to determine polymer grafting at the surface.

In the second part of the study, Thermal Gravimetric Analysis (TGA) was applied to determine grafting efficiency by analyzing solid RHA-rich fraction. Later, to obtain nanohybrid systems experiments were repeated with leaving RHA-rich fraction in polymer-rich fraction. Characterization of nanohybrids will be obtained by Hydrogen Nuclear Magnetic Resonance (HNMR), Differential Scanning Calorimetry (DSC), X-Ray Diffraction Analysis (XRD) and Scanning Electron Microscopy (SEM).

At the end of this study, PCL/RHA nanohybrids were successfully obtained with different grafting efficiencies as 8%, 4% and 14% for APTMS-activated, GPTMS-activated and APTES-activated RHA respectively. Moreover, molecular weights of these nanohybrids found as 4317, 4346 and 8916 g/mol. For usage of TMSPDA activated RHA molecular weight was 8446 g/mol. Crystallinity degrees of PCL/RHA nanohybrids were lower than PCL that obtained from previous studies. These crystallinities were 32%, 23%, 38.6% and 39.3% respectively. Lower crystallinity of nanohybrids show that these nanohybrids have amorphous structure so that usage of PCL/RHA nanohybrids are suitable for drug delivery applications.

In further studies, PCL/RHA nanohybrids may be used in tissue engineering or controlled drug delivery applications. Different studies about the usage of PCL/silica nanohybrids in medical applications exist in literature.

NANOHİBRİT SİSTEMLERİNİN GELİŞTİRİLMESİNDE SİLANLAMA AJANININ ETKİSİ

ÖZET

Biyobozunur polimerler, toksik olmamaları ve çevre dostu olmaları gibi önemli özelliklere sahip maddelerdir. Nanohibritler, polimer matrikslerinin inorganik maddeler ile doldurulması sonucu elde edilirler. Nanohibritlerin biyobozunur polimerlere göre daha iyi termal ve mekanik özelliklere sahip olmaları nedeniyle nanohibrit sistemlerinin geliştirilmesi önemli konulardan biri haline gelmiştir. Nanohibritler biyomedikal alanda doku mühendisliği ve kontrollü ilaç salınımı çalışmaları başta olmak üzere birçok uygulamada kullanılmaktadır. Alifatik biyopoliester olan polikaprolakton kimyasal katalizörler ya da enzimler kullanılarak ε-kaprolaktonun halka açılım reaksiyonu ile elde edilir. Enzimatik sentez kimyasal senteze göre birçok avantajı olduğu için daha çok tercih edilmektedir. Enzimatik sentezin en önemli avantajı enzimlerin toksik olmayan katalizörler olmasıdır. Bu çalışmanın amacı enzimatik polikaprolakton sentezi ve polikaprolakton içeren nanohibrit sistemlerinin geliştirilmesidir.

Hidrolazlar grubunda bulunan lipaz enzimi (EC 3. 1. 1. 3) başta trigliseritlerin gliserol ve yağ asitlerine hidrolizi olmak üzere ester sentezi gibi reaksiyonları da katalizlemektedir. Son dönemde endüstrilerdeki üretimin artmasıyla birlikte enzimler ve enzim immobilizasyonu daha önemli konular olmuştur. İmmobilizasyon enzimlerin aktivite, stabilite ve tekrar kullanılabilirliği gibi özelliklerini geliştirmektedir. Fiziksel adsorpsiyon, kovalent bağlanma, çapraz bağlanma ya da tutulma gibi farklı immobilizasyon yöntemleri bulunmaktadır. Fiziksel adsorpsiyon en çok kullanılan immobilizasyon yöntemidir. Fiziksel adsorpsiyon yönteminde enzimler katı bir destek materyaline van der Waals ya da hidrojen bağları gibi zayıf etkileşimlerle bağlanır. Fiziksel adsorpsiyon kolay ve ekonomik bir yöntemdir ancak zayıf etkileşimler enzimin kaçması gibi problemlere neden olabilmektedir. Enzimler kovalent bağlarla immobilize edilebilir ya da gluteraldehit gibi yardımcı bir maddenin varlığı ile çapraz bağlanma yöntemi ile de immobilize edilebilir. Enzim immobilizasyonu, enzimlerin bir kafes ya da polimer matriksi içerisinde tutuklanması ile de sağlanabilir.

Enzimler, yüzeyi aktifleşmiş destek materyalleri üzerinde tutuklanarak immobilize olurlar. Bu destek materyalleri polimerler, organik ya da inorganik maddeler olabilir.

İnorganik destek materyalleri silika içeriği yüksek maddelerdir. Pirinç kabuğunun yakılması ile elde edilen pirinç kabuğu külü yüksek silika içeriği ile uygun bir destek materyalidir. Pirinç kabuğu küllerinin yüzey modifikasyonu için 3-Aminopropil trimetoksisilan (3-APTMS), 3-Glisitiloksipropil trimetoksisilan (3-GPTMS), 3-Aminopropil trietoksisilan (APTES) and Trimetosilil propil etilendiamin (TMSPDA) gibi farklı silanlama ajanları kullanılmaktadır.

Bu çalışmada, *Candida antarctica* lipaz B (CALB) kullanarak PKL ve pirinç kabuğu üzerine aşılanmış PKL içeren nanokompozit sistemleri sentezlenmiştir. Buna ek olarak, Novozyme 435 kullanarak nanohibrit sistemleri elde edilmiştir.

CALB enzimi hem fiziksel adsorpsiyon hem de çapraz bağlanma yöntemi ile immobilize edilmiştir. Destek materyali olarak pirinç kabuğu külü ve yüzey modifikasyonu için de 3-APTMS, 3-GPTMS, APTES ve TMSPDA kullanılmıştır. İmmobilize CALB enzimi ε-kaprolaktonun halka açılım reaksiyonunda ve PKL/pirinç kabuğu külü nanohibritlerinin geliştirilmesinde kullanılmıştır.

Öncelikle, pirinç üretim tesisinden elde edilen pirinç kabukları 600 °C ve 6 saat boyunca pirinç kabuğu külü elde etmek için yakılmıştır. İmmobilizasyon işleminden önce pirinç kabuğu külleri ayrı bir şekilde dört farklı silanlama ajanı ile aseton içerisinde karıştırılmış ve su banyosunda bekletilerek yüzey modifikasyonunun gerçekleşmesi sağlanmıştır. Daha sonra, yüzeyi aktifleşen pirinç kabuğu külleri süzülmüş ve 60 °C'de kurutulmuştur. TMSPDA ile aktive edilen pirinç kabuğu külleri ek olarak gluteraldehit ile muamele edilmiştir. Yüzey modifikasyon işleminden sonra lipaz enzimi pirinç kabuğu külleri üzerine immobilize edilmiştir. ε-kaprolaktonun halka açılım reaksiyonu ve nanohibrit sistemlerinin geliştirilmesi enzim/monomer oranı %20 olacak şekilde immobilize enzimler ve Novozyme 435 kullanılarak gerçekleştirilmiştir. Reaksiyon sıcaklığı ve süresi farklı enzimler için değişmektedir. Reaksiyon sonunda pirinç kabuğu külüne aşılanmış PKL zincirleri, böylece PKL ve PKL/pirinç kabuğu külü nanohibritlerini içeren polimer kompozitinin elde edilmesi amaçlanmıştır. Reaksiyon sonunda yüzeydeki polimer aşılama etkinliğini belirlemek için karışım pirinç kabuğu külünce zengin ve polimerce zengin olmak üzere iki kısma ayrılmıştır.

Çalışmanın ikinci kısmında, aşılama etkinliğini belirlemek için pirinç kabuğunca zengin kısma Termal Gravimetrik Analiz (TGA) uygulanmıştır. Daha sonra, nanohibrit sistemi elde etmek için deneyler pirinç kabuğu külünce zengin kısım polimerce zengin kısım içerisinde bırakılarak tekrarlanmıştır. Nanohibritlerin karakterizasyonu Hidrojen Nükleer Manyetik Rezonans (HNMR), Diferansiyel Taramalı Kalorimetri (DSC), X-Işını Kırılması (XRD) ve Taramalı Elektron Mikroskobu (SEM) analizleri ile gerçekleştirilmiştir.

Yapılan çalışmanın sonunda, PKL/pirinç kabuğu külü nanohibritleri farklı aşılama etkinlikleri ile başarılı bir şekilde elde edilmiştir. Nanohibritlerin aşılama etkinlikleri sırasıyla APTMS, GPTMS ve APTES ile aktive edilmiş pirinç kabuğu külleri için %8, %4 ve %14'tür. Nanohibritlerin molekül ağırlıkları 4317, 4346 ve 8916 g/mol olarak bulunmuştur. TMSPDA ile aktive edilmiş pirinç kabuğu külü kullanıldığında ise molekül ağırlığı 8446 g/mol olarak hesaplanmıştır. PKL/pirinç kabuğu külü nanohibritlerinin kristaliniteleri polikaprolaktondan daha düşük olarak elde edilmiştir.

Bu kristalinite değerleri sırasıyla 32%, 23%, 38.6% ve 39.3%'tür. Nanohibritlerin düşük kristalinite değerine sahip olması amorf yapıda olduğunu göstermektedir bu nedenle PKL/pirinç kabuğu külü nanohibritlerinin ilaç taşınım çalışmalarında kullanımı uygun olmaktadır.

Gelecek çalışmalarda, PKL/pirinç kabuğu külü nanohibritleri doku mühendisliği veya kontrollü ilaç salınımı uygulamalarında kullanılabilir. Literatürde PKL/silika nanohibritlerinin biyomedikal uygulamalarda kullanıldığı çalışmalar bulunmaktadır.

1. INTRODUCTION

Biopolymers with their biodegradable, biocompatible and non-toxic properties can be used in many different applications especially in medical field. Nanohybrid systems based on these biopolymers have improved structural and functional properties than these polymers [1]. Biopolymers and nanohybrids based on these polymers can be synthesized by both chemical catalysts and biocatalysts (enzymes). Enzymes are environmentally friendly and biodegradable catalysts so they are preffered more than chemical catalysts in industries. They can catalyze reactions under mild conditions which means that they need lower temperature and lower pressure [2]. Enzymatically synthesized nanocomposite systems can be used in controlled drug release, drug targeting and tissue engineering studies [1].

Immobilization of enzymes becomes important topic with the increasing number of production in biotechnological industries. Enzymes are immobilized on support materials to increase their activity, stability and reusability. Moreover, immobilized enzymes can be recovered from reactions easier so that immobilization reduces contamination [3]. Enzymes can be immobilized by different reversible or irreversible methods. These methods may be physical adsorption, covalent bonding, cross-linking or entrapment. Enzymes are immobilized on support materials by weak interactions by physical adsorption. It is an economic, easy and reversible method for enzyme immobilization. Physical adsorption may have some problems like enzyme leakage from support. Cross-linking is a type of covalent bonding method that enzymes bound to support materials covalently with the help of a spacer arm like gluteraldehyde [4].

In immobilization, enzymes are bound to a support material. The support material is an economic, easily accessible, non-toxic, non-reactive and biocompatible material that may be organic or inorganic. Polymers, active carbon, metals and silica containing materials are used as support material [4]. Surface of these supports are activated before immobilization. 3-aminopropyl trimethoxysilane (3-APTMS), 3-glycidyloxypropyl trimethoxysilane (3-GPTMS), aminopropyl triethoxysilane

(APTES) and trimethoxycylyl propyl ethylenediamine (TMSPDA) are organosilanes that used in surface modification of support materials.

Lipase enzyme can catalyze both oil hydrolysis and polyester synthesis. Free or immobilized form of lipases are mostly used in poly(ε-caprolactone) (PCL) synthesis [5]. PCL is a biopolyester that can be obtained from ring opening polymerization (ROP) reaction of ε-caprolactone. PCL has semi-crystalline structure, good solubility and blend compatibility so that it is seen as potential for using in medical applications [6]. PCL mostly used in long-term implants and long term drug release applications because of its slow degradation property. PCL nanohybrids are also biocompatible materials used in drug delivery like controlled release of anti-cancer drugs. Nanohybrids can control release kinetics and minimize toxic side effects so that they improve therapeutic efficiency of drugs [1, 7, 8].

In this study, *Candida antarctica* lipase B (CALB) was immobilized on surface modified RHA to obtain nanocomposite system that include PCL and PCL/RHA nanohybrids. RHA that obtained by burning rice husks was preferred as support material because it contains high amount of silica [9]. Different silanization agents like 3-aminopropyl trimethoxysilane (3-APTMS), 3-glycidyloxypropyl trimethoxysilane (3-GPTMS), aminopropyl triethoxysilane (APTES) and trimethoxycylyl propyl ethylenediamine (TMSPDA) were used for surface activation of RHA. CALB was immobilized by cross-linking method for TMSPDA-modified RHA and by physical adsorption method for others. To develop PCL/RHA nanohybrids, these immobilized enzymes were used separately. Different nanohybrid systems were also obtained by Novozyme 435. Grafting efficiencies of these nanohybrids were determined by Thermal Gravimetric Analyses (TGA). Hydrogen Magnetic Resonance (HNMR), Differential Scanning Calorimetry (DSC), X-Ray Diffraction (XRD) and Scanning Electron Microscopy (SEM) were applied for characterization studies of these samples.

2. THEORETICAL STUDY

2.1 Lipase Enzyme

Lipases (EC 3. 1. 1. 3) that found in the group of hydrolases mainly catalyze triglycerides to glycerol and fatty acids. In addition to hydrolysis, lipases catalyze synthesis of esters. That's why they are used in industrial applications; mostly in chemical, food and pharmaceutical industries [5, 10]. Extracellular lipases can be obtained from different microorganisms like bacteria, fungi or yeast [5]. These microorganisms summarized in Table 1.

Table 2.1: Microorganisms that used in the production of lipases [5].

Source	Microorganisms
Bacteria	*Bacillus subtilis, Bacillus pumilus, Bacillus licheniformis, Bacillus coagulans, Pseudomonas sp.*
Fungi	*Rhizopus sp., Aspergillus sp., Penicillium sp., Geotrichum sp., Rhizomucor sp.*
Yeast	*Candida rugosa, Candida tropicalis, Candida antarctica, Candida cylindracea, Candida deformans*

Candida antarctica lipase B (CALB) is suitable for using in applications more than other lipases that given in Table 2.1 because it has better properties [11]. For example, it has high enantoselectivity and thermal stability. It is also stable in organic solvents. Moreover, CALB has broad substrate specificity and usage of CALB as a catalyst results in the formation of less complex structured products [11, 12]. CALB that founds in α/β hydrolases consists of two monomers in the asymmetric unit. The active site of CALB that composed of Serine (Ser), Histidine (His) and Aspartic acid/Glutamic acid (Asp/Glu) aminoacids is a catalytic triad. The aminoacid residues that are found in open and closed conformations of CALB can be seen from Figure 2.1 [11].

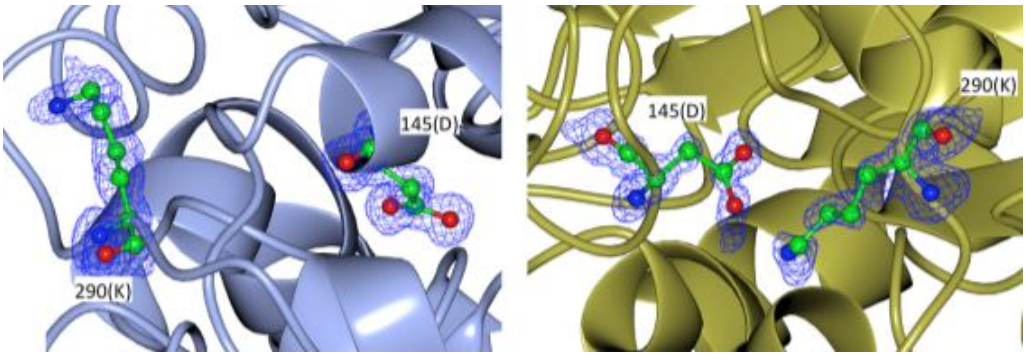

Figure 2.1: Open and closed conformations of CALB [11].

In Figure 2.1, the aminoacid residues (Asp145, Lys290) in open and closed forms of CALB can be seen respectively. The conformational changes in these aminoacid residues are important for the activity of lipase enzyme. The importance of conformational changes in these residues between open and closed states are given in Figure 2.2 in detail [11].

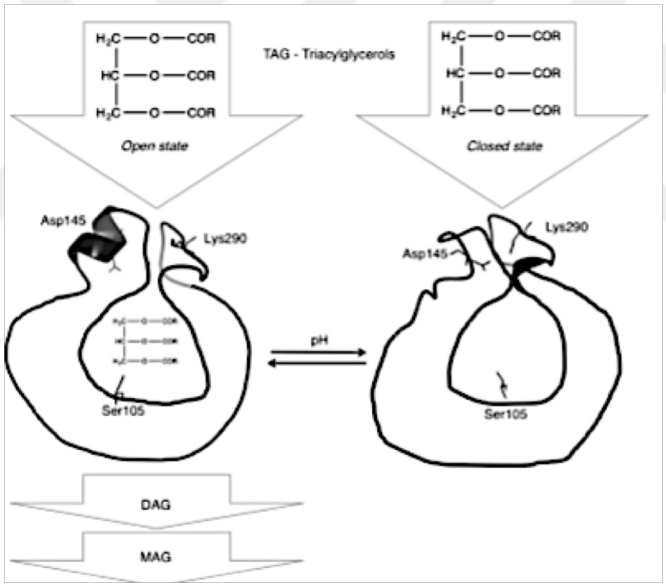

Figure 2.2: The mechanism of CALB catalysis [11].

As shown in Figure 2.2, in the open form of lipase enzyme triacylglycerol (TAG) converted to diacylglycerol and monoacylglcerol molecules respectively. In the closed form, there is a conformational change that Asp145 gets close to Lys290 and forms salt bridge. The formation of salt bridge prevents catalytic activity with blocking the access of substrates to catalytic site [11]. Main characteristics of CALB listed in Table 2.2 [12].

Table 2.2: Characteristics of CALB [12].

	CALB
Molecular Weight (kD)	33
Isoelectric Point (pI)	6.0
pH Optimum	7
Specific Activity (LU/mg)	435
Thermostability at 70 °C	15
pH Stability	7-10

Activity and substrate specificity are important for using an enzyme as a catalyst. These properties can be improved by different immobilization techniques to increase the usage of an enzyme in industrial applications as biocatalyst. The most important factor that limits the usage of CALB in industrial applications is that the immobilized product of CALB (Novozym 435) has high price [12]. Novozyme 435 is obtained by physical immobilization of CALB onto acrylic polymer resin [13]. New techniques are tried to obtain immobilized CALB with less price to increase its usage in industrial applications like polyesters and biodiesel synthesis [12].

2.2 Enzyme Immobilization

Enzymes are biocatalysts that can be used in many reactions. With the increase in production, the need for improving reusability and stability of enzymes also increase. Immobilization improves these properties of enzymes [14]. General advantages and disadvantages of immobilized enzymes are shown in Table 2.3 [4].

Table 2.3: Advantages and disadvantages of enzyme immobilization [4].

Advantages	Disadvantages
Catalyst reuse	Loss and reduction in activity
Easier reactor operation	Diffusional limitation
Easier product separation	Additional cost

Immobilized enzymes are generally used in lots of industries like pharmaceutical, food, cosmetics and biofuel [14].

2.2.1 Immobilization methods

Enzymes are attached to a support material for immobilization. Support materials may be biopolymers, synthetic polymers or inorganic materials. Enzymes are immobilized on support materials by reversible (physical adsorption, affinity binding) or

irreversible (covalent binding, entrapment) methods. [6, 7]. Reversible and irreversible immobilization methods can be seen from Figure 2.3 and Figure 2.4 respectively [4].

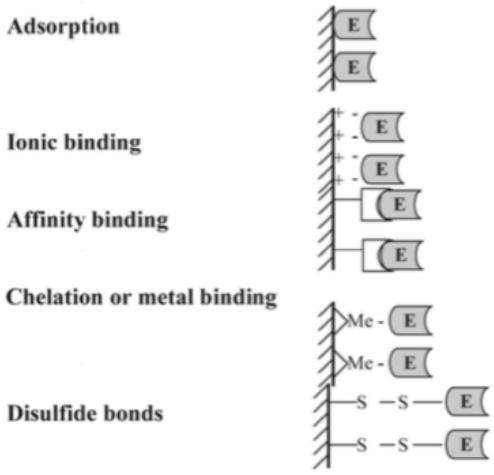

Figure 2.3: Reversible methods for enzyme immobilization [4].

As seen from Figure 2.3 adsorption, ionic binding, affinity binding, metal binding and disulfide bonds are reversible enzyme immobilization methods. Physical adsorption is an immobilization method that in which enzymes are attached to support materials by weak interactions like hydrogen binding, Van der Waals or hydrophobic interactions. It is mild, economic and easy to perform process. Adsorption protects activity of enzymes. If there are weak interactions between enzyme and matrix, it may bring about problems like enzyme leakage from matrix [4, 15].

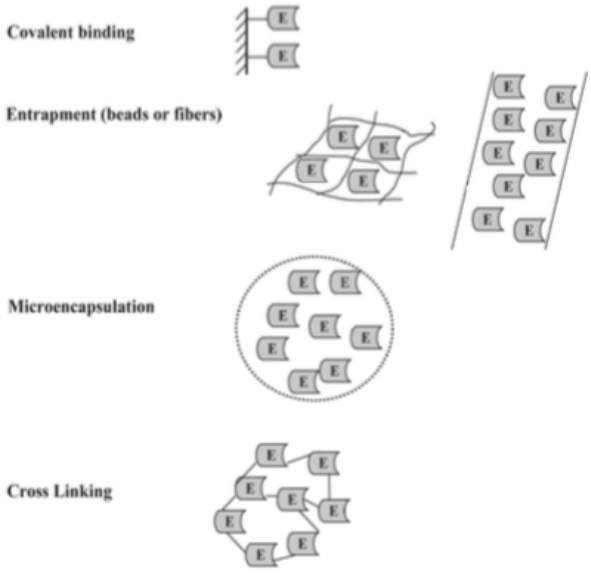

Figure 2.4: Irreversible methods for enzyme immobilization [4].

Covalent binding, entrapment, microencapsulation and cross linking are irreversible enzyme immobilization methods. In covalent binding method, enzymes can bind to supports covalently from their side chain amino acids (arginine, aspartic acid and histidine). The main advantage of covalent binding is formation of stable bonds between enzyme and matrix so that enzymes are not released into solution. Covalent binding by supports (mesoporous silica or chitosan) increases enzyme half-life and thermal stability [4, 15].

In entrapment method, enzymes are held by covalent or non-covalent bonds in gels or fibers. Entrapment method retains enzyme in polymeric network whereas allows substrate and products pass through. In addition to this, micro encapsulation is another type of entrapment [4, 15].

Glutaraldehyde, known as spacer arm, is a cross linker. Glutaraldehyde provides thermal stability to enzymes that are bound covalently. Cross-linking agents help formation of stable covalent bonds by inter- and intra- sububits [4, 15].

2.2.2 Support materials

For immobilization, enzymes may be attached to different support materials. These support materials can be divided into two main groups as organic and inorganic materials. Organic supports are synthetic polymers and biopolymers whereas inorganic supports are silica, mineral materials and carbon based materials. Support materials have similar features like high chemical and thermal stability, high affinity to enzymes, biocompatibility and reusability [2, 14].

Polysaccharides (cellulose, starch, chitosan) and proteins (albumin, gelatin) are biopolymers that can be used as support materials. Moreover Al, zeolites, ceramics, activated carbon and silica are inorganic supports. Lipase immobilization on silica granules are given in Figure 2.5 [14].

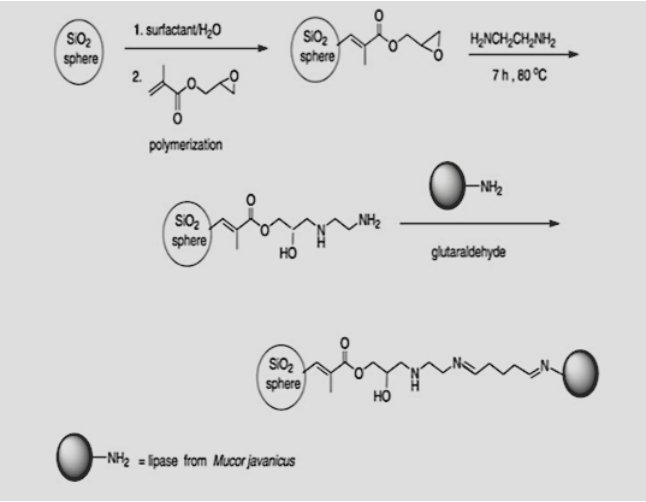

Figure 2.5: Lipase immobilization on silica granules [14].

As seen in Figure 2.5, lipase enzyme from *Mucor javanicus* immobilized on silica by cross-linking method. Before immobilization, silica surface was activated and treated with glutaraldehyde [14].

According to previous studies, it was found that immobilized lipases keep 91% and 96% of free enzyme activitiy on silica gel and mesoporous silica respectively [16].

Silica, that is a biocompatible material, has high thermal, chemical resistance and good mechanical properties so it is mostly used as inorganic support in immobilization. High surface area, porous structure and hydroxyl groups on surface of silica improves attachment of enzymes. With these structural properties silica surface can be modified by agents like gluteraldehyde or APTES to obtain stable enzyme-silica interactions. Silica gel supports modified by silanization to remove unreacted aldehyde groups [2, 16]. Sol gel silica and silica gel were used as supports in lipase immobilization. Their binding groups, cross-linking agents and immobilization types were given in Table 2.4 [16].

Table 2.4: Properties of silica-based materials used in lipase immobilization [16].

Support	Binding Group	Cross-linking Agent	Immobilization Type	Enzyme
Sol Gel Silica	-OH	-	Adsorption	Lipase from *Aspergillus Niger*
Silica Gel	-OH, C=O	Gluteraldehyde	Covalent binding	Commercial Lipase

According to Table 2.4, lipase from *Aspergillus Niger* immobilized on sol gel silica support by adsorption method and commercial lipase was immobilized on silica gel by covalent binding [16].

2.2.2.1 Rice husk ash as inorganic support material

Rice husk is a by-product obtained from rice production plants. Rice husk ash contains high amount of non-crystalline or amorphous silica with small amounts of inorganic salts produced from the burning of rice husks [9, 17]. Rice husk ashes obtained from burning of rice husks at different temperatures (600 °C, 800 °C and 1000 °C respectively) can be seen from Figure 2.6 [9].

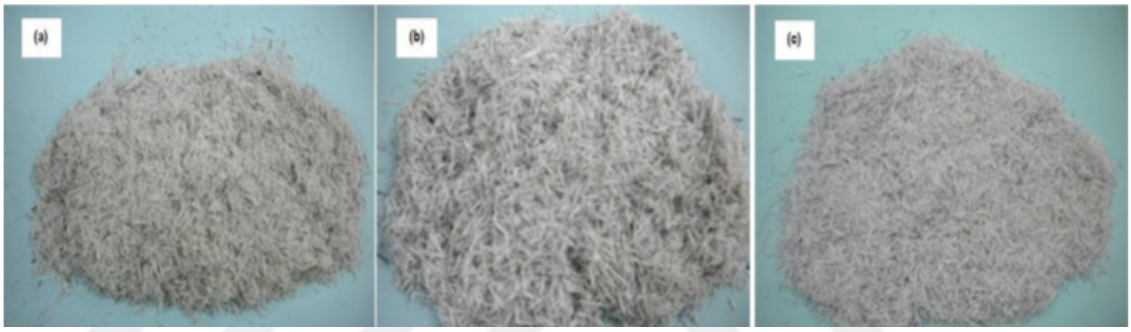

Figure 2.6: Rice husk ash burned at different temperatures (a) 600 °C (b) 800 °C (c) 1000 °C [9].

Burning of rice husk at 550-800 °C produces amorphous ash, in other words silica content of ash transformed into amorphous phase. Burning at higher temperatures, crystallization will occur [9, 17]. Chemical composition of rice husk ash is given in Table 2.5 [9].

Table 2.5: Chemical composition of rice husk ash [9].

Constituent	Amount in rice husk ash (wt%)
Silica (SiO_2)	80
Alumina (Al_2O_3)	3.93
Sulfur trioxide (SO_3)	0.78
Iron oxide (Fe_2O_3)	0.41
Calcium oxide (CaO)	3.84
Magnesium oxide (MgO)	0.25
Sodium oxide (Na_2O)	0.67
Potassium oxide (K_2O)	1.45
Loss on ignition at 850 °C	8.56

As seen from Table 2.5, rice husk ash contains high amount of silica so that it can be used as inorganic support material for enzyme immobilization [9].

2.2.3 Surface modification by silanization

Surface modification of silica is provided by amination of hydroxyl and reactive siloxane groups on silica surface. Silanization agents are used for surface modification of silica with reacting these groups. Methyl and polyvinyl alcohol groups are added so that bonds between enzyme and support material are strengthened. Surface modified silica can be grafted with monomers to obtain silica-polymer nanocomposites [18, 19].

Silica is found in many applications like drug delivery, drug targeting, tissue engineering, gene transfection and cell tracking. It is mostly used in drug delivery because silica can adsorb drugs and diffuse these drugs into tissues within time. Surface functionalized silica is preferred by increasing efficiency and decreasing toxicity. There are several studies in literature about the usage of surface modified silica in drug delivery applications. Different silanization agents are suitable for controlled release of different drugs because interactions between drug molecule and silica surface influence release properties. For example, 3-APTMS-modified silica is suitable for ibuprofen adsorption because carboxylic group (-COOH) that ibuprofen contains interact with amino groups (-NH2 and -NH) in surface modified silica and forms hydrogen bonding. On the other hand, for rhodamine materials that surface functionalized by organic groups are more suitable [20].

Trifunctional organosilanes (APTES, 3-APTMS and 3-GPTMS) are mostly used as silanization agents in surface modification of silica materials. These silanization agents react with silanol groups that found on surface through their alkoxy groups. APTES has ethoxy group, whereas 3-APTMS and 3-GPTMS have methoxy groups as alkoxy groups. Amine (-NH2) or epoxy ring obtained on the surface of modified-silica depending on the silanization agents. 3-GPTMS forms epoxy ring; APTES and 3-APTMS form amine. Immobilization of lipase enzyme on APTES-modified and 3-GPTMS-modified silica material can be seen from Figure 2.7 [21].

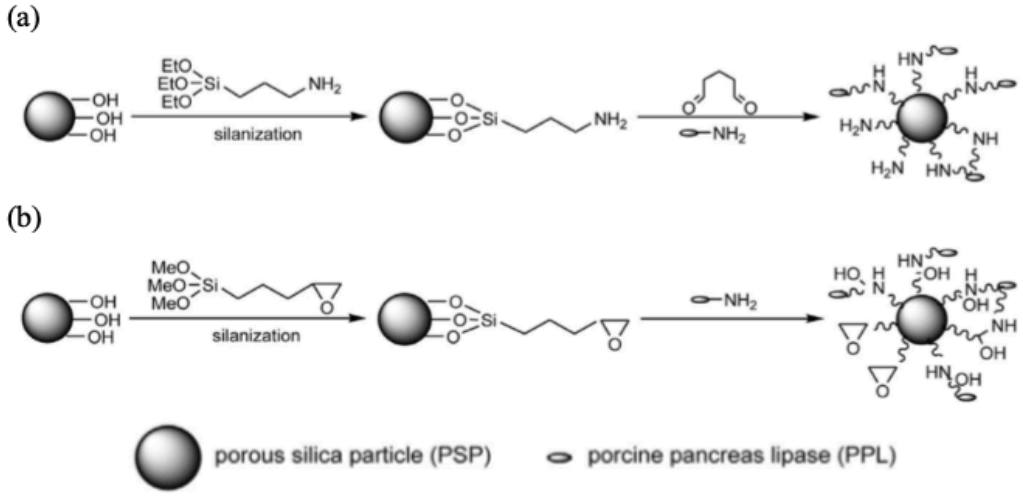

Figure 2.7: Immobilization on (a) APTES-modified (b) 3-GPTMS-modified materials [21].

As shown in Figure 2.7, for amine functional modified silica as in the modification by APTES gluteraldehyde is used a a crosslinking agent between support material and enzyme. Cross-linking is an alternative method for covalent bonding. Epoxy ring that formed in 3-GPTMS modification is reactive so that there is no need for an additional material. Enzyme can be immobilized on 3-GPTMS-modified silica by covalent bonds [21].

2.3 Biodegradable Polymers

Biodegradable polymers are polymer structures that can be degraded in nature by microorganisms. There are two main reasons that make biodegradable polymers more attractive than other ones. One of these reasons is the increase in environmental concerns. Biodegradable polymers can be a solution for growing waste problems because recycling of these polymers are easier and more economical. The other advantage of these polymers is that they can be obtained by bio-based materials whereas other polymers are fossil fuel based [22, 23].

Biodegradable polymers can be divided into two main groups as agro-polymers and biodegradable polyesters. The main classification of biopolymers can be seen from Table 2.3 [22].

Table 2.6: Classification of biodegradable polymers [22].

Agro-polymers (Biomass products)	Biopolyesters (From microorganisms)	Biopolyesters (From biotechnology)	Biopolyesters (From petrochemical products)
Polysaccharides Proteins	PHB PHBV	PLA	PCL Co-polyesters

Agro-polymers are biomass based polymers that include natural polymers like polysaccharides (starch, chitin&chitosan, pectin), proteins (soybean protein, corn protein, casein, gelatin) and lignin. Biopolyesters can be classified as obtained by extraction from microorganisms, synthesized from bio-derived monomers and synthesizing from synthetic monomers. Polyhydroxybutyrate (PHB) and Polyhydroxybutyrate-co-hydroxyvalerate (PHBV) are obtained from microorganisms; Polylactic acid (PLA) is synthesized from bio-derived monomers and Polycaprolactone (PCL) synthesized from petrochemical products [22]. Agro-polymers also called as natural polymers have an advantage that they can support cellular function with their biorecognition properties whereas they have low mechanial properties. On the other hand, synthetic polymers (biodegradable polyesters) show good mechanical strength and their degradation rate can be modified. Among these polyesters; PGA, PLA and their copolymers with their hydrophilic structure mostly used as scaffolds. However, PCL with its slow degradation property mostly used in long-term implants or controlled release [1].

2.3.1 Poly (ε-caprolactone)

Poly (ε-caprolactone) is an aliphatic polyester that produced by ring opening polymerization (ROP) of ε-caprolactone. It has semi-rigid structure at room temperature with low T_g (about -61 °C) and T_m (about 65 °C) values. The decomposition temperature of PCL is about at 350 °C so that it has high thermal stability. PCL can be used in blends and modified with copolymerization easily since it shows high compatibility with other materials [21-23]. Chemical structure of PCL is shown in Figure 2.3 [22].

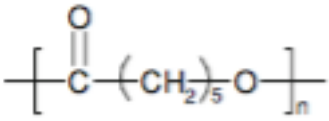

Figure 2.8: Chemical structure of PCL [22].

Enzymatically synthesized PCL, that is a non-toxic material, shows biodegradability and biocompatibility so that it can be used in biomedical applications like drug delivery, medical devices and tissue engineering scaffolds. PCL is advantageous for drug delivery systems and especially used in this field because it has slower degradation process than other biopolymers [24, 25].

2.4 Nanohybrid Systems

Nanohybrid systems formed from the combination of two or more materials include advantageous properties of each component. In other words, hybrids are multicomponent systems which show multifunctionality. For example, large polymeric structures that conjugated with inorganic materials are called as nanohybrids. Physical and chemical modifications with inorganic, organic or soft materials are used to obtain these heterostructures. The modifications can provide development of hybrid structures between silica based materials and others like polymers, peptides or proteins through Van der Waals, ionic or covalent bonds. Polyester properties can be improved by combination of polyester with inorganic material (silica) [26, 27].

2.4.1 Classification and applications of nanohybrid systems

Nanohybrids can be divided into four main classes as carbon-carbon, carbon-metal, metal-metal and organic molecule coated. Organic molecule coated nanohybrids contain polymeric materials. Their synthesis contain polymer grafting. These nanohybrids that consist of inorganic nanofiller and organic molecules (like silica and polymers) are especially important for medical applications because of their improved properties. Inorganic nanofillers provide increased mechanical strength to organic polymers. Moreover, these nanohybrids are lack of toxicity, show biocompatibility, biodegradability and improved stability [27, 28].

Electronics, environmental and medical applications are main usage areas of nanohybrids. Nanohybrid systems can be used in imaging or sensing applications, membrane technology, catalytic applications, cancer treatment and detection, delivery carriers and contolled drug release. Protective and targeted delivery obtained by controlled release of drugs from nanohybrids are produced from the interaction such as electrostatic or hyrogen bonding between drug and material [27, 29].

2.4.2 Development of PCL/RHA nanohybrid systems

PCL/RHA nanohybrid systems can be obtained by ROP of ε-caprolactone in the presence of RHA which is an inorganic material that contains silica. These nanohybrids that contain silica have advantageous behaviours like improved mechanical and rheological properties, thermal stability and barrier resistance than enzyme-catalyzed polymers [30]. Different types of polymer/silica structures are given in Figure 2.3 [26].

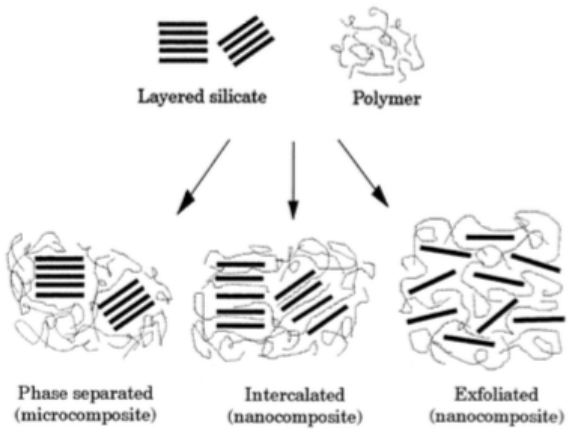

Figure 2.9: Different types of polymer/silica structures [31].

According to Figure 2.3, it can be seen that polymer/silica nanohybrids obtained by different structures. First of all if silica remains stacked onto each other, polymer and silica layers stay separately. In this structure, polymer-silica affinity is very low.

Secondly, polymer and silica partially pass in each other. Finally layered structure cannot be seen so that molecules dispersed well in matrix [31].

Schematic representation of polymer/RHA nanohybrids can be seen from Figure 2.5.

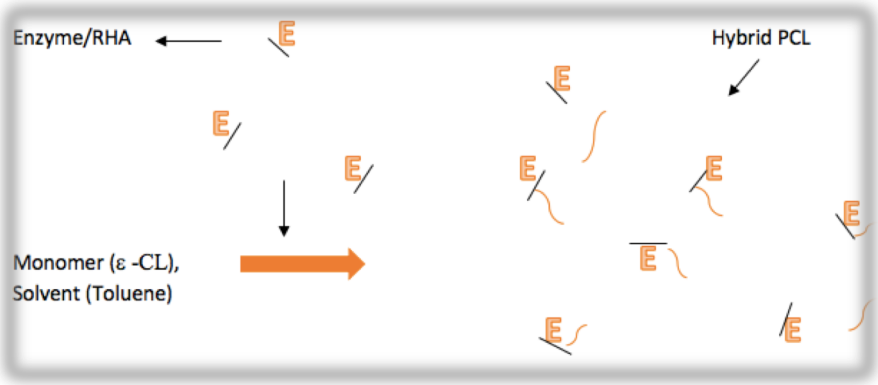

Figure 2.10: Polymerization reaction catalyzed by immobilized CALB on RHA

As shown in Figure 2.5 in the presence of a monomer, toluene and a biocatalyst (immobilized enzyme) polymer chains begin growing. When there is lipase immobilized on RHA in the environment, polymer chains graft on RHA. In this study, ε-caprolactone was used as monomer. Thus, it is aimed to obtain composites including PCL chains and PCL grafted on RHA (PCL/RHA nanohybrids).

Polymer/silica nanohybrids are generally suitable for nanomedicine applications. The examples of these nanohybrids that used in drug delivery processes are given in Table 2.3 [32].

Table 2.7: Polymer/silica hybrids for drug delivery [32].

Name of Hybrid System	Polymer Type	Drug	Description
MCF-PLA/PLGA	PLA and PLGA	Paliperidone	used in long acting release of paliperidone that is a drug used in treatment of biopolar disorder
P(NIPAM-co-MAA)/MGNSs	P(NIPAM-co-MAA)	Doxorubicin	controls release of doxorubicin under physical changes

As shown in Table 2.3, polymer/silica nanohybrids are important for controlled drug release applications. Main reasons for their usage in this field are that they maintain their stability even under harsh conditions and they have high density of active sites. The leakage of encapsulated compounds is prevented and controlled by the network between polymer and silica. In these nanohybrid systems polymers provide sustained

drug release, decrease side effects and increase therapeutic index of drugs as capping agents [32].

3. MATERIALS AND METHODS

3.1 Materials

Free CALB (Lipozyme®) from Novozymes and immobilized form of CALB (Novozyme 435®) from Sigma Aldrich were used as biocatalysts. Rice husk ash (RHA), that is high silica containing material, is considered to be a suitable material for enzyme immobilization. Rice husk ashes were obtained from the burning of rice husks. These rice husks can be obtained from rice production process as by-product. Because of being a by-product, they are cheap and easily accessible materials [33]. For this study, rice husks were provided from a rice production company in Edirne, Turkey. Surface of RHA modified with 3-Aminopropyl trimethoxysilane (3-APTMS) (95%, $C_6H_{17}O_3NSi$), 3-Glycidyloxypropyl trimethoxysilane (3-GPTMS) (98%, $C_9H_{20}O_5Si$), 3- Aminopropyl triethoxysilane (APTES) (99%, $C_9H_{23}NO_3Si$) and Trimethoxycylyl propyl ethylenediamine (TMSPDA), respectively. These silanization agents were purchased from Aldrich, Acros and Merck. 99% pure acetone (C_3H_6O), which was used as a solvent in surface modification, purchased from Riedel-de Häen. Phosphate buffer solution was prepared using monobasic sodium phosphate ($NaH_2PO_4.H_2O$) and dibasic sodium phosphate ($Na_2HPO_4.7H_2O$) salts. This solution was used for enzyme immobilization. These chemicals purchased from Carlo Erba and Merck, respectively.

ε-caprolactone (99%, $C_6H_{10}O_2$) used as monomer for polymerization reaction and obtained from Alfa Aesar. Toluene (99%, $C_6H_5CH_3$) and chloroform (99%, $CHCl_3$) were supplied from Merck and Sigma Aldrich, respectively. These chemicals were used in polymerization reaction of ε-caprolactone. Toluene was used as solvent and chloroform was used for termination. Methanol (99%, CH_3OH) from Merck was used to precipitate nanohybrids.

3.2 Methods

3.2.1 Support material preparation

Rice husks were washed with distilled water and then, dried in oven (Electro mag M3025P) for 24 h. To obtain RHA, rice husks were burned in furnace at 600-650 °C for 6 h. Before immobilization, surface of RHA modified with different silanization agents. For this modification, 250 mg RHA was mixed with 750 µl of 15 % (v/v) silanization agent (3-APTMS, 3-GPTMS, 3-APTES and TMSPDA, separately) in 5 ml acetone. The mixtures put in a water bath (Julabo SW22) and incubated at 50 °C, 160 rpm for 2 h. Then, surface activated RHA was filtered and washed with distilled water. RHA was dried at 60 °C for 4 h. Finally, TMSPDA activated RHA was mixed with 25 ml 2% (v/v) gluteraldehyde/phosphate buffer solution for 2 h.

3.2.2 Lipase enzyme immobilization

For immobilization of lipase enzyme two different methods were applied. Lipase enzyme immobilized on RHA samples that surface modification done by 3-APTMS and 3-APTES by physical adsorption. Lipase enzyme immobilized by cross-linking method on RHA that surface modification was done with 3-GPTMS and TMSPDA. For cross-linking immobilization, glutaraldehyde was used as coupling agent in support preparation for TMSPDA. When 3-GPTMS used as silanization agent, reactive epoxy ring formed on surface of support material so that enzyme can be immobilized covalently without using an additional cross-linker.

For immobilization of lipase enzyme, 250 mg surface-modified RHA was mixed with lipase enzyme (enzyme to support ratio of 2 µl/mg) and 25 ml 0.015 M pH 7.0 phosphate buffer solution on a magnetic stirrer at room temperature for 5 h. After mixing, immobilized enzyme was filtered and dried at 30 °C for 12h. Immobilized enzyme was stored at 4 °C. For phosphate buffer solution (0.015 M, pH 7.0), 29.25 ml of $NaH_2PO_4.H_2O$ solution was mixed with 45.75 ml of $Na_2HPO_4.7H_2O$ solution and mixture was completed to 1 L by distilled water.

3.2.3 Synthesis of PCL/RHA nanohybrids

The enzymes immobilized with four different silanization agents, and Novozyme 435 were used in the ROP of ε-caprolactone and development of nanohybrid systems. For polymerization reaction, 500 mg monomer (ε-caprolactone) was mixed with 1000 mg

toluene under dry nitrogen. Enzyme concentration (enzyme to monomer ratio) was 20% (w/w). For Novozyme 435, polymerization was also carried out with using higher amount (2000 mg) of toluene (NT). Reaction conditions for enzymes immobilized with using different silanization agents were given in Table 2.5. Reaction conditions were optimum temperature and time values for enzymatic PCL synthesis that obtained from previous studies.

Table 3.1: Reaction conditions for different silanization agents and Novozyme 435

Silanization Agents and Novozyme 435	Reaction Temperature (°C)	Reaction Time (h)
3-APTMS	40	48
3-GPTMS	40	48
3-APTES	60	48
TMSPDA	30	72
Novozyme 435	60	48
NT	60	48

At the end of the reactions it is aimed to obtain polycaprolactone (PCL) chains grafted onto RHA particles, thus polymer composites consisting of PCL and PCL/RHA nanohybrids. Reactions were terminated by the addition of chloroform. The reaction products were fractionated separately to obtain PCL rich and RHA rich fractions to prove that there were indeed PCL chains grafted onto RHA. For fractionation several chloroform washing and centrifugation steps were applied. PCL rich phases dried at 50 °C and RHA rich phases dried at 30 °C. RHA rich samples were characterized by Thermal Gravimetric Analysis (TGA) to determine grafting efficiencies. In later experiments, this procedure was replicated with these immobilized enzymes separately without fractionation. RHA rich phases left in the PCL rich phases to produce composite systems. The reactions were again terminated by chloroform and polymer composites precipitated with methanol. Polymer composites were dried at 30 °C. Hydrogen Nuclear Magnetic Resonance Spectroscopy (HNMR), Differential Scanning Calorimetry (DSC), X-Ray Diffraction Analysis (XRD) and Scanning Electron Microscopy (SEM) were applied for structural and thermal characterization of the composites.

PCL/RHA nanohybrids obtained after precipitation can be seen from Figure 2.6. Developments of these nanohybrids were catalyzed by immobilized enzymes and

surface modifications of these enzymes were provided by 3-APTES and TMSPDA respectively.

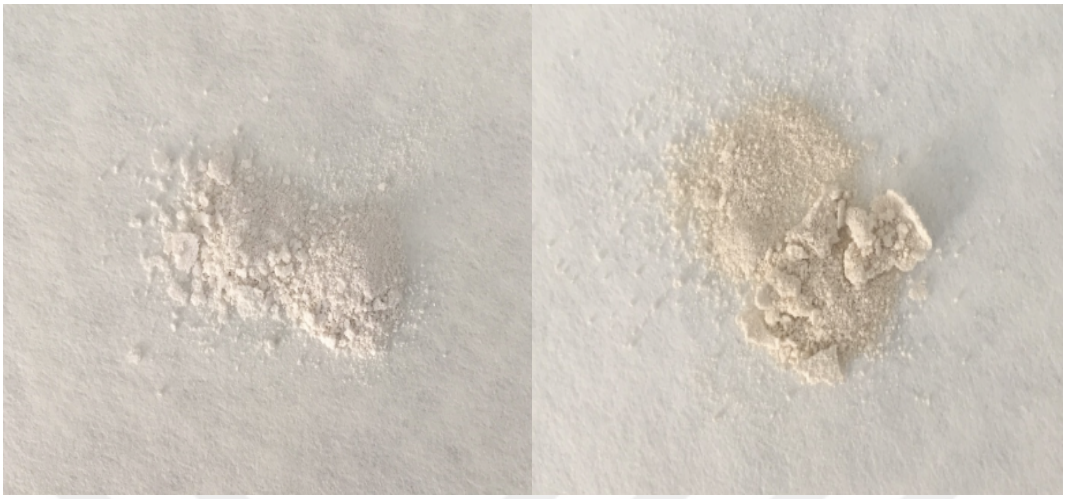

Figure 3.1: PCL/Silica Nanohybrids

3.3 Characterization Techniques

3.3.1 Thermal gravimetric analysis (TGA)

Thermal gravimetric analysis (SEIKO TG/DTA 6300) was applied to calculate grafting efficiencies of nanohybrids. 5-10 mg of samples heated to 1000 °C with changing temperature 10 °C/min and cumulative weight loss of samples were determined.

3.3.2 Proton nuclear magnetic resonance (HNMR)

Molecular weights and structural analysis of nanohybrids were determined by proton nuclear magnetic resonance (Bruker Ultrashield 300 MHz). For NMR analysis, 10 mg of samples weighed and dissolved in 750 µl of D-Chloroform in NMR tubes. Molecular weight of nanohybrids can be calculated from Equation 3.1 according to the areas of peaks obtained at (δ) values of 4.07 ppm (CH_2O) and 3.65 ppm (CH_2OH, end group) [34].

$$M_{n,NMR} = \frac{5xI(4.07)}{2xI(3.65)} x\, M_{\varepsilon-CL} \qquad (3.1)$$

According to Equation 3.1 $M_{\varepsilon\text{-CL}}$ is the molecular weight of ε-CL ($M_{\varepsilon\text{-CL}}$= 114 g/mol), $I_{4.07}$ and $I_{3.65}$ are integrated peak areas [31-34].

3.3.3 Differential scanning calorimetry (DSC)

Glass transition temperature (T_g), melting temperature (T_m) and crystallization temperature (T_c) of nanohybrid systems were measured and crystallinity degrees were determined by differential scanning calorimetry (SEIKO 7020 DSC). Thermal characterization of samples carried out between -70 and 200 °C with changing 10 °C in every minute by thermal cycles (heat-cool-heat). Crystalinity percentages (X_c) of samples can be calculated from the equation below [34].

$$Xc\ (\%) = \frac{\Delta Hm}{\Delta H°m} * 100 \qquad (3.2)$$

ΔH°m in Equation 3.2 is melting enthalpy of PCL with 100% crystalline structure (ΔH°m=139.3 J/g) [34].

3.3.4 X-ray diffraction (XRD)

For structural analysis and crystallinity comparison of nanohybrid systems, X-ray diffraction (PANalytical XPER'T PRO XRD apparatus with CuK α radiation source, λ=1.5406 Å) was used. XRD analyses performed between 15 and 30 °2 Theta values.

3.3.5 Scanning electron microscopy (SEM)

Scanning electron microscopy (JEOL JSM-6390LV SEM) was used to determine surface morphologies of nanohybrids. Scanning was performed at 5 kV with 5000X magnification.

4. RESULTS AND DISCUSSION

In this study, enzymes were immobilized on surface modified RHA by physical adsorption or cross-linking methods. For surface modification, different silanization agents (APTES, APTMS, GPTMS and TMSPDA) were used. Performance of immobilized enzymes evaluated according to immobilization efficiencies and specific activity values that obtained from previous studies. Immobilization efficiencies were found as 98%, 96%, 95%, 98% and specific activities were calculated as 4.4 U/mg, 3.6 U/mg, 3.7 U/mg, 2.8 U/mg for APTES, APTMS, GPTMS and TMSPDA lipases respectively [34, 35]. Characterization studies of PCL-based nanohybrids that synthesized by these immobilized lipases and Novozyme 435 were performed by different analyses.

4.1 Characterization studies of PCL/RHA nanohybrids and nanohybrids synthesized by Novozyme 435

Different analyses were applied for characterization of nanohybrids. Thermal Gravimetric Analyses (TGA) were conducted to RHA rich fraction for the determination of grafting efficiencies. Structural analyses were obtained and molecular weights of these nanohybrids were calculated by Hydrogen Magnetic Resonance (HNMR). Differential Scanning Calorimetry (DSC) was applied for thermal studies and by X-Ray Diffraction Analyses (XRD) crystallinity degrees were compared. Finally, surface morphologies of PCL/RHA nanohybrids were determined by Scanning Electron Microscopy (SEM).

4.1.1 Grafting efficiencies of PCL/RHA nanohybrids

Grafting efficiencies of PCL/RHA nanohybrids can be calculated from the difference between weight losses of RHA-rich fraction and surface modified-RHA. These weight losses can be determined by TGA analyses. Weight losses for RHA-rich fraction that surface of RHA modified by different silanization agents are given in Figure 4.1.

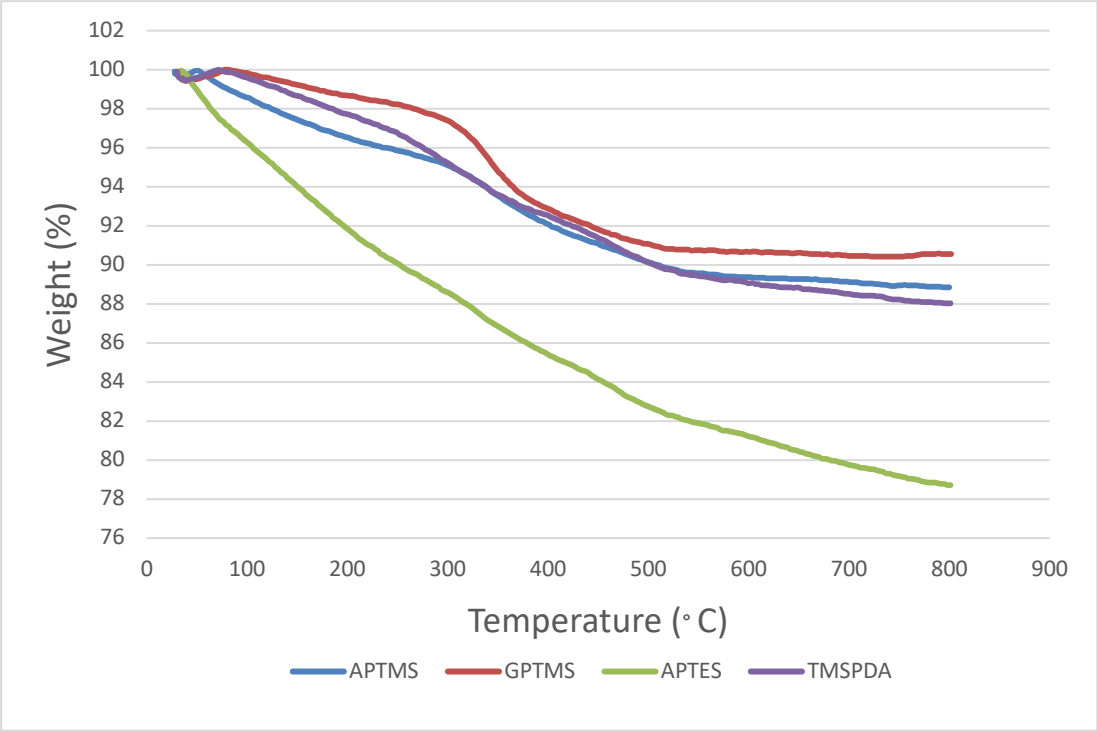

Figure 4.1: TGA curves of RHA-rich fractions with different silanization agents

As seen from Figure 4.1, weight losses for the samples are 9.5%, 11% and 21.5% for GPTMS-modified, APTMS-modified and APTES-modified respectively. For surface modified RHA samples with GPTMS, APTMS, APTES and TMSPDA; weight losses from TGA analyses in previous studies are 5.5%, 2.5%, 3.5% and 14%. These results show that higher grafting efficiency of PCL/RHA nanohybrids obtained as 18% for using APTES in surface modification of RHA. The grafting efficiencies of PCL/RHA nanohybrids for GPTMS-modified and APTMS-modified are similar and 4% and 8.5%. For samples that surface of RHA modified by TMSPDA, weight losses are almost same for RHA-rich fraction and TMSPDA-modified RHA. Thus, it can be said that there is no grafting observed for these samples.

4.1.2 Structural analysis of nanohybrids

HNMR spectrums of nanohybrids synthesized by different silanization agents (APTMS, GPTMS, APTES, TMSPDA), Novozyme 435 and NT are given from Figure 4.2 to Figure 4.7 respectively. Both structural analyses and molecular weights can be obtained from HNMR spectrums of PCL/RHA nanohybrids that synthesized by different immobilized enzymes and Novozyme 435. In HNMR spectrum, peaks at 4.07

ppm (t, CH_2O, main chain), 3.65 ppm (t, CH_2OH, end group), 2.32 ppm (t, CH_2CO), 1.6-1.7 ppm (m, CH_2) and 1.37 ppm (m, CH_2) are characteristics of PCL [36]. The following figures show that nanohybrids that synthesized by both immobilized lipases and Novozyme 435 include all characteristics of PCL in their structure as expected. Moreover, molecular weights of PCL/RHA nanohybrids were calculated from Equation (1) using areas of peaks at 4.07 ppm and 3.65 ppm in HNMR spectrums.

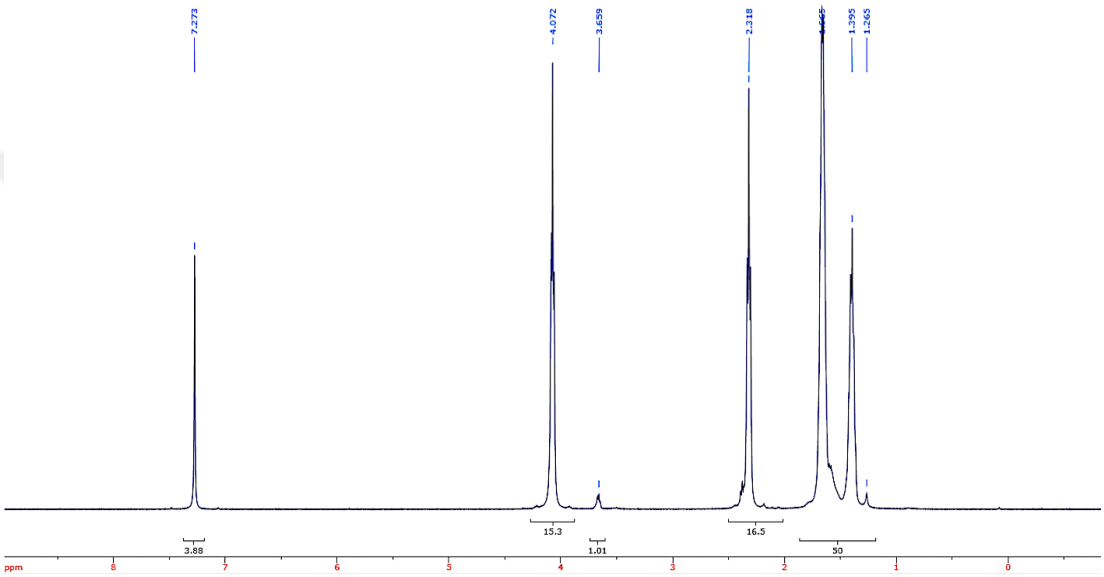

Figure 4.2: HNMR spectrum of PCL/RHA nanohybrids synthesized by APTMS lipase

HNMR spectrum for PCL/RHA nanohybrids synthesized by APTMS lipase can be seen in Figure 4.2. According to this spectrum, areas of peaks at 4.07 ppm and 3.65 ppm are 15.3 and 1.01 respectively. Molecular weight of these nanohybrids was calculated as in Equation (1) with using these peak areas and molecular weight of ε-CL. Molecular weight of ε-CL is 114 g/mol [34]. Thus, molecular weight of PCL/RHA nanohybrids synthesized by APTMS lipase was found 4317 g/mol.

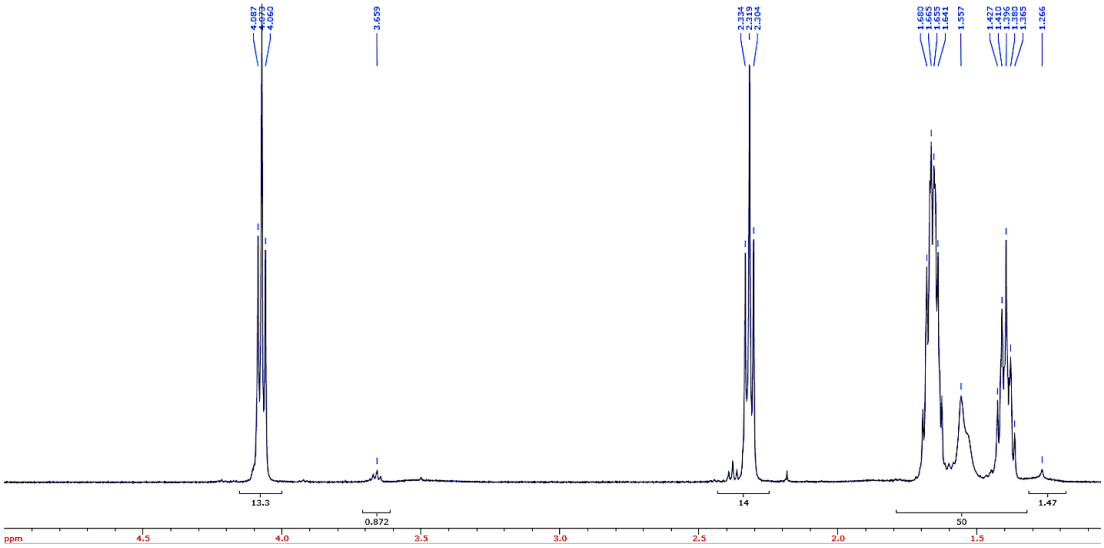

Figure 4.3: HNMR spectrum of PCL/RHA nanohybrids synthesized by GPTMS lipase

According to HNMR spectrum for PCL/RHA nanohybrids synthesized by GPTMS lipase as in Figure 4.3. molecular weight was calculated as 4346 g/mol.

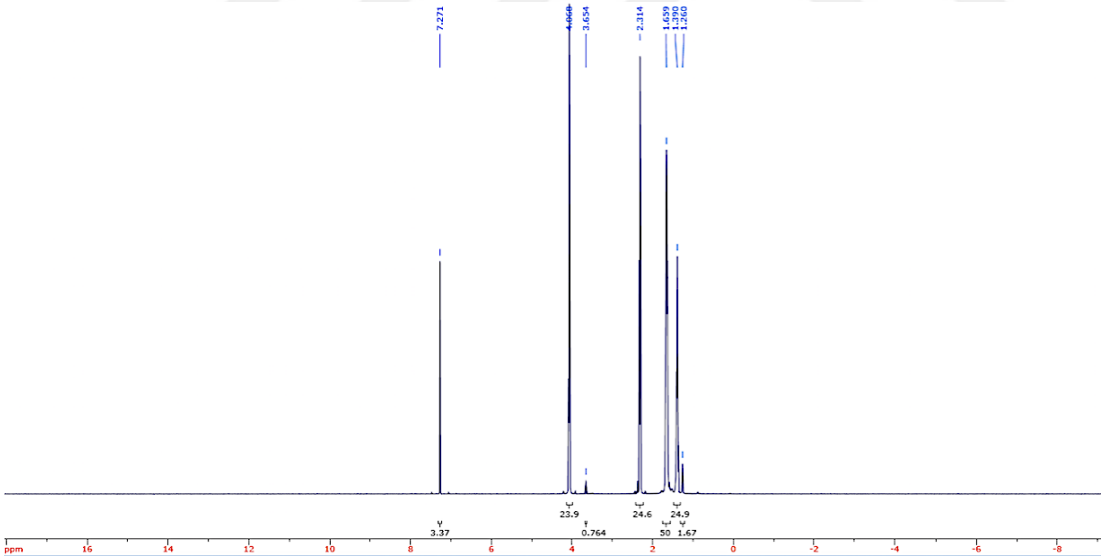

Figure 4.4: HNMR spectrum of PCL/RHA nanohybrids synthesized by APTES lipase

Molecular weight for PCL/RHA nanohybrids synthesized by APTES lipase was calculated with using peak areas of 4.07 ppm and 3.65 ppm in Figure 4.4. Molecular weight was found 8916 g/mol.

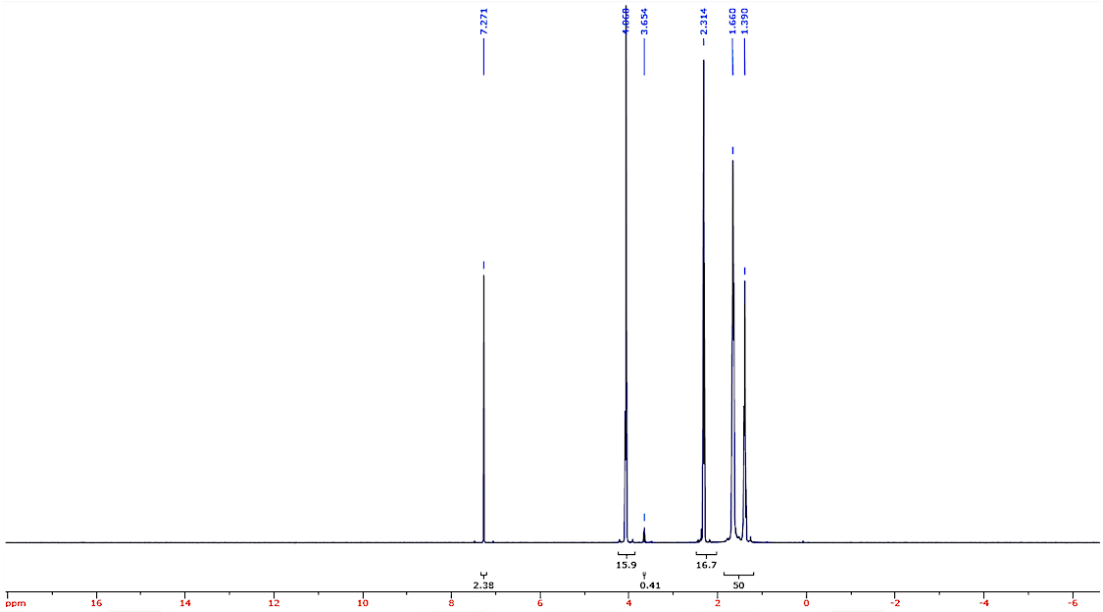

Figure 4.5: HNMR spectrum of PCL/RHA blends synthesized by TMSPDA lipase

HNMR spectrum of PCL/RHA blends synthesized by TMSPDA lipase can be seen in Figure 4.5 and their molecular weight was calculated as 11052 g/mol.

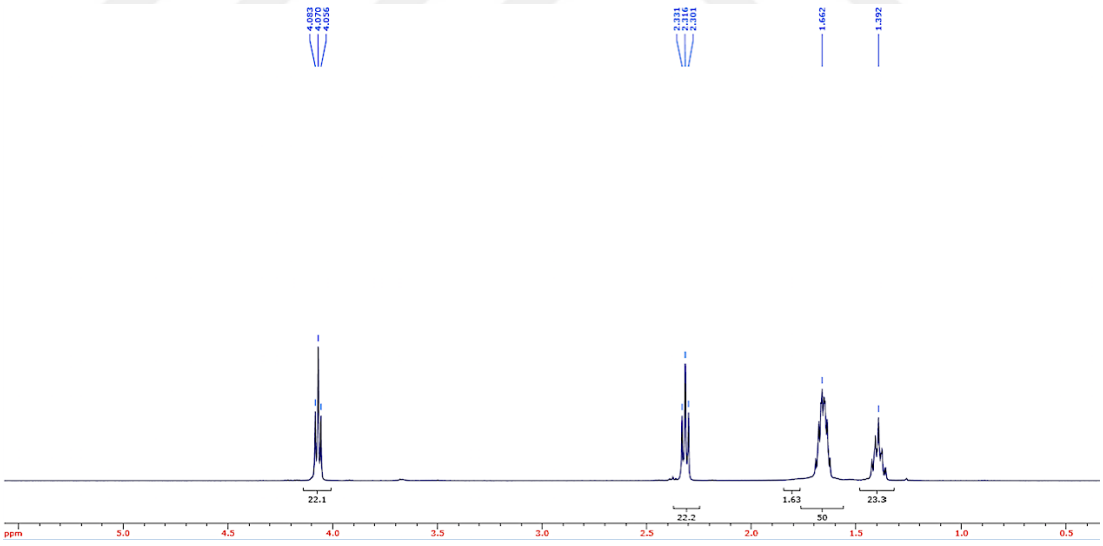

Figure 4.6: HNMR spectrum of nanohybrids synthesized by Novozyme 435

HNMR spectrum of nanohybrids synthesized by Novozyme 435 can be seen from Figure 4.6 and their molecular weight was calculated as 43140 g/mol.

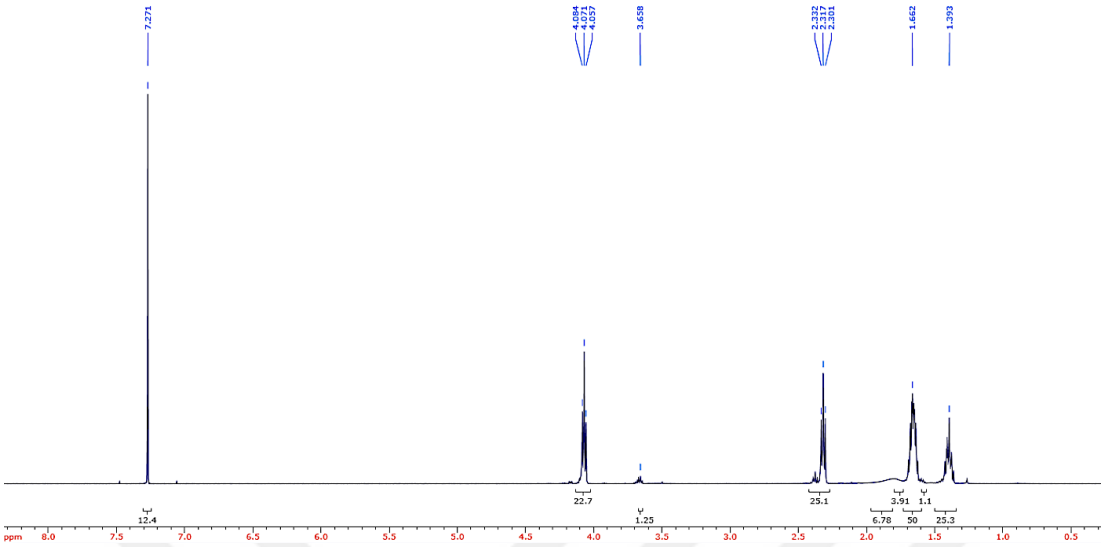

Figure 4.7: HNMR spectrum of nanohybrids synthesized by Novozyme 435 with higher amount of toluene (NT)

HNMR spectrum of synthesized by Novozyme 435 with using higher amount of toluene can be seen in Figure 4.7. Molecular weight was found 5176 g/mol.

4.1.3 Thermal characterization of nanohybrids

DSC thermograms of PCL/RHA nanohybrids synthesized with different silanization agents APTMS, GPTMS, APTES and TMSPDA can be seen in Figure 4.8 and nanohybrids synthesized by Novozyme 435 can be seen from Figure 4.9. Crystallinity degrees were calculated according to Equation (2) with using data obtained by DSC analyses. Thermal characterization and crystallinity degrees of PCL/RHA nanohybrids were summarized in Table 4.1.

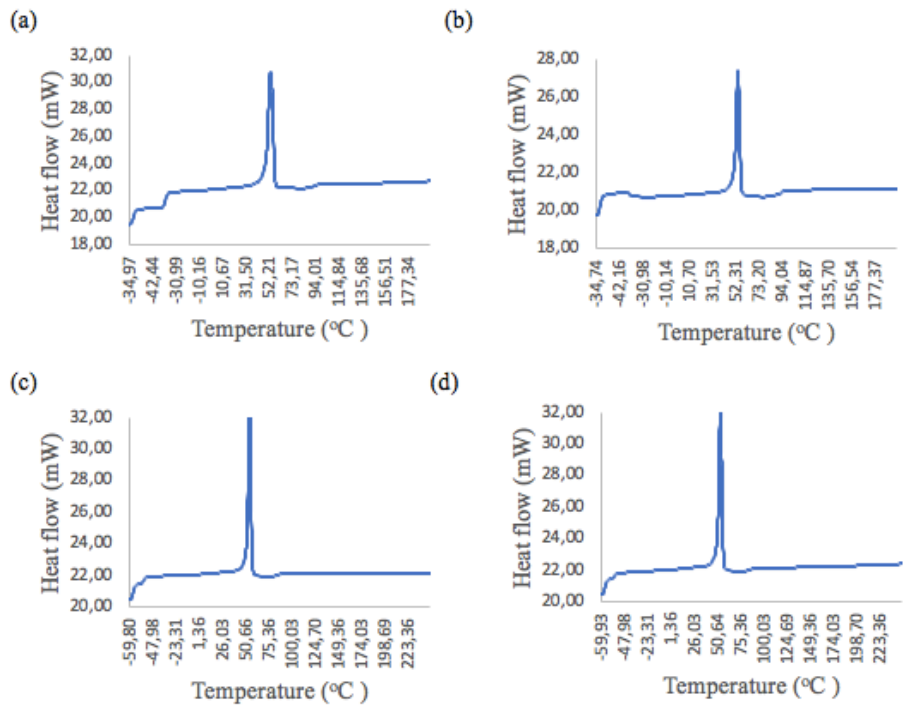

Figure 4.8: DSC thermograms of PCL/RHA nanohybrids with different silanization agents (a) APTMS, (b) GPTMS, (c) APTES and (d) TMSPDA

As seen from DSC thermograms that given in Figure 4.8, melting temperatures (T_m) of PCL/RHA nanohybrids synthesized by APTMS, GPTMS, APTES and TMSPDA lipases are almost same (54 °C). Their crystallization temperatures (T_c) are also found from DSC analyses. Crystallizaiton temperatures found very similar for both lipases (about 35 °C).

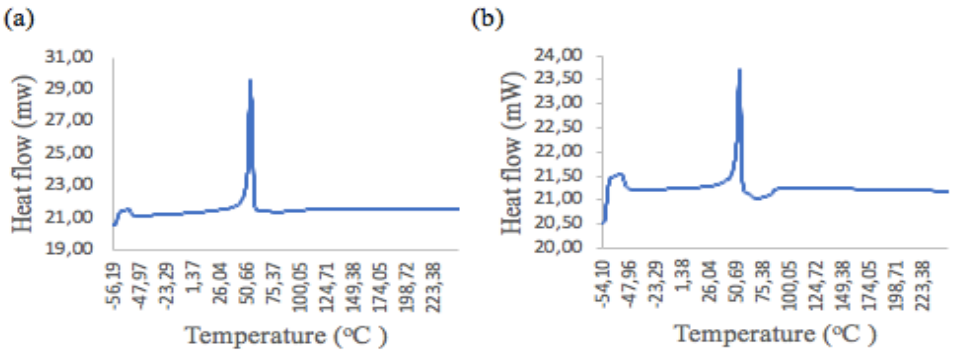

Figure 4.9: DSC thermograms of nanohybrids synthesized by (a) Novozyme 435 (b) NT

Melting temperatures of nanohybrids synthesized by Novozyme 435 and NT are also 54 °C as in immobilized lipases. Their crystallization temperatures are lower than PCL/RHA nanohybrids with the average value of 30 °C. According to literature, melting temperature for PCL blends changes from 50 °C to 60 °C and their crystallization temperature is about 40 °C [37, 38].

Table 4.1: Thermal properties of nanohybrid systems determined by DSC analyses

Nanohybrids	T_m (°C)	T_c (°C)	ΔH_f (J/g)	Crystallinity (%)
3-APTMS	53.7	34.4	44.2960	31.9
3-GPTMS	54.2	36.8	31.4395	22.6
3-APTES	54.7	33.9	53.6254	38.6
TMSPDA	53.7	34.2	54.5625	39.3
Novozyme 435	54.2	31.0	44.6972	32.1
NT	54.0	29.7	11.7520	8.4

As seen in Table 4.1, nanohybrids that synthesized with APTMS, APTES and TMSPDA lipases have similar crystallinity degrees. Their crystallization degrees change between 30% to 40%. However, nanohybrids that synthesized with GPTMS lipase have lower crystallinity degree as the value of 23%. According to literature, PCL/silica nanohybrids generally have crystallinity values between 35-55%. The interaction of PCL with silica nanoparticles may be the reason for the decrease in crystallinity [39]. In addition to these, crystallinity of nanohybrids that synthesized by Novozyme 435 have similar crystallinities with 32% but nanohybrids synthesized by Novozyme 435 with including more toluene (NT) give amorphous structure with very low crystallinity degrees as the value of 8 %.

4.1.4 Crystallinity structures of PCL/RHA nanohybrids

XRD patterns for PCL/RHA nanohybrids synthesized by APTMS, GPTMS, APTES and TMSPDA lipases are given in Figure 4.10.

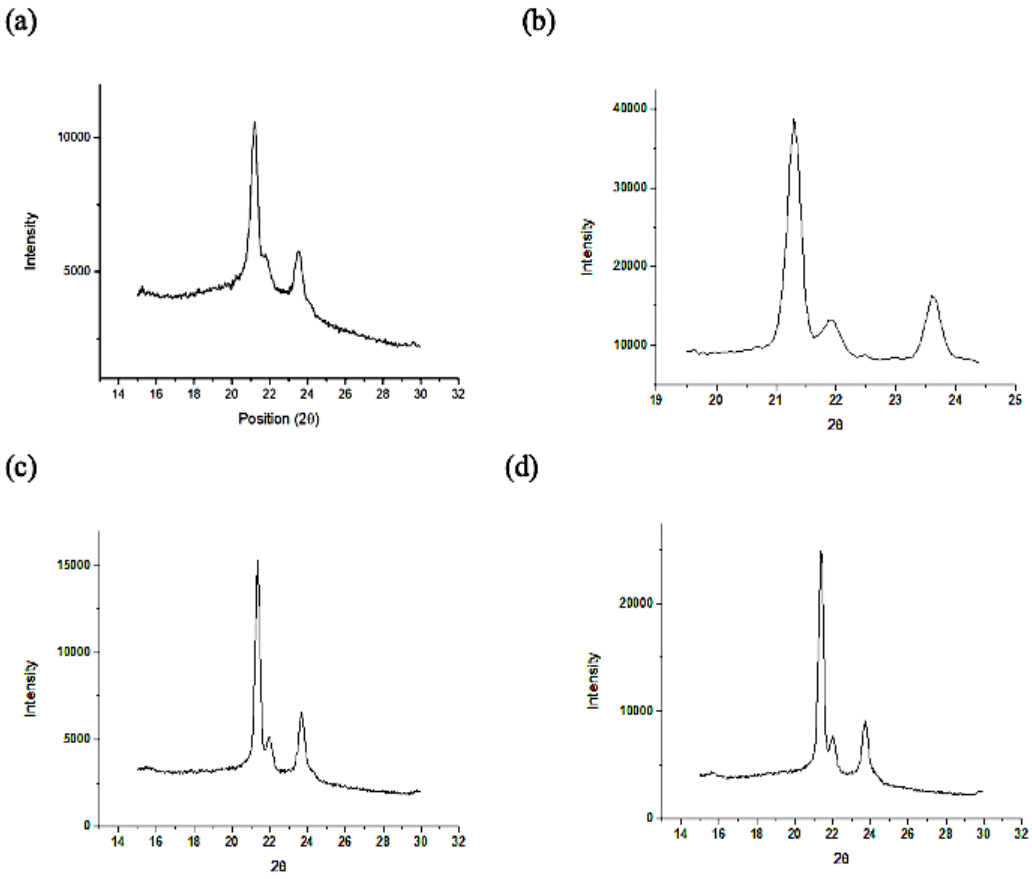

Figure 4.10: XRD patterns of PCL/RHA nanohybrids with different silanization agents (a) APTMS, (b) GPTMS, (c) APTES and (d) TMSPDA

Characteristics of PCL in crystalline structure give peaks at 21.3° and 23.7° in XRD pattern. The shoulder peak for PCL is at 22.0°. In addition to PCL, RHA give peak at about 21.0° in XRD pattern [38, 40]. As shown in Figure 4.10, PCL/RHA nanohybrids synthesized by both immobilized lipases give three peaks at 21.0°, 22.0° and 23.0°. The intensity values obtained from XRD patterns of PCL/RHA nanohybrids that synthesized by APTMS, GPTMS, APTES and TMSPDA lipases are 10000, 8000, 15000 and 25000 respectively. Intensity values in XRD patterns are proportional with crystallinity degrees. Higher intensity values show higher crystallinity values [39].

4.1.5 Surface morphology of PCL/RHA nanohybrids

Surface morphologies of PCL/RHA nanohybrids can be seen from SEM images in Figure 4.11.

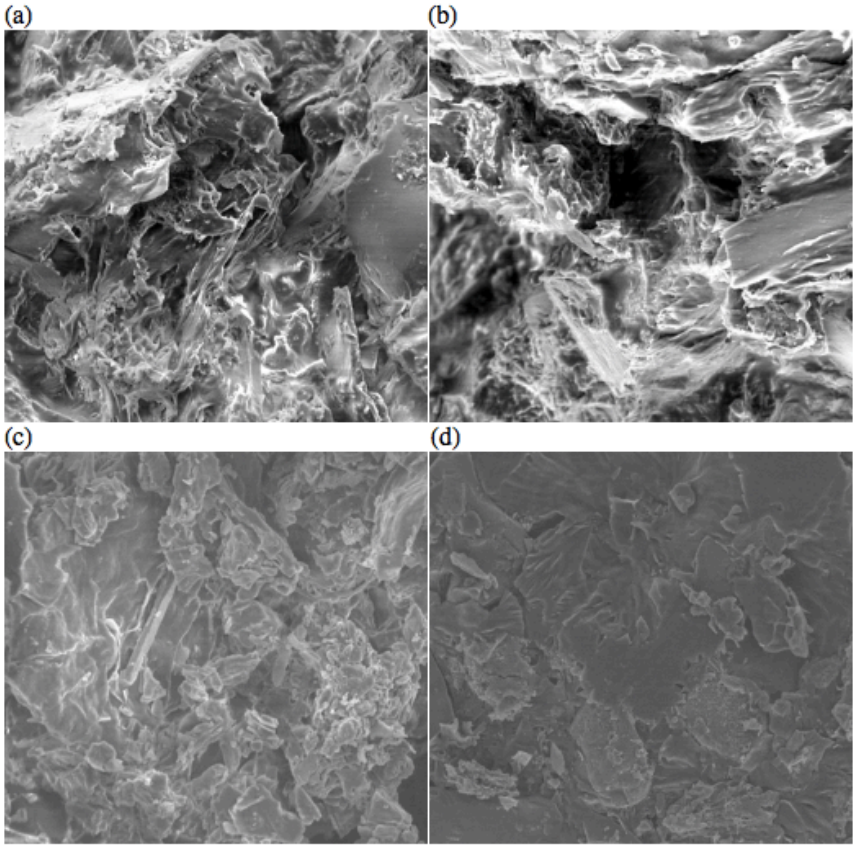

Figure 4.11: SEM images of PCL/RHA nanohybrids with different silanization agents (a) APTMS, (b) GPTMS, (c) APTES and (d) TMSPDA [5kv,5000x]

As seen from Figure 4.11, PCL/RHA nanohybrids have porous, foam-like structure with big holes on the surface. Porous morphology is important for both tissue engineering and drug delivery applications. Porous networks provide cell attachment, growth and proliferation. In addition to this, drugs entrapped with these pores and released with controlled mechanism. Pore sizes of these nanohybrids are important for drug delivery applications. These pores should be large enough to enable diffusion of therapeutic agents to use be used in controlled release systems [41, 42, 44]. RHA has also porous structure [34, 43]. Moreover, it can be seen from Figure 4.11 that

PCL/RHA nanohybrids synthesized by TMSPDA lipase are dispersed whereas others are agglomerated.

4.2 Effects of silanization agents on PCL/RHA nanohybrids and PCL

Characterization results for PCL/RHA nanohybrids that obtained by using different immobilized enzymes can be compared by PCL results that determined in previous studies.

4.2.1 Effects on molecular weight

Effects of different silanization agents on molecular weights of PCL and PCL/RHA nanocomposites for APTES-, APTMS-modified RHA and GPTMS-, TMSPDA-modified RHA are given in Figure 4.12 and Figure 4.13 respectively.

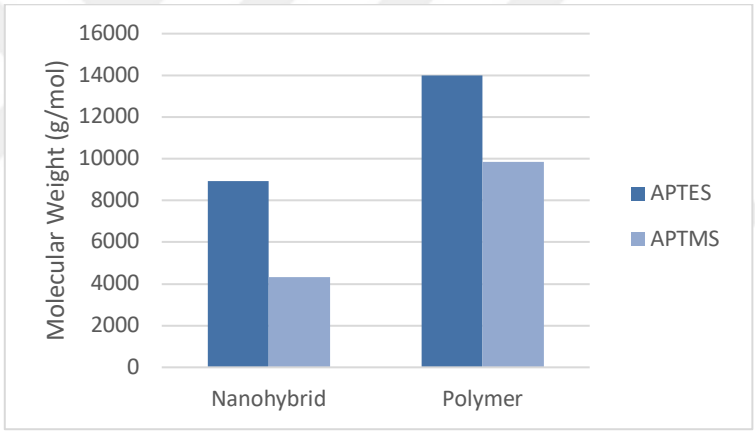

Figure 4.12: Effect of APTES- or APTMS-modified RHA on molecular weight of PCL/RHA nanohybrids and PCL

As mentioned before, for APTES-modified RHA molecular weight of PCL/RHA nanohybrids calculated as 8915.6 g/mol and for APTMS-modified it is 4317 g/mol. CALB enzyme immobilized on surface modified RHA by physical adsorption for both APTES-modification and APTMS-modification. Molecular weights of PCL for these silanization agents are 14000 g/mol and 9858 g/mol respectively. These results show that PCL/RHA nanohybrids are short-chained according to PCL. Moreover, it can be said that APTES-modified RHA is more suitable for the formation of PCL/RHA nanohybrids and PCL synthesis than APTMS-modified one since it gives higher molecular weight.

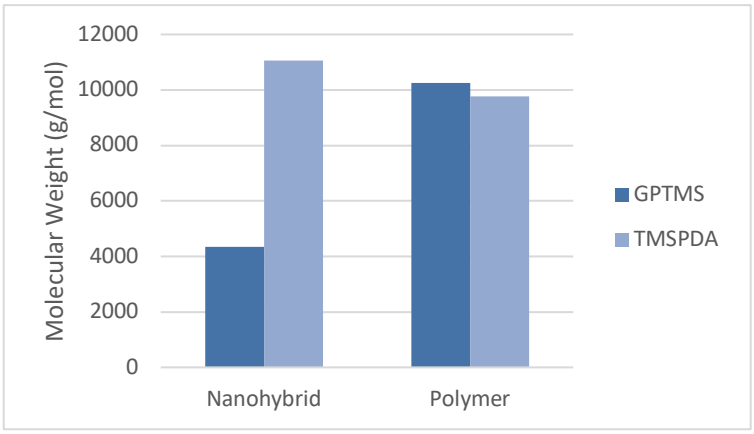

Figure 4.13: Effect of GPTMS- or TMSPDA-modified RHA on molecular weight of PCL/RHA nanohybrids and PCL

Molecular weight of PCL/RHA nanohybrids found as 4346 g/mol and 11052.4 g/mol for GPTMS-modified and TMSPDA-modified RHA. Molecular weights of PCL for these silanization agents are 10252 g/mol and 9772 g/mol respectively. According to these results, it can be said that there is no too much difference between molecular weights of PCL/RHA nanohybrids and PCL in the presence of TMSPDA-modified RHA. However, for using GPTMS-modified RHA molecular weight of PCL obtained higher than PCL/RHA nanohybrids. It shows that short-chained PCL/RHA nanohybrids synthesized from using GPTMS as silanization agent in surface modification of RHA.

Similar molecular weight results are found in literature for PCL/Cloisite 30B nanohybrids synthesized by CALB immobilized on Cloisite 30B. The molecular weights of nanohybrids change from 3000 g/mol to 8000 g/mol depending on the concentration of Cloisite 30B used [31].

4.2.2 Effects on crystallinity

Effects of different silanization agents on crystallinity degrees of PCL and PCL/RHA nanocomposites for APTES-, APTMS-modified RHA and GPTMS-, TMSPDA-modified RHA are given in Figure 4.14 and Figure 4.15 respectively.

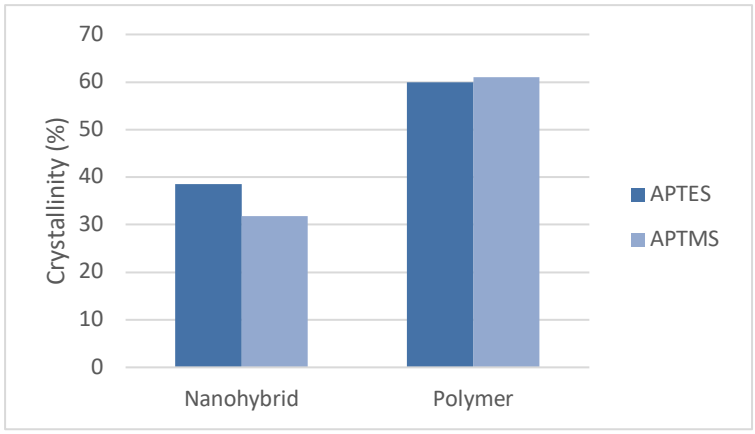

Figure 4.14: Effect of APTES- or APTMS-modified RHA on crystallinity of PCL/RHA nanohybrids and PCL

For APTES-modified RHA and APTMS-modified RHA, crystallinity degrees of PCL/RHA nanohybrids calculated as 39% and 32%. Crystallinity degrees of PCL that synthesized by CALB that immobilized on APTES-modified and APTMS-modified RHA are higher than results of nanohybrids with the values of 60% and 61%. Thus, it can be said that PCL/RHA nanohybrids show amorphous structures whereas PCL is semi-crystalline. Crystallinity degrees for these two silanization agents are very similar for both PCL/RHA nanohybrids and PCL in themselves.

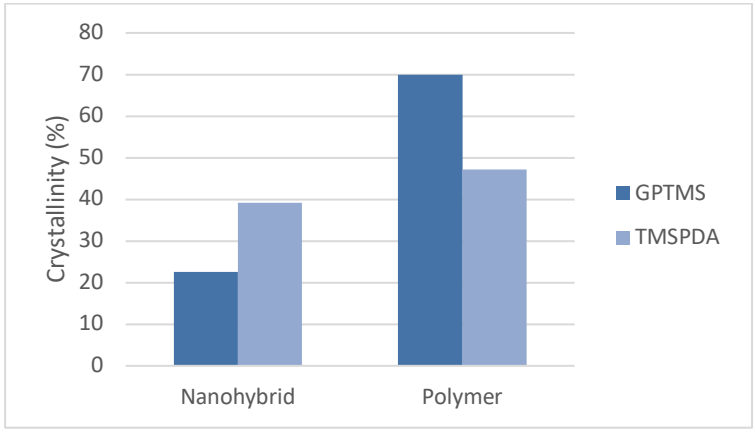

Figure 4.15: Effect of GPTMS- or TMSPDA-modified RHA on crystallinity of PCL/RHA nanohybrids and PCL

Crystallinity degrees of PCL/RHA nanohybrids calculated as 23% and 39% for GPTMS-modified RHA and TMSPDA-modified RHA. Crystallinity degrees of PCL that synthesized by CALB that immobilized on GPTMS-modified and TMSPDA-modified RHA are higher than crystallinities of nanohybrids with the values of 70%

and 47%. In the presence of TMSPDA, both PCL/RHA nanohybrids and PCL give similar crystallinity degrees. On the other hand, for GPTMS-modified RHA crystallinity of PCL/RHA nanohybrids obtained very low but PCL crystallinity becomes very high.

For both silanization agents, it was seen that crystallinity of PCL/RHA nanohybrids are lower than pure PCL. In other words, it can be said that crystallinity of PCL decreases in the presence of RHA. The possible reason for this situation is that crystal formation of PCL hindered by inorganic material (RHA) [45].

Crystallinity degrees of PCL/Cloisite 30B nanohybrids synthesized by CALB immobilized on inorganic Cloisite 30B are also similar but a little bit higher than crystallinities of PCL/RHA nanohybrids. The crystallinities for PCL/Cloisite 30B nanohybrids are calculated almost 50% [31]. According to these results, it can be said that PCL/RHA nanohybrids show more amorphous structure than PCL/Cloisite 30B nanohybrids.

According to all molecular weight and crystallinity results it can be seen that crystallization of PCL/RHA nanohybrids increase with the increase of molecular weights because free energy required for nucleation decreases with increasing molecular weights thus, crystallization becomes easier [45].

5. CONCLUSIONS AND RECOMMENDATIONS

This study includes two parts as lipase enzyme immobilization and development of PCL/RHA nanocomposite systems. In this study, CALB immobilized on surface activated RHA both by physical adsorption and cross-linking methods. Four different silanization agents (APTES, 3-APTMS, 3-GPTMS and TMSPDA) were used for the activation of surface of RHA. Enzymes immobilized on APTES- and 3-APTMS-modified RHA by physical adsorption and on 3-GPTMS- and TMSPDA-modified RHA by cross-linking methods. Nanocomposite systems that include PCL and PCL/RHA nanohybrids were synthesized with using these immobilized enzymes. In addition to PCL/RHA nanohybrids, PCL including nanohybrids were obtained with using Novozyme 435. Grafting efficiencies of these nanohybrids were determined by TGA analyses that applied RHA-rich fractions of samples. Molecular weights and structural analyses determined by HNMR. For crystallinity degrees, XRD analyses and for thermal properties, DSC analyses were applied. Finally, surface morphologies of these nanocomposites were determined by SEM analyses.

According to results obtained from TGA analyses, grafting efficiencies of PCL/RHA nanohybrids were found as 4%, 8.5% and 18% for GPTMS-, APTMS- and APTES-lipases respectively. It can also be said from TGA analyses that PCL was not grafted on RHA in the presence of TMSPDA-lipase. With grafting efficiency of 18%, it was seen that APTES is a suitable silanization agent for the development of PCL/RHA nanohybrids. Molecular weights of PCL/RHA nanohybrids were calculated from HNMR analyses. Molecular weights were found as 4317 g/mol, 4346 g/mol, 8916 g/mol and 11052 g/mol for APTMS-, GPTMS-, APTES- and TMSPDA-lipases respectively. In the presence of APTMS- and GPTMS-lipases, short-chained PCL was obtained. PCL/RHA nanohybrids synthesized by APTES-lipases are long-chained with higher molecular weight and show better polymerization. Crystallinity degrees that calculated from DSC analyses are 32%, 23%, 39% and 39% for APTMS-, GPTMS-, APTES- and TMSPDA-lipases respectively. These crystallinity degrees are also proportional to peak intensities obtained from XRD analyses. Higher crystallinity

in nanocomposites decreases diffusion rate because of more tortuous path. That's why, crystallinity in PCL/RHA nanohybrids should be low to increase in drug delivery systems [38]. GPTMS is suitable silanization agent for drug delivery applications with its lowest crystallinity degree. PCL/RHA nanohybrids give peaks at 21.3° and 23.7° in XRD analyses. These peaks are characteristics of PCL in crystalline structure. Finally, surface morphologies were obtained by SEM analyses. According to SEM results, it is seen that PCL/RHA nanohybrids are porous with big holes on surface for both immobilized lipases. However, nanohybrids synthesized by APTMS- and GPTMS-lipases are more agglomerated. On the other hand, others are more dispersed.

PCL based nanohybrids were also synthesized by Novozyme 435. Molecular weights of these nanohybrids were found 43140 g/mol and 5176 g/mol for Novozyme 435 and NT respectively by HNMR analyses. Therefore, it can be said that highest monomer conversion and best polymerization was obtained by Novozyme 435. In addition to this, crystallinity degrees for these nanohybrids were obtained 32% and 8% by DSC analyses.

In conclusion, PCL/RHA nanohybrids were obtained by APTMS-, GPTMS- and APTES-lipases so it can be said that these immobilized lipases are suitable for nanocomposite synthesis. PCL based nanohybrids, thus PCL/RHA nanohybrids can be used in tissue engineering with their biocompatible properties. Moreover, they can be used in controlled drug release with reducing toxic side effects and increasing efficiency of drugs [8].

Nanocomposites have improved mechanical properties, thermal resistance and chemical reagent resistance compared to pure PCL. Improved pysical properties of PCL/RHA nanohybrids provide that they can be used in broad range of areas [46-49]. In further studies, PCL/RHA nanohybrids may be used in controlled release of dug molecules.

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selectivity compared to lipase from Candida antractica B adsorbed on other hydrophobic supports, *Journal of Molecular Catalysis B: Enzymatic*, **57**, 171-176.

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- Kaptan, Y., Akkulak, M., Aydın, Y. A. and Avcibasi-Guvenilir, Y., 2018: Synthesis of PCL/silica nanohybrid systems via enzyme-catalyzed ROP. *17th Polymers and Organic Chemistry Conference,* June 3-7, 2018 Palavas, France. (Oral Presentation)
- Aydın, Y. A., Kaptan, Y., Akkulak, M. and Avcibasi-Guvenilir, Y., 2019: Lipase Mediated Synthesis of Polycaprolactone and Silica Based Nanohybrid. *Pure and Applied Chemistry,* https://doi.org/10.1515/pac-2018-1011.