ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL

DRUG LOADING OF G-PCL/RHA NANOHYBRID SYSTEM AND PH EFFECT ON DRUG RELEASE

M.Sc. THESIS

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Department of Chemical Engineering

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ISTANBUL TEKNİK ÜNİVERSİTESİ ★ LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

G-PCL/RHA NANOHİBRİT SİSTEMİNE İLAÇ YÜKLENMESİ VE İLAÇ SALIMINA PH ETKİSİ

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Çağla Menteşoğlu, a M.Sc. student of İTU Graduate School student ID 506191034, successfully defended the thesis entitled "DRUG LOADING OF G-PCL/RHA NANOHYBRID SYSTEM AND PH EFFECT ON DRUG RELEASE", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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To my family,

FOREWORD

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June 2021

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ABBREVIATIONS

3-GPTMS **:** 3-glycidyloxypropyl trimethoxysilane
CAL-B **:** *Candida antarctica* lipase B
DDS **:** Drug delivery system
DSC **:** Differential scanning calorimetry
ε-CL **:** Epsilon caprolactone
eROP **:** Enzymatic ring opening polymerization
FT-IR **:** Fourier transform infrared spectroscopy
G-PCL **:** Dissociated poly(ε-caprolactone) from nanohybrid
G-PCL/RHA **:** Poly(ε-caprolactone) and 3-GPTMS modified RHA nanohybrid
O/W **:** Oil in water
PCL **:** Poly(ε-caprolactone)
PVA **:** Polyvinyl alcohol
RHA **:** Rice husk ash
ROP **:** Ring opening polymerization
SEM **:** Scanning electron microscopy
TGA **:** Thermal gravimetric analysis
UV **:** Ultraviolet
w/w **:** Weight per weight ratio

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DRUG LOADING OF G-PCL/RHA NANOHYBRID SYSTEM AND PH EFFECT ON DRUG RELEASE

SUMMARY

Biodegradable polymers are used in various fields such as controlled drug delivery, anticancer drug delivery, protein and peptide delivery, gene delivery, and enzyme immobilization. They are commonly used in biomedical and pharmaceutical fields because of advantageous properties like tissue compatibility, biodegradability, low toxicity, sustained release property, unlike non-biodegradable polymers. Moreover, they degrade in the living body by in vivo enzymatic actions or chemical reactions after transform to biocompatible form and excrete from the body. Poly(ε-caprolactone) (PCL) is one of the biodegradable polymers that are used in numerous fields such as long-term drug delivery systems due to degrading in prolonged time and scaffolds in tissue engineering. PCL is mainly synthesized by ring-opening polymerization (ROP) of ε-caprolactone (ε-CL) monomer. Although metal-based catalysts are the most used catalysts for ROP of ε-CL, enzymes also gain importance for catalysis of polymerization. Enzymatic ROP (eROP) has advantages like not producing toxic products and operating under mild conditions. Candida antarctica lipase B (CAL-B) is an effective and high selective biocatalyst and can be used as the catalyst for eROP of ε-CL.

Drug delivery systems (DDS) are developed to prevent problems caused by conventional drug delivery systems in recent years. DDSs aim to keep the drug levels stable in a desired therapeutic range after a single dose, unlike conventional DDSs. In addition, DDSs enhance the efficacy of drugs with decreasing doses without side effects and prevent any harm on healthy tissue. There are criteria about these systems such as should be biospecific, non-toxic, biocompatible, and biodegradable. pH-sensitive DDSs are most widely used in the design of nano-systems in cancer therapy. pH values of tissues or organs vary along the gastrointestinal tract and can change during the disease conditions. Polymeric carriers are organic hosts that can be biodegradable or water-soluble polymer matrices used in DDSs and can be designed as stimuli-responsive. Microspheres are used for sustained controlled drug release and preparation techniques of microspheres offer to control drug administration. Chalcones are flavonoids that have two aromatic rings bonded by a three-carbon α,β-unsaturated carbonyl group and have lots of pharmacological and biological activities like anticancer activity.

In the previous study, rice husk ash (RHA), a support material, was obtained by burning rice husks. Surface modification of RHA was provided by silanization with 3-GPTMS, 3-APTMS and APTES organosilanes. After, the enzyme Candida antarctica lipase B (CAL-B) was immobilized on surface-modified RHA. In this study, only surface-modified RHA with organosilane 3-GPTMS was used. The immobilized enzyme CAL-B onto surface-modified RHA was catalyzed the ROP of ε-caprolactone. After this, obtained nanohybrid and pure PCL was named as G-PCL/RHA nanohybrid and pure G-PCL polymer respectively. Drug-loaded

microspheres were prepared by O/W emulsion-solvent evaporation method which chalcone was used as drug active substance. Drug release experiments were carried out in different pH conditions and drug release profiles were investigated.

The first part of the study, enzymatic ring-opening polymerization of ε-caprolactone was provided by immobilized enzyme Candida antarctica lipase B (CAL-B). To obtain G-PCL/RHA nanohybrid, after terminating the reaction with chloroform, the mixture was added to a stirring methanol solution and precipitation was observed. To obtain pure G-PCL polymer was also obtained by washing the mixture with chloroform and filtrate was dried. Microspheres were prepared by the O/W emulsion-solvent evaporation method. G-PCL/RHA nanohybrid and trans-chalcone were dissolved in chloroform and dichloromethane respectively for an organic phase. 1% PVA solution was prepared for an aqueous phase. The dissolved substances were added to the stirring PVA solution. After 24 h, evaporation of the solvent was observed, the mixture was centrifuged and dried. All of these experiments were also applied for pure G-PCL samples. After these procedures drug-loaded G-PCL/RHA and G-PCL microspheres were obtained. For calculations of encapsulation efficiency, drug-loaded microspheres and methanol were stirred for 3 h in a magnetic stirrer to solve the encapsulated drug. The mixture was filtrated to obtain the dissolved drug and analyzed by ultraviolet (UV). Encapsulated drug concentration and efficiency were calculated. For in vitro drug release experiments, different pH conditions were simulated by buffer solutions. The operating pH values were 3.6, 5.6, 7.4, and 8.0 throughout the study. Microspheres were added to buffer solutions in test tubes and test tubes were placed in a shaking water bath. At certain times, samples were collected from test tubes and analyzed by UV. The release drug concentrations and cumulative drug release were calculated. Characterization of drug-loaded G-PCL/RHA microspheres was done by Fourier transform infrared spectroscopy (FT-IR), thermal gravimetric analysis (TGA), differential scanning calorimetry (DSC), scanning electron microscopy (SEM), and water contact angle (WCA) measurement.

FT-IR results showed that characteristic PCL peaks in the G-PCL/RHA microsphere spectra. In addition, FT-IR spectra of G-PCL/RHA microsphere and trans-chalcone were compared. Accordingly, the presence of chalcone in G-PCL/RHA microspheres was proven. TGA results showed the decomposition temperatures and organic weight losses of drug-loaded G-PCL/RHA microspheres and chalcone. DSC results showed the melting temperature of G-PCL/RHA microspheres. Microsphere structures were observed in SEM pictures. Drug release results showed the highest cumulative drug release percentage was 97.3% at pH 3.6 following 98.0% at pH 5.6. Drug release was more efficient in acidic conditions. Pure G-PCL microspheres also showed the same drug release performance but not as high drug release as G-PCL/RHA nanohybrids.

The drug release results can be changed in vivo studies by the degradation of PCL by lipases and esterases. In further studies, these drug release experiments can be carried out in vivo. Also, for better understanding drug-polymer interactions and pH sensitivity of the drug release mechanism analyze methods can be developed. Such studies exist in the literature.

G-PCL/RHA NANOHİBRİT SİSTEMİNE İLAÇ YÜKLENMESİ VE İLAÇ SALIMINA PH ETKİSİ

ÖZET

Biyobozunur polimerler, kontrollü ilaç taşıyıcı sistem, antikanser ilaç taşıyıcılığı, protein ve peptit taşıyıcılığı, gen taşıyıcılığı ve enzim immobilizasyonu gibi çeşitli alanlarda kullanılmaktadır. Biyobozunur olmayan polimerlerden farklı olarak doku uyumluluğu, biyolojik olarak parçalanabilirlik, düşük toksisite, sürekli salım özelliği gibi avantajlı özelliklerinden dolayı biyomedikal ve farmasötik alanlarda yaygın olarak kullanılmaktadırlar. Ayrıca, biyouyumlu forma dönüşerek canlı organizmada in vivo enzimatik faaliyerler veya kimyasal reaksiyonlarla bozunurlar ve vücuttan atılırlar. Poli(ε-kaprolakton) (PCL), uzun sürede bozunması nedeniyle uzun süreli ilaç taşıyıcı sistemlerde ve doku mühendisliğinde yapı iskeleleri gibi birçok alanda kullanılan biyobozunur polimerlerden biridir. Polikaprolakton ana olarak ε-kaprolakton (ε-CL) monomerinin halka açılma polimerizasyonu (ROP) ile sentezlenir. Metal bazlı katalizörler ε-kaprolakton'un halka açılması polimerizasyonu için en çok kullanılan katalizörler olmasına rağmen, enzimler de polimerizasyonun katalizinde önem kazanmaktadır. Enzimatik halka açılma polimerizasyonu (eROP), toksik ürün üretmemesi ve ılımlı koşullarda çalışması gibi avantajlara sahiptir. Candida antarctica lipaz B (CAL-B) etkili ve yüksek seçici biyokatalizördür ve ε-kaprolakton'un enzimatik halka açılması polimerizasyonu için katalizör olarak kullanılabilir.

İlaç taşıyıcı sistemler (DDS), son yıllarda geleneksel ilaç salım sistemlerinden kaynaklanan sorunları önlemek için geliştirilmiştir. İlaç taşıyıcı sistemler geleneksel ilaç taşıyıcı sistemlerden farklı olarak, ilaç seviyelerini tek bir dozdan sonra istenen terapötik aralıkta sabit tutmayı amaçlar. Ayrıca ilaç taşıyıcı sistemler yan etki olmaksızın azalan dozlarla ilaçların etkinliğini arttırmakta ve sağlıklı dokuya zarar vermesini engellemektedir. Bu sistemlerin biyospesifik, toksik olmayan, biyouyumlu ve biyolojik olarak parçalanabilir gibi kriterleri vardır. pH duyarlı ilaç taşıyıcı sistemler, kanser tedavisinde kullanılan nano sistemlerin tasarımında en yaygın şekilde kullanılmaktadır. Doku veya organların pH değerleri gastrointestinal sistem boyunca değişmekte ve hastalık durumlarında değişebilmektedir. Polimerik taşıyıcılar, biyolojik olarak parçalanabilen veya ilaç taşıyıcı sistemlerde kullanılan suda çözünür polimer matrisler olabilen ve uyaranlara duyarlı olarak tasarlanabilen organik konaklardır. Mikroküreler, sürekli kontrollü ilaç salımı için kullanılır ve mikrokürelerin hazırlama teknikleri, kontrollü ilaç uygulaması sunar. Kalkonlar, üç karbonlu α,β-doymamış karbonil grubu ile birbirine bağlanmış iki aromatik halkaya sahip flavonoidlerdir ve antikanser aktivitesi gibi birçok farmakolojik ve biyolojik aktiviteye sahiptir.

Önceki çalışmada, pirinç kabuğu yakılarak bir destek malzemesi olan pirinç kabuğu külü (RHA) elde edilmiştir. RHA'nın yüzey modifikasyonu, 3-GPTMS, 3-APTMS ve APTES organosilanları ile sağlanmıştır. Daha sonra Candida antarctica lipaz B (CAL-B) enzimi, yüzeyi modifiye edilmiş RHA üzerine immobilize edilmiştir. Bu

çalışmada, sadece organosilan 3-GPTMS ile yüzeyi modifiye edilmiş RHA kullanılmıştır. Yüzeyi modifiye edilmiş RHA üzerine immobilize edilmiş enzim CAL-B, ε-kaprolaktonun halka açılması polimerizasyonunu katalizlemiştir. Daha sonra, elde edilen nanohibrit ve saf PCL, sırasıyla G-PCL/RHA nanohibrit ve saf G-PCL polimeri olarak adlandırılmıştır. İlaç yüklü mikroküreler, ilaç etken maddesi olarak kalkon kullanılan O/W emülsiyon-çözücü buharlaştırma yöntemiyle hazırlanmıştır. Farklı pH koşullarında ilaç salım deneyleri yapılmış ve ilaç salım profilleri incelenmiştir.

Çalışmanın ilk bölümünde, ε-kaprolakton'un enzimatik halka açılma polimerizasyonu immobilize Candida antarctica lipaz B (CAL-B) enzimi ile sağlanmıştır. G-PCL/RHA nanohibriti elde etmek için, reaksiyon kloroform ile sonlandırıldıktan sonra karışım, karışmakta olan metanol çözeltisine ilave edilmiştir ve çökelme gözlemlenmiştir. Ayrıca, saf G-PCL polimeri de elde etmek için, karışım kloroform ile yıkanmıştır ve süzüntü kurutulmuştur. Mikroküreler, O/W emülsiyon-çözücü buharlaştırma yöntemiyle hazırlanmıştır. Organik fazı oluşturan G-PCL/RHA nanohibrit ve trans-kalkon, sırasıyla kloroform ve diklorometan çözücülerinde çözdürülmüştür ve sulu fazı oluşturan %1'lik PVA çözeltisi hazırlanmıştır. Çözünen maddeler, karışmakta olan PVA çözeltisine ilave edilmiştir. 24 saat sonra çözücünün buharlaşması gözlemlenmiştir, karışım santrifüjlenip kurutulmuştur. Bu deneylerin tümü saf G-PCL örnekleri için de uygulanmıştır. Bu prosedürlerden sonra, ilaç yüklü G-PCL/RHA ve G-PCL mikroküreleri elde edilmiştir. Enkapsülasyon verimi hesaplamaları için ilaç yüklü mikroküreler ve metanol, enkapsüle edilmiş ilacı çözmek için manyetik bir karıştırıcıda 3 saat karıştırılmıştır. Karışım çözünen ilacı ayırmak için süzülip ultraviyole (UV) ile analiz edilmiştir. Enkapsüle edilmiş ilaç konsantrasyonu ve verimi hesaplanmıştır. In vitro ilaç salım deneyleri için, farklı pH koşulları tampon çözeltiler ile simüle edilmiştir. Deneyler boyunca çalışılan pH değerleri 3.6, 5.6, 7.4 ve 8.0'dır. Mikroküreler test tüplerindeki tampon çözeltilere eklenip test tüpleri çalkalamalı su banyosuna yerleştirilmiştir. Belirli zamanlarda test tüplerinden numuneler alınmış ve UV ile analiz edilmiştir. Salınan ilaç konsantrasyonları ve kümülatif ilaç salımı hesaplanmıştır. İlaç yüklü G-PCL/RHA mikrokürelerinin karakterizasyonu Fourier transform kızılötesi spektroskopisi (FT-IR), termal gravimetrik analiz (TGA), diferansiyel taramalı kalorimetri (DSC), taramalı elektron mikroskobu (SEM) ve su temas açısı (WCA) ölçümü ile yapılmıştır.

FT-IR sonuçları, G-PCL/RHA mikrokürelerinin spektrumunda karakteristik polikaprolakton piklerinin olduğunu göstermiştir. Ek olarak, G-PCL/RHA mikrokürelerinin ve trans-kalkon'un FT-IR spektrumları karşılaştırılmıştır. Buna göre, G-PCL/RHA mikrokürelerinde kalkon varlığı kanıtlanmıştır. TGA sonuçları, ilaç yüklü G-PCL/RHA mikrokürelerinin ve kalkonun bozunma sıcaklıklarını ve organik kütle kayıplarını göstermiştir. DSC sonuçları, G-PCL/RHA mikrokürelerinin erime sıcaklığını göstermiştir. SEM sonuçlarında mikroküre yapıları gözlemlenmiştir. İlaç salım sonuçları, en yüksek kümülatif ilaç salım yüzdesinin pH 5.6'da %98.0'i takiben pH 3.6'da %97.3 olduğunu göstermiştir. İlaç salımları asidik koşullarda daha verimli olmuştur. Saf G-PCL mikroküreleri de aynı ilaç salım performansını göstermiştir, ancak G-PCL/RHA nanohibritleri kadar yüksek ilaç salımı göstermemiştir.

İlaç salım sonuçları, in vivo çalışmalarda polikaprolaktonun lipazlar ve esterazlar tarafından bozunmasıyla değişebilir. Daha sonraki çalışmalarda ilaç salım deneyleri in vivo olarak gerçekleştirilebilir. Ayrıca ilaç-polimer etkileşimlerini ve ilaç salım

mekanizmasının pH duyarlılığını daha iyi anlamak için analiz yöntemleri geliştirilebilir. Literatürde bu tür çalışmalar mevcuttur.

1. INTRODUCTION

Biodegradable polymers have gained attention over the last two decades in the therapeutic field due to tissue compatibility, biodegradability, low toxicity, sustained release property. These are commonly used in various areas such as oral drug delivery and protein and peptide delivery. The biodegradable polymeric biomaterials are still developing for biomedical and pharmaceutical fields [1, 2]. There are several disadvantages of non-biodegradable polymers such as toxicity and accumulation in the body [1]. The degradation mechanism of the biodegradable polymers is provided by in vivo enzymatic actions or chemical reactions. After degradation, products are transformed into biocompatible form and excreted from the body [2]. There are some criteria in the design of biodegradable biomaterials such as not create a sustained inflammatory effect, degrade in proper time, have compatible properties, have nontoxic degradation products, have properties like permeability and processability [3]. The biodegradable polymers are classified into two main categories like natural and synthetic biodegradable polymers based on their origin [2].

Poly (ε-caprolactone) (PCL) is a biodegradable, biocompatible, and semicrystalline polymer and it is an aliphatic polyester and it has hexanoate repeat units [4, 5]. PCL is studied in several applications such as controlled drug delivery and tissue engineering because of its biocompatibility and biodegradability properties. PCL can be modified with other polymers effectively because, the structure of PCL allows changes in physical, chemical, and mechanical properties [6]. Moreover, PCL is a hydrophobic polymer but modification could make it hydrophilic [7]. The most common synthesis reaction of PCL is ring-opening polymerization (ROP) of cyclic monomer ε-caprolactone [4]. In addition, ROP occurs under mild conditions and short reaction times [8]. Candida antarctica lipase B (CAL-B) enzyme is an effective and high selective biocatalyst and can be used to catalyze ROP of ε-caprolactone [9]. Besides, enzymatic polymerization would not produce toxic products [10].

Drug delivery systems (DDS) has been developed in recent years to overcome the disadvantages of conventional drug delivery system. DDSs keep the drug levels

stable in a desired therapeutic range after a single dose and provide controlling drug release. Besides, DDSs enhance the efficacy of drugs with decreasing doses without side effects and prevent any harm on healthy tissue. There are criteria about these systems such as should be biospecific, non-toxic, biocompatible, and biodegradable [8, 11]. DDSs can be pH sensitive which shows performance in acidic or basic conditions by deformation or degradation [12]. In the living body, pH values vary along the gastrointestinal tract [13] and these pH values can stimulate the DDS. Polymers are used in pharmaceutical applications in DDSs as excipients for drug formulations, organic hosts for drug encapsulation, and matrices for inorganic nanostructured nanocomposites/hybrid materials [14]. These carriers are composed of biodegradable or water-soluble polymer matrices [8]. Polymeric microparticles take attention for being organic hosts. These particles have advantages like controlled particle size, specific surface, porosity, and reactivity [14]. Drug-loaded polymeric microspheres can be prepared by O/W (oil-in-water) emulsion-solvent method. In this method, the drug-active substance and polymer are dissolved in proper organic solvents. Polyvinyl alcohol (PVA) solution is generally used as an emulsifier in other words stabilizer. The dissolved substances are emulsified into the PVA solution and mixed. After evaporation of solvents, the microspheres are obtained [8]. Chalcones are open-chain flavonoids that have two aromatic rings bonded by a three-carbon α,β-unsaturated carbonyl group [15] and have pharmacological and biological activities such as anti-inflammatory, antibacterial, antifungal, antiviral, and antioxidant [16]. Additionally, chalcones have therapeutic potential like anticancer activity [17].

In this study, immobilized lipase enzyme Candida antarctica lipase B (CAL-B) was used as a catalyst for ROP of ε-caprolactone was prepared in the previous study [18]. After this, G-PCL/RHA nanohybrid and pure G-PCL were obtained. Drug-loaded microspheres were obtained by O/W emulsion-solvent evaporation method. Chalcone was used as drug active substance. Encapsulation efficiency was calculated. The buffer solutions that have different pH values (pH= 3.6, 5.6, 7.4, and 8.0) were prepared for in vitro drug release experiments for imitating pH values of the gastrointestinal tract. After this part, cumulative drug release values were calculated. GPCL/RHA and pure GPCL microspheres were analyzed by Fourier transform infrared spectroscopy (FT-IR), thermal gravimetric analysis (TGA),

differential scanning calorimetry (DSC), scanning electron microscopy (SEM), and water contact angle (WCA) measurement.

2. THEORETICAL STUDY

2.1 Biodegradable Polymers

Biodegradable polymers have gained attention over the last two decades in the therapeutic field due to tissue compatibility, biodegradability, low toxicity, sustained release property. These are commonly used in various areas such as controlled drug delivery, anticancer drug delivery, protein and peptide delivery, gene delivery, vaccine delivery, and enzyme immobilization. The biodegradable polymeric biomaterials are still developing for biomedical and pharmaceutical fields [1, 2]. The biodegradable polymers are classified into two main categories like natural and synthetic biodegradable polymers based on their origin [2]. Figure 2.1 shows polymer classification for biomedical and pharmaceutical applications. There are several disadvantages of non-biodegradable polymers such as toxicity and accumulation in the body [1]. Besides, the biodegradable polymer degrades in the living body through numerous biochemical processes [19]. The degradation mechanism of the biodegradable polymers is provided by in vivo enzymatic actions or chemical reactions [2]. Polymer chains are hydrolyzed to smaller compounds and the biodegradable polymer degrades [19]. After degradation, products are transformed into biocompatible form and excreted from the body [2]. Some of the biodegradable polymers, especially polyanhydrides and polyorthoesters, degrades only on the surface. According to this, the release rate is related to the surface area of the drug delivery system [19].

There are various parameters about the applications of biodegradable polymers in drug delivery and targeting such as compatibility of polymers with a living body and release kinetics of polymers [1]. The biodegradable biomaterials should not create a sustained inflammatory effect and should degrade in proper time, have properties like compatibility, permeability, and processability, and have nontoxic degradation products [1, 3]. In addition, biodegradable polymers should not harm the body and also the environment during excretion [1].

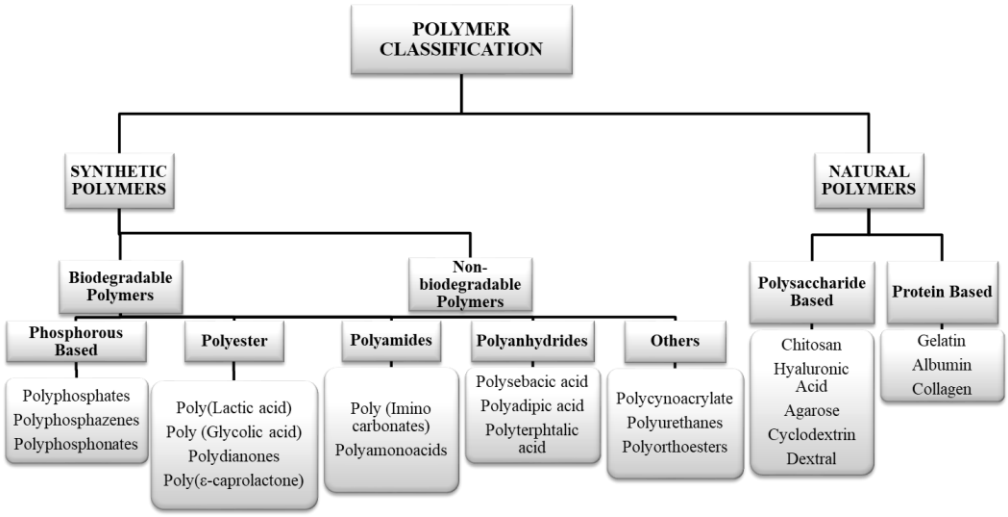

Figure 2.1 : Classification of polymers [1].

2.1.1 Poly(ε-caprolactone)

Polycaprolactone (PCL) is an aliphatic polyester and it has hexanoate repeat units. PCL is a biodegradable, biocompatible, and semicrystalline polymer that has a glass transition temperature -60°C and a melting point ranging 59 and 64°C [4, 5]. Its degree of crystallinity can reach 69% [4]. At room temperature, while chloroform, dichloromethane, carbon tetrachloride, benzene, toluene, cyclohexanone, and 2-nitropropane are good solvents for PCL, acetone, 2-butanone, ethyl acetate, dimethylformamide and acetonitrile are not good solvents for PCL. Also, PCL is not soluble in alcohol, petroleum ether, and diethyl ether [4, 5,7].

PCL has been used in a variety of fields mainly in long-term drug delivery systems [4, 5, 7], moreover; scaffolds in tissue engineering [4, 7], in microelectronics, in packaging, and finally as adhesives [4]. PCL has been approved by the Food and Drug Administration (FDA) by using implants and surgical absorbable sutures [7, 20].

There are numerous advantages of PCL. One of the advantages of PCL is miscibility. For improving stress crack resistance, adhesion and dyeability; PCL can be used with other polymers in the mixture [5]. In addition, it provides properties such as swelling, porosity, and stability to the mixture [20]. Its monomers have the potential to be obtained from renewable sources [4] and its production routes are relatively inexpensive [7]. Although PCL is hydrophobic, additional functional groups could

make the polymer more hydrophilic. Also, additional functional groups can be used for improving adhesivity and biocompatibility. Other than that PCL has superior rheological and viscoelastic properties like the ease of shaping [7].

There are two methods for the synthesis of PCL. One of them is the ring-opening polymerization of cyclic monomer ε-caprolactone and the other one is the condensation of 6-hydroxycaproic (6-hydroxyhexanoic) acid. ROP is the preferred route for PCL synthesis. Because after ROP, the obtained polymer will have higher molecular weight and a lower polydispersity [4]. Additionally, ROP occurs under mild conditions and short reaction times [8]. There are three different types of catalytic systems such as; metal-based, enzymatic and organic systems. Stannous octoate (stannous(II) ethylhexanoate, $Sn(Oct)_2$, that belongs to poor metal-based catalysts group, is the most used catalyst for ROP of ε-CL [4, 7]. It has various advantages such as being effective, commercially available, easy to handle and can be solved in common organic solvents. Besides, it has disadvantages like operating at high temperatures. It causes intermolecular and intramolecular esterification. To conclude, polydispersity will be enlarged [4]. Also, after polymerization metallic residues could remain and it may harm to the living body in medical applications [10].

Although metal-based catalysts are the most used catalysts for ROP of ε-CL, enzymes gain importance for catalysis of polymerization. Enzymatic polymerization would not produce toxic products. Besides, enzymes operate under mild conditions and have high enantio- and regio- selectivity. Also, they can be recycled [10]. Lipases (E.C. 3.1.1.3) are enzymes that catalyze the hydrolysis of lipids. Lipases are used for various industrial applications such as; pharmaceuticals, cosmetics, food and flavor making, synthesis of carbohydrate ester, amines, and amides. Candida antarctica lipase B (CAL-B) is an effective and high selective biocatalyst. It is used for industrial processes such as aminolysis, esterification, and transesterification. CAL-B is very stereospecific towards ester hydrolysis and synthesis [9]. Sivalingam et. al. have proved that Candida antartica lipase B (Novozym 435) is successful in six-seven membered ring-opening polymerization and showed better catalytic activity than other lipases [21].

Figure 2.2 shows the eROP of ε-CL below. In the first reaction, lipase reacts with ε-CL and produces a lipase-activated CL complex. In the second reaction, the alcohol

molecule reacts with this complex. After that, chain growth is observed and polymerization occurs [4].

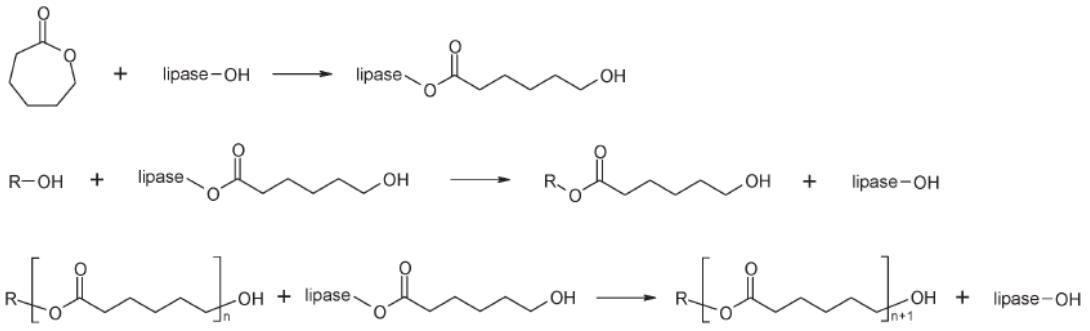

Figure 2.2 : Enzymatic ring-opening polymerization of ε-CL [4].

2.2 Drug Delivery Systems

Traditional drug delivery systems release the entire dose of the drug and this leads to significant fluctuation of plasmatic drug concentration in the body. So, the drug concentration in the body rises quickly, which sometimes can reach a toxic level, and then decreases. As a result of this, the patient needs the drug again and the therapy requires repetitive drug administration which is inconvenient for the patient. Each drug has a plasma level. Above plasma level, the drug becomes toxic while below plasma level, the drug is ineffective. Additionally, traditional drug delivery systems in other words conventional drug delivery systems are unpredictable and inefficient. For ensuring the drug reaches the site of action, a high dose of the drug is needed. To prevent these problems, solve these limitations, and increase therapeutic effect, drug delivery systems (DDS) are developed in recent years. The aim of DDS is to keep the drug levels stable in a desired therapeutic range after a single dose. The prolonged drug delivery systems maintain the therapeutic plasma concentration and release the drug at the right time and right media [8, 13, 14]. Figure 2.3 shows the comparison of traditional and prolonged drug delivery systems schematically.

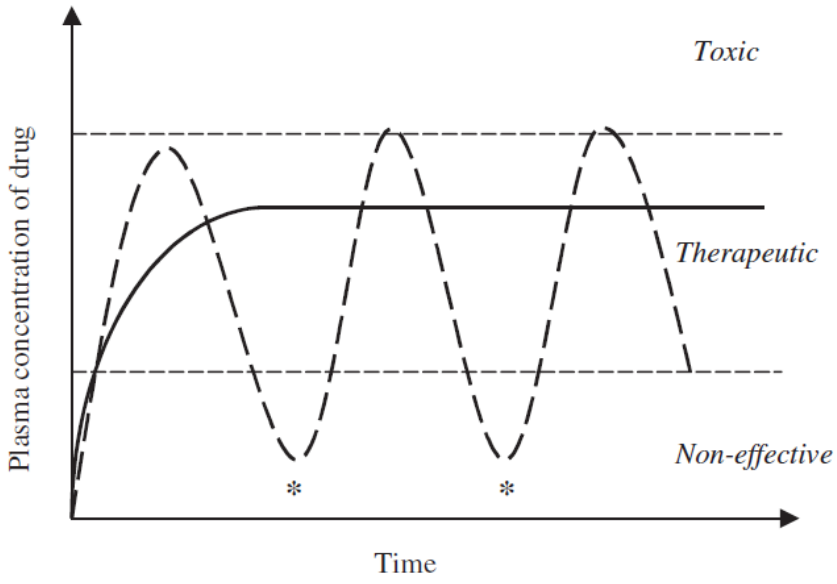

Figure 2.3 : The plasma concentration of drug as a function of time, (---) traditional drug delivery system, (-) prolonged drug delivery system [8].

DDS is a process that administering the pharmaceutical compound to achieve a therapeutic effect in humans or animals [11]. DDSs enhance the efficacy of drugs with decreasing doses without side effects and prevent any harm on healthy tissue [11, 19]. Controlled DDSs are used for delivering a small amount of drugs to a proper location and optimizing drug utilization. Biopharmaceutical and physicochemical properties of drug active substance and the needed concentration profile of the drug in the site of action are important for the selection and development of DDS [22]. Additionally, drug delivery systems should meet the criteria such as simple route for administration, targeting, and effective delivery of drugs. Moreover, drug delivery systems should be biospecific, non-toxic, biocompatible, and biodegradable [11].

Several approaches are performed in drug delivery devices. The drug administration route and the drug-release mechanism vary between the drug delivery devices [8]. One of them is polymers that are used in a variety of fields in pharmaceutical applications such as excipients for drug formulations, organic hosts for drug encapsulation, and matrices for inorganic nanostructured nanocomposites/hybrid materials [14]. Figure 2.4 shows applications of polymers in DDS.

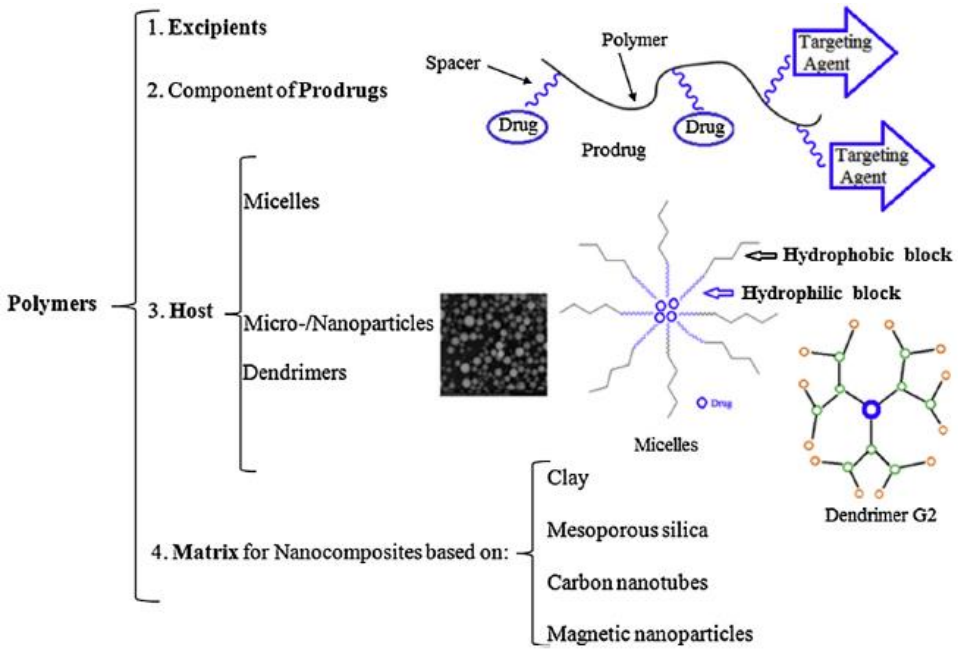

Figure 2.4 : Applications of polymers in DDS [14].

Polymeric carriers are organic hosts that can be biodegradable or water-soluble polymer matrices. These are can be in the form of colloidal-sized particles (such as microspheres or nanospheres) and rods or films [8]. The polymers are good ones for encapsulation of various substances such as drugs, hormones, proteins, peptides, antibodies, and nucleic acids [14]. In these matrice systems, drug active substance is trapped in the matrice but not chemically bonded to the polymer. During the polymer is dissolved or degraded, eroded, and resorbed the drug is released continuously [8].

Polymeric micro and nanoparticles take attention for being organic hosts. These particles have advantages like controlled particle size, specific surface, porosity, and reactivity. Polymeric particles are used for the encapsulation of drugs in order to reduce the unpleasant taste of drugs, protect the drug active substance from enzymatic degradation or pH variations until they reach desirable tissue, increase the encapsulation efficiency and improve the drug release profile [8, 14].

2.2.1 pH-sensitive drug delivery systems

Biomaterials are used for drug delivery in medical applications. pH-sensitive biomaterials have been widely used in the past decades. These materials show performance in acidic or basic conditions by deformation or degradation. pH-sensitive properties make the changes in intramolecular or intermolecular forces possible. As a result of this, the drug delivery system is stimulated and releases the

drug active substance [12]. This stimuli-responsiveness property provides additional control on the delivery of the encapsulated drug. These materials can keep the encapsulated drug until reaches the target area that stimulus allows the release of the drug. Inopportune drug release can be prevented through this system [23]. pH-sensitive DDSs are most widely used in the design of nano-systems in cancer therapy. In the living body, pH values vary along the gastrointestinal tract [13]. Oral drugs are tranferred from the oral cavity to the stomach, the small intestine and the large intestine. Each of them has different pH values and ionic concentration in the gastrointestinal system [24]. In addition, pH values of tissues or organs can change during the disease conditions such as ischemia, infection, inflammation, and tumorigenesis. Besides, the pH values of tumors are lower than normal tissues. The pH value of normal tissue is 7.4 while the pH values of tumors vary from 5.7 to 7.8 [13]. Table 2.1 shows pH values of gastrointestinal sites.

Table 2.1 : pH values of gastrointestinal sites.

Physiological fluid	pH	Reference
Saliva	6.0-7.0	[24]
Gastric fluid	1.2-3.7	[25]
Small-intestinal fluid	7.5-8.0	[24]
Large-intestinal fluid	5.5-7.0	[24]
Colon	6.0-7.5	[25]
Plasma	7.4	[24]

Polymers are one of the most important excipients that are often used in DDSs and pharmaceutical formulations and can be designed as stimuli-responsive [24] and encapsulate and protect the drug [13]. These can be pH or ion-sensitive according to changes in physiological conditions in the living body [24].

Briefly, there are two main different pH-sensitive mechanisms to release the encapsulated drugs. The first one is releasing the drugs through changed hydrophobicity or changing the charge of drug carrier by protonation/deprotonation through pH variations of the media. The other one is releasing the drugs through cleavage pH-sensitive chemical bonds of the drug carriers [12]. The first mechanism

represents ionizable chemical groups with nanomaterials. These are amines, phosphoric acids, and carboxylic acids which have different chemical properties and pKa values. These groups can accept or donate protons. In addition, the physical or chemical properties of these groups such as swelling ratio or solubility can change due to the pH variations. Thus, drug release occurs. The second mechanism includes acid-labile chemical bonds. These mechanisms are used to covalently attach the drug-active substance onto the drug carrier. Acid-labile bonds are hydrolyzed or degraded in acidic media. Acetal, orthoester, hydrazone, imine, and cis-aconyl bonds can be used as the acid-labile linkers according to various studies [13, 23].

2.2.2 Microspheres in drug delivery

The term microparticle refers to a particle size between 1-1000 micrometers. Microspheres are spherical microparticles while microcapsules are microparticles that have a core surrounded by a material different from the core. Generally, it is assumed that microparticles are a homogeneous mixture of polymer and active agents. However, microcapsules have one or more discrete domains of active agents [26]. Microspheres have been used for various fields such as graphic products, optics, agricultural chemicals, adhesives, perfumes, food, and flavorings [8]. The usage of microspheres in drug delivery has become widespread in the last decades [8, 20]. Figure 2.5 shows structures of microcapsules and microspheres schematically.

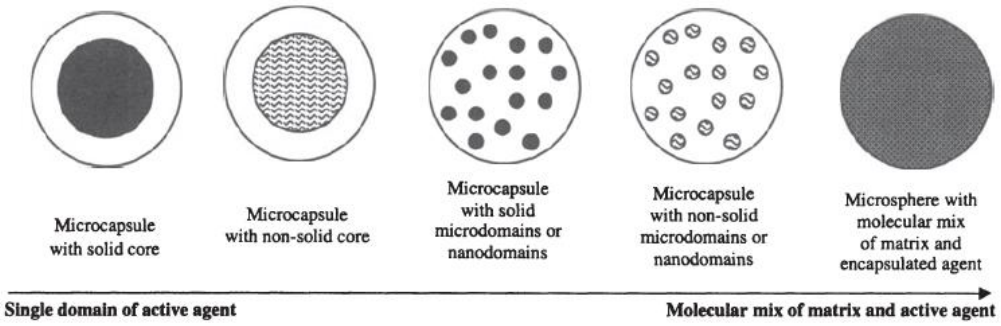

Figure 2.5 : Structures of microcapsules and microspheres [26].

Immunoglobulins serum proteins, liposomes, microspheres, microcapsules, and nanoparticles are used as drug carriers. Microspheres are used for sustained controlled drug release. The preparation techniques of microspheres offer to control drug administration. These techniques allow the right delivery of the proper amount

of drugs, reduced drug concentration except the target side, and the protection of the labile drugs before and after the administration site [27].

Biodegradable polymers are used for sutures, tissue-supporting scaffolds, and drug delivery devices. These polymers gradually degrade and then excrete from the body. Biodegradable polymers are large field but only several groups of polymers are suitable for drug delivery. There are number of methods for preparation of drug-loaded microspheres from biodegradable polymers. O/W (oil-in-water) emulsion/solvent technique is one of the most popular technique due to ease of performing [8].

In this method, the drug-active substance and polymer are dissolved in proper organic solvents. This mixing forms the organic phase. Polyvinyl alcohol (PVA) solution is generally used for an emulsifier in other words stabilizer. Stable emulsion forms as a result of immiscibility of the PVA solution and organic phase. The dissolved substances are emulsified into the aqueous PVA solution under high-speed stirring. This mixing forms oil-in-water (O/W) emulsion. The polymer microspheres precipitate after the evaporation of the solvent. Finally, the hardened microspheres are filtered and dried [5, 8, 20]. In addition, according to the research of Barbato et. al. (2001) after the emulsification, 100 ml of distilled water was added to the mixing. This leads to the increase diffusion of organic solvent to the aqueous phase so hardening of the microsphere [28]. Figure 2.6 shows microsphere preparation by O/W emulsion/solvent technique.

There are other methods such as W/O/W (water-oil-water) emulsion/solvent evaporation technique [5, 8, 27], coacervation [8, 27], spray drying [5, 8, 27] and hot-melt techniques [5, 8]. However, O/W emulsion/solvent technique has some advantages than other techniques like spray drying. O/W technique is performed at room temperature and constant stirring. These mild conditions provide preventing to decrease in the activity of drug active substance [29].

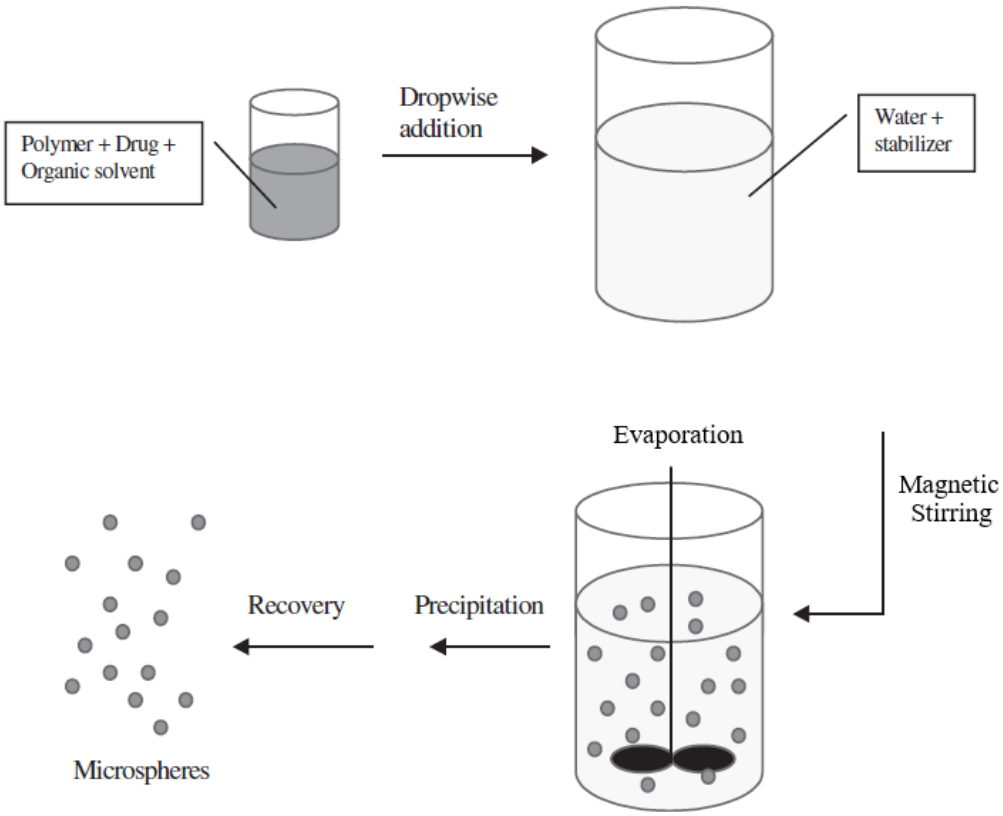

Figure 2.6 : Microsphere preparation by O/W emulsion/solvent technique [8].

2.2.3 Chalcones

Chalcones, 1,3-diaryl-2-propen-1-ones, are flavonoids that often found in fruits and vegetables, plant tissues, and yellow or orange pigments in some flowers. Plants that contain chalcone have been used as herbal medicines for years in traditional medicine in Asia, Africa, and South America [15, 16]. Chalcones are open-chain flavonoids that have two aromatic rings bonded by a three-carbon α,β-unsaturated carbonyl group. Figure 2.7 shows the general structure of the chalcone [15].

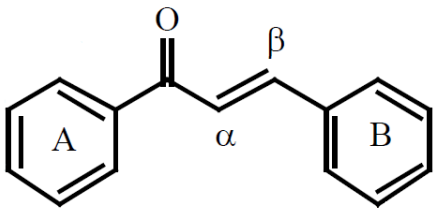

Figure 2.7 : General structure of chalcone [15].

In nature, most of the aromatic rings of chalcones are found as hydroxylated. The interest about consumption of plants that contain chalcone is increased because of free radical scavenging properties of phenol groups [16]. Moreover, chalcones have

lots of pharmacological and biological activities. These are anti-inflammatory, antibacterial, antifungal, antiviral, antioxidant, antineoplastic [16], analgesic, antiulcer, antimalarial, antihelmintic [30], anticancer [17], and antileishmanial [31]. Besides, chalcones synthesize easily and can be found in nature abundantly. Thus, chalcones have important therapeutic potential [17, 30]. In addition, various chalcone molecules anticancer activity has been studied and chalcone that have trimethoxyphenyl unit has been reported the most cytotoxic derivatives. A variety of clinically useful anticancer drugs can have genotoxic effect because of interaction with the amino groups in nucleic acids. Chalcones may not cause this side effect [17]. The α,β-unsaturated carbonyl groups of chalcones make them biologically active. Chalcones have shown antibacterial activity to various organisms such as S.aureus, E. coli, C. albicans and some other organisms [32].

Chalcones are precursors of all flavonoid groups so they are very important biosynthetic compounds. Chalcones change into the flavanones with catalysis of chalcone isomerase enzyme by stereospecific reaction in plants. Because of this, there is similar biogenetic and structural relation between chalcones and flavanones. So, these compounds are generally found together in nature [16]. Figure 2.8 shows the structure of flavone.

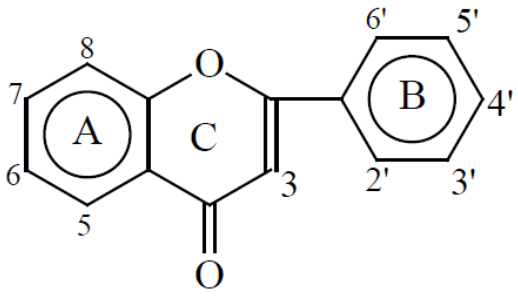

Figure 2.8 : Structure of flavone [16].

Chalcones can be natural or synthesize in the laboratory. There are numerous methods for synthesizing chalcones, but the most convenient method is Claisen-Schmidt condensation. This method requires equimolar amounts of acetophenone and benzaldehyde. Besides the obtained chalcones, little amount of flavanones can also be obtained end of this reaction. This reaction is catalyzed by bases or acids, but mostly bases such as aqueous alcoholic alkali, under homogeneous conditions. Basic

catalysts are more efficient than acidic catalysts [16, 30, 32]. Additionally, other catalysts such as basic alumina (Al_2O_3), zinc chloride ($ZnCl_2$) and Lewis acids, BF_3 and $AlCl_3$, can be used in this reaction [31]. Finally, this reaction has been carried out without any solvents as solid-state reaction. Chalcones were synthesized from the same reagents at high temperature water (200-350 °C) for the purpose of green chemistry synthesis [32]. Figure 2.9 shows Claisen-Schmidt condensation of acetophenone and benzaldehyde and the formation of flavanone.

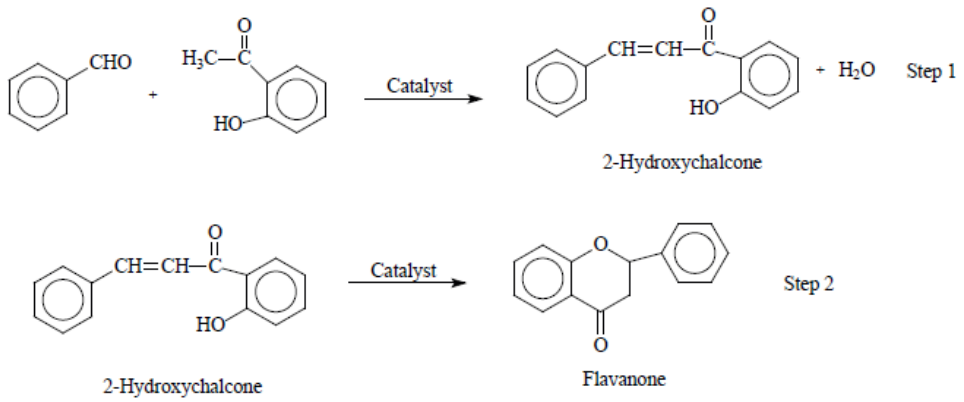

Figure 2.9 : Claisen-Schmidt condensation of acetophenone and benzaldehyde [16].

Chalcones have stereochemical properties such as conformational equilibrium between the hydrogen atoms of the Cα=Cβ bond. This bond can present cis or trans configuration. Thus, E- (trans) or Z- (cis) isomers of chalcone exist. The E- (trans) isomers of chalcone are thermodynamically more stable than others. It can be said that nearly all chalcones are isolated in this form [15, 16].

2.3 Silica/Organic Nanohybrid Systems

Nanocarriers that have organic and inorganic components are used for encapsulate or to adsorb drugs. Iron oxide nanoparticles, mesoporous silica nanoparticles, and double layered hydroxides are used as inorganic part while, liposomes, carbon nanotubes, dendrimers, micelles, solid lipid nanoparticles, polymeric nanoparticles, and nanogels are used for organic part of the nanocarriers. Figure 2.10 shows typical organic and inorganic components used in nanohybrids. It is known that, organic nanoparticles increase biocompatibility, versatility, and stability in loading hydrophilic and hydrophobic drugs [33]. Organic nanoparticles are used for minimize the side effects of therapy, targeting the specific side, reach the target side

without any harm to healthy tissue [33, 34]. However some organic nanoparticles can have disadvantages like non-chamical stability. Inorganic nanoparticles need to surface functionalization for increasing stability and dispersibility. Organic molecules are used for surface functionalities of inorganic nanoparticles to increase the biocompatibility and biodegradability [33].

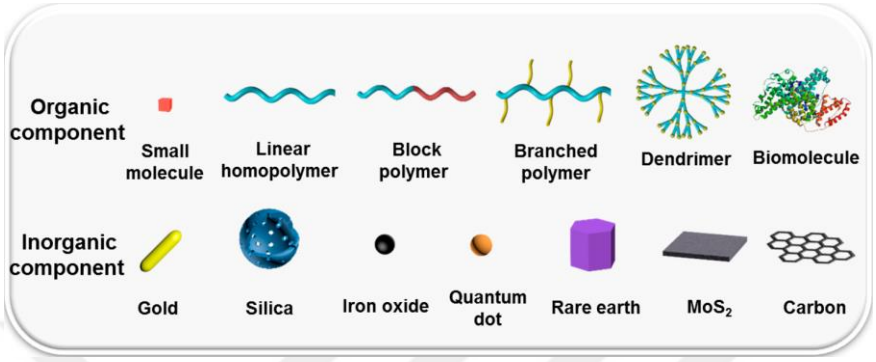

Figure 2.10 : Typical organic and inorganic components used in nanohybrids [34].

Organic/inorganic nanohybrids take attention for their favorable physicochemical properties. These hybrids show specific properties. Besides, they show new properties according to their synergistic effect [33, 34] such as stimuli-responsiveness, targeting, biodegradability, and controlled release [34]. Highly porous structure of inorganic components such as silica, and different functional groups of organic components leads to effective drug loading and controlled drug release [33]. Silica nanoparticles are commonly used in drug delivery due to their tunable pore structure, large surface area, easy surface functionalization, and drug loading ability properties. Despite mesoporous silica nanoparticles have advantageous properties like higher stability, multifunctionality, and effectice drug loading ability, have some disadvantages like lower biocompatibility, non-biodegradability, and inopportune drug release. The usage of silica/organic nanohybrids (SONH) overcome these problems. These nanohybrids can obtained by the surface modification or encapsulation of organic molecules into the silica matrix by bonding covalently or ionically. More drug molecules can attached to these modified hybrids. Stimuli-responsive nanohybrids take attention for using in lower pH conditions in cancer cells [33–35]. Silane chemistry is a preferred route for the production of silica based organic/inorganic nanohybrids. To obtain surface-modified nanohybrids small molecules like organosilanes such as 3-aminopropyl-trimethoxysilane (APTMS), 3-aminopropyltriethoxysilane (APTES) are used [18,

33–35]. These groups are attached to the surface via the formation of Si-O bonds [33]. Rice husk ash (RHA) can be used as source of silica in other words support material. It contains %97-92 of amorphous silica (SiO_2) and has large specific surface area. Poly (ε-caprolactone) and silica nanohybrid can be used for drug release [36].

3. MATERIALS AND METHODS

3.1 Materials

In this study, ε-caprolactone (99%, $C_6H_{10}O_2$) was used as a monomer for the polymerization process and purchased from Alfa Aesar. Toluene (99.8%, C_7H_8) which was the organic solvent of the polymerization reaction purchased from Merck. Chloroform (99.8%, $CHCl_3$) was used for terminating the polymerization reaction and purchased from Sigma-Aldrich. Methanol (99%, CH_3OH) was used for precipitating the mixture to obtain G-PCL/RHA nanohybrids and purchased from Sigma-Aldrich. Enzymatic ring-opening polymerization was catalyzed by an immobilized lipase enzyme which was immobilized in the previous study. Chalcone (trans-chalcone, 97%) was used as a drug for the preparation of drug-loaded microspheres and was purchased from Sigma-Aldrich. Microspheres were prepared by the emulsion-solvent evaporation method. Chalcone and G-PCL/RHA nanohybrid were dissolved in dichloromethane and chloroform respectively. Dichloromethane (CH2Cl2) was purchased from Merck. Dissolved chalcone and G-PCL/RHA nanohybrid were mixed and emulsified into the polyvinyl alcohol solution (1%). Polyvinyl alcohol (99%, $[C_2H_4O]n$) was used for the preparation of PVA solution and it was purchased from Sigma-Aldrich. Sodium acetate (CH_3COONa, Carlo Erba), acetic acid (CH_3COOH, Merck), sodium chloride (NaCl, Carlo Erba), potassium chloride (KCl, Merck), disodium hydrogen phosphate dihydrate ($Na_2HPO_4•2H_2O$, Carlo Erba), potassium dihydrogen phosphate (KH_2PO_4, Carlo Erba) and di-potassium hydrogen phosphate (K_2HPO_4, Merck) were used for the preparation of buffer solutions for drug release studies.

3.2 Methods

3.2.1 Support material and lipase immobilization

This part was performed in the previous study. The support material rice husk ash (RHA) was obtained by burning rice husks. Firstly, rice husks were washed with

distilled water, filtered and dried in an oven at 50 °C. After that, dried rice husks were burned in a furnace at 600 °C for 6 h. The obtained RHA was left to cool for 1 day and stored in a desiccator. Before lipase immobilization, RHA was modified with silanization agents. 250 mg RHA was added to test tubes and mixed with 3-GPTMS silanization agent 15% (v/v) in 5 ml acetone. Test tubes were placed in shaking water bath and silanization was performed at 50 °C and 160 rpm for 2 h. The same procedures were performed by using 3-APTMS and APTES as silanization agents. After silanization, surface modified RHA was washed with distilled water and filtered under vacuum. The filtered RHA was dried for 4 h at 60 °C in an oven then stored in desiccator.

In the enzyme immobilization part, free form of Candida antarctica lipase B (CAL-B) enzyme was immobilized on surface-modified RHA by physical adsorption. The surface-modified RHA and free CAL-B was mixed (enzyme/support: 2 µl/mg) and 25 ml of 0.015 M, pH 7.0 phosphate buffer solution was added to the mixture. The mixture was stirred for 5 h at room temperature on a magnetic stirrer. After 5 h, enzyme samples were washed with 0.015 M, pH 7.0 phosphate buffer solution. The filtered immobilized enzyme samples were dried at 30 °C in an oven for 12 h. Dry immobilized enzymes were stored in the fridge at 4 °C.

3.2.2 G-PCL/RHA nanohybrid system

In this study, only surface-modified RHA with organosilane 3-GPTMS was used. In this part, the immobilized CAL-B onto surface-modified RHA was catalyzed the ROP of ε-caprolactone. 100 mg of immobilized enzyme was stirred with 500 mg of ε-caprolactone monomer and 1000 mg toluene solvent in a glass flask for 48 h at 40 °C in a magnetic stirrer. Enzyme/monomer ratio was 20% (w/w) and enzyme/toluene ratio was 1:2 (w:w). Glass flask was filled with dry nitrogen gas to be an inert reaction environment before stirring. After 48 h, the reaction was terminated by adding chloroform. The mixture was added slowly to the stirring methanol solution. Precipitation of polymer particles was observed. After that, the mixture was filtered. The filtered sample was named as G-PCL/RHA nanohybrid because of using 3-GPTMS modified RHA and to prevent confusion between silanization agents names. To obtain pure PCL polymer, after terminating the reaction, the mixture was washed

with chloroform solvent and then filtrated. This filtrated sample was named as G-PCL. The filtrate was dried in an oven for 24 h at 50 °C.

3.2.3 Preparation of drug loaded microspheres

In this part, microspheres were prepared by the emulsion-solvent evaporation method. Chalcone (trans-chalcone, 97%), which is drug active substance, and G-PCL/RHA nanohybrid were dissolved in proper solvents. Chalcone was dissolved in dichloromethane (CH_2Cl_2) and G-PCL/RHA nanohybrid was dissolved in chloroform (99.8%, $CHCl_3$). Chalcone/solvent ratio was 10 mg/5 ml while nanohybrid/solvent ratio was 100 mg/5 ml. The dissolved substances were mixed. According to the emulsion-solvent evaporation method, there should be two phases; organic and aqueous phases. The mixture of dissolved chalcone and nanohybrid was an organic phase. Polyvinyl alcohol was an aqueous phase. For this reason 1% PVA solution was prepared in 100 ml volumetric flask. 50 ml of PVA solution (1%) was stirred in a magnetic stirrer and the organic phase was emulsified into it. 5 minutes after 50 ml of distilled water was added. The mixture was stirred at high speed for 24 h. After 24 h, evaporation of the solvent was observed. The mixture was centrifuged at 4000 rpm speed and the wet solid form was dried so that the drug-loaded microspheres were obtained. All of these experiments were also applied for G-PCL samples.

3.2.4 Calculations of encapsulation efficiency

Before calculating encapsulation efficiency, the calibration curve of chalcone was obtained. For calibration, 1 mg of chalcone and 100 ml of methanol were mixed. Solutions that between 0.001-0.01 mg/ml concentrations were prepared. Each solution was analyzed by ultraviolet (UV). Concentration-Absorbance graph was plotted and the calibration curve was obtained. These curve is given in Appendix A.

In this part, the efficiency of drug encapsulation was calculated. In order to solve the encapsulated drug, 10 mg of drug-loaded microspheres and 10 ml of methanol were stirred for 3 h in a magnetic stirrer. The mixture was filtrated to obtain the dissolved drug. 1 ml of filtrate and 9 ml of methanol were mixed and drug concentrations were determined by using ultraviolet (UV) analysis. Equation (3.1) shows the calculation of encapsulation efficiency [37].

$$\text{Encapsulation efficiency (\%)} = \frac{\text{Amount of drug at final state}}{\text{Amount of drug at initial state}} \times 100 \quad (3.1)$$

3.2.5 In vitro drug release experiments

Before starting drug release experiments, the calibration curve of chalcone was obtained. For calibration, 1 mg of chalcone and 100 ml of buffer solution were mixed. This part was repeated for each buffer solution (pH=3.6, 5.6, 7.4, 8.0). Solutions that between 0.001-0.01 mg/ml concentrations were prepared. Each solution was analyzed by ultraviolet (UV). Concentration-Absorbance graph was plotted and the calibration curve was obtained. These curves for each pH conditions are given in Appendix A.

For in vitro drug release experiments, different pH conditions were simulated by buffer solutions. The operating pH values were 3.6, 5.6, 7.4, and 8.0 throughout the study. For pH 3.6 acetate buffer solution (0.1 M), 396 mg of sodium acetate and 2.72 ml of acetic acid were dissolved in 800 ml of distilled water. The solution was transferred to a volumetric flask and completed to 1 l of distilled water. For pH 5.6 acetate buffer solution (0.1 M), 7.721 g of sodium acetate and 336 µl of acetic acid were used. The process was the same with pH 3.6 acetate buffer preparation. For pH 7.4 phosphate buffer solution (0.1 M), 8 g of sodium chloride, 0.2 g of potassium chloride, 1.81 g of disodium hydrogen phosphate dihydrate, and 0.24 g of potassium dihydrogen phosphate were dissolved in 800 ml of distilled water. The solution was transferred to a volumetric flask and completed to 1 l of distilled water. For pH 8.0 phosphate buffer solution (0.1 M), 16.28 g of di-potassium hydrogen phosphate and 888 mg of potassium dihydrogen phosphate were dissolved in 800 ml of distilled water. The solution was transferred to a volumetric flask and completed to 1 l of distilled water.

For drug release experiments, 10 mg of microspheres and 10 ml of buffer solution were transferred to test tubes. Test tubes were placed in the shaking water bath. At certain times, 1 ml of samples were collected from test tubes and 1 ml of buffer solution was transferred to test tubes instead. 1 ml of samples and 9 ml of buffer solution were mixed and concentrations were analyzed by ultraviolet (UV).

Cumulative drug release values were calculated and the graphs of drug release were obtained. Equation (3.2) shows the calculation of drug release values [37].

$$\text{Drug release (\%)} = \frac{\text{Amount of drug at any time}}{\text{Amount of drug at initial state}} \times 100 \quad (3.2)$$

3.2.6 Kinetic modelling of drug release

Several mathematical models were used to evaluate the drug release kinetics and mechanism. Zero Order, First Order, Higuchi and Korsmeyer-Peppas release models were used for interpretation of nonlinear release profiles. The correlation coefficients "R2" of the models were compared with each other and the model with the highest correlation coefficient was considered to be the model most compatible with the release data. Fitting of the models and related model calculations were made by OriginLab® OriginPro 8.5 software.

3.3 Characterization Techniques

3.3.1 Ultraviolet (UV) spectrophotometry

The efficiency of drug encapsulation was calculated by measuring absorbance values with a UV spectrophotometer (UV mini 1240 SHIMADZU). Drug-loaded microspheres that were dissolved in methanol were filtrated. 1 ml of filtrate and 9 ml of methanol were mixed and analyzed by UV spectrophotometer. The blank sample was methanol. The concentrations were calculated by absorbance values and efficiency was obtained. The absorbance values for the part of obtaining calibration curve were determined by UV spectrophotometer. The blank samples were each buffer solution (pH=3.6, 5.6, 7.4, 8.0). Each solution was analyzed by a UV spectrophotometer. In drug release experiments, samples that were collected from test tubes and were mixed with buffer solutions were analyzed by UV spectrophotometer. The blank samples were each buffer solution. The absorbance values of the drug were obtained. Drug concentrations were calculated by using the calibration curve. All of the measurements were recorded at 308 nm.

3.3.2 Fourier transform infrared spectroscopy (FT-IR)

Chalcone (trans-chalcone, 97%) and drug-loaded microspheres were characterized by FTIR analysis (Perkin-Elmer, Spectrum One). Spectra were recorded between 4000-650 cm-1 at 2 cm^{-1} resolutions. The characteristic peaks of chalcone and poly-ε-caprolactone were observed in FT-IR spectra. Chemical structures of these can be defined from FT-IR spectra.

3.3.3 Thermal gravimetric analysis (TGA)

Thermal properties of drug-loaded microspheres were determined by thermal gravimetric analysis (TGA). Cumulative weight loss and degradation temperatures of chalcone and poly-ε-caprolactone were measured by SEIKO TG/DTA 6300. 10 mg of samples were heated from 30 °C to 550 °C with a rate of 10 °C/min under nitrogen flow.

3.3.4 Differential scanning calorimetry (DSC)

Other thermal properties of drug-loaded microspheres were determined by differential scanning calorimetry (DSC, Perkin-Elmer Pyris). The operation temperature of DSC was -80-150 °C at a rate of 10 °C/min under a continuous nitrogen flow. Thermograms were obtained with heat/cool/heat cycle and melting point (Tm) was determined by DSC heating curves.

3.3.5 Scanning electron microscopy (SEM)

Microsphere structures were observed by scanning electron microscopy (SEM) analysis. Scanning was performed at 15 kV with x250 magnification by using TESCAN VEGA 3 SEM.

3.3.6 Water contact angle (WCA) measurements

Wettability of the pure G-PCL, G-PCL/RHA nanohybrid and G-PCL/RHA microspheres were determined by water contact angle (WCA) measurements with Attension (KSV).

4. RESULTS AND DISCUSSION

4.1 In Vitro Drug Release Studies of G-PCL/RHA and G-PCL Microspheres

Efficiency of drug encapsulation was calculated before starting drug release experiments. According to calculations, encapsulation efficiency of G-PCL/RHA and G-PCL microspheres were 74.4±1.2(%) and 60.7±9.9(%), respectively.

The drug release studies were performed at different pH conditions (3.6, 5.6, 7.4, and 8.0) for G-PCL/RHA and G-PCL microspheres. Cumulative drug release (%) and time (h) plots for different pH conditions can be seen in Figure 4.1. At this part, drug release of G-PCL/RHA nanohybrid and G-PCL microspheres were compared.

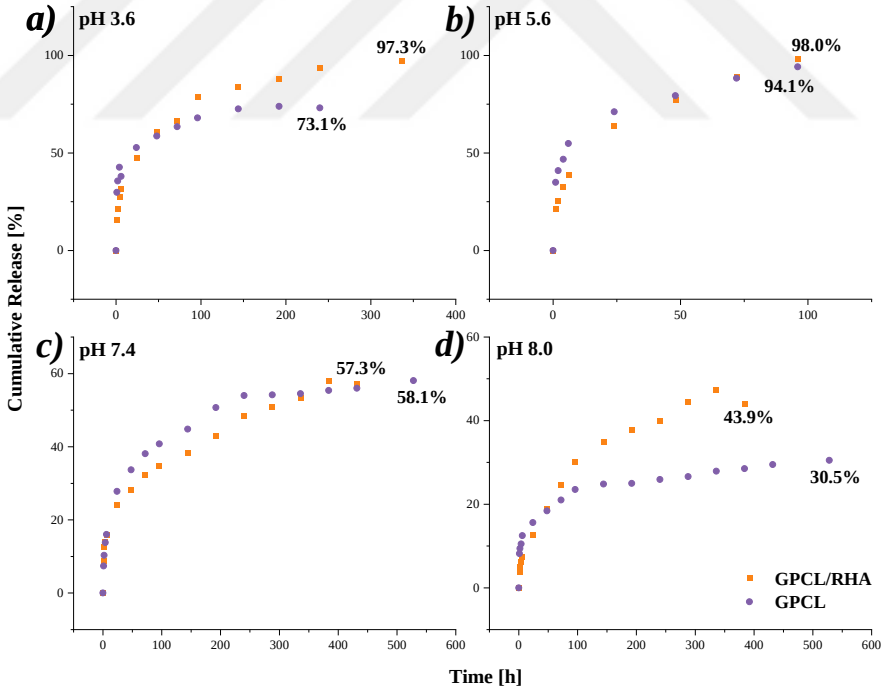

Figure 4.1 : Effect of pH conditions on cumulative drug release of drug-loaded G-PCL/RHA and G-PCL microspheres, a) 3.6, b) 5.6, c) 7.4, and d) 8.0 pH values.

For pH 3.6, the cumulative drug release percentage of G-PCL/RHA microspheres was 97.3% and GPCL microspheres were 73.1%; pH 5.6, the cumulative drug release percentage of G-PCL/RHA microspheres was 98.0% and G-PCL microspheres was 94.1%; pH 7.4, the cumulative drug release percentage of G-PCL/RHA microspheres was 57.3% and G-PCL microspheres was 58.1%; pH 8.0, the cumulative drug release percentage of G-PCL/RHA microspheres was 43.9% and G-PCL microspheres was 30.5%.

In pH 3.6 and 8.0 condition; there are obvious differences between cumulative release (%) of G-PCL/RHA nanohybrid and G-PCL microspheres while this difference is slight in pH 5.6 and 7.4. In addition it can be said that G-PCL/RHA nanohybrid system is more efficient than pure polymer G-PCL at pH 3.6, 5.6 and 8.0 conditions. It can be concluded that microspheres obtained from nanohybrid system are more successful than pure polymer microspheres in drug release.

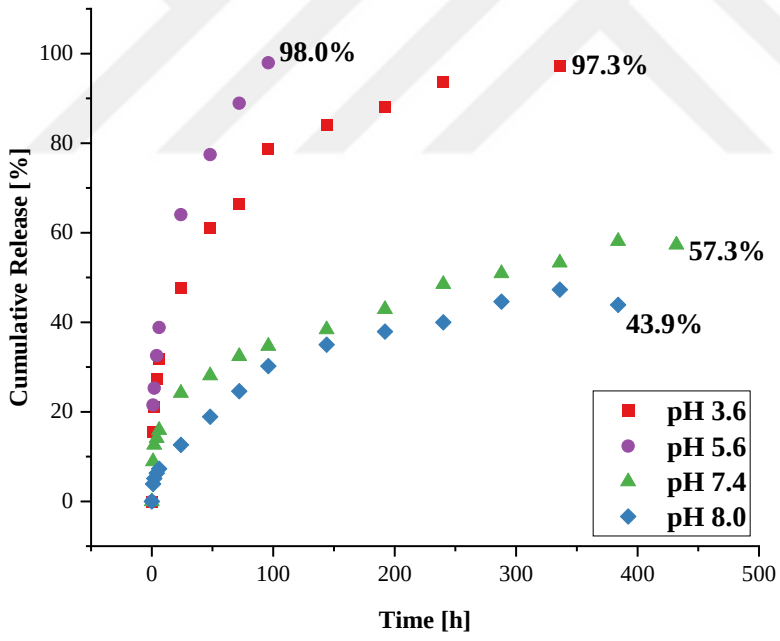

Figure 4.2 : Cumulative drug release of drug-loaded G-PCL/RHA microspheres for pH=3.6, 5.6, 7.4, and 8.0.

In Figure 4.2. the cumulative drug release percentages of G-PCL/RHA microspheres were compared in different pH conditions (3.6, 5.6, 7.4, and 8.0). The cumulative drug release percentages are the highest in pH 3.6 and 5.6 conditions. The drug release which is named initial burst effect seem to be sharp for both curves at pH 3.6

and 5.6 conditions within 48 h [38]. Decreasing pH conditions were observed to increase drug release efficiency. It can be said that this drug delivery system is more suitable in acidic conditions [37]. G-PCL/RHA microspheres showed drug releases at pH 3.6 and 5.6, 97.3% at day 14 and 98.0% at day 4 respectively. These results can lead to develop further in vivo experiments of the microspheres [39].

4.2 Kinetic Modelling Results of G-PCL/RHA and G-PCL Microspheres

Drug release profiles were fitted to Korsmeyer-Peppas, Higuchi, Zero Order and First Order release models. The parameters of the models were calculated for release profiles of G-PCL/RHA microspheres at different pH conditions (3.6, 5.6, 7.4, and 8.0). In addition, model parameters of release profile of G-PCL microspheres at 7.4 pH condition were calculated for comparing as well. Table 4.1 shows the parameters of the kinetic models.

Table 4.1 : Parameters of kinetic models fitted to chalcone release from G-PCL/RHA and G-PCL microspheres.

Model	Korsmeyer-Peppas			Higuchi		Zero Order		First Order	
Sample	K_{KP} (h^{-n})	n	R^2	K_H ($h^{-1/2}$)	R^2	K_0 (h^{-1})	R^2	K_1 (h^{-1})	R^2
G-PCL (pH:7.4)	10.66	0.29	0.980	3.27	0.794	0.0978	0.7278	0.0031	0.5466
G-PCL/RHA (pH:3.6)	19.47	0.29	0.988	6.37	0.792	0.2515	0.7696	0.0194	0.8783
G-PCL/RHA (pH:5.6)	21.76	0.33	0.998	11.05	0.879	0.8747	0.8581	0.0591	0.8634
G-PCL/RHA (pH:7.4)	8.66	0.31	0.993	3.09	0.879	0.1146	0.8672	0.0027	0.5541
G-PCL/RHA (pH:8.0)	4.04	0.42	0.982	2.61	0.970	0.1183	0.8655	0.0022	0.8187

As can be seen in Table 4.1, the highest correlation coefficients were calculated from Korsmeyer-Peppas release model. This model can be considered as the best model that fits the drug release profiles of microspheres. Active substance release generally can be controlled by dissolution, diffusion, swelling, and erosion. Korsmeyer-Peppas model are used for describing the release from a polymeric system and also describes release mechanisms such as diffusion of the water into the polymer matrix, swelling

and dissolution of the matrix simultaneously. The "n" value of this model defines how to active substance released from the matrix. If the particles are spherical shape and the "n" value is less than 0.43, the drug release is governed by the Fickian diffusion mostly. Diffusion is defined as spontaneous transport of active substance from higher concentrations to lower concentrations. Diffusion of active substance is a prominent function of the matrix systems [40]. Fickian diffusion mechanism occurs through the molecular diffusion of the drug due to a chemical potential gradient. Other diffusion mechanisms of the Korsmeyer-Peppas model can be include polymer disentaglement and erosion [41]. According to Table 4.1, all of the "n" values of Korsmeyer-Peppas release model are lower than 0.43. Thus, it can be commented that drug release mechanism of the microspheres were governed by Fickian diffusion mechanism. In this case, diffusional mechanism was more dominant than polymer relaxation or erosion. In addition, Higuchi model did not fit with the drug release profiles. This result can be show that microspheres have non-swellable character.

4.3 FT-IR Results of G-PCL/RHA Nanohybrid, G-PCL/RHA Microspheres and Chalcone

Fourier transform infrared spectroscopy (FT-IR) was used for observing intensities of functional groups and chemical bonds. Drug-loaded microspheres and commercial trans-chalcone were characterized by FT-IR spectroscopy. Figure 4.3 shows FTIR spectrum of drug-loaded microspheres below.

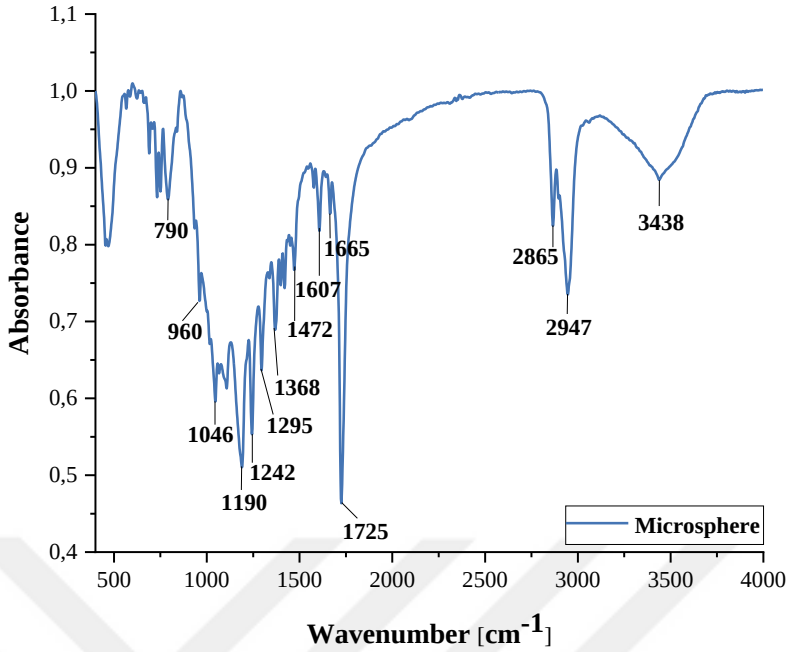

Figure 4.3 : FTIR spectrum of drug-loaded microspheres.

The region between 790-1607 cm^{-1} represents the skeletal structure of the polymer chain. This region shows bending, wagging, and stretching of the methylene groups and trans isomerization of ester groups [42]. The peaks between 790-960 cm^{-1} represent C-C stretching and C-H bending, while the peaks between 1190-1295 cm^{-1} represent C-O stretching. C-H symmetric and asymmetric deformation can be seen between 1368-1472 cm^{-1} [42,43]. The strong peak at 1725 cm^{-1} shows typical stretching C=O bonds [42–44]. The peak at 2865 cm^{-1} and its shoulder at 2947 cm^{-1} represent stretching of the C-H bonds of the methylene groups [42,43]. In addition 790 cm^{-1} region represents Si-CH_3 rocking and epoxy ring of 3-GPTMS from modified RHA and at around 1046 cm^{-1} peak represent Si-O-Si asymmetric stretching from silica based material RHA. The peak 1665 cm^{-1} represents C=O stretching and NH_2 deformation of the CALB and also the peak 3438 cm^{-1} represents –OH stretching vibrations of carboxylic acid in CALB [18].

Figure 4.4 shows FTIR spectra of commercial trans-chalcone, drug-loaded G-PCL/RHA microspheres and G-PCL/RHA nanohybrid together to determine the existence of chalcone molecules in microspheres and interactions of drug and polymer.

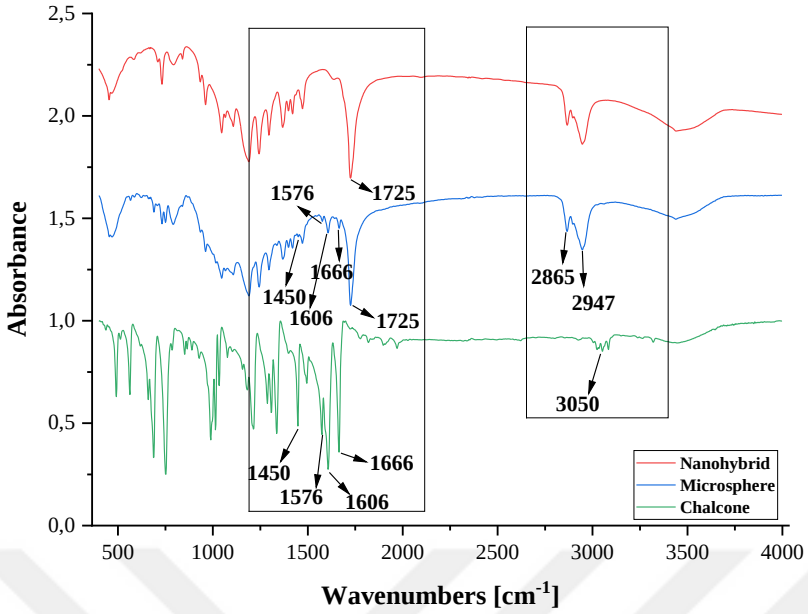

Figure 4.4 : FTIR spectra of trans-chalcone, drug-loaded G-PCL/RHA microspheres and G-PCL/RHA nanohybrid.

In the chalcone spectrum, the peak 1450 cm^{-1} represents the in-plane deformation of the =C-H bond [30]. The peaks between 1495 and 1606 cm^{-1} represent vibrations of the aromatic ring such as stretching mode of the aromatic C=C and C-C bonds [30,45]. And also, these peaks can be observed in microsphere spectrum compared to nanohybrid spectrum as expected. These peaks may be show the interactions of chalcone and polymer. In the microsphere spectrum, peaks 1666 and 1725 cm^{-1} indicate α-β unsaturated C=O bond and stretching bands of the ester respectively [30,45]. Thus, the presence of chalcone in microspheres was proven. In addition, the peak at 1666 cm^{-1} in the chalcone spectrum has upshifted and conjugated with the peak at 1725 cm^{-1} in the microsphere spectrum. The peaks at 2865 and 2947 cm^{-1} represent stretching of the C-H bonds of the methylene groups in the nanohybrid and microsphere spectra [42,43]. The peak at around 3050 cm^{-1} represents asymmetric and symmetric stretching vibrations of the aromatic C-H bonds in the chalcone spectrum [30] and this peak has downshifted and conjugated the the peaks at 2865 and 2947 cm^{-1}. This shifting can be show the interactions between C-H bonds of both the methylene group and aromatic ring.

4.4 TGA Results of G-PCL/RHA Microspheres

Drug-loaded G-PCL/RHA microspheres were also characterized by thermal gravimetric analysis (TGA) to observe the presence of drug active substance chalcone. Figure 4.5. shows TGA and DTG analysis of G-PCL/RHA microspheres.

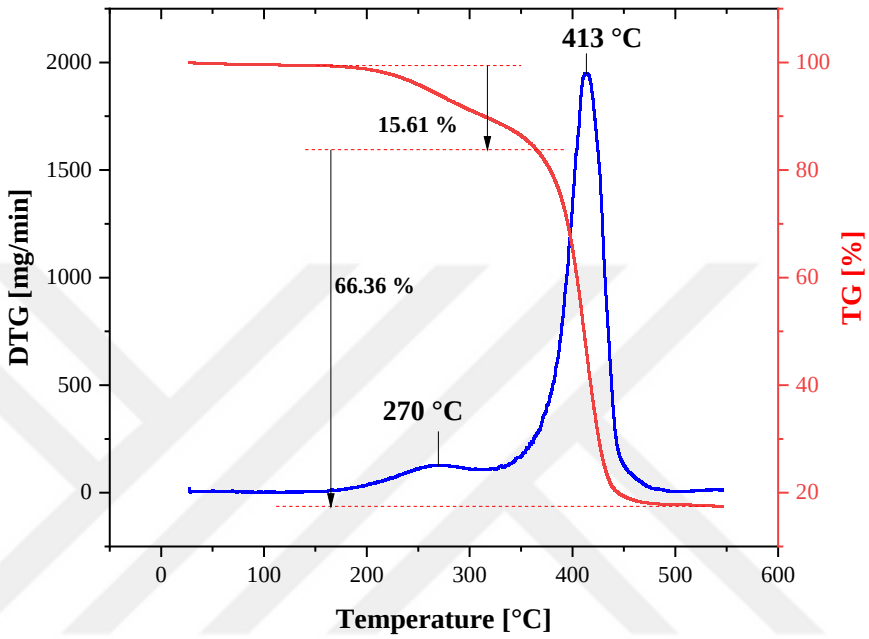

Figure 4.5 : TGA and DTG analysis of G-PCL/RHA microspheres.

As can be seen in Figure 4.5. the peaks at 270 and 413 °C can be observed. In the DTG curve, there is a peak between 165-340°C interval that represents the decomposition temperature of chalcone [31]. It can be said that in the TG curve 15.61% of weight loss represents the quantity of chalcone. The peak that is between 350-450 °C interval represents the degradation temperature of PCL polymer [46]. 66.36% of weight loss represents the quantity of PCL polymer. In addition to this, other organic matter contents can be contained in the percentage of weight loss.

4.5 DSC Results of G-PCL/RHA Microspheres

DSC melting thermogram of G-PCL/RHA microspheres can be seen in Figure 4.6. The melting point (Tm) of the microsphere is determined by DSC melting thermogram. According to the thermogram, there is a peak at 52 °C that represents the melting temperature of PCL polymer [47].

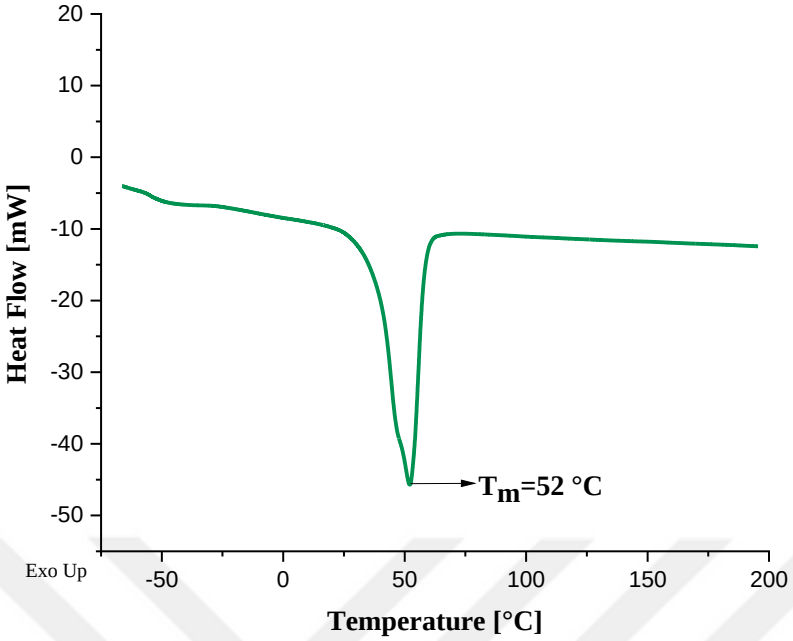

Figure 4.6 : DSC melting thermogram of G-PCL/RHA microspheres.

In previous study, crystallinity percentage of G-PCL/RHA nanohybrid was 23% while GPCL was 70% and molecular weight of G-PCL/RHA nanohybrid and pure G-PCL was 4346 and 10252 g/mol respectively. A study showed that encapsulation efficiency increased with the decreasing molecular weight of the polymer [48]. Also melting temperature of G-PCL/RHA nanohybrid was 54 °C while pure PCL was 60 °C [49]. In addition, the melting temperature of G-PCL/RHA microspheres almost remained unchanged.

4.6 SEM Results of G-PCL/RHA and G-PCL Microspheres

The surface morphologies of G-PCL/RHA and G-PCL microspheres were characterized by scanning electron microscopy (SEM). Figure 4.7 shows SEM images of G-PCL/RHA and G-PCL microspheres.

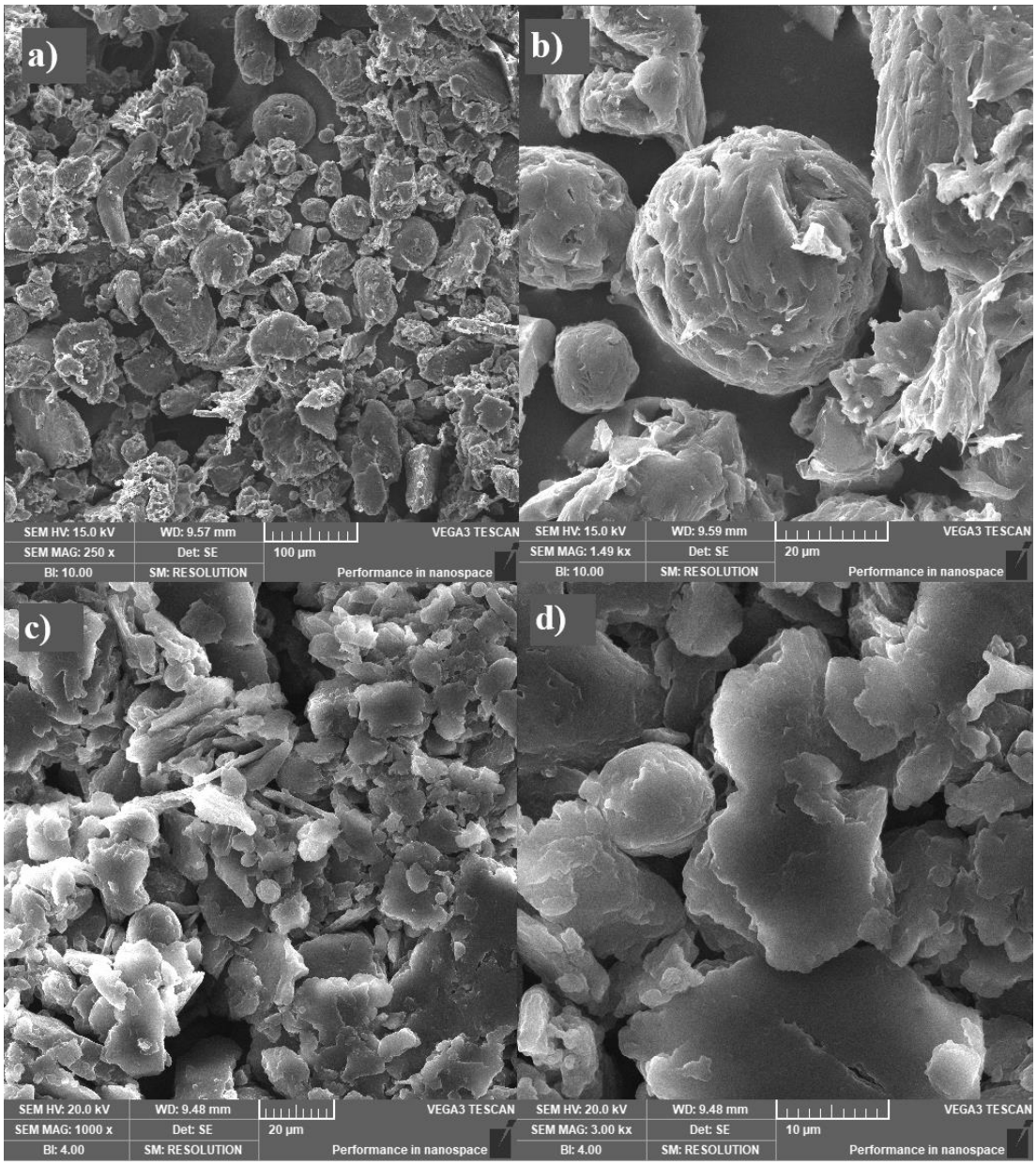

Figure 4.7 : SEM images of G-PCL/RHA microspheres: a) 250x, b)1.49 kx and G-PCL microspheres c)1.00 kx, d) 3.00 kx.

Particles of the G-PCL/RHA microspheres were appeared to be spherical as expected and sphere-like structures with various size can be seen. The surface of these microspheres were highly porous according to G-PCL microspheres. In Figure 4.7 (b), microsphere size was about 20 µm was observed. However, agglomeration and adherence can be seen in between microspheres in Figure 4.7 (a). G-PCL microspheres SEM images can be seen in Figure 4.7 (c) and (d). According to this results, G-PCL microspheres were more crystalline and smooth than G-PCL/RHA microspheres. In addition, microsphere structures cannot be easily seen in G-PCL microspheres. Microspheres have a porous surface structure which provides

advantages like have higher drug loading capacity. In addition, spherical shapes with various sizes provide microspheres permability for drug active substance and higher surface area [37].

4.7 WCA Results of G-PCL Polymer, G-PCL/RHA Nanohybrid and G-PCL/RHA Microspheres

Hydrophilic characters of the microparticles were investigated by water contact angle measurements. Water contact angle (WCA) measurements of the G-PCL polymer, G-PCL/RHA nanohybrid and G-PCL/RHA microspheres can be seen in Figure 4.8.

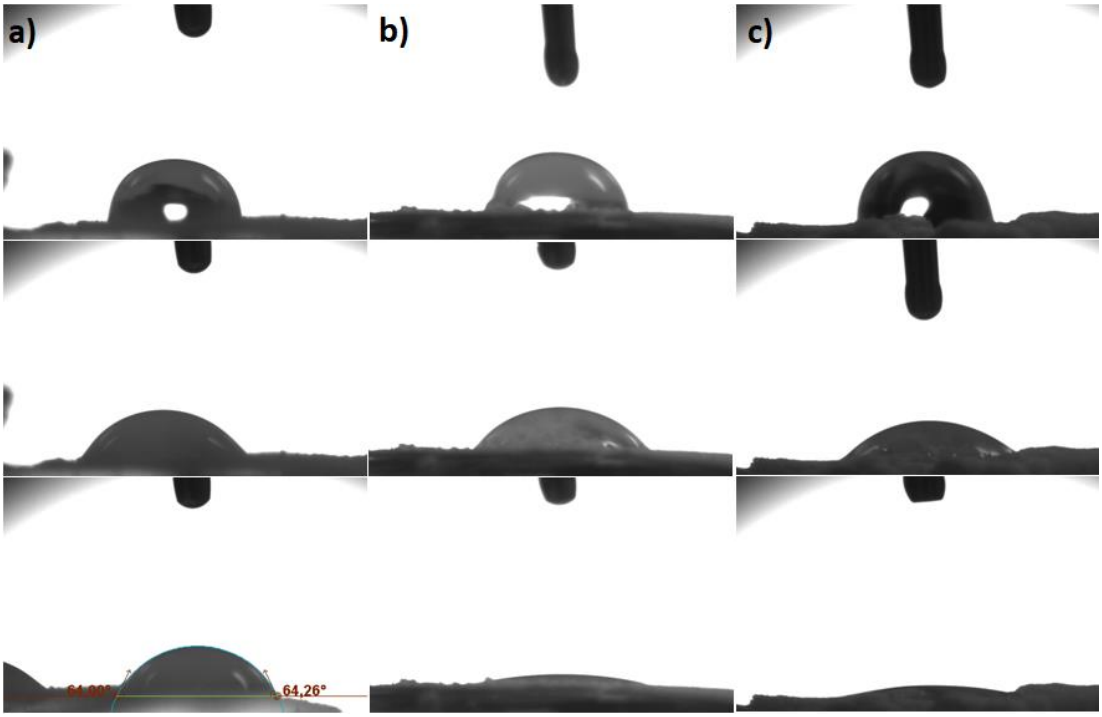

Figure 4.8 : The water contact angle measurements of a) G-PCL, b)G-PCL/RHA, c) G-PCL/RHA microspheres.

As can bee seen in the Figure 4.8, G-PCL polymer has hydrophobic property while G-PCL/RHA nanohybrid and microspheres have hydrophilic property. It can be said that nanohybrid system changed the hydrophobicity of the PCL. Hydroxyl groups of the RHA provide hydrophilic property of G-PCL/RHA nanohybrid and microspheres. In addition, hydrophilic matrices are used for controlled drug release [41].

5. CONCLUSIONS AND RECOMMENDATIONS

The first part of this study, enzymatic ring-opening polymerization (eROP) of ε-caprolactone was provided by immobilized lipase enzyme Candida antarctica lipase B (CAL-B). Free CALB was immobilized into 3-GPTMS modified rice husk ash (RHA) in the previous study [18]. eROP was terminated by adding chloroform which can solve the PCL. This mixture was precipitated in the stirring methanol which cannot solve the PCL. After evaporation and drying, G-PCL/RHA nanohybrid was obtained. Pure PCL polymer was also prepared for comparison with G-PCL/RHA nanohybrid. For this, after terminating the reaction, the mixture was washed with chloroform and the filtrate was dried to obtain pure PCL. In the second part, drug-loaded microspheres were prepared by O/W emulsion/solvent evaporation method. Dissolved G-PCL/RHA nanohybrid and trans-chalcone were mixed and this mixture was an organic phase and, 1% PVA solution was used for the aqueous phase. Dissolved substances were added to the stirring PVA solution after that, distilled water was added to the mixture to obtain hardened microspheres. The same procedure was applied to the pure PCL. Finally, G-PCL/RHA and G-PCL drug-loaded microspheres were obtained. In the last part, drug release experiments were carried out. Different pH conditions (3.6, 5.6, 7.4, and 8.0) were simulated by buffer solutions. The release profiles of the drug-loaded G-PCL/RHA and G-PCL microspheres were determined and cumulative drug release percentages were calculated.

According to drug release results, the highest cumulative drug release percentage was 97.3% at pH 3.6 following 98.0% at pH 5.6. Besides, cumulative drug release percentages of microspheres at pH 7.4 and 8.0 were 57.3% and 43.9% respectively. Apparently, drug release was more efficient in acidic conditions. These results indicate that the chalcone release from G-PCL/RHA microspheres was affected by pH conditions of the media. Besides, G-PCL microspheres also showed the same drug release performance but not as high drug release as G-PCL/RHA nanohybrids. The sharp curve at pH 3.6 and 5.6 conditions within 48 h was the initial burst effect.

The drug molecules that are found near to the surface of the microsphere may lead to a small initial burst release. Low water permeability of the PCL hinders the penetration of the water into the polymer matrix and the diffusion of the drug to the media is retarded and this results burst release. After this part, the drug release profile slows down and provides sustained drug release [38, 48] with the help of hydrophobic properties of PCL and this leads to drug release prolonged time [50]. In addition, it is known that although PCL is biodegradable polymer, it degrades quite slowly according to the other biodegradable polymers. The surface degradation of the PCL is not observed or very slow during the drug release because of its hydrophobicity. So, the drug release is possible by the diffusional mechanism. The diffusion occurs by penetrating of the water into the amorphous region [39, 48]. The drug release profiles were fitted to several mathematical models. The best fitted model was Korsmeyer-Peppas. In addition, Fickian diffusion mechanism is present if the "n" value of the model for the spherical particles is less than 0.43. According to release model results, all of the "n" values are less than 0.43. So, the drug release only occurs by diffusional mechanism in the G-PCL and G-PCL/RHA microspheres. Thus, diffusional mechanism was more dominant than polymer disentanglement or erosion [41]. However, the degradation of PCL can be occurred by lipases and esterases in vivo which leads to faster degradation than in vitro [39].

Functional groups and chemical bonds were determined by FT-IR analysis. FT-IR spectra of trans-chalcone, G-PCL/RHA nanohybrid and drug-loaded G-PCL/RHA microspheres were compared and the presence of chalcone in the microspheres was demonstrated. 1666 and 1725 cm^{-1} peaks can be observed in both G-PCL/RHA and chalcone FT-IR spectra. These peaks show α-β unsaturated C=O bond and stretching bands of the ester respectively [45]. In addition, the peak 1725 cm^{-1} shows typical C=O bonds in the PCL polymer. The fact that 1725 cm^{-1} peak is too strong in the G-PCL/RHA spectrum can be interpreted as the overlap of the two peaks. Moreover, the peaks 1495 and 1579 cm^{-1} represents vibrations of the aromatic ring-like stretching mode of the aromatic C=C and C-C bonds [30, 45]. The peak 3056 cm^{-1} represents asymmetric and symmetric stretching vibrations of the aromatic C-H bonds from the chalcone molecule [30] and has downshifted and conjugated the the peaks at 2865 and 2947 cm^{-1} that represent methylene group of the PCL. This shifting can be show the interactions between C-H bonds of both the methylene

group and aromatic ring. According to a study, hydrophobic interactions between drug and polymer may be associated between the aromatic ring of chalcone and methylene groups of PCL [54].

Thermal properties and weight loss of G-PCL/RHA microspheres were analyzed by TGA analysis. In DTG graph, two peaks were observed. 270 °C represents decomposition temperature of chalcone [31] while 413 °C represents degradation temperature of PCL polymer [46]. According to these, 15.61% and 66.36% percentage show quantity of chalcone and PCL respectively, including some other organic matter contents. G-PCL/RHA microspheres were analyzed by also DSC. According to DSC analysis, the peak at 52 °C represents the melting temperature of PCL polymer [47]. In a previous study, the crystallinity percentage of G-PCL/RHA nanohybrid was 23% while G-PCL was 70% and the molecular weight of G-PCL/RHA nanohybrid and pure G-PCL was 4346 and 10252 g/mol respectively. Also melting temperature of G-PCL/RHA nanohybrid was 54 °C while pure PCL was 60 °C [49]. It can be said that the nanohybrid structure decreased the crystallinity. According to this, G-PCL/RHA nanohybrid was more amorphous than G-PCL. Decreasing molar weight, crystallinity, and melting temperature have proven this. The encapsulation efficiency of G-PCL/RHA and G-PCL microspheres was 74.4±1.2(%) and 60.7±9.9(%), respectively. A study showed that encapsulation efficiency increased with the decreasing molecular weight of the polymer [48]. More drug was loaded in G-PCL/RHA than G-PCL microspheres because of G-PCL/RHA microspheres were more amorphous and have less molecular weight. In drug release experiments, the initial burst release can be affected that. In addition, the melting temperature of G-PCL/RHA microspheres almost remained unchanged. Thus, it can be commented that the crystallinity of G-PCL/RHA nanohybrid was not affected by the microencapsulation process [39]. Microsphere structures were observed by SEM analysis. Spherical and sphere-like structures with various sizes can be seen in SEM images of the G-PCL/RHA microspheres. According to these images, the microspheres have an advantageous porous surface structure that can be caused by the RHA content. Porous structures have higher surface area and provide higher drug loading capacity and permeability for drug active substance. However, agglomeration and adherence are observed in the images [37]. According to research of Chen et.al. (2000) PVA solution was hydrophilic that used in the microsphere

production process. This leads to coagulation of emulsion microdroplets so irregular microspheres and uneven particle size distribution [51]. The more crystalline structure can be seen in the SEM images of the G-PCL microspheres. This may leads to less formation of the microsphere structures so, this structures cannot be easily seen in G-PCL microspheres.

According to contact angle measurement; although PCL is a hydrophobic polymer [7], G-PCL/RHA nanohybrid and microspheres are hydrophilic. It can be said that nanohybrid system changed the hydrophobicity of the PCL. Hydrophobic adsorption plays a crucial role for drug loading. The hydrophobic core of nanoparticles including hydrophilic drugs show quite slow drug release [52]. Chalcones are known as hydrophobic [53] and G-PCL/RHA nanohybrid is hydrophilic. Drug release results demonstrated that drug-loaded G-PCL/RHA microspheres release the drug faster than pure G-PCL microspheres. Besides, encapsulation efficiency of hydrophilic G-PCL/RHA microspheres was higher than G-PCL microspheres. Hydrophilic matrices are used for controlled drug release [41]. According to mathematical release model, drug was released by a diffusional mechanism in this study. Drug release may carried out by hydrophilic and hydrophobic interactions between polymer nanohybrid and chalcone. Besides, pH effect on the drug release can be explained by the reactivity of epoxy ring of 3-GPTMS and protonation of amine groups that located in enzyme CAL-B in acidic media [12]. For better understanding drug-polymer interactions and pH effect on the drug release mechanism, analysis methods can be developed in further studies. Besides, drug release of microspheres can be carried out in vivo. Such studies exist in the literature.

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APPENDICES

APPENDIX A: Calibration Curves

APPENDIX A

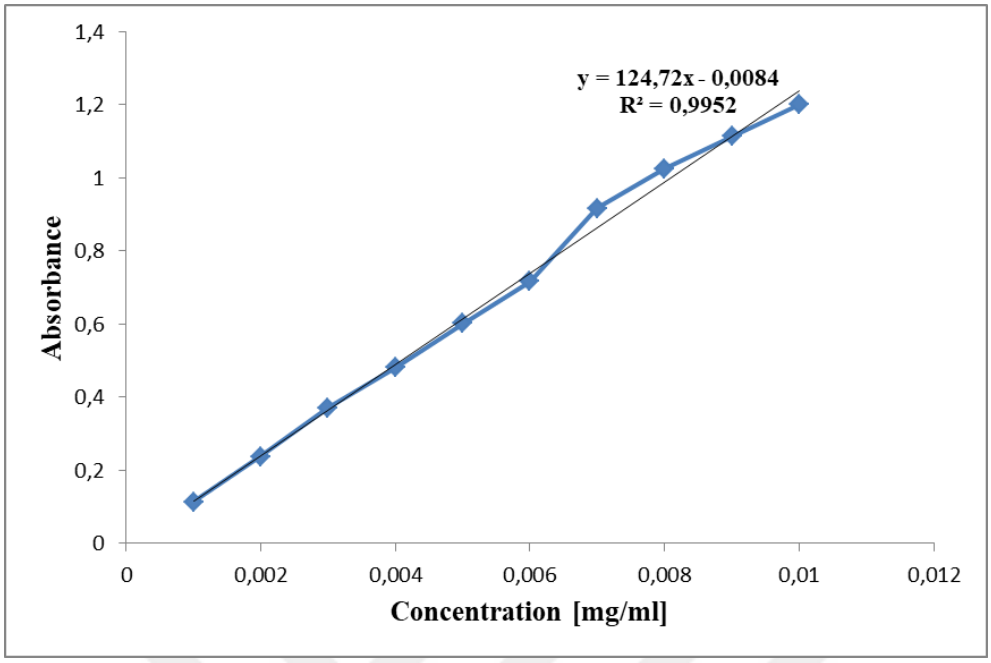

Figure A.1 : Calibration curve for calculating encapsulation efficiency of microspheres.

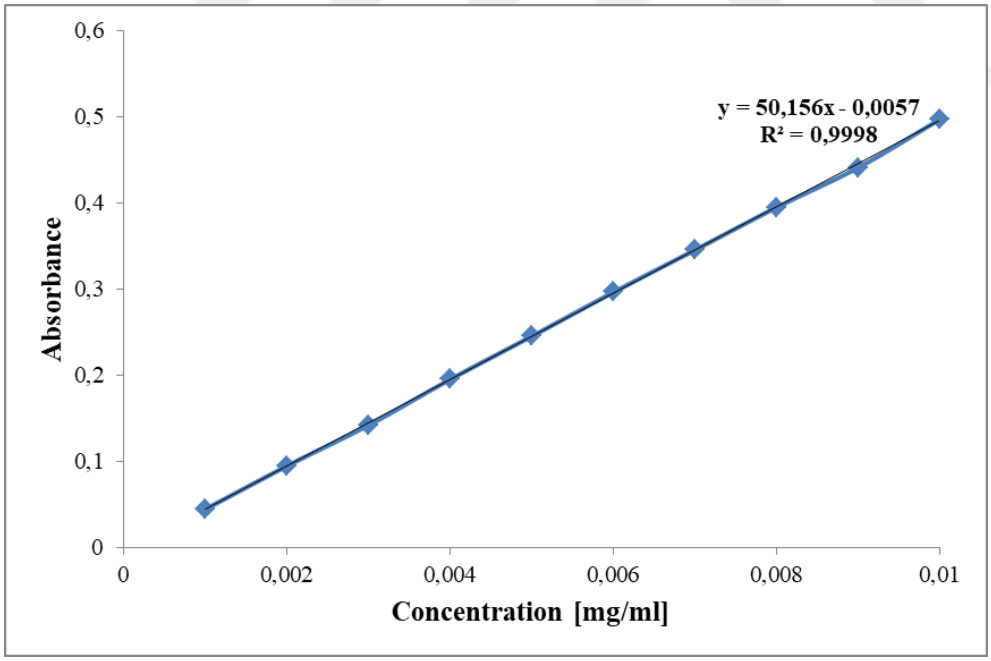

Figure A.2 : Calibration curve for pH 3.6 condition.

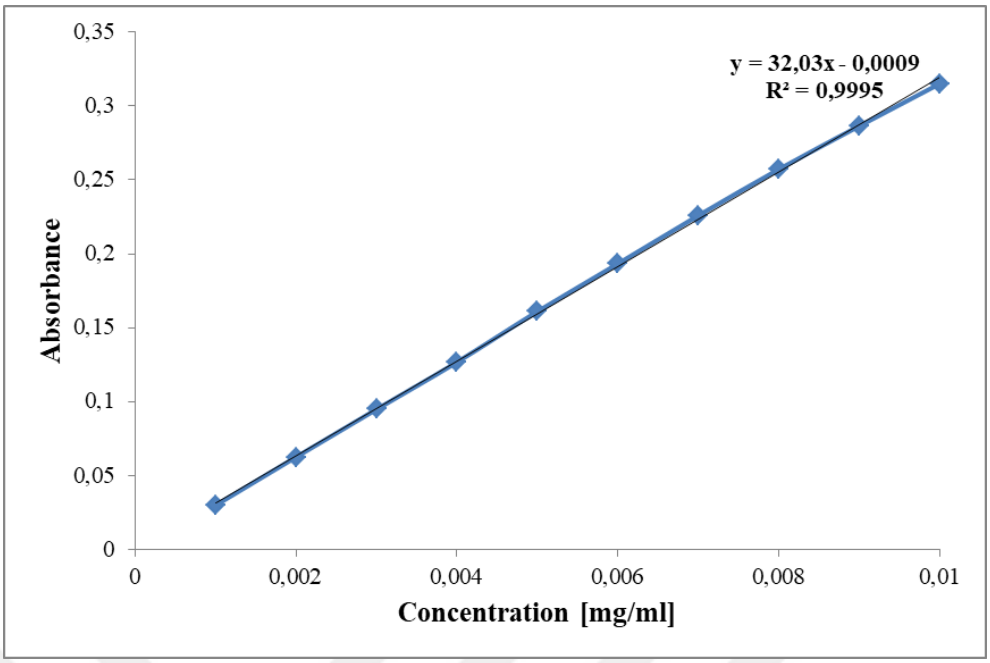

Figure A.3 : Calibration curve for pH 5.6 condition.

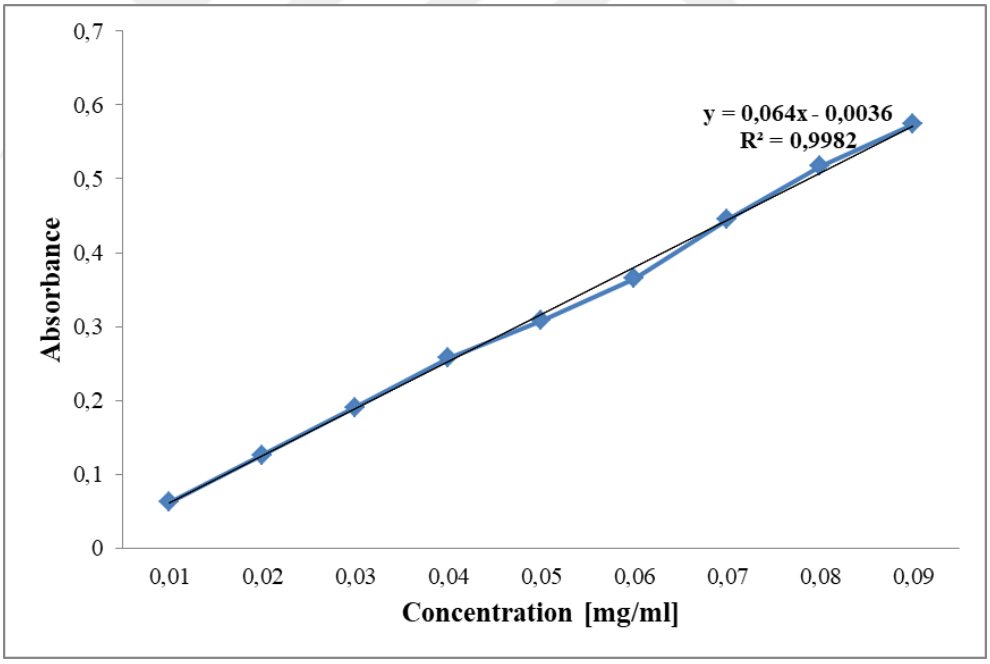

Figure A.4 : Calibration curve for pH 7.4 condition.

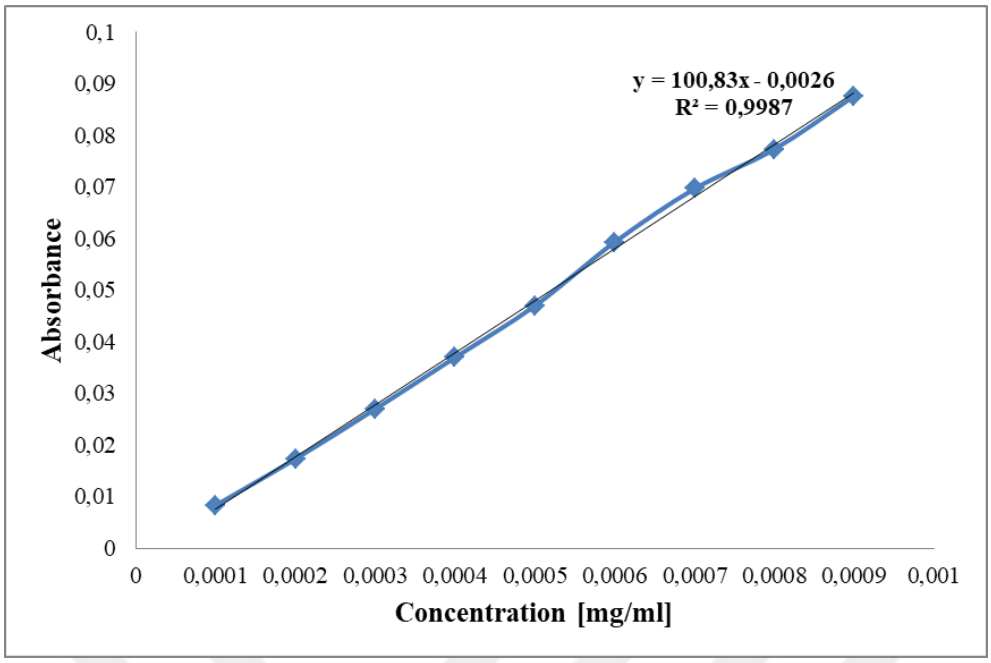

Figure A.5 : Calibration curve for pH 8.0 condition.

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