

ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL

**CONTROLLED DELIVERY OF CHALCONE VIA BIOPOLYESTER
NANOHYBRID**



PhD. THESIS

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Department of Chemical Engineering

Chemical Engineering Programme

NOVEMBER 2022

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Thesis Advisor: Prof. Dr. F. Yüksel GÜVENİLİR

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To everyone,



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ABBREVIATIONS

3-APTES	: (3-Aminopropyltriethoxysilane
3-APTMS	: (3-Aminopropyl)trimethoxysilane
3-GPTMS	: (3-Glycidyloxypropyl)trimethoxysilane
3-TMSPDA	: N-[3-(Trimethoxysilyl)propyl]ethylenediamine
A-RHA	: RHA activated with 3-APTMS
AP-RHA	: RHA activated with 3-APTES
CALB	: <i>Candida antarctica</i> Lipase B
DSC	: Differential scanning calorimetry
ϵ-CL	: Epsilon-caprolactone
e-ROP	: Enzymatic ring opening polymerization
FT-IR	: Fourier transform infrared spectrophotometry
G-RHA	: RHA activated with 3-GPTMS
¹H-NMR	: Proton nuclear magnetic resonance
HLB	: Hydrophilic lipophylic balance
PCL	: Polycaprolactone
PCL-A-RHA	: Nanohybrid synthesized with immobilized CALB on A-RHA
PCL-AP-RHA	: Nanohybrid synthesized with immobilized CALB on AP-RHA
PCL-G-RHA	: Nanohybrid synthesized with immobilized CALB on G-RHA
PCL-T-RHA	: Nanohybrid synthesized with immobilized CALB on T-RHA
PVA	: Polyvinyl alcohol
RHA	: Rice husk ash
SEM	: Scanning electron microscopy
T-RHA	: RHA activated with 3-TMSPDA
TC	: Trans-chalcone
TGA	: Thermogravimetric analysis
XRD	: X-ray diffraction



SYMBOLS

K₀	: Release constant of Zero Order model
K₁	: Release constant of First Order model
K_H	: Release constant of Higuchi model
K_{KP}	: Release constant of Korsmeyer-Peppas model
M_n	: Number average molecular weight
n	: Release exponent of Korsmeyer-Peppas model
R²	: Coefficient of determination
t	: Time
T_c	: Crystallinity temperature
T_g	: Glass transition temperature
T_m	: Melting temperature
v/v	: Volume to volume ratio
w/v	: Weight to volume ratio
w/w	: Weigh to weight ratio
X_c	: Crystallinity percentage
XL	: Inductive reactance
ΔH⁰_m	: Heat of fusion



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CONTROLLED DELIVERY OF CHALCONE VIA BIOPOLYESTER NANOHYBRID

SUMMARY

In recent years, biodegradable polymers have received great attention especially in medical applications and have begun to replace traditional petroleum-based synthetic polymers. Durable polymeric materials with superior physical, mechanical and chemical properties are highly demanded for medical applications. It is crucial that these materials can survive and perform in the harsh conditions of the human body, such as very low or very high pH environments and mechanical stress. One approach to manufacturing such advanced medical devices is the use of hybrid polymeric materials. In simple terms, a hybrid polymer is material consisting of two compartments, one of which is a natural or synthetic polymer, interacting with each other at the molecular level. The formation of an organic/inorganic hybrid system allows us to take advantage of the advantageous properties of each component or to create enhanced properties, sometimes synergistically. Especially in drug delivery and controlled release applications, several inorganic materials such as iron oxide nanoparticles, gold nanoparticles, silver nanoparticles, mesoporous silica and various types of clay are widely used either individually or in combination with polymers. These inorganic materials are preferred because of their small particle sizes and improved optical, electrical and mechanical properties. Despite the superior properties of inorganic materials, the use of such inorganic particles as drug carriers has some drawbacks. The main disadvantage is that they require surface modifications to ensure stability and good dispersion. Generally, these inorganic particles are used in drug delivery applications by coating or grafting with biodegradable and biocompatible polymers or forming composites. This approach also increases the biocompatibility of particles, which is one of the key features in the development of successful drug delivery system. These polymers can be synthetic or natural, and the most commonly used polymers in organic/inorganic hybrid systems for medical applications are polycaprolactone, polyvinyl alcohol, poly(d,l-lactide-co-glycolide), polyethylene glycol. Organic/inorganic hybrid systems developed with a polymer and inorganic particles can be classified under two main groups depending on the interactions between the two components. In Class 1 hybrid systems, inorganic particles are trapped or encapsulated in the polymer matrix by weak intermolecular forces such as Van der Waals, electrostatic interactions, and hydrogen bonding. Class 2 organic/inorganic hybrid system is formed by covalent or ionic bonding between organic and inorganic components. This covalent bonding can be formed by two different approaches: polymer in situ synthesis in the presence of inorganic particles, in situ formation of inorganic material, or a combination of polymer and inorganic material, both of which are produced ex situ. Interface characteristic is an important factor that changes the characteristics of the developed hybrid system.

PCL has high biocompatibility as its degradation products can be metabolized in the body or excreted directly from the body. Because of its biodegradability and biocompatibility, PCL has been approved by the US Food and Drug Administration

for use in medical applications. PCL-based materials have been successfully used in bone tissue regeneration, skin tissue engineering and vascular tissue engineering applications. In addition, several drug release studies using PCL-based drug delivery systems have been reported. PCL can be synthesized both chemically and enzymatically via ring-opening polymerization (ROP). Industrially, tin octoate is used as a metallic catalyst. Metallic catalysts operate at high temperature and pressure. Also, the end product may be toxic due to unremoved metallic residue, thus reducing the chances of PCL's medical applications. On the other hand, enzymes are non-toxic and can catalyze reactions in milder conditions. Lipase enzymes catalyze the polymerization of ϵ -caprolactone (CL).

Drug delivery system (DDS) designs improve drug pharmacokinetics and biodistribution and provide a sustained release profile. DDS provides some exceptional properties compared to conventional drug formulations. The major disadvantage of active substances used in the treatment of diseases is that some molecules agglomerate in body fluids due to their hydrophobic character. Conventional drug formulations provide a solution to this complexity by using appropriate additives. However, these additives can have adverse effects on their intended site of action. The drug carrier used in such designs also protects the targeted area from the toxic effects of active molecules by controlling the dosage and keeping it below the toxic limit. Another function of the carrier material is to protect active molecules from premature degradation and rapid degradation by body metabolism. Smart drug delivery systems (SDDS) are systems designed and developed to deliver active substances to the desired site of action and to release them when stimulated by a physical or chemical change. The main purpose of using SDDS is to control the release kinetics so that the active material can be delivered to the desired site of action without causing any side effects to the non-targeted sites. Controlled release of the active ingredient is usually provided by stimuli-responsive polymers. Such polymers can undergo structural changes when exposed to different physical conditions that facilitate drug release. These changes in the physical environment, or 'stimulants', can be light radiation, temperature, pH, and magnetic stimuli.

Chalcones are open-chain molecules naturally found in plants. Their chemical structure consists of two aromatic rings with a three-carbon α,β -unsaturated carbonyl system between them. The chemical structure of chalcones can be varied by adding functional groups to aromatic rings. Trans-chalcone (TC) has attracted attention in recent years in terms of its biological activities, due to its abundance in nature, its preparation and its simple structure. TC has been proven to have anticancer activity against several types. The anti-leishmania activity of trans-chalcone has been widely studied. TC is also anti-inflammatory and acts by reducing oxidative stress caused by various inflammatory diseases. However, there are limitations to the clinical use of TC, mainly due to its water-insoluble and thus low bioavailability. TC is a plant-based chemical, so its toxicity in the body is relatively low compared to synthetic drug molecules. However, one extremely risky aspect of TC accumulation in the body is that TC is a proestrogen. TC is metabolically activated to many other chemicals. These compounds have been shown to have estrogenic activity. Many adverse health effects may occur in mammals due to this estrogenic activity of xenobiotic compounds, such as precocious puberty in females, obesity, decreased sperm count, changes in reproductive organs and sexual behavior, and an increase in certain types of cancer. Therefore, it is very important to control the dosage of TC therapy and prevent the accumulation of TC molecules in the body.

This study aimed to synthesize a new hybrid polymer based on PCL and silica particles with low crystallinity and hydrophilic character. The synthesis reaction was in situ ring-opening polymerization of ϵ -caprolactone catalyzed by immobilized *Candida antarctica* Lipase B. In this study, the free form of *Candida antarctica* Lipase B was immobilized on rice husk ash by physical adsorption. The specificity and stability of CALB were increased by providing enzyme immobilization. The support material on which the enzyme was immobilized was first prepared by burning rice husks in an oven at 650 °C for 6 hours. The produced RHA is a material with a high silica content, which plays a very important role in the formation of the nanohybrid system in the next steps. In order to add functional groups that will facilitate enzyme adsorption to the RHA surface, the surface was modified using four different organosilane compounds, 3-APTES, 3-APTMS, 3-GPTMS and 3-TMSPDA, before enzyme immobilization. Results from the analysis of TGA curves found that different organosilane compounds behave differently. Surface modification percentages were calculated as 1.2%, 0.8%, 3.7% and 10.1% for 3-APTES, 3-APTMS, 3-GPTMS and 3-TMSPDA, respectively. This reaction took place through the –OH groups of RHA and the methoxy or ethoxy groups of the silanization agents used, and Si-O-Si bonds were formed. After CALB immobilization on surface modified RHA, the resulting catalytic systems were used to catalyze the ROP of ϵ -caprolactone and to synthesize PCL-based nanohybrid systems in situ. During this reaction, short PCL chains were grafted from the free –OH groups of surface-modified RHA as well as the long, aliphatic chains of pure PCL. Therefore, it is very important to keep the surface modification at an optimum level in order to achieve PCL grafting from silica. Evidence from this analysis shows that increasing the percentage of silanization by a given amount increases the grafting efficiency. Previous studies suggest an inverse relationship between PCL chain length and the number of surface Si-OH groups. The findings of this study are in line with those of previous studies that suggested the role of silanol groups as co-initiators for the polymerization reaction resulting in a high number of growing chains. A significant decrease in percentage of crystallinity was observed for all nanohybrid samples, which was associated with low molecular weight and inhibition of crystal formation by silica in the nanohybrids. Also increased glass transition temperature due to restricted mobility caused by grafted PCL. PCL-based nanohybrids were hydrophilic. The hydrophilic character of nanohybrids can markedly increase the bioavailability of poorly water soluble drug molecules. The second aim of this study is to develop TC-loaded microspheres with O/W emulsion and nanospheres with interfacial polymer deposition method and to investigate the loading efficiency and in vitro release behavior. PCL-based nanohybrids synthesized in the first part of this study were used as polymeric carriers in these drug delivery systems. The result of this research showed that there are optimum microsphere formulations with 60-75% encapsulation efficiency. One of the more important findings from this study is that TC release was prolonged in a controlled manner to 22-57 days. It is an important property of our hydrophilic microspheres as it can increase the bioavailability of poorly water-soluble TC. Similar results were obtained with TC-loaded nanospheres produced by interfacial polymer deposition or nano-deposition method. Higher encapsulation efficiency (80-83%) was obtained with nanospheres. TC release from the nanosphere formulation was increased relative to the microsphere formulations; cumulative emissions reached 83-90%. The nanospheres showed pH-dependent release behavior; the acidity of the release medium increased the release. The TC release has been extended to 28 days under neutral conditions. Water contact angle measurements also revealed the hydrophilic character of the nanospheres.



BİYOPOLİESTER NANOHİBRİT İLE KALKONUN KONTROLLÜ SALIMI

ÖZET

Son yıllarda biyolojik olarak parçalanabilen, biyobozunur polimerler özellikle medikal uygulamalarda büyük ilgi görmüş ve geleneksel petrol temelli sentetik polimerlerin yerini almaya başlamıştır. Polikaprolakton (PKL), biyolojik olarak parçalanabilme özelliğine sahip alifatik bir polyesterdir. Yapısındaki alifatik ester bağları hidrolize duyarlıdır ve bu sayede vücut içerisinde biyolojik olarak parçalanabilmektedir. Bununla birlikte, biyolojik olarak parçalanması vücutta oldukça yavaştır. Uygulama alanına göre biyobozunurluğun artırılması gerektiği takdirde çeşitli yöntemler kullanılarak biyobozunma hızı artırılabilir. Bu yöntemler arasında farklı polimerler ile harman, kompozit ya da kopolimerler oluşturularak veya farklı üretim yöntemleri kullanılarak kristalinite yüzdesinin azaltılması sayılabilir.

Hibrit polimerler, iki farklı polimer tipinden veya bir polimer ve bir inorganik dolgu malzemesinden oluşur. Bu tür hibrit polimerler sayesinde hibrit sistemin bileşenlerinin kendi başlarına kullanılmasıyla elde edilemeyen özellikler elde edilebilir. Üstün fiziksel, mekanik ve kimyasal özelliklere sahip dayanıklı polimerik malzemeler tıbbi uygulamalar için oldukça talep görmektedir. Bu malzemelerin, çok düşük veya çok yüksek pH ortamları ve mekanik stres gibi insan vücudunun zorlu koşullarında dayanım ve performans gösterebilmesi çok önemlidir. Bu tür gelişmiş tıbbi cihazların üretilmesine yönelik yaklaşımlardan biri, hibrit polimerik malzemelerin kullanılmasıdır. Basit bir ifadeyle, bir hibrit polimer, biri doğal veya sentetik bir polimer olan ve moleküler düzeyde birbirleriyle etkileşime giren iki bölmeden oluşan malzemedir. Organik/inorganik hibrit bir sistemin oluşumu, her bir bileşenin avantajlı özelliklerinden yararlanılmasına veya bazen sinerjik olarak gelişmiş özellikler yaratılmasına izin verir. Özellikle ilaç verme ve kontrollü salım uygulamalarında, demir oksit nanoparçacıkları, altın nanoparçacıkları, gümüş nanoparçacıkları, gözenekli silika ve çeşitli kil türleri gibi birkaç inorganik malzeme ya ayrı ayrı ya da polimerlerle birleşerek yaygın olarak kullanılmaktadır. Bu inorganik malzemeler küçük partikül boyutları ve gelişmiş optik, elektriksel ve mekanik özellikleri nedeniyle tercih edilmektedir. İnorganik malzemelerin üstün özellikleri olmasına rağmen, bu tür inorganik parçacıkların ilaç taşıyıcıları olarak kullanılmasının bazı sakıncaları vardır. Ana dezavantaj, stabilite ve iyi dağılım sağlamak için yüzey modifikasyonları gerektirmeleridir. Genel olarak, bu inorganik partiküller, biyobozunur ve biyouyumlu polimerlerle kaplama, aşılama veya kompozitler oluşturma yoluyla ilaç salım uygulamalarında kullanılır. Bu yaklaşım aynı zamanda başarılı ilaç salım sisteminin geliştirilmesinde anahtar özelliklerden biri olan partiküllerin biyouyumluluğunu da artırır. Bu polimerler sentetik veya doğal olabilir ve tıbbi uygulamalar için organik/inorganik hibrit sistemlerde en yaygın olarak kullanılan polimerler polikaprolakton, polivinil alkol, poli(d,l-laktid-ko-glikolid), polietilen glikoldür. Bir polimer ve inorganik partiküller ile geliştirilen organik/inorganik hibrit sistemler, iki bileşen arasındaki etkileşimlere bağlı olarak iki ana grup altında sınıflandırılabilir. Sınıf 1 hibrit sistemlerde, inorganik parçacıklar, Van der Waals, elektrostatik

etkileşimler ve hidrojen bağı gibi zayıf moleküller arası kuvvetler tarafından polimer matrisinde tutulur veya matris içerisine kapsülendir. Organik/inorganik hibrit malzemelerin bu sınıfı, yerinde veya yerinde olmayan yöntemlerle sentezlenebilir. 2. Sınıf organik/inorganik hibrit sistem, organik ve inorganik bileşenler arasındaki kovalent veya iyonik bağ ile oluşturulur. Bu kovalent bağlanma, inorganik partiküllerin varlığında polimerin yerinde sentezi, inorganik malzemenin yerinde oluşturulması veya her ikisi de ex situ olarak üretilen polimer ve inorganik malzemenin kombinasyonu olmak üzere iki farklı yaklaşımla oluşturulabilir. Arayüz karakteristiği, geliştirilen hibrit sistemin özelliklerini değiştiren önemli bir faktördür.

PKL, bozunma ürünleri vücutta metabolize edilebildiği veya doğrudan vücuttan atılabildiği için biyouyumluluğu yüksek bir polimerdir. Biyobozunurluğu ve biyouyumluluğu nedeniyle PKL, tıbbi uygulamalarda kullanım için ABD Gıda ve İlaç Dairesi tarafından onaylanmıştır. PKL tabanlı materyaller, kemik dokusu rejenerasyonu, cilt dokusu mühendisliği ve vasküler doku mühendisliği uygulamalarında başarıyla kullanılmıştır. Ek olarak, PKL bazlı ilaç salım sistemlerini kullanan çeşitli çalışmalar yapılmıştır.

PKL, halka açma polimerizasyonu (ROP) yoluyla hem kimyasal hem de enzimatik olarak sentezlenebilir. Endüstriyel üretimde kalay oktoat metalik bir katalizör olarak kullanılır. Metalik katalizörler yüksek sıcaklık ve basınçta çalışır. Ayrıca, son ürün, uzaklaştırılmamış metalik kalıntı nedeniyle toksik olabilir ve bu nedenle PKL'nin tıbbi uygulamalarda kullanılma şansını azaltır. Öte yandan, enzimler toksik değildir ve daha hafif koşullarda reaksiyonları katalize edebilir. Lipaz enzimleri, ϵ -kaprolaktonun (KL) polimerizasyonunu katalize eder. Bu reaksiyonun ilk basamağında lipaz enzimi ϵ -kaprolakton ile reaksiyona girerek enzim-KL kompleksini oluşturur. Daha sonra ortamdaki hidroksil uçlu molekül ara ürün ile reaksiyona girer ve polimerin ilk zinciri oluşur. Daha sonra zincir büyümesi bu molekül aracılığıyla devam eder.

İlaç taşıma sistemi (DDS) tasarımları, ilacın farmakokinetiğini ve biyolojik dağılımını geliştirir ve sürekli bir salım profili sağlar. DDS, geleneksel ilaç formülasyonlarına kıyasla bazı istisnai özellikler sağlar. Hastalıkların tedavisinde kullanılan aktif maddelerin en büyük dezavantajı, bazı moleküllerin hidrofobik karakterde olması nedeniyle vücut sıvılarında aglomere olmasıdır. Geleneksel ilaç formülasyonları, uygun katkı maddeleri kullanarak bu karmaşıklığa bir çözüm getirmektedir. Bununla birlikte, bu katkı maddelerinin amaçlanan etki alanı üzerinde olumsuz etkileri olabilir. DDS tasarımlarında genellikle ipek fibroin, kolajen, jelatin, albümin, nişasta, selüloz, kitosan, poli(laktik asit), polikaprolakton, poliglikolik asit ve polilaktik glikolik asit gibi biyouyumlu polimerler kullanılmakta ve bu da katkı maddesi ihtiyacını ortadan kaldırmaktadır. Bu tür tasarımlarda kullanılan ilaç taşıyıcı da dozajı kontrol ederek ve toksik limitin altında tutarak hedeflenen bölgeyi aktif moleküllerin toksik etkilerinden korur. Taşıyıcı malzemenin bir başka işlevi, aktif molekülleri vücut metabolizması tarafından erken ve hızlı bozulmadan korumaktır.

Akıllı ilaç taşıma sistemleri (SDDS), aktif maddeleri istenen etki alanına iletmek ve fiziksel veya kimyasal bir değişiklik ile uyarıldığında serbest bırakmak için tasarlanmış ve geliştirilmiş sistemlerdir. SDDS'yi kullanmanın temel amacı, aktif malzemenin, hedeflenmeyen bölgelere herhangi bir yan etkiye neden olmadan istenen eylem bölgesine iletebilmesi için salım kinetiğini kontrol etmektir. Aktif maddenin kontrollü salımı genellikle uyaranlara tepki veren polimerler tarafından sağlanır. Bu tür polimerler, ilaç salımını kolaylaştıran farklı fiziksel koşullara maruz kaldıklarında yapısal değişikliklere uğrayabilirler. Fiziksel ortamdaki bu değişiklikler veya "uyarıcılar", ışık radyasyonu, sıcaklık, pH ve manyetik uyaranlar olabilir. SDDS, pH'a duyarlı sistemlerin oldukça araştırıldığı kanser tedavisinde özellikle yararlıdır.

Kalkonlar, bitkilerde doğal olarak bulunan açık zincirli moleküllerdir. Kimyasal yapıları, aralarında üç karbonlu α,β -doymamış karbonil sistemi bulunan iki aromatik halkadan oluşur. Bu yapı, kalkonlar ve diğer flavonoidler arasındaki temel farktır. Kalkonların kimyasal yapısı, aromatik halkalara fonksiyonel grupların eklenmesiyle çeşitlendirilebilir. Bitkilerde kalkon molekülleri, ayırt edici, yoğun sarı bir renge sahip oldukları için pigment görevi görür. Trans-kalkon (TC), doğada bol miktarda bulunması, kolay elde edilebilmesi ve basit yapısı nedeniyle biyolojik aktiviteleri açısından son yıllarda dikkatleri üzerine çekmiştir. TC'nin çeşitli tiplere karşı antikanser aktivitesi olduğu kanıtlanmıştır. Trans-kalkonun anti-leishmania aktivitesi geniş çapta araştırılmıştır. Çalışmalar, *Leishmania amazonensis* kaynaklı kutanöz leishmaniasis tedavisi için bileşiğin etkisini göstermiştir. TC ayrıca anti-inflamatuardır ve çeşitli inflamatuvar hastalıkların neden olduğu oksidatif stresi azaltarak etki eder. Bununla birlikte, TC'nin klinik kullanımında, esas olarak suda çözünemez olması ve dolayısıyla düşük biyoyararlanımı olmak üzere sınırlamalar vardır. TC bitki bazlı bir kimyasaldır, bu nedenle vücuttaki toksisitesi sentetik ilaç moleküllerine kıyasla nispeten düşüktür. Bununla birlikte, vücutta TC birikiminin son derece riskli bir yönü, TC'nin bir proöstrojen olmasıdır. TC, metabolik olarak diğer birçok kimyasala dönüştürülür. Bu bileşiklerin östrojenik aktiviteye sahip olduğu gösterilmiştir. Dişilerde erken ergenlik, obezite, sperm sayısında azalma, üreme organlarında ve cinsel davranışlarda değişiklikler ve bazı kanser türlerinde artış gibi ksenobiyotik bileşiklerin bu östrojenik aktivitesine bağlı olarak memelilerde birçok olumsuz sağlık etkisi meydana gelebilir. Bu nedenle, TC tedavisinin dozajını kontrol etmek ve TC moleküllerinin vücutta birikmesini önlemek çok önemlidir.

Bu çalışma, düşük kristallik ve hidrofilik karaktere sahip PKL ve silika partiküllerine dayalı yeni bir hibrit polimer sentezlemeyi amaçlamıştır. Sentez reaksiyonu, immobilize edilmiş *Candida antarctica* Lipase B ile katalize edilen ϵ -kaprolaktonun in situ halka açma polimerizasyonudur. Bu çalışmada, *Candida antarctica* Lipaz B'nin serbest formu fiziksel adsorpsiyon ile pirinç kabuğu külü üzerine immobilize edilmiştir. Enzim immobilizasyonu sağlanarak CALB'nin özgülüğü ve stabilitesi artırılmıştır. Enzimin immobilize edildiği destek materyali önceden temizlenmiş pirinç kabuklarının 650 °C'de 6 saat fırında yakılmasıyla hazırlanmıştır. Üretilen RHA, daha sonraki adımlarda nanohibrit sisteminin oluşumunda çok önemli bir rol oynayan yüksek silika içeriğine sahip bir malzemedir. RHA yüzeyine enzim adsorpsiyonunu kolaylaştıracak fonksiyonel gruplar eklemek için enzim immobilizasyonundan önce 3-APTES, 3-APTMS, 3-GPTMS ve 3-TMSPDA olmak üzere dört farklı organosilan bileşiği kullanılarak yüzey modifikasyonu gerçekleştirilmiştir. TGA eğrilerinin analizinden elde edilen sonuçlar, farklı organosilan bileşiklerinin farklı davrandığını göstermiştir. Yüzey modifikasyon yüzdeleri 3-APTES, 3-APTMS, 3-GPTMS ve 3-TMSPDA için sırasıyla %1,2, %0,8, %3,7 ve %10,1 olarak hesaplanmıştır. Bu reaksiyon, RHA'nın -OH grupları ve kullanılan silanizasyon ajanlarının metoksi veya etoksi grupları aracılığıyla gerçekleşmiş ve Si-O-Si bağları oluşmuştur. Yüzeyi modifiye edilmiş RHA üzerinde CALB immobilizasyonundan sonra, elde edilen katalitik sistemler, ϵ -kaprolaktonun polimerizasyonunu katalize etmek ve PKL bazlı nanohibrit sistemlerin yerinde sentezini gerçekleştirmek için kullanılmıştır. Bu reaksiyon sırasında, saf PKL'nin uzun, alifatik zincirlerinin yanı sıra yüzeyi modifiye edilmiş RHA'nın serbest -OH gruplarına aşılansın kısa PKL zincirleri sentezlenmiştir. Bu nedenle, silikadan PKL aşılansının başarılabilmesi için yüzey modifikasyonunu optimum seviyede tutmak çok önemlidir. TGA eğrileri ile yapılan hesaplamalar silanizasyon yüzdesi ile aşı yüzdesi arasındaki ilişkiyi kanıtlamıştır. Silanizasyon yüzdesi %10,1 olan T-RHA aşılı PKL üretememiştir. Bu analizden elde edilen kanıtlar,

belirli bir miktarda artan silanizasyon yüzdesinin aşılama verimliliğini arttırdığını göstermektedir. Önceki çalışmalar, PKL zincir uzunluğu ile yüzey Si-OH gruplarının sayısı arasında ters bir ilişki olduğunu öne sürmektedir. Bu çalışmanın bulguları, silanol gruplarının yüksek sayıda büyüyen zincirle sonuçlanan polimerizasyon reaksiyonu için yardımcı başlatıcılar olarak davrandığını öneren önceki çalışmaların bulgularıyla uyumludur. Nanohibritlerde düşük moleküler ağırlık ve silika tarafından kristal oluşumunun engellenmesi ile ilişkili olarak tüm nanohibrit numunelerin kristallik yüzdesinde önemli bir düşüş gözlemlenmiştir. Ayrıca aşılınmış PKL'nin neden olduğu kısıtlı hareketliliğe bağlı olarak artan camsı geçiş sıcaklığı gözlemlenmiştir. PKL bazlı nanohibritler hidrofiliktir. Nanohibritlerin hidrofilik karakteri, yüklü ilaç moleküllerinin biyoyararlanımını belirgin bir şekilde artırabilir, bu nedenle nanohibritler, ilaç salım uygulamaları için bir potansiyele sahiptir. Bu çalışmanın devamında, O/W emülsiyonu yöntemi ile mikroküreler ve arayüzey polimer biriktirme yöntemi ile nanoküreler geliştirilmiş ve yükleme etkinliği ve in vitro salım davranışı araştırılmıştır.

Bu çalışmanın ilk bölümünde sentezlenen PKL tabanlı nanohibritler, bu ilaç salım sistemlerinde polimerik taşıyıcı olarak kullanılmıştır. Bu araştırmanın sonucu, %60-75 kapsülleme verimliliğine sahip optimum mikroküre formülasyonlarının olduğunu göstermiştir. Bu çalışmadan ortaya çıkan daha önemli bulgulardan biri, TC salınımının kontrollü bir şekilde 22-57 güne uzatılmış olmasıdır. Suda çözünmeyen bir molekül olan TC'nin biyoyararlanımını artırabildiğinden, hidrofiliklik üretilen mikrokürelerin önemli bir özelliğidir. Ara yüzey polimer biriktirme veya nano çökeltme yöntemiyle üretilen TC yüklü nanoküreler ile de benzer sonuçlar elde edilmiştir. Nanoküreler ile daha yüksek kapsülleme verimliliği (%80-83) elde edilmiştir. Nanokürelerden TC salımı, mikroküre formülasyonlarına göre arttırılmıştır; kümülatif salım %83-90'a ulaşmıştır. Nanoküreler pH'a bağlı salım davranışı göstermiştir; salım ortamının asitliği ilaç salımını arttırmıştır. Asidik koşullar altında nanokürelerden artan TC salımının, asidik koşullar altında flavilyum katyonlarının oluşumu nedeniyle TC'nin artan çözünürlüğünden kaynaklandığı düşünülmektedir. TC salımı, nötral pH'da 28 güne uzatılmıştır. Su temas açısı ölçümleri, nanokürelerin hidrofilik karakterini de ortaya çıkarmıştır.

Bu çalışmanın bulguları, hastalıkların uzun süreli tedavisinin sağlanmasında mikrokürelerin ve nanokürelerin olası bir uygulamasını önermektedir. Asidik salım ortamı ilaç salımını desteklemiş ve daha hızlı salım kinetiği ve artan kümülatif salım ile sonuçlanmıştır. Mikroküre ve nanoküre formülasyonlarından pH ile tetiklenen ilaç salımı, kanser tedavisinde tümör hedefli ilaç taşınımı ve salımı için kullanılabilir. Mevcut çalışma, akıllı ilaç salımında söz konusu hibrit polimerin uygulanmasına yönelik gelecekteki araştırmalar için zemin hazırlamaktadır. Sitotoksiste, hücre canlılığı ve in vivo farmakokinetiği belirlemek için kapsamlı bir çalışmanın yapılması gerekmektedir.

1. INTRODUCTION

Recently, biodegradable polymers have attracted a great deal of attention and have been replacing conventional petroleum-based polymers. Polycaprolactone (PCL) is aliphatic polyester with biodegradable nature. The aliphatic ester bonds in its structure are susceptible to hydrolysis, causing biodegradability [1]. However, its biodegradation is rather slow in body and it can be accelerated by reducing crystallinity by either blending with other polymers [2] or utilizing different production methods [3] if a faster degradation is needed.

PCL is also biocompatible since its degradation products are can be either metabolized in the body or removed from the body directly [1]. Due to its biodegradability and biocompatibility, PCL has been approved by US Food and Drug Administration for use in medicinal practices [4]. PCL-based materials have been successfully used bone tissue regeneration [5, 6], skin tissue engineering [7] and vascular tissue engineering [8] applications. Additionally, a variety of drug release studies has been reported utilizing PCL-based drug release systems [9 - 13].

PCL can be synthesized both chemically and enzymatically, via ring opening polymerization (ROP) [14]. Industrially, tin octoate is used as a metallic catalyst. Metallic catalysts function at high temperature and pressure. Moreover, the end product may be toxic due to unremoved metallic residue thus reducing the chance of medicinal practices of PCL. On the other hand, enzymes are not toxic and can catalyze reactions in milder conditions [15]. Lipase enzymes catalyze polymerization of ϵ -caprolactone (CL). At the first step of this reaction, lipase enzyme reacts with ϵ -caprolactone and forms enzyme-CL complex. Later, hydroxyl-ended molecule in the medium reacts with the intermediate and the first chain of polymer is formed. Then the chain growth continues through this molecule.

Chalcones are open-chained molecules, which are naturally found in plants. Their chemical structure consist of two aromatic rings with three-carbon α , β -unsaturated carbonyl system in between [16] as given in Figure 1. Such structure is the main

difference between chalcones and other flavonoids [17]. The chemical structure of chalcones can be diversified by addition of functional groups to the aromatic rings [18]. In plants, chalcone molecules act as pigments since they possess a distinctive, intense yellow color [19].

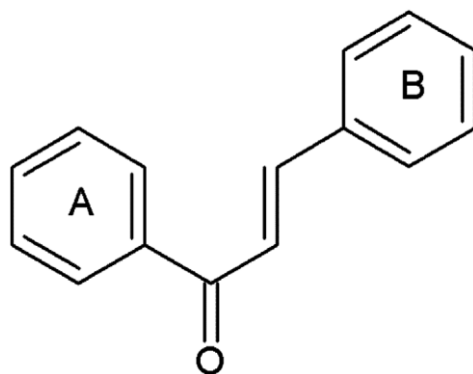


Figure 1.1 : General structure of chalcone molecule [18].

In recent years, chalcones have attracted attention in terms of their biological activities due to their abundance in nature, ease of preparation and their simple structure [20]. Several chalcones and chalcone derivatives are known to have anticancer activity towards several types such as breast cancer [21 – 23], colon cancer [24], ovarian cancer [25], bladder and urinary tract cancer [17], antifungal [26], ant tuberculosis [27], anti-AIDS [28] and antidiabetic [29] activity. Chalcones can be chemically synthesized by Claisen-Schmidt condensation reaction. The reactants are acetophenone and benzaldehyde, and the reaction is catalyzed by a base, generally NaOH or KOH [16, 30].

Drug delivery systems (DDS) are developed for the transportation of drugs to targeted sites in the body and consist of a carrier material (generally lipid or polymers) and the desired drug [31]. The aim of DDS design is to enhance pharmacokinetics and biodistribution of the drug and provide a sustained release profile [31 – 33].

DDS has some advantages over conventional drugs. Poor solubility of the drugs may cause problems in conventional formulations; for example, hydrophobic drugs may precipitate in aqueous media. Also, additive that are used to improve solubility may have toxic effects in vivo. DDS enhances drug solubility. DDS also protects the body from adverse effects of the active ingredient [32] and the drug from premature degradation and rapid breakdown [31] thus lower doses of drug are required in DDS. DDS also enhances drug specificity and reduce side effects in non-targeted tissues [31].

Biopolymers in DDS are preferred over synthetic polymers since they provide biocompatibility, biodegradability and low immunogenicity. Biopolymers such as silk fibroins, collagen, gelatin, albumin, starch, cellulose and chitosan are the mostly used ones [34]. Synthetic polymers such as PLA, PCL, polyglycolic acid (PGA), and polylactic glycolic acid (PLGA), and their blends with natural polymers are used for production of drug delivery systems [33, 35, 36].

There is a variety of methods to fabricate DDS such as electrospinning, spray-drying, layer-by-layer self-assembly, freeze-drying, formation of nanocapsules and microcapsules [34, 37, 38]. Such drug carrier systems can be used in ocular, transdermal, dental or intranasal delivery systems [34].

In this study, it is aimed to use polycaprolactone/silica nanocomposites synthesized by enzymatic ring opening polymerization as a drug delivery system. The catalysis of this reaction will be provided by *Candida antarctica* lipase B enzyme immobilized on a silica material (rice husk ash) in our previous study [39]. By preparing a composite containing inorganic material, crystallinity is aimed to be reduced. Low and moderate crystallinity is a desired property for potential drug delivery applications since high crystalline PCL decreases the mobility of the drug thus causes the drug to be located at the surface [40]. Additionally, enzymatic synthesis of such composite material will provide an economic and green process.

The second aim of this study is to develop TC-loaded microspheres with O/W emulsion and nanospheres with interfacial polymer deposition method and to investigate the loading efficiency and in vitro release behavior. PCL-based nanohybrids synthesized in the first part of this study is used as polymeric carriers in these drug delivery systems. It is expected to extend drug release in a controlled manner from these formulations. It is also thought that hydrophilic microspheres and nanospheres can increase the bioavailability of poorly water-soluble TC. Ph-dependency of TC release from microspheres and nanospheres are also investigated.



2. LITERATURE REVIEW

2.1 Hybrid Polymers

Hybrid polymers consists of two different types of polymers or a type of polymer and an inorganic reinforcement. Such hybrid polymer are constructed since advance properties can be obtained which cannot be attained by using the components of the hybrid system on their own [41].

Resistible polymeric materials with distinguished physical, mechanical and chemical properties have been highly demanded for medical applications. It is critical that these materials can survive and perform in the harsh conditions of the human body such as extreme pH environments and mechanical stress. One of the approaches to produce such advanced medical devices is to utilize hybrid polymeric materials. By simple term, a hybrid polymer is the material consisted of two compartments, one being a natural or synthetic polymer, interacting with each other at molecular level. Formation of an organic/inorganic hybrid system allows us to exploit the advantageous properties of each component or sometimes create advanced properties synergistically. Especially in drug delivery and controlled release applications, several inorganic materials are widely used either separately or by incorporating with polymers, such as iron oxide nanoparticles, gold nanoparticles, silver nanoparticles, mesoporous silica and several types of clays. These inorganic materials are preferred due to their small particle size and advanced optical, electrical and mechanical properties [42, 43]. Iron oxide particles are one of the widely used inorganic material for drug loading and release especially for their advanced targeting due to magnetic properties. Iron oxide nanoparticles were successfully employed for the sustained release of drugs like doxorubicin [44, 45]. A group of researchers successfully developed a magnetically triggered release system for vancomycin delivery and release by utilizing an organic/inorganic hybrid system based on chitosan as the organic content and superparamagnetic iron oxide nanoparticles [46]. Silver nanoparticles are becoming widely used for drug delivery applications. In a recent study, it was shown that silver nanoparticles embedded in natural rubber latex facilitated the release of metronidazole,

a synthetic antibiotic, increasing drug release from 14.24 % to 27.27 % [47]. By silver nanoparticle incorporation in the PEG-PLA polymer matrix, the release of silibinin can be strictly controlled by external stimuli [48]. Biosynthesized silver nanoparticles also exhibited antibacterial activity and were proved to be prominent for the release of resveratrol, especially in acidic conditions [49].

Although inorganic materials have superior properties, there are some drawbacks of utilizing such inorganic particles as drug carriers. The main disadvantage is that they require surface modifications in order to provide stability and well-dispersion [42]. Generally, these inorganic particles are used in drug delivery applications by coating or grafting with or forming composites with biodegradable and biocompatible polymers. This approach also increases biocompatibility of the particles which is one of the key feature to the development of successful drug delivery system. These polymers can be synthetic or natural ones and the most widely used polymers in organic/inorganic hybrid systems for medical applications are polycaprolactone [51-54], polyvinyl alcohol [55, 56], poly(d,l-lactide-co-glycolide) [57, 58], polyethylene glycol [59-61].

Organic/inorganic hybrid systems developed with a polymer and inorganic particles can be classified under two main groups depending on the interactions between the two components. In Class 1 hybrid systems, inorganic particles are entrapped or encapsulated in the polymer matrix by weak intermolecular forces such as Van der Waals, electrostatic interactions and hydrogen bonding. This Class of organic/inorganic hybrid materials can be synthesized either by in situ or ex situ methods. Class 2 organic/inorganic hybrid system is constructed by covalent or ionic bonding between the organic and inorganic components. This covalent bonding can be constructed by two different approaches as in situ synthesis of polymer in the presence of inorganic particles, in situ formation of inorganic material, or combination of polymer and inorganic material both produced ex situ. The interface characteristic is an important factor that alters the properties of the developed hybrid system [62].

2.1.1 Synthesis of organic/inorganic hybrid materials

There are great number of production methods to obtain organic/inorganic hybrid materials. These methods can be classified under several subgroups depending on the interactions between the components of the organic/inorganic hybrid material and

whether the formation of the components are in situ or ex situ. Figure 2.1 summarizes the possible strategies for producing polymer/inorganic hybrid materials. The first strategy is to use polymer and inorganic material, which were prepared earlier. This strategy is an ex situ method and both covalently and noncovalently attached hybrids can be obtained. The second strategy is the in situ synthesis of polymer component in the presence of ex situ synthesized inorganic material. In this strategy polymer can be covalently bonded or “grafted from” the surface of the inorganic material. Ex situ synthesized inorganic particles can be encapsulated within the monomer droplet and nanocomposite particles are produced at the end of polymerization reaction. Likewise, inorganic component of the hybrid system can be synthesized in situ by sol gel process.

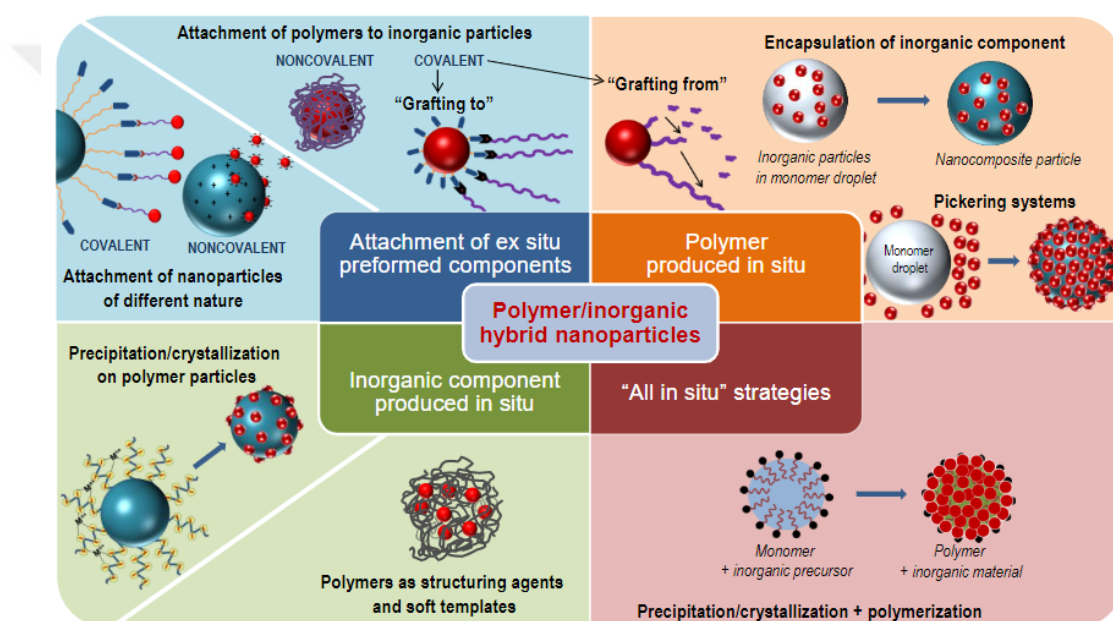


Figure 2.1 : Synthesis strategies of polymer/inorganic hybrid materials [63].

Covalent attachment of ex situ-synthesized polymer and ex situ synthesized inorganic material, also called “grafting to” approach, should involve the surface functionalization of the inorganic particle so that the polymer can be grafted to the surface. He et al. synthesized poly(ethyleneimine)/silica hybrid by grafting presynthesized PEI on the surface of chloropropyltriethoxysilane functionalized silica particles for Ni removal. They showed that PEI grafting was successfully achieved after surface functionalization of silica particles and BET surface area decreased due to PEI grafting. The synthesized PEI/silica hybrid demonstrated fast adsorption kinetics, high adsorption capacity and regeneration capability [64].

In a recent study, researchers developed several organic/inorganic hybrids by grafting Dawson type polyoxometalates to porphyrin with different stereoisomerism (Figure 2.2) and studied the optical properties of the hybrids. The hybrids showed improved nonlinear optical properties and large fluorescence quenching compared to ungrafted porphyrins [65].

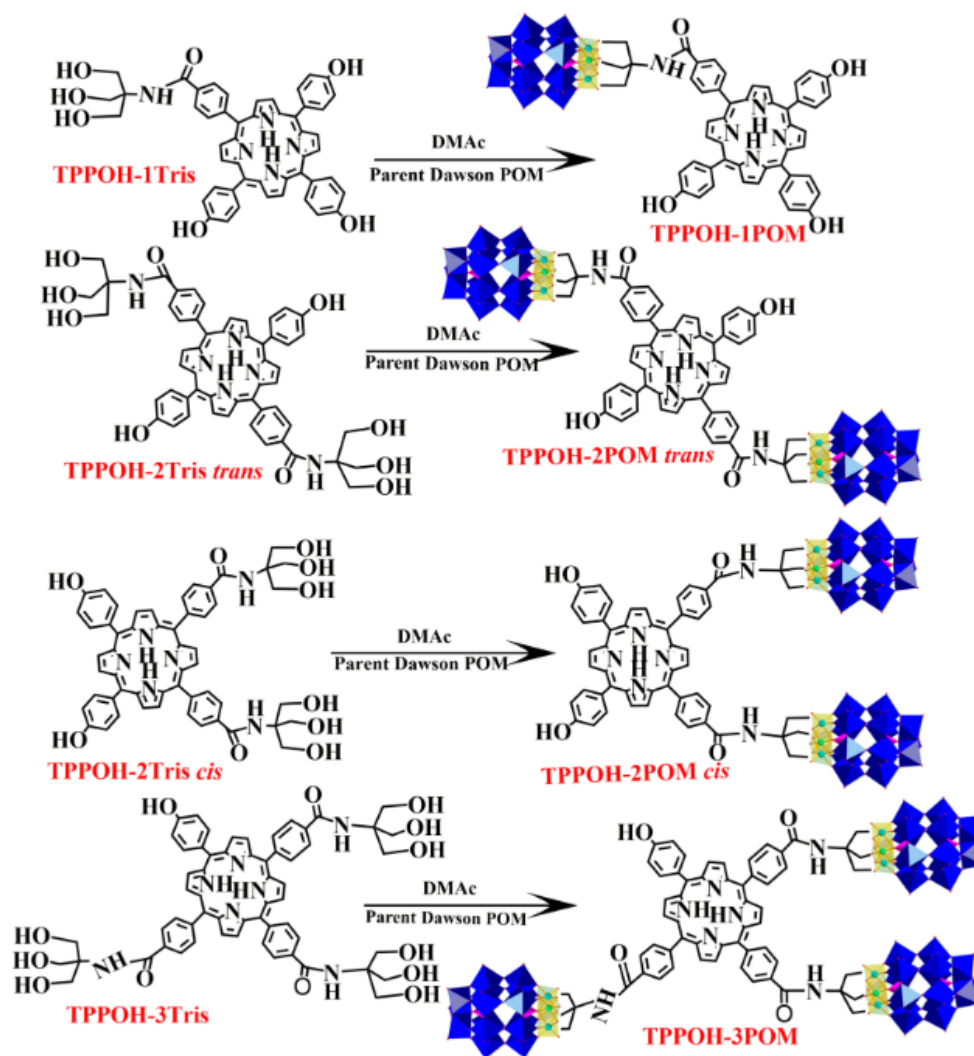


Figure 2.2 : Synthesis of organic/inorganic hybrids; Dawson type polyoxometalate grafting to ex situ-synthesized porphyrins [65].

One of the preferred methods for polymer/inorganic hybrid material synthesis is the one-step in situ polymerization of the organic material in the presence of inorganic nanoparticles. This method simply applies a procedure of polymerization reaction of a monomer taking place in the presence of the desired inorganic material. Babazadeh et al. had synthesized Class 1 Polypyrrole/TiO₂ hybrid by one-step in situ polymerization of pyrrole in the presence of TiO₂ nanoparticles. The researchers have showed that the morphology of the product is strictly dependent on the amount of TiO₂ nanoparticles.

SEM images indicated that TiO_2 nanoparticles acted as a nucleating agent and facilitated the encapsulation of the nanoparticles in the PPy matrix (Figure 2.3). They also reported that the PPy/ TiO_2 nanocomposites were synthesized with higher thermal stability with respect to the pure PPy and the electrical conductivity was enhanced by the incorporation of low TiO_2 content [53].

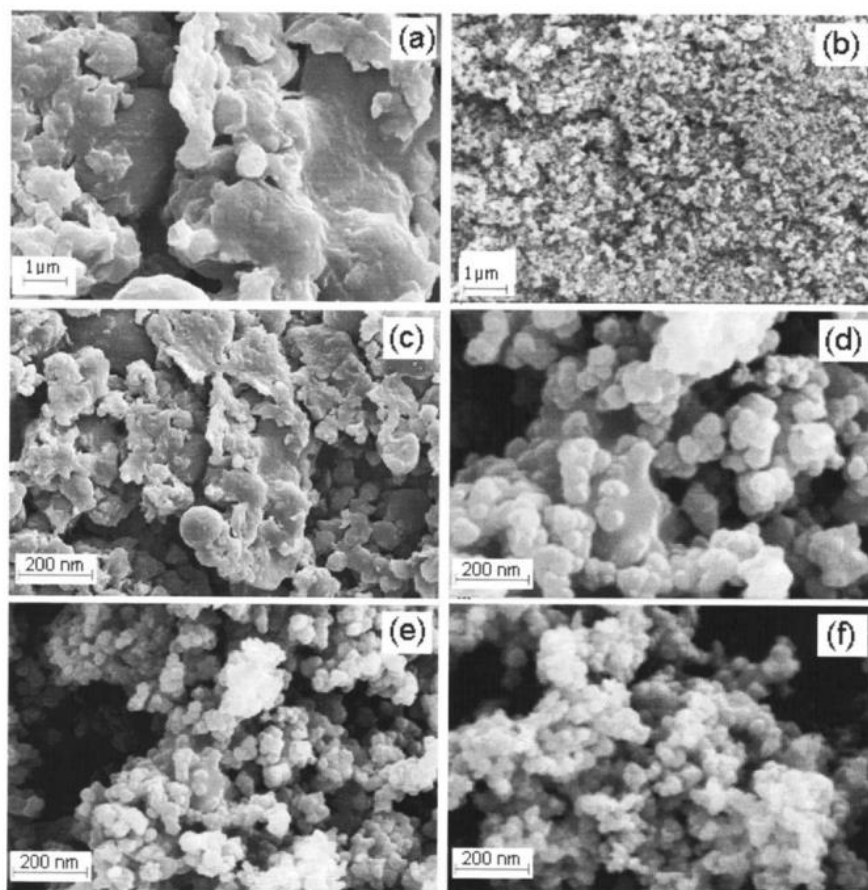


Figure 2.3 : SEM images of (a) pure PPy, (b) pure TiO_2 , (c) PPy/ TiO_2 (0.025), PPy/ TiO_2 (0.05), PPy/ TiO_2 (0.075), and PPy/ TiO_2 (0.1) nanocomposites [53].

Using this approach, Öztürk-Düşkünkörür et al. developed an organic/inorganic nanohybrid system consisted of PCL and several types of nanoclays (montmorillonite, organophilic modified montmorillonite and sepiolite). They conducted in situ ring opening polymerization of ϵ -caprolactone in the presence of nanoclays. The data they obtained demonstrated that the surface functionality affects the properties of the developed hybrid system; organophilic modified montmorillonite with $-\text{OH}$ groups on its surface yielded a hybrid system where PCL chains were grafted from the surface of the clay Figure 2.4 shows the SEM images of PCL-grafted organophilic modified montmorillonite. These images indicated the well dispersion of nanoclays within the PCL matrix [66].

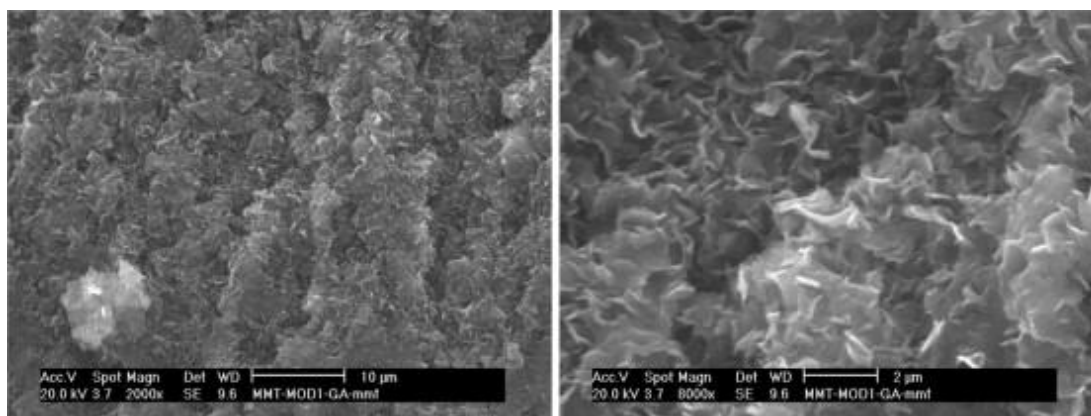


Figure 2.4 : SEM images of the PCL-clay nanohybrid showing the PCL chains grafted on clay surface at 2000× magnification (left) and 8000× magnification (right) [66].

Radharkrishnan et al. grafted block copolymers of polystyrene, poly(*tert*-butyl acrylate) and poly(methyl acrylate) from the surface of functionalized silica nanoparticles. They have utilized tetraethoxysilane (TEOS) for silica nanoparticle synthesis in the presence of a reactive stabilizer, [3-(2-bromoisobutryl)propyl]-triethoxysilane (BPTS) and [3-(2-bromoisobutryl)propyl]ethoxydimethylsilane (BPMS) and an ATRP initiator was added to the noncondensable end of the silica nanoparticles (Figure 2.5). The mentioned polymers were grafted from the initiator-end of the silica nanoparticles by atom transfer radical polymerization (ATRP). They have managed to graft polymer chains from the surface of the functionalized silica nanoparticles almost monodisperse (polydispersity index between 1.1-1.3) and molecular weight ranging between 6000 and 27000 g.mol⁻¹ [67].

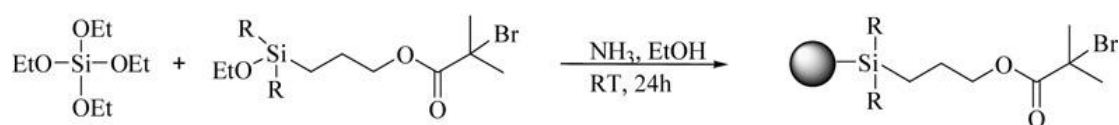


Figure 2.5 : Surface functionalization of silica nanoparticles [67].

Similar strategy to produce poly((2-dimethylamino) ethyl methacrylate)/ Fe₃O₄-Br hybrid system was pursued by Zhou et al. Their study introduced the synthesis of pH-responsive PDMAEMA-coated magnetite nanoparticles by ATRP for drug delivery applications for which the reaction route was given in Figure 2.6. The researchers showed the pH-dependency of the synthesized hybrid nanoparticles by determining hydrodynamic radius of the nanoparticles under different pH medium. They have determined that hydrodynamic radius decreased with increasing pH. In acidic medium, polymer chains grafted from the magnetite nanoparticles are protonated and stretched

which increased the hydrodynamic radius. On the other hand, basic conditions caused the polymer chains to aggregate and it resulted in decreased hydrodynamic radius. This phenomenon proved the pH-response of the hybrid nanoparticles.

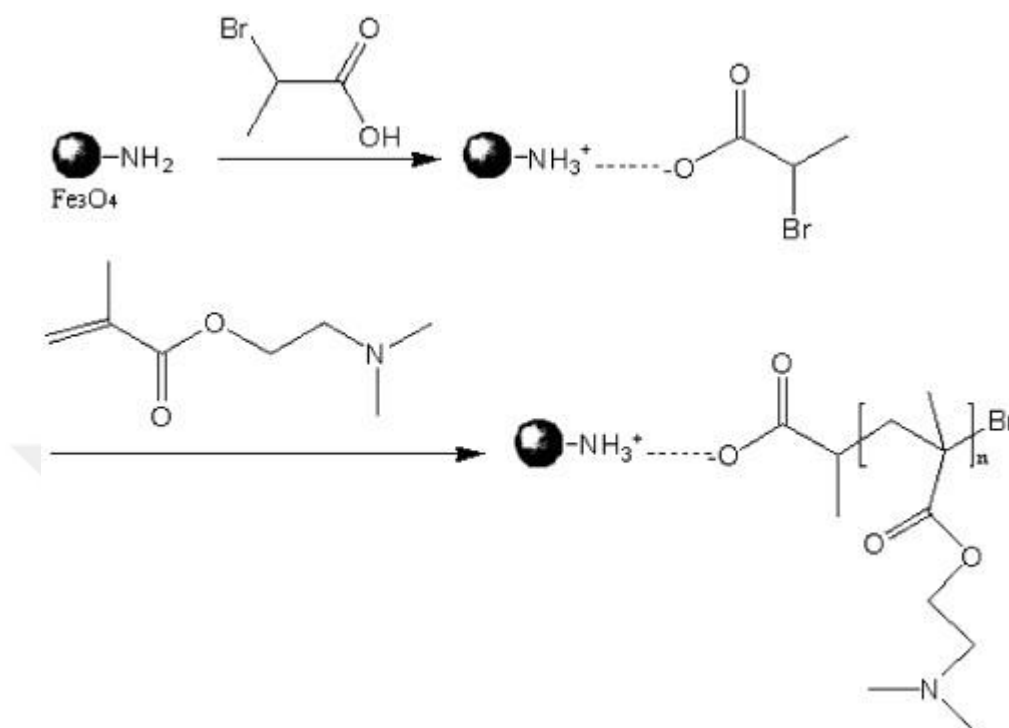


Figure 2.6 : Synthesis of PDMAEMA-coated Fe_3O_4 nanoparticles [68].

Later on pH-responsive drug release from the hybrid nanoparticles by using phenolphthalein as a model drug. The release kinetics under different pH conditions were given in Figure 2.7. As can be seen in the figure, in acidic medium drug release was much faster (56.4 %) than under neutral pH condition (26.4 %). This difference was attributed to the conformational changes in the structure of the polymer component of the hybrid nanoparticles. Under acidic conditions, polymer chains grafted from the magnetite particles expanded and facilitated the drug diffusion towards the release environment whereas under neutral or basic pH condition polymer chains were more compact which obstructed drug diffusion. The study also showed that the hybrid nanoparticles were superparamagnetic which is an important property for drug release applications. The saturation magnetization of the hybrid nanoparticles were strong enough to release the loaded drug controlled by an external stimuli [68].

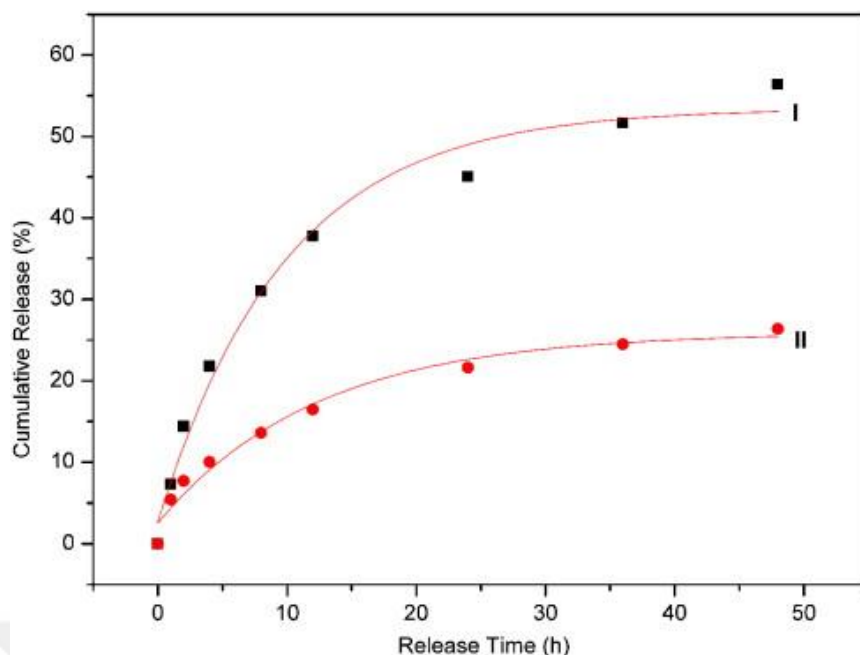


Figure 2.7 : Drug release kinetics of PDMAEMA-coated magnetite nanoparticles at I) pH 3.0, II) pH 7.0 [68].

Another approach to create organic/inorganic hybrid materials is the in situ synthesis of inorganic material by sol-gel method. Basically, sol gel process is the hydrolysis and condensation reactions of metal alkoxides and two main strategies may be followed during this process. The first one is to simply mix the organic material (polymer) with the inorganic material, which is generally a metal alkoxide or alkoxy silane, in the presence of a suitable solvent. In this approach covalent bonds cannot be obtained, rather hydrogen bonding may occur between the phases. To obtain covalently-bonded organic/inorganic hybrid system (Class 2), hydrolysis and condensation reaction takes place and covalent bonds are formed between the polymer matrix and the metal alkoxide or alkoxy silane. This hydrolysis and condensation reaction may be either of alkoxy silane with organic group substitution or of polymer substituted with alkoxy silane. The reaction route of hydrolysis and condensation of an alkoxy silane is shown in Figure 2.8.



Figure 2.8 : Reaction route of hydrolysis and condensation of an alkoxy silane: (1) Hydrolysis reaction, (2) and (3) Condensation reactions producing siloxane bonds (Si-O-Si) [69].

These reactions are affected by several factors such as the pH of the reaction medium, the type of solvent and the presence of water in the reaction medium. The presence of water at a certain amount facilitates the hydrolysis reaction and determines the morphology of the final product. The pH of the reaction medium alters the reaction kinetics and thus the final structure of the synthesized organic/inorganic hybrid; acidic medium favors the hydrolysis reaction and yields long linear polymer chains as seen in Figure 2.9a whereas in the basic conditions of the reaction medium nucleated clusters of spherical particles can be obtained by the facilitated condensation reaction (Figure 2.9b).

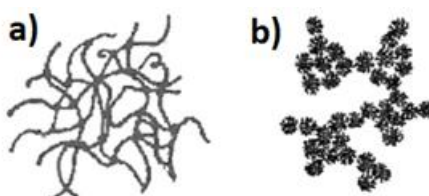


Figure 2.9 : Organic/inorganic hybrid material synthesized in a) acidic medium, b) basic medium [69].

There have been great number of studies investigating the synthesis of organic/inorganic hybrid materials by sol gel method throughout the past years. Xiong and a group of researchers synthesized acrylic resin/titania organic/inorganic hybrid by two different approaches: 1st one is the mechanical blending the two componets and 2nd one is the in situ polymerization by sol gel process. They compared several properties of the two differently synthesized hybrids such as mechanical, thermal and morphological and have found that blending method created a more homogeneous matrix with finely dispersed phases with respect to in situ method. They explained this finding by the aggregation titania nanoparticles at the in situ polymerization temperature causing phase separation. The aggregation of the titania network also caused the hybrid morphology to be rougher. The mechanical properties of the in situ polymerized hybrid systems were investigated and the stroge modulus was found to be increasing with increasing titania content (Figure 2.10a). Based on the stroge modulus, the researchers calculated crosslinking densities of the hybrid systems. They have found that crosslinking density of the hybrid systems increased with increasing titania content which was also observed in the shift of $\tan\delta$ curves (Figure 2.10b) [70].

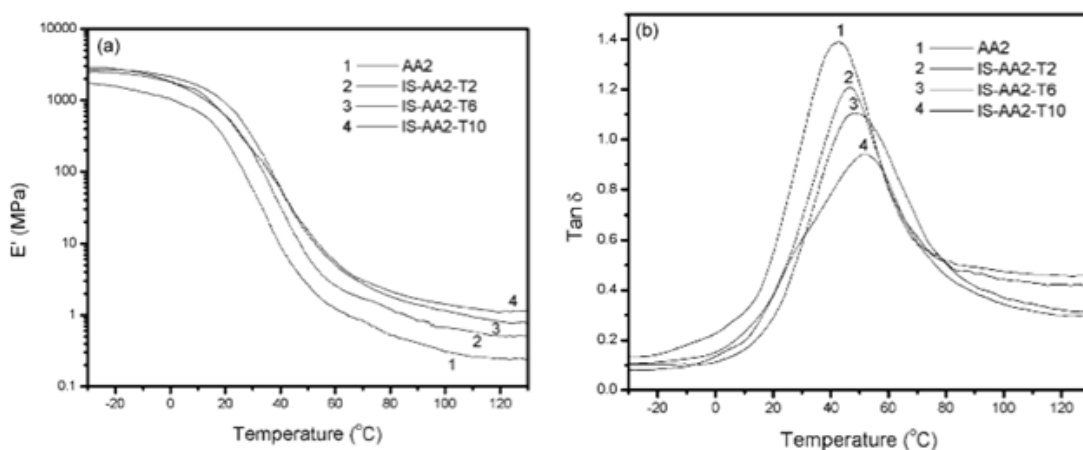


Figure 2.10 : DMA curves of acrylic acid and the hybrid systems with increasing titania content, a) storage modulus, b) loss tangent [70].

2.1.2 PCL-based organic/inorganic hybrid materials

Polycaprolactone (PCL) is a semicrystalline aliphatic polyester with biodegradable nature [52]. With its biocompatibility and convenient processing, PCL is widely used in medical applications such as drug delivery and release systems and tissue engineering. PCL has a low glass transition temperature which makes it tough and mechanically stiff under physiological conditions [71]. However, its biodegradation is rather slow in body and it can be accelerated by reducing crystallinity by either blending with other polymers or inorganic materials which may also tune the mechanical properties [2, 71].

The final properties of the PCL-based organic/inorganic hybrid systems are dependent on how finely dispersed the inorganic material is in the polymer matrix. Cobos et al. prepared polycaprolactone/polyhedral oligomeric silsesquioxane (PCL/POSS) hybrid and studied the effect of POSS type and content in the hybrid on morphology, thermal and mechanical properties [72]. PCL/POSS hybrids were prepared by solution casting and melt blending both being *ex situ* methods. The researchers showed that out of several different types of POSS shown in Figure 2.11, PIVOSS-PCL-PIOVOSS masterbatch had the best dispersion in the polymer matrix. Thermal behavior was also affected by the presence of POSS particles and the preparation method. For both melt blending and solution casting methods, study had shown that the presence of all POSS types enhanced the thermal stability of PCL increasing the degradation temperature by 50-60 °C. The mechanical properties of the hybrids were also investigated by the determination of Young's modulus, tensile strength and elongation at break values.

The Young's modulus of the solution-casted hybrids were decreased compared to neat PCL whereas the hybrids prepared by melt mixing did not show any significant difference in terms of Young's modulus. Solution-casting method yielded less brittle and stiff material. One of the noticeable result of the study was that the hydrophobicity of PCL was increased by the addition of POSS particles.

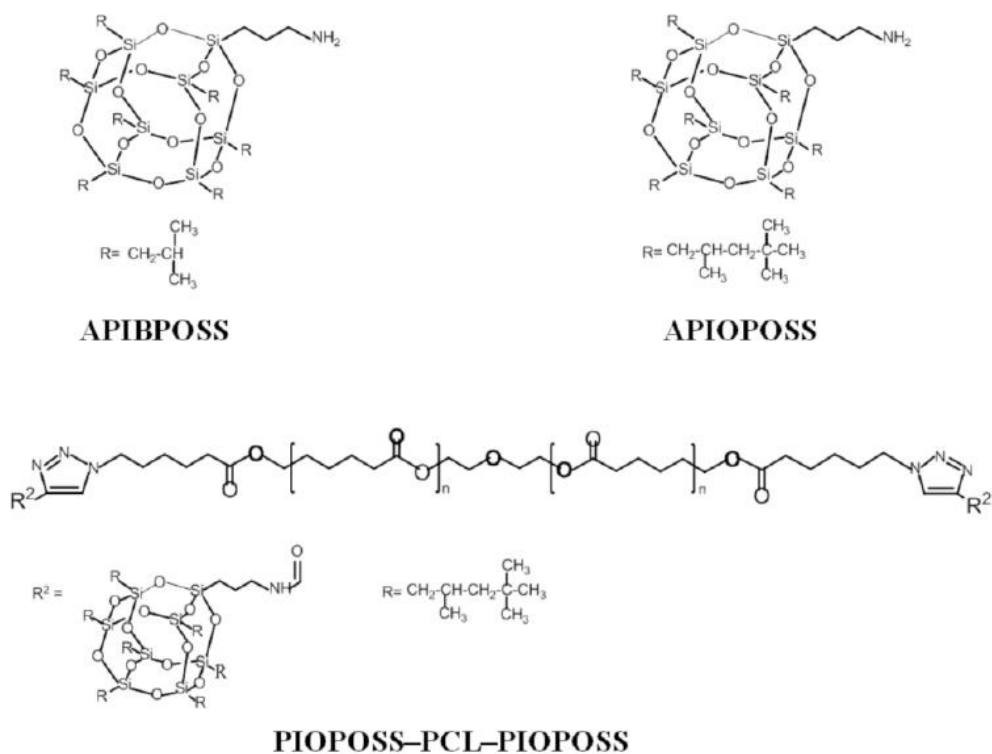


Figure 2.11 : Structures of different types of POSS used in PCL/POSS hybrids [72].

PCL/inorganic hybrid materials can be promising for tissue engineering applications especially if the surface morphology can be tuned for cell proliferation. In a recent study, researchers developed PCL/hydroxyapatite (PCL/HAp) hybrid for osteogenesis and showed that the PCL/HAp hybrid had better osteogenicity compared to HAp nanoparticles [54]. The PCL/HAp hybrids were prepared by emulsification-solvent evaporation by method where both PCL and HAnp solution were emulsified and the final hybrids were obtained with no chemical interactions between the components (Class 1 hybrids). TEM images revealed the signs of stem cells differentiation into osteoblasts (Figure 2.12) which proved that the PCL/HAp hybrids developed in this study were eminently suitable for osteogenesis.

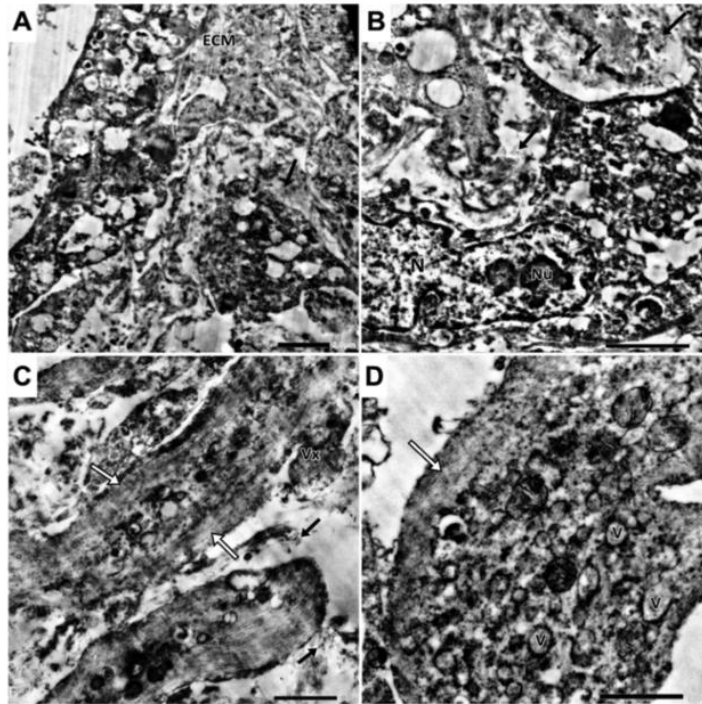


Figure 2.12 : TEM micrographs of stems cells in PCL/HAp hybrids (A–D), scale bar for A, C: 2 μ m and B, D: 1 μ m. (N): irregular euchromatic nucleus, (Nu): prominent nucleolus, (ECM): extracellular matrix, (black arrow): collagen fibrils, (white arrow): stress fibers, (V): coated vesicles, (Vx): exocytotic matrix vesicles [54].

Another research group had studied PCL/hydroxyapatite hybrids for bone tissue regeneration with gelatin added to the polymeric component [73]. They improved the preparation method of Gelatin-PCL-nHAp electrospun hybrids by employing surface deposition of nHAp on the PCL/gelatin electrospun fibers. By this modification, it was ensured that nHAp had better dispersion and deposition on the surface of the electrospun fiber mats. The morphology of the hybrid scaffolds were imaged by FESEM (Figure 2.13). It was observed that nHAp deposition on the surface of the hybrid scaffolds increased with the increasing treatment time period of the scaffolds with nHAp solution. The untreated PCL/gelatin scaffold had a smooth surface whereas the nHAp-treated scaffolds had rougher surface structure. The longer the treatment time, the denser the deposition was which decreased the density of the pores in the structure. Such decreased porosity is not desired for tissue engineering application thus the researchers suggested that 30 min of nHAp treatment is not suitable for osteogenesis. The scaffolds were tested for cell viability and cell proliferation and the researchers reported that the highest viability and proliferation of human osteoblast cells were achieved on the Gelatin-PCL-nHAp scaffolds. They also imaged the cell adhesion on the scaffolds by FESEM, given in Figure 2.14. The images revealed that

the polygonal-shaped osteoblast cells adhered and proliferated on the Gelatin-PCL-nHAp scaffolds after 5 days of seeding. The researchers concluded that the developed Gelatin-PCL-nHAp hybrid scaffolds provided a suitable environment for the osteoblast cells to proliferate thus they are promising for potential bone tissue regenerative applications *in vivo*.

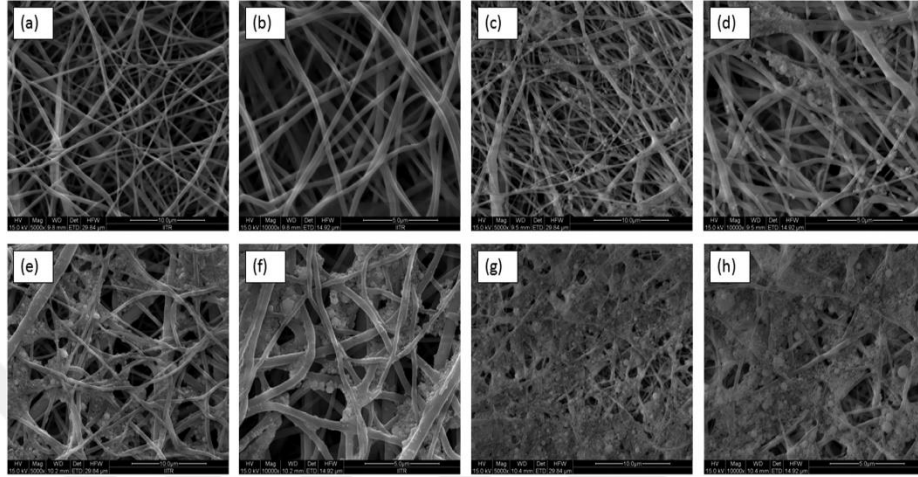


Figure 2.13 : FESEM images of Gelatin-PCL-nHAp scaffolds treated with nHAp at 5000x and 10000x magnification; (a, b) no treatment, (c, d) treated for 10 min, (e, f) treated for 20 min, (g, h) treated for 30 min [73].

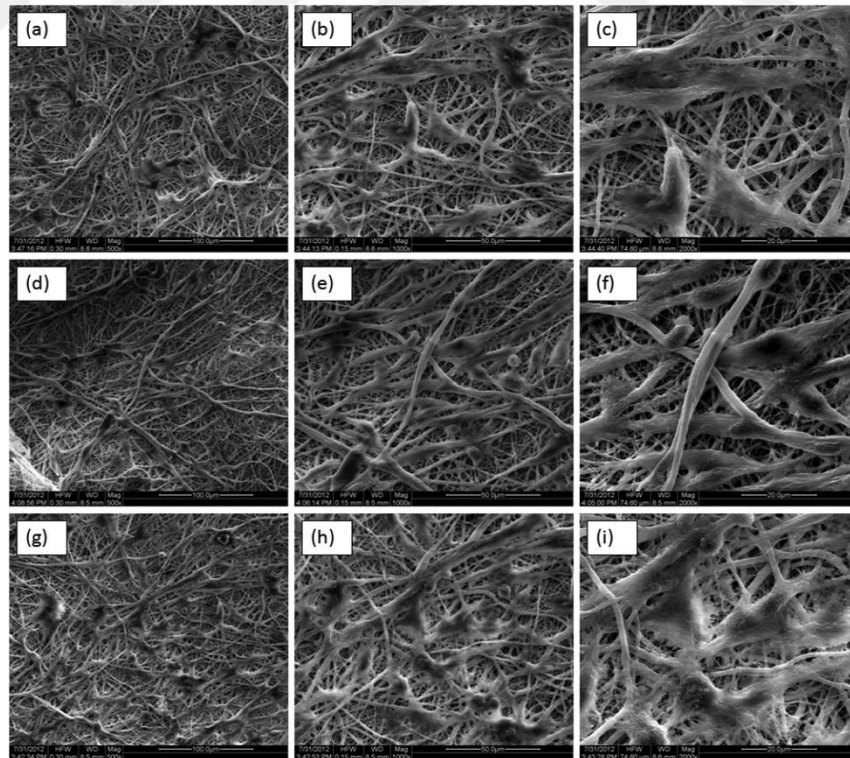


Figure 2.14 : FESEM images of osteoblast-seeded Gelatin-PCL-nHAp hybrid scaffolds at 500x, 1000x and 2000x magnification: (a, b, c) 1 day after seeding, (d, e, f) 3 days after seeding, (g, h, i) 5 days after seeding [73].

3D printing of the PCL-based hybrids for bone tissue engineering applications is attracting attention since PCL provides a solid scaffold which the active bio-inks cannot provide. The morphology and porous structure of the 3D-printed scaffolds can be more strictly controlled with respect to the conventional solvent casting or electrospinning. A recent study by Radhakrishnan et al. encapsulated the in situ reduced silver nanoparticles (AgNps) into PCL matrix. This hybrid was then extruded into filaments and used for the 3D printing of the scaffolds seen in Figure 2.15 [74].



Figure 2.15 : Photograph of the PCL and PCL/AgNp 3D-printed scaffolds [74].

The SEM images of the scaffolds revealed uniform pores with sizes larger than 300 μm which is suitable for osteoblast infiltration. The images also displayed morphologies giving insights of the mechanical properties of the scaffolds. The structure of the 3D-printed filaments of PCL/AgNp hybrid were smoother compared to the PCL filaments which was the indication of increased stiffness with the addition of AgNps. The enhancement of the rigidity was also observed from the changes in Young's modulus. The Young's modulus of PCL was determined as 0.35 GPa whereas this value was measured as 1 GPa for PCL/AgNp hybrid scaffold containing 3 % AgNps. This study had also tested the cytocompatibility and the antimicrobial activity of the hybrid scaffolds. It was demonstrated that the hybrid scaffolds were cytocompatible and showed antimicrobial activity against *E.coli* (Figure 2.16) which was attributed to the diffusion of silver towards the test environment. The researchers concluded that the hybrid scaffold containing 3 % AgNps was the most promising antimicrobial scaffold formulation for bone tissue engineering applications.

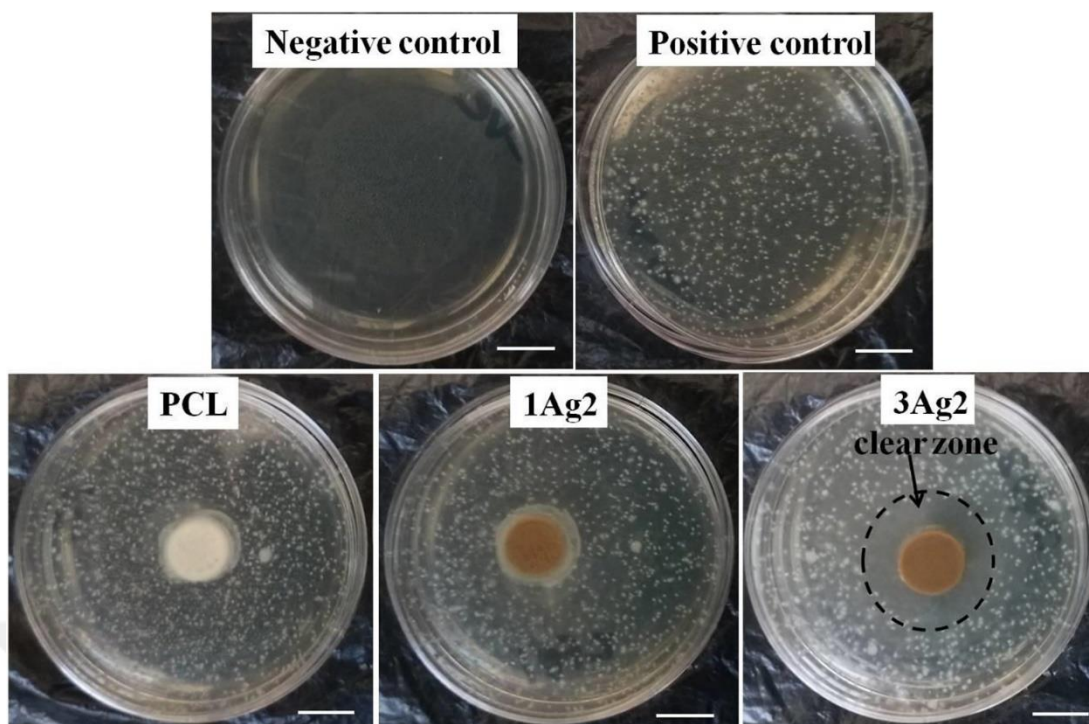


Figure 2.16 : Incubation of the 3D-printed PCL/AgNp hybrid scaffolds in *E. coli* inoculated soft agar plates for 24 h [74].

PCL-based hybrid materials are efficient for controlled drug release applications and extensively studied by many researchers. PCL, being a biocompatible and biodegradable polymer, is generally preferred for the development of smart drug delivery and release systems. However, the hydrophobic nature of PCL restricts its applications as drug delivery and release systems due to inefficient release and cellular uptake of the active material. Hydrophilicity can be obtained by the addition of inorganic materials into the polymer matrix and creating hybrid materials. For example, Singh et al. tuned the morphology, hydrophilicity, thermal and mechanical properties of PCL by using surface modified nanoclay and employed the hybrid in the controlled release of dexamethasone, an anticancer drug [75]. The alterations in the structure of the hybrids due to the type of surface modification of nanoclays (methyl tallow bis-hydroxyethyl quaternary ammonium (30B) and dimethyl-octadecylamine (C18)) caused significant difference in mechanical properties of the hybrids. The elasticity of the PCL-C18 hybrid was higher than that of PCL-30B hybrid since C18 was intercalated within the PCL matrix whereas 30B was exfoliated. Both of the hybrids possessed sufficient toughness required for medical applications. The hybrids had enhanced biodegradation with respect to pure PCL which was attributed to the decreased crystallinity. The hybrids did not show cytotoxicity or hemotoxicity thus are

potentially suitable for in vivo applications. The release of a model drug, dexamethasone, was sustained compared to pure PCL. This sustained drug release was associated with drug molecules interacting not only with PCL matrix but also with the nanoclays. Also, the configuration of the nanoclays within the polymer matrix played a role in sustained release. The authors offered a convincing explanation that the presence of dispersed nanoclays increased the diffusional path as seen in Figure 2.17 and resulted in delayed release.

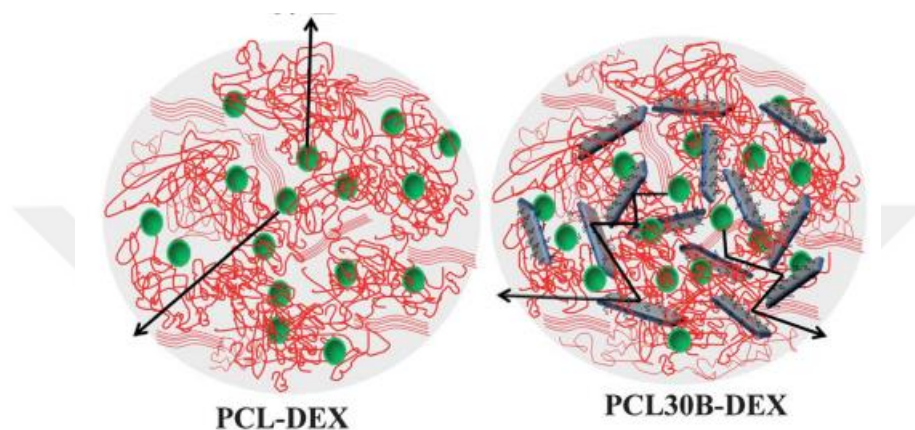


Figure 2.17 : Schematic representation of drug release from nanostructures (blue plates: nanoclay, green spheres: drug molecules, arrows: the probable path of drug diffusion) [75].

2.1.3 Silica-based organic/inorganic hybrid materials

Silica nanoparticles are very widely used inorganic materials in drug delivery applications. They possess advantageous features such as large surface area, high stability and high drug loading capacity. However, the main two drawbacks that limit the application of silica nanoparticles in drug release are the low biocompatibility and rapid release of the loaded active material [76]. One approach to overcome these drawbacks is to synthesize silica/polymer hybrids, by doing so the polymer coated or grafted on the silica particles acts as a protective agent preventing drug leakage and provides sustained drug release [77]. A group of researchers showed that mesoporous silica nanoparticle/thermogel hybrid slowed down the release of anti-VEGF bevacizumab. This slower release of the drug was modulated by the structure of the release system in which the drug molecules were loaded into the mesoporous silica nanoparticles (MSN). The diffusional path of the drug was increased and the drug was exposed to higher resistance since the loaded MSN were incorporated in the thermogel matrix thus the release was slower compared to the release from loaded-MSN [78].

Polymers also are suitable materials for stimuli-responsive drug delivery [79] and their incorporation into a silica-based hybrid material provide similar properties to the hybrid system. In a recent study, researchers used a hybrid consisted of poly(hydroxyethylmethacrylate) grafted onto mesoporous silica nanoparticles for the development of a pH-triggered drug release system [80]. In vitro release studies showed the pH dependency of the release of the cancer drug fluorouracil (5-FU). Drug release was significantly enhanced at pH 7.4 reaching a cumulative release of 86 % whereas at acidic pH (pH 2) cumulative drug release was 46 %. They also conducted in vitro cytotoxicity tests on the release system against cancer cells. The results indicated that the drug-loaded release system was more effective than the free drug in terms of cytotoxicity against U87 glioblastoma cells. Depending on these results, the researchers suggested that the developed release system would have application in cancer therapy.

Yuan and his coworkers developed a silica/polymer hybrid and successfully utilized the hybrid in doxorubicin (DOX) release [81]. Mesoporous silica nanoparticles obtained by sol-gel process, was hybridized with hydrazine-ended morpholin-4-yl-acetyl-poly(ethylene glycol)-b-poly(lactic acid) (MOPNHNH₂). The polymer and surface-modified MSN were synthesized separately. The polymer was synthesized by the copolymerization of morpholin-4-yl-acetyl-poly(ethylene glycol) and polylactic acid catalyzed by stannous octanoate (Figure 2.18A). MSN was modified with APTES and then pyruvic acid (PA) so that the hydrazine-ended polymer can be grafted onto MSN through this moiety (Figure 2.18B). Finally, MOPNHNH₂ was grafted onto the surface modified MSN which acted as a protective layer over the pores of MSN. One additional aim of polymer grafting was to achieve pH triggered release. As represented by the researchers in Figure 2.19, the surface charge of the capped polymer can be altered in different pH environments facilitating the cellular uptake of the release system. Once the system taken into the cells, the grafted polymer degrades at endosomal pH which enhances and facilitated drug release.

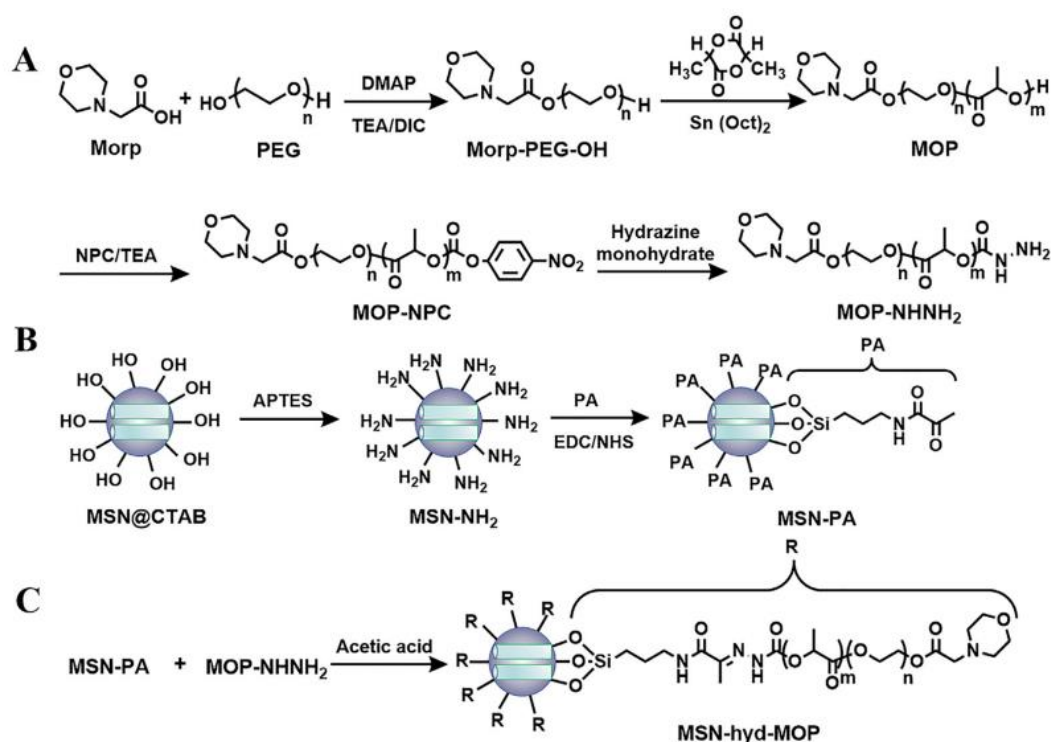


Figure 2.18 : Reaction steps of MSN-hyd-MOP synthesis, A) Synthesis of MOP-NHNH₂, B) Surface modification of MSN and C) Synthesis of MSN-hyd-MOP [81].

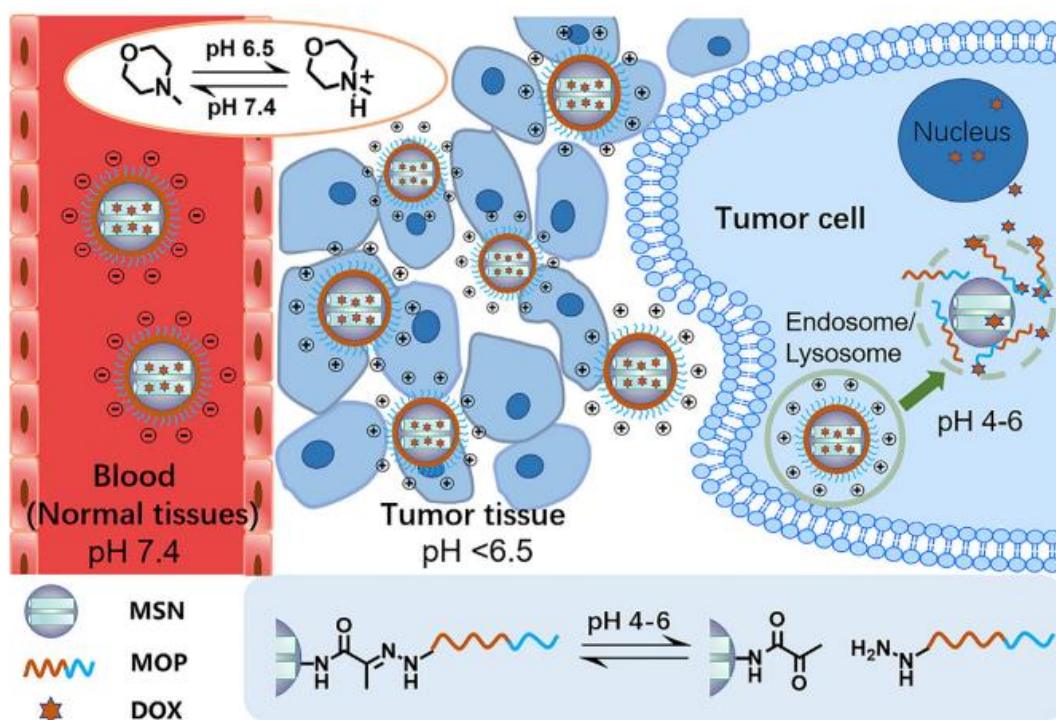


Figure 2.19 : Schematic representation of pH-triggered release from DOX-loaded MSN-hyd-MOP [81].

TEM images of MSN-hyd-MOP given in Figure 2.20 revealed a thin layer of polymer which proved the successful grafting of MOPNHNH₂ onto surface modified MSN. The particle size was determined as 150-200 nm which was considered to be suitable for cellular uptake of the spherical particles. Moreover, pH dependent charge alteration of MSN-hyd-MOP was demonstrated with zeta potential measurements, which showed that the particles were negatively charged at pH 7.4 whereas the zeta potential increased as the pH value decreased to acidic values. These findings also had impacts on drug release and in vitro cytotoxicity. It was found that DOX release from the hybrid particles were pH-triggered; at pH 7.4 and 6.5 release was slow and suppressed whereas at pH 5.0 release was increased by 6-folds. Additionally, polymer grafting retarded drug release; DOX cumulative release at pH 5.0 reached to more than 60 % from MSN-PA particles alone while this value was 43 % for MSN-hyd-MOP particles. Cellular uptake and cytotoxicity studies confirmed the charge alteration under changing pH values enhancing cytotoxicity and cellular uptake, which indicated the potential utilization of the hybrid particles in cancer therapy.

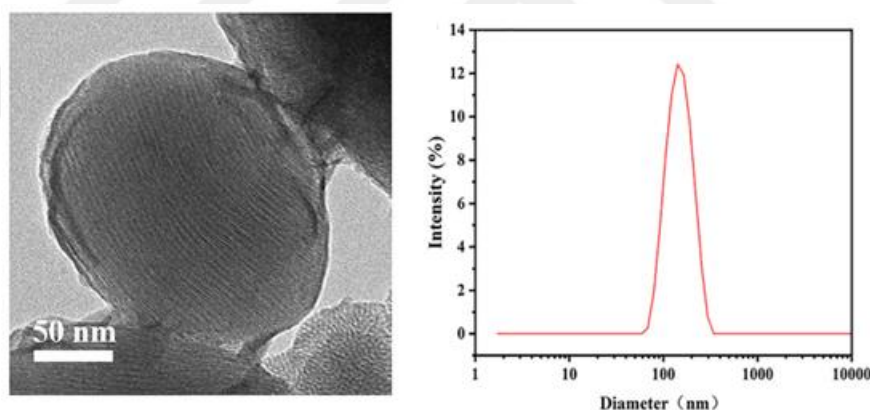


Figure 2.20 : TEM image of MSN-hyd-MOP and size distribution [81].

Another study investigating controlled DOX release utilized polymer coated MSN for stimuli triggered release [82]. The MSNs were loaded with the model drug, DOX, and then electrostatically coated with perylene- and iodomethane-functionalized poly(dimethylaminoethyl methacrylates) (PDMAEMA). The polymer coating is supposed to respond to two stimuli: visible light and pH and thus the drug release is expected to be enhanced under optimal light stimulus and pH value. The proposed mechanism of DOX loading, polymer coating and stimuli triggered DOX release was represented in Figure 2.21.

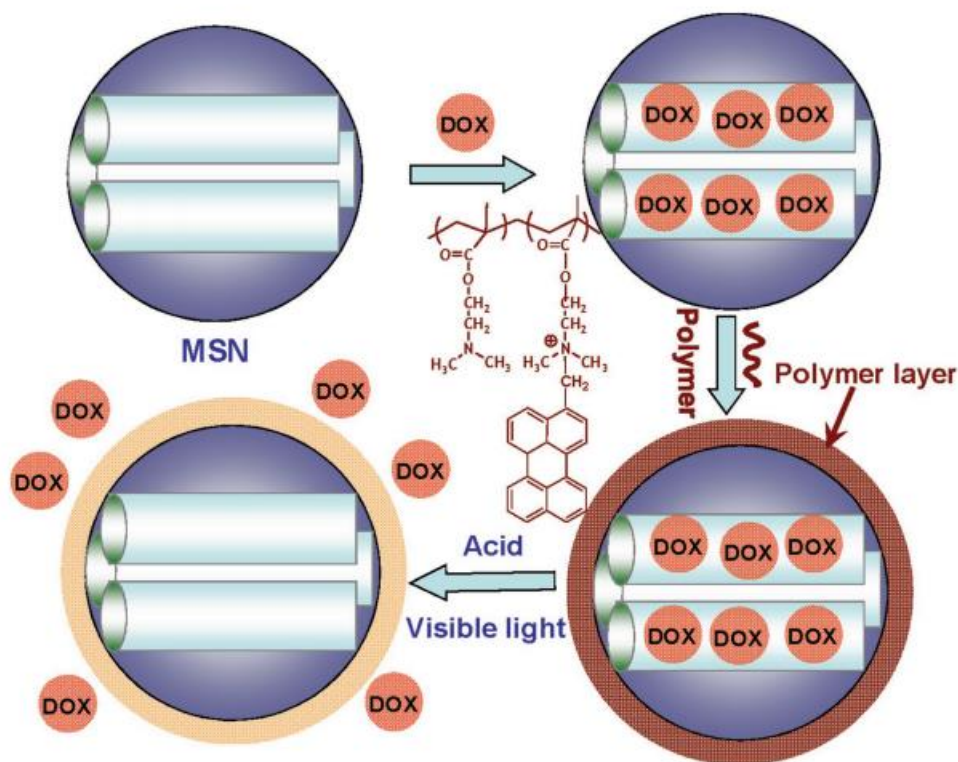


Figure 2.21 : Schematic representation of DOX-loading, polymer coating and stimuli triggered release [82].

Drug release studies have confirmed the response of the polymer coated MSN to the visible light stimulation (450 nm , 20 mW/cm^2) and change in pH value (Figure 2.22). As can be seen in the figure below, acidic pH triggered release from perylene-functionalized polymer-coated MSNs since the polymer was protonated under acidic pH and opened the nanopores of the MSN. Similar response was observed for the iodomethane-functionalized polymer coated MSNs. However, in the case of light stimulation perylene-functionalized polymer-coated MSNs were found to be responsive. A synergistic effect was observed such that at lower pH, protonation of PDMAEMA left the photoinduced carboxylic acid moieties of the polymer non-crosslinked. This, in return, led to an increase in the number of opened nanopores after irradiation by visible light, resulting in enhanced drug release. This finding was highlighted by the researchers since these mild stimulations can be more applicable compared to harsh conditions for triggering release.

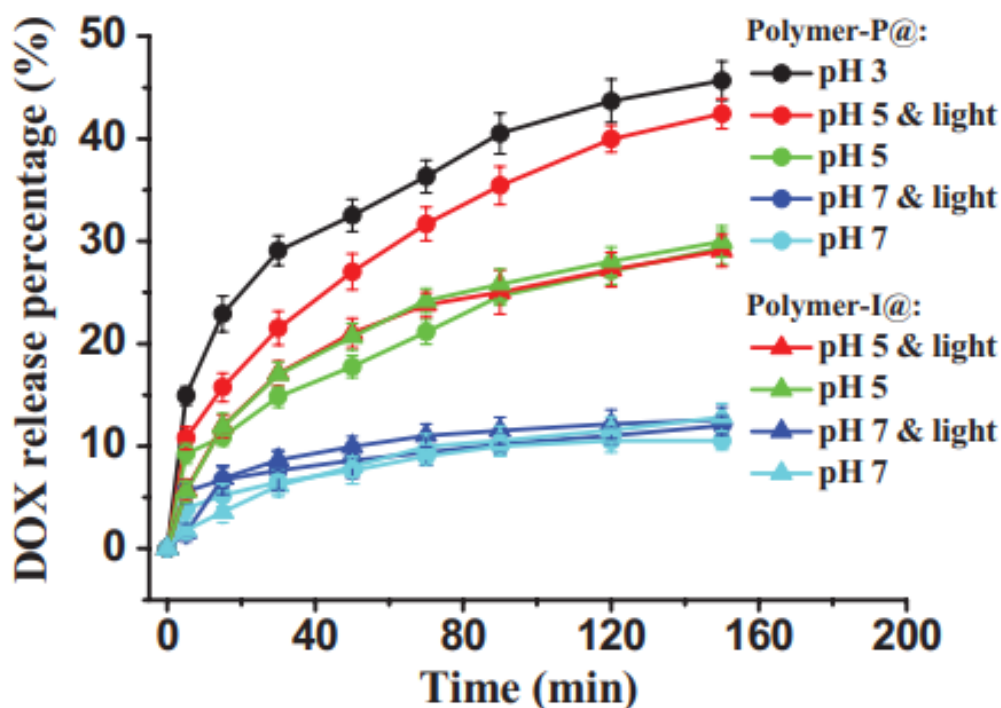


Figure 2.22 : Cumulative DOX release (Polymer-P@: perylene-functionalized polymer-coated MSNs; Polymer-I@: iodomethane-functionalized polymer-coated MSNs) [82].

2.2 Smart Drug Delivery Systems

Drug delivery system (DDS) designs enhance pharmacokinetics and biodistribution of the drug and provide a sustained release profile [32]. DDS provides some exceptional features compared to the conventional drug formulations. The major drawback of active materials used in treatment of diseases is the hydrophobic character of some molecules since these molecules may precipitate in body fluids. Conventional drug formulations bring a solution to this complexity by using suitable additives. However, these additives may have adverse effects on the intended site of action. DDS designs generally utilize biocompatible polymers such as silk fibroin, collagen, gelatin, albumin, starch, cellulose, chitosan, poly(lactic acid) (PLA), polycaprolactone (PCL), polyglycolic acid (PGA), and polylactic glycolic acid (PLGA) to overcome this issue which eliminates the necessity of additives. Drug carrier used in such designs also protects the targeted site from the toxic effects of the active molecules by controlling and keeping the dosage under the toxic limit [32]. Another function of the carrier material is to protect the active molecules from premature degradation and rapid breakdown by body metabolism [31].

Smart drug delivery systems (SDDS) are systems that are designed and developed to deliver active materials to the desired site of action and release it when stimulated by a physical or chemical change. The main purpose of employing SDDS is to control release kinetics so that the active material can be delivered to the desired site of action without causing any side effects to the non-targeted sites. The controlled release of the active material is generally achieved by stimuli-responsive polymers. Such polymers may undergo structural changes when exposed to different physical conditions which facilitates drug release. These changes in the physical environment or “stimulations” can be light radiation, temperature, pH and magnetic stimuli. SDDS are especially useful in cancer therapy in which pH-responsive systems are quite explored [83].

Drug delivery with these systems can be achieved with several types of release modes or profiles. These concentration profiles, whatever the mode is, should fall in between an upper and a lower limit ($C_{\max}$ and $C_{\min}$, respectively) which is called therapeutic window (Figure 2.23). The lower limit, $C_{\min}$, represents the minimum concentration limit at which the active material shows therapeutic effect on the targetted site. $C_{\max}$ is the upper concentration limit at which the active material is not therapeutic any longer, showing rather toxic effects on the target site [84]. One of the most preferred release modes is biphasic release (Figure 2.23a) which basically consists of two phases of release, burst release followed by a sustained release phase. This simple release mode decreases the need for frequent administration since it controls the dosage at the desired level by sustainin the release of the active material [85, 86]. Pulsed release (Figure 2.23b) is obtained when the system is objected to an external stimulus (pH change, magnetism, temperature change, etc.). During stimulation, the release of the active material is increased, other than that the release is kept at minimum. This release mode minimizes the possible side effects of the active material on the non-targetted sites [84]. A multi-effect release is the result of multiple mechanisms having part in the total release of the active material (Figure 2.23c). The most common formulation resulting in multi-effect release is the formulation with polymeric carriers from which the release is controlled by drug diffusion and matrix erosion. The first phase seen in Figure 2.23c is due to drug diffusion through the polymer matrix towards the environment whereas the second phase is due to the degradation of the polymer [84]. Liposomal release of active materials is a more specialized release from liposomes which consist of an aqueous core and several layer of phospholipids surrounding the

core. Liposomes keep the drug molecules in the core without releasing. The drug molecules are released from the liposome only when the liposome reaches to the specific site. The release profile of such behaviour is given in Figure 2.23d.

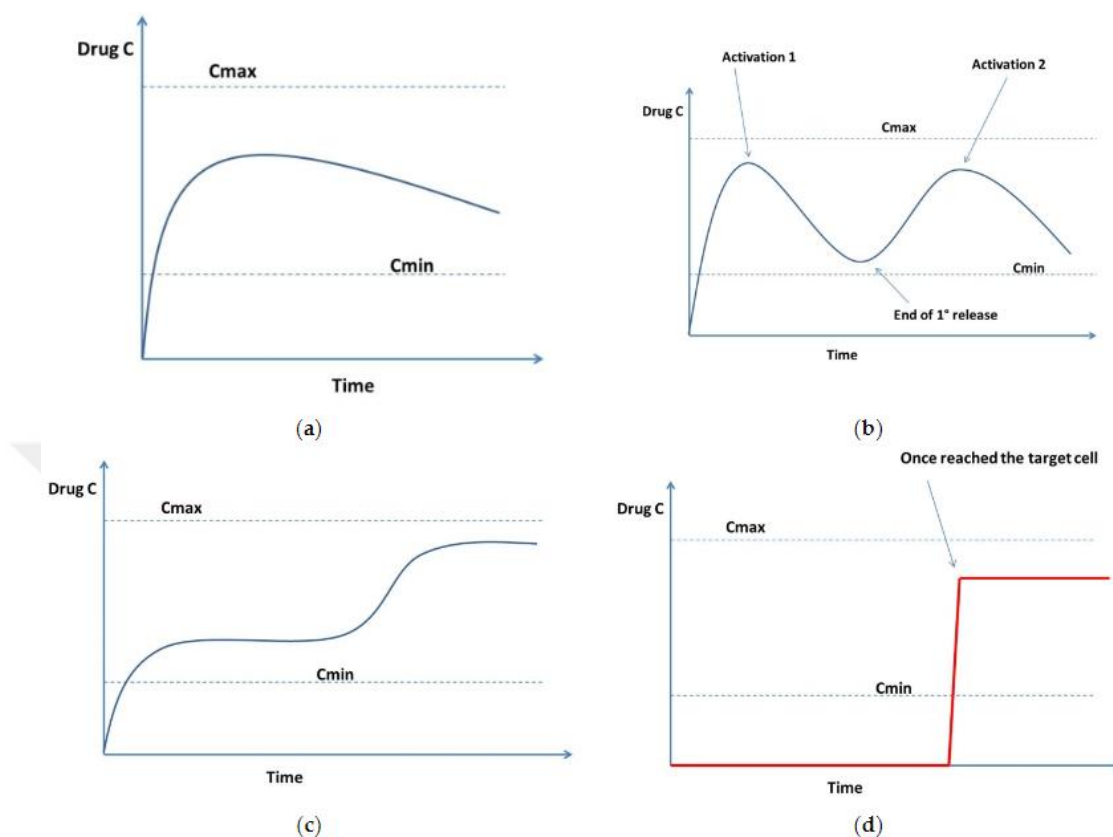


Figure 2.23 : Different modes of drug release, a) biphasic release, b) pulsed release, c) multi-effect release, d) liposomal release [84].

There may be different types of drug delivery such as microspheres and microcapsules, nanospheres and nanocapsules, liposomes, hydrogels and electrospun fiber mats (Figure 2.24). Depending on the administration model and desired release mode, different types of delivery can be selected. Some of the administration types are listed as: oral; rectal; parenteral administration such as subcutaneous injection, intramuscular injection, intravenous injection, intra-arterial injection; transmucosal; transnasal; pulmonary; transdermal and intra-osseous infusion delivery [87]. Since this study investigates microspheres and nanospheres, these systems will be exhaustively discussed. The drug release from these microsphere and nanosphere systems can be achieved mainly by five different mechanisms; diffusion through polymer matrix, diffusion through water-filled pores, osmotic pumping, surface erosion and bulk erosion which are schematically represented in Figure 2.25.

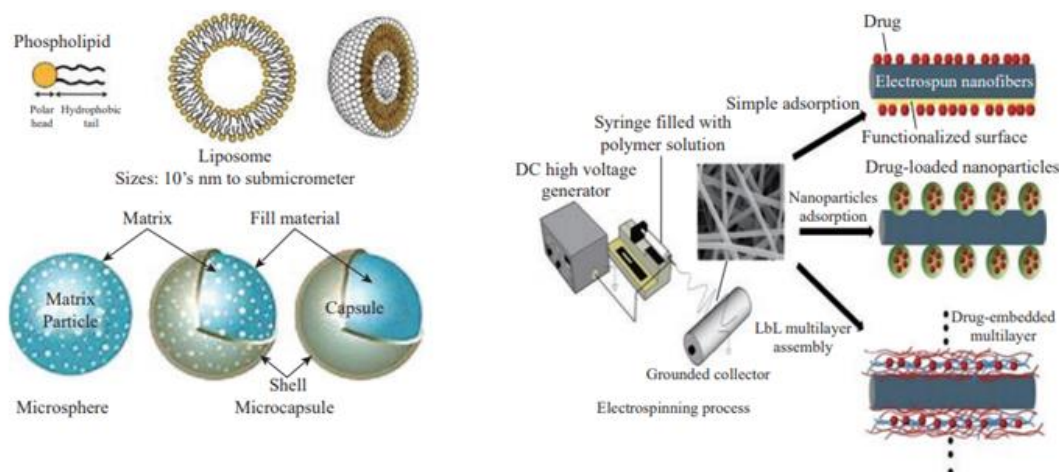


Figure 2.24 : Types of drug delivery and release formulations [87].



Figure 2.25 : Drug release mechanisms for polymeric micro and nanospheres: (a) diffusion through the polymer matrix, (b) diffusion through water-filled pores, (c) osmotic pumping, (d) surface erosion, and (e) bulk erosion (Red dots: drug) [88].

2.2.1 Microspheres as drug delivery systems

Microspheres are particles with spherical geometry and particle size in the range of 1-1000 μm . The particles can have solid or hollow form, this case they are called microspheres [89]. Drug molecules are dispersed within a polymer matrix (Figure 2.26) and controlled drug release can be achieved by either diffusion through the polymer matrix or by the degradation of the polymer matrix [90].

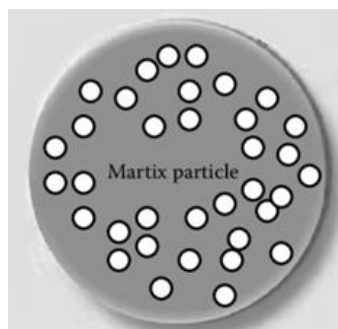


Figure 2.26 : Structure of microspheres [91].

The particles can be either formed as microcapsules which have an entrapped material in an inner core surrounded by the polymeric wall. The microcapsules may have different inner structure as shown in Figure 2.27.

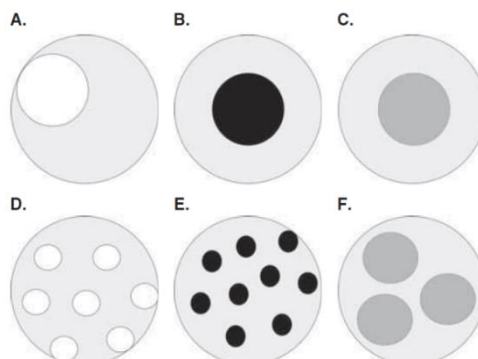


Figure 2.27 : Structure of microcapsules [37].

Microspheres have some advantageous properties in terms of efficacy. The spherical geometry and small particle size ease the administration of the drug delivery system in the body. Another feature small particle size provides is the increased surface area which is especially effective in increasing the potency of poorly soluble drugs. Microspheres can be produced by several methods such as single emulsion solvent evaporation, double emulsion solvent evaporation, sedimentation polymerization, spray-drying, electrospraying or phase separation technique [90].

2.2.1.1 Emulsion/solvent evaporation method

Emulsion/solvent evaporation method is the simplest method for drug-loaded microsphere fabrication. The emulsion can be single or double depending on the solubilities of polymer and drug. Single O/W (oil-water) emulsion/evaporation method involves an organic (internal or dispersed) phase and an aqueous (external or continuous) phase. The organic phase consists of polymer and drug dissolved in an organic solvent and is emulsified with the aqueous phase. Aqueous phase is an aqueous solution of a high hydrophilic-lipophilic balance (HLB) surfactants; generally aqueous polyvinyl alcohol (PVA) solution is used as the continuous phase. It was shown that PVA solution as the continuous phase significantly increased drug encapsulation compared to other types of surfactants [92]. The emulsion is stirred until the organic solvent is evaporated. Alternatively, the solvent can be evaporated under reduced pressure by using a rotary evaporator. Lastly, the microspheres are collected by suitable methods such as filtration or centrifugation and dried (Figure 2.28).

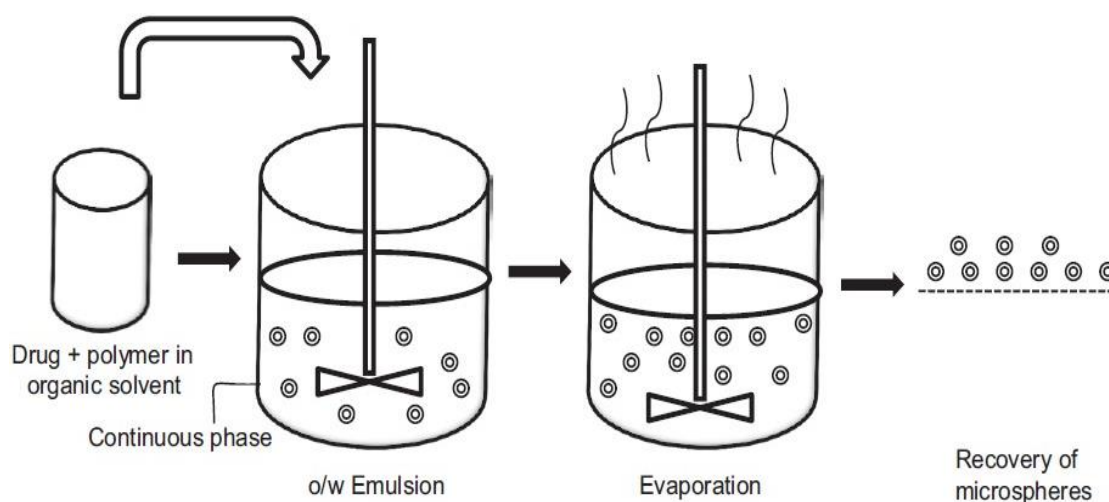


Figure 2.28 : Formation of microcapsules by single O/W emulsion/solvent evaporation method [93].

Double emulsions are needed when the drug to be encapsulated is not soluble in the organic phase. Aqueous solutions of hydrophilic drugs are firstly emulsified in the polymer solution in an organic solvent to form the primary W/O emulsion. This W/O emulsion is then added to the water phase (an aqueous solution of a hydrophilic surfactant) which forms the final $W_1/O/W_2$ emulsion [94]. A double emulsion can be also a $O_1/W/O_2$ type. The material to be encapsulated is dissolved in an organic solvent and then added to the aqueous solution of the polymer to form O_1/W emulsion. This primary emulsion then added to the second oil phase (O_2) [95].

The technique of emulsion solvent evaporation has several advantages over methods such as spray drying, sonication, emulsion diffusion or emulsion polymerization, because it requires mild conditions (room temperature and constant stirring). Thus, the activity of the drug is not compromised [96]. With this method, microspheres with high drug encapsulation can be obtained for lipophilic active materials. However, encapsulation of hydrophilic drugs is not sufficient. This drawback can be overcome by applying double emulsions which are quite efficient for both hydrophobic and hydrophilic drug encapsulation. The ease of adjusting the particle size of the microspheres by altering process parameters such as concentrations of the phases, the type of the surfactant or homogenization speed, renders the emulsion/solvent evaporation method preferable to the other methods. The main disadvantage of the method is that it is hard to scale up for large productions [97].

The parameters affecting the final properties of microspheres have been investigated widely. Especially for emulsion containing material with poor water solubility (such

as chalcone in this study [98]), it was found that increasing stirring rate decreases both particle size and size distribution which affect drug release profile. Additionally, encapsulation efficiency was determined by stirring rate [99]. Another parameter that affects drug release profile is the crystallinity degree of the polymer drug carrier. In a drug release study utilizing homopolymer poly-D-lactic acid and copolymer polylactic-co-glycolic acid, it was found that copolymerization reduced the crystallinity degree. It was deduced that due to this reduction in crystallinity degree, the matrix degradation and drug release profiles were higher in case of copolymer carrier [100].

For the fabrication of microspheres, many types of biopolymers like silk fibroin, collagen, gelatin, albumin, starch, cellulose and chitosan, or synthetic polymers such as poly(lactic acid) (PLA), polycaprolactone (PCL), poly(glycolic acid) (PGA), and poly(lactic glycolic acid) (PLGA) are widely investigated. PCL, being a FDA-approved synthetic polymer readily being used in medical applications, is widely utilized in the formation of drug loaded microspheres. PCL gains prominence as a material suitable for in vivo medical appliances due to another important aspect. Since PCL is degrades in the body much slower than other widely used, synthetic polymers such as PLA, PGA and PLGA which provides sufficient time for the removal of the metabolites. Additionally, the degradation products of PCL is less acidic than the degradation products of PLA, PGA and PLGA [101].

The types of solvent and surfactants used in the formulation of microspheres and the nature of drug affects the particle size and encapsulation efficiency. Table 2.1 shows numerous microsphere formulations prepared by emulsion/solvent evaporation method with PCL or PCL-based materials as the encapsulating agent.

Table 2.1 : Microsphere formulations prepared by emulsion/solvent evaporation method, with PCL or PCL-based materials as the encapsulating agent.

Polymer	Drug	Oil Phase	Water Phase	Particle Size (μm)	Encapsulation Efficiency (%)	Drug Release	Reference
PCL	Valsartan	Methylene Chloride	PVA	51	79 $\pm$ 1.6	pH 1.2: 8 h, ~65 % pH 6.8: 8 h, ~97 %	102
PCL	Allopurinol	Dichloromethane	PVA	77	69 $\pm$ 1.7	pH 1.2: 8 h, 71 %	103
PCL	Erythromycin	Dichloromethane	Gelatin	112	95	pH 7.4: 25 h, 96 %	104
PCL/carbon dots	Rifapentin	Dichloromethane	PVA	4.60	81	pH 7.4: 7 days, 56.7 %	105
PCL-PEG-PCL	Ibuprofen	Methylene Chloride	PVA	90-236	88.86 $\pm$ 3.77	-	106
PCL PCL/Graphene nanopowder	Amoxicillin	Chloroform	PVA	19.02 $\pm$ 6.41 (PCL) 212.48 $\pm$ 68.74 (PCL/G) 58 (P-nitroaniline)	37 (PCL) 65 (PCL/G)	pH 7.4: 168 h, ~60 % (PCL) pH 7.4: 168 h, 86 % (PCL/G)	89
PCL	P-nitroaniline Rhodamine B	Dichloromethane	Methylcellulose	252 (Rhodamine B)	41 (P-nitroaniline) 12 (Rhodamine B)	pH 7.4: 30 days, ~88 % (P-nitroaniline); 30 days, ~48 % (Rhodamine B)	107
PCL/F68 blend	Paclitaxel	Dichloromethane	PVA	24.32 $\pm$ 10.68	90.12	pH 7.4: 60 days, ~23 %	108

Table 2.1 (continued) : Microsphere formulations prepared by emulsion/solvent evaporation method, with PCL or PCL-based materials as the encapsulating agent.

Polymer	Drug	Oil Phase	Water Phase	Particle Size (µm)	Encapsulation Efficiency (%)	Drug Release	Reference
PCL Star-shaped PCL	Ibuprofen	Dichloromethane	PVA	79-154	83-93	pH 7.4: 24 h, ~58-70 %	109
PCL	Aripiprazole	Dichloromethane	PVA	49.4-387.1	70-95	pH 7.4: 45 days, 87-95	110
PCL	Metrodinazole	Dichloromethane/Methanol	Paraffin oil/Span 80	131-280	11-34	pH 7.4: 80 min, ~93 %	111
PCL	Erlotinib Hydrochloride	Dichloromethane	PVA	1.42 ±0.8-5.52±1.2	76.3-87.37	pH 7.4: 24-48 h, ~95-100 %	112
PCL	Doxorubicin	Ethyl acetate	PVA	15-60	42.2	-	113
PCL	Rasagiline-mesylate	Dichloromethane	PVA	24.18- 47.87	7.68 ± 0.26-27.47 ± 1.60	pH 7.4: 45 days, ~60-75 %	114
PCL/ cellulose acetate phthalate	Tripolidine hydrochloride (aqueous solution)	Dichloromethane/Isopropyl alcohol	PVA	11 ± 9-19 ± 11	48.76 ± 1.8-56.81 ± 2.8	pH 1.2: 24 h, ~45-65 % pH 7.4: 24 h, ~90-95 %	115
PCL	Piroxicam	Dichloromethane	PVA	0.658±0.052-2.812±0.460	47.9±0.78-91.0±0.45	pH 7.4: 6 h, ~5-45 µg/ml	116

2.2.2 Nanospheres

Polymeric nanoparticles are widely used materials used in drug delivery applications with a diameter of 1-1000 nm. As microparticles nanoparticles also can be in two different forms: nanocapsules and nanospheres whose structures are represented in Figure 2.29. Nanocapsules are formed by two distinctive layers; a liquid inner core of dissolved active material and a polymer membrane layer surrounding the inner core. On the other hand nanospheres have a monolithic structure and the active material is encapsulated within the polymer matrix or adsorbed on the surface of the polymeric nanoparticle.

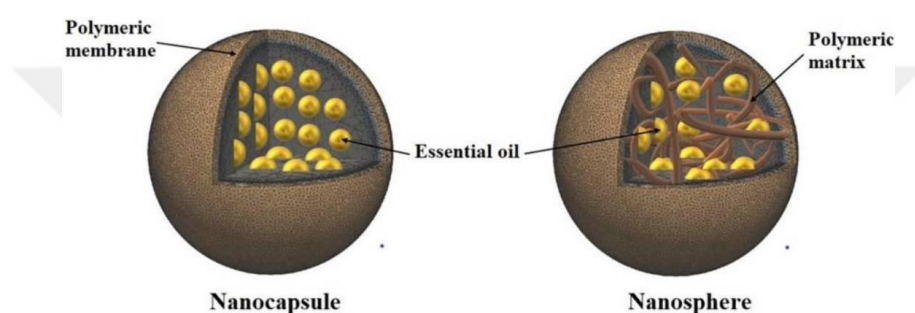


Figure 2.29 : Structures of nanospheres and nanocapsules [117].

Due to increased surface area/volume ratio with decreasing particle size, release from nanoparticles are significantly enhanced with respect to microparticles. Small particle size of the nanospheres and nanocapsules allows them to be administered by injection [118] and also increases the cellular uptake of the particles [38, 119]. Nanoparticle systems can improve the stability of the active materials and the biocompatibility can be enhanced by using biocompatible polymers as the carrier matrix [38]. The biodistribution of the particles is inversely proportional to the diameter of the nanoparticles. Biodistribution is also strictly dependent on the functionalization of the particles [120]. Nanospheres and nanocapsules can be formed by numerous methods although the most common is interfacial polymer deposition which is also known as nanoprecipitation or solvent displacement.

2.2.2.1 Interfacial polymer deposition (nanoprecipitation)

The interfacial polymer deposition method, also known as nanoprecipitation, was introduced by Hatem Fessi in 1989. The method requires both solvent and non-solvent phases [121]. The solvent phase or organic phase includes a polymer solution in a

solvent, the therapeutic material, oil and w/o surfactant. The non-solvent phase or aqueous phase consists of a non-solvent for the polymer and a surfactant in which non-solvent is generally water [38].

Generally, biodegradable polyesters are used, such as poly-ε-caprolactone (PCL), poly(lactide) (PLA) and poly(lactide-co-glicolide) (PLGA). Capric/caprylic triglycerides are often used as the oily core ingredient [37]. Also some vegetable oils such as argan oil, sunflower seed oil and soybean oil can be used [37, 38]. Sorbitan esters and phospholipids are generally preferred as w/o surfactants. In most cases, acetone is used as polymer solvent and water is chosen as non-solvent material. Surfactant material in aqueous phase is generally is poloxamer 188 or polysorbate [38].

In interfacial polymer deposition method (Figure 2.30), the organic phase is injected dropwise to the aqueous phase with moderate stirring. The nanocapsules are obtained as a colloidal suspension. The important parameters of the procedure that will affect the properties of the nanocapsules are organic phase injection rate, aqueous phase agitation rate, the method of organic phase addition and the organic phase/aqueous phase ratio.

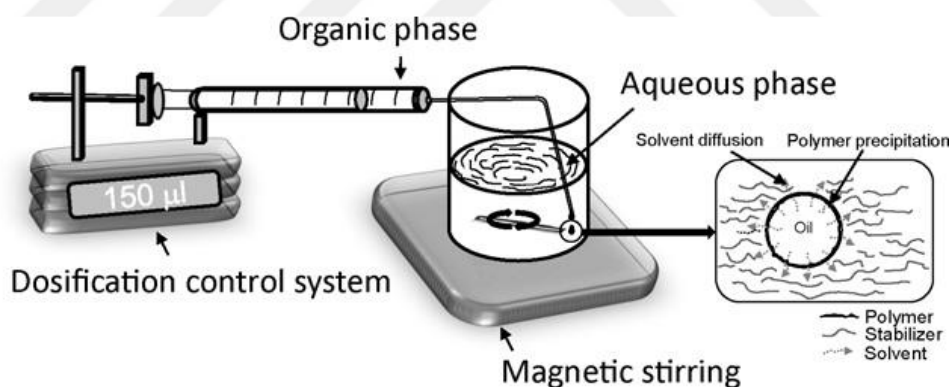


Figure 2.30 : Interfacial polymer deposition [38].

The addition of the oil in the organic phase allows the active material to be dissolved in the oily core of the nanoparticle and results in a nanocapsule structure in which the drug is reservoired in the liquid core as a solution surrounded by the polymer membrane. If the oil is omitted from the formulation, a solid matrix of polymer loaded with drug molecules can be obtained. In this case the particles obtained are called nanospheres in which the drug molecules are encapsulated within the polymer matrix or adsorbed on the surface of the nanoparticles [122].

Table 2.2 : Nanosphere formulations prepared by nanoprecipitation method, with PCL or PCL-based materials as the encapsulating agent.

Polymer	Drug	Oil Phase	Water Phase	Particle Size (nm)	Encapsulation Efficiency (%)	Drug Release	Reference
PCL	Lidocaine	Chloroform/Acetone	PVA	449.6 ± 0.5	93.3 ± 2.1	pH 7.4: 350 min, 60 %	123
PCL	5-fluorouracil	Acetone	TWEEN	-	38.57	pH 7.4: 72-96 h, ~75-100 %	124
PCL	Curcumin	Acetone	PVA or P407	210.4±2.3-326.8±7.0	9.7±6.1- 80.5±0.3	-	125
PCL	Alendronate sodium	Acetone	PVA	195-447	0.36 ± 0.25-18.80 ± 0.70	pH 7.4: 750-2000 h, ~90-95 %	126
PCL-PEG-PCL/Iron oxide	Doxorubicin	Dichloromethane	PVA	70-150	95	Higher release rate was obtained at pH 5.8 compared to pH 7.4	127
PCL	Cefotaxime	Dichloromethane	PVA	216	-	pH 5.5: 48 h, 56 % pH 7.4: 48 h, 83 %	128
PCL	Paclitaxel	Acetone	Poloxamer 407®	142.5 ± 3.53; 142.1 ± 0.56 and 156.4 ± 0.42 nm	~100	-	129

2.3 Trans-Chalcone

Chalcones are open-chained molecules, which are naturally found in plants. Their chemical structure consist of two aromatic rings with three-carbon α,β -unsaturated carbonyl system in between [16] as given in Figure 2.31. Such structure is the main difference between chalcones and other flavonoids [17]. The chemical structure of chalcones can be diversified by addition of functional groups to the aromatic rings [18]. In plants, chalcone molecules act as pigments since they possess a distinctive, intense yellow color [19].

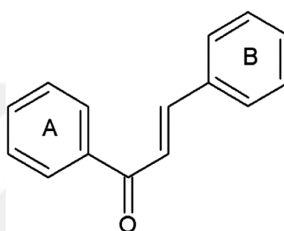


Figure 2.31 : General structure of trans-chalcone [18].

Chalcones are precursors of flavones. Indeed, 2-hydroxychalcones can be easily converted into flavanones through Michael addition and into aurones [19].

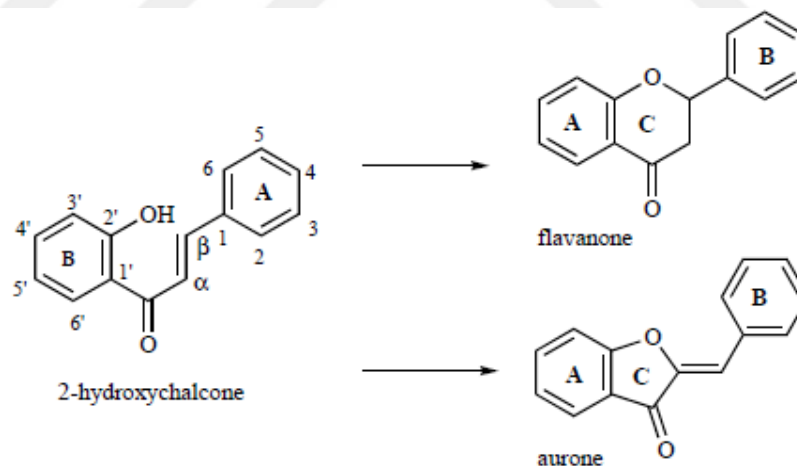


Figure 2.32 : Conversion of chalcones into flavanones and aurones [19].

In recent years, trans-chalcone (TC) have attracted attention in terms of its biological activities due to its abundance in nature, ease of preparation and its simple structure [16]. TC has been proved to have anticancer activity towards several types such as breast cancer (MCF-7 cell line) [130], bone cancer (U2OS cell line) [131, 132] and liver cancer (HuH7.5 cell line) [133]. Anti-leishmanial activity of trans-chalcone has been widely explored. Studies showed the potency of the compound for Leishmania

amazonensis-induced cutaneous leishmaniasis treatment [134-136]. TC is also anti-inflammatory, acting by reducing oxidative stress induced by various inflammatory diseases [137-140]. Lamoke et al. showed that retinal neovascularization process (major cause of which is vascular inflammation and oxidative stress) in ischemic retina of mice was suppressed by TC [141]. However, there are limitations for clinical use of TC, mainly water insolubility and thus low bioavailability [142]. TC is a plant-based chemical thus its toxicity in body is relatively low compared to synthetic drug molecules. Cytotoxicity values of TC on several cells lines are presented in Table 2.3 [143]. However, an extremely risky aspect of TC accumulation in the body is that TC is a proestrogen. TC is metabolically activated to several other chemicals, which are mainly 4'-hydroxychalcone, 4-hydroxychalcone and 2-hydroxychalcone. It was shown that these compounds have estrogenic activity [144]. Many adverse health effects in mammals can occur due to this estrogenic activity of xenobiotic compounds such as precocious puberty in females, obesity, reduced sperm count, alterations in reproduction organs and sexual behaviors, and increase in some cancer types [145]. Thus, it is critical to control the dosage of TC treatment and avoid the accumulation of TC molecules in the body.

Table 2.3 : Cytotoxicity of TC [143].

Cell Lines	Half-maximal effective concentration (μM)	Half-maximal effective mass (in 10 ml, μg)
MDBK	293.9 ± 2.9	612
NIH/3T3	434.4 ± 2.7	905
HFF	498 ± 3.1	1037

2.3.1 Synthesis of chalcones

Chalcones can be chemically synthesized by Claisen-Schmidt condensation reaction (Figure 2.33). The reactants are acetophenone and benzaldehyde, and the reaction is catalyzed by a base, generally NaOH or KOH [146].

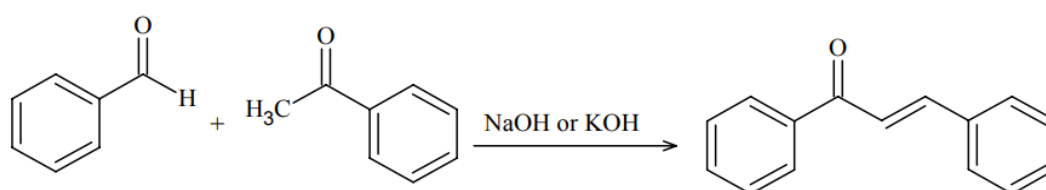


Figure 2.33 : Claisen-Schmidt condensation of acetophenone and benzaldehyde [146].

A solventless method for Claisen-Schmidt condensation was reported by Ballini *et. al.* This reaction was conducted between acetophenone and benzaldehyde at 130 °C. It was found that an acidic clay as a catalyst yields best performance [147].

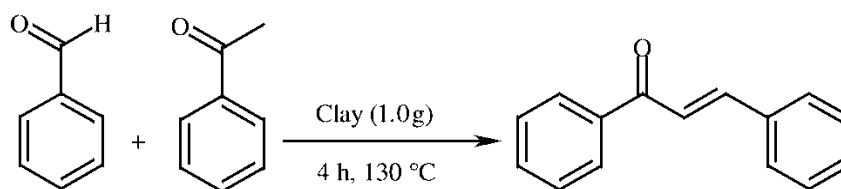


Figure 2.34 : Solventless Claisen-Schmidt condensation [147].

Suzuki reaction, given in Figure 2.35, is another method for chalcone synthesis. This reaction occurs between activated cinnamic acids and phenylboronic acids in the presence of palladium [18].

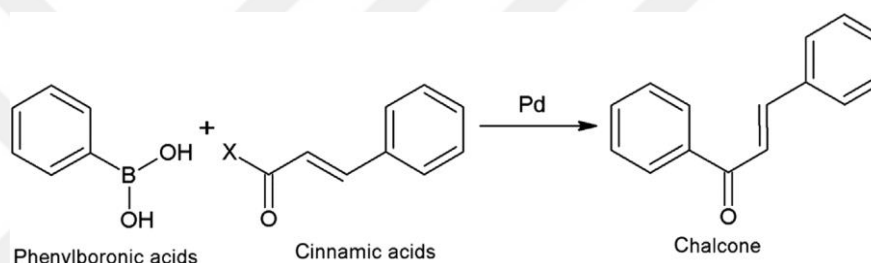


Figure 2.35 : Chalcone synthesis by Suzuki reaction [18].

Another reaction that produces chalcone takes place between arylboronic acids and benzoic anhydride in the presence of PdCl_2 and Na_2CO_3 in H_2O /acetone [18].

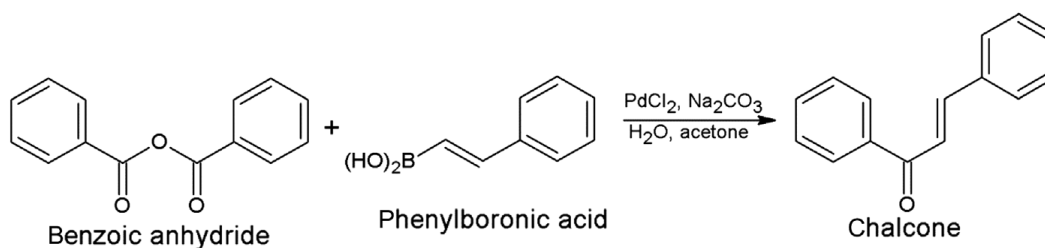


Figure 2.36 : Synthesis of chalcone with arylboronic acids and benzoic anhydride [18].

Chalcones can be also synthesized enzymatically. In plants, chalcone synthase (CHS, EC 2.3.1.74) is an important enzyme in flavonoid biosynthesis pathway. 4,2',4',6'-tetrahydroxychalcone, or naringenin chalcone is a product of the condensation reaction between p-coumaroyl-CoA and malonyl-CoA, which is the first step of the flavonoid biosynthesis pathway [148].

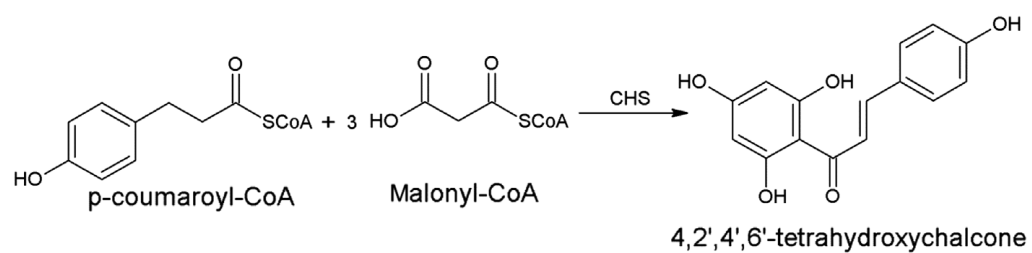


Figure 2.37 : Biosynthesis of naringenin chalcone [18].

3. MATERIALS AND METHODS

3.1 Materials

Rice husks were kindly supplied by a local rice production facility (Edirne, Turkey) and treated to obtain rice husk ash (RHA). (3-Aminopropyl)trimethoxysilane (3-APTMS, $C_6H_{17}NO_3Si$, 95 %, Acros Organics), (3-Aminopropyl)triethoxysilane (3-APTES, $C_9H_{23}NO_3Si$, 98 %, Sigma-Aldrich), (3-Glycidyloxypropyl)trimethoxysilane (3-GPTMS, $C_9H_{20}O_5Si$, 98 %, Acros Organics) and N-[3-(Trimethoxysilyl)propyl]ethylenediamine (3-TMSPDA, $C_8H_{22}N_2O_3Si$, 97 %, Acros Organics) were used in the experiments for surface modification of RHA. Acetone (C_3H_6O , 100 %, Merck) was used as medium in surface modification of RHA. ϵ -caprolactone (ϵ -CL) ($C_6H_{10}O_2$, 99 %, Alfa Aesar) was used as monomer in polymerization reactions. It was dried over molecular sieves to avoid moisture. Toluene (C_7H_8 , 99.8 %, Merck) was used as organic solvent in polymerization reactions. Reactions were catalyzed by immobilized *Candida antarctica* Lipase B (CALB) enzyme (E.C. 3.1.1.3, recombinant, expressed in *Aspergillus niger*, Sigma). Chloroform ($CHCl_3$, 99 %, Merck) was used to terminate polymerization reactions and as solvent for PCL hybrids during microsphere and nanosphere formation. Trans-chalcone (TC, $C_{15}H_{12}O$, 97 %, Sigma-Aldrich), model drug, was purchased from Sigma-Aldrich. During preparation of TC-loaded microspheres, dichloromethane (CH_2Cl_2 , 99 %, Carlo Erba) was used as solvent of TC, in the internal phase. Trans-chalcone was also synthesized chemically with Claisen-Schmidt condensation, by using benzaldehyde (C_7H_6O , 99 %, Merck) and acetophenone (C_8H_8O , 99 %, Sigma Aldrich) as monomers. Polyvinyl alcohol ($[C_2H_4O]_n$, 99 %, Merck) was used to prepare the external phase. For nanosphere preparation, Poloxamer 188 solution (Poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol), 10 %, Sigma-Aldrich) and Span 80 (Sorbitane monooleate, 60 %, Sigma-Aldrich) were used as surfactant materials in the external and the internal phases, respectively. Methanol (CH_3OH , 99.8 %, Merck) was used in determination of loading efficiency

of the microspheres. Potassium di-hydrogen phosphate (KH_2PO_4 , 100 %, Carlo Erba) and di-potassium hydrogen phosphate (K_2HPO_4 , 100 %, Merck), sodium chloride (NaCl , 99.6 %, Carlo Erba), potassium chloride (KCl , 100 %, Merck), disodium phosphate dihydrate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 100 %, J. T. Baker), sodium dihydrogen phosphate (NaH_2PO_4 , 100 %, Merck), acetic acid (CH_3COOH , 99.8 %, Merck) and sodium acetate (CH_3COONa , 99 %, GPR) were used to prepare buffer solutions for enzyme immobilization and drug release studies. For the preparation of proton nuclear magnetic resonance (^1H -NMR) samples, deuterated chloroform (CDCl_3 , Merck, 99.8%) was used.

3.2 Methods

3.2.1 Preparation and surface modification of RHA

Rice husk ash was obtained and surface-modified as described in our previous study [149]. Briefly, rice husks were rinsed with distilled water and dried in an oven at 30 °C overnight. Then, the cleaned husks were burned at 600 °C for 6 h, increasing the temperature stepwise until the desired temperature was reached. Surface modification was realized by using various silanization agents (3-APTES, 3-APTMS, 3-GPTMS and 3-TMSPDA). 15 % (v/v) related silanization agent in acetone was added to the RHA and the mixtures were incubated at 50 °C for 2 h. Later, the modified RHA were filtered, rinsed with distilled water and dried.

3.2.2 CALB immobilization

PCL-based nanohybrid was enzymatically synthesized by using immobilized CALB onto surface-modified RHA as catalytic system. For this purpose, free CALB was physically adsorbed onto modified RHA as described earlier [149]. The surface-modified RHA was mixed with free lipase enzyme (enzyme to support ratio of 2 $\mu\text{L}/\text{mg}$) in phosphate buffer solution (0.015 M, pH 7.0) for 5 h. Immobilized enzyme samples were filtrated under vacuum and stored at 4 °C.

3.2.3 Synthesis of PCL-based nanohybrid systems

Immobilized CALB was utilized in the ROP of ϵ -caprolactone and development of nanohybrid systems [150]. The polymerization reaction was conducted in toluene as reaction medium with monomer to enzyme ratio of 5 (w:w). At the end of the specified

reaction time, polymerization reaction was terminated with chloroform. Reaction conditions (time and temperature) were determined by optimization experiments in earlier laboratory studies. Reaction product was precipitated with methanol to obtain PCL-based nanohybrid system. A hybrid system was successfully formed consisting of pure PCL chains, short PCL chains grafted on modified RHA (through –OH groups) and ungrafted RHA. In order to determine the actual PCL grafting on RHA, methanol precipitation step was omitted and the reaction product was fractionated by centrifugation cycles to separate PCL rich and RHA rich phases. Both samples were dried at 30 °C. The reaction steps of nanohybrid synthesis were visualized in Figure 3.1.

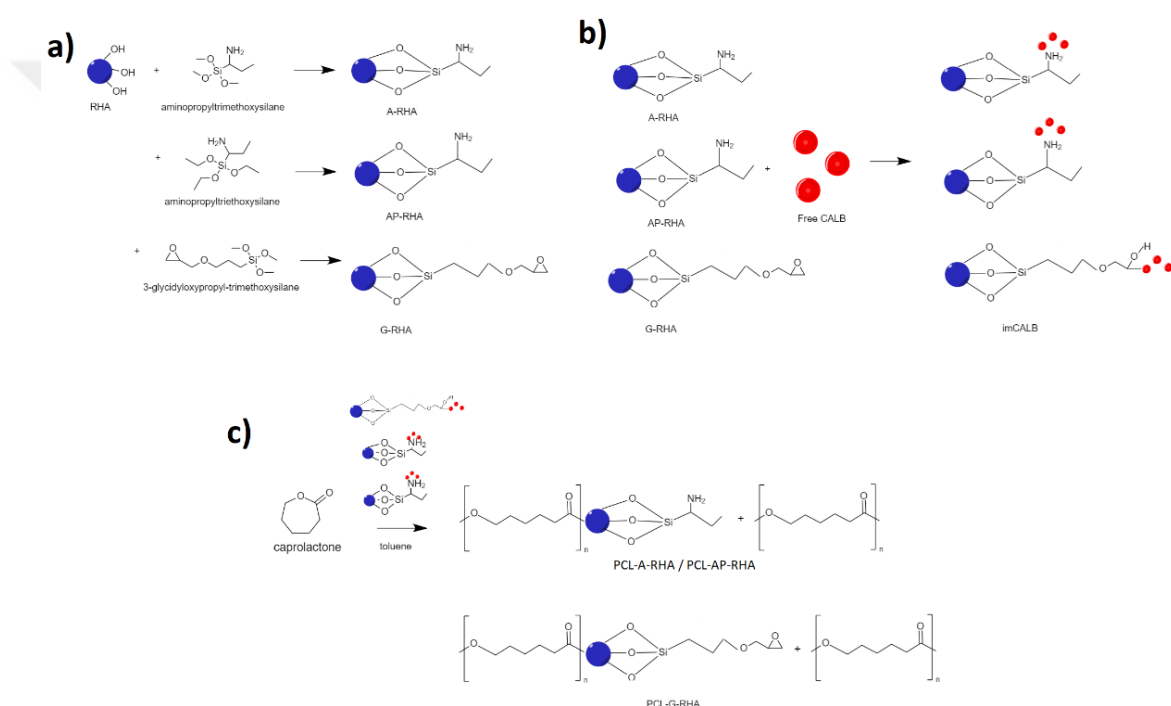


Figure 3.1 : Reaction steps of nanohybrid synthesis, a) Surface modification of RHA, b) Immobilization of CALB onto surface modified RHA and c) Enzymatic ROP of ϵ -caprolactone catalyzed by imCALB and nanohybrid synthesis.

3.2.4 Synthesis of TC

Trans-chalcone (TC) was chemically synthesized to observe the possible differences between the commercial and synthesized TC in drug loading and release studies. The reaction was Claisen Schmidt condensation reaction utilizing acetophenone and benzaldehyde as reactants. This reaction was catalyzed by sodium hydroxide (NaOH) and ethanol. The reactants were fed equimolar (0.1 mol).

3.2.5 Preparation of TC-loaded microspheres and nanospheres

PCL-based nanohybrid systems were used to prepare TC-loaded microsphere structures [151]. Microspheres were prepared by conventional O/W emulsion/solvent evaporation method [152]. Internal phase consisted of TC solution in dichloromethane (DCM) and PCL-based nanohybrid solution in chloroform (CH). Amount of PCL-based nanohybrid system was varied, maintaining the TC:PCL-G-RHA ratio at 1:10, 1:5 and 1:2.5. External phase was PVA solution with varying concentrations (0.1, 0.5 and 1 (w/v) %) to be able to observe the effects of different concentrations on TC loading and release. First, TC solution and PCL-based nanohybrid solution were well mixed by a vortex mixer. This solution was added to 50 ml of PVA solution under agitation at 1500 rpm for 5 min to form a milky white emulsion. Then the emulsion was diluted with 50 ml of distilled water and left under agitation for 24 h in order to allow solvents to evaporate. After evaporation, the emulsion was centrifuged in order to remove the PVA and the noncapsulated TC and the collected microspheres were dried overnight in an oven at 30 °C. Encapsulation efficiencies (EE) and loading capacities (LC) of the microspheres were measured directly by extracting drug content of the microspheres with methanol [153]. Methanol dissolves chalcone that was encapsulated in the particles but not the polymer thus the polymer did not cause disturbance in absorbance measurement by UV spectrophotometer [154]. The resulting suspension was filtered and absorbance was measured by UV spectrophotometer at 308 nm, with methanol as blank solution (Calibration curve was provided in Appendices A). Encapsulation efficiency and loading capacity were calculated by using the following equations.

$$EE (\%) = \frac{\text{weight of TC in the microspheres}}{\text{weight of TC in the emulsion}} \times 100 \quad (3.1)$$

$$LC (\%) = \frac{\text{Actual weight of TC in the microspheres}}{\text{Total weight of microspheres}} \times 100 \quad (3.2)$$

Nanospheres were formed by interfacial polymer deposition method. Nanohybrid systems and trans-chalcone (constant amount; 10 mg) were dissolved separately in chloroform and dichloromethane, respectively. These two mixtures were vortexed for 1 min in order to form organic phase. In some formulations, sorbitane monooleate (Span 80; HLB=4.3) was added to the organic phase as an oily core material. Aqueous

phase was 1 % aqueous solution of Poloxamer 188 (HLB=29) as stabilizing agent. Organic phase was added to the aqueous phase dropwise and emulsified at ~1000 rpm. Later, the emulsion was left under agitation overnight and solvent evaporation occurred at atmospheric pressure. After the solvent evaporation was completed, the nanospheres were centrifuged, rinsed with distilled water and dried at room temperature.

3.2.6 In vitro TC release from loaded microspheres and nanospheres

Drug release profiles of microspheres and nanospheres were studied. Drug release studies were conducted in PBS solution (pH 7.4, 0.1 M) as release media. 10 mg of loaded micro or nanospheres were suspended in PBS solution and shaken in an incubator at 37 °C and 120 rpm. At specified time intervals, 1 ml aliquot was drawn and same volume of fresh PBS solution was added. The aliquot was filtered with syringe filters if necessary to remove suspended micro or nanospheres that was present in the sample. The withdrawn sample was diluted to 10 ml with PBS and absorbance value was read by UV spectrophotometer. Ph-responsive release behavior of the loaded micro or nanospheres was investigated by changing the release medium with acetate buffer solutions (pH 3.6 and 5.6) potassium buffer solution (pH 8.0). All release experiments were conducted under sink conditions. Related calibration curves were presented in the Appendices A.

3.2.7 Kinetic model fitting

The drug release profiles were fitted to kinetic models in order to understand the mechanism of drug release for each sample. Zero Order, First Order, Higuchi and Korsmeyer-Peppas kinetic models were fitted to the data by nonlinear regression and the related kinetic parameters were calculated. OriginLab® OriginPro 8.5 data analysis and graphing software was used model fitting and related calculations. The equations used for the kinetic models were given below.

$$\text{Zero Order: } Q = Q_0 + K_0 \times t \quad (3.3)$$

$$\text{First Order: } Q/Q_0 = 1 - e^{(-K_1 \times t)} \quad (3.4)$$

$$\text{Higuchi: } Q = K_h \times t^{0.5} \quad (3.5)$$

$$\text{Korsmeyer-Peppas: } Q/Q_0 = K_{kp} \times t^n \quad (3.6)$$

3.2.8 Characterization of microspheres and nanospheres

Chemical structure of the nanohybrids and TC-loaded micro- and nanospheres were determined by Fourier Transform Infrared Spectroscopy (FT-IR). Spectra were recorded between 4000-400 cm^{-1} at 2 cm^{-1} resolutions (Perkin-Elmer, Spectrum One). Samples were analyzed by KBr pellet. For thermal analysis, thermogravimetric analysis (TGA, Seiko TG/DTA 6300, 30 to 550 $^{\circ}\text{C}$ for TC, microspheres and nanospheres, 30 to 800 $^{\circ}\text{C}$ for nanohybrid systems at a heating rate of 10 $^{\circ}\text{C min}^{-1}$ under nitrogen flow) and differential scanning calorimetry (DSC, Perkin-Elmer Pyris) were used. For thermogravimetric analysis, 10 mg of sample was heated to 550 $^{\circ}\text{C}$ or 800 $^{\circ}\text{C}$ with a rate of 10 $^{\circ}\text{C/min}$ under N_2 atmosphere. The operation temperature of DSC was -80-150 $^{\circ}\text{C}$ at a rate of 10 $^{\circ}\text{C/min}$ and thermograms were obtained with heat/cool/heat cycle to clear polymer's thermal history [155,156]. Crystallinity percentages were calculated by the equation below, where ΔH_m is the heat of fusion per gram of the samples and ΔH_m^0 is the heat of fusion per gram of the 100 % crystalline form. ΔH_m^0 is 135.31 j/g and 86.521 j/g for 100 % crystalline PCL and TC, respectively [157, 158].

$$X_c = \frac{\Delta H_m}{\Delta H_m^0} \times 100 \quad (3.7)$$

Molecular weight calculations and structural analysis of PCL-based nanohybrids were conducted with end-group analysis by proton nuclear magnetic resonance ($^1\text{H-NMR}$, Bruker Ultrashield 300 MHz). $^1\text{H-NMR}$ samples were prepared in d-chloroform. Wettability of the micro- and nanospheres as well as PCL-based nanohybrid systems and neat PCL was determined by water contact angle (WCA) measurements with Attension (KSV) apparatus. Particle geometry was determined by optical microscopy imaging and the images were analyzed by CellProfiler software to measure particle size. Surface structure of the micro- and nanospheres were observed by Scanning Electron Microscopy (SEM, TESCAN VEGA 3, at 15-20 kV). Coating of the samples were conducted with Au/Pd (SC7620 sputter coater, Quorum Technologies Ltd, UK). Determination of drug content of micro- and nanospheres was achieved by UV spectrophotometric analysis (UV mini 1240 SHIMADZU). X-ray diffraction analysis (PANalytical XPER'T PRO XRD apparatus with $\text{CuK}\alpha$ radiation source, $\lambda=1.5406 \text{ \AA}$) was performed between 15 and 30 $^{\circ}2\theta$ values for structural analysis of PCL-based nanohybrid systems.

3.2.9 In vitro cytotoxicity studies

3.2.9.1 Cell culture and stock solution

L929 cell line, with the passage number of 18, was kept in a 25-cm² flask, in Eagle's Minimum Essential Medium (EMEM) containing 10 % fetal bovine serum (FBS; Pan Biotech P30-1301) and checked twice a day until 70 % or more confluency was reached (Doubling Time: 28-32 hours; L929 Cell line originated from ATCC CCL-1). Stock solution of 20 mg/mL concentration was prepared in DMSO. The samples were sonicated (10 minutes at 100% power - 20 minutes at 50% power) in the medium. Then, the samples were incubated on rollers at 25 rpm for 24 hours at room temperature. After 24 hours, the samples were centrifuged at 2500 rpm for 10 minutes and the supernatant was separated for cytotoxicity testing.

3.2.9.2 Inoculation of cells into 96-well plates

500 µL of Trypsin-EDTA (Gibco, 15400054) was added to cells that reached 70 % or more confluency in a 25-cm² flask and incubated at 37 °C for 3-5 minutes in 5 % CO₂. As soon as the cells are observed to dissociate under an inverted microscope (Zeiss Primovert, Germany), the medium containing 10 % FBS is added. The sample was centrifuged at 300 xg for 5 minutes to discard the medium. Then, 1 mL of EMEM medium was added. Counting was performed on the Logos Luna II instrument using Trypan blue (Gibco, 1525061). A medium with 10% FBS is added at 10⁴ cells per well.

3.2.9.3 In vitro cytotoxicity test

When 70% confluence is achieved in each well, the medium in the culture dish is withdrawn. EMEM Medium containing 10% FBS containing active ingredient at 5 different concentrations (100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL) was used.

All tests groups with 5 different concentrations were conducted in triplicate in three replicate wells. After the sample is applied, the cells are kept in incubation for 22 hours. At the end of 22 hours, 10% of the well volume (20 µL) WST-1 solution is added. The cell culture dish is wrapped with aluminum foil and left in the incubator for another 2 hours. After 2 hours, measurement are taken at 450 nm and 630 nm

wavelengths in the cell culture dish. The results are formulated and the % viability is determined by using Equation 3.8.

$$\% \text{ Cell Viability} = \frac{(\text{Sample well} - \text{Blank})}{(\text{Positive control} - \text{Blank})} * 100 \quad (3.8)$$

3.2.10 Statistical analysis

All experiments were conducted as triplicate sets unless it was mentioned otherwise. The results were expressed as mean±standard deviation. The data were statistically analyzed and the differences among experimental groups were investigated by one-way ANOVA with Tukey HSD as a post-hoc test. Related calculations were executed in GraphPad Prism 8 software. OriginLab® OriginPro 8.5 data analysis and graphing software was used for data visualization.

4. RESULTS AND DISCUSSION

4.1 Synthesis and Characterization of Nanohybrid Systems

PCL-based nanohybrid system were synthesized by e-ROP of ϵ -caprolactone utilizing immobilized CALB mentioned in Section 3.2.3. The reaction conditions of the polymerization reaction was predetermined by earlier optimization studies (Table 4.1).

Table 4.1 : Predetermined reaction condition of e-ROP of ϵ -caprolactone.

Hybrid Sample	Catalytic system	Reaction Time (h)	Reaction Temperature (°C)
PCL-AP-RHA	Immobilized CALB onto AP-RHA	48	60
PCL-A-RHA	Immobilized CALB onto A-RHA	48	40
PCL-G-RHA	Immobilized CALB onto G-RHA	48	40
PCL-T-RHA	Immobilized CALB onto T-RHA	72	60

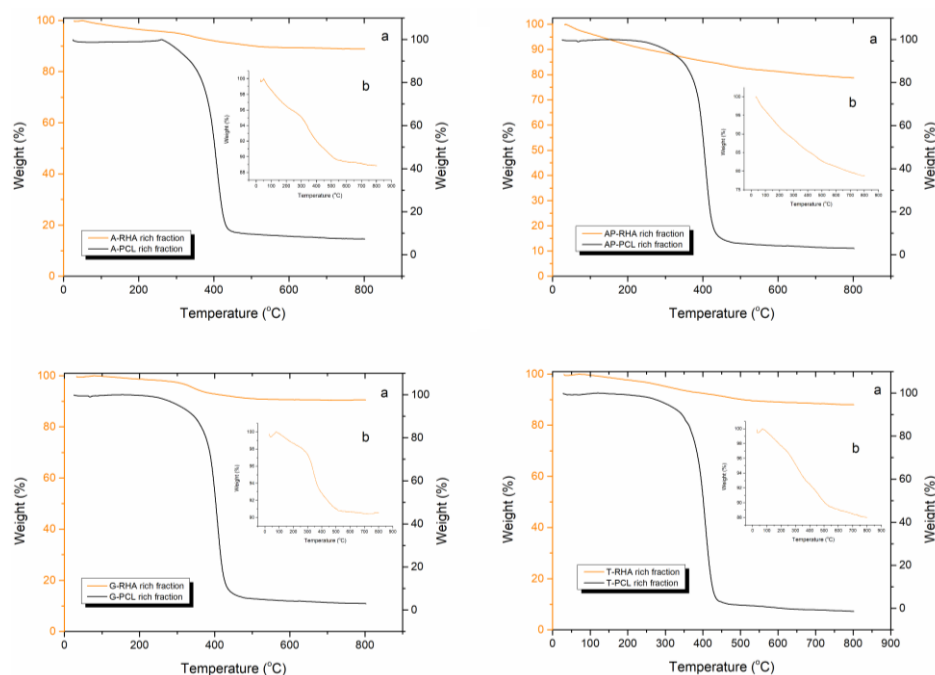
PCL grafting upon activated RHA was calculated through TGA of RHA-rich fraction of which preparation was described in Section 3.2.3. PCL grafting efficiencies of PCL-based nanohybrids can be calculated from the difference between weight losses of RHA-rich fraction and activated RHA. The calculated grafting percentages were given in Table 4.2. PCL grafting efficiency of PCL-T-RHA was calculated as 0 % which means grafting was unsuccessful. In this case the product obtained at the end of the reaction cannot be defined as a hybrid system; it is rather PCL with an inorganic filler material (silica of RHA). Since both activation of RHA with silanization agents and PCL grafting propagates through Si-OH groups on the surface of RHA, high silanization percentage caused lowered PCL grafting which was more emphasized in PCL-T-RHA.

Table 4.2 : PCL grafting percentages in nanohybrid systems.

Hybrid Sample	Grafting Percentage (%) ^a	Silanization Percentage (%) ^a
PCL-AP-RHA	14	1.2
PCL-A-RHA	8.5	0.8
PCL-G-RHA	4	3.7
PCL-T-RHA	0	10.1

^a Calculated by TGA

Furthermore, RHA-rich fractions were distinguished from PCL rich fraction in terms of their thermal degradation behaviors (Figure 4.1). The degradation of RHA-rich fraction consisted of two consecutive weight loss zones ascribed to silanization agent and grafted-PCL constituents. However, such consecutive zoned structure cannot be observed in TGA thermogram of T-RHA rich fraction which may be another indication of unsuccessful PCL grafting. Since the product PCL-T-RHA with no grafted PCL does not satisfy the definition of nanohybrid system, it was omitted from further characterization, and drug loading and release studies. Meanwhile, the degradation of PCL rich fraction was typical with a one-step degradation within 400–450 °C [159]. The ambiguity in the initial part of TGA curves of RHA-rich fractions were neither due to PCL nor activated RHA, but rather representative of an artefact since the mass loss which occurred in the range 32–50 °C was completely recovered with continued heating up to 79 °C, approximately.

**Figure 4.1 : TGA thermograms of (a) PCL- and RHA-rich fractions (b) Inset: Enlarged thermograms of RHA-rich fractions.**

The chemical structure of PCL-based nanohybrid samples and their molecular weights and monomer conversions were exposed by ^1H -NMR analysis. Characteristic signals of PCL chain (peaks at 4.07 ppm (t, CH_2O , main chain), 3.65 ppm (t, CH_2OH , chain end), 2.32 ppm (t, CH_2CO), 1.6-1.7 ppm (m, CH_2) and 1.37 ppm (m, CH_2)) were observed in spectra of all nanohybrid samples (Spectra were shown in Figure 4.2-4.4).

The calculated monomer conversion and molecular weight values were given in Table 4.3. Despite the high activity of the catalytic systems [39], small PCL chains were synthesized. Previous investigations by Öztürk-Düşkünkörür et al. have suggested an inverse relationship between PCL chain length and the number of surface Si-OH groups. Similar output obtained in this study, supported the proposed action of silanol groups as co-initiators for polymerization reaction resulting with high number of growing chains [66].

Table 4.3 : M_n and monomer conversions of hybrid samples.

Hybrid Sample	M_n – nanohybrid (g/mol) ^a	Monomer Conversion (%) ^a	M_n – neat PCL (g/mol)
PCL-AP-RHA	8700	99.4	14000
PCL-A-RHA	4800	99.4	12000
PCL-G-RHA	5500	99.7	10300

^a Calculated by ^1H -NMR.

^b Calculated by GPC.

Chemical structures of the nanohybrid systems were investigated by FT-IR analysis and the related spectra were presented in Figure 4.5. All three nanohybrid systems had the characteristic peaks of the main components which are PCL and RHA. The peaks are assigned as listed: at 800 cm^{-1} , 1050 cm^{-1} and 1259 cm^{-1} which are caused by Si- CH_3 rocking, asymmetric stretching of Si-O-Si and symmetric bending in Si- CH_3 , respectively [160] (RHA component); at 1293 cm^{-1} , 1238 cm^{-1} and 1167 cm^{-1} observed in the spectra belonging to C-O and C-C bonds seen in crystalline phase, asymmetric stretch of C-O-C and symmetric stretch of C-O-C, at $\sim 1720\text{ cm}^{-1}$ belonging to carbonyl bond (aliphatic), the peak of asymmetric CH_2 bonds at 2945 cm^{-1} , symmetric CH_2 bonds at 2866 cm^{-1} , peak at 3500 cm^{-1} of stretching vibrations of O-H end group (PCL component).

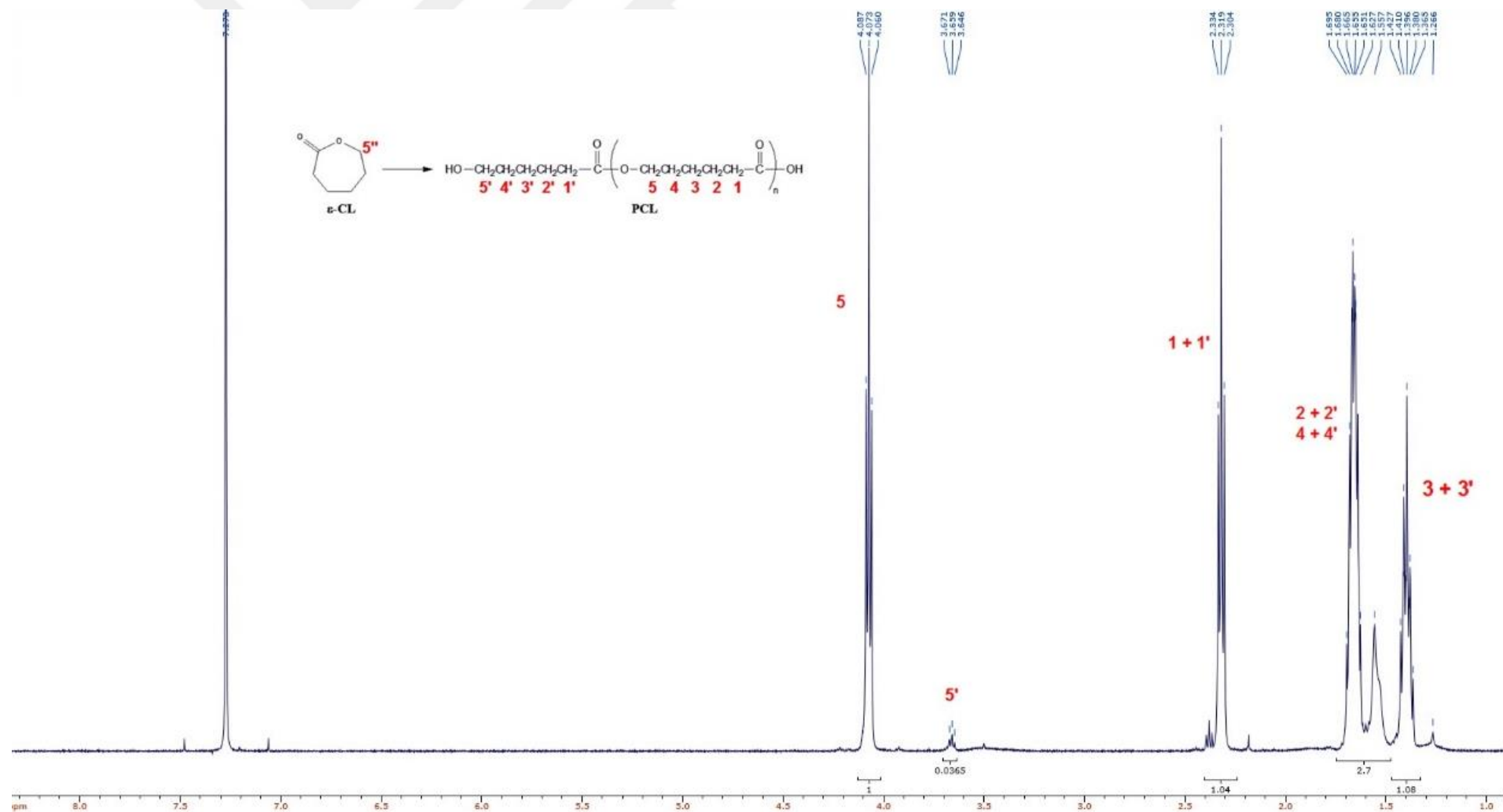


Figure 4.2 : ^1H -NMR spectrum of PCL-G-RHA.

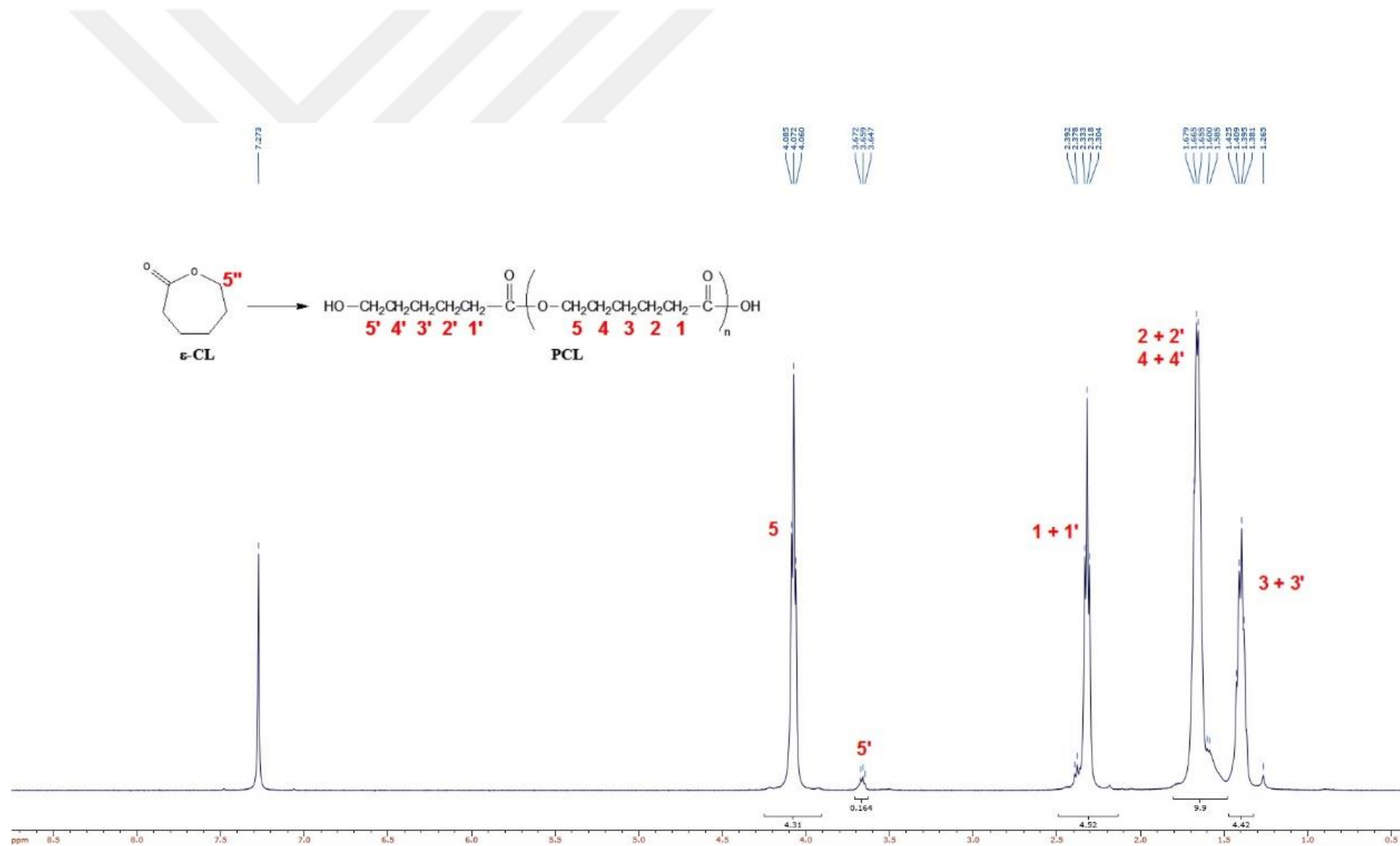


Figure 4.3 : ^1H -NMR spectrum of PCL-A-RHA.

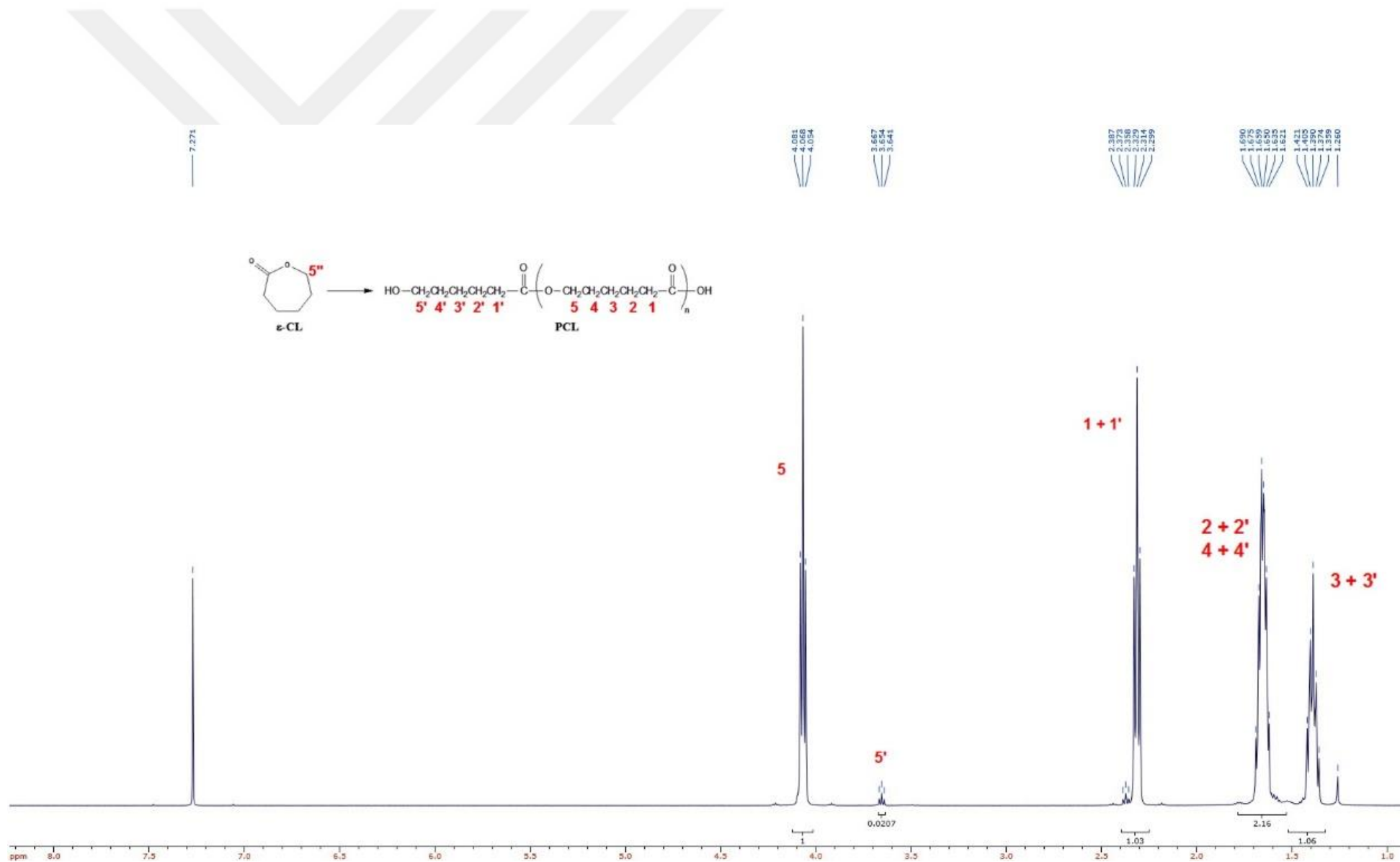


Figure 4.4 : $^1\text{H-NMR}$ spectrum of PCL-AP-RHA.

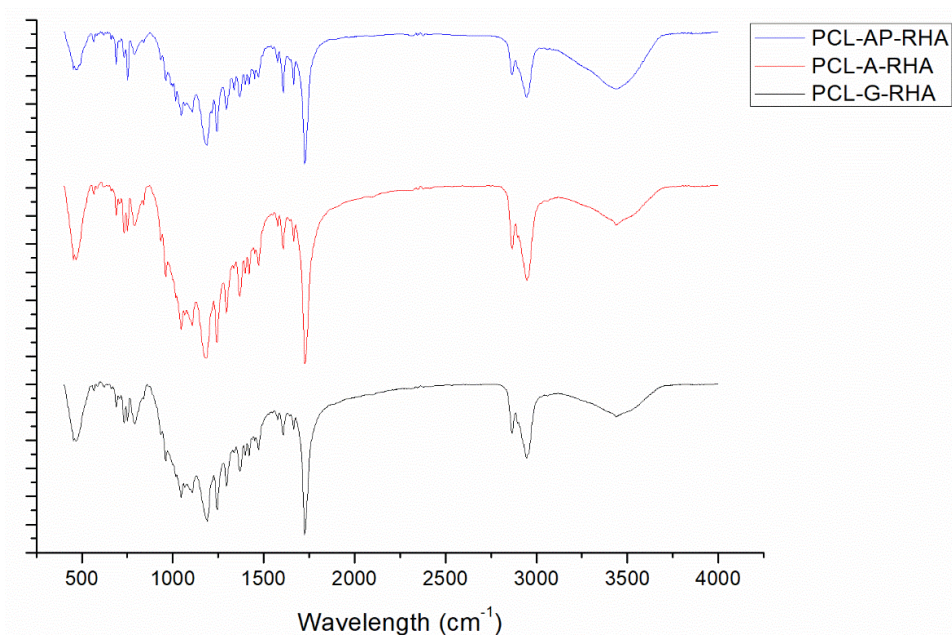


Figure 4.5 : FT-IR spectra of nanohybrid systems.

DSC results of PCL-based nanohybrid systems are provided in Table 4.4. Based on the DSC scan, T_m values were determined as approximately 55 °C. As compared to pure PCL properties, a significant decrease of crystallinity percentage was observed for all of the nanohybrid samples. This could be related to the low molecular weight of PCL in the nanohybrid system, which is known to affect the physical, thermal, mechanical properties of PCL [161, 162]. The decreased crystallinity percentage was also the result of silica incorporation in the nanohybrids hindering the crystal formation of PCL [162]. Second heating scans of the nanohybrid systems revealed a high glass transition temperature, T_g (Figure 4.6 - Inset). This finding was a consequence of RHA presence since short chains of PCL grafted on the surface of RHA restricted chain mobility and thus created a high thermal transition temperature with respect to its aliphatic counterpart (neat PCL) [163, 164]. This high T_g did, in return, increased the stability of the nanohybrids against recrystallization at mild temperatures below the high T_g [165].

Table 4.4 : DSC results.

Hybrid Sample	T_m (°C)	T_c (°C)	T_g (°C)	Crystallinity (%)
PCL-AP-RHA	55	34	85	39
AP-PCL rich	53	n.d.	n.d.	60
PCL-A-RHA	53	34	93	32
A-PCL rich	55	29	n.d.	61
PCL-G-RHA	54	37	93	23
G-PCL rich	60	36	n.d.	65

Figure 4.7 shows the XRD pattern of the PCL-based nanohybrid systems. The characteristic peaks of PCL crystal structures were observed positioned at 21.3° , 22.0° and 23.7° corresponding to (110), (111) and (200) reflection planes of the orthorhombic crystal structure of PCL, respectively [162, 166]. Moreover, broadened peak pattern between $20\text{--}30^\circ$, especially observed in PCL-G-RHA and PCL-A-RHA patterns, indicates the amorphous structure of silica [167]. The sharp reflection observed in the pattern of neat PCL indicated the high crystallinity degree. The reflection peaks of PCL-A-RHA and PCL-G-RHA was broader and their intensity was lowered. Additionally, the peak belonging to (111) lattice plane merged to its neighboring peak of (110) lattice plane in the patterns of the aforementioned nanohybrids. This broadening and merging of the peaks is the indication of decreased crystallinity, which is also consistent with the findings of DSC analysis. On the other hand, PCL-AP-RHA nanohybrid had a sharp reflection pattern similar to neat PCL which also supports the results obtained from DSC thermograms.

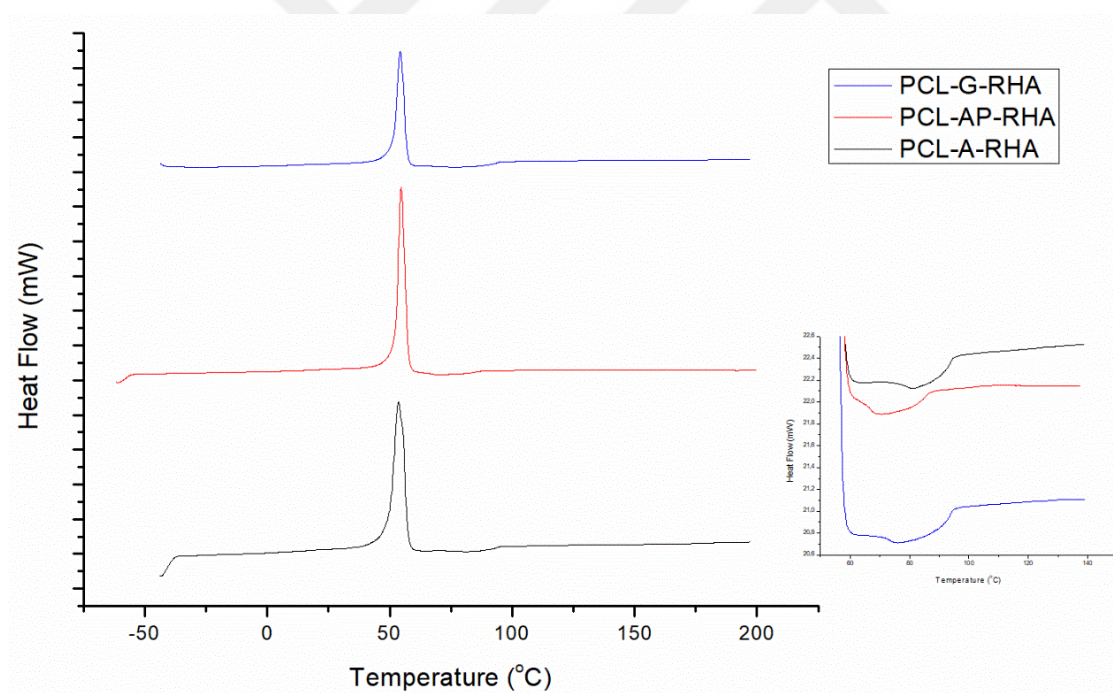


Figure 4.6 : DSC thermograms of the nanohybrid systems, demonstrating melting endotherm (second heating cycle). Inset: close-up view between $50\text{--}140^\circ\text{C}$.

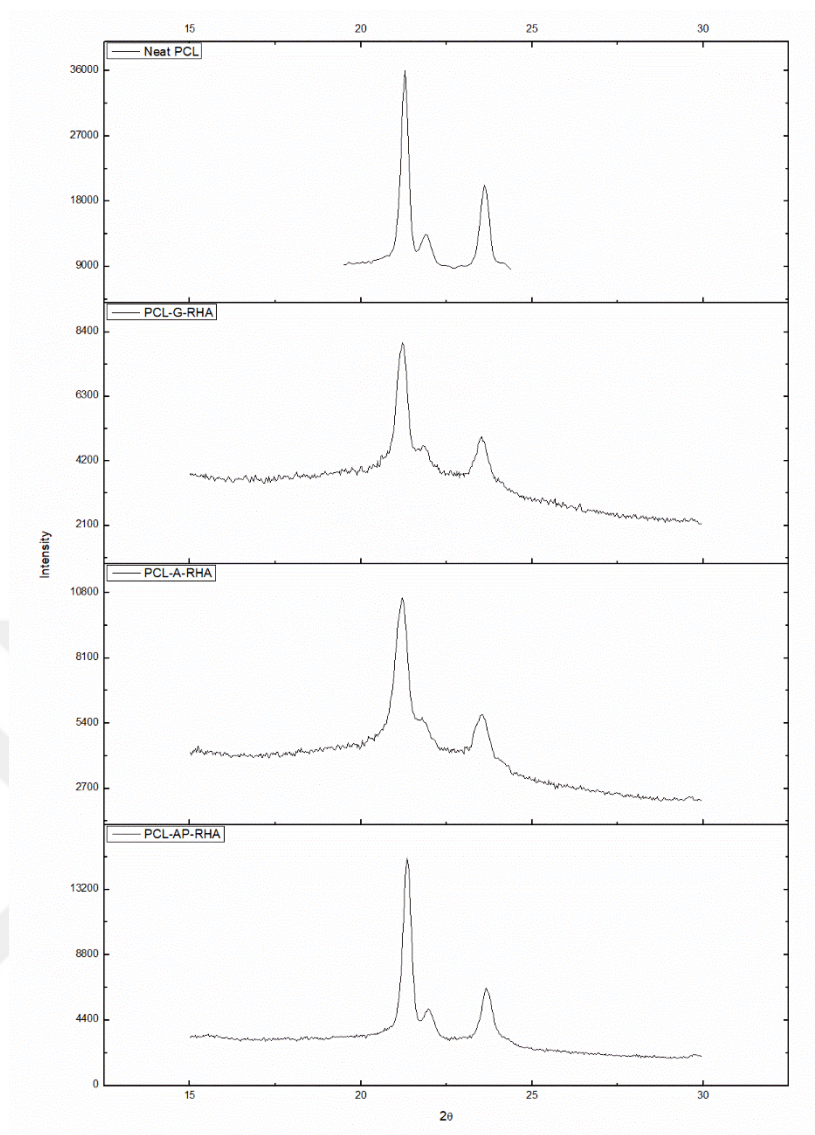


Figure 4.7 : XRD patterns of PCL-based nanohybrid systems.

Hydrophilic character of the nanohybrid systems were investigated by water contact angle measurements (Figures 4.8-4.10). PCL is known to be highly hydrophobic which was also proved by WCA. Addition of RHA in the hybrid system changed the hydrophobic character of PCL owing to the hydroxyl groups on the surface of RHA. Thus, it becomes clear why PCL-AP-RHA was more hydrophobic since PCL grafting through hydroxyl groups was higher in PCL-AP-RHA hybrid system (18 %). The hydrophilic ethylene oxide end-group of 3-GPTMS in the structure of PCL-G-RHA also contributed to the hydrophilicity of the nanohybrid.

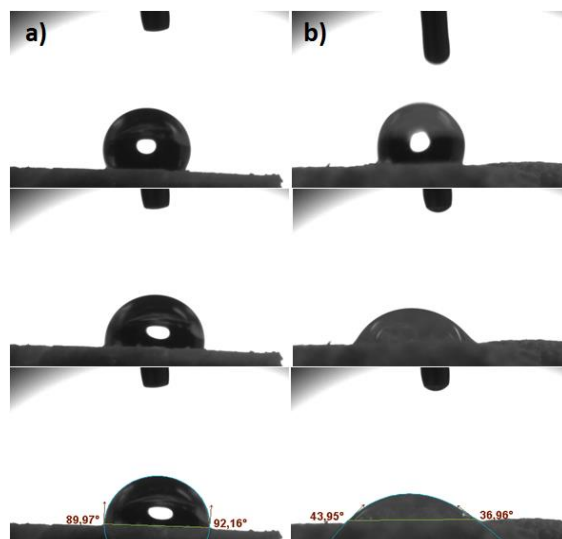


Figure 4.8 : Water contact angle a) APPCL, b) PCL-AP-RHA.

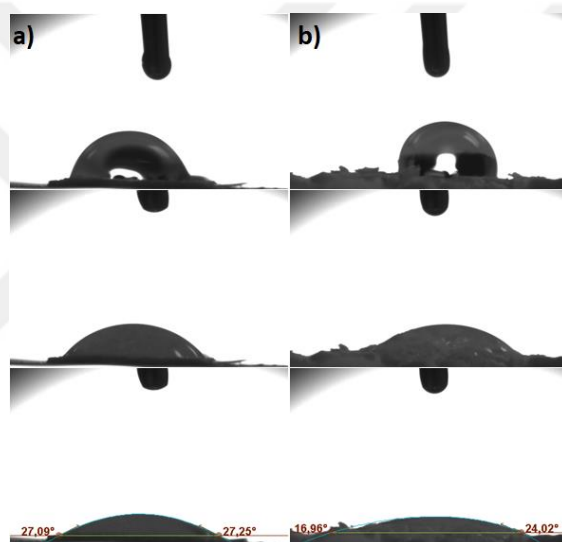


Figure 4.9 : Water contact angle a) APCL, b) PCL-A-RHA.

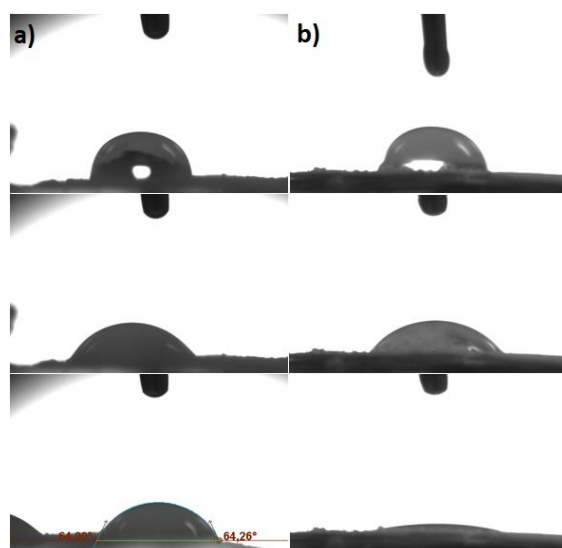


Figure 4.10 : Water contact angle a) GPCL, b) PCL-G-RHA.

SEM images of PCL-based nanohybrid systems revealed a porous and grainy surface structure (Figure 4.11). The silica particles of RHA was seen in the images as light spots contrasting the dark PCL matrix [168]. Porous surface morphology may contribute to controlled drug release mechanism by entrapping the loaded therapeutic agent within the carrier matrix. The grainy surface of the nanohybrids implied that short polymer chains growing from the silanized RHA coated the surface.

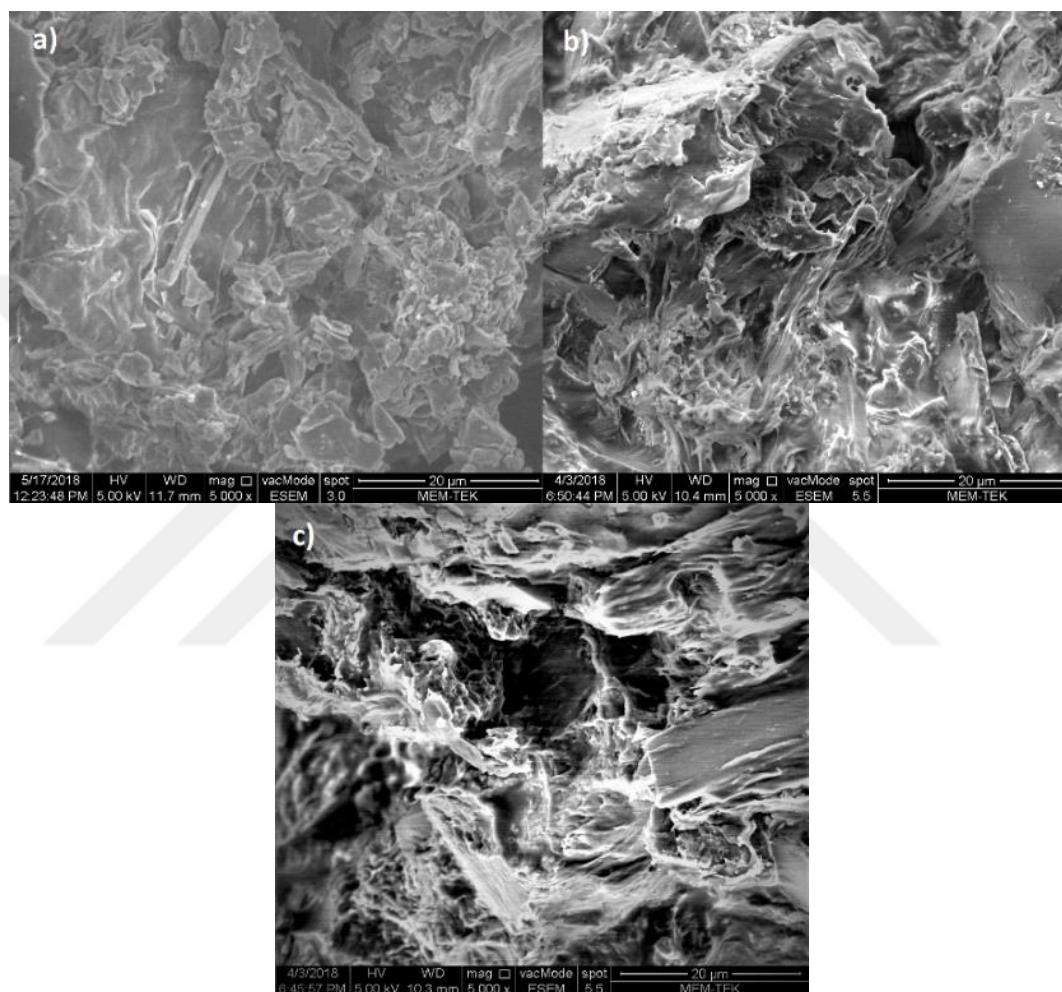


Figure 4.11 : SEM images of PCL-based nanohybrids a) PCL-AP-RHA; b) PCL-A-RHA and c) PCL-G-RHA (Magnification 5000x).

4.2 TC Synthesis and Characterization

TC was chemically synthesized by Claisen-Schmidt condensation reaction, with acetophenone and benzaldehyde as monomers. The yield of this reaction was 93.7 %. The synthesized TC and commercial TC were characterized by FT-IR, TGA and DSC to observe possible differences in terms of structural and thermal properties.

Figure 4.12 shows the FT-IR spectra of the two TC. The characteristic peaks of TC molecule were observed in both of the spectra which proved that the end product of the Claisen-Schmidt condensation was indeed TC and indicated the purity of the product. The characteristic peaks were detected at 675-900 cm^{-1} (oop C-H bending, aromatic), 1011 cm^{-1} (in-plane C-H bending), 1400-1500 cm^{-1} (C-C stretching, aromatic), 1605 cm^{-1} (C=C), 1663 cm^{-1} (α , β unsaturation in ketones), and multiple peaks at 3030, 3052 and 3082 cm^{-1} (C-H, aromatic). No difference was observed in the characteristic peaks of chemically synthesized TC and commercial TC.

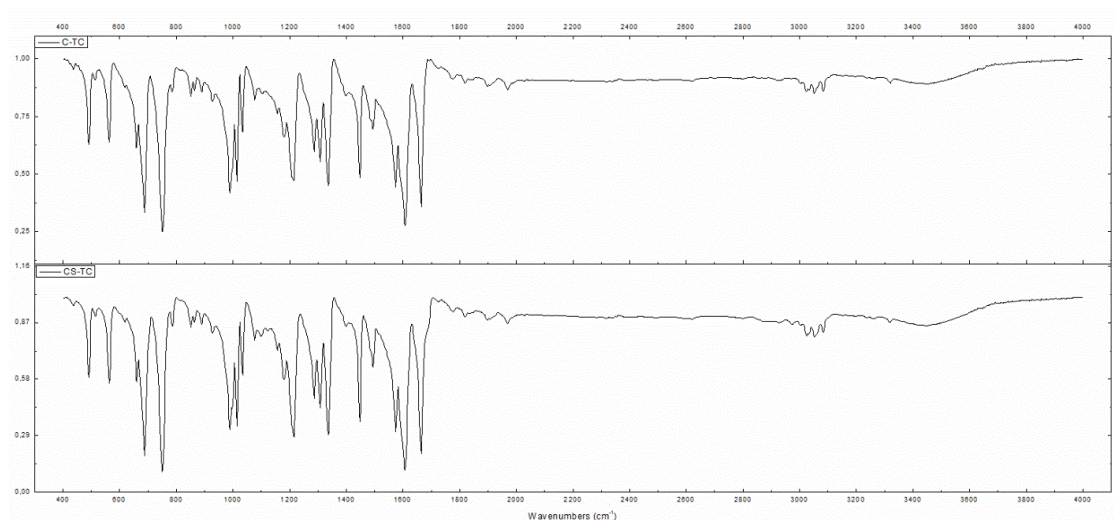


Figure 4.12 : FT-IR spectra of commercial TC (C-TC) and chemically synthesized TC (CS-TC).

Proton NMR spectra of the commercial TC and chemically synthesized TC were presented in Figure 4.13. ^1H -NMR analysis was conducted to further observe structural properties of the two chalcones which were characterized by FT-IR analysis earlier and did not show structural differences. However, by comparing ^1H -NMR spectra of the two chalcones, an important structural difference was detected. As can be seen in Figure 4.10a, commercially available chalcone had all the characteristic peaks of trans configuration. The ^1H -NMR spectrum of chemically synthesized chalcone had additional signals to the signals of trans configuration at 7.960, 7.945 ppm, 7.386, 7.371, 7.356 ppm and 7.309, 7.925 ppm. These additional peaks belong to cis-configuration of chalcone molecules [169]. The low intensity of the mentioned peaks showed that the chemically synthesized chalcone consisted of mainly trans configuration with a low amount of cis-configuration.

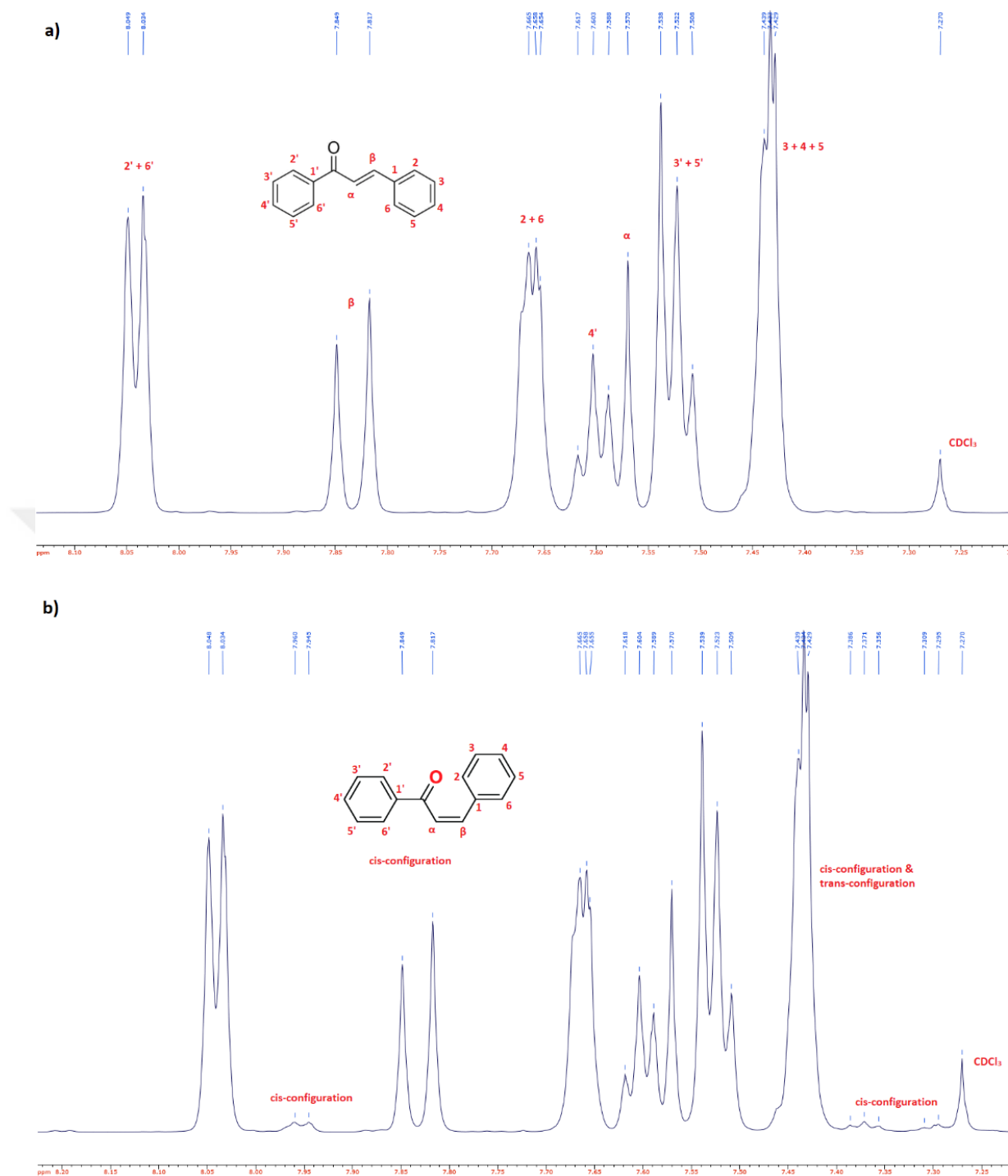


Figure 4.13 : ¹H-NMR spectra of a) commercial chalcone, b) chemically synthesized chalcone.

Thermal characterization of commercial and chemically synthesized TC with TGA was given in Figure 4.14. Thermal decomposition behavior of the two TC molecules were similar. Commercial and chemically synthesized TC had decomposition peak at 227 °C and 238 °C, respectively. The purity of the chemically synthesized TC was demonstrated with TGA since no additional decomposition peak was observed in the thermogram.

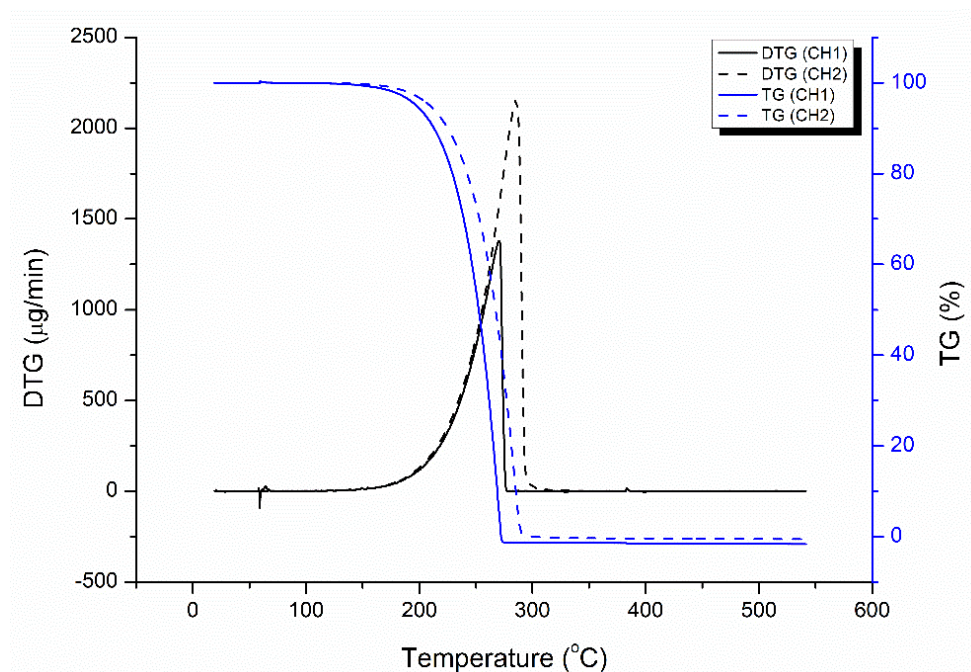


Figure 4.14 : TG and DTG curves of commercial TC (CH1) and chemically synthesized TC (CH2).

Thermal characteristics such as melting temperature (T_m), crystallization temperature (T_c) and crystallinity percentage (X_c) of the commercial and chemically synthesized TC were determined by DSC analysis. The melting temperatures of the two TC molecules did not differ from each other significantly (Figure 4.15 and Figure 4.17). However, commercial TC had a crystallization temperature of 5 °C (Figure 4.16) whereas chemically synthesized TC showed cold crystallization endotherm at -4.7 °C (Figure 4.17), which is an indication of its semi-crystalline structure. Indeed, the crystallinity percentage of the chemically synthesized TC was calculated as 77 %, whereas the crystallinity percentage of the commercial TC was 100 % (calculated by using Equation 3.3). This difference in crystalline structures of the two TC might affected the encapsulation efficiency of the commercial and chemically synthesized TC and release behavior of these molecules from the polymeric microspheres and nanospheres which will be discussed later in this section.

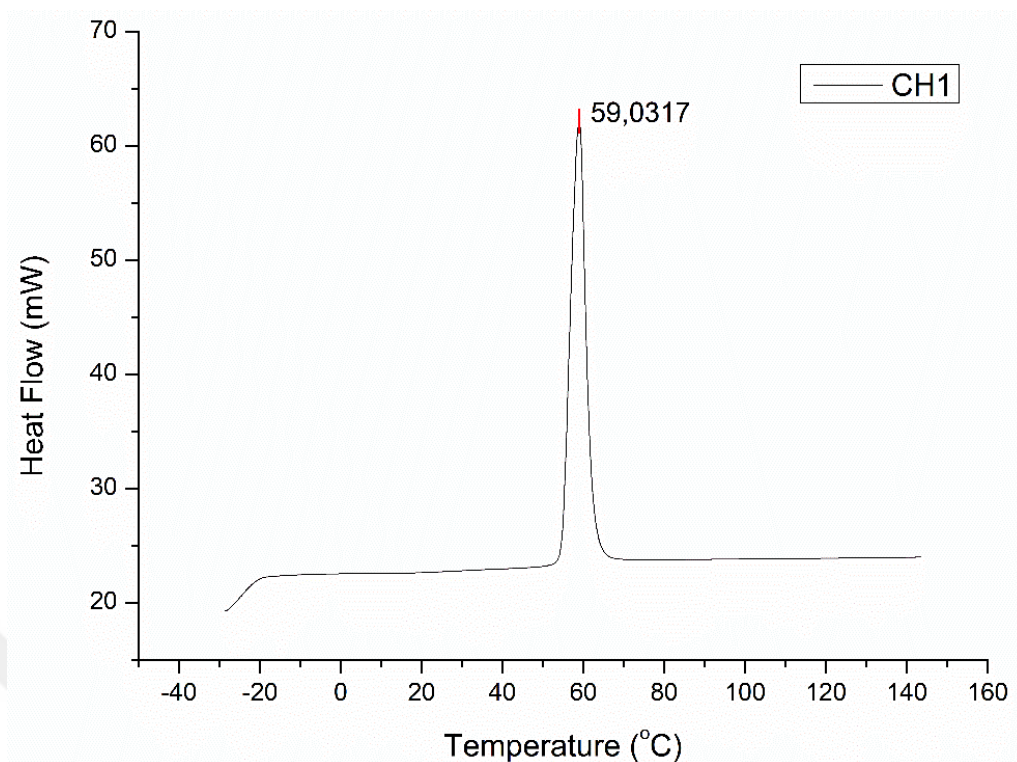


Figure 4.15 : DSC thermogram of commercial TC, demonstrating melting endotherm (second heating cycle).

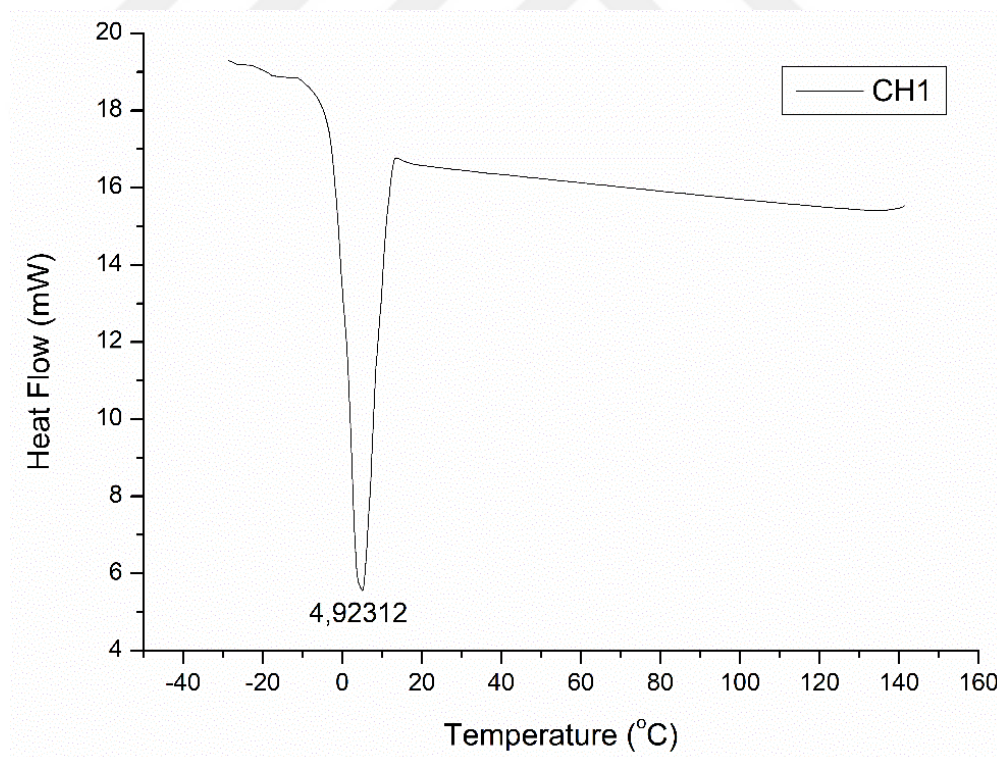


Figure 4.16 : DSC thermogram of commercial TC, demonstrating crystallization endotherm (cooling cycle).

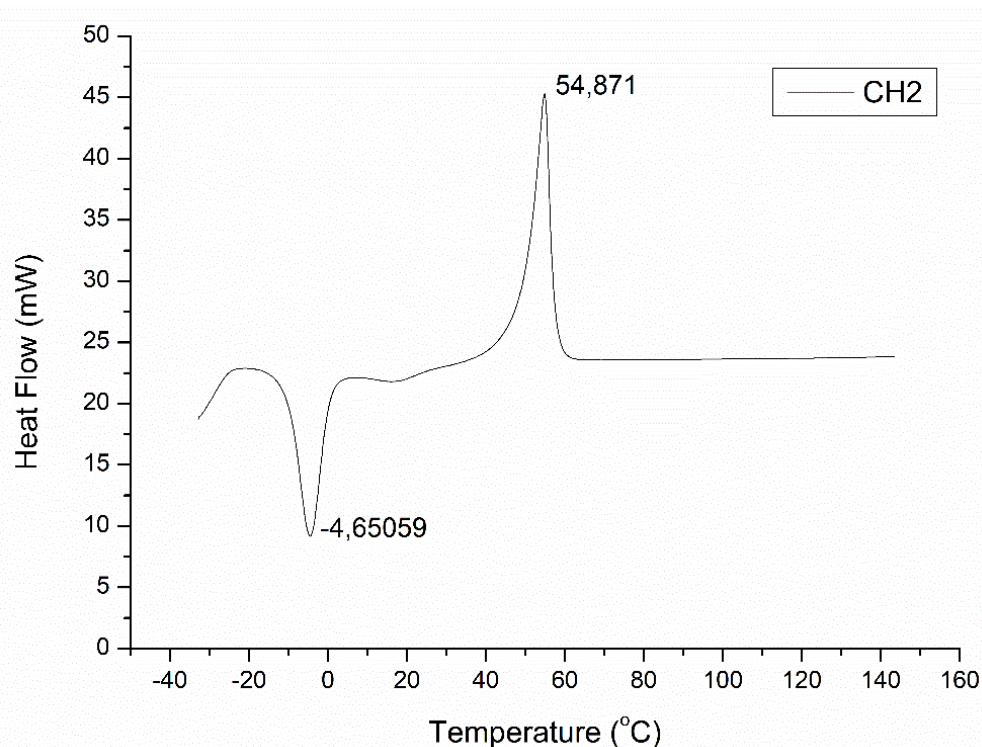


Figure 4.17 : DSC thermogram of chemically synthesized TC, demonstrating cold crystallization endotherm and melting endotherm (second heating cycle).

4.3 PCL-based Nanohybrid Microspheres

4.3.1 Effect of process parameters on TC loading to microspheres of PCL-based nanohybrid systems

PCL-based nanohybrid systems were used to prepare TC-loaded microsphere structures by emulsion/solvent evaporation method. Various microsphere formulations were prepared to investigate the effect of the process parameters.

4.3.1.1 Effect of external phase concentration

The external phase of the microsphere formulations was PVA with varying concentrations (0.1, 0.5 and 1 %). The conditions of microsphere formation and calculated encapsulation efficiencies, loading capacities and mean particle diameters of the microspheres were summarized in Table 4.5. Particle size is an important parameter affecting encapsulation efficiency. The mean diameters changed in the range of 15-30 μm . A decrease in the external phase concentration reduces the capability of emulsification of PVA thus resulting in larger droplet size [170]. This phenomenon can be clearly seen with PCL-AP-RHA microspheres. When the concentration in the external phase was 0.1 % PVA (AP1), the mean particle diameter

of the microspheres were measured $21 \pm 7 \mu\text{m}$. Increasing the concentration to 0.5 % and 1 % reduced the mean particle diameter to $15 \pm 3 \mu\text{m}$ and $15 \pm 2 \mu\text{m}$, respectively. Changing the silanization agent also had effect on mean particle diameter. When PVA concentration was low, the difference between silanization agents was not emphasized. However, in the case of 0.5 % and 1 % PVA in the external phase, PCL-AP-RHA formed smaller microspheres (AP2: $15 \pm 3 \mu\text{m}$, AP3: $15 \pm 2 \mu\text{m}$). It seems that nanohybrid system incorporated with 3-APTES had contributed to emulsifying capability of PVA whether due to its higher molecular weight or higher crystallinity.

Table 4.5 : Microsphere formulations, encapsulation efficiencies, loading capacities and mean particle diameters.

Sample Code	Formulation	Encapsulation Efficiency (%) ^a	Loading Capacity (%) ^a	Mean Particle Diameter (μm) ^b
A1	0.1 % PVA	48 ± 1	4.4 ± 0.03	23 ± 4
	1:10 TC/polymer			
G1	0.1 % PVA	74 ± 6	6.9 ± 0.8	20 ± 4
	1:10 TC/polymer			
AP1	0.1 % PVA	41 ± 3	3.8 ± 0.2	21 ± 7
	1:10 TC/polymer			
A2	0.5 % PVA	60 ± 5	5.4 ± 0.4	29 ± 6
	1:10 TC/polymer			
G2	0.5 % PVA	72 ± 10	6.6 ± 0.7	21 ± 3
	1:10 TC/polymer			
AP2	0.5 % PVA	53 ± 4	4.8 ± 0.3	15 ± 3
	1:10 TC/polymer			
A3	1 % PVA	56 ± 12	5.1 ± 0.9	14 ± 4
	1:10 TC/polymer			
G3	1 % PVA	66 ± 6	6.0 ± 0.5	28 ± 5
	1:10 TC/polymer			
AP3	1 % PVA	63 ± 7	5.7 ± 0.5	15 ± 2
	1:10 TC/polymer			

TC amount in the internal phase was fixed at 10 mg.

^a mean $\pm$ SD; n=3.

^b Determined by optical microscope images (mean $\pm$ SD; n=20).

Effect of PVA concentration in the external phase on encapsulation efficiency was investigated (Figure 4.18). According to the results obtained, encapsulation efficiency of PCL-G-RHA microspheres were significantly higher than the other two ($p < 0.001$, n=3). This difference might be the result of the different structural natures of the silanization agents used during the nanohybrid synthesis. 3-APTES and 3-APTMS have ammonium groups in their structure as end-group whereas 3-GPTMS does not and this may have caused the entrapped drug to diffuse out to the external phase during microsphere formation with PCL-A-RHA and PCL-AP-RHA nanohybrid systems

[171]. It was also observed that PCL-AP-RHA microspheres were more susceptible to the changes in PVA concentration. 1 % PVA concentration in the external phase caused higher encapsulation efficiency which can be attributed to the increasing viscosity which limits the diffusion of drug towards the external phase.

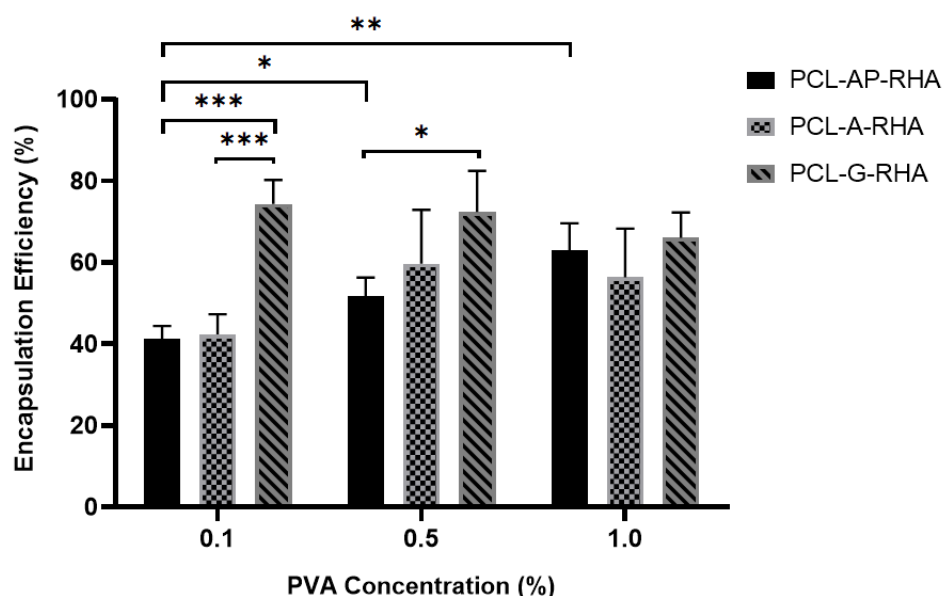


Figure 4.18 : Effect of PVA concentration in the external phase on encapsulation efficiency of the microspheres (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$).

4.3.1.2 Effect of internal phase concentration

To determine the optimum drug/polymer ratio, PVA concentration was used for each sample (PCL-A-RHA: 0.5 % PVA, PCL-AP-RHA: 1 % PVA; PCL-G-RHA: 0.1 % PVA). For PCL-A-RHA microspheres 60 % encapsulation efficiency was obtained with 0.5 % PVA in the external phase. For PCL-AP-RHA and PCL-G-RHA microspheres, highest encapsulation efficiency was obtained with formulations of 1 and 0.1 % PVA, respectively.

As presented in Table 4.6, decrease in polymer amount in the internal phase caused decrease in mean particle diameter (G1-G4-G5). This, in return, led to decline in encapsulation efficiency since surface drug loss from lower particles is higher than larger particles. When the polymer concentration in the internal phase decreased by 4-folds (G1 to G5), the particle mean diameter decreased from $20\pm4\ \mu\text{m}$ to $15\pm5\ \mu\text{m}$ ($p<0.01$). The increasing viscosity of the internal phase due to increasing polymer concentration caused the formation of larger particles [172]. Similar decrease was also observed for PCL-A-RHA microspheres. The mean particle diameter reduced to $19\pm3\ \mu\text{m}$ ($p<0.0001$) and $14\pm3\ \mu\text{m}$ ($p<0.0001$) by lowering the polymer amount in the

internal phase from 100 mg to 50 mg and 25 mg, respectively. However, PCL-AP-RHA microspheres showed an opposite effect, particles became larger as the polymer concentration in the internal phase decreased.

Table 4.6 : Microsphere formulations, encapsulation efficiencies, loading capacities and mean particle diameters.

Code	Formulation	Encapsulation Efficiency (%) ^a	Loading Capacity (%) ^a	Mean Particle Diameter (μm) ^b
G1	0.1 % PVA 1:10 drug/polymer ratio	74±6	6.9±0.8	20±4
A2	0.5 % PVA 1:10 drug/polymer ratio	60±5	5.4±0.4	29±6
AP3	1 % PVA 1:10 drug/polymer ratio	63±7	5.7±0.5	15±2
A4	0.5 % PVA 1:5 drug/polymer ratio	64±8	10.6±1.2	19±3
G4	0.1 % PVA 1:5 drug/polymer ratio	76±6	12.7±0.9	16±4
AP4	1 % PVA 1:5 drug/polymer ratio	64±4	10.7±0.6	21±4
A5	0.5 % PVA 1:2.5 drug/polymer ratio	62±9	17.5±2.2	14±3
G5	0.1 % PVA 1:2.5 drug/polymer ratio	67±6	19.1±1.4	15±5
AP5	1 % PVA 1:2.5 drug/polymer ratio	32±6	9.0±1.4	27±6

^a mean±SD; n=3.

^b Determined by optical microscope images (mean±SD; n=20).

The highest encapsulation efficiency was found at 1:10 (0.1) drug/polymer ratio for both PCL-A-RHA and PCL-G-RHA microspheres (Table 4.6). As can be seen in Figure 4.19, for PCL-AP-RHA microspheres drug/polymer ratio of 1:5 (0.2) yielded highest efficiency. PCL-A-RHA and PCL-G-RHA samples were not seem to be significantly affected by the change in the amount of polymer in the internal phase. Whereas increasing the amount of polymer in the internal phase (decreasing drug/polymer ratio) significantly increased encapsulation efficiency for PCL-AP-RHA samples ($p<0.01$). The possible reason of such effect is the lowered drug concentration gradient between the internal and the external phases which retards the diffusion from emulsion droplets towards the external phase [173]. Furthermore, high polymer amount provides a protective barrier and slows down the TC leaching from the internal phase towards the external phase during fast microsphere solidification [174, 175].

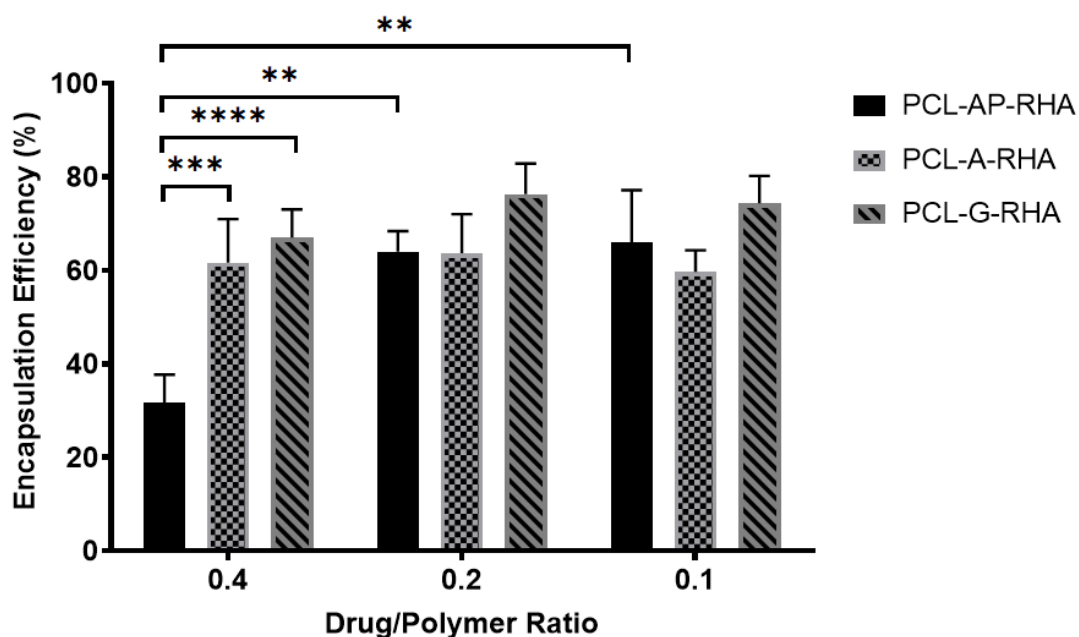


Figure 4.19 : Effect of polymer concentration in the internal phase on encapsulation efficiency of the microspheres. Drug content was fixed at 10 mg. (**: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$).

4.3.2 Effect of process parameters on TC release from microspheres of PCL-based nanohybrid systems

TC release studies in pH 7.4 PBS medium were conducted to investigate the effect of the external and internal phase concentrations.

4.3.2.1 Effect of external phase concentration

The effect of PVA concentration in the external phase on the release profiles of the microspheres were given in the following figures (Figure 4.20-4.25). According to the given graphics, high level of PVA in the aqueous phase (1 %) decreases both the released amount of drug, especially in PCL-A-RHA and PCL-AP-RHA microspheres. The possible reason of such effect can be the change in the mean diameter of the microsphere samples. Increasing particle mean diameter causes the released amount of drug to decline [99]. Additionally, PVA in the external phase as a stabilizer creates a protective surrounding coating around the microspheres. The PVA layer could not be fully removed from the surface [170]. Thus, increasing PVA amount in the external phase increases diffusional path causing reduced cumulative release.

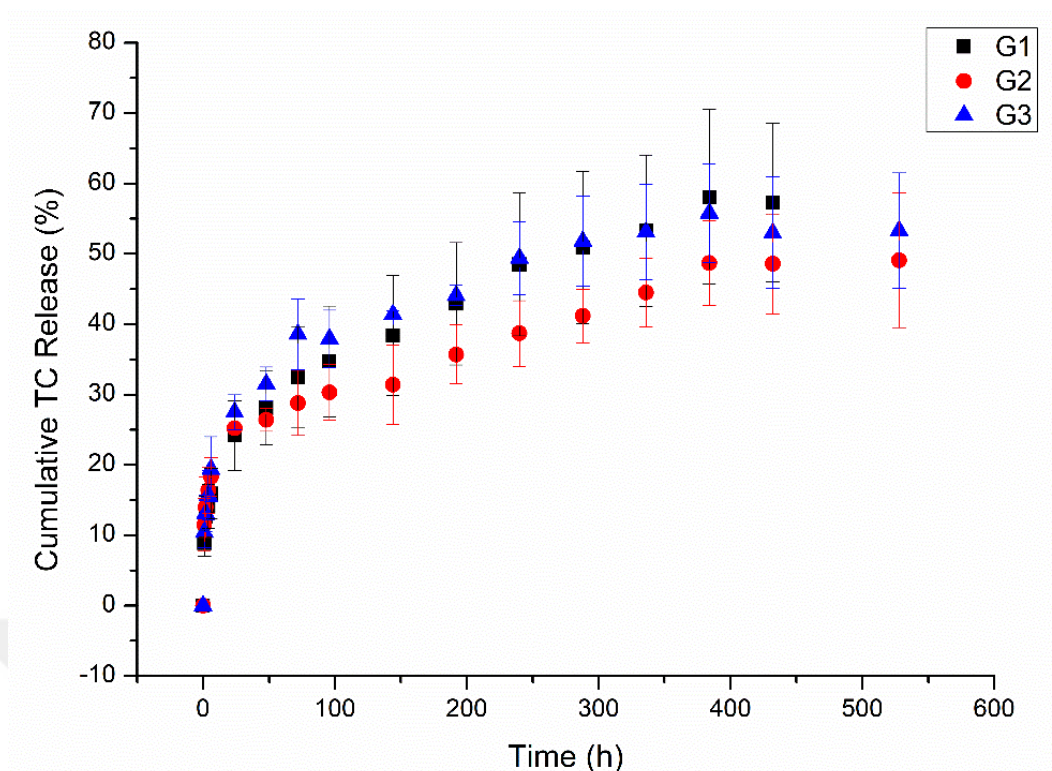


Figure 4.20 : Effect of PVA concentration on TC cumulative release (PCL-G-RHA, TC/polymer ratio=1:10; G1: 0.1 % PVA, G2: 0.5 % PVA, G3: 1 % PVA).

The release rates of the PCL-G-RHA microspheres revealed the differences in terms of the burst release behaviors (Figure 4.21) [151]. 1 % PVA in the external phase caused the initial release within the first hour to reduce due to the fact that PVA on the surface of the microspheres extends the diffusional path through the surface. Due to a 5-fold decrease in PVA concentration (G2-G1), the initial TC release rate reduced from approximately 12 % to 9 %. TC release rates of the PCL-G-RHA microspheres did not seem to be depended on the concentration of TC and release was achieved in a controlled manner with constant release rate after a short burst release in the first 4 h of the release (G1: 14 %, G2: 16 %, G3: 16 %). When compared to the total cumulative release from the microsphere formulations, it was observed that burst release of G1 formulation was 25 % of the total cumulative release at the end of 432 h which was 57 %. The burst release values of the other two formulations (G2 and G3), on the other hand, were 33 % and 29 % of their total cumulative release, respectively; which were considerably higher than G1 formulation. Release rates at the stable phase of the release did not significantly changed with varying PVA concentration.

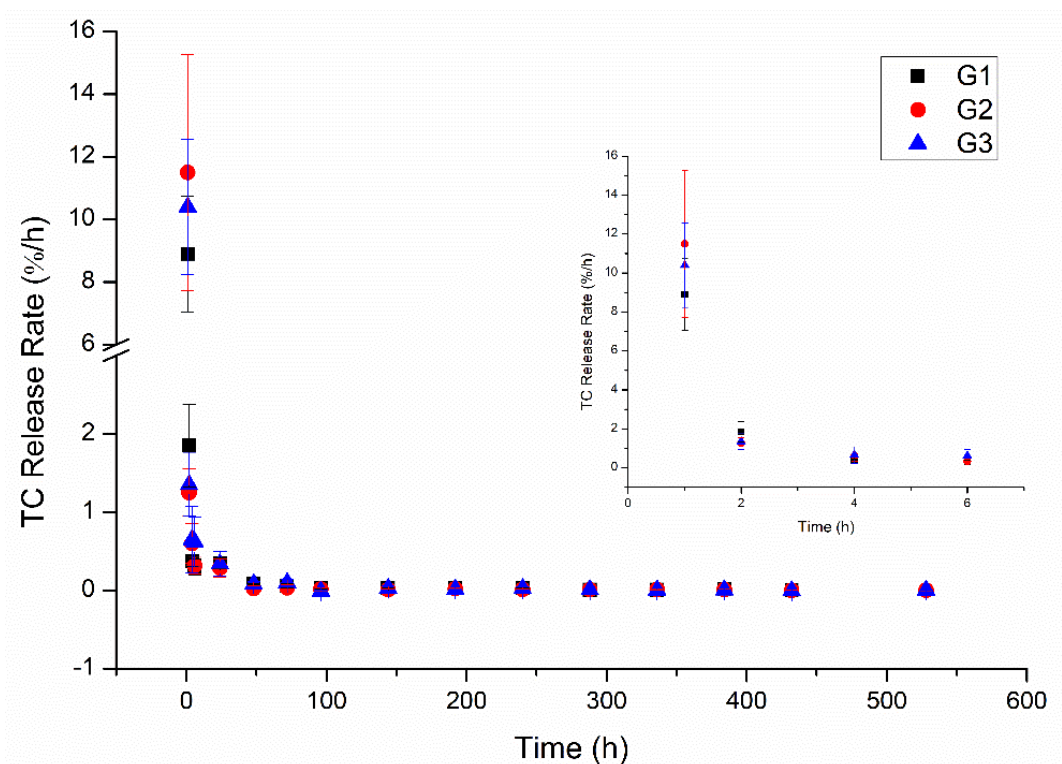


Figure 4.21 : Effect of PVA concentration on TC release rate (PCL-G-RHA, TC/polymer ratio=1:10; G1: 0.1 % PVA, G2: 0.5 % PVA, G3: 1 % PVA).

Figure 4.22 shows the effect of PVA concentration in the external phase on the release rates of the PCL-A-RHA microspheres. The increasing PVA concentration had a similar effect on the initial release as in PCL-G-RHA microspheres (Figure 4.23). The highest initial release rate was obtained when the PVA concentration was 1 % (16 %/h) and it was reduced to 13 %/h and 14 %/h as the PVA concentration decreased to 0.5 % and 0.1 %, respectively. A stable release phase was reached after the 2nd h of the release at which burst releases of the microspheres were 15 % and 16 % for A1 and A2 microsphere formulations whereas A3 formulation had a burst effect after the 4th h reaching 27 % cumulative release. However, burst release of A3 formulation with respect to the total cumulative release was significantly higher than that of A1 and A2 formulations. A3 formulation had a burst release of 39 % of the total cumulative release (which was 69,1 %) whereas A1 and A2 formulations had 16 % and 24 %, respectively. Similar results were also obtained for G microsphere formulations (G1, G2 and G3) burst release to total cumulative release ratio was increased as the PVA concentration in the aqueous phase was increased.

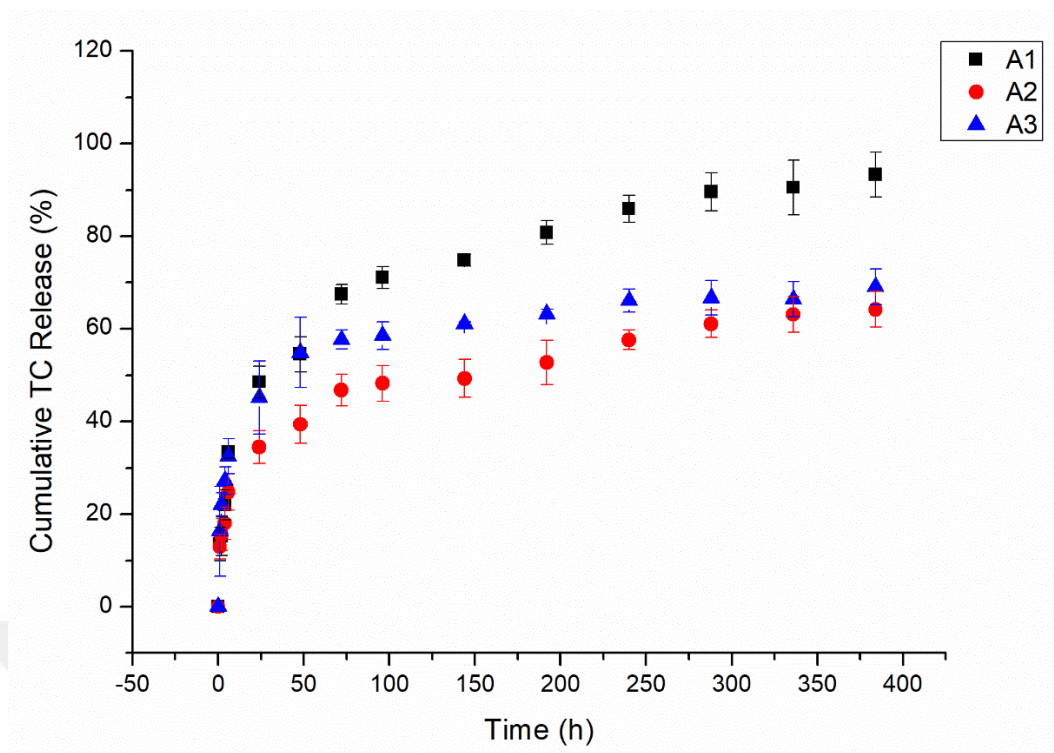


Figure 4.22 : Effect of PVA concentration on TC cumulative release (PCL-A-RHA, TC/polymer ratio=1:10; A1: 0.1 % PVA, A2: 0.5 % PVA, A3: 1 % PVA).

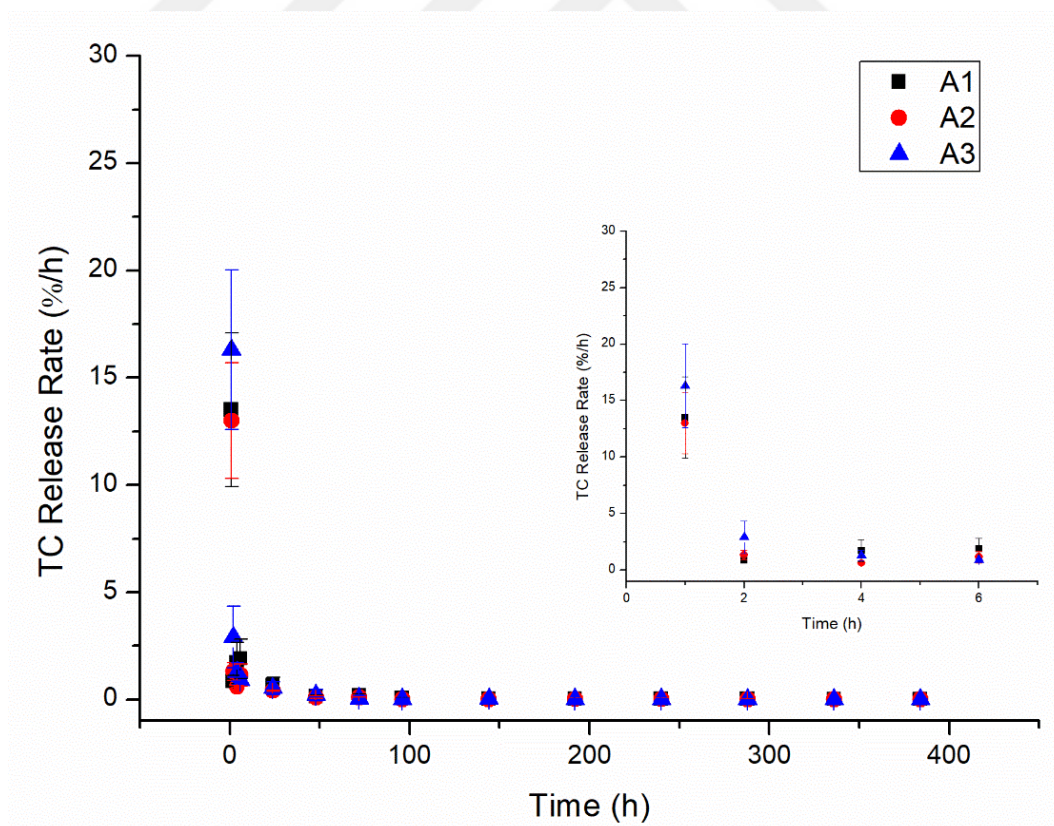


Figure 4.23 : Effect of PVA concentration on TC release rate (PCL-A-RHA, TC/polymer ratio=1:10; A1: 0.1 % PVA, A2: 0.5 % PVA, A3: 1 % PVA).

The release profiles and rates of the PCL-AP-RHA microspheres were presented in Figure 4.24 and Figure 4.25. The initial release rate of the AP3 microsphere (1 % PVA concentration) was higher than that of the other two formulations (AP3: 1 % PVA, 10 %/h; AP2: 0.5 % PVA, 7 %/h; AP1: 0.1 % PVA, 7 %/h). In spite of the retarding effect of PVA on the release, high PVA concentration caused a higher initial release due to the small particle diameter and also to the TC molecules adsorbed on the surface or near the surface of the microspheres which is known to be factor affecting burst release behavior [107, 176]. Moreover, initial release from the PCL-AP-RHA microspheres were lower than those of PCL-A-RHA and PCL-G-RHA microspheres. The reason of such difference can be the higher hydrophobicity of the PCL-AP-RHA nanohybrid which retards the aqueous release medium to diffuse through the microsphere surface [177]. This nature of the PCL-AP-RHA microspheres also affected burst release. The release rate of the microspheres reached a constant-rate phase after 2nd hour of the release (AP1: 9 % cumulative release; AP2: 10 % cumulative release; AP3: 11 % cumulative release). However, varying PVA concentration in the external phase did not affect the burst release behavior of the microspheres.

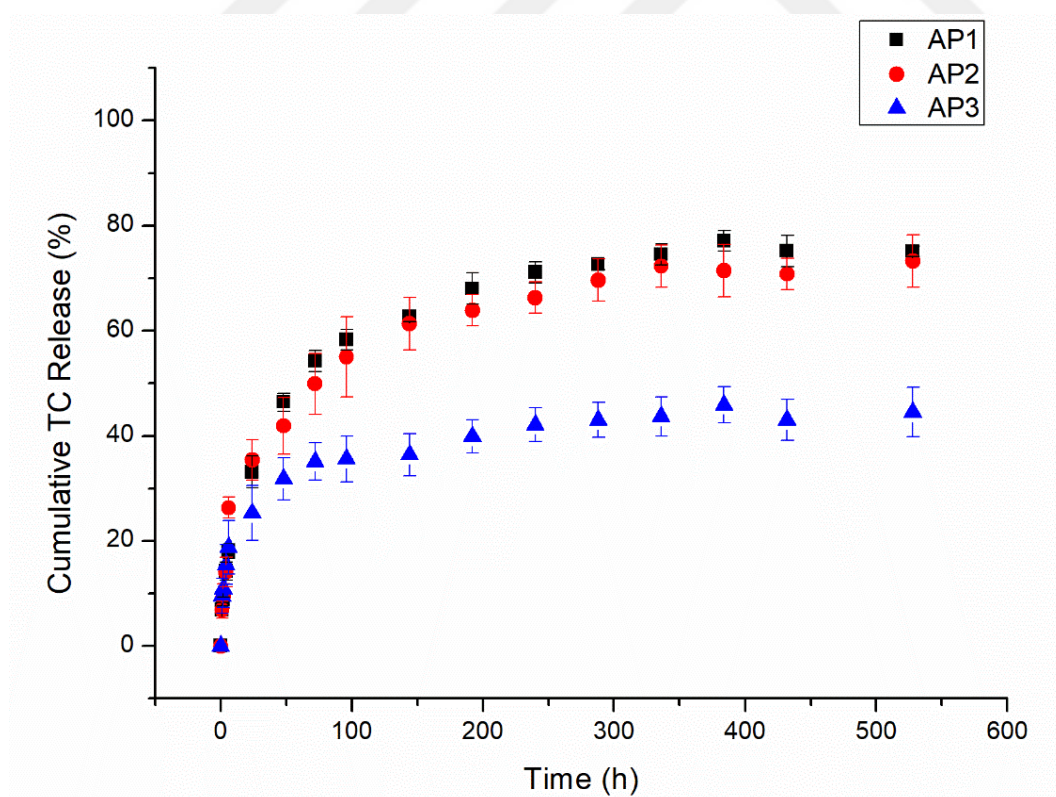


Figure 4.24 : Effect of PVA concentration on cumulative TC release (PCL-AP-RHA, TC/polymer ratio=1:10; AP1: 0.1 % PVA, AP2: 0.5 % PVA, AP3: 1 % PVA).

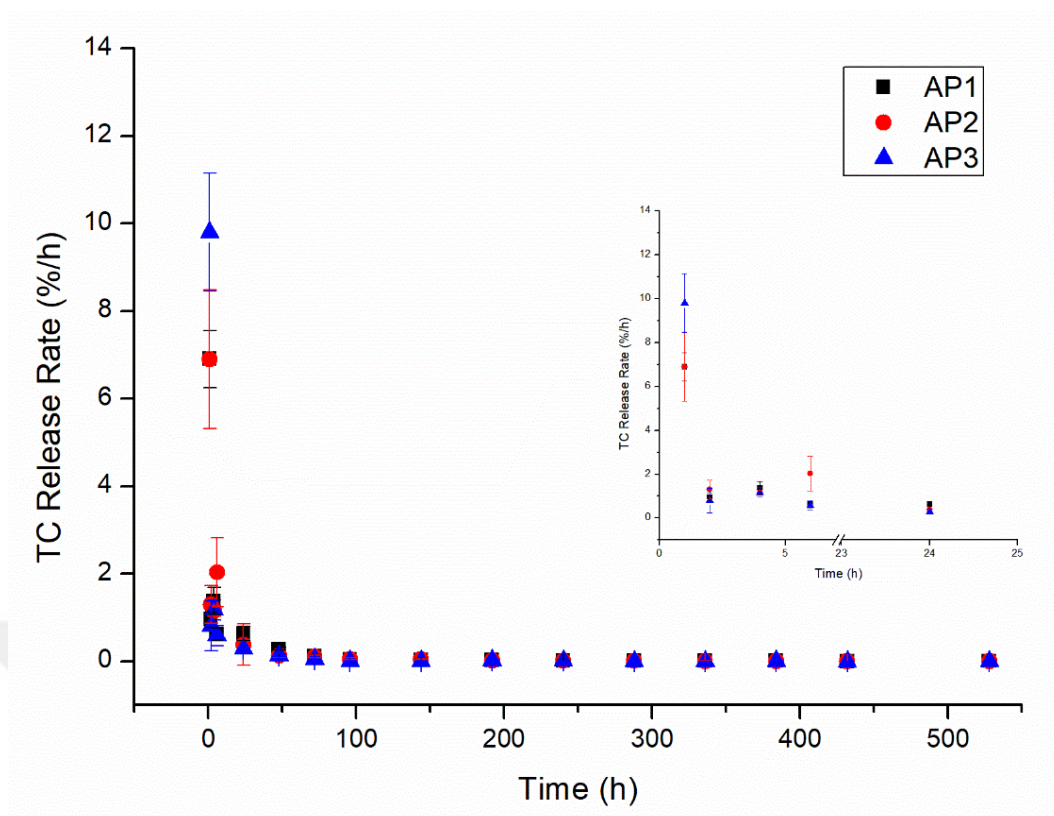


Figure 4.25 : Effect of PVA concentration on TC release rate (PCL-AP-RHA, TC/polymer ratio=1:10; AP1: 0.1 % PVA, AP2: 0.5 % PVA, AP3: 1 % PVA).

4.3.2.2 Effect of internal phase concentration

The effect of polymer concentration in the internal phase on release profiles were given in the following figures (Figure 4.26-4.31). In Figure 4.26, release profiles of PCL-G-RHA microspheres with varying polymer concentration in the internal phase show that cumulative TC release was significantly higher when drug/polymer ratio is low; in other words, increasing polymer amount in a fixed volume of emulsion increases release amount. The viscosity of the organic phase was increased by increasing the polymer amount, mean diameter of the microspheres were expected to be increased and caused slower release [99]. However, PCL-G-RHA microspheres were inversely affected by the increasing polymer amount in the internal phase. Unexpectedly, the TC release from G1 microsphere formulation (high polymer concentration) lasted 432 h (18 days) whereas the other two formulations with lower polymer concentration (G4 and G5) released the drug in 528 h (22 days). Normally, the release from microspheres with high polymer amount tends to be slower since the drug molecules were trapped deeper into the polymer matrix [178].

The initial release rate decreased as the polymer concentration in the internal phase decreased (Figure 4.27). Moreover, the release rate of the microspheres reached to the stable release phase faster when the polymer concentration was lower. G1 microspheres with higher polymer amount released the loaded TC in a controlled manner after the 4th hour of the release time. By the 4th hour, cumulative release of TC reached 14 ± 1 % [151].

The released amount of TC from the PCL-A-RHA microspheres decreased with decreasing polymer concentration in the internal phase (Figure 4.28). The decreasing polymer concentration in the internal phase caused the mean particle diameter of the microspheres to decrease, as discussed in Section 4.2.2. This decrease in the mean particle diameter affected the released amount of TC in a way that was not expected. Higher polymer amount in the internal phase resulted in earlier completion of the release in 384 h (16 days). On the other hand, the TC release from low polymer concentration formulation took place in 528 h (22 days). Furthermore, the cumulative TC release from A2 formulation (64 ± 14 % cumulative release) was higher than the other two formulations A4 and A5 which reached 48 ± 5 % and 41 ± 13 % cumulative release.

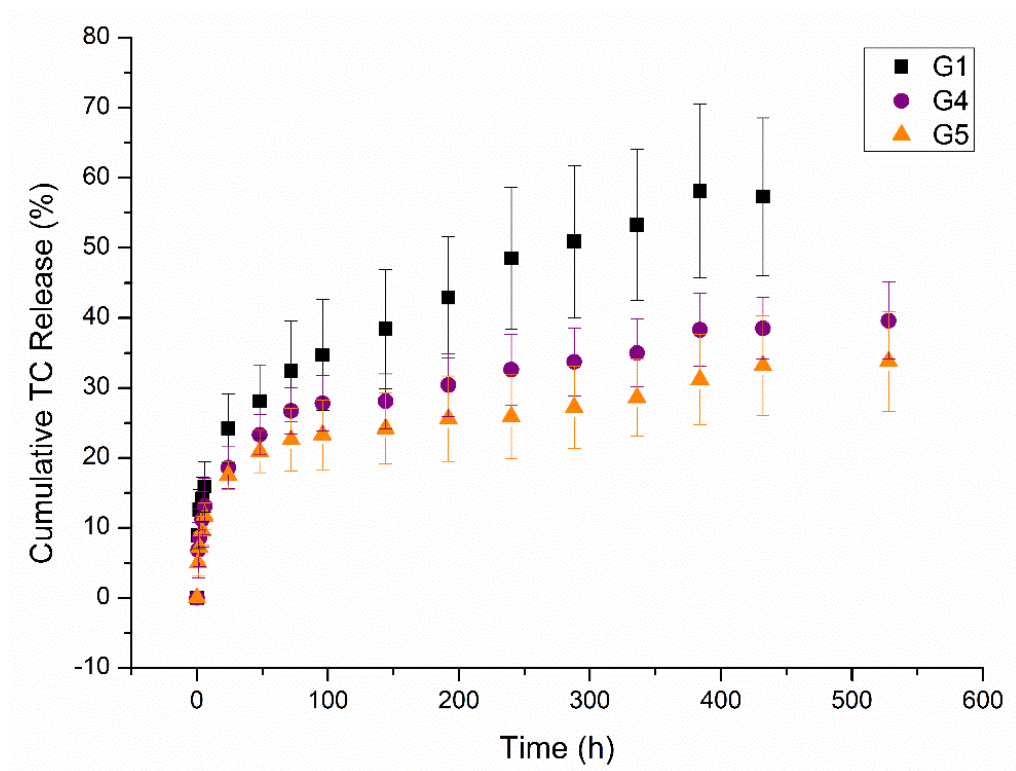


Figure 4.26 : Effect of polymer concentration on cumulative TC release (TC=10 mg; 0.1 % PVA; G1: 1:10; G4: 1:5; G5: 1:2.5 TC/polymer ratio).

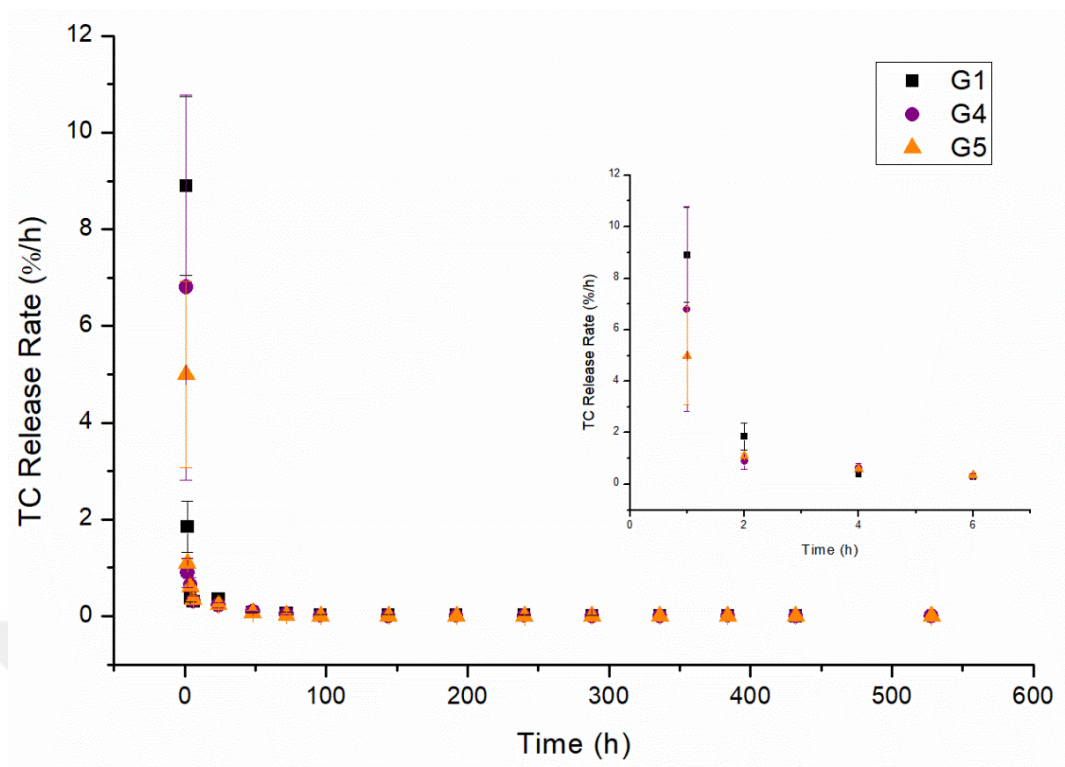


Figure 4.27 : Effect of polymer concentration on TC release rate (TC=10 mg; 0.1 % PVA; G1: 1:10; G4: 1:5; G5: 1:2.5 TC/polymer ratio).

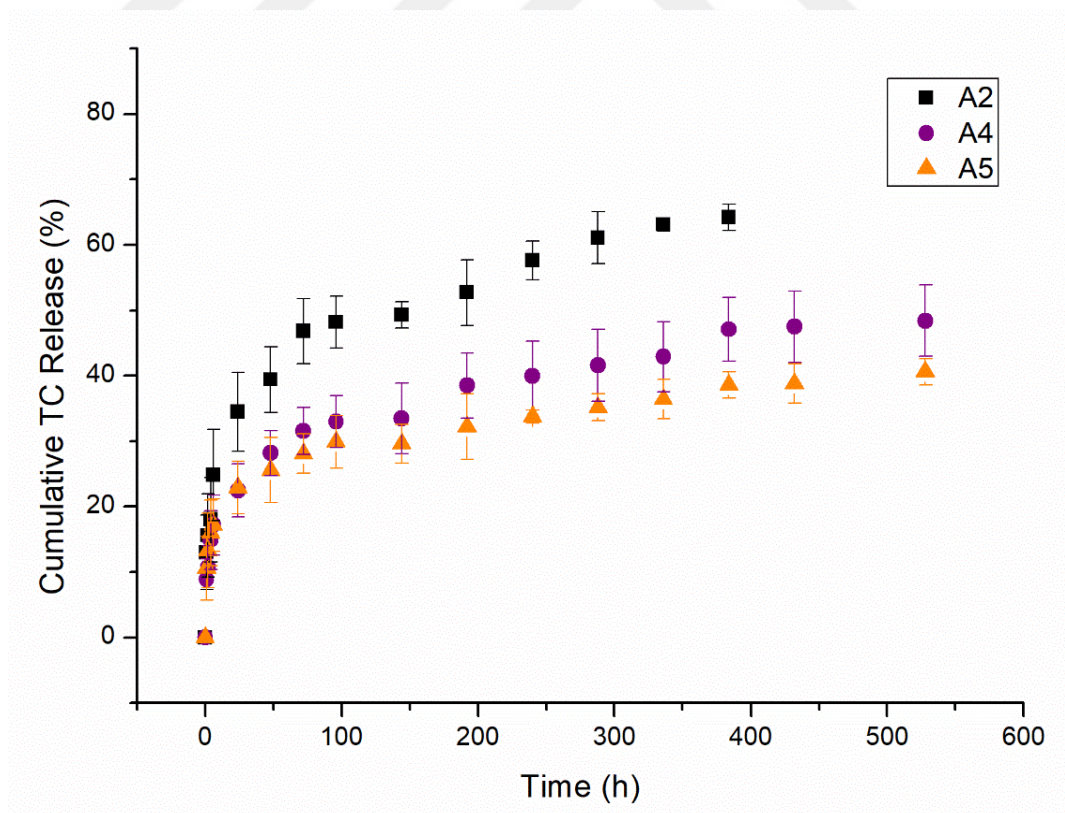


Figure 4.28 : Effect of polymer concentration on cumulative TC release (TC =10 mg; 0.5 % PVA; A2: 1:10; A4: 1:5; A5: 1:2.5 TC/polymer ratio).

The effect of changing polymer concentration in the internal phase on the release rate of the PCL-A-RHA microspheres was shown in Figure 4.29. The initial release rates of the microspheres were inversely proportional to polymer concentration in the internal phase. The release reached to a constant-rate phase after the 2nd hour of the release time (A2: 16±6 %; A4: 11±3 % and A5: 13±6 %).

TC release from PCL-AP-RHA microsphere formulations was significantly improved by reducing polymer amount in the organic phase, as expected (Figure 4.30). The smaller microspheres of AP3 formulation reached lower cumulative release (47±5 %) with respect to low polymer concentration formulations (AP4: 61±7 % and AP5: 58±7 %). Moreover, release of low polymer concentration formulations (AP4: 888 h – 37 days; AP5: 672 h – 28 days) lasted longer than high polymer concentration formulation (AP3: 528 h – 22 days).

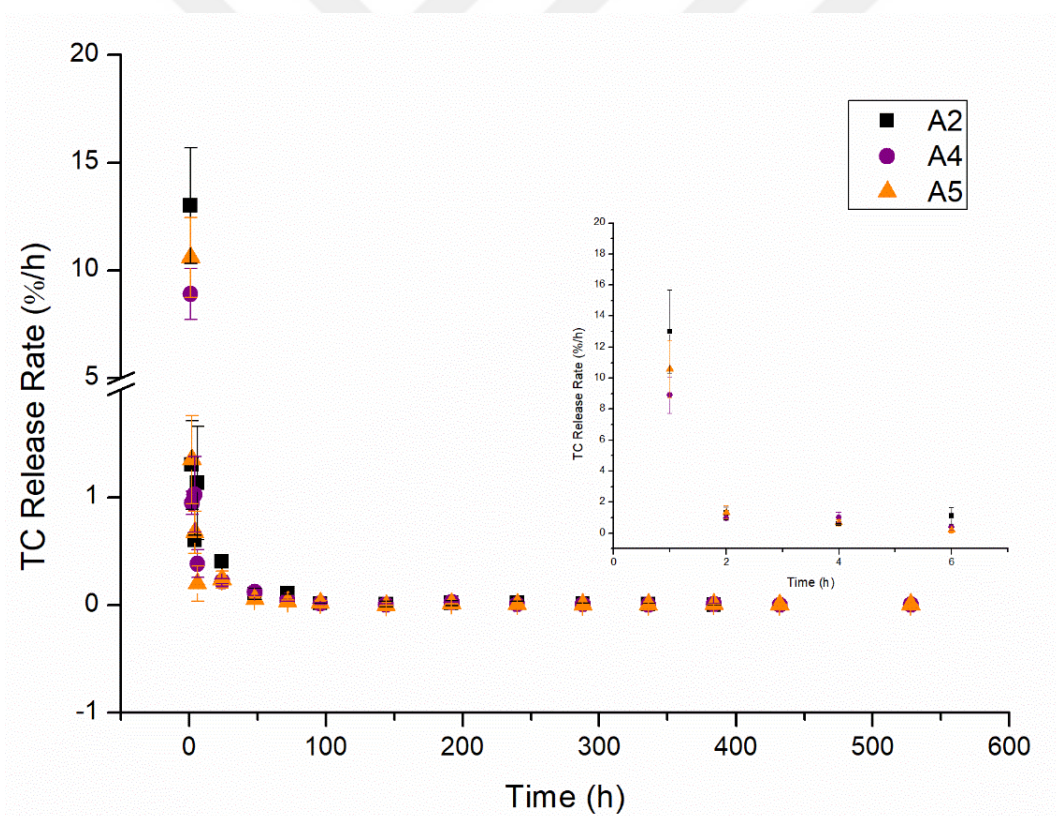


Figure 4.29 : Effect of polymer concentration on TC release rate (TC=10 mg; 0.5 % PVA; A2: 1:10; A4: 1:5; A5: 1:2.5 TC/polymer ratio).

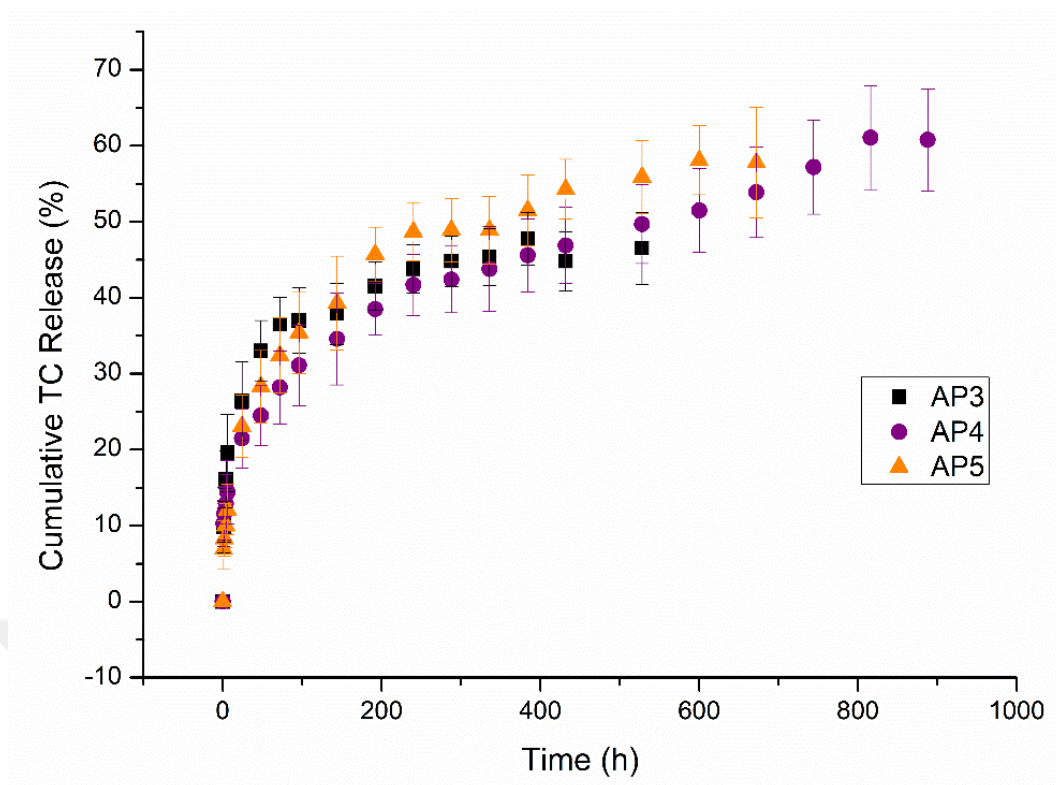


Figure 4.30 : Effect of polymer concentration on cumulative TC release (TC amount in the internal phase was fixed at 10 mg; 1 % PVA; AP3: 1:10; AP4: 1:5; AP5: 1:2.5 TC/polymer ratio).

In Figure 4.31, the effect of polymer concentration on the release rates from the AP microspheres can be seen. The initial release rate of the AP5 formulation was obviously lower than AP3 and AP4 formulation. This finding was consistent with the encapsulation efficiency calculation since the encapsulation efficiency of the AP5 formulation was much lower than those of the other two formulations. The release rate of the microspheres did not significantly affected by the decreasing polymer concentration in the internal phase. The release rate reached to a plateau after the 1st hour of the total release time (AP3: 10 ± 3 %; AP4: 10 ± 3 % and AP5: 7 ± 3 % cumulative release).

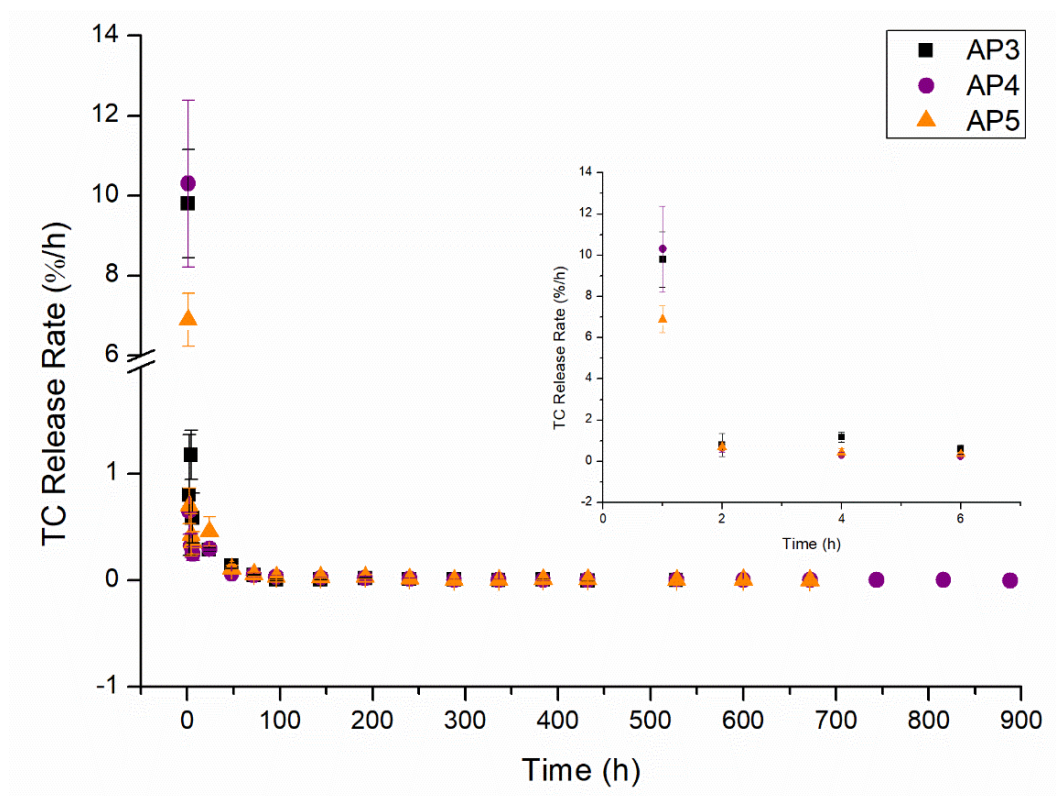


Figure 4.31 : Effect of polymer concentration on TC release rate (TC amount in the internal phase was fixed at 10 mg; 1 % PVA; AP3: 1:10; AP4: 1:5; AP5: 1:2.5 TC/polymer ratio).

4.3.3 Kinetic modelling of release profiles

Additionally, the drug release profiles were fitted to several kinetic models in order to understand the mechanism of drug release for each sample. The parameters calculated for the fitted kinetic model were given in Table 4.7. Highest correlation coefficient was found for Korsmeyer-Peppas kinetic model. For spherical geometries, n value is lower than 0.43 when the drug release is controlled by mainly Fickian diffusion [179]. When n value is between 0.43 and 0.85, the dominating release mechanism is anomalous transport or non-Fickian diffusion, which involves both diffusion and polymer erosion. N values higher than 0.85 indicates super case-II transport, which is controlled by the erosion of the polymeric chain [180]. Most of the microsphere formulations given in the table below had n value lower than 0.43. The release from these microspheres were dominated by Fickian diffusion. Only the release from AP1 microsphere involved polymer erosion besides Fickian diffusion.

Table 4.7 : Kinetic model parameters calculated for microspheres.

Code	Korsmeyer-Peppas			Higuchi		Zero Order		First Order	
	K_{KP} (h ⁻ⁿ)	n	R ²	K_H (h ^{-0.5})	R ²	K_0 (h ⁻¹)	R ²	K_1 (h ⁻¹)	R ²
G1	0.161	0.30	0.995	2.59	0.977	0.125	0.871	0.004	0.718
G2	0.239	0.22	0.983	1.82	0.941	0.076	0.822	0.003	0.678
G3	0.196	0.27	0.994	2.49	0.943	0.117	0.795	0.004	0.644
G4	0.188	0.27	0.990	1.54	0.926	0.062	0.759	0.003	0.634
G5	0.185	0.27	0.968	1.26	0.904	0.051	0.738	0.003	0.603
A1	0.147	0.39	0.953	4.22	0.911	0.181	0.729	0.004	0.541
A2	0.210	0.27	0.983	2.88	0.921	0.133	0.755	0.004	0.588
A3	0.273	0.28	0.981	2.69	0.801	0.110	0.585	0.003	0.439
A4	0.201	0.26	0.990	1.87	0.932	0.076	0.772	0.003	0.628
A5	0.299	0.20	0.991	1.46	0.879	0.063	0.712	0.003	0.608
AP1	0.093	0.48	0.994	3.46	0.898	0.136	0.696	0.004	0.540
AP2	0.113	0.43	0.954	3.42	0.912	0.147	0.729	0.004	0.551
AP3	0.237	0.25	0.969	1.74	0.854	0.067	0.645	0.003	0.534
AP4	0.153	0.27	0.994	1.78	0.970	0.056	0.840	0.002	0.671
AP5	0.121	0.35	0.992	2.39	0.952	0.097	0.791	0.004	0.638

4.3.4 Characterization of microspheres

A series of optical microscopy images were used for observation of particle geometry and particle size measurement (Figure 4.32). Spherical geometry was observed for all microsphere formulations. SEM images of individual microspheres were studied. PCL-G-RHA microspheres had spherical geometry with highly porous and rough surface structure, which is an expected feature for atmospheric solvent evaporation method [99]. Low polymer content in the microspheres resulted in smaller pore size (Figure 4.32Bd and 4.32Be) which can explain the reduced cumulative TC release from these microspheres [151].

PCL-A-RHA microspheres, as PCL-G-RHA microspheres, had highly porous and rough surface structure as a result of atmospheric solvent evaporation. A1 and A3 formulations did not created neat spherical geometries; instead, rough and sphere-like particles were formed (Figure 4.33Ba and Figure 4.33Bc). PCL-AP-RHA microspheres had smooth and less porous surface structure, which can explain the slower release rate (Figure 4.34). This smooth surface structure can be attributed to the higher PCL grafting of PCL-AP-RHA (18 %), thus increasing crystallinity (39 %) [181]. SEM image of AP5 formulation revealed an agglomerated structure instead of individual microspheres as can be seen in Figure 4.34Be.

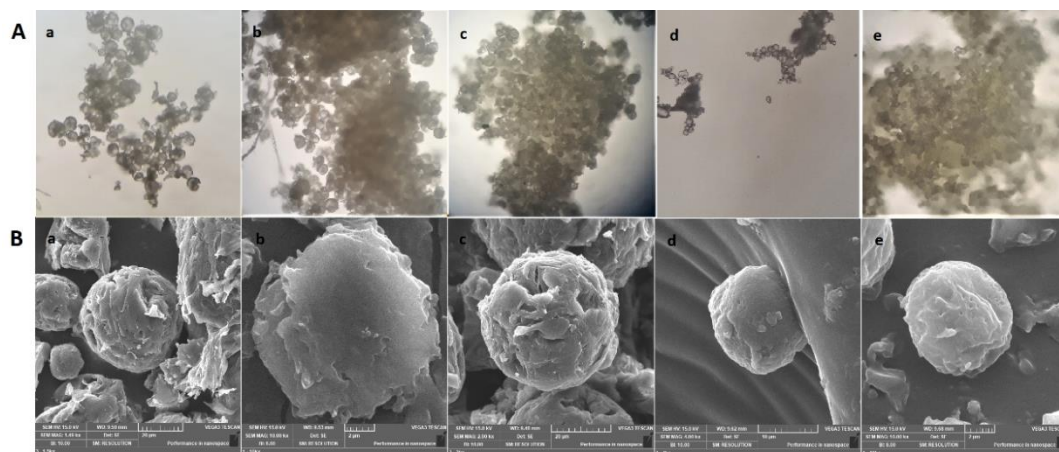


Figure 4.32 : A) Optical microscopy images of a) G1, b) G2, c) G3, d) G4, e) G5 (Magnification 40x); B) SEM images of a) G1, b) G2, c) G3, d) G4, e) G5 (Magnification 5000x).

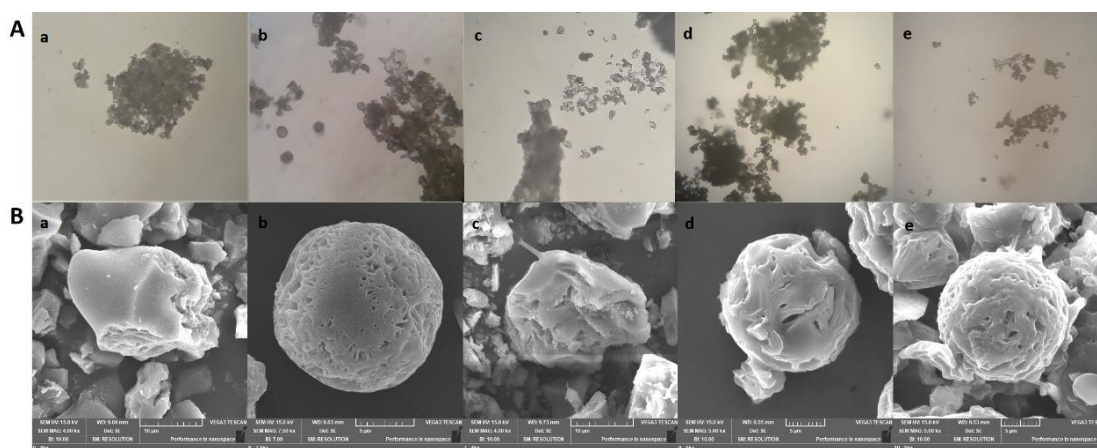


Figure 4.33 : A) Optical microscopy images of a) A1, b) A2, c) A3, d) A4, e) A5 (Magnification 40x); B) SEM images of a) A1, b) A2, c) A3, d) A4, e) A5 (Magnification 5000x).

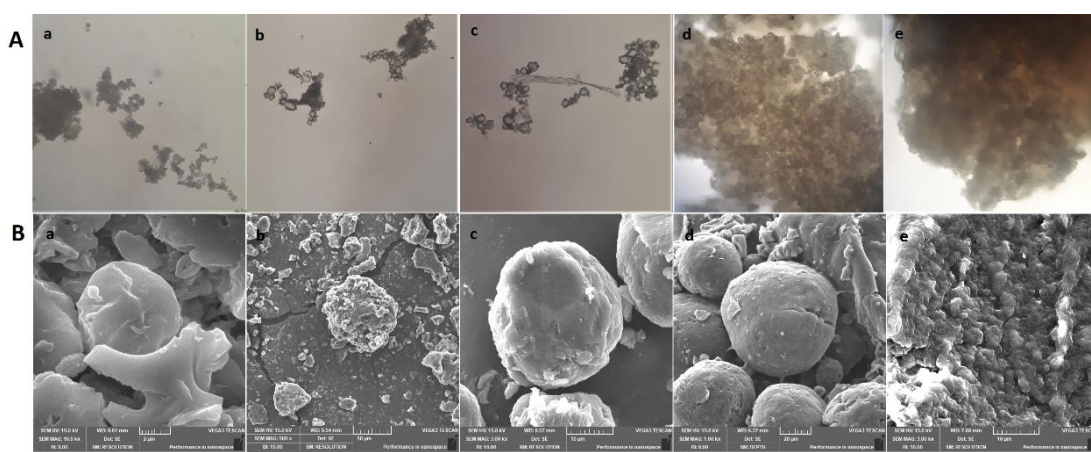


Figure 4.34 : A) Optical microscopy images of a) AP1, b) AP2, c) AP3, d) AP4, e) AP5 (Magnification 40x); B) SEM images of a) AP1, b) AP2, c) AP3, d) AP4, e) AP5 (Magnification 5000x).

Structural and thermal characterization of the microspheres were carried out with the optimal process parameter formulations which were determined in terms of their effect on TC loading and release. The further characterization of the microsphere samples were conducted with the optimum formulations chosen for 3 types of nanohybrid systems (PCL-G-RHA: G1 microsphere formulation, PCL-A-RHA: A2 microsphere formulation, PCL-AP-RHA: AP4 microsphere formulation). The microspheres were structurally characterized by FT-IR in order to observe the presence of the components in the loaded spheres (Figure 4.35-4.41). G1 showed two peaks at 800 and 1050 cm^{-1} which are caused by Si-CH₃ rocking and asymmetric stretching of Si-O-Si. These peaks proved the presence of RHA in the hybrid systems. The characteristic peak of TC molecule (α , β unsaturation in ketones, 1663 cm^{-1}) can be observed. Another characteristic peak of TC (=C-H stretching, aromatic, multiple peaks at 3100-3200 cm^{-1}) has been detected in the spectrum. The weakening of the -CH₂ wagging peak of oxirane ring (1150 cm^{-1}) [182] in the spectrum of G1 may be considered as the proof of ring opening of oxirane of 3-GPTMS. TC is a molecule with low redox potential [183]. Thus, it may be possible that it exhibited ionic interactions with free -OH groups present in the structure of PCL-G-RHA hybrid system as the result of such ionic interactions. The peak belonging to C=C stretching in aromatic compounds at 1600 cm^{-1} and C-C stretching in aromatic compounds at 1400-1500 cm^{-1} was observed in G1 spectrum. These mentioned peaks altogether were the indication of the successful TC loading. Furthermore, peaks at 1293 cm^{-1} , 1238 cm^{-1} and 1167 cm^{-1} observed in the spectra belonging to C-O and C-C bonds seen in crystalline phase, asymmetric stretch of C-O-C and symmetric stretch of C-O-C in PCL structure, respectively. The peaks of asymmetric CH₂ bonds at 2945 cm^{-1} , symmetric CH₂ bonds at 2866 cm^{-1} were observed. The broad peak at 3442 cm^{-1} was observed in the spectra of PCL-G-RHA and G1 microspheres and was assigned to stretching vibrations of H-bonded -OH of PCL-G-RHA hybrid system. Lastly, aliphatic carbonyl bond peak at 1726 cm^{-1} has showed the presence of PCL in the nanohybrid system.

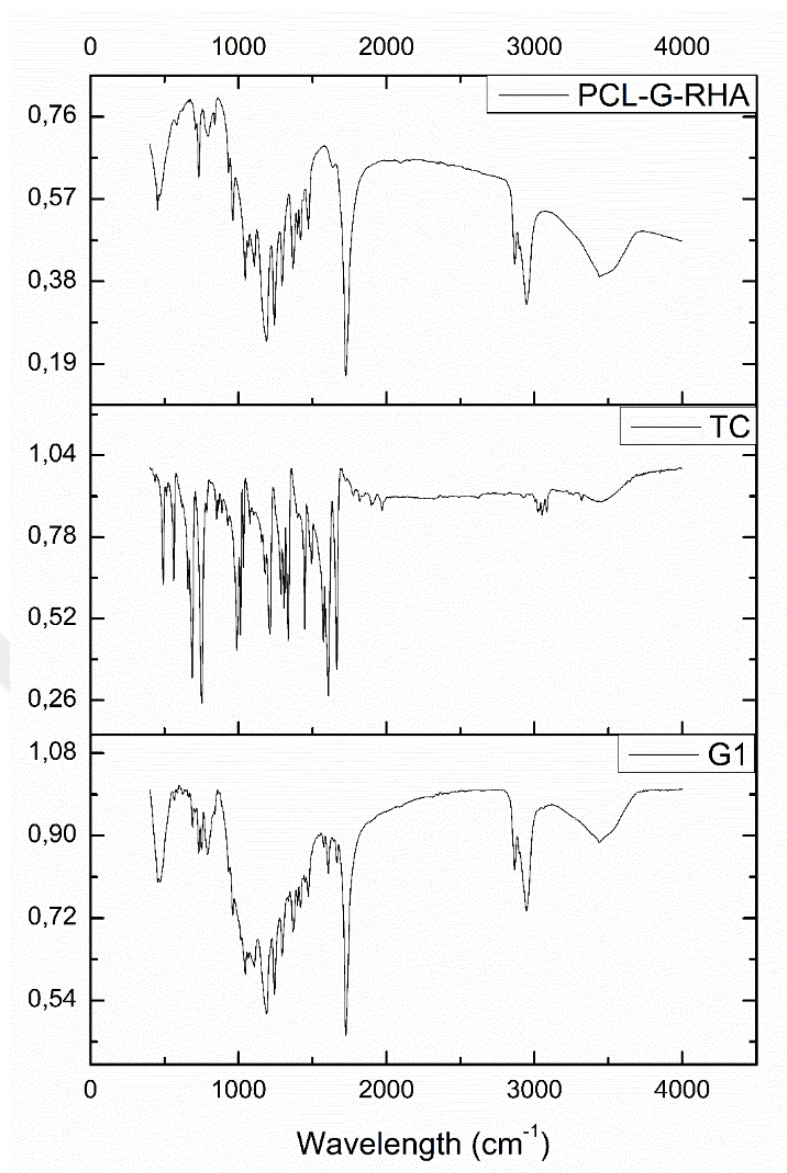


Figure 4.35 : FT-IR spectra of G1, PCL-G-RHA and TC (PCL-G-RHA: Hybrid system containing 3-GPTMS; TC: Trans-chalcone; G1: 0.1 % PVA, 1:10 TC:PCL-G-RHA).

The possible interactions between the hybrid systems and the TC molecules were furtherly investigated by comparing the FT-IR spectra of microspheres and the physical mixtures of the components of the microspheres with the same ratios. As presented in insets in Figure 4.36, a significant difference was detected between the two spectra, at 840 cm^{-1} . This peak was assigned to oxirane ring [184] and the disappearance of it in microsphere spectrum suggested that oxirane ring in the structure of PCL-G-RHA hybrid system was protonated in the microspheres. The characteristic peak of TC at 1662 cm^{-1} (α, β unsaturated ketone) shifted to 1667 cm^{-1} in the spectrum of G1 microspheres. Also, in the spectrum of the physical mixture of the formulation, signals of both free and intramolecular H-bonded -OH groups can be detected at 3442

cm^{-1} and 3536 cm^{-1} , respectively. However, in the spectrum of G1 microspheres free $-\text{OH}$ stretching was not visible. This may be due to interaction of α, β unsaturation of TC and free $-\text{OH}$ groups of PCL-G-RHA which have contributed to higher drug loading compared to PCL-A-RHA and PCL-AP-RHA and prolonged TC release.

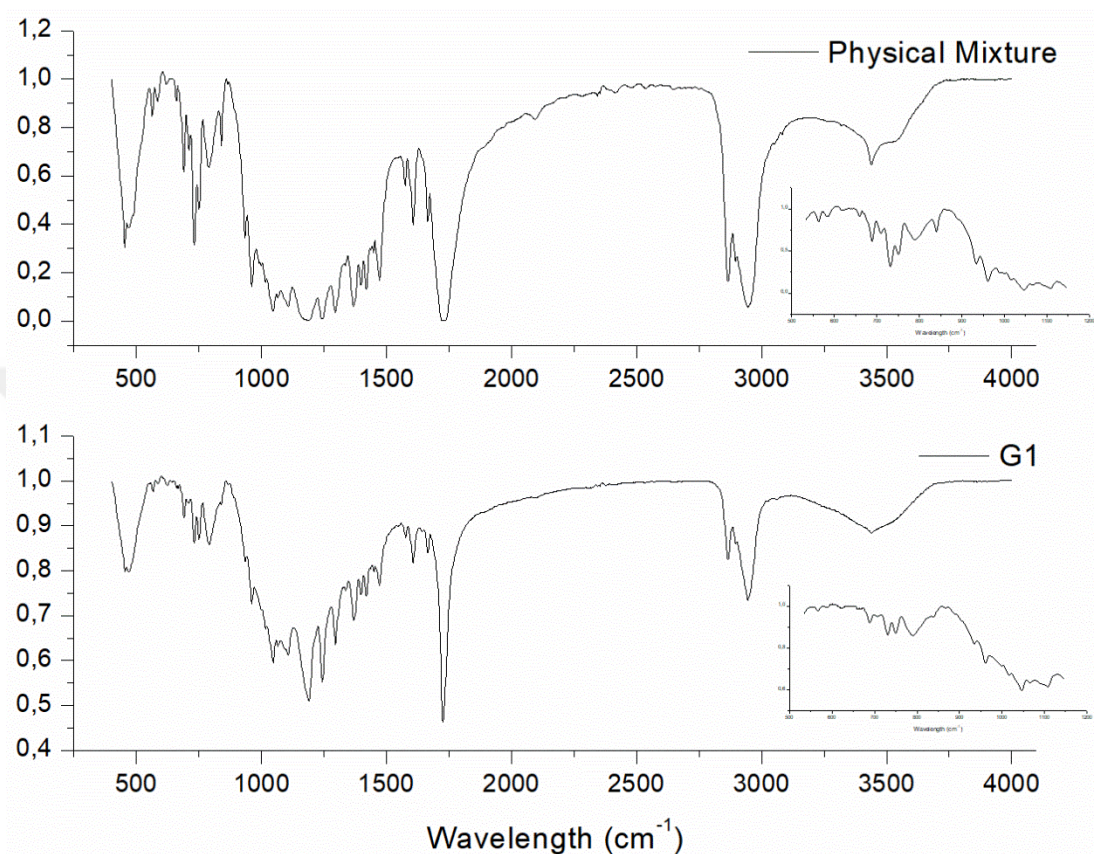


Figure 4.36 : FT-IR spectra of G1 microspheres and the physical mixture of PCL-G-RHA and TC (G1: 0.1 % PVA, 1:10 TC:PCL-G-RHA; Physical mixture: Mixture of 1:10 (w:w) TC:PCL-G-RHA).

Similarly, all of the characteristic peaks of the main components of the A2 microsphere, which are PCL-A-RHA and TC, in the FT-IR spectrum as presented in Figure 4.37. These peaks were mainly as listed: peaks of RHA present in the structure of PCL-A-RHA at 788 cm^{-1} and 1050 cm^{-1} belonging to Si-CH_3 rocking and asymmetric stretching of Si-O-Si ; TC peaks at $1400\text{--}1500 \text{ cm}^{-1}$ belonging to C-C stretching in aromatic compounds, 1605 cm^{-1} belonging to C=C stretching in aromatic compounds, 1662 cm^{-1} assigned to α, β unsaturation in ketones, 3059 cm^{-1} belonging to $=\text{C-H}$ stretching in aromatics; PCL peaks at 1293 cm^{-1} , 1238 cm^{-1} and 1167 cm^{-1} C-O and C-C bonds seen in crystalline phase, asymmetric stretch of C-O-C and symmetric stretch of C-O-C in PCL structure, respectively, at 1726 cm^{-1} belonging to carbonyl bond (aliphatic), symmetric CH_2 bonds at 2868 cm^{-1} asymmetric CH_2 bonds at 2949 cm^{-1} .

A weak shoulder at 3512 cm^{-1} partly conjugated with --OH stretching at 3480 cm^{-1} was assigned to --NH stretching (primary amine).

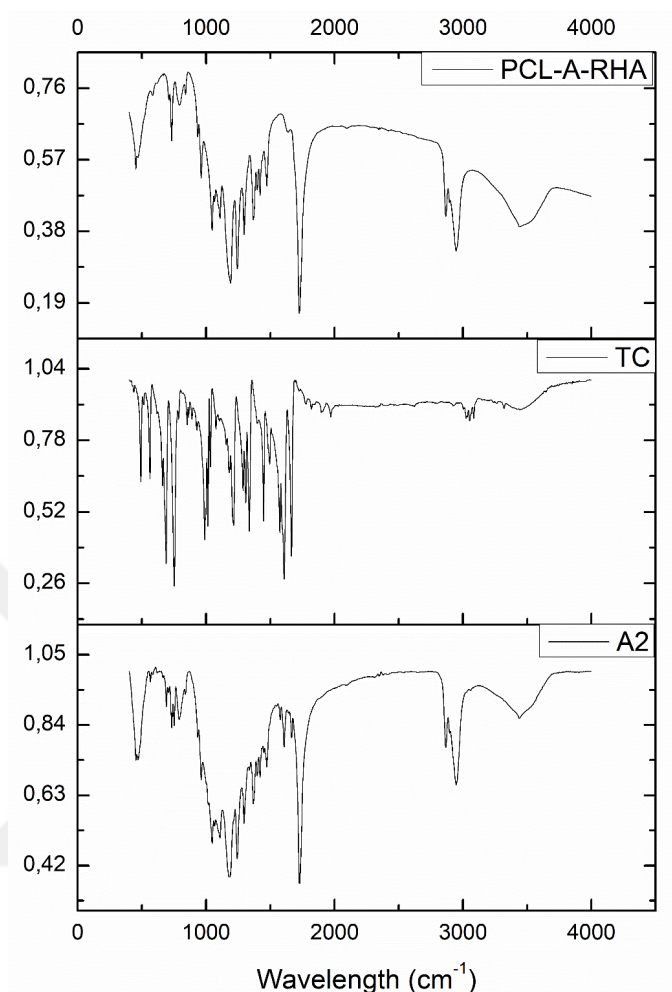


Figure 4.37 : FT-IR spectra of A2, PCL-A-RHA and TC (PCL-A-RHA: Hybrid system containing 3-APTMS; TC: Trans-chalcone; A2: 0.5 % PVA, 1:10 TC:PCL-A-RHA).

However, when the spectra of A2 microspheres and physical mixture of PCL-A-RHA and TC were compared, no significant difference was observed (Figure 4.38). Both signals expected to involve in drug/polymer interactions, N-H stretching which appears as a shoulder peak and were intact in both spectra. This finding indicated that chemical interactions did not take any part in drug loading to the PCL-A-RHA microspheres.

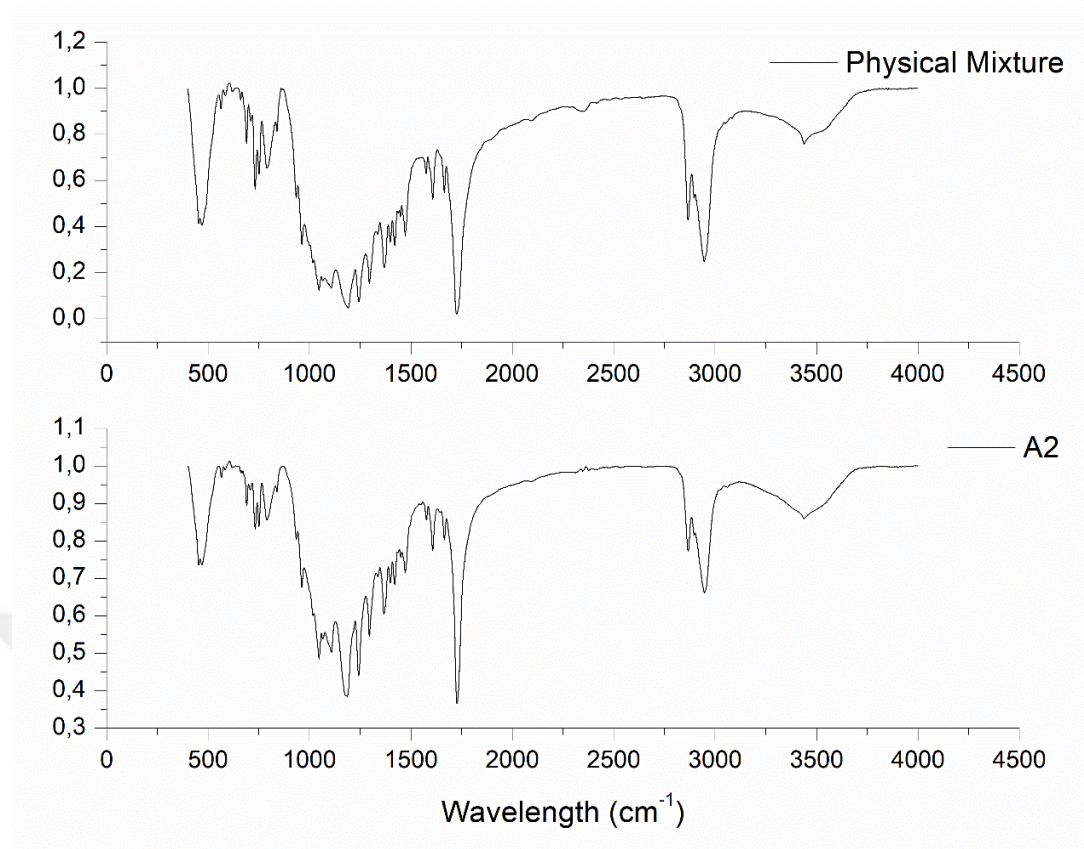


Figure 4.38 : FT-IR spectra of A2 microspheres and the physical mixture of PCL-A-RHA and TC (A2: 0.5 % PVA, 1:10 TC:PCL-A-RHA; Physical mixture: Mixture of 1:10 (w:w) TC:PCL-A-RHA).

In the given spectra below (Figure 4.39), several sharp peaks were detected at 1727 cm^{-1} , 2859 and 2950 cm^{-1} which were assigned to carbonyl bond (aliphatic), symmetric and asymmetric CH_2 bonds in PCL structure, respectively. Symmetric and asymmetric CH_2 bond peak have conjugated with the characteristic signal of TC; $=\text{C-H}$ stretching (aromatic) at 3083 cm^{-1} which may be an indication of hydrogen bonding between TC and PCL-AP-RHA. In the spectrum of PCL-AP-RHA there is a low intensity shoulder at 3510 cm^{-1} which is assigned to N-H stretching in primary amines. Additionally, peaks at 1293 cm^{-1} , 1240 cm^{-1} and 1188 cm^{-1} C-O and C-C bonds seen in crystalline phase, asymmetric stretch of C-O-C and symmetric stretch of C-O-C were assigned to the PCL structure in the hybrid system, respectively. Moreover, peaks at 753 and 1043 cm^{-1} belonging to Si- CH_3 rocking and asymmetric stretching of Si-O-Si were caused by the RHA and 3-APTES content of the PCL-AP-RHA system. TC in the microspheres can also be identified from the peaks at 1362 cm^{-1} belonging to C-C stretching in aromatic compounds, 1606 cm^{-1} belonging to C=C stretching in aromatic compounds and α,β unsaturation at 1662 cm^{-1} .

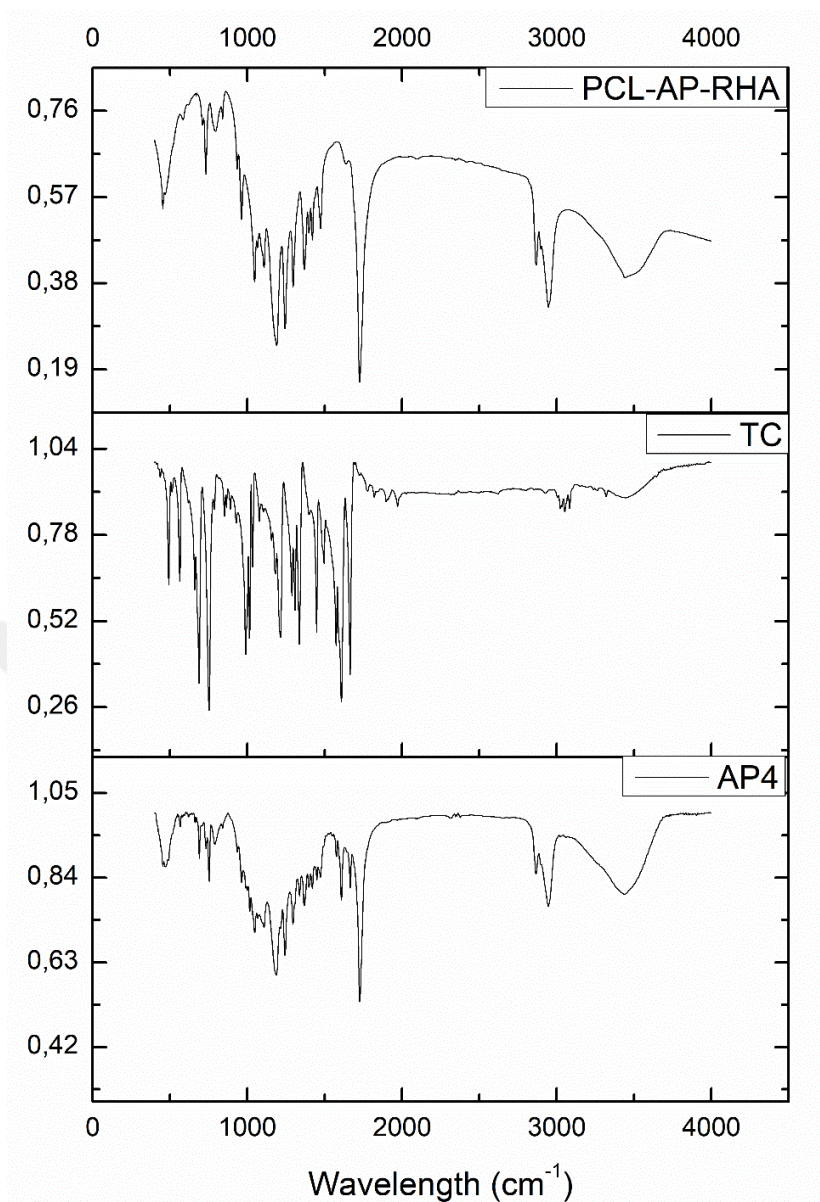


Figure 4.39 : FT-IR spectra of AP4, PCL-AP-RHA and TC (PCL-AP-RHA: Hybrid system containing 3-APTES; TC: Trans-chalcone; AP4: 1 % PVA, 1:5 TC:PCL-AP-RHA).

The physical mixture of the components of the related PCL-AP-RHA microspheres (AP4) was also investigated spectrophotometrically in order to understand possible interactions between PCL-AP-RHA and TC molecules. As compared in Figure 4.40, α , β -unsaturation peak of TC at 1662 cm^{-1} in the spectrum of the physical mixture had shifted to 1666 cm^{-1} in the spectrum of AP4 microsphere formulation. This difference also shows the possible interaction between TC and PCL-AP-RHA during microsphere formation, namely hydrogen bonding between carbonyl group of TC and amine group of PCL-AP-RHA [185]. The slight shouldering peak at 3510 cm^{-1} assigned to primary amines had also disappeared in the spectrum of AP4 microspheres.

due to this hydrogen bonding whereas it was visible in the spectrum of the physical mixture. Strong H-bonding between the TC molecules and PCL-AP-RHA may be the explanation of prolonged TC release from AP4 microsphere formulation (888 h – 37 days).

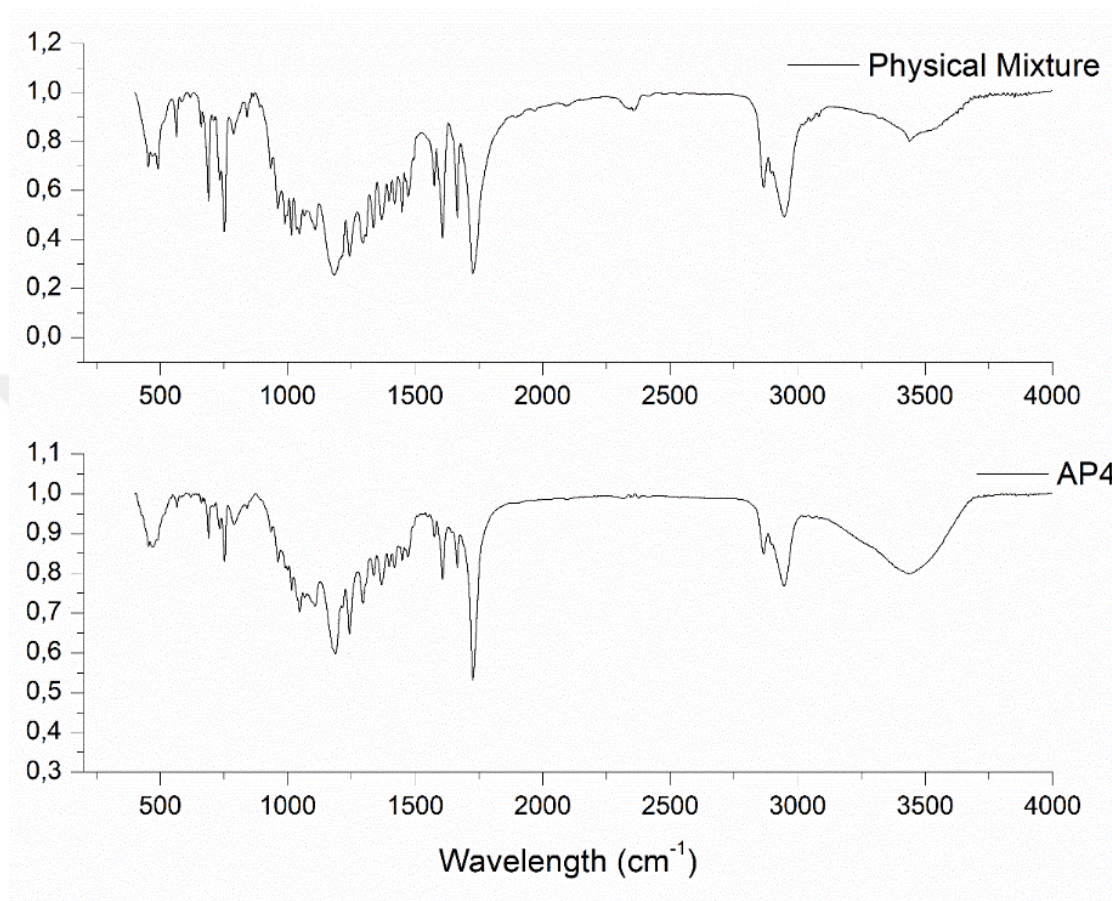


Figure 4.40 : FT-IR spectra of AP4 microspheres and the physical mixture of PCL-AP-RHA and TC (AP4: 1 % PVA, 1:5 TC:PCL-AP-RHA; Physical mixture: Mixture of 1:5 (w:w) TC:PCL-AP-RHA).

A2, G1 and AP4 microsphere formulations were thermally characterized by TGA and DSC. TGA and DTG curves were given in Figure 4.41. Thermograms showed two main peaks; ~225-275 °C and ~400 °C. The first peak at 275 °C, 248 °C and 225 °C in the thermograms of G1, A2 and AP4, respectively belongs to TC degradation [186]. The peak at 414 °C (G1), 418 °C (A2) and 406 °C (AP4) is the degradation temperature of PCL. Thermogram of AP4 formulation also revealed slight and broad peak belonging to RHA at approximately 500 °C [187]. The relatively high intensity of the peak at ~250 °C in the A2 thermogram might be caused by the 3-APTMS degradation coinciding with TC degradation [188].

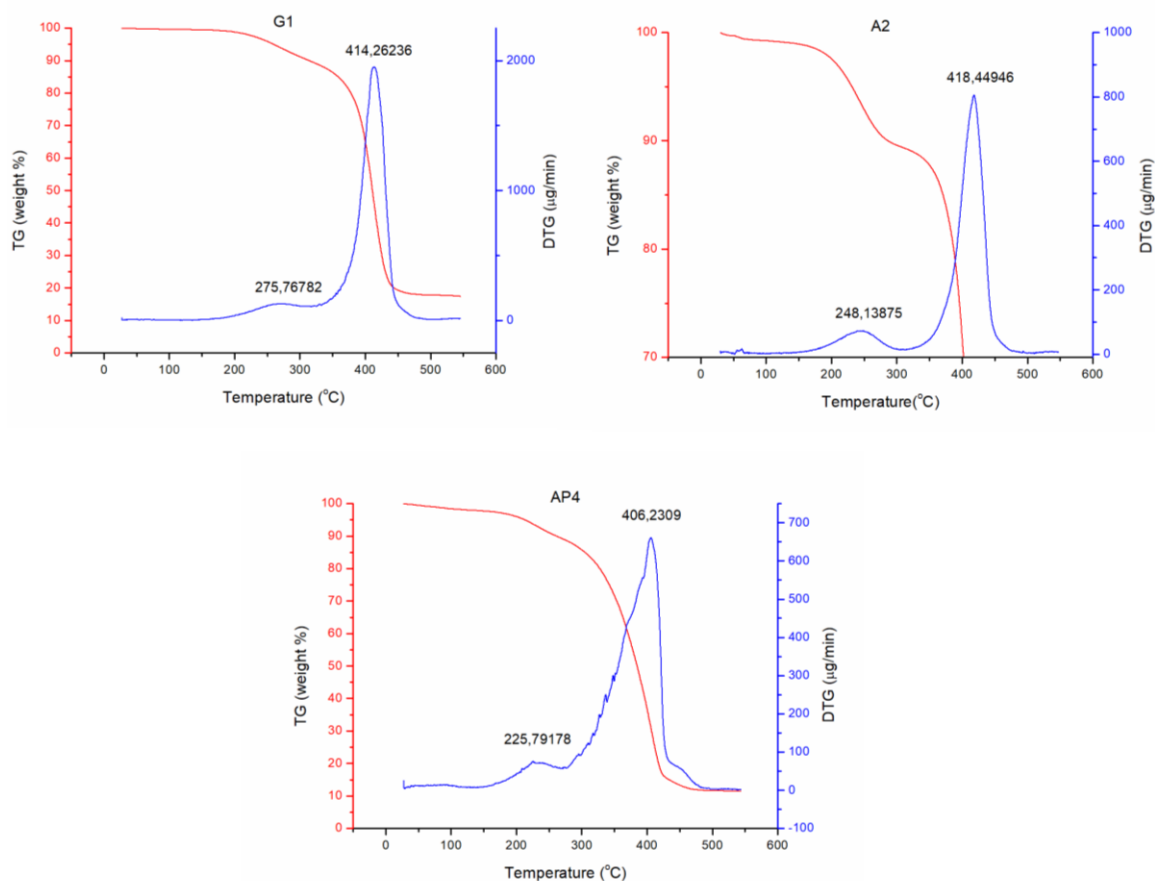


Figure 4.41 : TG and DTG curves of microspheres (G1: 0.1 % PVA, 1:10 TC:PCL-G-RHA; A2: 0.5 % PVA, 1:10 TC:PCL-A-RHA; AP4: 1 % PVA, 1:5 TC:PCL-AP-RHA).

Melting, crystallization and glass transition temperatures of microspheres were determined by DSC analysis (Table 4.8). Melting temperatures of the microspheres were at the same range with the melting temperature of the hybrid polymers and trans-chalcone. Crystallization temperature was increased by microsphere formation; T_c of commercial trans-chalcone is approximately 5 °C.

Table 4.8 : DSC data of microspheres.

Sample	T_m (°C)	T_c (°C)
AP4	51	26
A2	51, 54	32
G1	52	28

Microspheres and the physical mixtures of the components of the microspheres with the same ratios were thermally characterized by DSC (Figure 4.42). The endothermic peak of G1 microsphere was observed at 52 °C which is a temperature lower than the melting temperatures of the two component of the microsphere (PCL-G-RHA: 54 °C;

TC: 59 °C). This indicates that a eutectic system was formed between TC and PCL-G-RHA [189]. The hydrogen bonding of which indications were observed in FT-IR spectrum of G1 may have increased the miscibility of the two components [190]. The formation of a single endothermic peak in G1 second heating scan indicated the molecular dispersion of TC within PCL-G-RHA matrix. On the other hand, the physical mixture of TC and PCL-G-RHA at the same ratio as the microsphere formulation had two distinctive endothermic melting peak of TC and PCL-G-RHA with a slight decrease compared to pure forms. The melting temperature of TC in the physical mixture has slightly decreased due to the dilution of drug [191]. Crystallization temperature of microspheres was determined to be in between the values of TC and PCL-G-RHA (G1: 28 °C; TC: 5 °C; PCL-G-RHA: 37 °C). As presented in previous sections, PCL-G-RHA has a high glass transition temperature. This property provides stability since recrystallization of the loaded drug can be avoided due to the decreased molecular motion by the polymer [192]. Such effect was observed in the release behaviors of PCL-G-RHA and PCL microspheres as discussed in the following section.

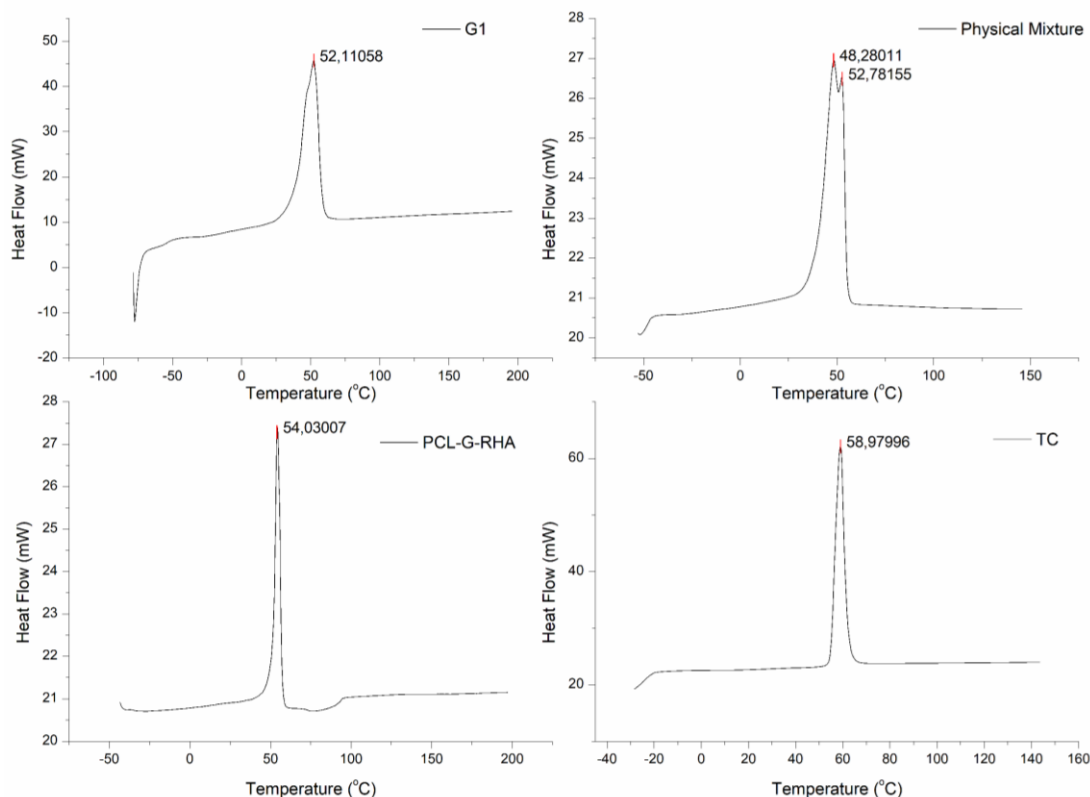


Figure 4.42 : DSC thermogram of the PCL-G-RHA, TC, G1 and physical mixture, demonstrating melting endotherm (second heating cycle), G1: 0.1 % PVA, 1:10 TC:PCL-G-RHA.

Similar comparison between A2 microsphere and the physical mixture of its components was made. As can be seen in Figure 4.43, the melting endotherm of A2 microsphere showed a twin peak at 50 °C and 54 °C. The presence of such double peak means that both PCL-A-RHA and TC preserved their thermal properties with a slight decrease. This result supports the findings of FT-IR spectra indicating that TC was encapsulated in the microspheres rather than molecularly dispersed within the polymer matrix. The double melting peak of the physical mixture was conjugated and became broader than individual PCL-A-RHA and TC.

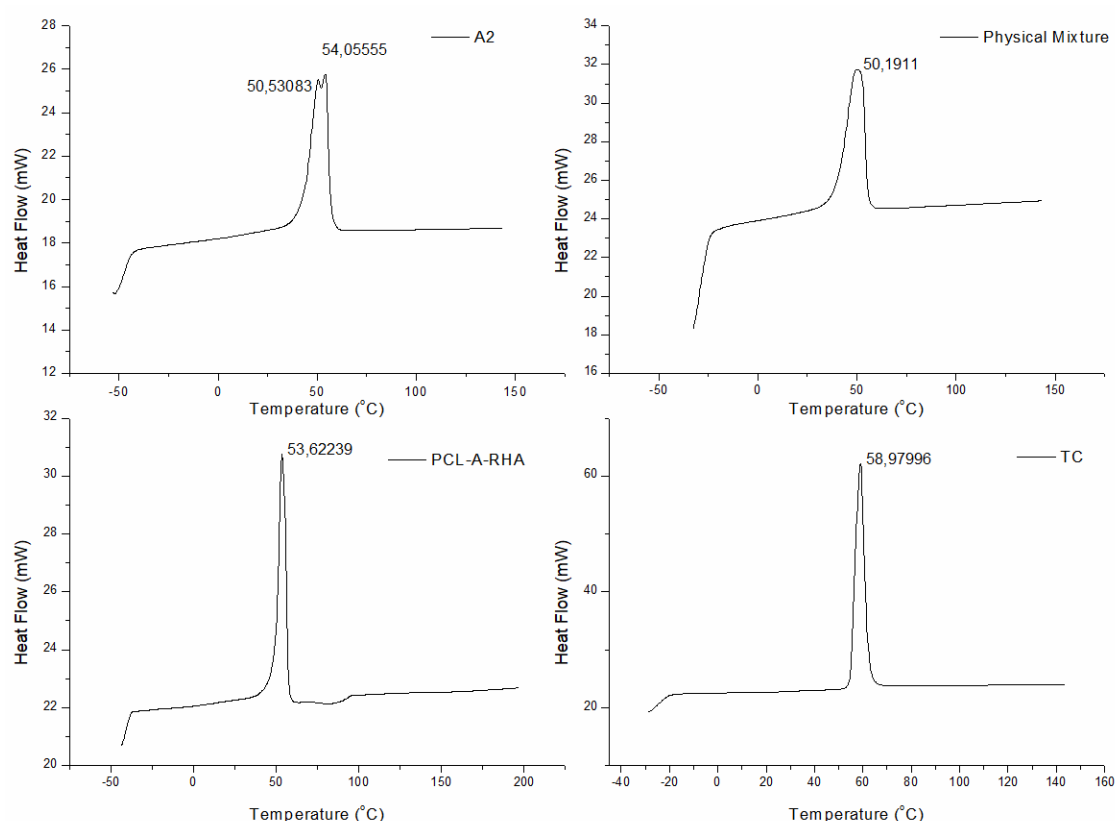


Figure 4.43 : DSC thermogram of the PCL-A-RHA, TC, A2 and physical mixture, demonstrating melting endotherm (second heating cycle), A2: 0.5 % PVA, 1:10 TC:PCL-A-RHA.

Figure 4.44 shows the melting endotherms of AP4 microspheres, the physical mixture of the components of this microsphere formulation, PCL-AP-RHA polymer and TC. AP4 microspheres had a single melting peak at approximately 51 °C, a value lower than the melting temperature of both of the components. This situation was similar with G1 microspheres of which melting endotherms revealed a eutectic system. Such single endothermic peak indicates, as disappearance of some TC characteristic peaks in FT-IR spectrum, molecular dispersion of TC within the polymer matrix. However,

the melting endotherm of the physical mixture of PCL-AP-RHA and TC showed an indistinct double melting peak at lower temperatures than the melting temperature of pure PCL-AP-RHA and pure TC.

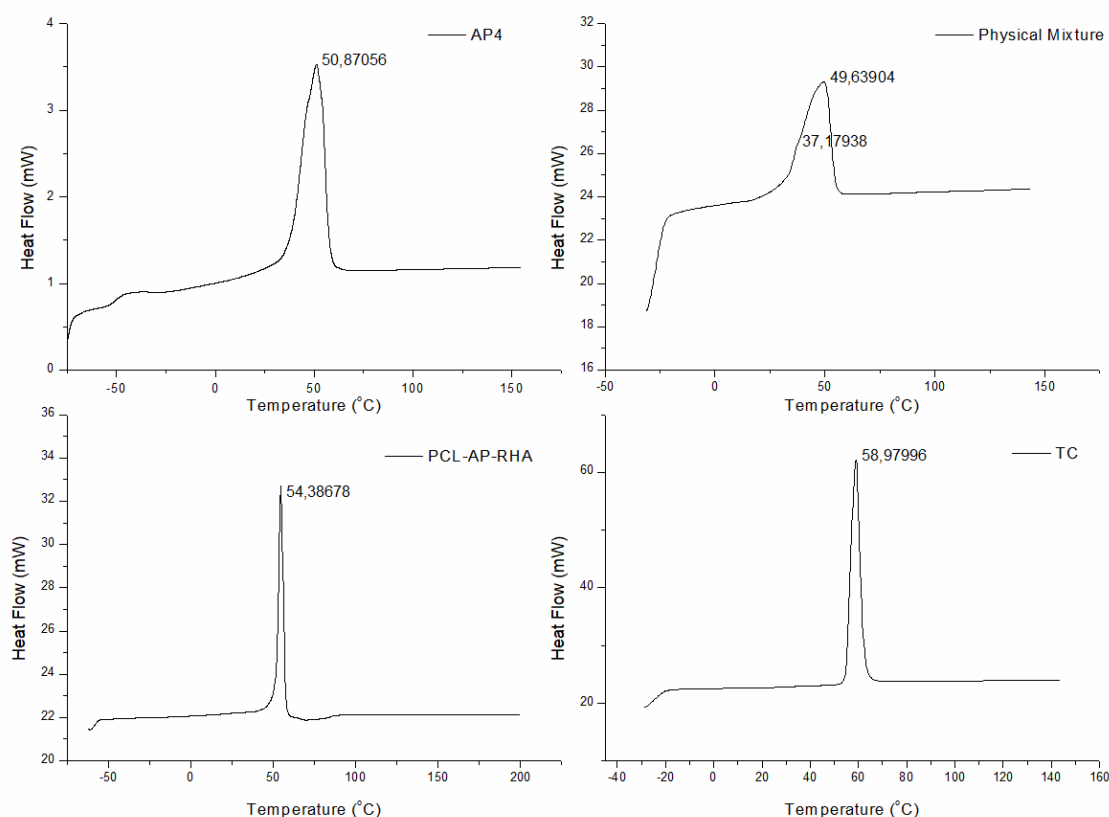


Figure 4.44 : DSC thermogram of the PCL-AP-RHA, TC, AP4 and physical mixture, demonstrating melting endotherm (second heating cycle), AP4: 1 % PVA, 1:5 TC:PCL-AP-RHA.

Hydrophilic character of the microspheres were investigated by water contact angle measurements (Figures 4.45, 4.46 and 4.47). Pure PCL is known to be highly hydrophobic which was also proved by WCA. Addition of RHA in the hybrid system changed the hydrophobic character of PCL owing to the hydroxyl groups on the surface of RHA. Thus, it becomes clear why PCL-AP-RHA was more hydrophobic since PCL grafting through hydroxyl groups was higher in PCL-AP-RHA hybrid system (18 %). Microspheres were highly hydrophilic, which is an advantageous feature for drug delivery systems especially in case of poor water-soluble drugs, such as trans-chalcone. Hydrophilic systems can also reduce the effect of opsonization process in the body so can avoid being removed from body circulation by reticuloendothelial system [193].

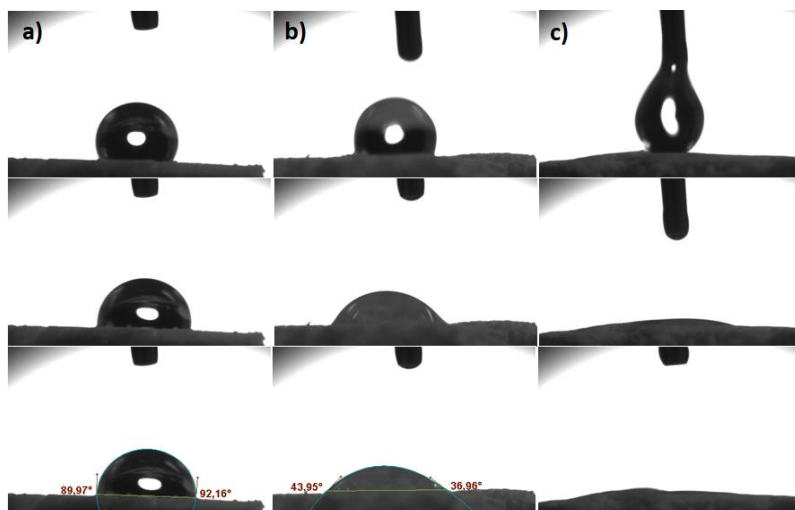


Figure 4.45 : Water contact angle a) APPCL, b) PCL-AP-RHA, c) AP4 microsphere.

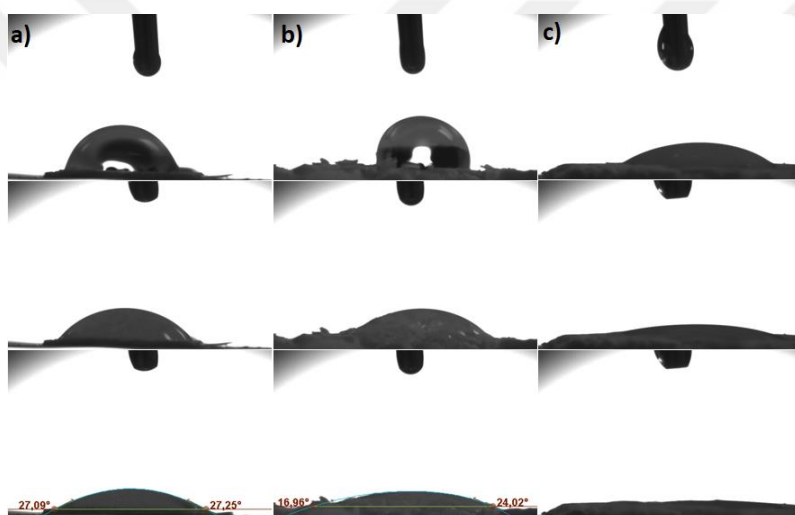


Figure 4.46 : Water contact angle a) APCL, b) PCL-A-RHA, c) A2 microsphere.

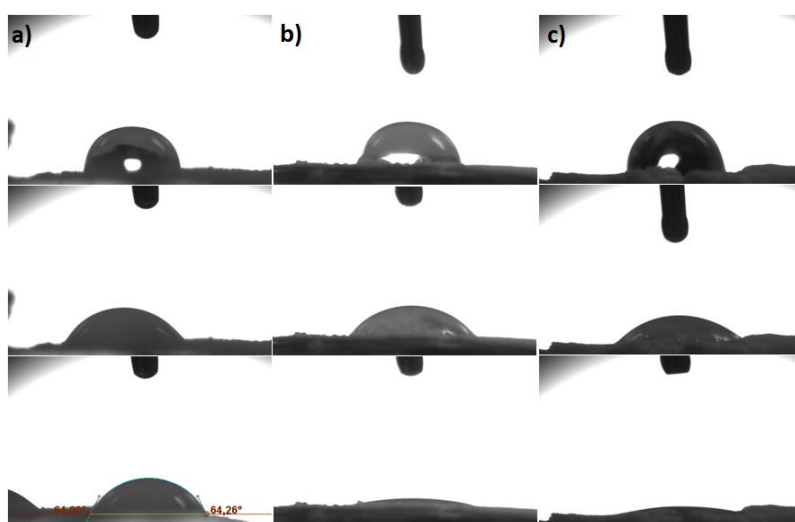


Figure 4.47 : Water contact angle a) GPCL, b) PCL-G-RHA, c) G1 microsphere.

4.3.5 Effect of silica incorporation in the polymer matrix

In order to understand the involvement of silica in the hybrid system in drug loading, TC encapsulation experiments were also conducted by using neat PCL synthesized with immobilized lipases. Encapsulation formulations were chosen to be the optimum concentrations of PVA and polymer in the external and internal phases. These concentrations were chosen as 0.5 % PVA and 1:10 drug/polymer ratio for PCL-A-RHA; 1 % PVA and 1:5 drug/polymer ratio for PCL-AP-RHA; 0.1 % PVA and 1:10 drug/polymer for PCL-G-RHA.

Encapsulation efficiencies were calculated to be higher when PCL-RHA nanohybrid systems were used as the polymer matrix (Table 4.9). The main two differences between hybrid system and the neat polymer are the crystallinity percentage and molecular weight. As mentioned previously, crystallinity percentages of PCL-AP-RHA, PCL-A-RHA and PCL-G-RHA were 39 %, 32 % and 23 %, respectively. On the other hand, crystallinity of neat PCL were much higher. A study investigating drug release behavior of PCL showed that decreasing molecular weight of the polymer increased encapsulation efficiency [177]. The presence of silica in the hybrid systems contributed to interactions between –OH of silanol group and PCL fraction in the nanohybrid system and α,β -unsaturated ketone group in the structure of TC and resulted in higher encapsulation efficiencies [194, 195].

Table 4.9 : Comparison of encapsulation efficiencies (mean $\pm$ SD, n=3).

	A2	APCL	AP4	APPCL	G1	GPCL
Polymer	PCL-A-RHA	Neat A-PCL	PCL-AP-RHA	Neat AP-PCL	PCL-G-RHA	Neat G-PCL
Encapsulation Efficiency (%) ^a	60 $\pm$ 5	42 $\pm$ 5	64 $\pm$ 4	57 $\pm$ 9	74 $\pm$ 6	61 $\pm$ 10
Loading Capacity (%) ^a	5.4 $\pm$ 0.4	3.8 $\pm$ 0.4	10.7 $\pm$ 0.6	9.4 $\pm$ 1.3	6.9 $\pm$ 0.8	5.5 $\pm$ 0.7
Mean Particle Diameter (μ m) ^b	29 $\pm$ 6	18 $\pm$ 4	21 $\pm$ 4	28 $\pm$ 4	20 $\pm$ 4	24 $\pm$ 5

^a mean $\pm$ SD; n=3.

^b Determined by optical microscope images (mean $\pm$ SD; n=20).

Drug release amount did not seem to be affected significantly by the addition of silica in the polymer; nanohybrid systems having slightly more sustained release profiles

with respect to pure PCL. This slight difference may be the result of several factors cancelling out each other's effects: (1) High molecular weight of pure PCL increased release rate, (2) Low crystallinity of the hybrid systems increased release rate and (3) Hydrophilicity of the hybrid systems enhanced drug release.

Figure 4.48 shows the cumulative release profiles of the PCL-G-RHA and neat GPCL microspheres. The cumulative release of the two microsphere formulations were the same (58 ± 11 and 58 ± 9 % cumulative release for PCL-G-RHA and GPCL, respectively). However, the burst release from PCL-G-RHA microspheres lasted for 4 h and reached to 14 ± 1 % cumulative release. The burst release of the neat GPCL microspheres on the other hand was completed at the end of 6th h of the release, with 16 ± 2 % cumulative release (Figure 4.49). In addition, TC release from the PCL-G-RHA microspheres completed earlier than neat GPCL microspheres. This delayed release and burst release behavior of neat GPCL microspheres were due to the hydrophobicity of the polymer, which slows down the diffusion of water and drug molecules through the microsphere surface [177].

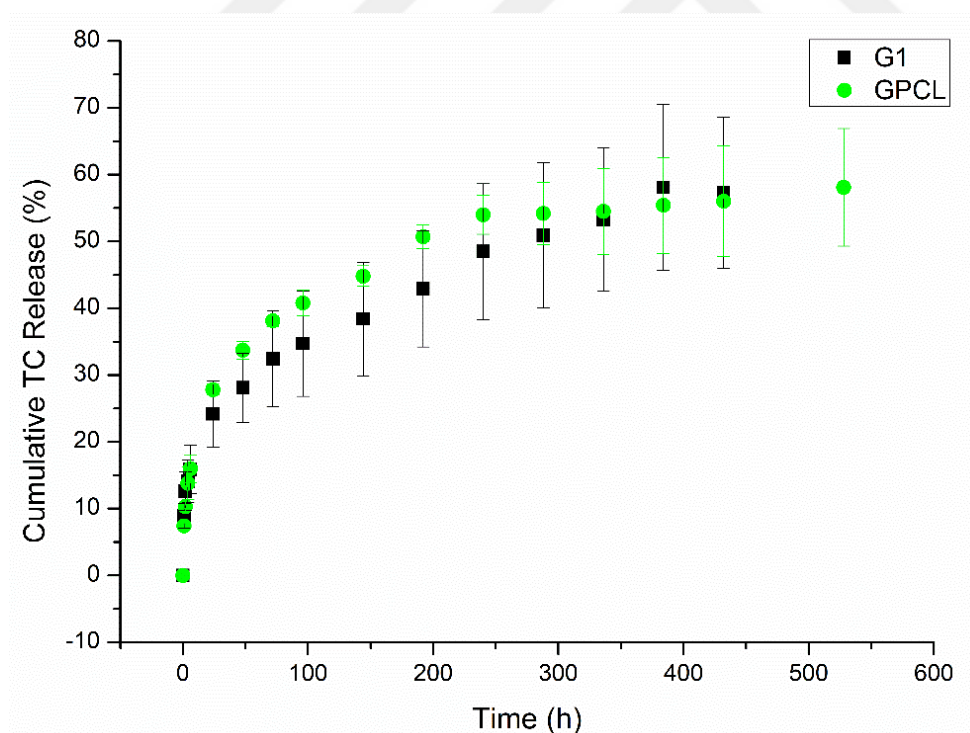


Figure 4.48 : Effect of silica incorporation on cumulative TC release (TC amount in the internal phase was fixed at 10 mg; 0.1 % PVA and 1:10 TC/polymer ratio; G1: PCL-G-RHA; GPCL: neat GPCL).

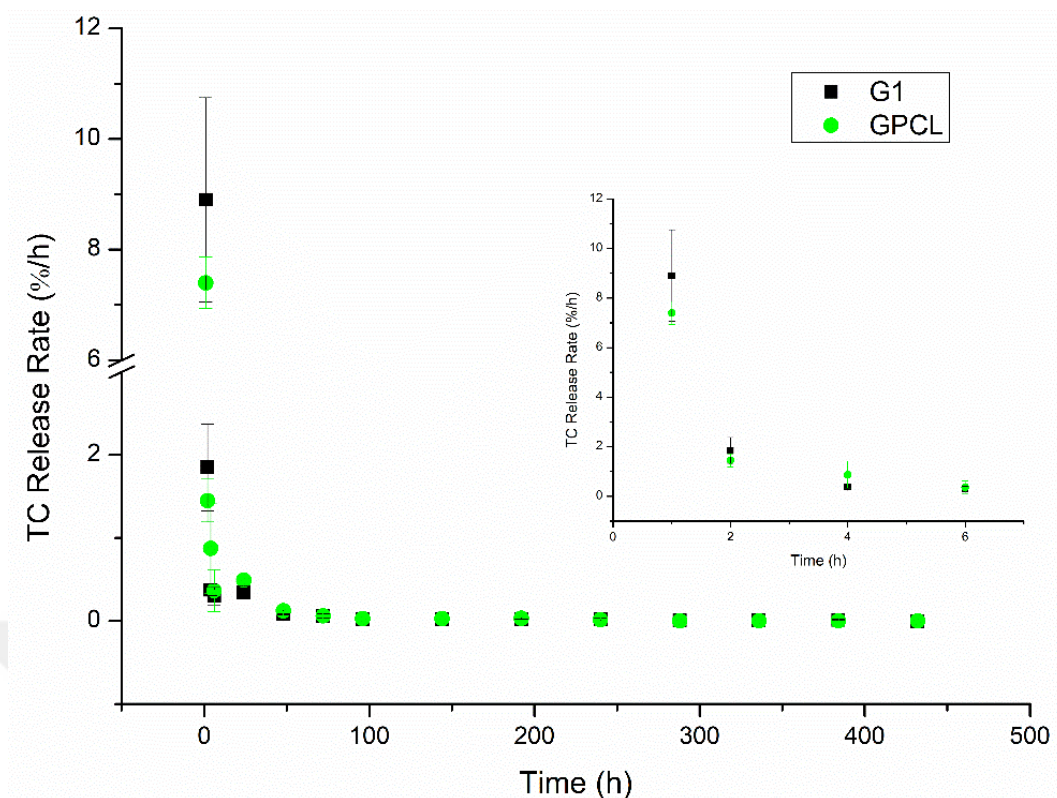


Figure 4.49 : Effect of silica incorporation on TC release rate (TC amount in the internal phase was fixed at 10 mg; 0.1 % PVA and 1:10 TC/polymer ratio; G1: PCL-G-RHA; GPCL: neat GPCL).

The cumulative release and the TC release rate of the PCL-A-RHA and neat APCL microspheres did not show significant difference (Figure 4.50 and 4.51). The cumulative release of PCL-A-RHA and neat APCL microspheres were 65 ± 14 and 66 ± 14 %, respectively. Both of the microsphere formulations completed TC release in 384 h – 16 days. The release rate of both microsphere formulations stabilized within 2 h, having completed 16 ± 6 % (PCL-A-RHA microspheres) and 14 ± 0 % (neat APCL microspheres) cumulative release.

Moreover, neat APCL microspheres facilitated TC release compared to neat GPCL microspheres due to its higher hydrophilicity demonstrated in WCA measurements (WCA of neat APCL was calculated as $\sim 27^\circ$ whereas WCA of neat GPCL was calculated as $\sim 64^\circ$).

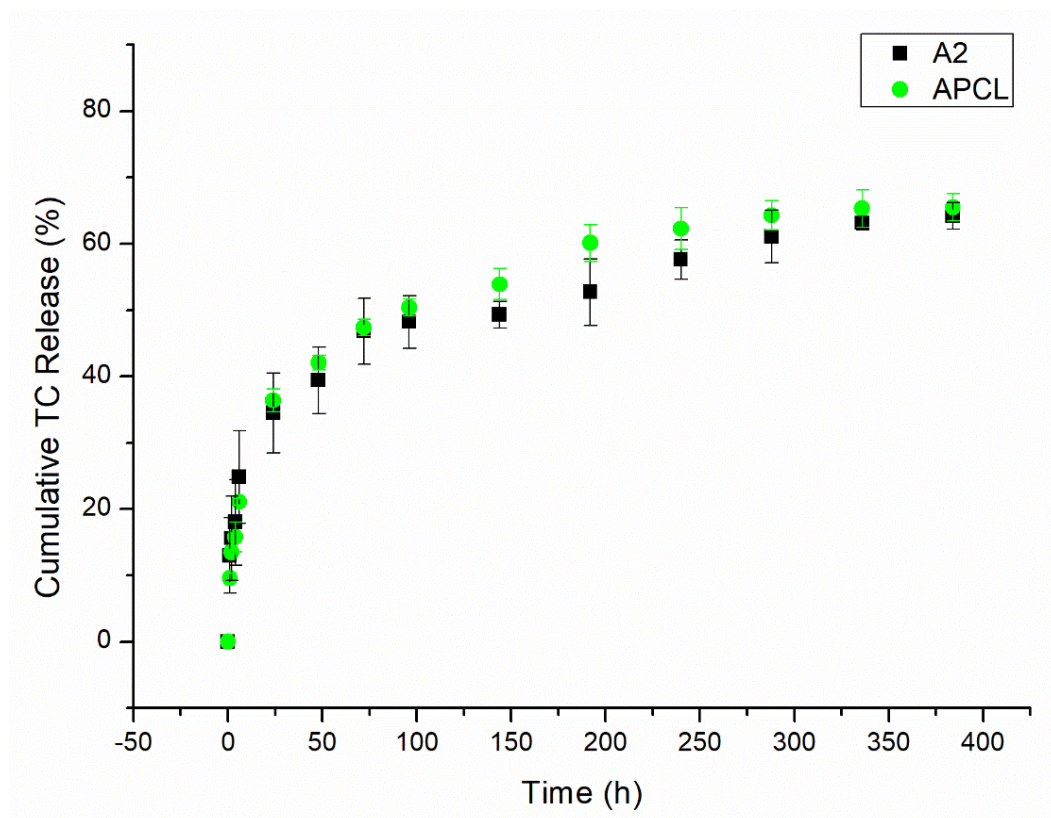


Figure 4.50 : Effect of silica incorporation on cumulative TC release (TC=10 mg; 0.5 % PVA and 1:10 TC/polymer ratio; A2: PCL-A-RHA; APCL: neat A-PCL).

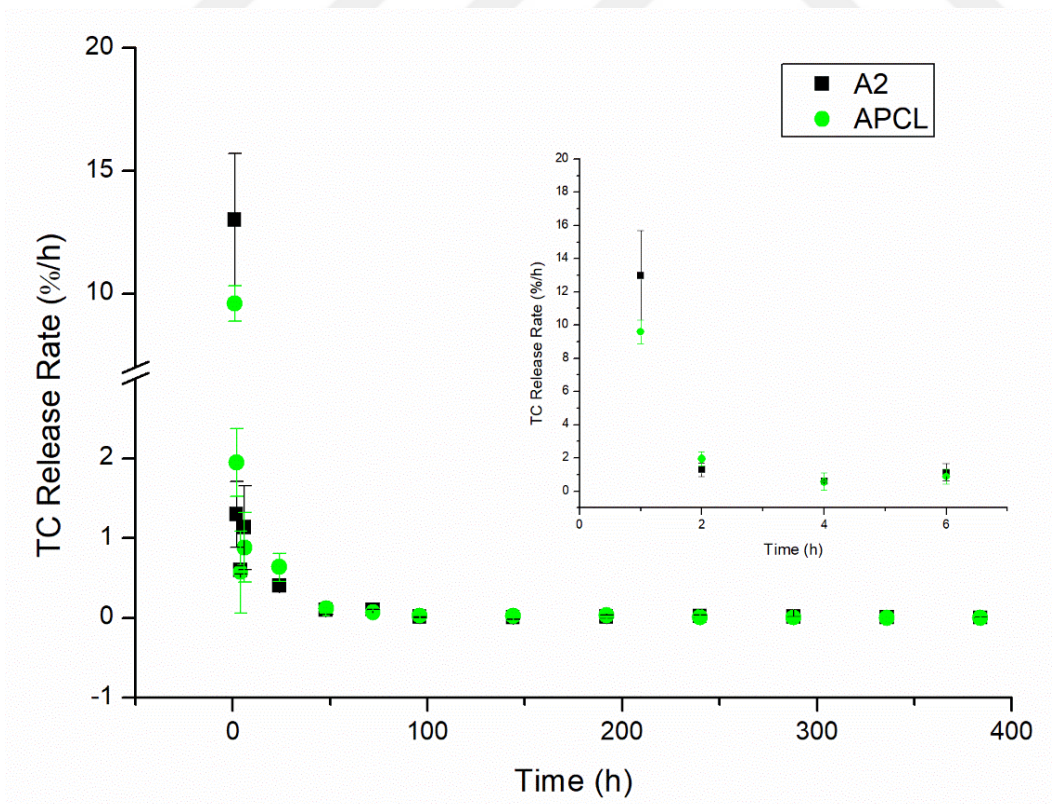


Figure 4.51 : Effect of silica incorporation on TC release rate (TC=10 mg; 0.5 % PVA and 1:10 TC/polymer ratio; A2: PCL-A-RHA; APCL: neat A-PCL).

The release profiles of the PCL-AP-RHA and neat APPCL microspheres differed from each other as seen in Figure 4.52. The release time of the neat APPCL microspheres (1368 h – 57 days) was much longer than the release time of the PCL-AP-RHA microspheres (888 h – 37 days) although the final cumulative release of both of the microsphere formulations were approximately the same (AP4: 61 ± 7 % and APPCL: 62 ± 13 % cumulative release). Higher crystallinity and hydrophobicity of the neat APPCL retarded the penetration of release medium to the microspheres since this phenomenon happens through the amorphous regions and it promotes drug diffusion through the microspheres [177, 196].

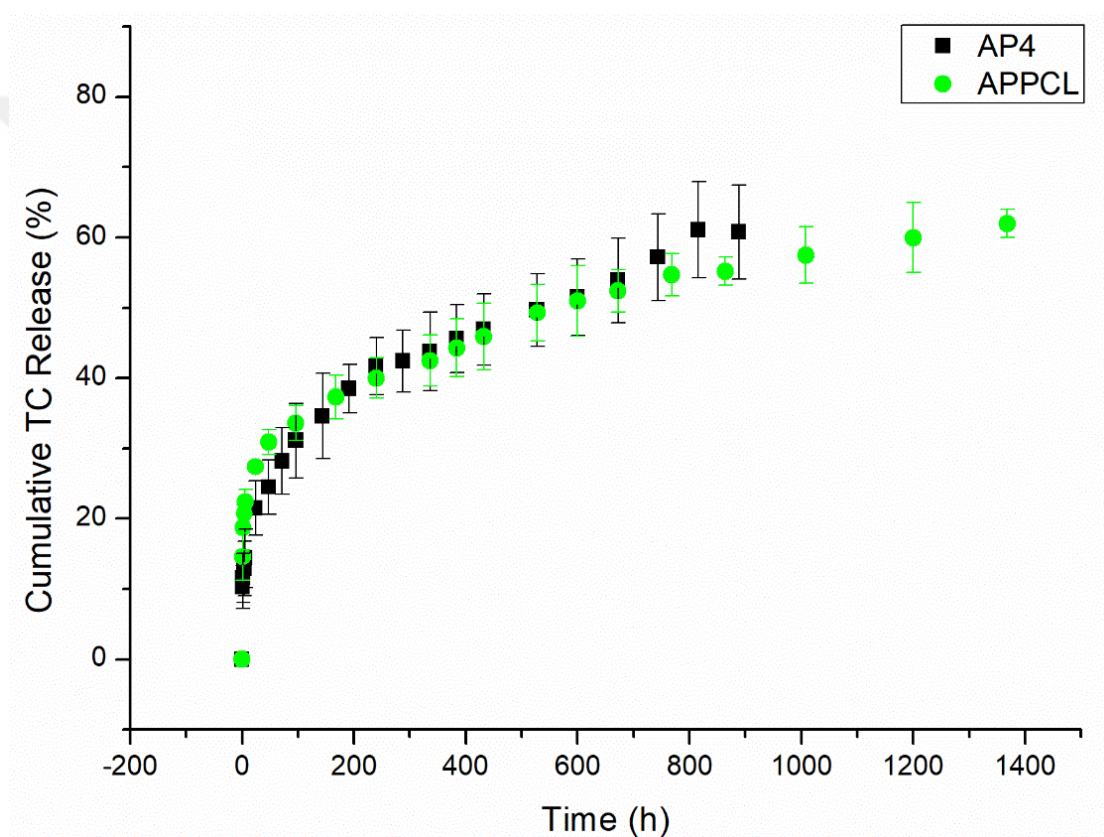


Figure 4.52 : Effect of silica incorporation on cumulative TC release (TC=10 mg; 1 % PVA and 1:5 TC/polymer ratio; AP4: PCL-A-RHA; APPCL: neat AP-PCL).

As given in Figure 4.53, initial release rate of the neat APPCL microspheres (15 ± 4 % cumulative release) were higher than the initial release rate of the PCL-AP-RHA microspheres (10 ± 3 % cumulative release). The crystallinity of the neat APPCL was measured as 60 % whereas the crystallinity of the PCL-AP-RHA was 39 %. This difference between the crystallinity percentages of the polymers created the different initial release rates of the two microspheres. High crystalline polymers have a tendency to localize the drug molecules on the surface of the microspheres which affects the

burst release behavior [40]. The constant-rate phase was reached in 4 h for neat APPCL microspheres completing 21 ± 2 % cumulative release. The same stable phase for PCL-AP-RHA microspheres was obtained in 2 h with 12 ± 4 % cumulative release.

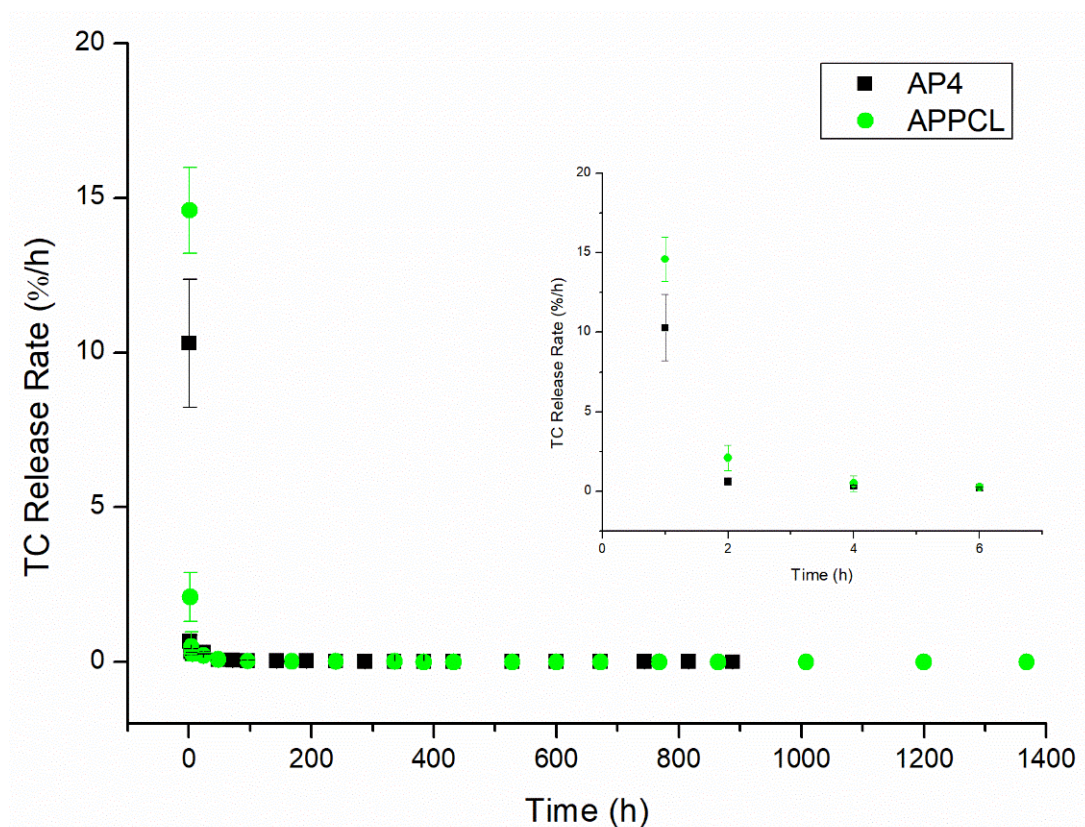


Figure 4.53 : Effect of silica incorporation on TC release rate (TC=10 mg; 1 % PVA and 1:5 TC/polymer ratio; AP4: PCL-A-RHA; APPCL: neat AP-PCL).

Several kinetic models were fitted to the release profiles of neat PCL microspheres to determine the dominant release mechanism and make a comparison with the release from nanohybrid polymer microspheres. The calculated parameters of each kinetic model were given in Table 4.10. Similar to nanohybrid microspheres, release profiles of the neat PCL microspheres best fitted to Korsmeyer-Peppas kinetic model. GPCL, APCL and APPCL microspheres released the drug by Fickian diffusion. The rate constants of Korsmeyer-Peppas model (K_{KP}) revealed differences in initial release amounts of the microspheres. GPCL and APCL microspheres had higher release amounts at the beginning of the release time with respect to G1 and A2. On the other hand, APPCL released higher amount of drug at time zero than AP4 formulation.

Table 4.10 : Kinetic model parameters calculated for neat PCL microspheres.

Code	Korsmeyer-Peppas			Higuchi		Zero Order		First Order	
	K_{KP} (h^{-n})	n	R^2	K_H ($h^{-0.5}$)	R^2	K_0 (h^{-1})	R^2	K_1 (h^{-1})	R^2
G1	0.161	0.30	0.995	2.59	0.977	0.125	0.871	0.004	0.718
GPCL	0.149	0.33	0.983	2.47	0.917	0.098	0.728	0.003	0.581
A2	0.210	0.27	0.983	2.88	0.921	0.133	0.755	0.004	0.588
APCL	0.160	0.35	0.986	3.21	0.919	0.147	0.738	0.004	0.595
AP4	0.153	0.27	0.994	1.78	0.970	0.056	0.840	0.002	0.671
APPCL	0.245	0.18	0.986	1.29	0.923	0.030	0.752	0.001	0.549

Optical microscopy and SEM images of the neat PCL microspheres revealed the spherical geometry of the particles as shown in Figure 4.54. The similar rough surface structure was observed neat PCL microspheres which is characteristic property of atmospheric solvent evaporation method. Surface pores of the neat PCL microspheres seemed to be lowered compared to nanohybrid microspheres.

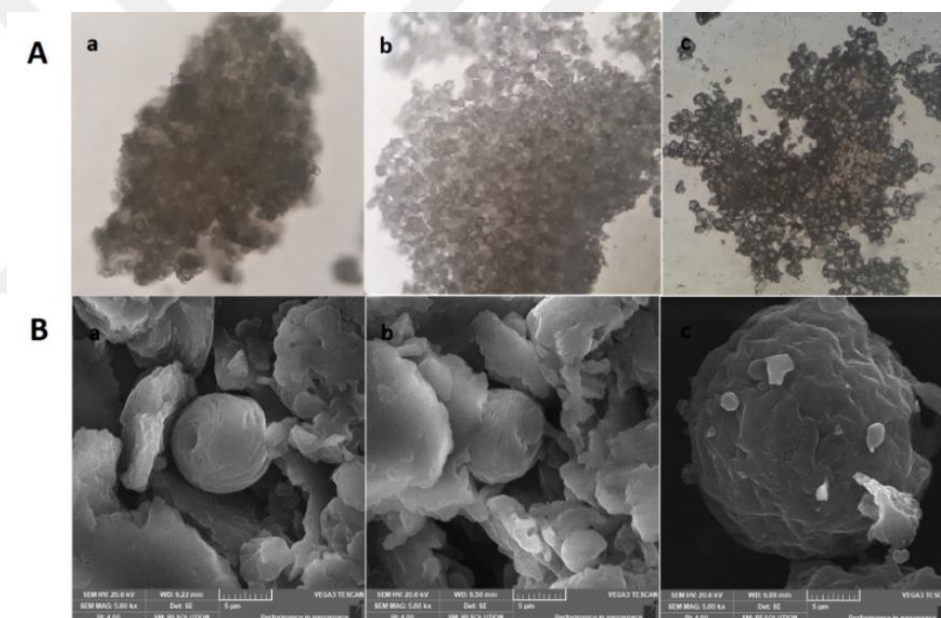


Figure 4.54 : A) Optical microscopy images of a) APCL, b) GPCL, c) APPCL (Magnification 40x); B) SEM images of a) APCL, b) GPCL, c) APPCL (Magnification 3000x).

The neat PCL microspheres were structurally characterized by FT-IR in order to observe the presence of the components in the loaded spheres and the structural differences between nanohybrid and neat PCL microspheres (Figure 4.55-4.60). As G1 microspheres GPCL microspheres also the characteristic peaks of PCL and TC. The two characteristic signals were observed belonging to TC; α , β unsaturation in ketones at 1662 cm^{-1} and a slight peak at $3100\text{-}3200\text{ cm}^{-1}$ of aromatic $=\text{C-H}$ stretching, respectively. Moreover, peaks at $1425\text{-}1471\text{ cm}^{-1}$ and 1610 cm^{-1} assigned to aromatic

C-C and aromatic C=C stretching proved the presence of TC in the microsphere structure. The main signals of PCL were at 1726 cm^{-1} (aliphatic carbonyl bond), 2868 cm^{-1} (symmetric CH_2) and 2949 cm^{-1} (asymmetric CH_2), respectively. Additional peaks belonging to PCL were also detected at 1193 cm^{-1} , 1245 cm^{-1} and 1297 cm^{-1} belonging to C-O and C-C asymmetric stretch, C-O-C asymmetric stretch and C-O-C symmetric stretch, respectively. In contrast to G1 microspheres, GPCL microsphere spectrum did not revealed peak at 800 cm^{-1} which was assigned to Si- CH_3 rocking in the structure RHA. This finding was expected since neat G-PCL did not contain RHA in its structure. Moreover, CH_2 wagging belonging to oxirane ring at approximately 1150 cm^{-1} was not present in the spectra of both GPCL and the physical mixture of the formulation.

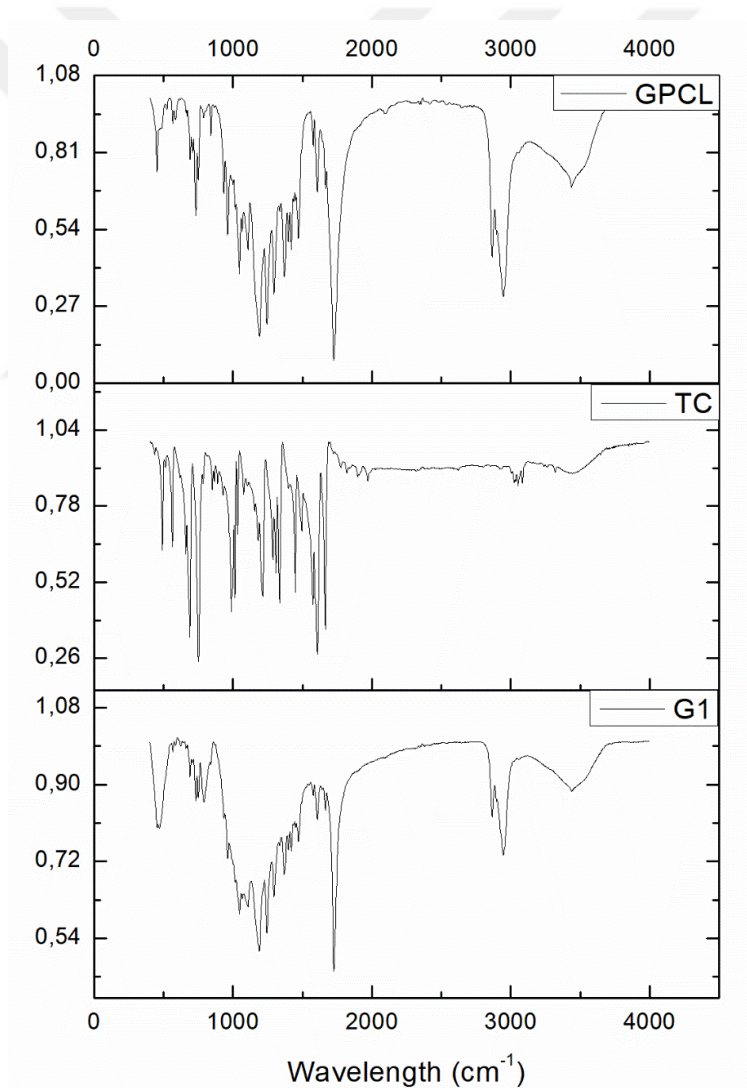


Figure 4.55 : FT-IR spectra of GPCL, G1 and TC (GPCL: 0.1 % PVA, 1:10 TC:G-PCL; G1: 0.1 % PVA, 1:10 TC:PCL-G-RHA; TC: Trans-chalcone).

Similar to the spectrum of G1 microsphere formulation, the spectrum of GPCL microsphere formulation did not contain the signal of free –OH groups of PCL whereas the signal was detected in the spectrum of the physical mixture of the formulation at 3536 cm^{-1} . However, there was no shifts observed in the peak of α , β unsaturation of TC which was the functional group associated with the drug/polymer interactions earlier due to its electrophilic character. The nonexistence of such shift in the peak of α , β unsaturation of TC shows that no chemical interactions have taken place between G-PCL and TC and TC molecules were entrapped in the microspheres without any significant change in its structure and stability [178]. The disappearance of the free –OH peak may be due to intramolecular H-bonding in PCL structure.

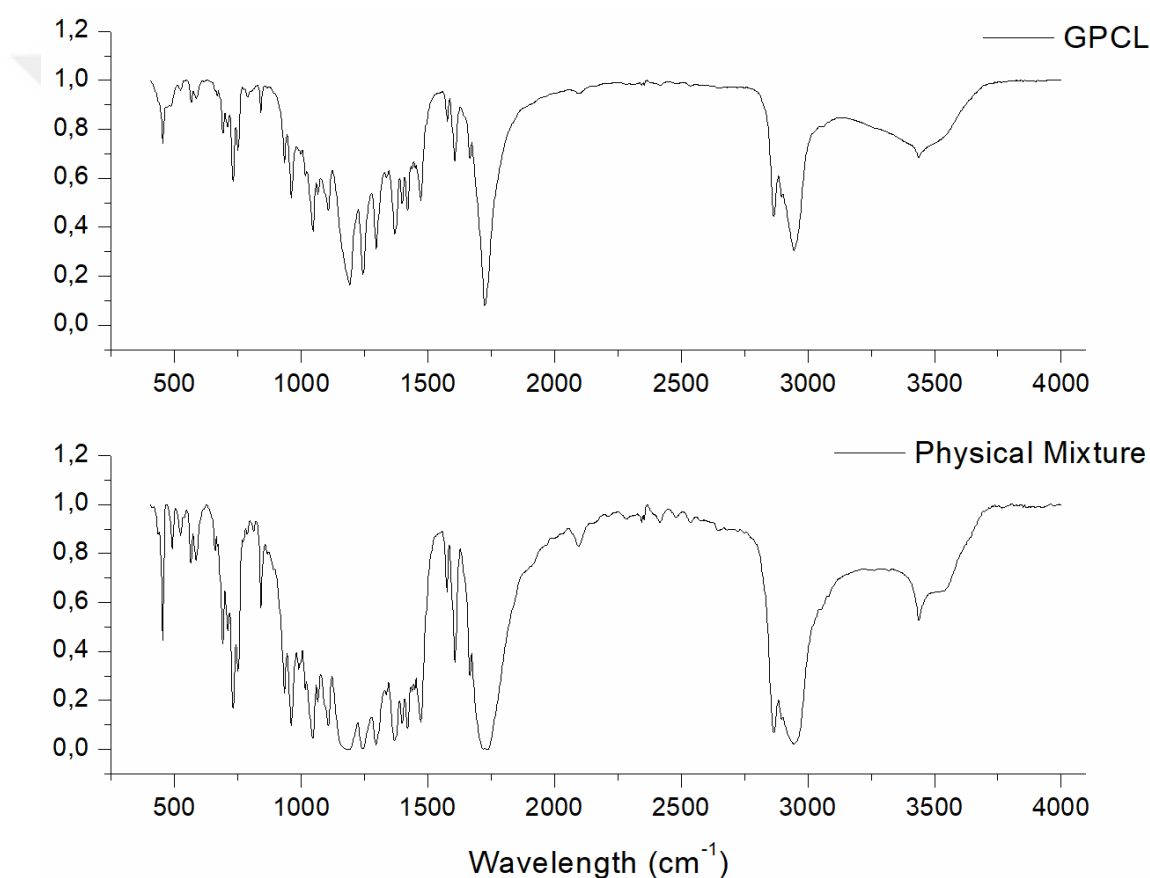


Figure 4.56 : FT-IR spectra of GPCL microspheres and the physical mixture of G-PCL and TC (GPCL: 0.1 % PVA, 1:10 TC:G-PCL; Physical mixture: Mixture of 1:10 (w:w) TC:G-PCL).

As presented in Figure 4.57 APCL microspheres TC and PCL characteristic peaks are clearly visible. Peaks at 1452 cm^{-1} (aromatic C-C stretching), 1606 cm^{-1} (aromatic C=C stretching), 1665 cm^{-1} (α , β unsaturation in ketones) and the slight peak at 3100 cm^{-1} (aromatic =C-H stretching) are the signs of the presence of TC. The main signals of PCL were at 1726 cm^{-1} (aliphatic carbonyl bond), 2868 cm^{-1} (symmetric CH_2) and

2949 cm^{-1} (asymmetric CH_2), respectively. PCL peaks were also observed at 1193 cm^{-1} , 1245 cm^{-1} and 1297 cm^{-1} belonging to C-O and C-C asymmetric stretch, C-O-C asymmetric stretch and C-O-C symmetric stretch, respectively. In contrast to A2 microspheres, in the spectrum of APCL microsphere peak at around 800 cm^{-1} was not observed, which was assigned to Si- CH_3 rocking in the structure RHA. Additionally the peak at 1050 cm^{-1} which is caused by asymmetric stretching of Si-O-Si was not visible in the spectrum of APCL. This finding was expected since neat A-PCL did not contain RHA in its structure.

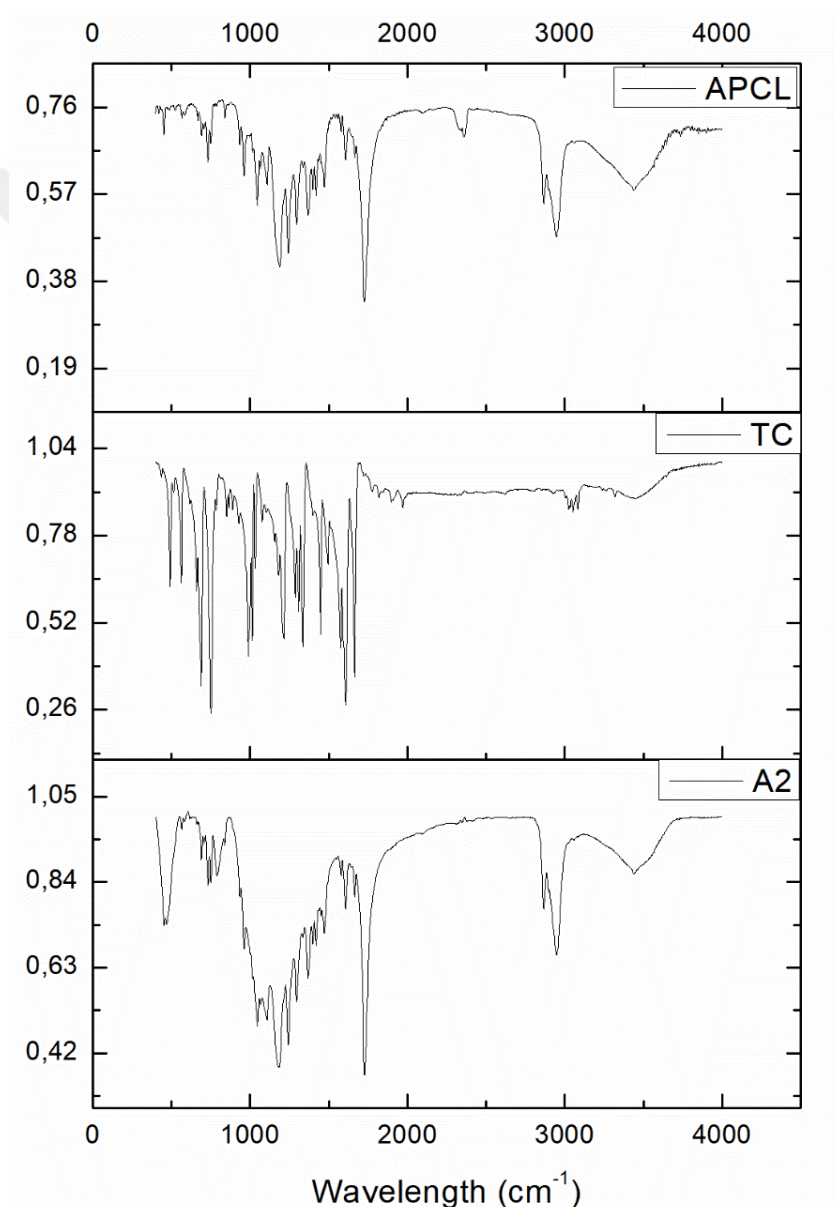


Figure 4.57 : FT-IR spectra of APCL, A2 and TC (APCL: 0.5 % PVA, 1:10 TC:A-PCL; A2: 0.5 % PVA, 1:10 TC:PCL-A-RHA; TC: Trans-chalcone).

The FT-IR spectrum of the physical mixture of A-PCL and TC revealed differences in the signals. A shift to higher wavelength was observed in the signal of α, β unsaturation of TC. In the spectrum of APCL microsphere formulation the mentioned peak was at 1665 cm^{-1} whereas in the spectrum of the physical mixture of this formulation it was detected at 1660 cm^{-1} . Moreover, free $-\text{OH}$ band disappeared in the spectrum of APCL microsphere formulation which was visible in the spectrum of the physical mixture. As discussed before these changes may be the indication of drug/polymer interaction between the mentioned functional groups.

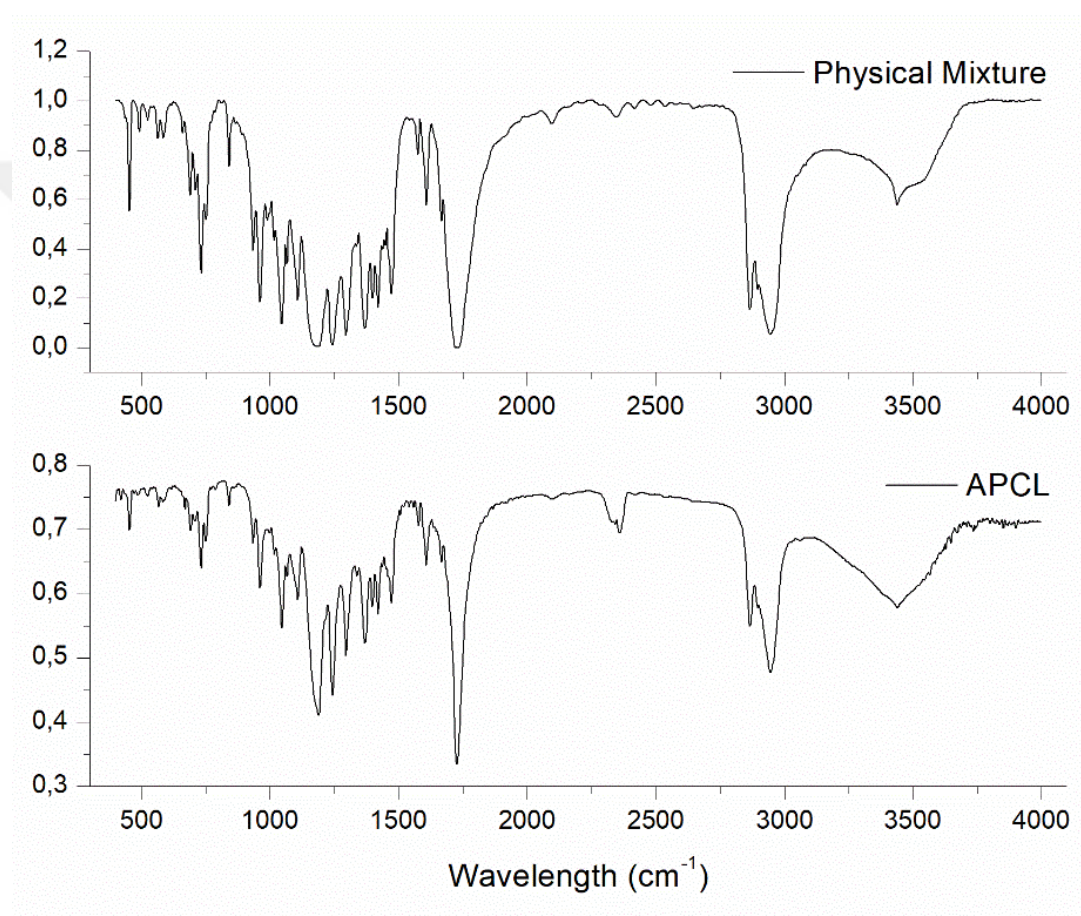


Figure 4.58 : FT-IR spectra of APCL microspheres and the physical mixture of A-PCL and TC (APCL: 0.5 % PVA, 1:10 TC:A-PCL; Physical mixture: Mixture of 1:10 (w:w) TC:A-PCL).

Figure 4.59 and Figure 4.60 show the FT-IR spectra of APPCL microspheres and the physical mixture of AP-PCL and TC. In Figure 4.55, characteristic peaks of PCL and TC mentioned previously were observed in the spectrum of APPCL microspheres. Peaks at 1469 cm^{-1} (aromatic C-C stretching), 1603 cm^{-1} (aromatic C=C stretching), 1667 cm^{-1} (α, β unsaturation in ketones) and the slight peak at 3100 cm^{-1} (aromatic $=\text{C}-\text{H}$ stretching) are the signs of the presence of TC. The main signals of PCL were at

1727 cm^{-1} (aliphatic carbonyl bond), 2867 cm^{-1} (symmetric CH_2) and 2944 cm^{-1} (asymmetric CH_2), respectively. PCL peaks were also observed at 1187 cm^{-1} , 1246 cm^{-1} and 1295 cm^{-1} belonging to C-O and C-C asymmetric stretch, C-O-C asymmetric stretch and C-O-C symmetric stretch, respectively. This meant that two main component of the APPCL microsphere were actually PCL and TC.

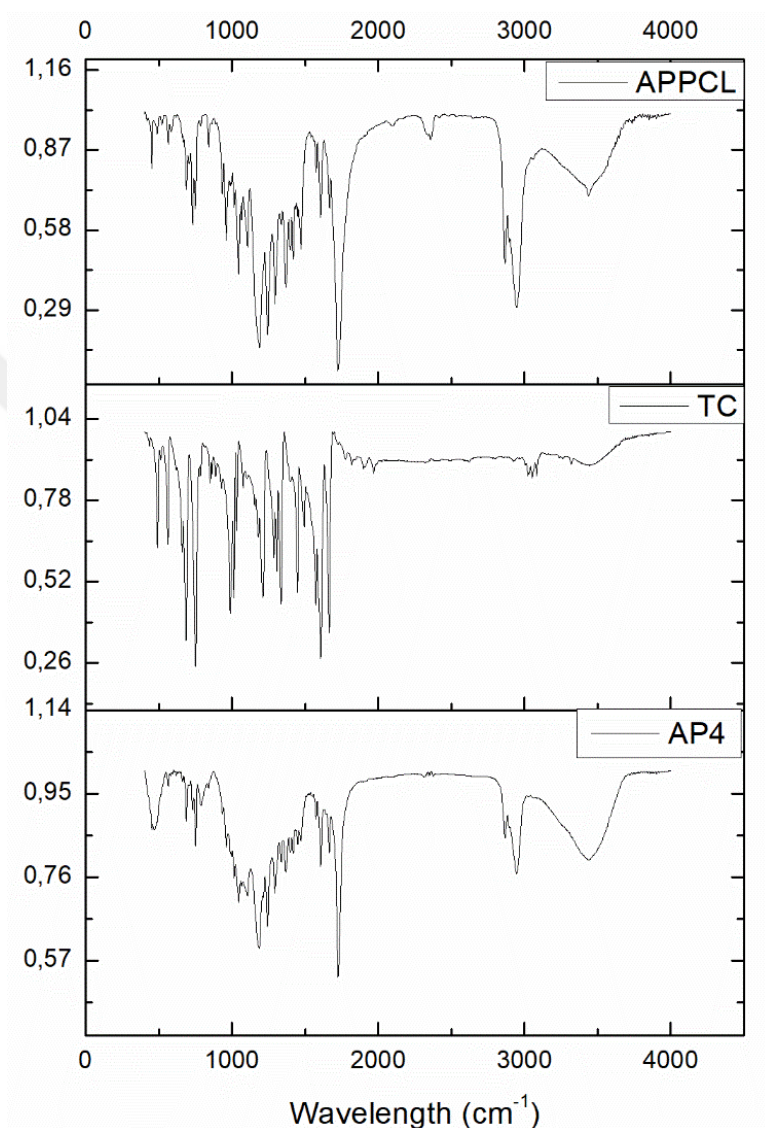


Figure 4.59 : FT-IR spectra of APPCL, AP4 and TC (APPCL: 1 % PVA, 1:5 TC:AP-PCL; AP4: 1 % PVA, 1:5 TC:PCL-AP-RHA; TC: Trans-chalcone).

The physical mixture of this formulation did not differ from the microsphere form structurally as the FT-IR spectrum in Figure 4.60 did not reveal any significant changes or shifts in the characteristic peaks of TC and PCL. This means that the TC molecules were encapsulated in the microspheres of neat AP-PCL polymer without undergoing structural changes. The TC molecules in the microspheres did not involve in an interaction with AP-PCL.

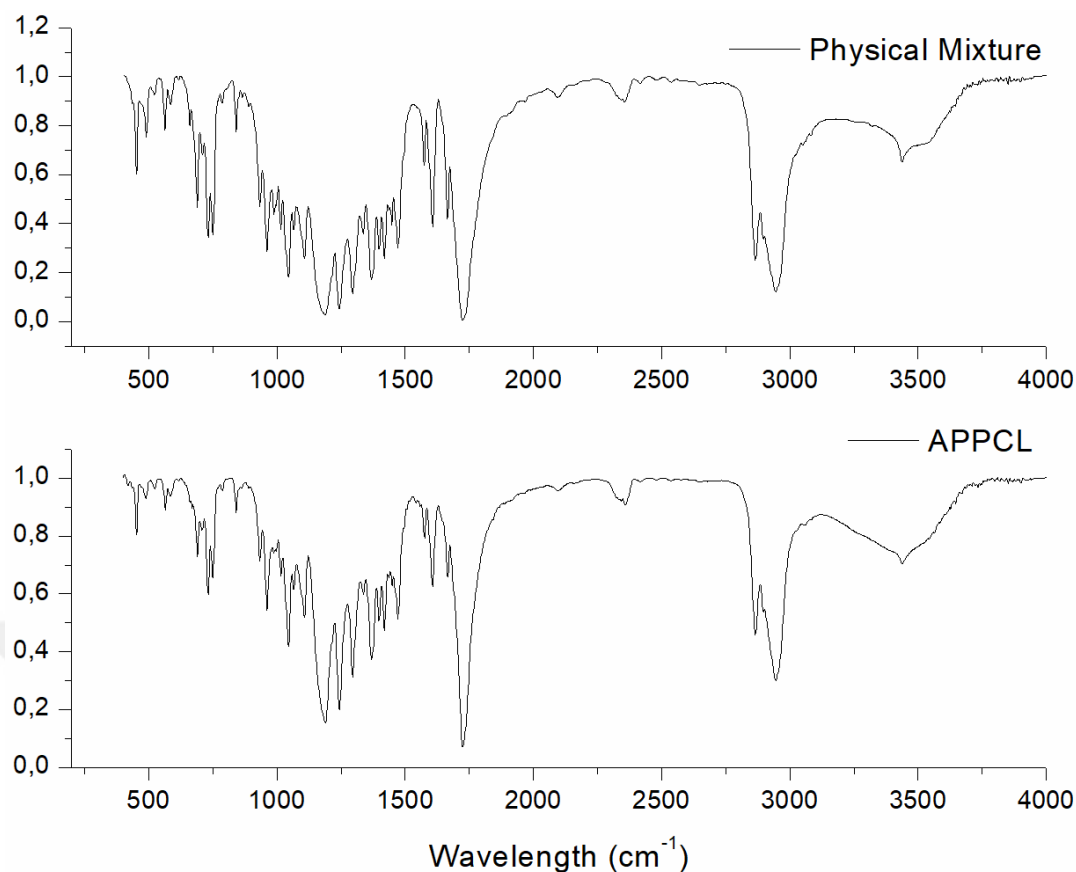


Figure 4.60 : FT-IR spectra of APPCL microspheres and the physical mixture of AP-PCL and TC (APPCL: 1 % PVA, 1:5 TC:AP-PCL; Physical mixture: Mixture of 1:5 (w:w) TC:AP-PCL).

The melting endotherm of GPCL microspheres showed two peaks at 50 °C and 54 °C, similar to the physical mixture of this formulation which showed two endothermic peaks at 46 °C and 51 °C (Figure 4.61). The melting endotherms of GPCL formulation and the physical mixture of this formulation did not significantly changed which supported the idea of TC is encapsulated in the microspheres as its crystalline form [197]. As discussed earlier in this section, TC molecules were dispersed within the polymer matrix in the case of microspheres of PCL-G-RHA hybrid (G1). This dispersion was the result of hydrogen bonding and ionic interactions between TC molecules and the functional group of PCL-G-RHA hybrid polymer which was oxirane ring. The difference in loading mechanism of PCL-G-RHA hybrid polymer and neat G-PCL polymer was due to the difference in the functionalities of the two polymer; neat G-PCL did not have any functional group in its structure to interact with TC molecules.

Similar findings were obtained for APCL microspheres and the physical mixture of this formulation. As given in Figure 4.62, the melting endotherms of the two mentioned samples displayed two peaks belonging to A-PCL polymer and TC. As in GPCL microspheres, TC was encapsulated in the APCL microspheres in its crystalline form. This result was also obtained by investigating FT-IR spectra of the related samples as presented previously.

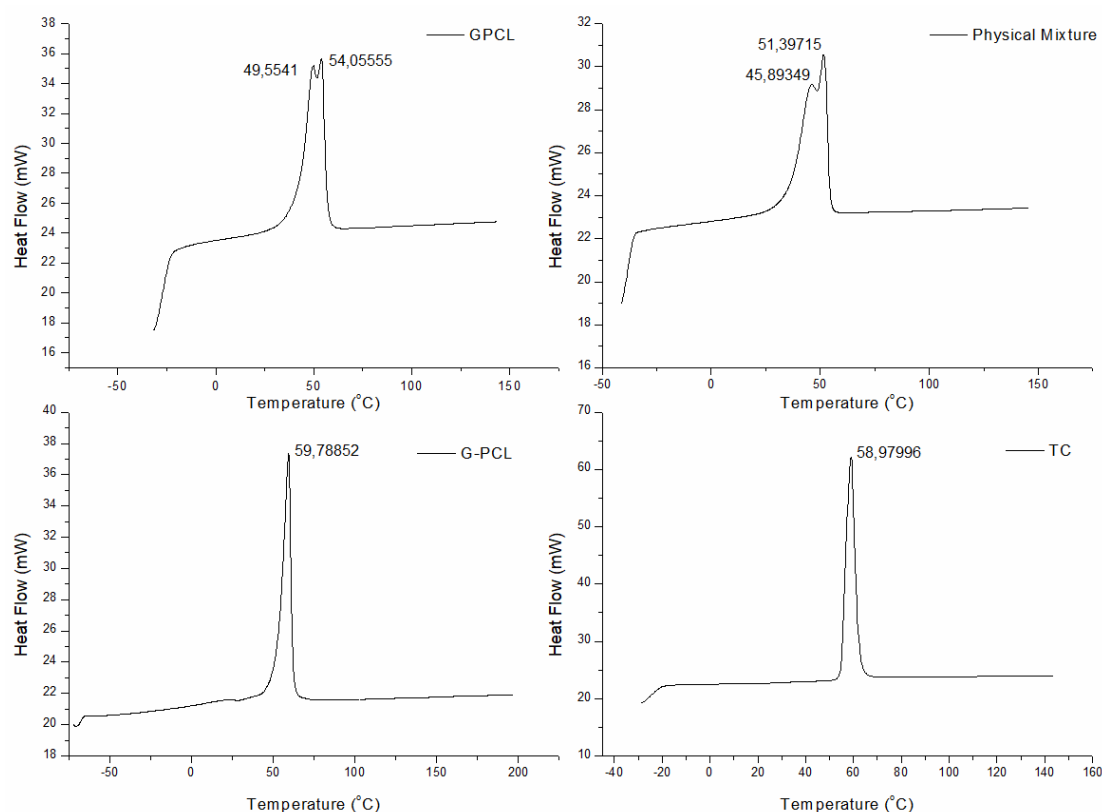


Figure 4.61 : DSC thermogram of the G-PCL, TC, GPCL and physical mixture, demonstrating melting endotherm (second heating cycle), GPCL: 0.1 % PVA, 1:10 TC:G-PCL; G-PCL: neat PCL enzymatically synthesized with CALB immobilized onto G-RHA.

TC molecules did not disperse within the neat AP-PCL matrix. Rather they were encapsulated without any structural change in its crystal form, as in the case of GPCL and APCL microspheres. This conclusion was derived from the twin endothermic peaks observed in the thermograms of APPCL and the physical mixture, belonging to TC and AP-PCL (Figure 4.63).

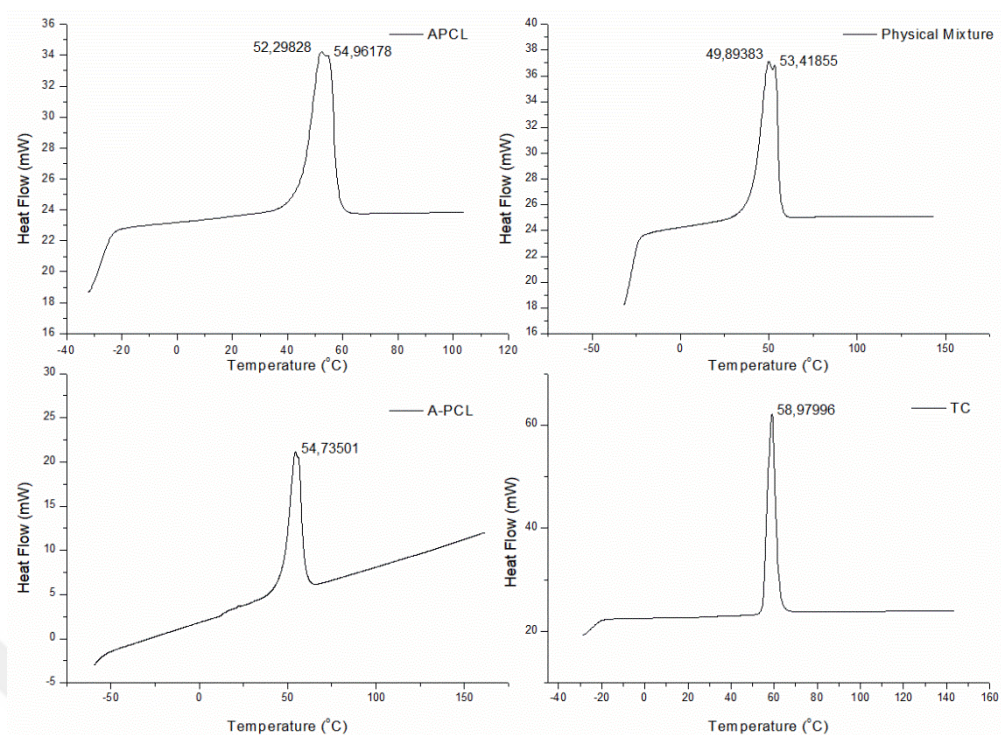


Figure 4.62 : DSC thermogram of the A-PCL, TC, APCL and physical mixture, demonstrating melting endotherm (second heating cycle), APCL: 0.5 % PVA, 1:10 TC:A-PCL; A-PCL: neat PCL enzymatically synthesized with CALB immobilized onto A-RHA.

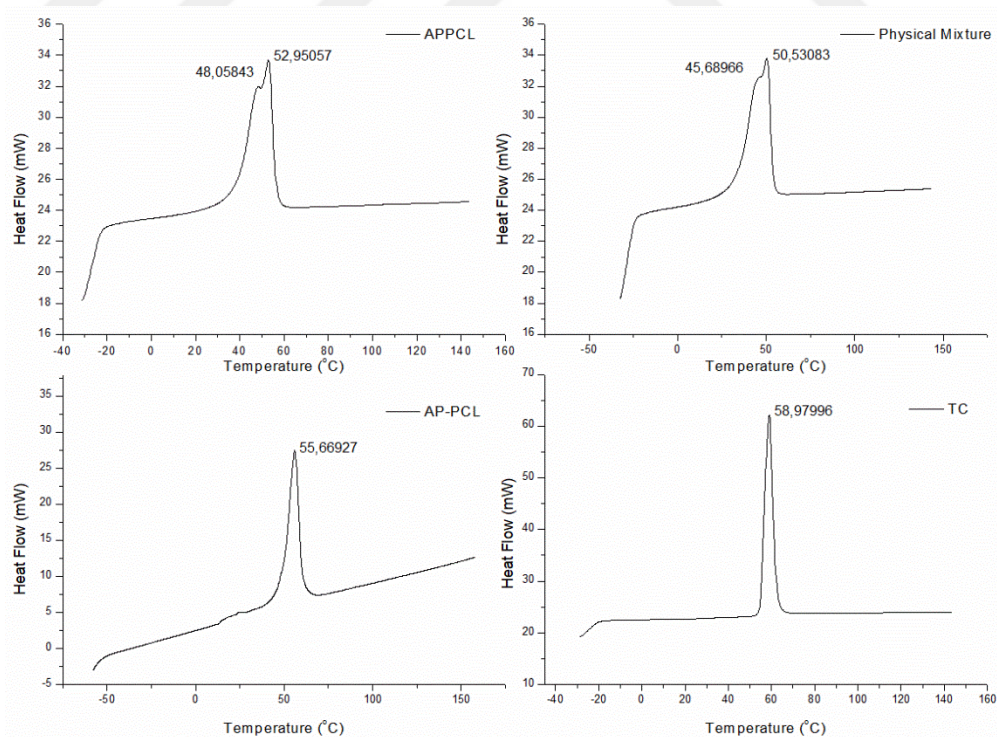


Figure 4.63 : DSC thermogram of the AP-PCL, TC, APPCL and physical mixture, demonstrating melting endotherm (second heating cycle), APPCL: 1 % PVA, 1:5 TC:AP-PCL; AP-PCL: neat PCL enzymatically synthesized with CALB immobilized onto AP-RHA.

4.3.6 Effect of the loaded TC

TC loading studies were conducted by using both commercially available TC and chemically synthesized TC. Encapsulation efficiencies, loading capacities and mean particle diameters related to synthesized TC was given in Table 4.11. Encapsulation efficiency of chemically synthesized TC into PCL-A-RHA hybrid was significantly higher than that of PCL-G-RHA hybrid. Encapsulation of CS-TC was better when the polymer shell was constructed from PCL-A-RHA and PCL-AP-RHA hybrids and was inefficient when the shell was PCL-G-RHA. It is possible that interactions between the end groups of the hybrid systems and the chalcones show differences. PCL-A-RHA and PCL-AP-RHA nanohybrid systems have amino end groups in their structures whereas PCL-G-RHA nanohybrid system has epoxy ring, which interact with the chalcones differently. If the drug to be encapsulated has strong interactions with the polymer shell, the encapsulation efficiency is likely to be higher [198]. Thus, we comment that amino end groups of PCL-A-RHA and PCL-AP-RHA hybrids interact with the synthesized trans-chalcone more strongly than they interact with the commercial one. This might be caused by the different crystallization behavior of the two trans-chalcones as DSC thermograms revealed. It is also possible to assert that the interactions between the hybrids and commercial trans-chalcone was dominated by the ionic interactions yielding higher encapsulation efficiency for PCL-G-RHA hybrid with higher amount of free –OH groups on its surface (4 % PCL grafting). On the other hand, the interactions between the hybrids and synthesized trans-chalcone was mainly the result of hydrophobic interactions. This yielded high encapsulation efficiency values for more hydrophobic PCL-A-RHA and PCL-AP-RHA hybrids (shown by WCA measurements).

Table 4.11 : Comparison of chalcone encapsulation (mean±SD, n=3).

	PCL-A-RHA		PCL-AP-RHA		PCL-G-RHA	
	TC	CS-TC	TC	CS-TC	TC	CS-TC
Microspheres	A2	ACH	AP4	APCH	G1	GCH
Encapsulation Efficiency (%) ^a	60±5	84±7	53±4	71±5	74±6	58±8
Loading Capacity (%) ^a	5.4±0.4	7.6±0.6	10.7±0.6	10.3±2	6.9±0.8	5.3±0.6
Mean Particle Diameter (µm) ^b	29±6	18±4	21±4	29±4	20±4	26±6

TC: trans-chalcone (purchased)

CS-TC: trans-chalcone (synthesized)

^a mean±SD; n=3.

^b Determined by optical microscope images (mean±SD; n=20).

The result of the release studies were given in the following figures (Figure 4.64-4.69). What stands out in these figures is that the release of chemically synthesized TC from the microspheres lasted much longer than the release of commercial TC (G1: 432 h, GCH: 864 h; A2: 384 h, ACH: 672 h; AP4: 888 h, APCH: 1368 h). This difference in release times may be explained by the fact that the crystallization properties. A study had shown that the drug release rate decreases with decreasing crystallization of the drug molecules within the polymer matrix [199]. Considering this finding, it can be commented that CS-TC was molecularly dispersed and not crystallized within the polymer matrix without any interactions.

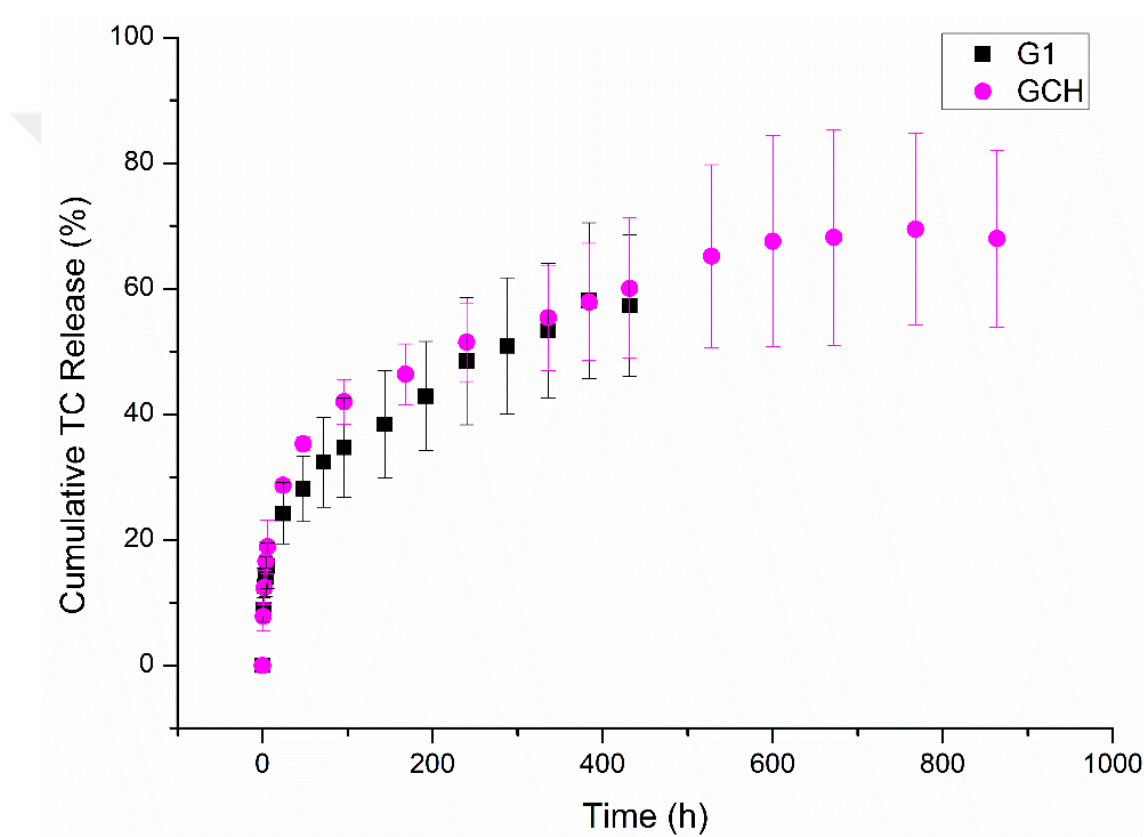


Figure 4.64 : Effect of loaded-TC on cumulative release (TC=10 mg; 0.1 % PVA and 1:10 TC/polymer ratio; G1: commercial TC; GCH: chemically synthesized TC).

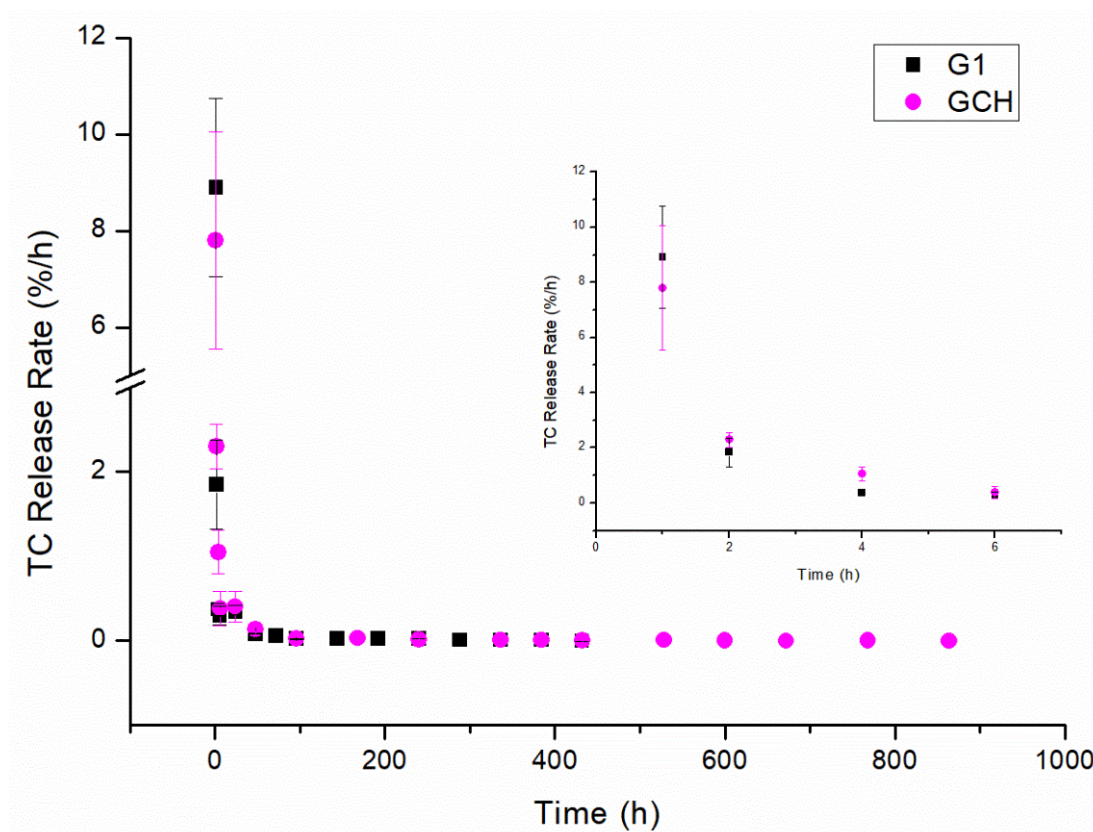


Figure 4.65 : Effect of loaded-TC on release rate (TC=10 mg; 0.1 % PVA and 1:10 TC/polymer ratio; G1: commercial TC; GCH: chemically synthesized TC).

As can be seen in Figure 4.66 the amount of synthesized chalcone released from the ACH microspheres (55 ± 4 %) was lower compared to the A2 formulation with commercial TC (65 ± 14 %). Furthermore, the release was significantly retarded in the case that model drug was synthesized chalcone. The release from ACH formulation expired in 28 days (672 h) whereas the release from PCL-A-RHA microspheres was much faster, completed in 16 days (384 h). Such retardation effect was also observed with the GCH and APCH formulations. This retarded release of synthesized TC from the microspheres may be explained by investigating the drug-polymer interactions. However, released drug amount of GCH microspheres reached to a higher value (70 ± 14 %) than that of G1 formulation (58 ± 11 %).

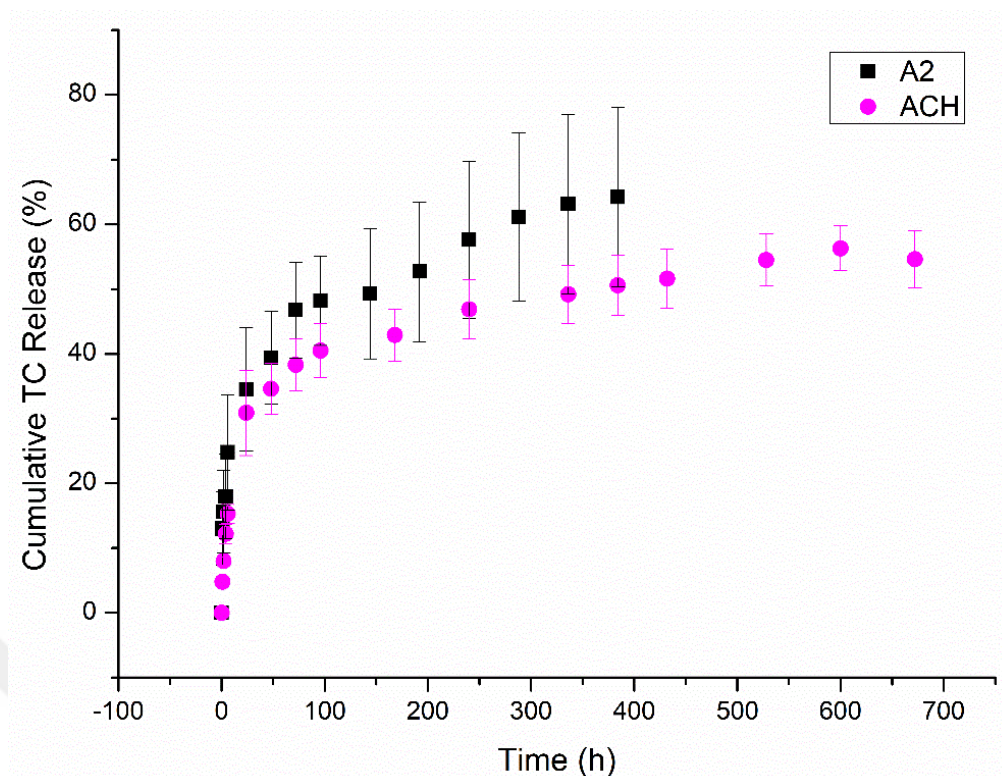


Figure 4.66 : Effect of loaded-TC on cumulative release (TC=10 mg; 0.5 % PVA and 1:10 TC/polymer ratio; A2: commercial TC; ACH: chemically synthesized TC).

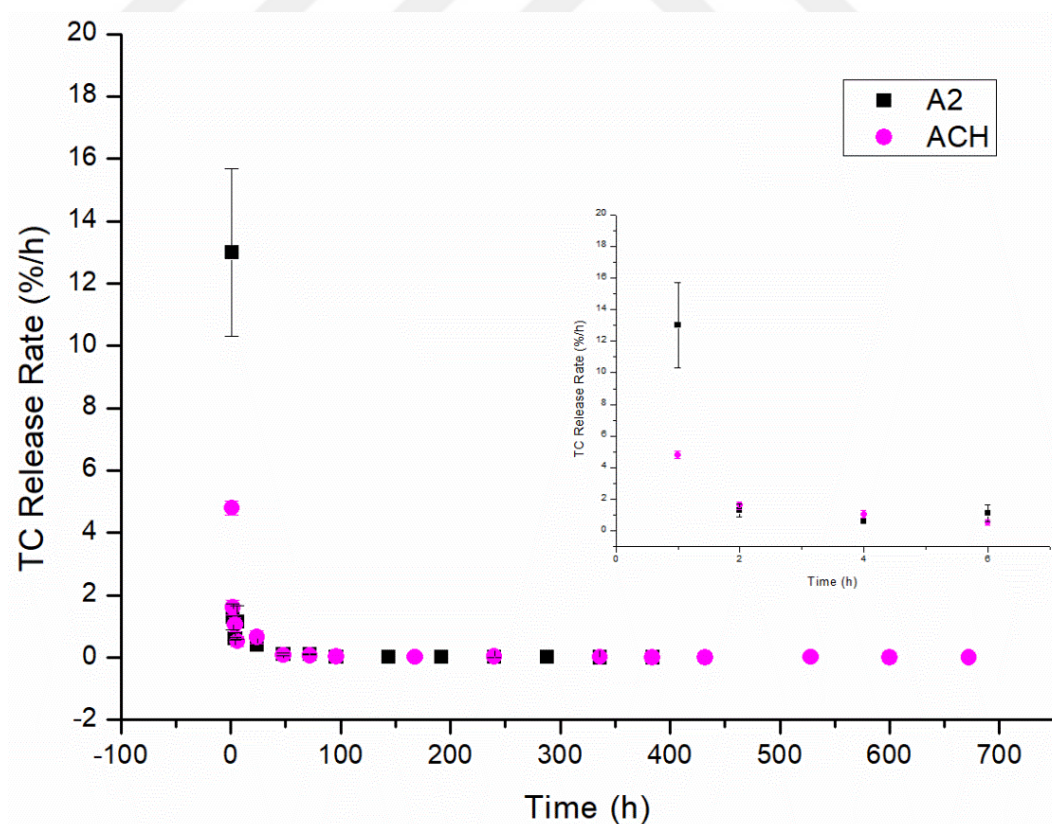


Figure 4.67 : Effect of loaded-TC on release rate (TC=10 mg; 0.5 % PVA and 1:10 TC/polymer ratio; A2: commercial TC; ACH: chemically synthesized TC).

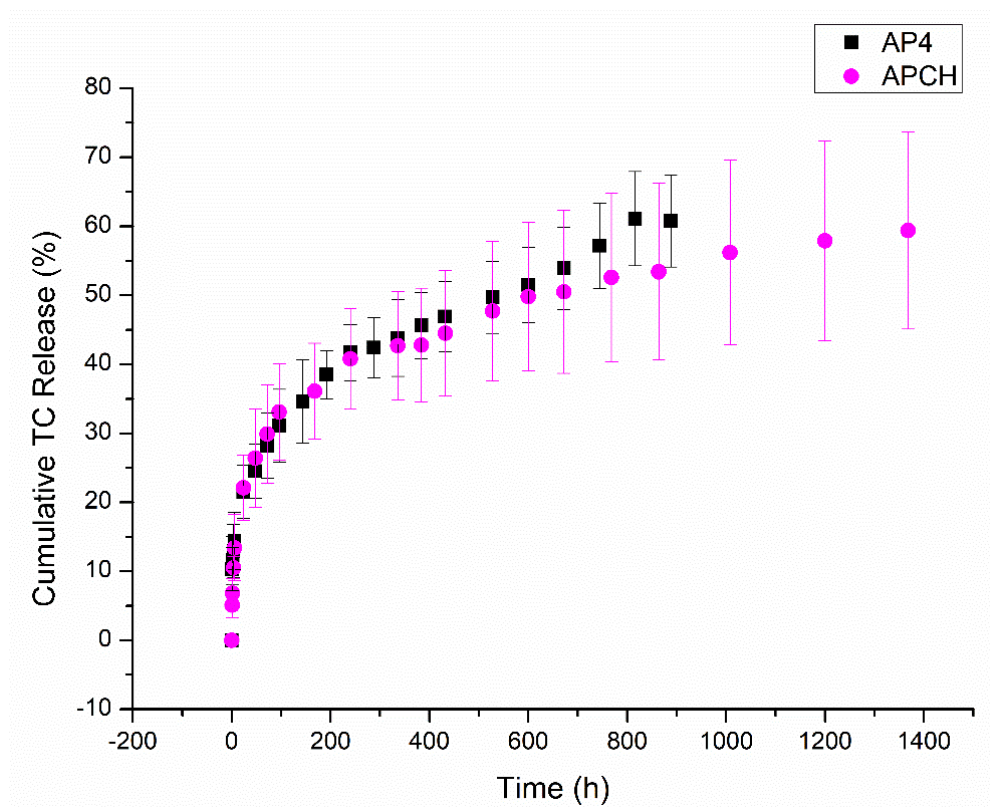


Figure 4.68 : Effect of loaded-TC on cumulative release (TC=10 mg; 1 % PVA and 1:5 TC/polymer ratio; AP4: commercial TC; APCH: chemically synthesized TC).

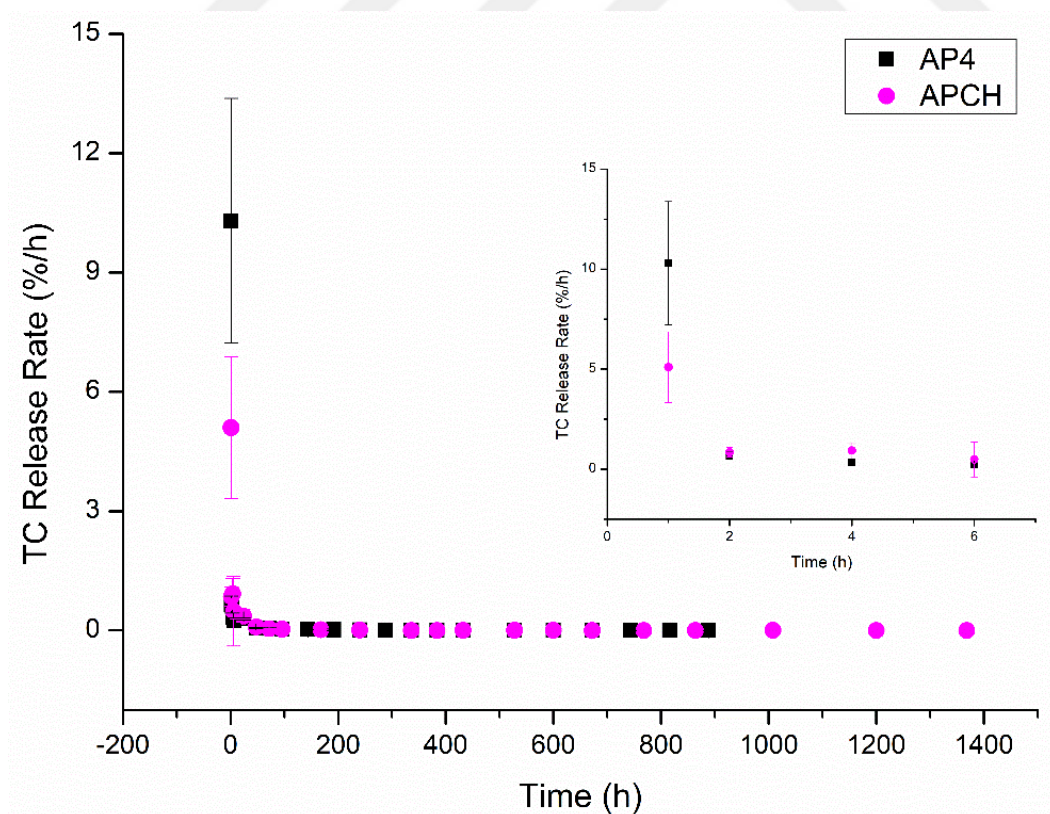


Figure 4.69 : Effect of loaded-TC on release rate (TC=10 mg; 1 % PVA and 1:5 TC/polymer ratio; AP4: commercial TC; APCH: chemically synthesized TC).

Kinetic modelling was also conducted for microspheres containing chemically synthesized TC. The calculated parameters were presented and compared with microspheres containing commercial TC in Table 4.12. Korsmeyer-Peppas model was the best fitting model for all of the formulation. The main release mechanism of all CS-TC loaded microspheres were Fickian diffusion according to the calculated n values. n values below 0.43 for spherical geometries indicates release by Fickian diffusion. The rate of polymer erosion can be neglected with respect to the rate of drug diffusion towards the release environment and did not contributed to drug release from the microsphere formulations.

Table 4.12 : Kinetic model parameters calculated for neat PCL microspheres.

Code	Korsmeyer-Peppas			Higuchi		Zero Order		First Order	
	K_{KP} (h^{-n})	n	R^2	K_H ($h^{-0.5}$)	R^2	K_0 (h^{-1})	R^2	K_1 (h^{-1})	R^2
G1	0.161	0.30	0.995	2.59	0.977	0.125	0.871	0.004	0.718
GCH	0.146	0.31	0.980	2.19	0.931	0.068	0.752	0.002	0.552
A2	0.210	0.27	0.983	2.88	0.921	0.133	0.755	0.004	0.588
ACH	0.116	0.41	0.952	2.46	0.884	0.111	0.693	0.004	0.515
AP4	0.153	0.27	0.994	1.78	0.970	0.056	0.840	0.002	0.671
APCH	0.111	0.32	0.973	1.50	0.916	0.037	0.711	0.001	0.495

The chemically synthesized TC loaded microspheres were imaged by both optical microscopy and SEM to visualize the surface properties of the microspheres. Figure 4.66 shows the images taken. The first thing to be noticed from the images was that ACH formulation did not created spherical particles. GCH and APCH microsphere formulations were spherical particles as visualized in optical microscopy and SEM images presented in Figure 4.70. GCH microspheres were hollow particles, partially deformed with large openings on the surface. This may have contributed to increased TC release from this formulation with respect to G1 microsphere formulation. APCH microspheres had meandering surface structure with expectedly increased surface area which may have contributed to increased TC loading as presented in Table 11.

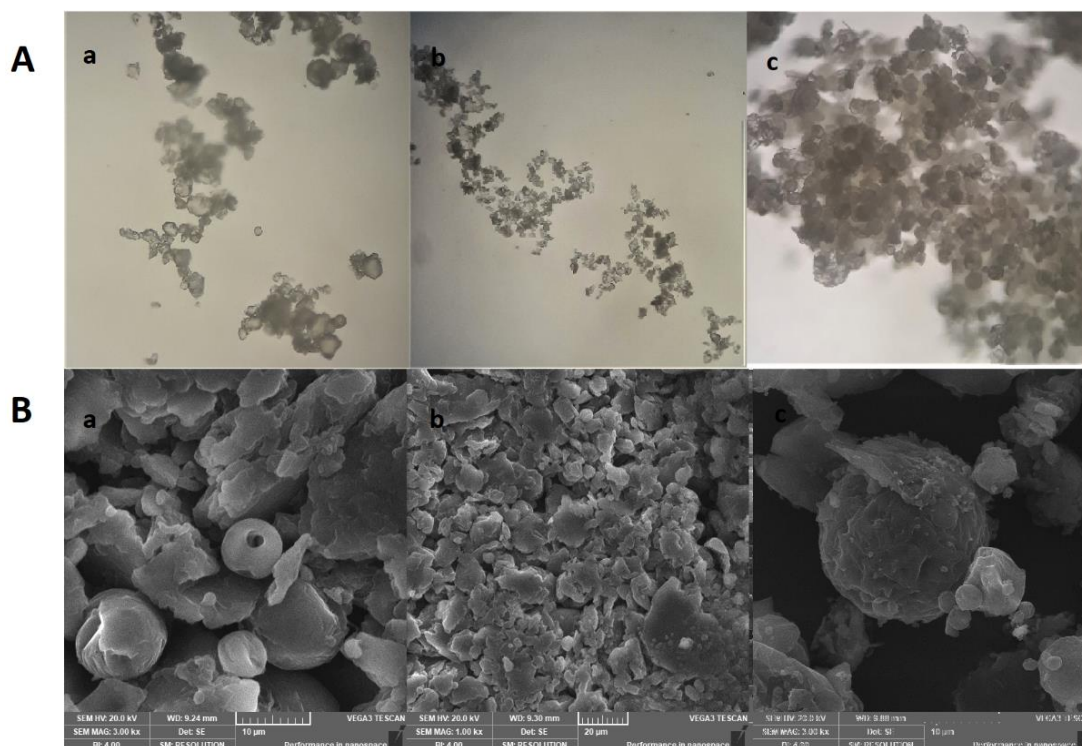


Figure 4.70 : A) Optical microscopy images of a) ACH, b) GCH, c) APCH (Magnification 40x); B) SEM images of a) ACH, b) GCH, c) APCH (Magnification 3000x).

Microspheres loaded with chemically synthesized TC were structurally characterized by FT-IR spectroscopy. In Figure 4.71 FT-IR spectra of GCH, TC and PCL-G-RHA were presented. No indication of drug polymer interaction was detected from the spectra since no significant change occurred in the signals. The spectrum of GCH showed all the characteristic peaks of TC and PCL-G-RHA as listed below:

- 800 cm^{-1} Si-CH₃ rocking, 1050 cm^{-1} asymmetric stretching of Si-O-Si, 1150 cm^{-1} -CH₂ wagging peak of oxirane ring, 840 cm^{-1} oxirane ring of PCL-G-RHA
- $1400\text{-}1500\text{ cm}^{-1}$ C-C stretching in aromatic compounds, 1600 cm^{-1} C=C stretching in aromatic compounds, 1663 cm^{-1} α, β unsaturation in ketones, 3100 cm^{-1} aromatic =C-H stretching of TC
- 1293 cm^{-1} C-O and C-C, 1238 cm^{-1} asymmetric stretch of C-O-C, 1167 cm^{-1} symmetric stretch of C-O-C, 1726 cm^{-1} aliphatic carbonyl bond, 2866 cm^{-1} symmetric CH₂, 2945 cm^{-1} asymmetric CH₂, 3500 cm^{-1} stretching vibrations of -OH of PCL-G-RHA structure

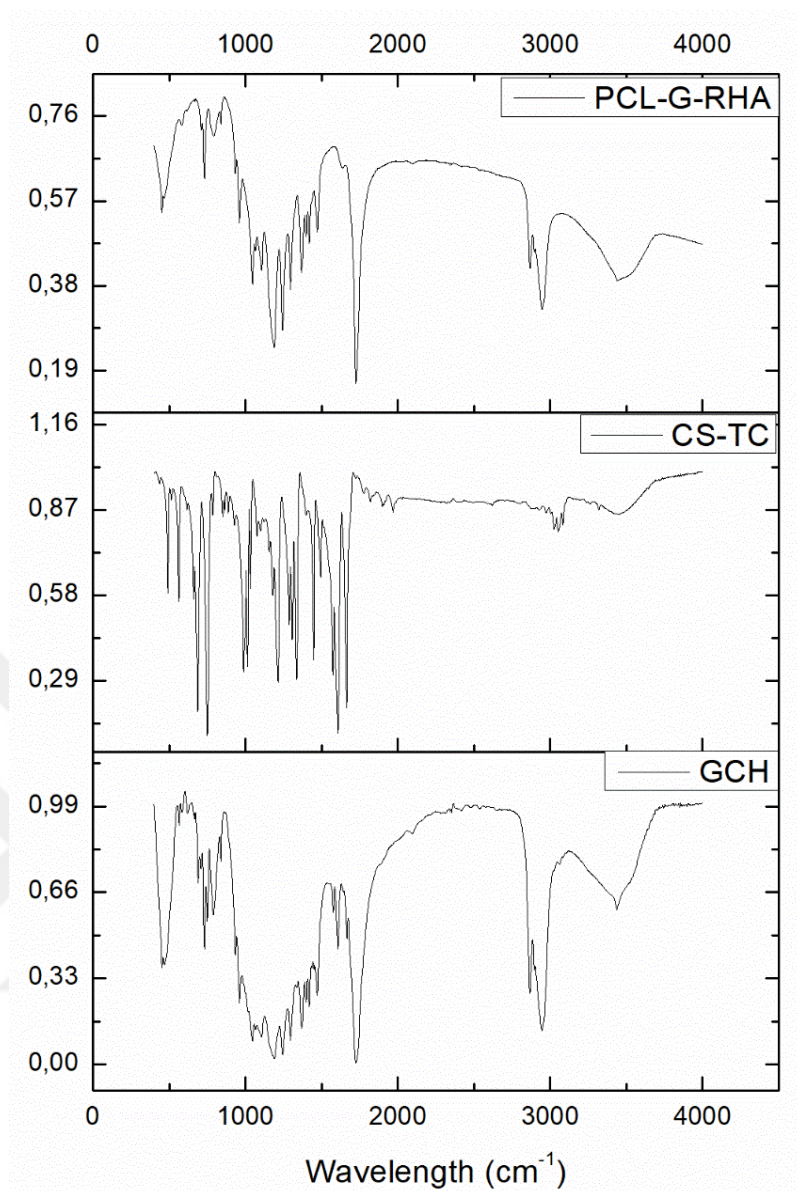


Figure 4.71 : FT-IR spectra of GCH, PCL-G-RHA and CS-TC (GCH: 0.1 % PVA, 1:10 CS-TC:PCL-G-RHA).

The FT-IR spectrum of the physical mixture of GCH formulation did not differ from the FT-IR spectrum of GCH microspheres as given in Figure 4.72. In the FT-IR spectrum of G1 microspheres, there were indications of interactions between TC molecules and PCL-G-RHA. However, GCH spectrum showed no sign of drug/polymer interactions. This indicates that during microsphere formation CS-TC molecules did not interact with PCL-G-RHA. Drug/polymer interactions is an important factor to affect the loading efficiency and release behavior of the microspheres. Indeed, the lack of interactions between CS-TC and PCL-G-RHA can explain the reduced encapsulation efficiency obtained for this formulation compared to G1 microspheres (58 ± 8 %).

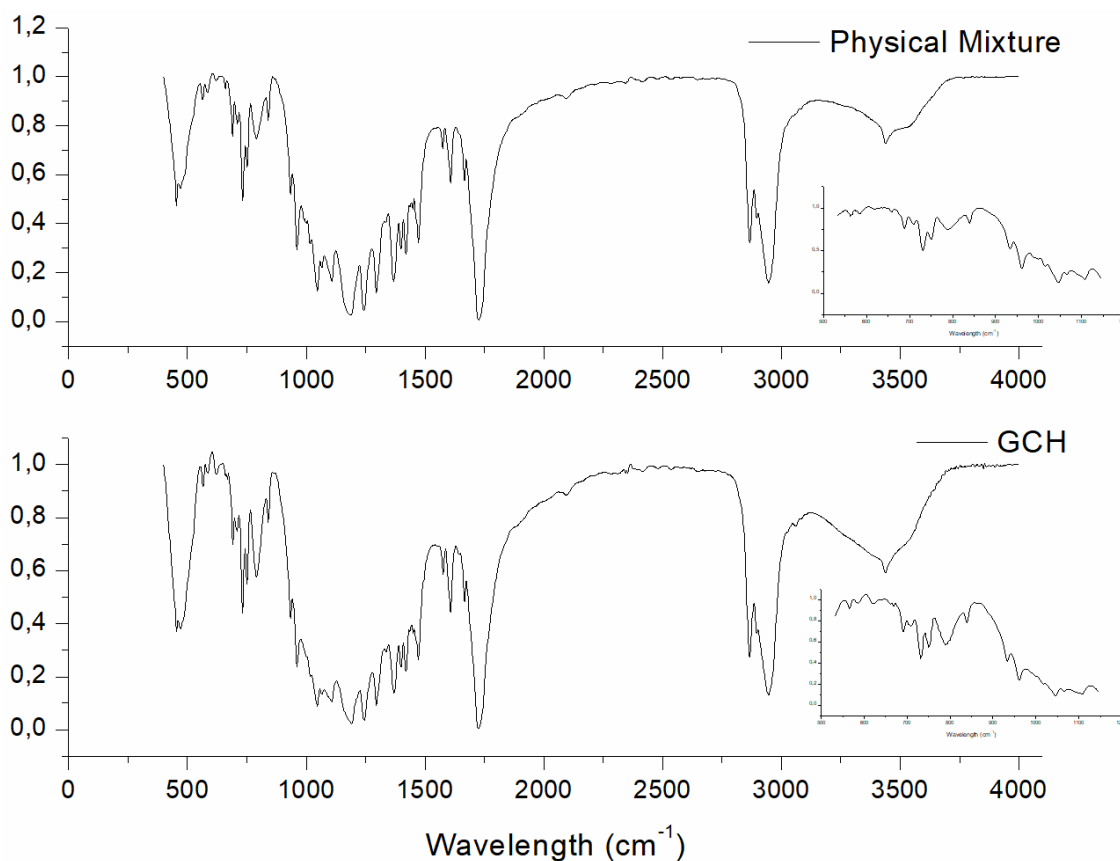


Figure 4.72 : FT-IR spectra of GCH microspheres and the physical mixture of PCL-G-RHA- and CS-TC (GCH: 0.1 % PVA, 1:10 CS-TC:PCL-G-RHA; Physical mixture: Mixture of 1:10 (w:w) CS-TC:PCL-G-RHA).

The FT-IR spectrum of ACH microspheres in Figure 4.73 had every characteristic peak of loaded TC (chemically synthesized) and PCL-A-RHA. These peaks are 800 cm^{-1} Si-CH₃ rocking, 1050 cm^{-1} asymmetric stretching of Si-O-Si, 1293 cm^{-1} C-O and C-C, 1238 cm^{-1} asymmetric stretch of C-O-C, 1167 cm^{-1} symmetric stretch of C-O-C, 1726 cm^{-1} aliphatic carbonyl bond, 2866 cm^{-1} symmetric CH₂, 2945 cm^{-1} asymmetric CH₂, 3500 cm^{-1} stretching vibrations of O-H of PCL-G-RHA; 1400-1500 cm^{-1} C-C stretching in aromatic compounds, 1606 cm^{-1} C=C stretching in aromatic compounds, 1663 cm^{-1} α , β unsaturation in ketones. However, multiple peaks at around 3100 cm^{-1} aromatic =C-H stretching of CS-TC was not visible in the spectrum of ACH microsphere formulation.

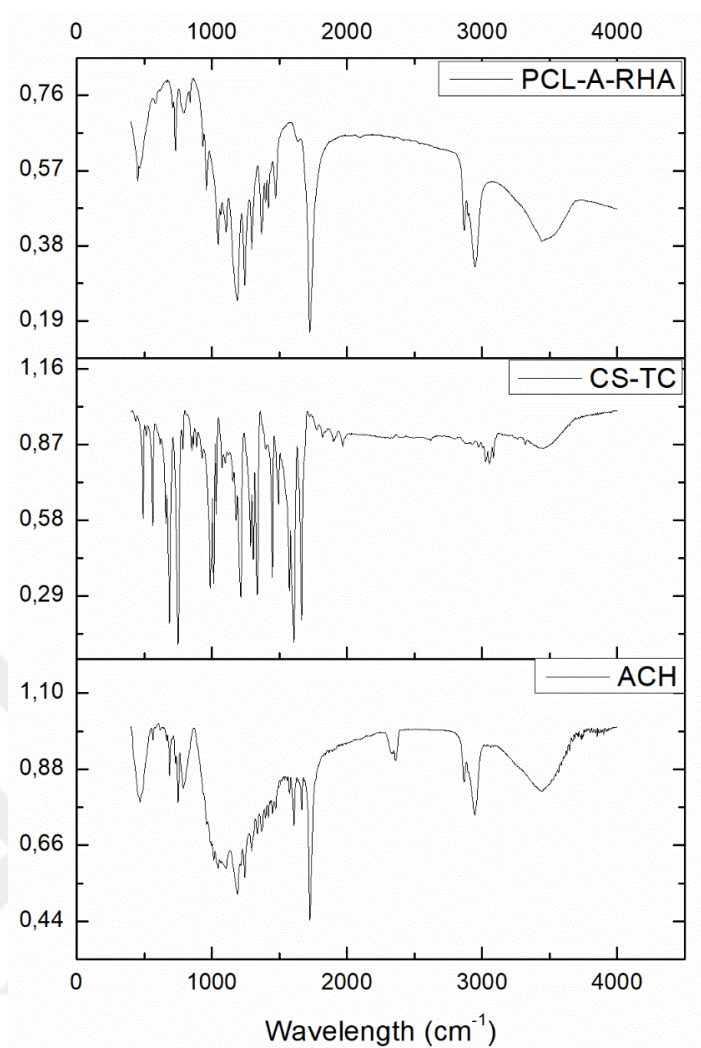


Figure 4.73 : FT-IR spectra of ACH, PCL-A-RHA and CS-TC (ACH: 0.5 % PVA, 1:10 CS-TC:PCL-A-RHA).

The FT-IR spectrum of the physical mixture of ACH formulation revealed an important difference at peaks at 934 and 966 cm^{-1} which are assigned to NH_2 and N-H wagging in primary amines (Figure 4.74). These peaks were almost disappeared and the peak at 966 cm^{-1} in the spectrum of physical mixture shifted to 961 cm^{-1} in the spectrum of ACH microspheres. This alteration in the peaks may indicate that amine groups of PCL-A-RHA nanohybrid was involved in the interactions between CS-TC and PCL-A-RHA. TC is considered to be open to nucleophilic attacks due to the α , β unsaturated ketone group in its structure and can be involved in Michael addition reaction with the NH_2 end-groups of PCL-A-RHA [200]. This strong covalent interactions between PCL-A-RHA and CS-TC may explain the higher encapsulation efficiency of ACH microspheres with respect to GCH and APCH microspheres. The release from ACH microspheres were slower than A2 formulation (with commercial TC) which may be the result of such strong hydrogen bonding.

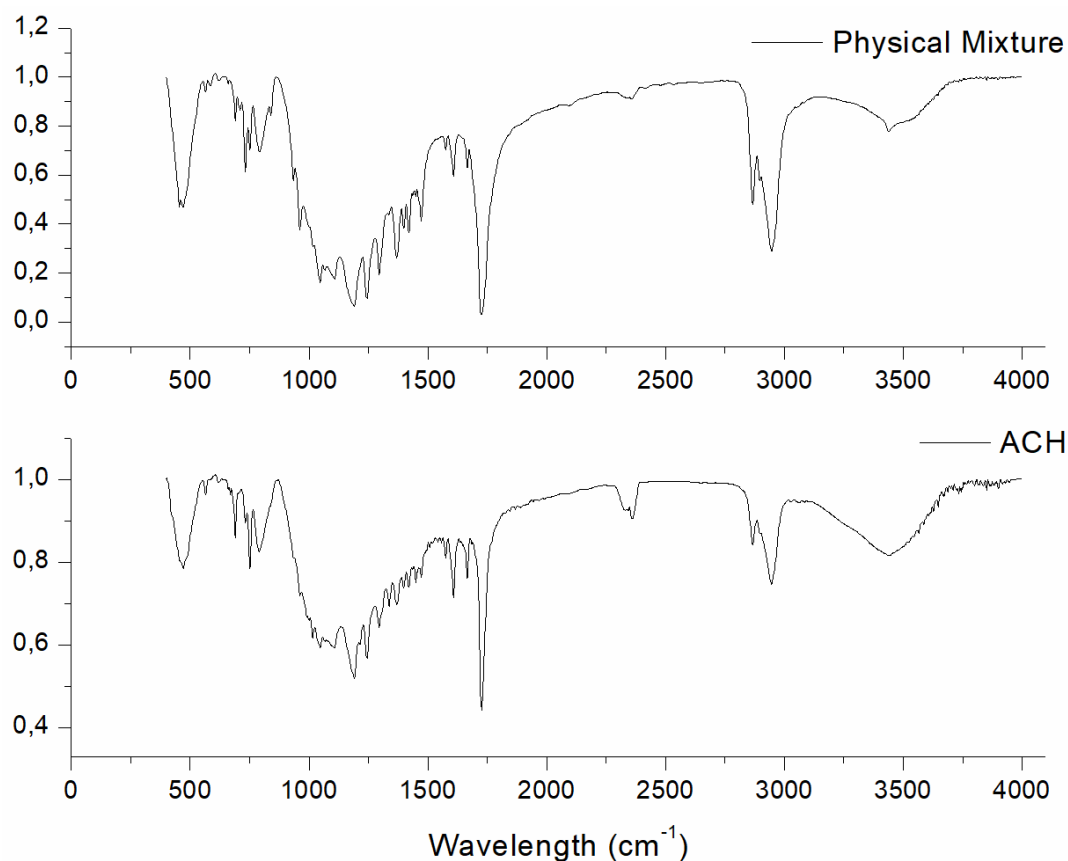


Figure 4.74 : FT-IR spectra of ACH microspheres and the physical mixture of PCL-A-RHA- and CS-TC (ACH: 0.5 % PVA, 1:10 CS-TC:PCL-A-RHA; Physical mixture: Mixture of 1:10 (w:w) CS-TC:PCL-A-RHA).

The FT-IR spectrum of APCH revealed the expected peaks mainly belonging to CS-TC and PCL-AP-RHA. Peaks at 800 cm^{-1} Si-CH₃ rocking, 1050 cm^{-1} asymmetric stretching of Si-O-Si, 1293 cm^{-1} C-O and C-C, 1238 cm^{-1} asymmetric stretch of C-O-C, 1167 cm^{-1} symmetric stretch of C-O-C, 1726 cm^{-1} aliphatic carbonyl bond, 2866 cm^{-1} symmetric CH₂, 2945 cm^{-1} asymmetric CH₂, 3500 cm^{-1} stretching vibrations of O-H of PCL-AP-RHA; $1400\text{--}1500\text{ cm}^{-1}$ C-C stretching in aromatic compounds, 1600 cm^{-1} C=C stretching in aromatic compounds, 1663 cm^{-1} α , β unsaturation in ketones, 3100 cm^{-1} aromatic =C-H stretching of CS-TC were clearly observed in the spectrum of APCH, as can be seen in Figure 4.75. In the spectrum of APCH, a peak belonging to CS-TC structure almost disappeared at 1560 cm^{-1} assigned to phenyl ring moiety. Such disappearance of the mentioned peak was most likely the consequence of hydrophobic interactions between CS-TC and PCL-AP-RHA [201]. As discussed previously, hydrophobic interactions between CS-TC and PCL-AP-RHA had contributed to the high encapsulation efficiency and prolonged release compared to commercial TC.

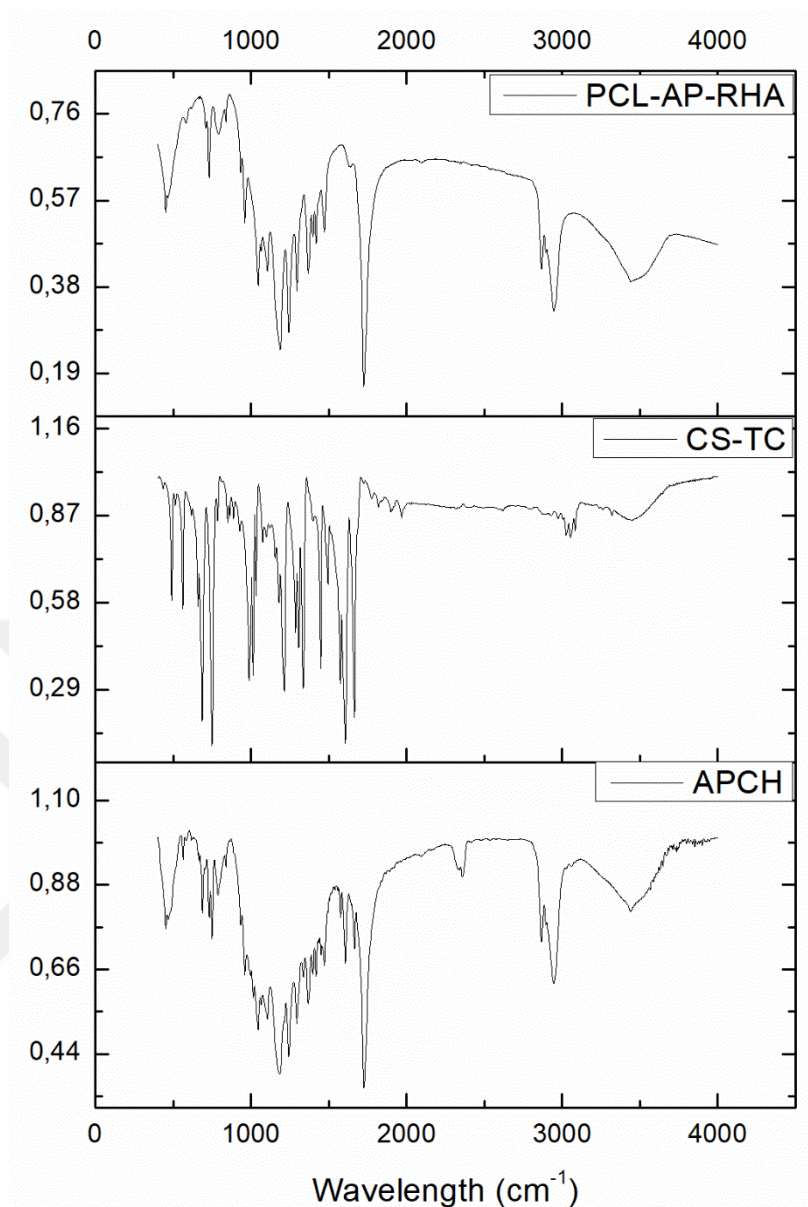


Figure 4.75 : FT-IR spectra of APCH, PCL-AP-RHA and CS-TC (APCH: 1 % PVA, 1:5 CS-TC:PCL-A-RHA).

Differently from ACH microspheres, in the APCH microspheres PCL-AP-RHA and CS-TC were not involved in any kind of interactions since no significant difference in the peaks was detected in the FT-IR spectra of APCH microspheres and the physical mixture of this formulation (Figure 4.76). Previously, while interpreting the FT-IR spectra of ACH and the physical mixture of ACH formulation, the disappearance of peaks at 934 and 966 cm^{-1} were associated with the interactions of primary amine groups of 3-APTMS in the structure of PCL-A-RHA. Such indications were not observed in the spectra of APCH and the physical mixture of this formulation; peaks at 929 and 961 cm^{-1} were clearly visible in both of the FT-IR spectra. Thus, it was suggested that no interactions between CS-TC and PCL-AP-RHA were involved in

the drug loading to the microspheres. However, drug loading and release suggested that some kind of interaction occurred between PCL-AP-RHA and CS-TC since encapsulation efficiency of APCH microspheres (71 ± 5) was higher than the encapsulation efficiency of AP4 microspheres (53 ± 4). It may be hydrophobic interactions between hydrophobic PCL-AP-RHA hybrid and hydrophobic CS-TC.

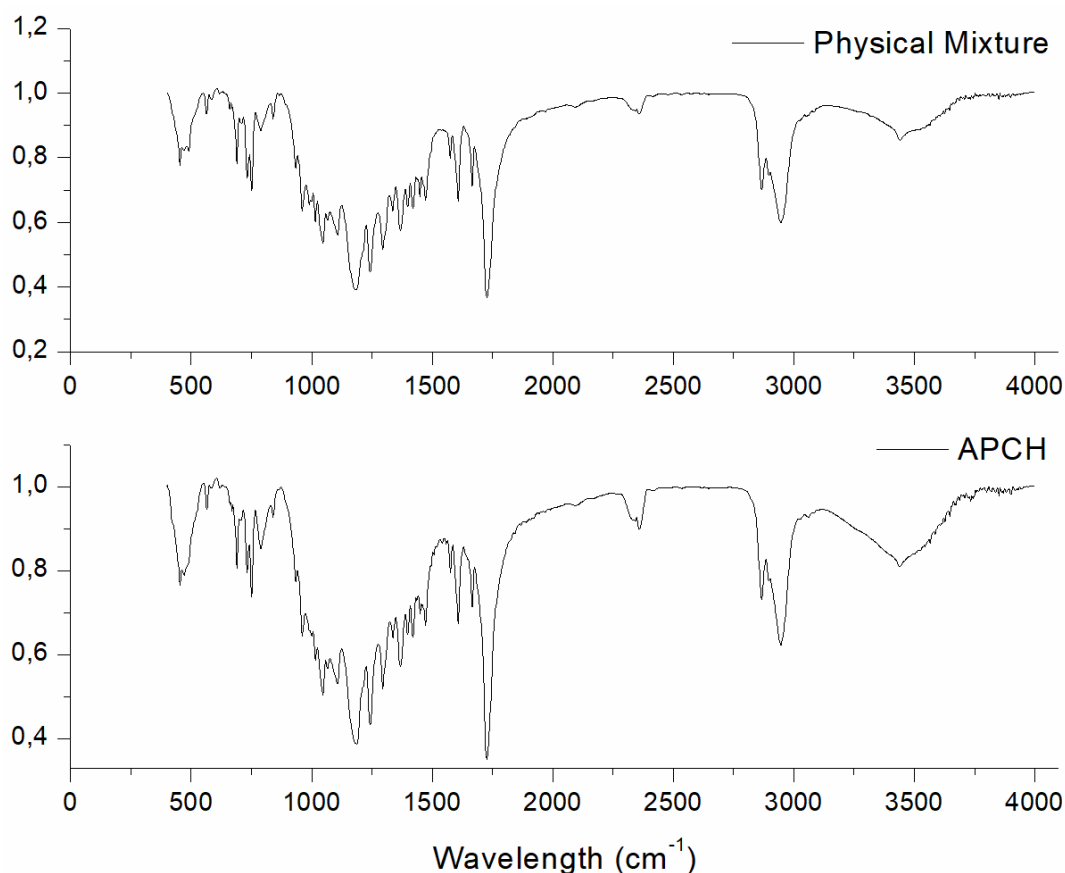


Figure 4.76 : FT-IR spectra of APCH microspheres and the physical mixture of PCL-AP-RHA- and CS-TC (APCH: 1 % PVA, 1:5 CS-TC:PCL-AP-RHA; Physical mixture: Mixture of 1:5 (w:w) CS-TC:PCL-AP-RHA).

The DSC scanning of the chemically synthesized TC loaded microspheres supported the findings of FT-IR spectra. Figure 4.77 shows the melting endotherms of GCH microspheres and the physical mixture of the formulation. The melting peak of GCH microspheres consisted of a single peak at 50 °C. This, as earlier discussed, indicated that CS-TC was molecularly dispersed within the microspheres. However, the physical mixture had a two separate melting peaks which indicates that thermal properties of CS-TC and PCL-G-RHA did not altered by physical mixing whereas microsphere formation did altered melting behavior of CS-TC.

On the other hand, melting endotherms of ACH microspheres and the physical mixture of this formulation revealed a significant difference (Figure 4.78). The physical mixture of the related formulation had a twin peak at 42 °C and 48 °C whereas ACH microsphere had a single melting peak close to the melting peaks of both PCL-A-RHA and CS-TC. This difference in melting endotherms was associated with the CS-TC being molecularly dispersed within the polymer matrix during microsphere formation.

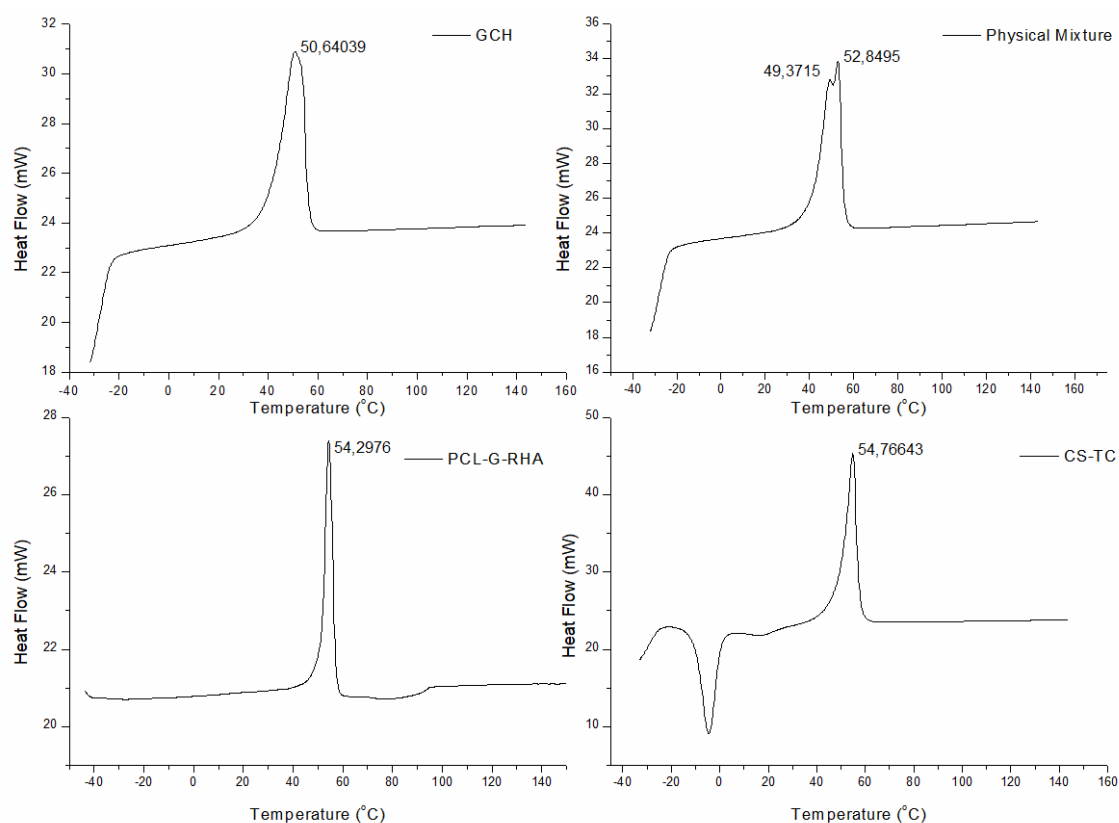


Figure 4.77 : DSC thermogram of the PCL-G-RHA, CS-TC, GCH and physical mixture, demonstrating melting endotherm (second heating cycle), GCH: 0.1 % PVA, 1:10 CS-TC:PCL-G-RHA.

Unlike the GCH and ACH microsphere formulations, the melting endotherm of APCH microsphere formulation had two partly conjugated peaks at 47 °C and 52 °C. The physical mixture of this formulation had a similar characteristic in terms of melting behavior. The physical mixture had two conjugated peaks at 48 °C and 51 °C. This finding together with the findings of FT-IR analysis indicated that CS-TC was encapsulated in the microspheres of PCL-AP-RHA in its crystalline form without undergoing any chemical change or without any interactions with the polymer matrix.

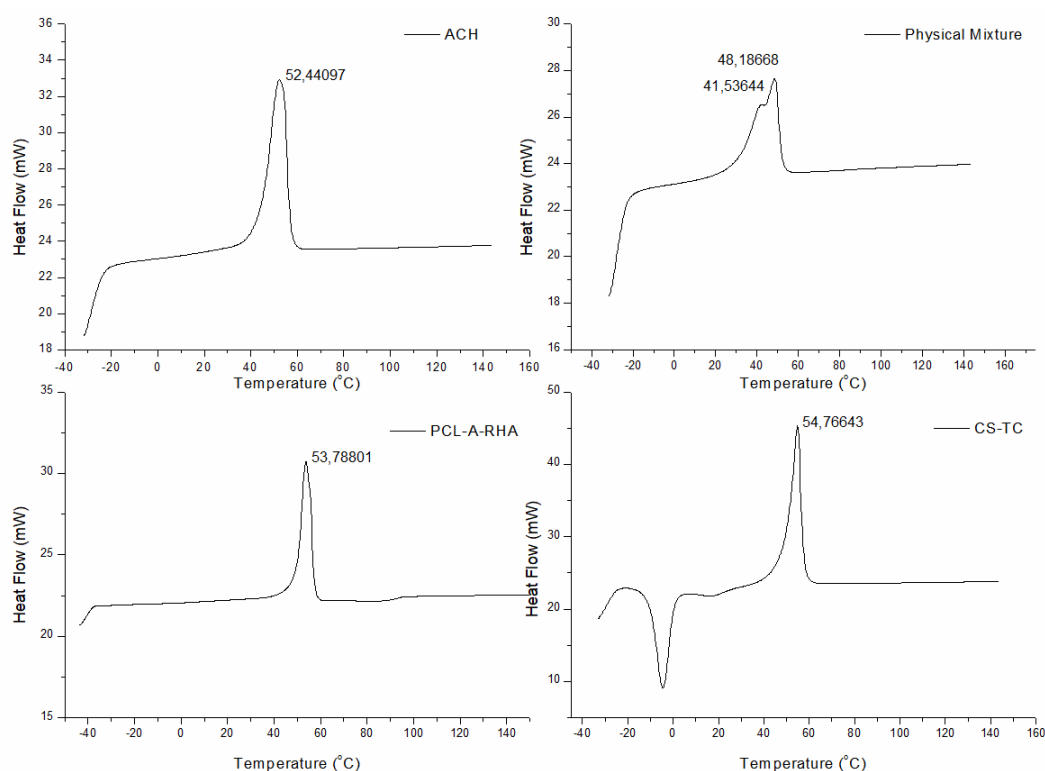


Figure 4.78 : DSC thermogram of the PCL-A-RHA, CS-TC, ACH and physical mixture, demonstrating melting endotherm (second heating cycle), ACH: 0.5 % PVA, 1:10 CS-TC:PCL-A-RHA.

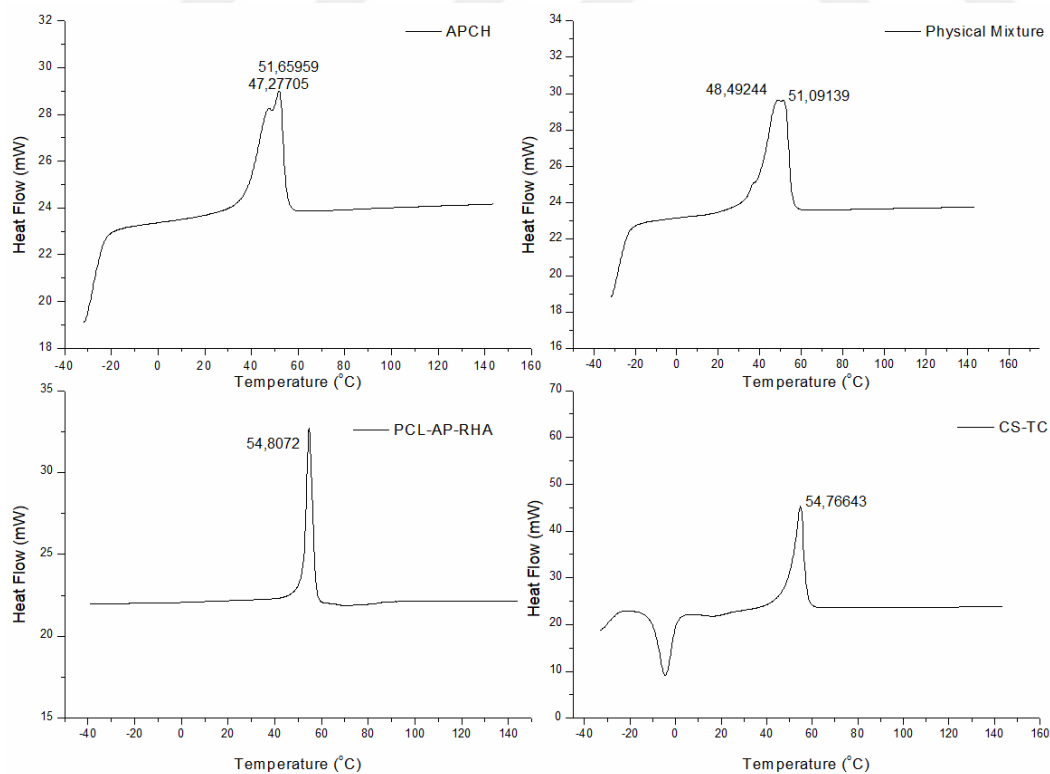


Figure 4.79 : DSC thermogram of the PCL-AP-RHA, CS-TC, APCH and physical mixture, demonstrating melting endotherm (second heating cycle), APCH: 1 % PVA, 1:5 CS-TC:PCL-AP-RHA.

4.3.7 pH dependent release from microspheres

The properties of the release medium significantly affect the rate, amount and mechanism of the drug release. One of these properties is the pH, of which effects on the release from the microspheres were investigated. Several pH values were chosen accordingly to simulate the conditions of the different parts of the human body (pH 8: distal small intestine; pH 3.6: lower stomach [120]; pH 5.6: microenvironment of tumor cells [202]).

Figure 4.80 shows the release behavior of G1 microsphere formulation at varying pH conditions. Released drug amount significantly increased as the medium became more acidic. Release in pH 5.6 medium was significantly faster, lasting only 4 days. Basic to neutral conditions caused the release of TC to extend over two weeks. Noting that hydrogen bonding tends to be weaker at acidic conditions [203], the possible reason of such difference in release behavior may be the hydrogen bonding interactions between PCL-G-RHA and TC. This fast release in acidic conditions may be advantageous in targeted drug delivery [204]. Comparison between PCL-G-RHA nanohybrid and G-PCL polymer in terms of their release behavior was conducted at varying pH conditions. In pH 3.6 and pH 8.0 medium, cumulative TC release was enhanced when the microspheres were fabricated with PCL-G-RHA. In pH 5.6 release medium, TC release from PCL-G-RHA microspheres did not differ significantly from G-PCL microspheres. In general, TC release was achieved in a slower manner from PCL-G-RHA microspheres in media with varying pH.

Figure 4.81 shows the release profiles of various microsphere formulations in different release media. As in the case of G1, GPCL and GCH microspheres, TC release from A2, APCL and ACH microsphere formulations were faster and higher in acidic release medium (pH 3.6 and 5.6). This improvement in the cumulative release was attributed to the protonation of NH_2 groups of PCL-A-RHA which may have affected the water permeability of microspheres [199]. However, TC release from these microspheres in pH 3.6 medium were lower with respect to their G-counterparts (G1: 97 ± 6 % A2: 80 ± 2 %, GPCL: 73 ± 6 % APCL: 71 ± 9 %, GCH: 97 ± 9 % ACH: 83 ± 10 %). On the other hand, differences in terms of cumulative percent release were not significant in pH 5.6 medium (G1: 98 ± 3 % A2: 99 ± 5 %, GPCL: 94 ± 7 % APCL: 99 ± 5 %, GCH: 100 ± 7 % ACH: 93 ± 7 %). As the pH of the release medium was increased to neutral or basic values, cumulative release decreased gradually. In accordance with this result,

previous study investigating a hydrophobic drug have demonstrated that decreased drug release at slightly basic medium was due to the drug molecules localized on the surface of the microspheres tended to be more stable [201].

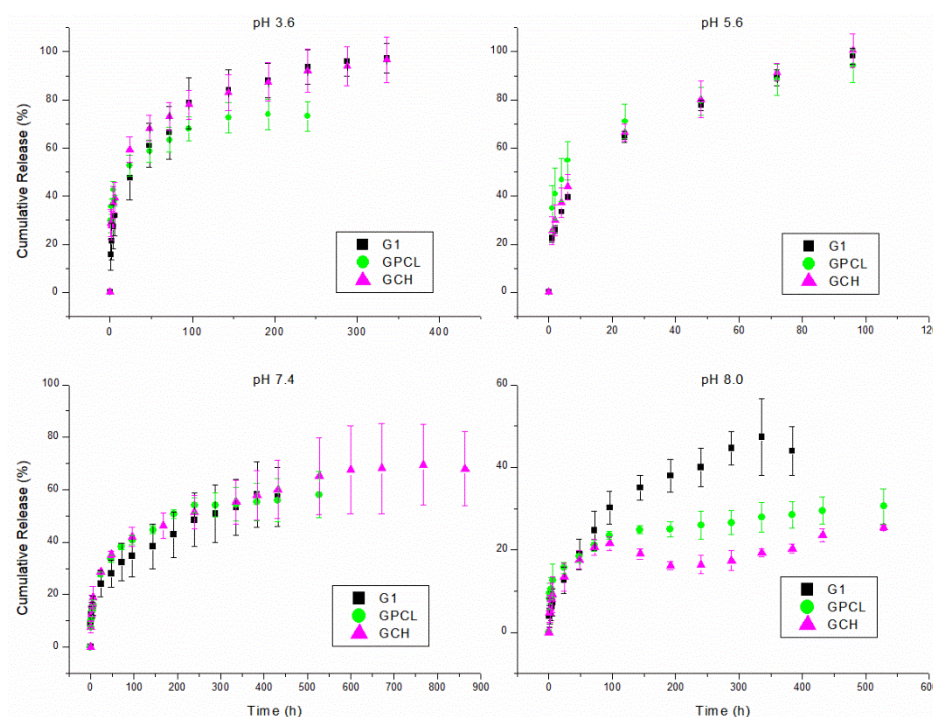


Figure 4.80 : TC release in different pH media; G1: 0.1 % PVA, 1:10 TC:PCL-G-RHA; GPCL: 0.1 % PVA, 1:10 TC:G-PCL; GCH: 0.1 % PVA, 1:10 CS-TC:PCL-G-RHA.

Ph-dependent release from PCL-AP-RHA and AP-PCL microspheres was also investigated (Figure 4.82). Similarly, TC release from these microsphere formulations was enhanced in acidic release medium (pH 3.6 and 5.6). Protonation of carboxylic groups of PCL moiety of PCL-AP-RHA in acidic medium (especially pH 5.6) may have contributed to the enhanced cumulative release and faster release [205]. In the figure given below, there is a clear trend of increased TC release from AP4 with respect to APPCL and APCH in several release media. The difference between AP4 and APPCL in terms of release behavior may be explained by the fact that hydrophobic character and relatively high crystallinity of neat PCL retarded drug diffusion out of the microsphere [206]. The FT-IR investigation of AP4 formulation revealed a possible hydrogen bonding between carbonyl group of TC and amine group of PCL-AP-RHA, as discussed earlier. The weakening of hydrogen bonds in acidic conditions which may have contributed to the increased TC release with respect to APCH formulation. Not very surprisingly, TC release in pH 8.0 medium was significantly lowered.

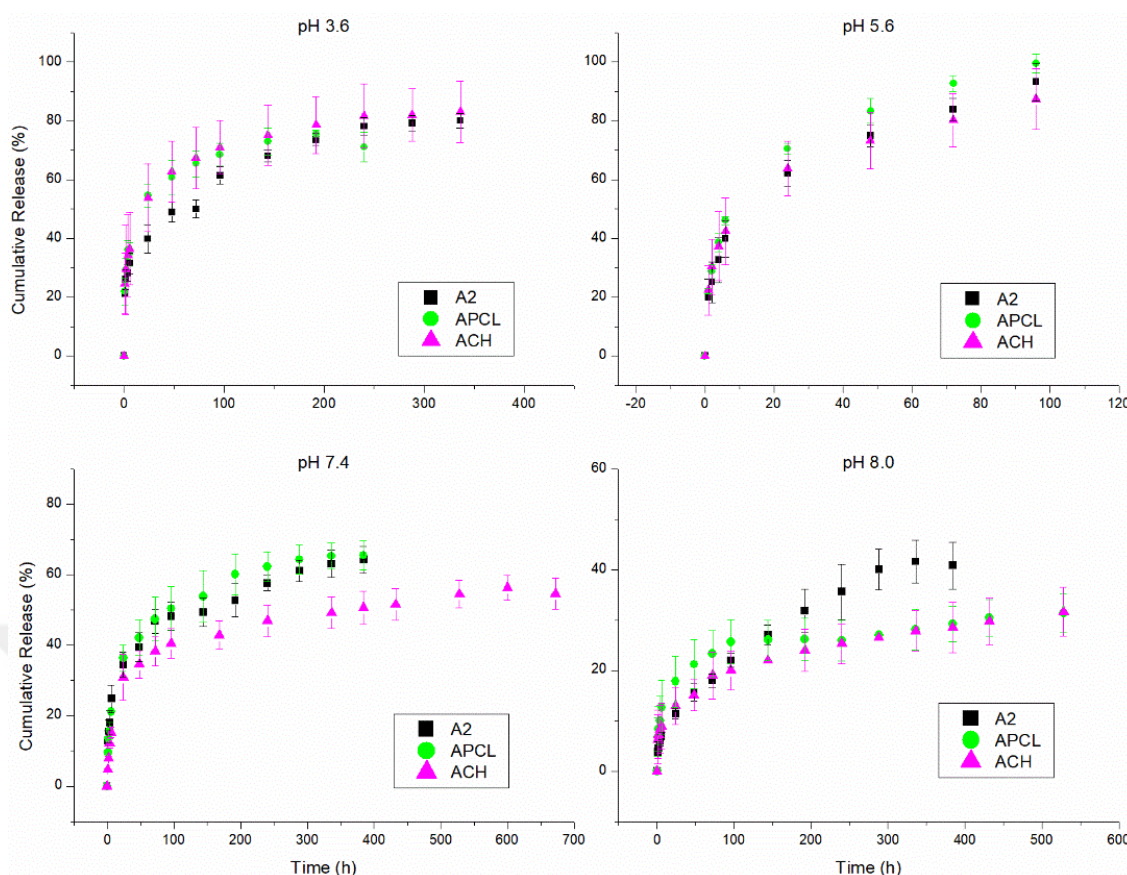


Figure 4.81 : TC release in different pH media; A2: 0.5 % PVA, 1:10 TC:PCL-A-RHA; APCL: 0.5 % PVA, 1:10 TC:A-PCL; ACH: 0.5 % PVA, 1:10 CS-TC:PCL-A-RHA.

Such fast release in acidic medium and prolonged release behavior in neutral or basic medium indicated a pH-triggered release from G1, A2 and AP4 microsphere formulations. This pH-triggered release from these microsphere formulations might be exploited for targeted delivery in cancer treatment (TC was shown to have anti tumorous activity). In GI tract, transit time of particles through acidic environment (pH 1.2-5.8) was 1-2 h [175, 207]. For our microsphere formulations, 20-25 % cumulative release and 25-35% cumulative release was completed within 2 h, which is a considerable amount of drug to be lost before reaching the neutral or slightly basic part of the GI tract. Thus the formulations studied in this thesis may not be very suitable for colon-targeted oral delivery.

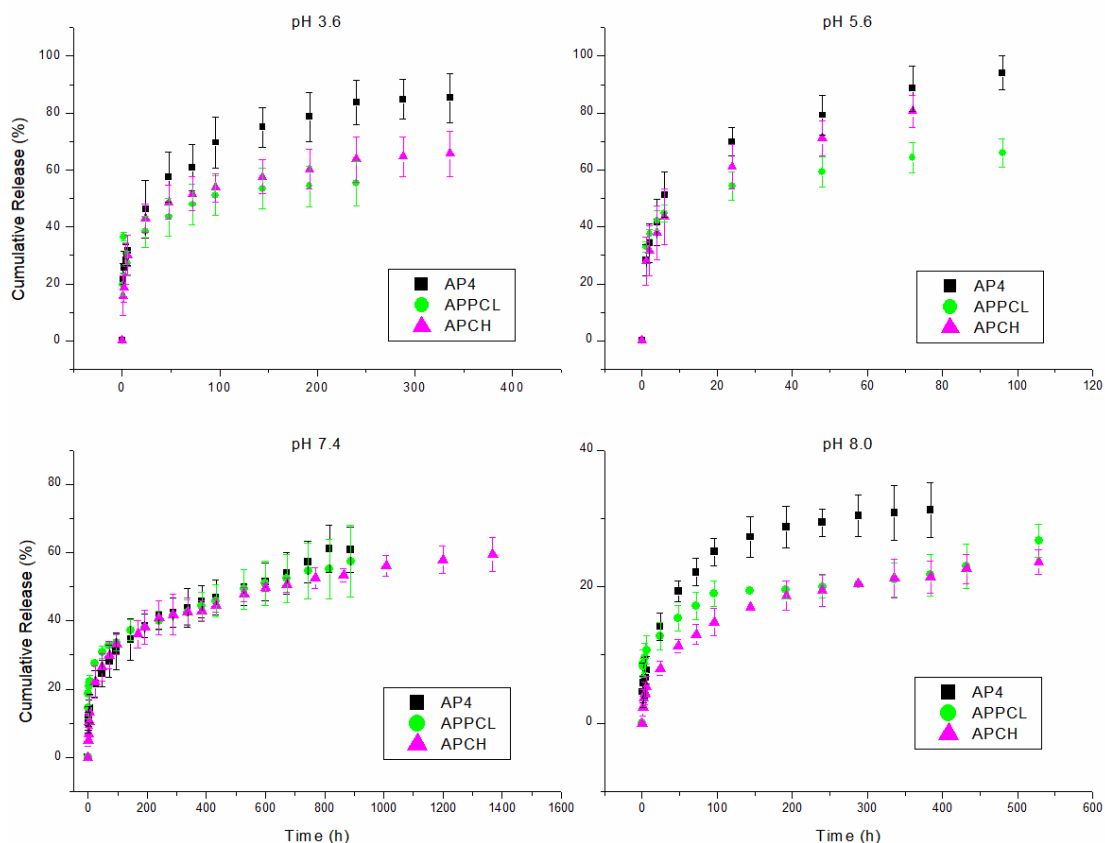


Figure 4.82 : TC release in different pH media; AP4: 1 % PVA, 1:5 TC:PCL-AP-RHA; APPCL: 1 % PVA, 1:5 TC:AP-PCL; APCH: 1 % PVA, 1:5 CS-TC:PCL-AP-RHA.

Kinetic modelling was also applied to the release data of the microsphere formulations in different release media. Most of the formulations were best fitted to Korsmeyer-Peppas model, with the indication of Fickian diffusion ($n < 0.43$). Only release from A2 microsphere formulation in pH 8.0 medium fitted to Higuchi kinetic model which also describes the release behavior is mainly controlled and dominated by Fickian diffusion. Changes in pH of release media did not affected the dominant release mechanism of the microsphere formulations.

Table 4.13 : Kinetic model parameters of release in pH 3.6 medium.

Code	Korsmeyer-Peppas			Higuchi		Zero Order		First Order	
	K_{KP} (h^{-n})	n	R^2	K_H ($h^{-0.5}$)	R^2	K_0 (h^{-1})	R^2	K_1 (h^{-1})	R^2
G1	0.169	0.34	0.995	5.080	0.939	0.252	0.770	0.005	0.568
GPCL	0.421	0.17	0.942	3.726	0.801	0.210	0.604	0.005	0.377
GCH	0.270	0.25	0.936	4.464	0.889	0.217	0.704	0.004	0.468
A2	0.272	0.46	0.99	3.842	0.937	0.193	0.793	0.004	0.723
APCL	0.328	0.26	0.974	4.101	0.824	0.229	0.607	0.004	0.589
ACH	0.295	0.24	0.997	3.794	0.861	0.183	0.668	0.003	0.640
AP4	0.245	0.25	0.994	4.197	0.921	0.207	0.746	0.005	0.538
APPCL	0.433	0.16	0.852	2.643	0.755	0.149	0.579	0.004	0.404
APCH	0.265	0.25	0.968	3.038	0.865	0.147	0.677	0.004	0.506

Table 4.14 : Kinetic model parameters of release in pH 5.6 medium.

Code	Korsmeyer-Peppas			Higuchi		Zero Order		First Order	
	K_{KP} (h^{-n})	n	R^2	K_H ($h^{-0.5}$)	R^2	K_0 (h^{-1})	R^2	K_1 (h^{-1})	R^2
G1	0.218	0.34	0.994	9.323	0.972	0.869	0.854	0.018	0.615
GPCL	0.368	0.24	0.982	7.854	0.871	0.704	0.708	0.014	0.416
GCH	0.248	0.31	0.996	9.291	0.962	0.862	0.838	0.017	0.572
A2	0.193	0.36	0.996	7.239	0.941	0.476	0.768	0.008	0.674
APCL	0.214	0.37	0.993	7.370	0.905	0.475	0.709	0.007	0.626
ACH	0.253	0.32	0.992	6.021	0.886	0.377	0.687	0.006	0.631
AP4	0.250	0.32	0.981	5.051	0.875	0.247	0.701	0.004	0.448
APPCL	0.437	0.15	0.995	3.367	0.795	0.175	0.613	0.004	0.366
APCH	0.269	0.25	0.995	4.616	0.887	0.226	0.716	0.004	0.471

Table 4.15 : Kinetic model parameters of release in pH 8.0 medium.

Code	Korsmeyer-Peppas			Higuchi		Zero Order		First Order	
	K_{KP} (h^{-n})	n	R^2	K_H ($h^{-0.5}$)	R^2	K_0 (h^{-1})	R^2	K_1 (h^{-1})	R^2
G1	0.081	0.44	0.992	2.466	0.980	0.118	0.866	0.006	0.718
GPCL	0.269	0.21	0.994	1.099	0.891	0.044	0.709	0.002	0.650
GCH	0.203	0.24	0.897	0.799	0.769	0.029	0.617	0.002	0.531
A2	0.084	0.42	0.992	2.167	0.994	0.107	0.928	0.006	0.770
APCL	0.242	0.24	0.971	1.247	0.839	0.055	0.631	0.003	0.559
ACH	0.195	0.26	0.994	1.292	0.957	0.061	0.821	0.003	0.747
AP4	0.139	0.38	0.990	1.586	0.926	0.073	0.745	0.004	0.643
APPCL	0.313	0.18	0.983	0.772	0.869	0.028	0.695	0.002	0.675
APCH	0.109	0.38	0.963	1.014	0.963	0.041	0.809	0.003	0.640

4.4 PCL-based Nanohybrid Nanospheres

Drug loading and release of microsphere formulations gave promising results; highest encapsulation efficiency of ~75 % was reached with PCL-G-RHA microspheres. The PCL-G-RHA microspheres formed highly porous surface structure and hydrophilic

character. Drug release of these microspheres reached ~60 % in 432 h (18 days) and acceptably sustained. There can be mainly two strategies to improve drug release: (1) using polymer matrix with faster dissolution and biodegradation (since drug release of PCL microspheres controlled by drug diffusion not by dissolution of polymer matrix) or (2) increasing surface area: volume ratio, thus diffusion rate. Second strategy was chosen for improvement of drug release and decided to form nanospheres using the same hybrid systems. It was also decided to continue the experiments with PCL-G-RHA nanohybrid because of the promising results obtained in microsphere formation.

Encapsulation efficiencies of the nanospheres were presented in Table 16. Addition of Span 80 in the organic phase decreased encapsulation efficiency, significantly ($p < 0.05$). Previous studies evaluating low concentrations of Span 80 in the organic phase have observed encapsulation efficiency of the desired active material varying between 30-40 % [208-211]. A strong relationship between Span 80 amount and the mean particle diameter have been reported as increasing Span 80 concentration reduces the mean particle diameter [209, 212]. This effect is created by the reduced surface tension between the aqueous and organic phases by the addition of Span 80 [213]. However, below a certain concentration the particles tends to aggregate [210, 213], which is the case for the formulation in this study (as observed in SEM images). Increasing polymer amount in the formulation slightly decreased the encapsulation efficiency as expected due to increasing viscosity. Moreover, this increased viscosity have affected the mean particle diameter, increasing it from 254 ± 3 nm to 946 ± 12 nm [214].

Additionally, encapsulation efficiency was significantly increased by applying nanoprecipitation method with respect to the formulations prepared according to emulsion/solvent evaporation procedure. Such difference is due to TC leakage during primary O/O emulsion of polymer/TC, which is also consistent with the previously reported results [215]. Also, pH of the aqueous phase used in these methods may have affected the encapsulation efficiency. PVA aqueous solutions that were used in emulsion/solvent evaporation method were slightly acidic with pH value between 5.1-5.5 whereas 1 % Poloxamer 188 aqueous solution in the nanoprecipitation method had pH value of 3.5. Acidic environment increases the strength of hydrogen bonds thus the more acidic conditions of Poloxamer 188 aqueous solution may have contributed to increased encapsulation efficiency compared to microspheres of the same nanohybrid

system. Such difference may have also affected the release rate which was significantly increased for nanospheres with respect to microspheres of the same nanohybrid system.

Table 4.16 : Nanosphere formulations, encapsulation efficiencies, loading capacities and mean particle diameters.

Sample Code	Formulation	Encapsulation Efficiency (%) ^a	Loading Capacity (%) ^a	Mean Particle Diameter (nm) ^b
Gn1	1 % Poloxamer 1:10 TC/polymer	79±4	6.7±0.4	254±3
Gn2	1 % Poloxamer (w phase), Span 80 (o-phase, 0.5 % (w/v)) 1:10 TC/polymer	48±2	4.3±0.7	872±7
Gn3	1 % Poloxamer 1:20 TC/polymer	72±3	3.4±0.1	946±12

^a mean±SD; n=3.

^b Determined by SEM images (mean±SD; n=20).

TC release profiles of the nanospheres and the release rates were compared in figures given below (Figure 4.83 and Figure 4.84). Lipid-core nanosphere formulation (Gnano2) showed very low drug release since presence of Span 80 might have added an extra diffusive layer to the surface of the nanosphere and increased diffusive path of the drug. TC release from this formulation reached 39±1 % in 240 h. However, formulation with the same amount of polymer and without Span 80 in the continuous phase (Gn1) released 84±5 % of the total TC incorporated in the nanospheres in 600 h. Increased polymer amount in the continuous (organic) phase did not changed the TC release profile significantly. Cumulative TC release from Gn3 nanosphere formulation was 89±5 % in 672 h. Nanospheres of 1:10 drug/polymer ratio released 84±5 % of the drug in 600 h (25 days). On the other hand, microspheres of the same ratio released 58±11 % of the drug in 432 (18 days). Thus, we can easily conclude that the strategy of nanosphere formation increased the diffusion rate of the drug and the amount of the diffused drug towards the release environment.

Figure 4.84 below illustrates the release rates of the nanosphere formulations. The release rates from this figure can be compared with the release rates in Figure 4.21, which shows the release rates of microsphere formulations with the same type of polymer. This comparison revealed that nanospheres demonstrated different burst release behavior than the microsphere formulations. Microsphere formulations had

biphasic release profiles reaching to constant-rate phase after a short burst release within 4 h. Nanosphere formulations also had biphasic release profiles. However, the constant rate phase was reached at 24th h (Gn1: 33±3 %, Gn2: 29±1, Gn3: 36±2). An increase in burst release is an expected phenomenon for nanospheres due to the increased surface area to volume ratio [88]. However, the release rate of TC from Gn1 nanosphere formulation decreases gradually and in a more sustained manner. Then reaches to constant rate phase in 24 h of release. On the other hand, oily core nanosphere formulation (Gn2), though the burst phase was gradual, 58 % of the total released amount was released at the end of 6 h. Burst release of Gn1 and Gn3 formulation at the end of 6h were 32 % and 33 % of the total cumulative release, respectively (Gn1: 83,6 % and Gn3: 88,7 %). The reason of such high amount of sudden release was the TC molecules adsorbed on the surface of the Gn2 nanospheres.

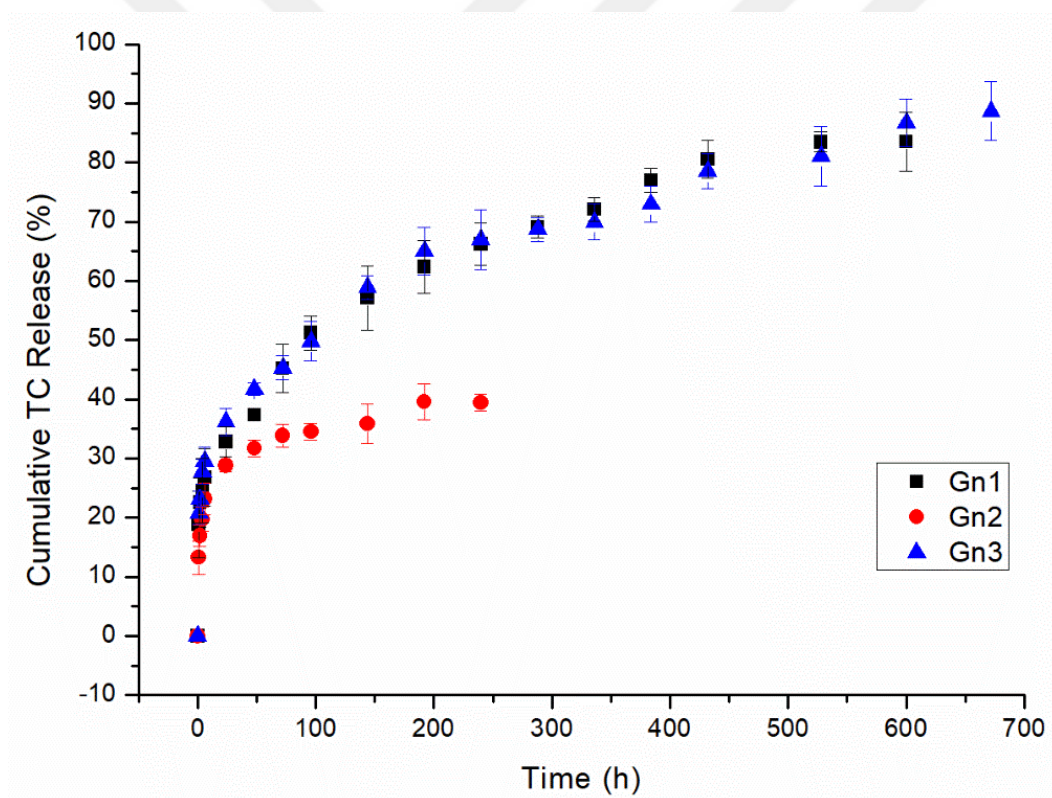


Figure 4.83 : TC cumulative release from nanospheres (TC=10 mg; 1 % Poloxamer 188; Gn1: 1:10 TC/PCL-G-RHA; Gn2: 5 % w/v Span 80, 1:10 TC/PCL-G-RHA; Gn3: 1:5 TC/PCL-G-RHA).

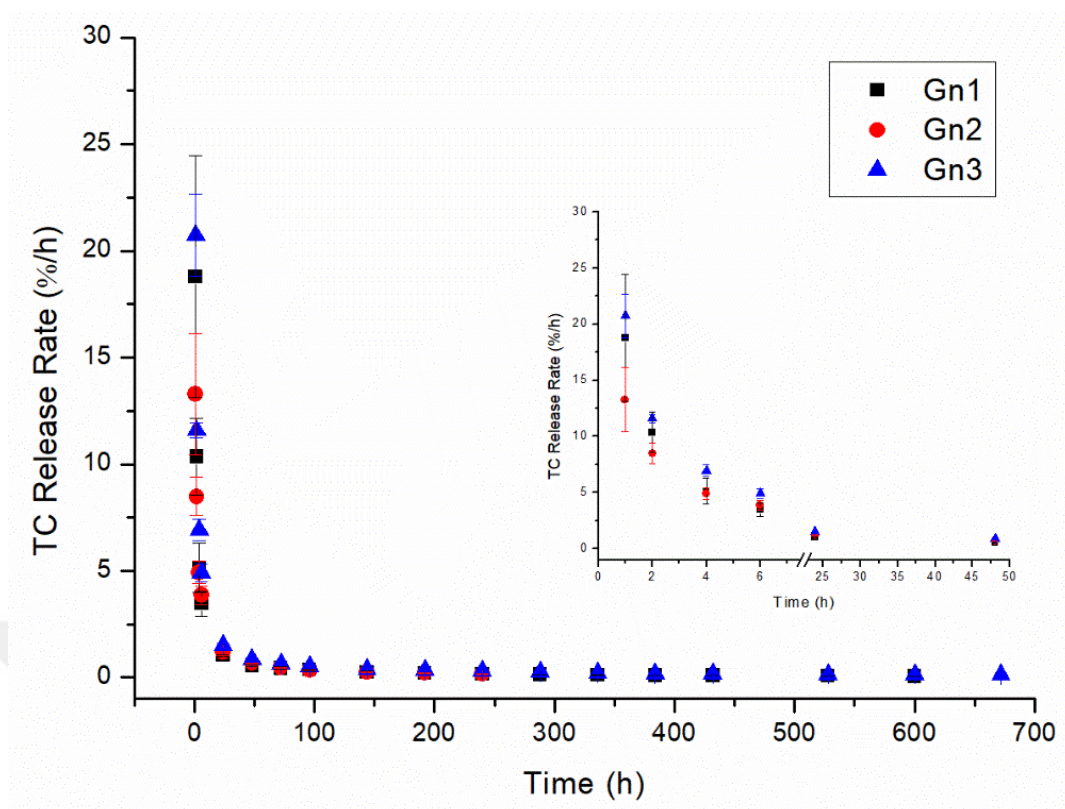


Figure 4.84 : TC release rate of nanospheres (TC=10 mg; 1 % Poloxamer 188; Gn1: 1:10 TC/PCL-G-RHA; Gn2: 5 % w/v Span 80, 1:10 TC/PCL-G-RHA; Gn3: 1:5 TC/PCL-G-RHA).

Kinetic modelling of the release data showed that TC release from the nanosphere formulations were controlled by Fickian diffusion. As presented in Table 4.17, calculated parameters of Korsmeyer-Peppas kinetic model, n value was lower than 0.43 which indicates Fickian diffusion-controlled drug release.

Table 4.17 : Kinetic model parameters of nanosphere formulations.

Code	Korsmeyer-Peppas			Higuchi		Zero Order		First Order	
	K_{KP} (h^{-n})	n	R^2	K_H ($h^{-0.5}$)	R^2	K_0 (h^{-1})	R^2	K_1 (h^{-1})	R^2
Gn1	0.219	0.22	0.972	3.060	0.962	0.118	0.829	0.002	0.760
Gn2	0.379	0.19	0.973	2.017	0.807	0.114	0.609	0.004	0.623
Gn3	0.231	0.20	0.985	2.862	0.953	0.104	0.818	0.002	0.752

Optical microscopy and SEM images of nanospheres revealed spherical particles of nanometer scale diameter. As mentioned earlier, presence of Span 80 in the oily core of the nanospheres avoids them to aggregate but there is a concentration limit below which this effect becomes the opposite [210, 213]. This phenomenon can be observed in the SEM image of oily core formulation (Gn2) in which the aggregated particles are clearly visible.

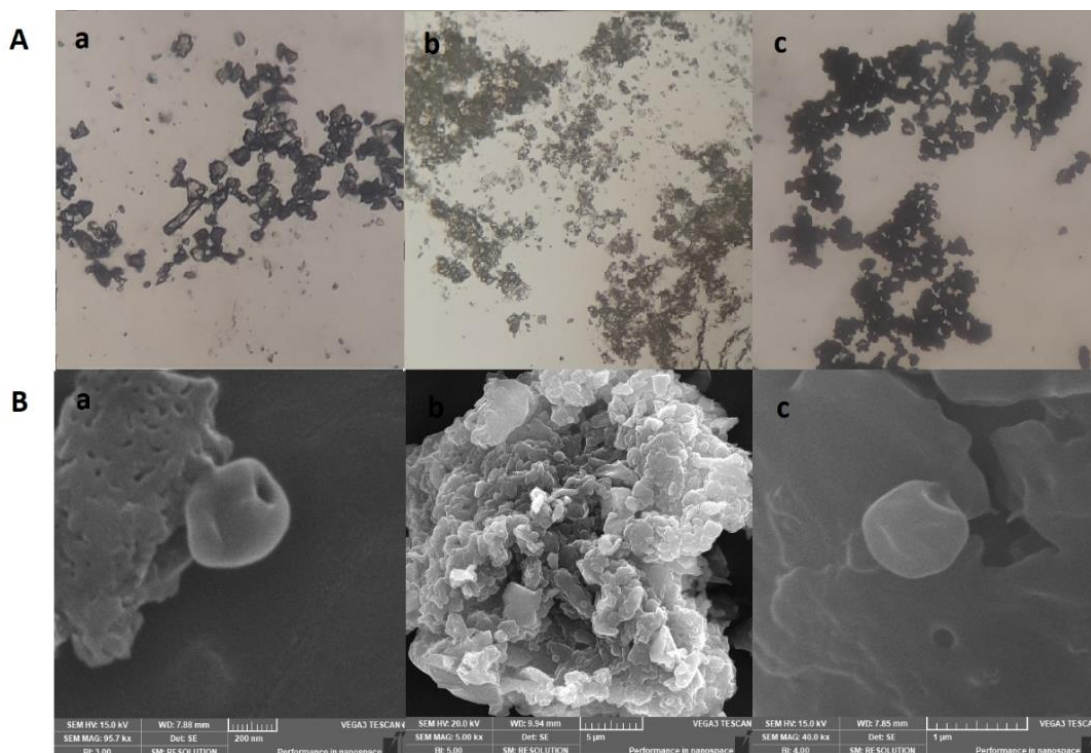


Figure 4.85 : A) Optical microscopy images of a) Gn1, b) Gn2, c) Gn3 (Magnification 100x); B) SEM images of a) Gn1 (Magnification 95000x), b) Gn2 (Magnification 5000x), c) Gn3 (Magnification 40000x).

FT-IR spectra given in Figure 4.86 displays the the characteristic absorption peaks of PCL-G-RHA and TC. Peaks at 794 cm^{-1} and 1043 cm^{-1} were assigned to Si-CH_3 rocking and asymmetric stretching of Si-O-Si which are present in the structure of RHA part of PCL-G-RHA nanohybrid. C-O and C-C bonds in the structure of PCL content showed absorbance peaks at 1297 cm^{-1} . Additionally, symmetric and asymmetric stretching of C-O-C can be observed at 1190 cm^{-1} and 1244 cm^{-1} , respectively. The peaks of asymmetric CH_2 bonds at 2945 cm^{-1} , symmetric CH_2 bonds at 2863 cm^{-1} were observable in the spectrum of Gn1 which were caused by the PCL content of the nanohybrid. The broad peak at 3441 cm^{-1} was observed in the spectra of PCL-G-RHA and Gn1 nanospheres was assigned to stretching vibrations of intramolecularly H-bonded $-\text{OH}$ group of PCL-G-RHA hybrid system. Very slight and broad signal was detected, which was conjugated with the peak of intramolecularly H-bonded $-\text{OH}$ group, at 3533 cm^{-1} assigned to free $-\text{OH}$ content of PCL-G-RHA. Lastly, aliphatic carbonyl bond peak at 1728 cm^{-1} has showed the presence of PCL in the PCL-G-RHA nanohybrid system. TC molecules were proved to be in the structure of nanospheres. Absorbance peaks at 1660 cm^{-1} belonging to α, β unsaturation in ketones, at 1601 cm^{-1} belonging to $\text{C}=\text{C}$ stretching and at 1475 cm^{-1} belonging to C-

C stretching in aromatic compounds. Another characteristic peak of TC ($=C-H$ stretching, aromatic, multiple peaks at $3100-3200\text{ cm}^{-1}$) was slightly visible in the spectrum of nanospheres. These mentioned peaks altogether indicated the presence and the successful encapsulation of TC.

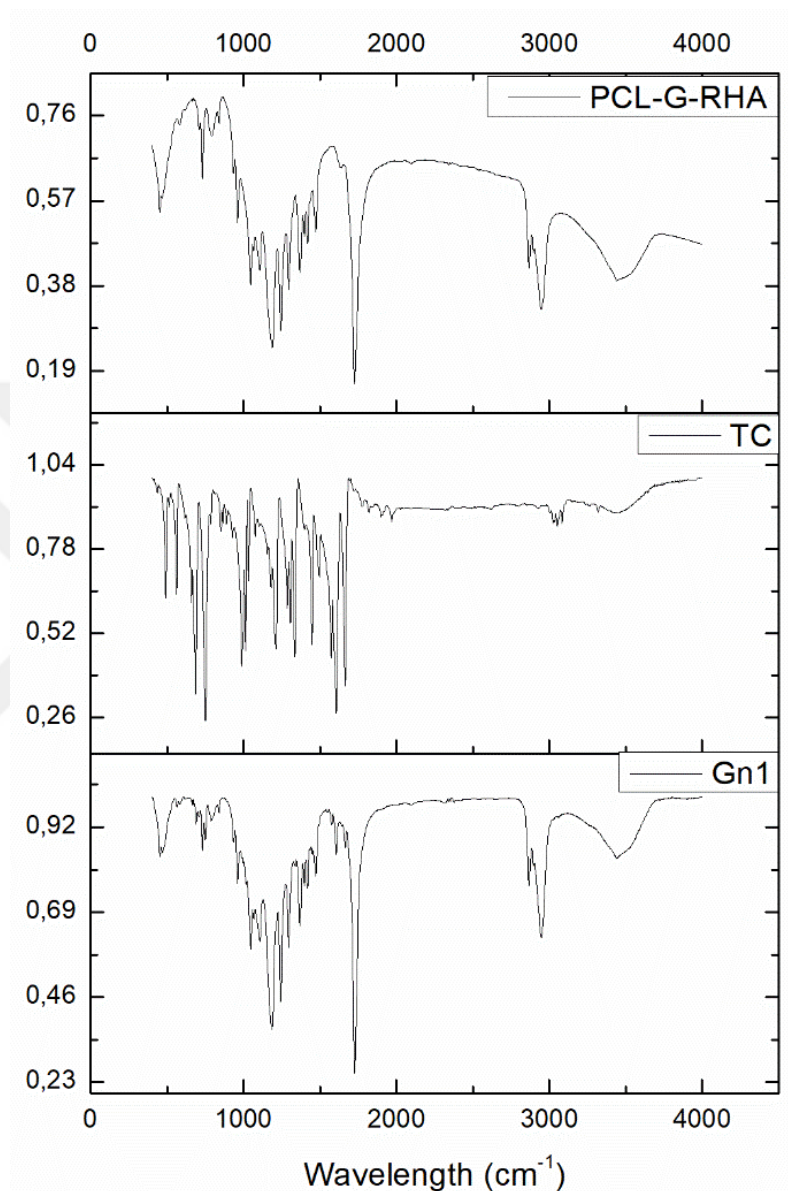


Figure 4.86 : FT-IR spectra of Gn1, PCL-G-RHA and TC (Gn1: 1 % Poloxamer 188, 1:10 TC:PCL-G-RHA).

The spectra of nanospheres and the physical mixture of the nanosphere formulation were compared in Figure 4.87. Two distinctions were observed between the spectra of nanospheres and the physical mixture of the formulation. Firstly, absorbance peak of oxirane ring at 840 cm^{-1} in the spectrum of the physical mixture shifted to 839 cm^{-1} and the intensity of the peak had significantly decreased. This may indicate that TC

molecules have partially interacted with the oxirane ring which is present in 3-GPTMS content of the PCL-G-RHA nanohybrid system. The second distinction between the two spectra also supports this suggestion. In the spectrum of the physical mixture, a broad peak at 1150-1200 cm^{-1} was observed in which the signal of CH_2 wagging was conjugated with symmetric stretching of C-O-C bonds in PCL. However, the signal of CH_2 wagging had completely disappeared and this broad peak was reduced to a single sharp peak at 1190 cm^{-1} which was assigned to symmetric stretching of C-O-C. These findings may be due to the protonation and ring opening of oxirane ring of 3-GPTMS in the structure of PCL-G-RHA nanohybrid. Additional to these changes, the peak at 3536 cm^{-1} in the spectrum of the physical mixture completely disappeared in the spectrum of Gn1 nanosphere formulation. This peak was assigned to free -OH groups of PCL-G-RHA. The disappearance of this peak suggest that α , β unsaturated ketone of TC molecules was exposed to nucleophilic attack by the free -OH groups of PCL-G-RHA [216] and these interactions between the polymer and TC molecules contributed to high drug loading.

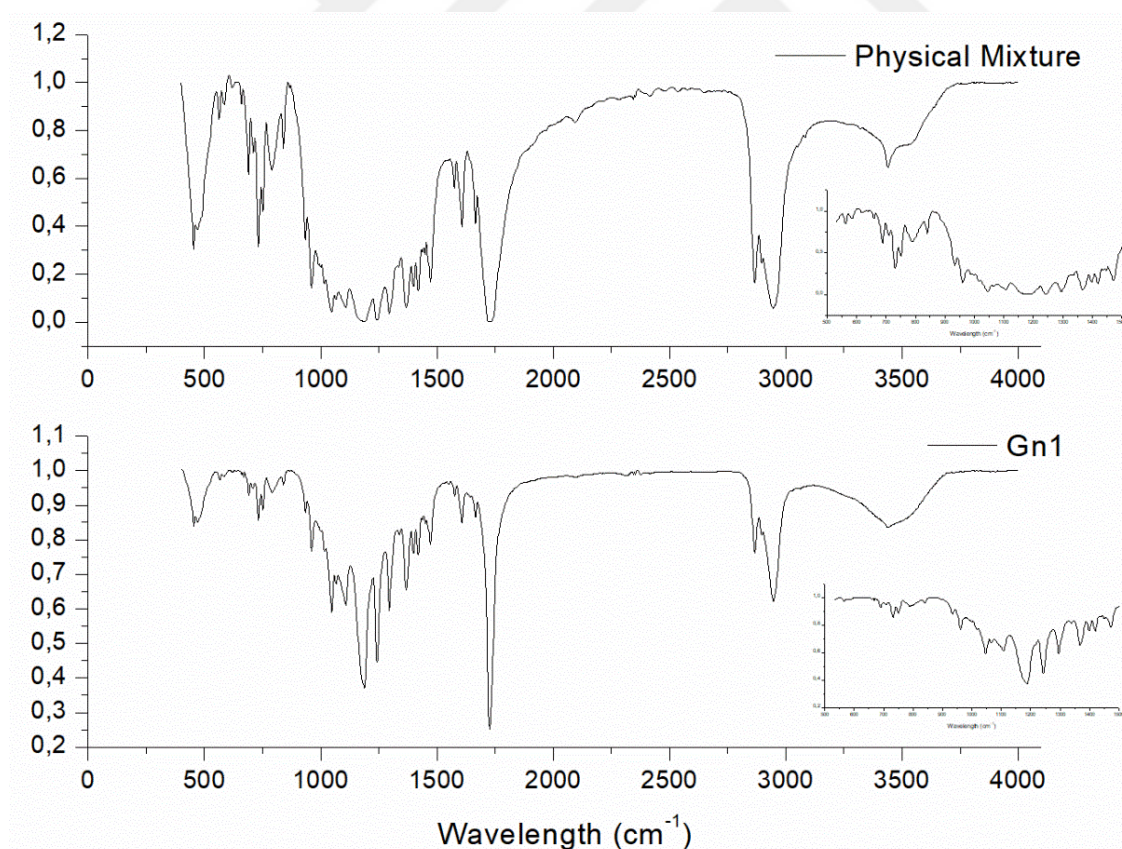


Figure 4.87 : FT-IR spectra of Gn1 nanospheres and the physical mixture of PCL-G-RHA and TC (Gn1: 1 % Poloxamer 188, 1:10 TC:PCL-G-RHA; Physical mixture: Mixture of 1:10 (w:w) TC:PCL-G-RHA).

Nanospheres were also characterized by DSC in order to understand the thermal properties and to reveal whether TC molecules were dispersed in the polymer matrix or not. The DSC scans of the melting endotherms (second heating cycle) of Gn1 nanospheres, physical mixture of the Gn1 nanosphere formulation, PCL-G-RHA nanohybrid and TC were presented in Figure 4.88. The second heating of the nanospheres revealed two distinctive melting peaks at 48 °C and 52 °C. Such double melting peak indicated that TC molecules were encapsulated in the nanospheres at its crystalline forms rather than molecularly dispersed within the polymer matrix.

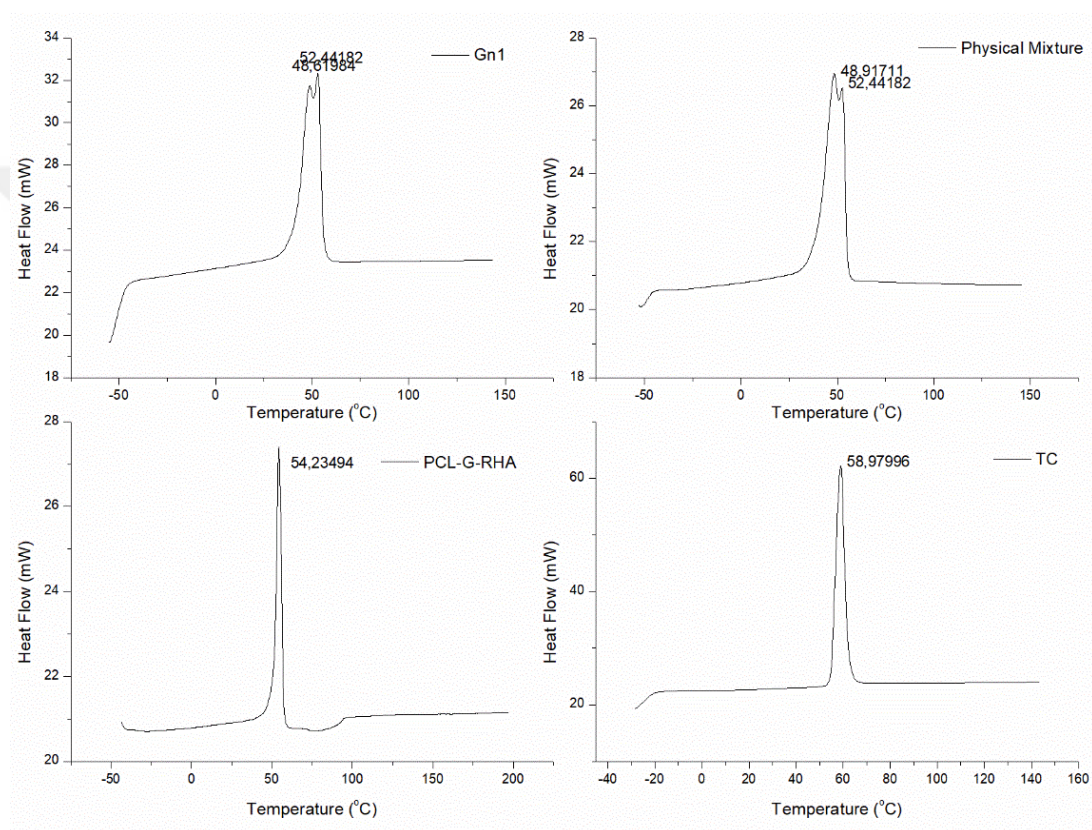


Figure 4.88 : DSC thermogram of the PCL-G-RHA, TC, Gn1 and physical mixture, demonstrating melting endotherm (second heating cycle), Gn1: 1 % Poloxamer 188, 1:10 TC:PCL-G-RHA.

4.4.1 Effect of silica incorporation in the polymer matrix

Nanospheres of neat PCL were fabricated with the same formulation in order to understand how the presence of silica in the PCL-G-RHA nanohybrid system affected drug loading and release behavior. Table 4.18 shows the comparison between neat PCL nanospheres (GnPCL) and PCL-G-RHA nanospheres (Gn1) in terms of encapsulation efficiency, drug loading capacity and mean particle diameter. It is apparent from the table that mean particle diameter was significantly increased when

the polymer matrix of the nanospheres was neat PCL (G-PCL). This increase may be due to the difference in the molecular weight of the two polymers. As presented previously the molecular weight of G-PCL was 10300 g/mol whereas the molecular weight of PCL-G-RHA was calculated as 5500 g/mol. Increased molecular weight in the emulsion during nanosphere formation had increased the viscosity and resulted in higher particle size since the increased viscosity led to the formation of larger emulsion droplets during nanosphere formation [217]. Encapsulation efficiency and loading capacity did not significantly affected by the changing molecular weight of the polymer.

Table 4.18 : Nanosphere formulations, encapsulation efficiencies, loading capacities and mean particle diameters.

Sample Code	Formulation	Encapsulation Efficiency (%) ^a	Loading Capacity (%) ^a	Mean Particle Diameter (nm) ^b
Gn1	1 % Poloxamer 1:10 TC/PCL-G-RHA	79±4	7.2±0.4	254±3
GnPCL	1 % Poloxamer 1:20 TC/G-PCL	79±8	7.2±0.7	1102±14

TC cumulative release profiles of Gn1 and GnPCL nanospheres were given in Figure 4.89. As can be clearly seen from the figure, nanospheres constructed with PCL-G-RHA nanohybris system (Gn1) released TC more efficiently than nanospheres with G-PCL as the polymer matrix (GnPCL). Gn1 nanosphere formulation reached 84±5 % cumulative release of the total TC within the nanospheres whereas GnPCL released only 51±3 % of hte total TC. Additionally, TC release from Gn1 nanospheres were slower than TC release from GnPCL nanospheres and reached to completion in 600 h (25 days). However, release from GnPCL was completed in 384 h (16 days). This slow release may be explained by the smaller particle size which decreases the surface area for diffusion of the drug molecules [218].

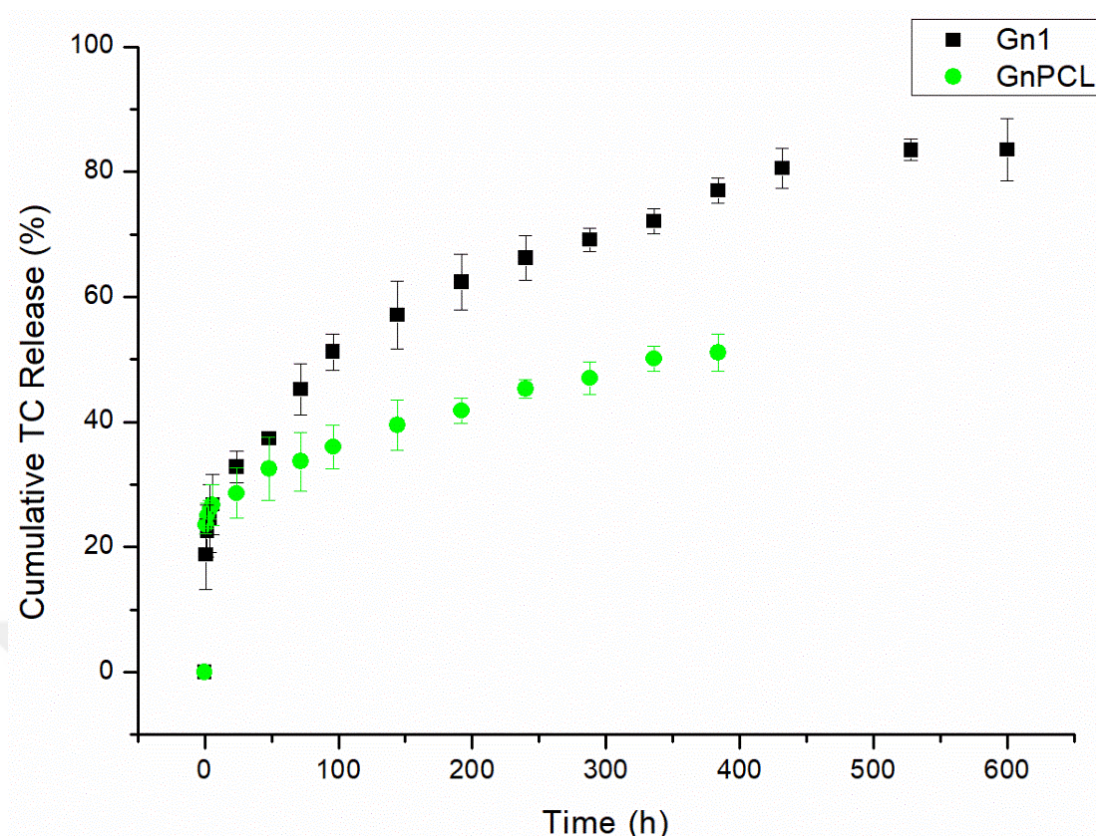


Figure 4.89 : TC cumulative release from nanospheres (TC=10 mg; 1 % Poloxamer 188; Gn1: 1:10 TC/PCL-G-RHA; GnPCL: 1:10 TC/G-PCL).

Calculated release rates of the two nanosphere formulations can be seen in Figure 4.90. TC release rates of GnPCL nanospheres in the first few hours of the release were slightly higher than that of Gn1 nanospheres. However, burst release behavior of both of the formulations were similar. Both nanosphere formulations had biphasic release consisted of a burst release and slow release phase, and reached to a steady release phase in the first 24 h of release time. The burst release of the drug molecules from nanoparticles is mostly associated with the drug molecules adsorbed on the surface or located close to the surface of the particles [219]. Thus the burst release is directly affected by the type of the polymer used in the formulations. High crystallinity of the polymers generally localize the drug molecules on the surface of the particles [40]. Thus, the increased burst release of GnPCL nanospheres can be explained by the drug molecules localized on the surface due to higher crystallinity of G-PCL (as determined by DSC).

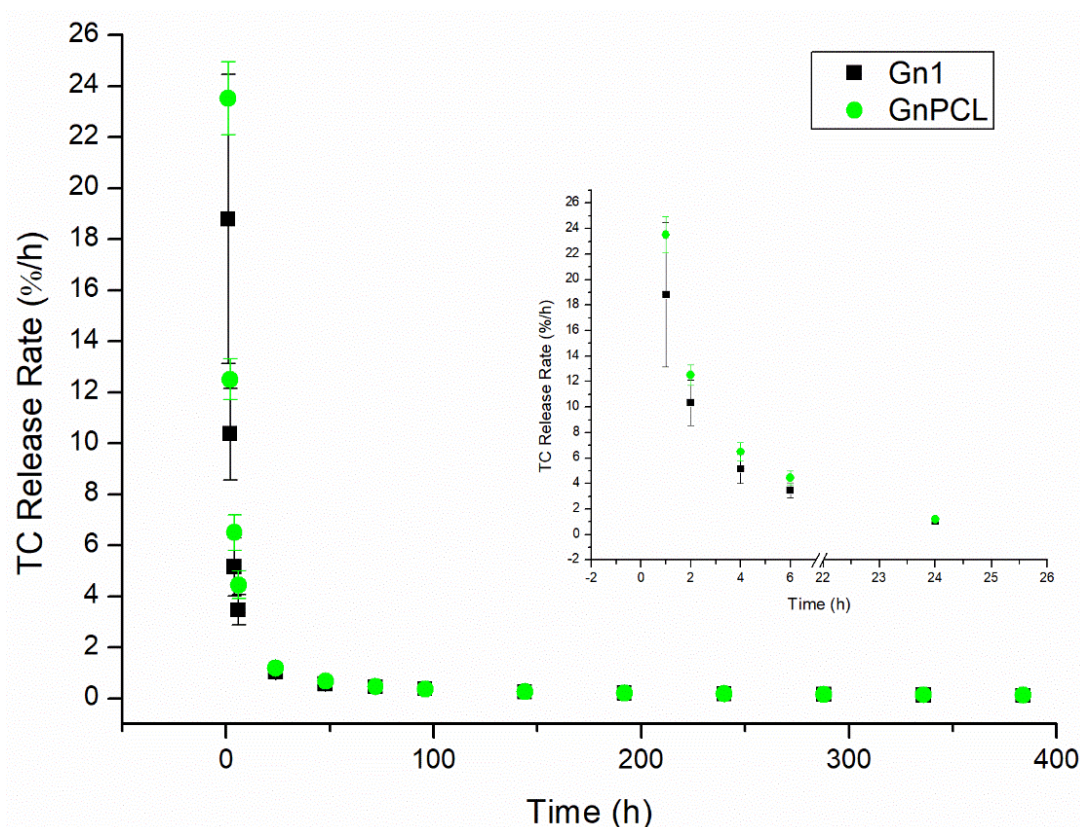


Figure 4.90 : TC release rate of nanospheres (TC=10 mg; 1 % Poloxamer 188; Gn1: 1:10 TC/PCL-G-RHA; GnPCL: 1:10 TC/G-PCL).

Several kinetic models were fitted to the release profiles of Gn1 and GnPCL nanospheres. The best fitting model was determined to be Korsmeyer-Peppas with a regression coefficient of 0,972 and 0,922, respectively. The calculated n value indicated that TC release from both of the nanosphere formulations were controlled by Fickian diffusion and polymer erosion was not effective on the release.

Table 4.19 : Kinetic model parameters of nanosphere formulations.

Code	Korsmeyer-Peppas			Higuchi		Zero Order		First Order	
	K_{KP} (h^{-n})	n	R^2	K_H ($h^{-0.5}$)	R^2	K_0 (h^{-1})	R^2	K_1 (h^{-1})	R^2
Gn1	0.219	0.22	0.972	3.060	0.962	0.118	0.829	0.002	0.760
GnPCL	0.422	0.13	0.922	1.761	0.831	0.084	0.791	0.002	0.912

GnPCL nanospheres were characterized by SEM, FT-IR and DSC analyses in order to determine and compare some properties such as surface structure, drug/polymer interactions and melting behavior. Optical microscopy and SEM images revealed spheric geometries of the nanospheres as expected (Figure 4.91). It can be clearly seen from the SEM images (Figure 4.91B) surface of the nanospheres were smooth and less porous than microspheres.

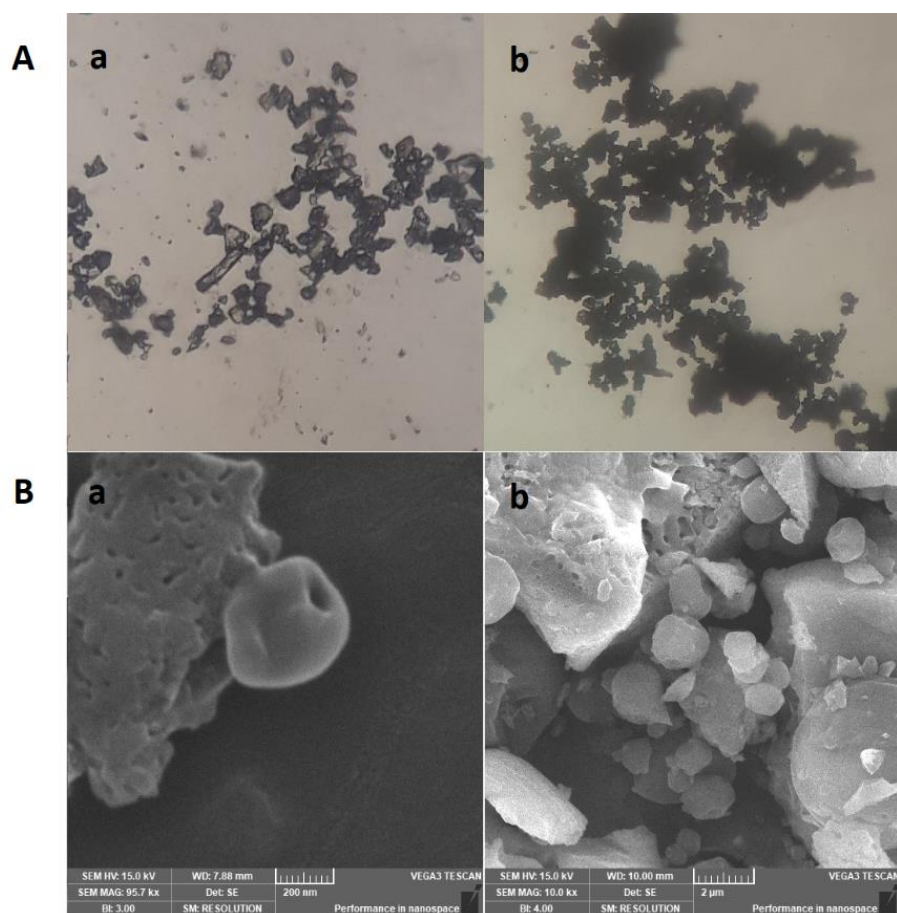


Figure 4.91 : A) Optical microscopy images of a) Gn1, b) GnPCL (Magnification 100x); B) SEM images of a) Gn1 (Magnification 95000x), b) GnPCL (Magnification 10000x).

FT-IR spectrum of GnPCL given in Figure 4.92 was compared with the spectra of TC and Gn1 nanosphere formulation. Peaks associated with silica in the structure of RHA were not present in the spectrum of GnPCL. This nonexistence is expected indeed since neat G-PCL does not contain RHA in its structure. Presence of PCL was verified by the peaks observed in the FT-IR spectrum of GnPCL. These peaks are listed as: C-O and C-C bonds at 1295 cm^{-1} , symmetric and asymmetric stretching of C-O-C at 1185 cm^{-1} and 1240 cm^{-1} , respectively, aliphatic carbonyl bond at 1729 cm^{-1} , asymmetric CH_2 bonds at 2950 cm^{-1} , symmetric CH_2 bonds at 2867 cm^{-1} , stretching vibrations of -OH end group at 3439 cm^{-1} . The spectrum also shows the successful loading of TC to the GnPCL nanosphere formulation. Absorbance peak at 1661 cm^{-1} was assigned to α , β unsaturation in ketones which is the primary structure in TC. Additionally, peaks at 1601 cm^{-1} belonging to $\text{C}=\text{C}$ stretching and at 1473 cm^{-1} belonging to C-C stretching in aromatic compounds are the bond structures of benzene ring of TC molecule.

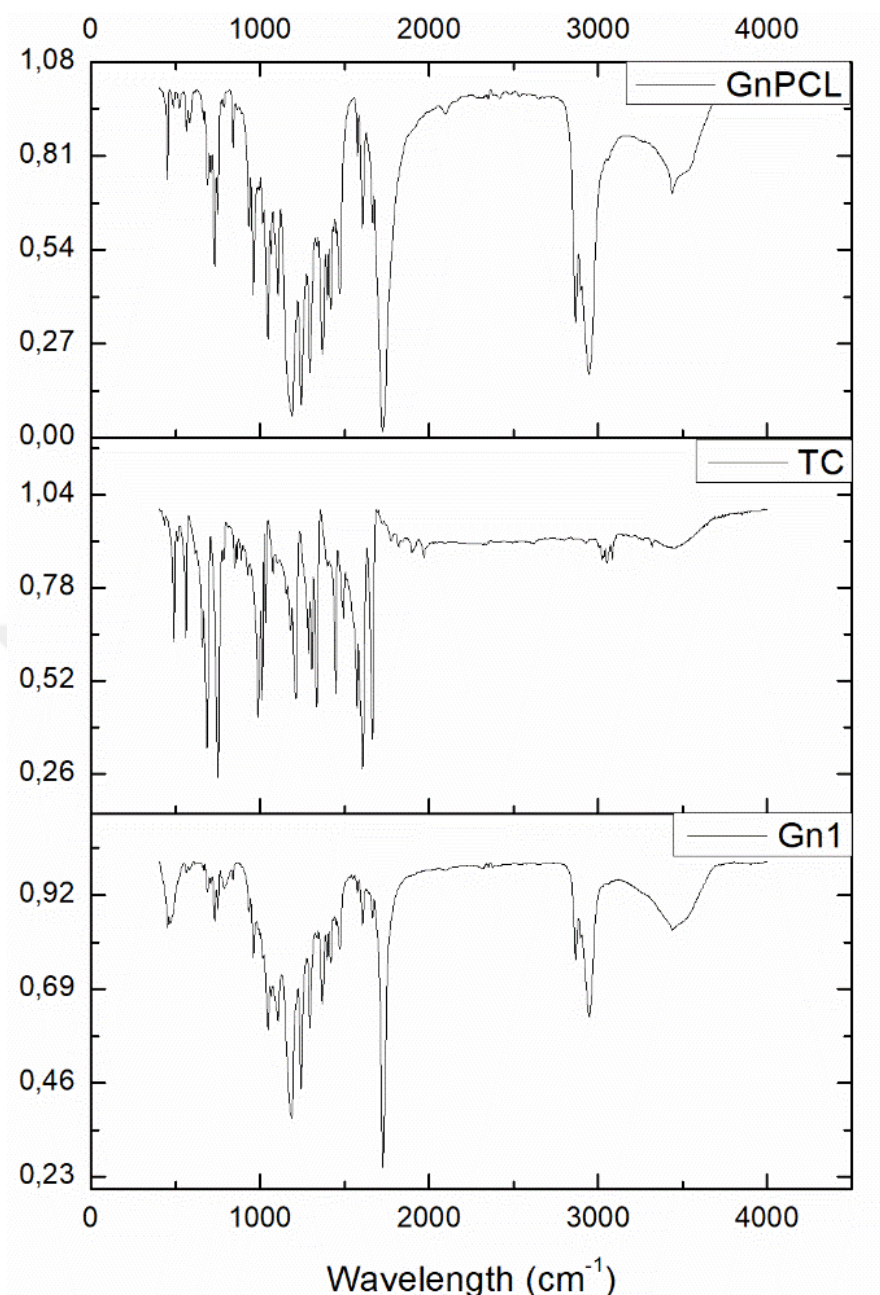


Figure 4.92 : FT-IR spectra of GnPCL, Gn1 and TC (Gn1: 1 % Poloxamer 188, 1:10 TC:PCL-G-RHA; GnPCL: 1 % Poloxamer 188, 1:10 TC:G-PCL).

Figure 4.93 shows the comparison between the spectra of GnPCL and the physical mixture of the nanosphere formulation. This comparison did not show any indications of polymer/drug interactions due to nanosphere formation. Shifts were not observed in two characteristic absorbance peaks of TC and G-PCL which are considered to be the possible moieties for interactions. The peak of TC associated with α , β unsaturated ketone group located at 1667 cm^{-1} in the spectrum of the physical mixture. This peak was also detected at 1667 cm^{-1} in the spectrum of GnPCL nanosphere formulation. Moreover, the peak at 3536 cm^{-1} observed in the spectrum of the physical mixture had

been detected in the spectrum of GnPCL. This peak was assigned to free –OH groups of G-PCL. These findings suggested that intermolecular interactions were not present between α , β unsaturated ketone group of TC and –OH end group of PCL.

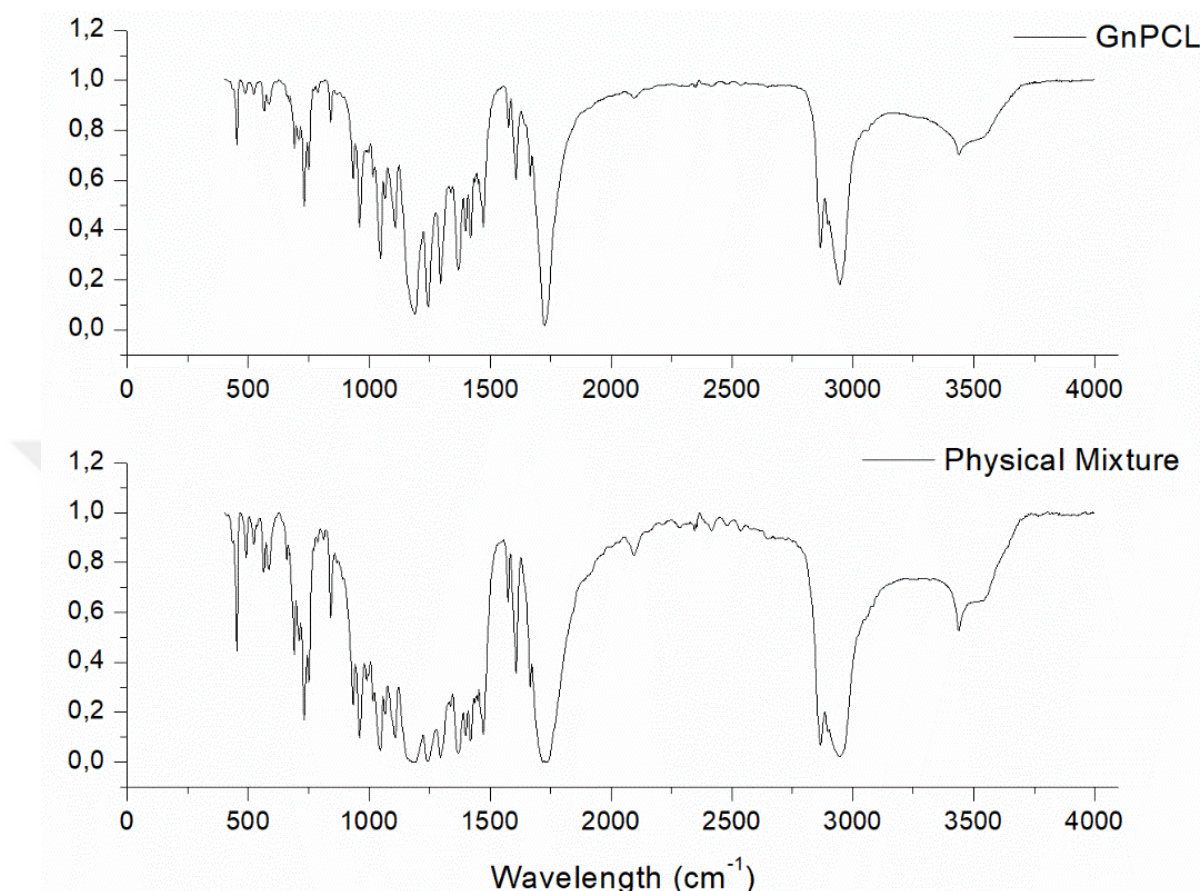


Figure 4.93 : FT-IR spectra of GnPCL nanospheres and the physical mixture of G-PCL and TC (GnPCL: 1 % Poloxamer 188, 1:10 TC:G-PCL; Physical mixture: Mixture of 1:10 (w:w) TC:G-PCL).

DSC scanning was also applied to GnPCL and the physical mixture of this formulation in order to understand the interactions between the drug and the polymer, and crystalline form of the drug within the nanospheres depending on their melting behaviors. As can be seen in Figure 4.94, GnPCL showed the double melting peak as the physical mixture of the same formulation did which is characteristic to the drug molecules encapsulated within the polymeric spheres in crystalline form rather than molecular dispersion [220].

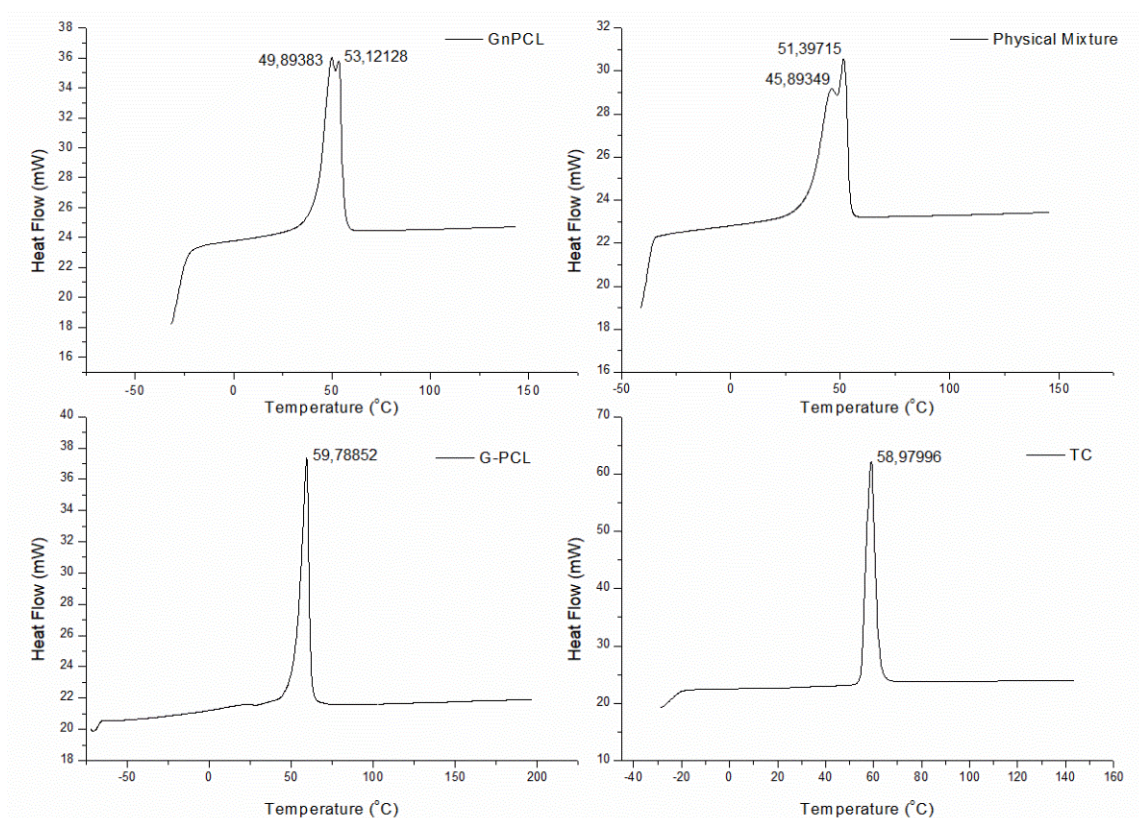


Figure 4.94 : DSC thermogram of the G-PCL, TC, GnPCL and physical mixture, demonstrating melting endotherm (second heating cycle), GnPCL: 1 % Poloxamer 188, 1:10 TC:G-PCL.

4.4.2 Effect of the loaded TC

Properties of the loaded drug molecules also affects the encapsulation efficiency and drug release behavior of nanospheres. For this reason, drug loading and release studies were conducted with CS-TC to observe possible differences in loading release properties of nanospheres. Table 20 shows the comparison between CS-TC loaded nanospheres and C-TC loaded nanospheres in terms of encapsulation efficiency, loading capacity and mean particle diameter. Encapsulation efficiency and loading capacity of the two formulation with C-TC and CS-TC loading did not differ from each other significantly. However, spheres with CS-TC loading were significantly larger than C-TC loaded spheres with mean particle diameter in micrometric scale.

Table 4.20 : Nanosphere formulations, encapsulation efficiencies, loading capacities and mean particle diameters.

Sample Code	Formulation	Encapsulation Efficiency (%) ^a	Loading Capacity (%) ^a	Mean Particle Diameter (nm) ^b
Gn1	1 % Poloxamer 1:10 C-TC/PCL-G-RHA	79±4	7.2±0.4	254±3
GnCH	1 % Poloxamer 1:20 CS-TC/PCL-G-RHA	75±8	6.4±0.7	2418±24

Drug release profiles of the two nanosphere formulations with C-TC and CS-TC were presented in Figure 4.95. Release profiles of the two formulations did not differ from each other significantly. However, cumulative release was higher for Gn1 nanosphere formulation (C-TC-loaded; 84±5 %) than that of GnCH nanosphere formulation (CS-TC-loaded; 75±5 %). Release time from the nanospheres were significantly different. When the loaded drug was commercial TC, drug release reached to completion in 600 h (25 days). The two factors affecting drug release are the crystallization behavior of polymer matrix and the dispersion of the drug molecules within the polymer matrix. The differences in released amount and release time of the nanosphere formulations might be due to the different crystalline properties of the loaded drug. As presented earlier CS-TC has lower crystallinity degree (70 %) than C-TC (100 %). An earlier study had shown that the presence of molecularly dispersed drug within PCL microspheres increased the degree of crystallinity of PCL which led to slow drug release [221]. Thus, it is necessary to determine the dispersion state of CS-TC within the PCL-G-RHA matrix and compare it to C-TC to fully understand the differences in release behavior of the formulations.

Figure 4.96 shows the release rates of Gn1 and GnCH nanosphere formulations. Both formulations had biphasic profile with a short burst release period followed by constant-rate phase. As can be seen in the figure, release rate in the burst release phase in 6 h was slightly higher for GnCH nanosphere formulation. After the burst release phase, drug release rates of both of the formulations were similar.

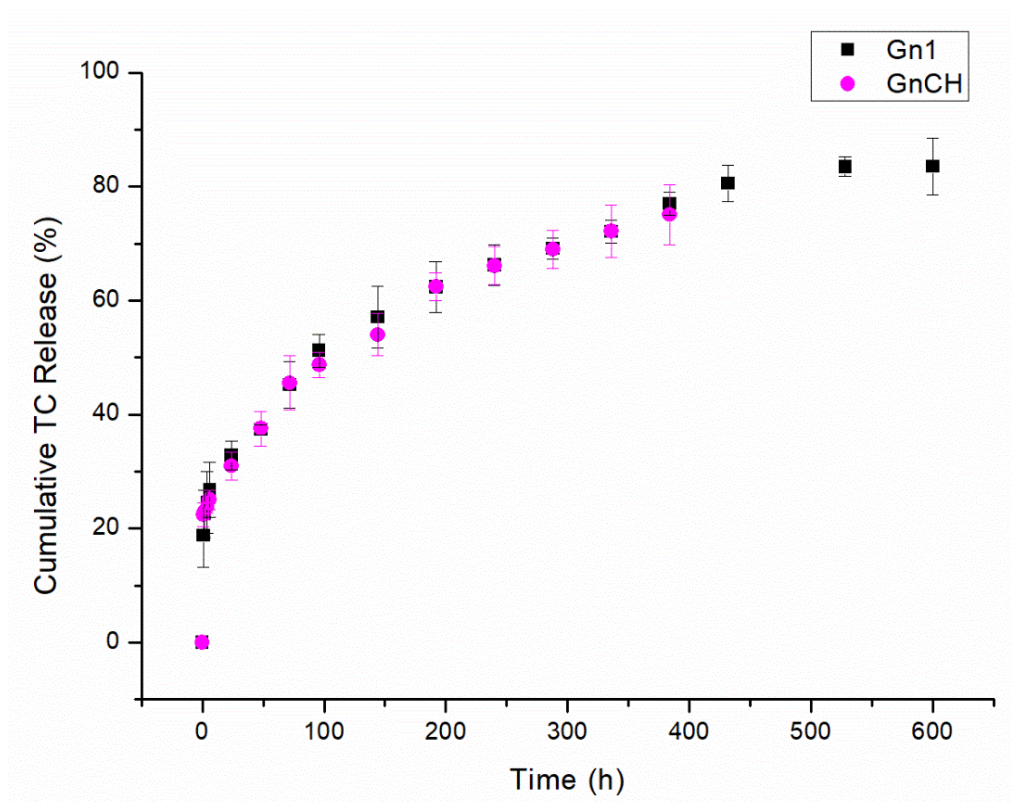


Figure 4.95 : TC cumulative release from nanospheres (TC=10 mg; 1 % Poloxamer 188; Gn1: 1:10 TC/PCL-G-RHA; GnCH: 1:10 CS-TC/PCL-G-RHA).

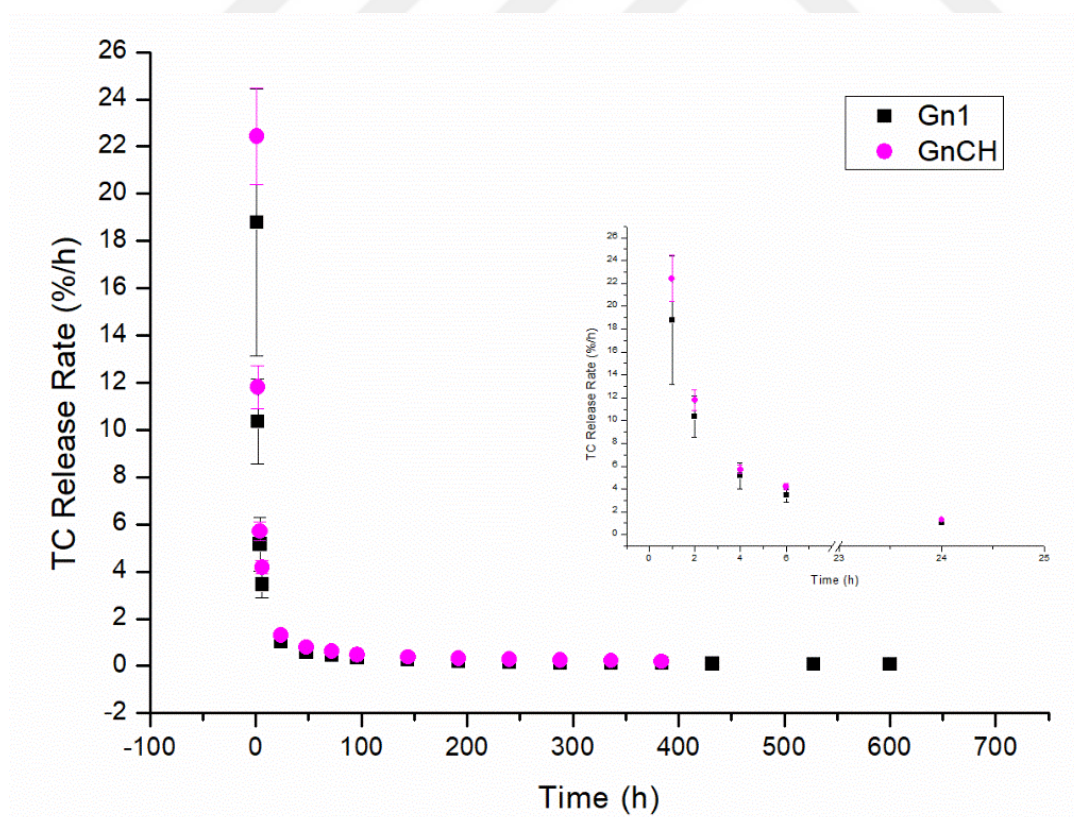


Figure 4.96 : TC release rate of nanospheres (TC=10 mg; 1 % Poloxamer 188; Gn1: 1:10 TC/PCL-G-RHA; GnCH: 1:10 TC/PCL-G-RHA).

Kinetic modelling of release profiles of the GnCH formulation was done to determine the dominant release mechanism and the calculated parameters were presented in Table 4.21 with the calculated kinetic parameters of Gn1 formulations for comparison. The best fitting kinetic model for GnCH nanopshere formulation was determined to be Higuchi model with a correlation coefficient of 0.958. This model applies to Fickian diffusion of drug molecules from insoluble poylmer matrix. This result also indicates that polymer chain erosion was not dominant mechanism of release and did not contribute to TC release from the nanospheres significantly.

Table 4.21 : Kinetic model parameters of nanosphere formulations.

Code	Korsmeyer-Peppas			Higuchi		Zero Order		First Order	
	K_{KP} (h^{-n})	n	R^2	K_H ($h^{-0.5}$)	R^2	K_0 (h^{-1})	R^2	K_1 (h^{-1})	R^2
Gn1	0.219	0.22	0.972	3.060	0.962	0.118	0.829	0.002	0.760
GnCH	0.262	0.19	0.931	3.239	0.958	0.156	0.851	0.003	0.840

Optical microsocopy images of nanosphere formulations were taken to observe surface structure and the geometries of the formulations. Both SEM and optical microscopy images showed that the particles obatined from both formulations had spherical shapes as expected. Both of the nanosphere formulations had large holes on their surfaces which may have contributed to drug diffusion towards release medium. SEM images also revealed particle size of the nanosphere formulations. GnCH nanospheres were significantly larger than Gn1 nanospheres which may be explained by the differences in dispersion state of the drug molecules withinh the nanosphere formulations. The dispersion state of the drug molecules withinh the nanosphere formulations will be investigated and discussed later in this section with DSC results.

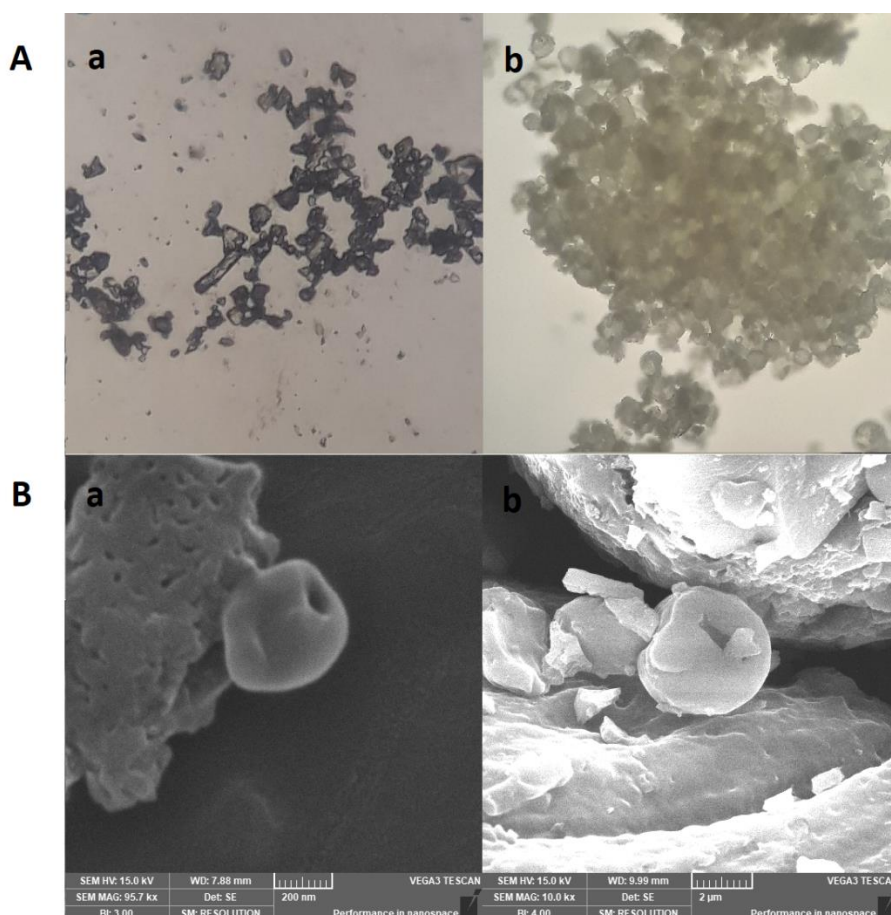


Figure 4.97 : A) Optical microscopy images of a) Gn1, b) GnCH (Magnification 100x); B) SEM images of a) Gn1 (Magnification 95000x), b) GnCH (Magnification 10000x).

Nanosphere formulation loaded with CS-TC were structurally characterized by FT-IR spectroscopy in order to reveal potential drug/polymer interactions. In Figure 4.98 FT-IR spectra of GnCH, CS-TC and PCL-G-RHA were presented. The spectrum of GnCH showed the characteristic peaks of CS-TC and PCL-G-RHA as listed: 789 cm^{-1} Si-CH₃ rocking, 1043 cm^{-1} asymmetric stretching of Si-O-Si, 838 cm^{-1} oxirane ring of PCL-G-RHA; 1474 cm^{-1} C-C stretching in aromatic compounds, 1606 cm^{-1} C=C stretching in aromatic compounds, 1669 cm^{-1} α , β unsaturation of TC; 1293 cm^{-1} C-O and C-C, 1244 cm^{-1} asymmetric stretch of C-O-C, 1185 cm^{-1} symmetric stretch of C-O-C, 1728 cm^{-1} aliphatic carbonyl bond, 2863 cm^{-1} symmetric CH₂, 2946 cm^{-1} asymmetric CH₂, 3440 cm^{-1} stretching vibrations of intramolecularly H-bonded -OH of PCL-G-RHA structure.

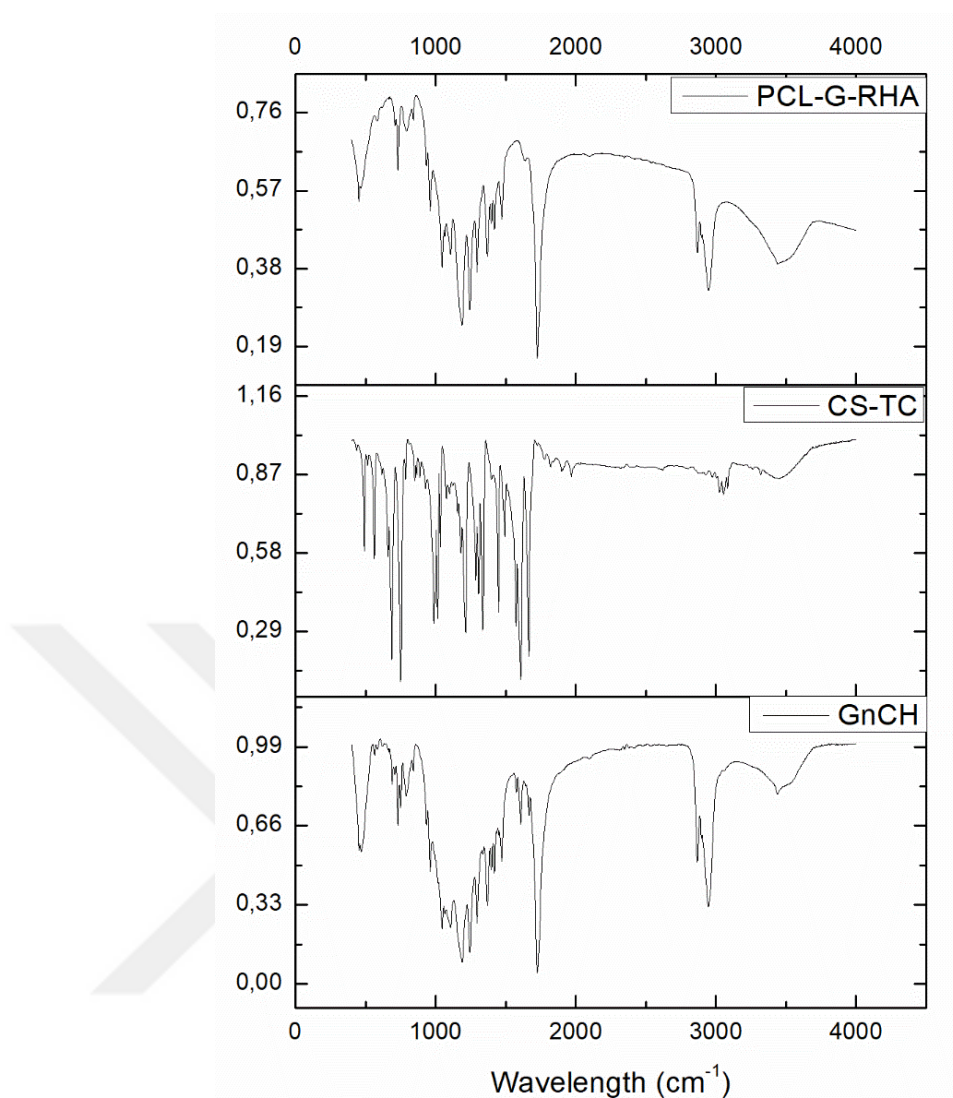


Figure 4.98 : FT-IR spectra of GnCH, PCL-G-RHA and CS-TC (Gn1: 1 % Poloxamer 188, 1:10 TC:PCL-G-RHA; GnCH: 1 % Poloxamer 188, 1:10 CS-TC:PCL-G-RHA).

Comparison of the FT-IR spectra of GnCH nanosphere formulation and the physical mixture of this formulation revealed some shifts in the characteristic peaks of CS-TC and PCL-G-RHA (Figure 4.99). In the spectrum of the physical mixture, the peak at 843 cm^{-1} was assigned to the oxirane ring of PCL-G-RHA which was added to the nanohybrid structure by modification with 3-GPTMS. It was observed that this peak had shifted to 838 cm^{-1} in the spectrum of GnCH nanospheres. Additionally, α , β unsaturation in the structure of CS-TC created an absorbance at 1669 cm^{-1} in the spectrum of the physical mixture. Due to nanosphere formation by nanoprecipitation method, this peak had shifted to a lower wavelength at 1665 cm^{-1} . These shifts in the characteristic peaks of the main components indicated interactions between CS-TC molecules and PCL-G-RHA.

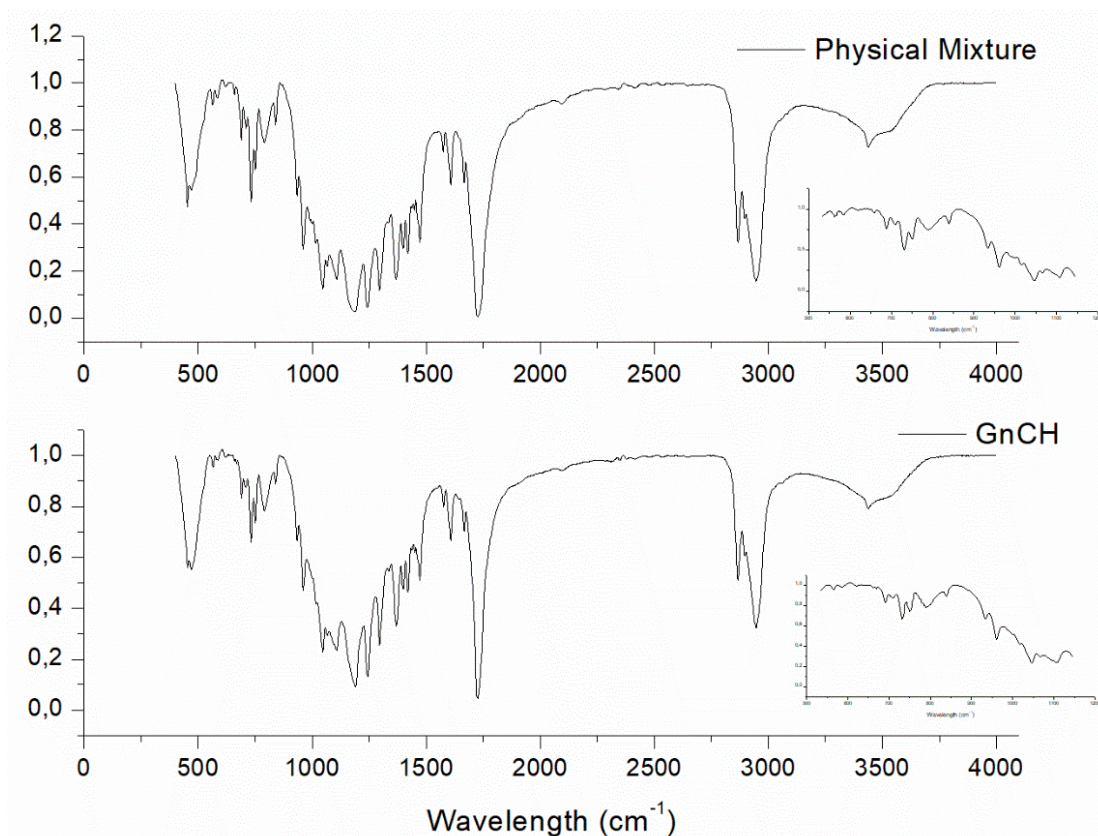


Figure 4.99 : FT-IR spectra of GnCH nanospheres and the physical mixture of PCL-G-RHA and CS-TC (GnCH: 1 % Poloxamer 188, 1:10 TC:PCL-G-RHA; Physical mixture: Mixture of 1:10 (w:w) CS-TC:PCL-G-RHA).

Dispersion state of CS-TC molecules within the GnCH nanosphere formulation was determined by studying melting endotherms of nanospheres and the physical mixture of the formulation. Physical mixture of the components of GnCH formulation had two distinctive melting peak belonging to CS-TC and PCL-G-RHA with a slight melting point depression. However, the single melting peak observed in the DSC thermogram of GnCH indicated that CS-TC molecules were molecularly dispersed as amorphous form within the nanospheres [222]. The dispersed state of CS-TC within the nanospheres contributed to the slow release from this nanosphere formulation as presented earlier. This effect was attributed to the increase in polymer crystallinity due to molecularly dispersed drug [221].

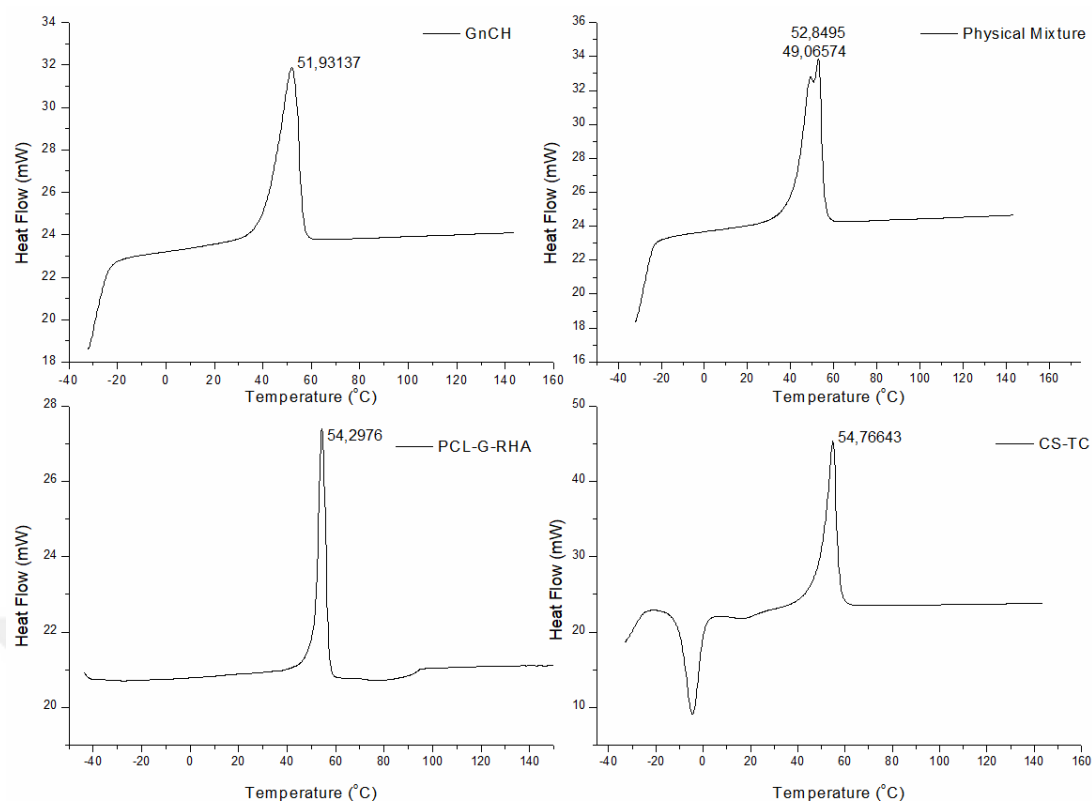


Figure 4.100 : DSC thermogram of the PCL-G-RHA, CS-TC, GnCH and physical mixture, demonstrating melting endotherm (second heating cycle), GnCH: 1 % Poloxamer 188, 1:10 CS-TC:PCL-G-RHA.

4.4.3 pH dependent release from nanospheres

PH of the release medium is a factor that affects the release behavior of the nanoparticles. For this investigation, several pH values in addition to pH 7.4 as the simulated extracellular environment were chosen to mimic different parts of the body; pH 3.6 medium was used as simulated gastric fluid [223], pH 5.6 simulated the microenvironment of tumour cells [224] and pH 8.0 simulated the ileum [225, 226]. First thing to be noticed in Figure 4.97 is that TC release is significantly enhanced with decreasing pH. The reason of such enhancement may be that the solubility of the polymer increases in acidic medium and polymer chain erosion starts to contribute to drug release. However, the dominant drug release mechanism is determined as Fickian diffusion under both acidic and basic conditions. TC molecules can be converted to flavylum cation and there is an equilibrium between two compounds. This equilibrium shifts towards the flavylum cation with decreasing pH and vice versa [227]. As explained earlier, PCL-G-RHA has oxirane end group in its structure and oxirane ring is subjected to nucleophilic ring opening under acidic conditions becoming positively charged [228]. Thus, the presence of cations of both TC and PCL-G-RHA under acidic

conditions may have created electrostatic repulsion and increased TC release [229]. For Gn1 and GnCH formulation, this effect was emphasized as can be seen in Figure 4.101. TC release gradually decreased as pH of the release medium increased. As for GnPCL nanosphere formulation similar result was obtained. However, the mechanism of such decrease in TC release as pH of the release medium increases must be different since GnPCL formulation did not contain oxirane ring. FT-IR analysis did not show any kind of interactions between the polymer and TC molecules. Thus the increased drug release from GnPCL nanospheres under acidic conditions may be caused by increased solubility of TC due to formation of flavylum cations under acidic conditions.

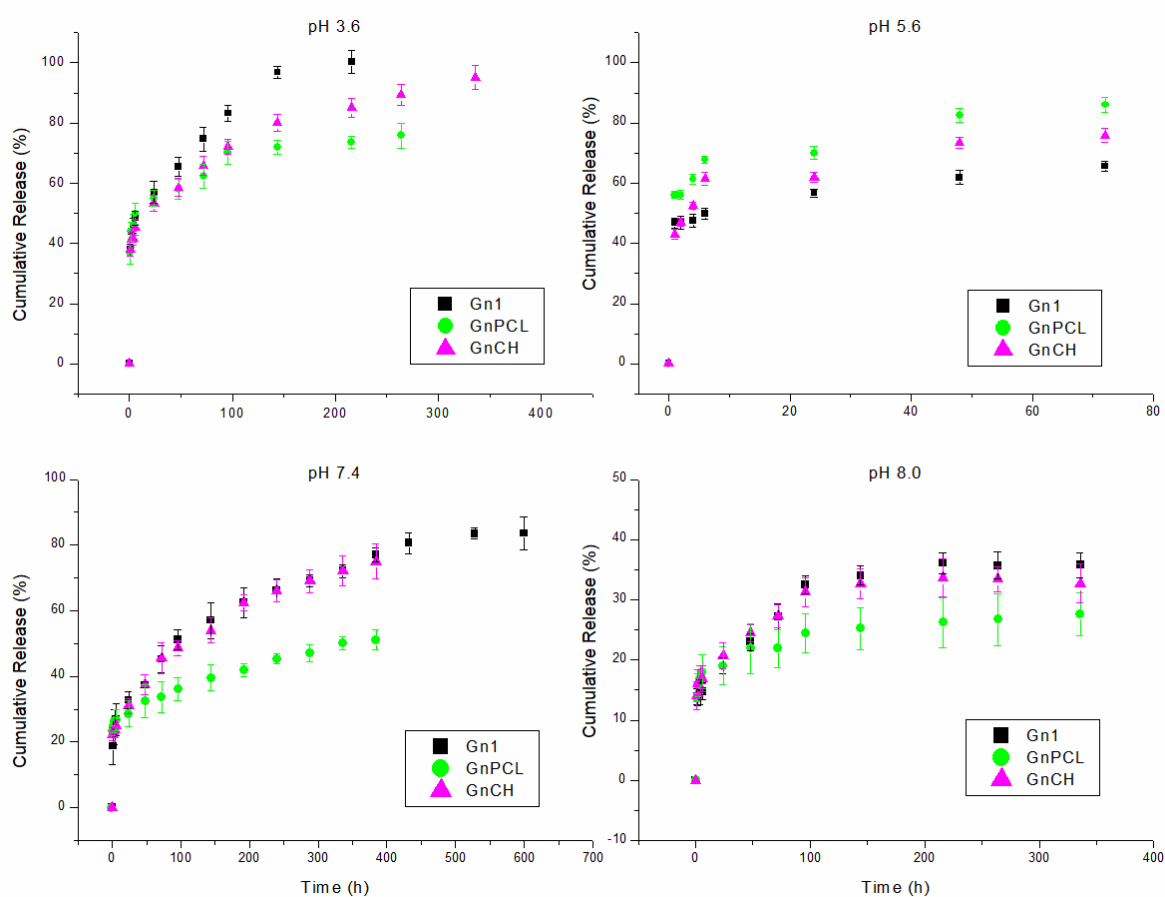


Figure 4.101 : TC release in different pH media; Gn1: 1 % Poloxamer 188, 1:10 TC:PCL-G-RHA; GnPCL: 1 % Poloxamer 188, 1:10 TC:G-PCL; GnCH: 1 % Poloxamer 188, 1:10 CS-TC:PCL-G-RHA.

Kinetic models were fitted to the release profiles of nanosphere formulations in different pH media. The calculated parameters were presented in Table 4.22, Table 4.23 and Table 4.24. According to these parameters, release from most of the formulations in different release media were best explained by Korsmeyer-Peppas kinetic model. The calculated n value for these formulations indicated a Fickian diffusion drug release model. As an exception, release from Gn1 formulation in pH 5.6 medium was best fitted to First Order model with a correlation coefficient of 0.968. First order model describes a concentration-dependent model and generally applies to release of poorly water-soluble drug molecules from water-soluble matrices [230].

Table 4.22 : Kinetic model parameter of release in pH 3.6 medium.

Code	Korsmeyer-Peppas			Higuchi		Zero Order		First Order	
	K_{KP} (h^{-n})	n	R^2	K_H ($h^{-0.5}$)	R^2	K_0 (h^{-1})	R^2	K_1 (h^{-1})	R^2
Gn1	0.388	0.28	0.975	5.499	0.876	0.349	0.730	0.004	0.846
GnPCL	0.534	0.11	0.936	3.511	0.689	0.208	0.502	0.003	0.730
GnCH	0.393	0.11	0.977	3.911	0.866	0.197	0.716	0.003	0.826

Table 4.23 : Kinetic model parameter of release in pH 5.6 medium.

Code	Korsmeyer-Peppas			Higuchi		Zero Order		First Order	
	K_{KP} (h^{-n})	n	R^2	K_H ($h^{-0.5}$)	R^2	K_0 (h^{-1})	R^2	K_1 (h^{-1})	R^2
Gn1	0.965	0.09	0.921	4.927	0.545	0.695	0.399	0.005	0.968
GnPCL	0.630	0.10	0.941	6.777	0.597	0.642	0.417	0.006	0.867
GnCH	0.579	0.13	0.970	6.338	0.646	0.608	0.463	0.007	0.776

Table 4.24 : Kinetic model parameter of release in pH 8.0 medium.

Code	Korsmeyer-Peppas			Higuchi		Zero Order		First Order	
	K_{KP} (h^{-n})	n	R^2	K_H ($h^{-0.5}$)	R^2	K_0 (h^{-1})	R^2	K_1 (h^{-1})	R^2
Gn1	0.327	0.20	0.944	1.832	0.893	0.098	0.729	0.004	0.770
GnPCL	0.511	0.12	0.979	1.065	0.711	0.055	0.540	0.002	0.757
GnCH	0.412	0.17	0.969	1.562	0.824	0.081	0.635	0.003	0.723

4.4.4 In vitro cytotoxicity of the nanospheres

In order to assess the cytotoxicity of the nanosphere on the L929 cell line, in vitro cytotoxicity testing was applied. According to the results given in Figure 4.102, an increase was observed in the viability of cells treated with Gn1 nanosphere sample with varying concentrations for 24 h compared to the control group. The cell viability reached to 153 % when treated with 5 μ g/ml of sample-containing medium and it did not changed significantly as the concentration increased stepwise. However, 100 μ g/ml

concentration caused a significant decrease in cell proliferation. The viability decreased to 132 % with the 100 $\mu\text{g/ml}$ concentration although the mentioned concentration still favored cell proliferation with respect to positive control. By contrast, 48 h treatment did not cause any significant improvement in terms of cell viability but still provided cell proliferation as much as the positive control. The possible reason of this decrease in cell viability at different testing time intervals might be the geometrical restriction; a decrease in available surface for cell growth as the treatment time increases [231]. Such effect was clearly visible between the treatment times of 5 $\mu\text{g/ml}$ sample; a statistically significant decrease in cell viability was observed when the exposure time was 48 h ($p < 0.0001$). Overall, these results indicate that the nanosphere sample does not cause cytotoxicity and is biocompatible, thus is suitable for biomedical applications.

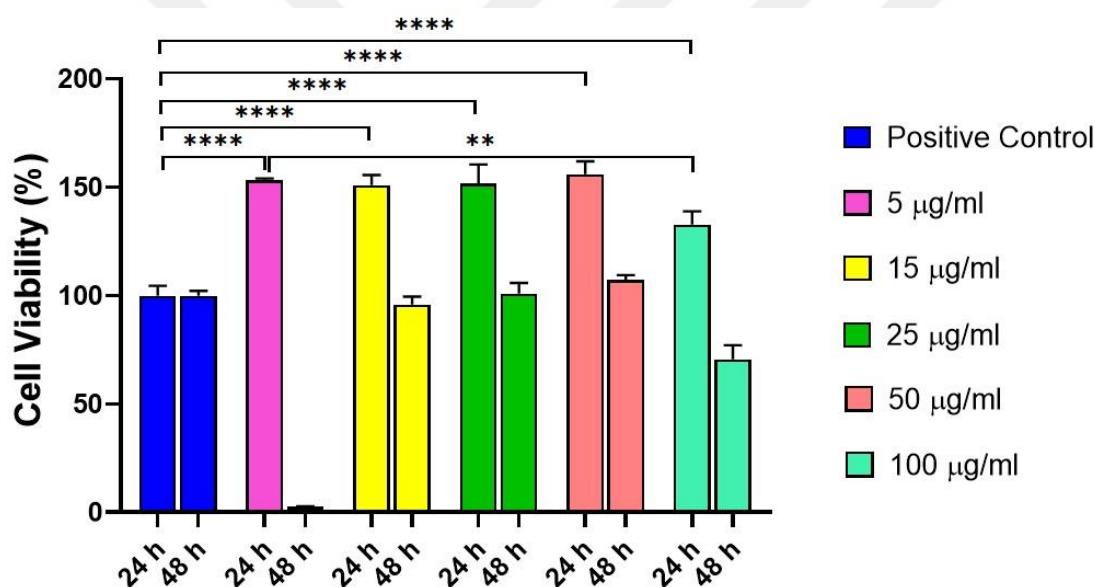


Figure 4.102 : Cell viability of L929 treated with Gn1 nanospheres for 24 and 48 h, positive control: EMEM/FBS , ****: $p < 0.0001$, **: $p < 0.005$.

5. CONCLUSIONS

This study set out to synthesize a novel hybrid polymer based on PCL and silica particles with low crystallinity and hydrophilic character. The synthesis reaction was in situ ring opening polymerization (ROP) of ϵ -caprolactone, which was catalyzed by immobilized *Candida antarctica* Lipase B. In this study, free form of *Candida antarctica* Lipase B was immobilized onto rice husk ash (RHA) by physical adsorption of which process details were reported earlier [149]. By achieving enzyme immobilization, the specificity and stability of CALB was enhanced. The support material, on which the enzyme was immobilized, was prepared firstly by burning cleaned rice husks in a furnace at 650 °C for 6 h. The produced RHA is a material with high silica content, which plays a crucial role in the formation of the nanohybrid system in later steps. RHA was surface modified prior to enzyme immobilization by using four different organosilane compounds, 3-APTES, 3-APTMS, 3-GPTMS and 3-TMSPDA in order to add functional groups to the surface of RHA that would facilitate the adsorption of enzyme to the surface. The results obtained from the analysis of TGA curves, it was found that different organosilane compounds acted differently. Surface modification percentages were calculated as 1.2 %, 0.8 %, 3.7 % and 10.1 % for 3-APTES, 3-APTMS, 3-GPTMS and 3-TMSPDA, respectively. This reaction took place through the –OH groups of RHA and methoxy or ethoxy groups of the silanization agents used, and Si-O-Si bonds were formed. After CALB immobilization onto surface modified RHA, the obtained catalytic systems were used to catalyze ROP of ϵ -caprolactone and in situ synthesis of PCL-based nanohybrid systems. During this reaction, short PCL chains were grafted from the free –OH groups of surface-modified RHA besides the long, aliphatic chains of neat PCL. Therefore, it is crucial to keep the surface modification at an optimum level so that PCL grafting from the silica can be achieved. The calculations performed with TGA curves proved the relation between the silanization percentage and grafting percentage. T-RHA with a silanization percentage of 10.1 % failed to produce grafted PCL. The evidence from this analysis suggests that a certain amount of increased silanization percentage increases grafting

efficiency. Previous studies have suggested an inverse relationship between PCL chain length and the number of surface Si–OH groups. The findings of this study is in consistence with those of earlier studies proposing the role of silanol groups as co-initiators for polymerization reaction resulting with high number of growing chains. A significant decrease of crystallinity percentage was observed for all of the nanohybrid samples, which was associated to the low molecular weight and the hinderance of crystal formation by the silica in the nanohybrids. Also an increased glass transition temperature due to restricted mobility caused by the grafted PCL. PCL-based nanohybrids were hydrophilic. Hydrophilic character of the nanohybrids may prominently increase bioavailability of the loaded drug molecules thus the nanohybrids have a potential for drug delivery applications.

The second aim of this study is to develop microspheres by O/W emulsion and nanospheres by interfacial polymer deposition method for TC administration and investigate the loading efficiency and in vitro release behavior. The PCL-based nanohybrids synthesized in the first part of this study were employed as the polymeric carrier in these drug delivery systems. The result of this investigation showed that optimum microsphere formulations had 60-75 % encapsulation efficiency. One of the more significant findings to emerge from this study is that TC release was extended to 22-57 days, in a controlled manner. Hydrophilicity was an important feature of our microspheres since it can enhance bioavailability of poor water soluble TC.

Similar results were obtained with the TC-loaded nanospheres fabricated by interfacial polymer deposition or nanoprecipitation method. Higher encapsulation efficiency was achieved (80-83 %) with nanospheres. TC release from the nanosphere formulation was enhanced with respect to the microsphere formulations; the cumulative release reached to 83-90 %. Nanospheres showed pH-dependent release behavior; acidity of the release medium increased release. The increased TC release from the nanospheres under acidic conditions may be caused by increased solubility of TC due to formation of flavylum cations under acidic conditions. TC release was extended to 28 days under neutral conditions. Water contact angle measurements revealed the hydrophilic character of the nanospheres as well. The results of MTT assay showed that nanospheres did not negatively affect cell viability of L929 cell line, indicating the biocompatibility of the TC-loaded nanospheres.

The findings of this study suggest a possible application of the microspheres and nanospheres in providing long-term treatment of diseases. Acidic release medium promoted the release and resulted in faster release kinetics and enhanced cumulative amount. This pH-triggered release from these microsphere and nanosphere formulations might be exploited for targeted delivery in cancer treatment. The present study lays the groundwork for future research into the application of the subject hybrid polymer in smart drug delivery. Considerably more work will need to be done to determine cytotoxicity, cell viability and in vivo pharmacokinetics.





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APPENDICES

APPENDIX A: Calibration Curves



APPENDIX A: Calibration Curves

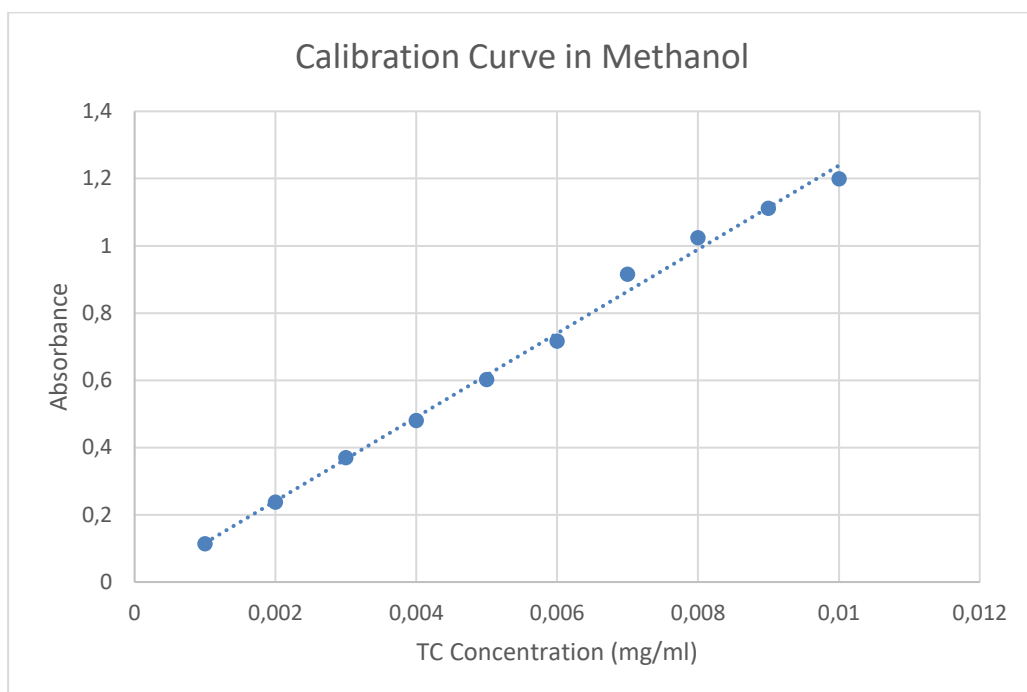


Figure A.1 : Calibration curve of TC in methanol.

$$y = 124.72 \times x - 0.0084$$

$$R^2 = 0.9952$$

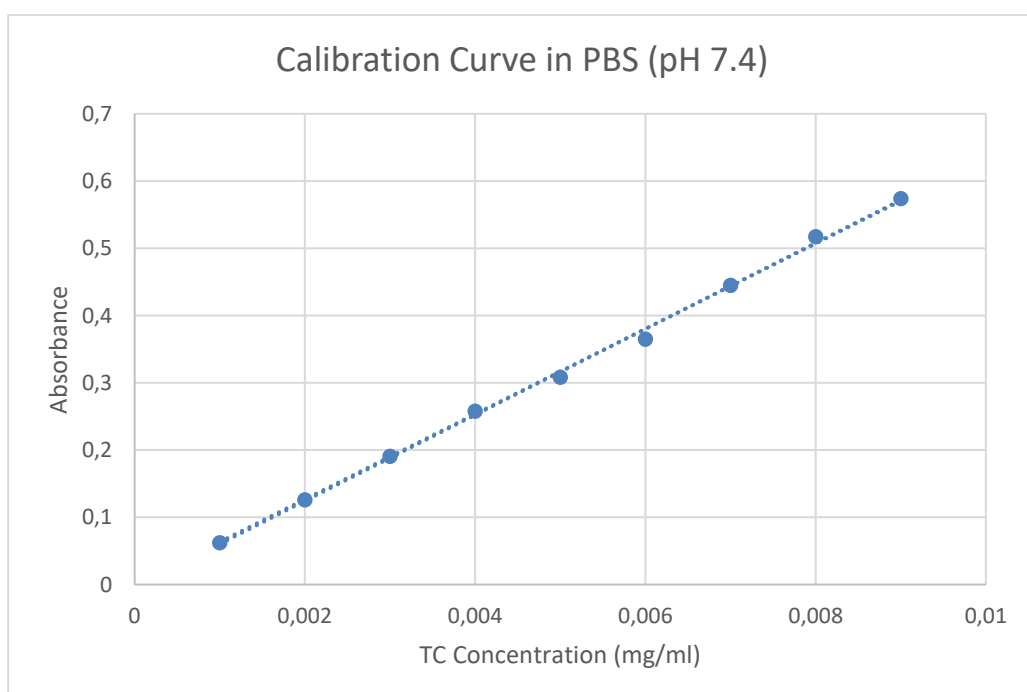


Figure A.2 : Calibration curve of TC in PBS (pH 7.4).

$$y = 63.95 \times x - 0.0036$$

$$R^2 = 0.9982$$

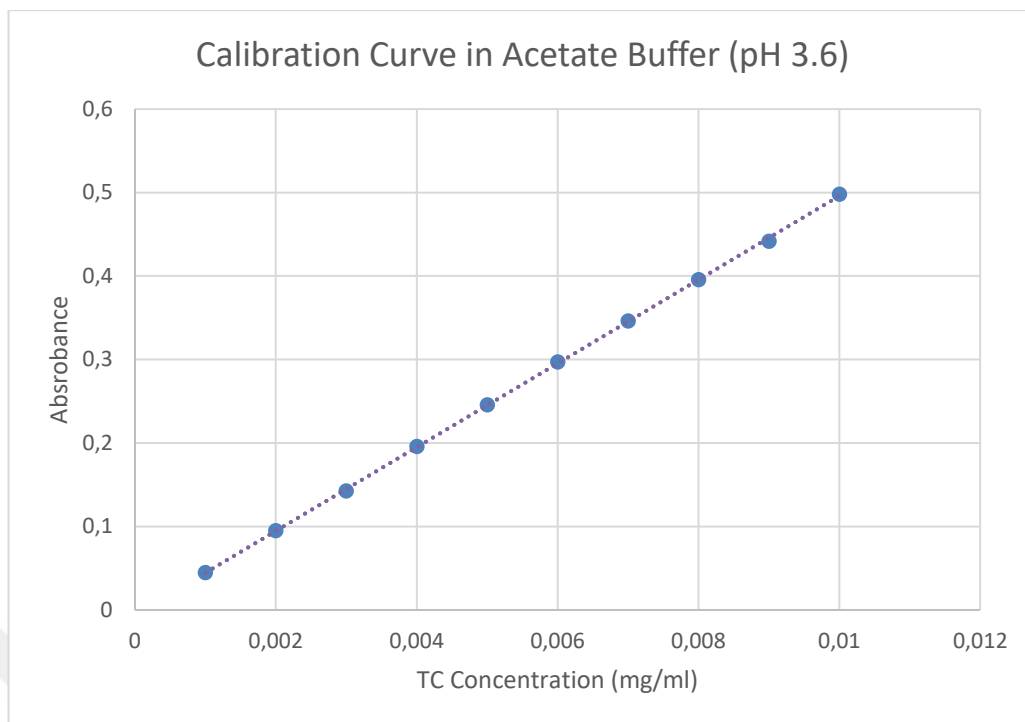


Figure A.3 : Calibration curve of TC in acetate buffer solution (pH 3.6).

$$y = 50.156 \times x - 0.0057$$

$$R^2 = 0.9998$$

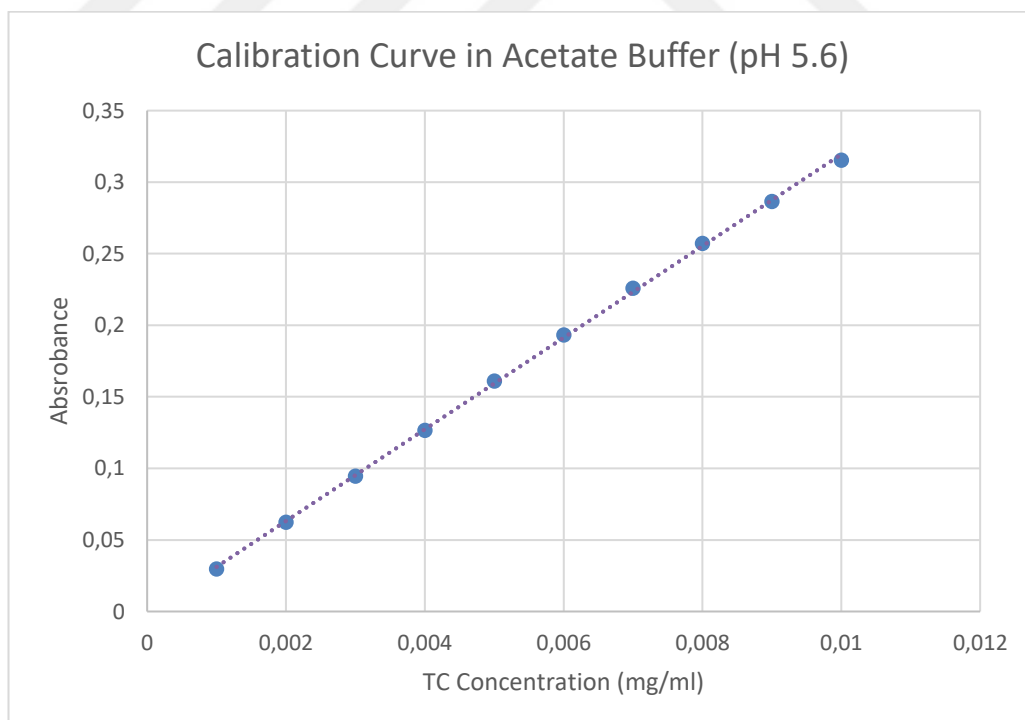


Figure A.4 : Calibration curve of TC in acetate buffer solution (pH 5.6).

$$y = 32.03 \times x - 0.0009$$

$$R^2 = 0.9995$$

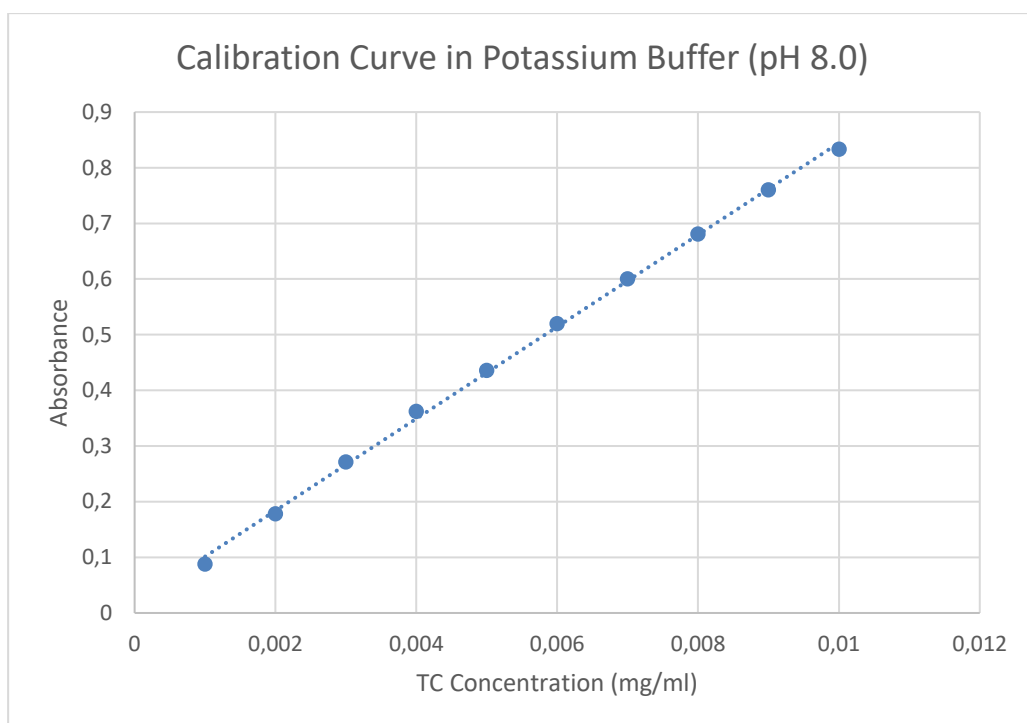


Figure A.5 : Calibration curve of TC in potassium buffer solution (pH 8.0).

$$y = 82.588 \times x + 0.0187$$
$$R^2 = 0.9989$$

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