

**FORMULATION AND EVALUATION OF EFFICACY OF
ANTICANCER DRUG DOCETAXEL WITH ORALLY
ADMINISTERED ANIONIC OR CATIONIC POLYMERIC
NANOCAPSULES**

**ANTİKANSER İLAÇ DOSETAKSELİN ANYONİK VE
KATYONİK POLİMERİK NANOKAPSÜLLERLE
FORMÜLASYONU VE ORAL UYGULAMA SONRASI
ETKİNLİĞİNİN DEĞERLENDİRİLMESİ**

DEMET DAŞKIN

PROF. DR. EREM BİLENSOY
Supervisor

Submitted to Graduate School of Science and Engineering of Hacettepe University
as a Partial Fulfillment to the Requirements for the Award of the Degree of Doctor
of Philosophy in Division of Nanotechnology and Nanomedicine

2016

This work entitled “**Formulation and Evaluation of Efficacy of Anticancer Drug Docetaxel with Orally Administered Anionic or Cationic Polymeric Nanocapsules**” by **DEMET DAŞKIN** has been approved as a thesis for the Degree of **DOCTOR OF PHILISOPHY IN DIVISION OF NANOTECHNOLOGY AND NANOMEDICINE** the below mentioned Examining Committee Members.

Prof. Dr. Fatma Nevin ÇELEBİ

Head

.....

Prof. Dr. Erem BİLENSOY

Supervisor

.....

Prof. Dr. Mehmet MUTLU

Member

.....

Prof. Dr. İmran VURAL

Member

.....

Doç. Dr. Tansel ÇOMOĞLU

Member

.....

This thesis has been approved as a thesis for Degree of **DOCTOR OF PHILISOPHY IN DIVISION OF NANOTECHNOLOGY AND NANOMEDICINE** by Board of Directors of the Institute of Science.

Prof. Dr. Salih Bülent ALTEN

Director of the Institute of
Graduate School of Science and Engineering



*To my family
and little sweet love...*

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16/05/2016

DEMET DAŞKIN

ÖZET

ANTİKANSER İLAÇ DOSETAKSELİN ANYONİK VE KATYONİK POLİMERİK NANOKAPSÜLLERLE FORMÜLASYONU VE ORAL UYGULAMA SONRASI ETKİNLİĞİNİN DEĞERLENDİRİLMESİ

Demet DAŞKIN

Doktora, Nanoteknoloji ve Nanotıp Anabilim Dalı

Tez Danışmanı: Prof. Dr. Erem BİLENSOY

Mayıs 2016, 146 sayfa

Bu tezin amacı, suda az çözünen hidrofobik bir antikanser ilaç olan dosetakselin oral uygulama için anyonik ve katyonik polimerik nanokapsül formülasyonlarını geliştirmek ve etkinliğini *in vitro* ve *in vivo* olarak test etmektir. Dosetaksel gibi hidrofobik antikanser ilaçların düşük biyoyararlanımı ve dolaşımdaki stabilite sorunları nedeniyle nanoteknolojik yaklaşımlar sıklıkla benzer çalışmalarda kullanılmaktadır. Ancak, dosetakselin polimerik oral formülasyonları üzerine yapılan çalışmalar oldukça azdır. Bu çalışma ile de güvenilir bir oral nano-formülasyon oluşturularak etkinliği üzerine çalışmalar yapmak amaçlanmıştır.

Bu çalışmada, öncelikle boş polikaprolakton nanokapsüller, nanoçöktürme yöntemi ile hazırlanarak amaca göre optimizasyonu sağlanmıştır. Daha sonra dosetaksel yüklü nanokapsül formülasyonları hazırlanmış, polietilen glikol ve kitosan modifikasyonları ile farklı yüzey yüklerine sahip nanokapsüller elde edilmiştir. Kapsül boyutları, polidispersite indeks değerleri ve zeta potansiyel ölçümleri yapılarak nanokapsüller *in vitro* karakterize edilmiştir. Yüzey morfolojisi incelemesi ve boyut doğrulaması Taramalı Elektron Mikroskopu (SEM) ile yapılmıştır. Dosetaksel yüklü ortalama partikül boyutunun 180-210 nm arasında değiştiği gözlenmiştir. Nanokapsüller düşük polidispersite indeksine sahip ve anyonik, nötr ve katyonik yüzey yükü ile uygun bir stabilite değer aralığında elde edilmiştir. Kısa süreli stabilite değerlendirmeleri, nanokapsül dispersiyonlarındaki görsel değişiklik ile partikül boyutu ve polidispersite indeksi değişimleri bakımından yapılmış, belirgin bir görsel değişiklik gözlenmediği gibi oldukça tutarlı boyut değerleri ve dar aralıkta boyut dağılımı ölçülmüştür. HPLC ölçümlerine yönelik dosetakselin validasyon yöntemi geliştirilmiş ve bu yöntem ile nanokapsüllere yüklenen ilaç

miktarı hesaplanmıştır. Hazırlanan formülasyonlara oldukça yüksek oranda ilacın enkapsüle edilebildiği yükleme etkinliği çalışması ile gösterilmiştir. Oral formülasyonların, kimyasal ortamı değişkenlik gösteren gastrointestinal kanal içerisindeki stabilitesi, asidite bakımından mide ve bağırsak sıvılarına benzer özellikler gösteren simüle gastrik sıvı (SGF) ve simüle intestinal sıvıda (SIF) gerçekleştirilmiştir. Simüle sıvıların nanokapsül formülasyonlarının fizikokimyasal özellikleri üzerindeki etkileri partikül büyüklüğü, polidispersite indeksi ve yüzey yükü bakımından incelenmiş ve elde edilen sonuçlar, söz konusu parametreler üzerinde büyük bir değişikliğin olmadığını göstermiştir. Nanokapsül formülasyonlarının gastrointestinal kanal boyunca mukus ile etkileşimi turbidimetrik çalışmalar ile incelenmiş, müsin-nanokapsül etkileşimi hem ölçülen absorpsiyon değerleri hem de boyut ve yük değişimleri ile doğrulanmıştır. Dosetakselin nanokapsüllerden *in vitro* salımı diyaliz yöntemiyle sink koşullarda gerçekleştirilmiştir. 24 saat boyunca sürekli salım gözlemlenmiş, kapsüllerden zaman içinde salınan ilaç miktarı HPLC ile ölçülmüştür.

In vitro hücre kültürü çalışmalarında, nanokapsül formülasyonlarının farklı hücre kültürleri üzerindeki etkileri incelenmiştir. Sitotoksikite çalışmaları L929 hücre hattı üzerinde gerçekleştirilmiş ve boş nanokapsüllerin belirlenen konsantrasyonlarda güvenilir olduğu gösterilmiştir. MCF-7 hücre hattı etkinlik çalışmasında kullanılmış, dosetaksel yüklü nanokapsüllerin antikanser etkinliğinin, aynı konsantrasyondaki serbest dosetaksel uygulamasına göre anlamlı düzeyde farklı olduğu izlenmiştir ($p<0,05$). Oral nanokapsüllerin permeabilite özellikleri Caco-2 hücre hattı üzerinde çalışılmıştır. Nanokapsüllerin intestinal permeabilitesinin, dosetaksel çözeltisine göre yaklaşık 2 ila 3 kat arttığı ve kitosan kaplı nanokapsüllerin PEG kaplı olanlara göre farkının daha büyük olduğu gözlemlenmiştir. Ayrıca, floresan madde Nil Kırmızısı yüklü nanokapsüllerin intestinal emilimi *in vivo* hayvan modelinde incelenmiştir. Nanokapsül formülasyonlarına yüklü halde oral yoldan verilen floresan maddenin özellikle kitosan kaplı nanokapsüller yoluyla bağırsaktan oldukça yüksek oranda alındığı gösterilmiştir.

Anahtar Kelimeler: dosetaksel, oral nanokapsül, oral kemoterapi, polikaprolakton, kitosan, polietilen glikol, intestinal permeabilite, GI emilim

ABSTRACT

FORMULATION AND EVALUATION OF EFFICACY OF ANTICANCER DRUG DOCETAXEL WITH ORALLY ADMINISTERED ANIONIC OR CATIONIC POLYMERIC NANOCAPSULES

Demet DAŞKIN

**Doctor of Philosophy, Department of Nanotechnology and
Nanomedicine**

Supervisor: Prof. Dr. Erem BİLENSOY

May 2016, 146 pages

The objective of this thesis is to develop docetaxel-loaded anionic or cationic oral nanocapsule formulations and investigate the efficacy of these nanoparticles *in vitro* and *in vivo*. Nanotechnological approaches are often used in similar studies because of low bioavailability and stability problems of poorly soluble and hydrophobic anticancer drugs such as docetaxel. However, there are only a few studies on polymeric oral formulations of docetaxel currently. This thesis aims to design a safe oral nanoformulation of docetaxel and investigate its efficiency.

In this study, first of all blank poly(ϵ -caprolactone) nanocapsules were prepared by nanoprecipitation method and optimized to obtain desired final properties. Then, docetaxel-loaded nanocapsule formulations were prepared and nanocapsules having different surface charges were obtained by surface modifications with polyethylene glycol or chitosan. *In vitro* characterization was performed by measurements of nanocapsule size, polydispersity index and zeta potential. Morphological investigation and size verification were realized by Scanning Electron Microscope (SEM). The mean particle size was found to be in the range of 180 to 210 nm for docetaxel-loaded nanocapsules. Nanocapsules were obtained with a low polydispersity index, anionic, neutral or cationic surface charge depending on formulation and an optimal stability. Short-term stability evaluation was scored by any visual change in nanocapsule dispersions, difference in particle size and size distributions. The results showed that nanocapsules were consistent in size and had low polydispersity index values. A validation method was

developed for docetaxel quantification in HPLC and the nanoparticle-bound drug amount was calculated with a validated HPLC quantification method. With drug entrapment efficiency study, nanocapsule formulations were demonstrated to have significantly high encapsulation efficiency. The stability of oral nanocapsule formulations in gastrointestinal passage were evaluated within simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) to determine any change in particle size, polydispersity index and surface charge. The results of this analysis represented that there was no significant change on those parameters under biorelevant media.

Mucus interaction of nanocapsule formulations through gastrointestinal passage was evaluated by turbidimetric studies. Mucin-nanocapsule interaction was verified with turbidity absorbance, size and surface charge differences in the presence of mucin. *In vitro* release of docetaxel was performed by dialysis membrane method under sink conditions. Sustained release within 24 hours was observed and the drug amount released in time from nanocapsules was measured by HPLC.

The effects of nanocapsule formulations on different cell lines were investigated by cell culture studies. Cytotoxicity of nanocapsules was determined against L929 cell line and empty nanocapsules were verified as safe within defined concentration range. Anticancer efficacy of docetaxel-loaded nanocapsules was studied on MCF-7 cell line. It was observed that anticancer efficacy of formulations was significantly different from docetaxel solution in the same concentration ($p < 0.05$). The intestinal permeability of oral nanocapsules was studied on Caco-2 cell line. The permeability of nanocapsules were 2 to 3 fold higher than docetaxel solution and chitosan-modified nanocapsules resulted in higher permeability for docetaxel than PEG-modified ones.

Finally, the *in vivo* intestinal uptake of fluorescent Nile Red-loaded nanocapsules was evaluated in a mouse model after application with oral gavage. It was demonstrated that fluorescent dye was intestinally absorbed with a quite high ratio especially by chitosan-modified oral nanocapsules.

Keywords: docetaxel, oral nanocapsule, oral chemotherapy, polycaprolactone, chitosan, polyethylene glycol, intestinal permeability, GI absorption

ACKNOWLEDGEMENTS

I would like to express my heartfelt gratitude to Prof. Dr. Erem Bilensoy for her continuous inspiring guidance, endless patience, insight, encouragements and critical discussion at every stage of this thesis. I am grateful to her not only being my advisor, but also providing precious and sincere support with her belief in me to complete my study.

My sincere thanks to Prof. Dr. Emir Baki Denkbař and Prof. Dr. Mehmet Mutlu for their excellent understanding throughout my thesis. It was a great opportunity to have their valuable guidance with a friendly atmosphere. The committee members Prof. Dr. Fatma Nevin elebi, Prof. Dr. İmran Vural and Assoc. Prof. Dr. Tansel omoęlu are also very appreciated for their contributions, comments and advices.

I would like to extend my thanks to Prof. Dr. Alper İskit for his pleasant collaboration and valuable helps in *in vivo* studies. I wish to express my appreciation to Prof. Dr. Pelin Kelicen Uęur for her kind help when I needed.

A special gratitude goes to Aslı aplı Kara for her help and invaluable support throughout my cell culture studies. I also would like to thank Dr. Glsen Osmanbařoęlu, Mahmut zer, Pınar & Giray Bulut specifically for their help and willing cooperation in statistical work in this thesis.

I am most grateful to my dearest friends, Dr. Hale nal, Gamze & Cem Varan for their sincere friendship, spiritual supports, invaluable helps, comments and patience to my endless questions and requests. I also thank Dr. Nazlı Erdoęar and Adem řahin for their support, suggestions and encouragement at laboratory work. My special thanks go to all proffessors, all my friends and my colleagues in the Division of Pharmaceutical Technology for their help and support, and also for creating enjoyable atmosphere during my hard lab-working period. Specifically I wish to express my deepest thanks to Prof. Dr. Sema alıř for her support and confidence.

My special thanks go to my friends in Health Sciences Research Grant Committee (SBAG), at The Scientific and Technological Research Council of Turkey (TUBITAK) for their support. I am grateful to Prof. Dr. Sevim Aydın who makes my thesis period more livable in TUBITAK and for her excellent understanding, confidence and leading. Moreover, it was an honor for me to get valuable comments by Prof. Dr. Zafer Evis on my thesis. Furthermore, I would like to thank to my group members in Basic Sciences Research Grant Committee (KBAG) for their support in my hard times during period of adjustment while writing this thesis.

Deepest thanks accompanied with my love are extended to my husband Ahmet Dařkın and my little sweetly prince Ahmet Bera who makes life worth living by inspiring, praising and sharing. Finally, thanks go to my family for their endless love, trust, support and patience. My whole family is my life and worths everything to me...

Demet Dařkın

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SYMBOLS AND ABBREVIATIONS

Symbols

β	beta
μ	micro
ε	epsilon
λ	wavelength
Ω	ohm

Abbreviations

ABS	Absorbance
ACN	Acetonitrile
AFM	Atomic force microscopy
AP	Aqueous phase
ATP	Adenosine triphosphate
AUC	Area under curve
BCS	Biopharmaceutical Classification System
CO ₂	Carbondioxide
CS	Chitosan
CV	Coefficient of variation
CYP3A4	Cytochrome P450 3A4
Da	Dalton
DAD	Diode array detector
DCM	Dichloromethane
dH ₂ O	deionized water
DLS	Dynamic light scattering
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DOC	Docetaxel
EMA	European Medicines Agency
EPR	Enhanced permeability and retention
FBS	Fetal bovine Serum

FDA	US Food and Drug Administration
g	Gram
GI	Gastrointestinal
GMP	Good manufacturing practice
H ₂ O	Water
HA-CE	Hyaluronic acid-ceramide
HBSS	Hank's balanced salt solution
HCl	Hydrochloric acid
HEPES	n-(2-hydroxyethyl) piperazine-n-(2-ethanesulfonic acid)
HGC	Glycol-chitosan-5 β -cholanolic-acid
HPLC	High performance liquid chromatography
HPMA	N-(2-Hydroxypropyl)methacrylamide
h	Hours
ICH	The International Council for Harmonisation
IUPAC	International Union of Pure and Applied Chemistry
i.m.	Intramuscular
i.t.	Intratracheal
i.v.	Intravenous
KH ₂ PO ₄	monobasic potassium phosphate
LOD	Limit of detection
LOQ	Limit of quantification
MDR	Multi drug resistance
mg	Milligram
min	Minute
mL	Milliliter
mm	Millimeter
MPS	Mononuclear phagositic system
MTT	(3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide; thiazolyl blue)
mV	Millivolt
Mw	Molecular weight
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NBCDs	Non-biological complex drugs

NCI	National Cancer Institute
nm	Nanometer
NNI	National Nanotechnology Initiative
NP	Nanoparticle
NR	Nile red
OP	Organic phase
P_{app}	Apparent permeability coefficient
PBS	Phosphate buffer saline
PCL	Poly- ϵ -caprolactone
PdI	Polydispersity index
PDLLA	Poly(<i>D,L</i> -lactide)
PEG	Polyethylene glycol
PEG-b-PBD	polyethylene glycol- <i>block</i> -polybutadiene
PEO	Polyethylene oxide
PLA	Poly(lactic acid)
PLGA	Poly(lactide-co-glycolide)
POE	Polyoxyethylene
PTX	Paclitaxel
P-gp	P-glycoprotein
RES	Reticuloendothelial system
rpm	Revolutions per minute
R%	Percent recovery
R^2	Correlation coefficient
SD	Standard deviation
SEM	Scanning electron microscopy
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
s.c.	Subcutaneous
TEER	Transepithelial electrical resistance
TEM	Transmission electron microscopy
T _g	Glass transition point
TPGS	Tocopheryl polyethylene glycol 1000 succinate
USP	United States Pharmacopoeia
UV	Ultraviolet

v	Volume
w	Weight
λ_{em}	Emmission wavelenght
λ_{ex}	Excitation wavelenght
μg	Microgram
μL	Microliter
μm	Micrometer
3D	Three-dimensional
$^{\circ}\text{C}$	Centrigrade degree celcius



1. INTRODUCTION

Cancer is a genetic disorder and the disease itself can be considered as mainly the general name of the collection of related diseases. Cancer is fatal because abnormal cells proliferate in an uncontrolled manner. As a result, it can cause serious illnesses and even death within a short period of time. Cancer cells also tend to grow differently and continuously which is quite different from normal cells. They are also known to invade other tissues remarkably. These two characteristic behaviors make a cell a cancerous cell.

Although there are hundreds of studies carried out about cancer, there has been no definite solution of cancer treatment yet. Chemotherapy is one of the common ways for the cancer cells to be killed or to stopped from proliferating

The most common route of chemotherapy is intravenous administration. Although it is the most common way since direct application of chemotherapeutic agents into the circulation and even within the solid tumor tissue is possible, oral chemotherapy is a challenge and offers hope for a more patient-compliant and economic cancer treatment in the future. Regarding oral chemotherapy, there are many advantages such as the ease of administration, possibility of self-medication, better patient compliance and the last but not least; significantly lower cost.

However, oral chemotherapy necessitates the development of special formulations that can overcome the specific barriers and conditions throughout the gastrointestinal (GI) route. Mainly, possible mucosal interactions and pH differences during GI transit constitute a strong barrier for the fate of anticancer drug. Furthermore, bioavailability of orally administered chemotherapeutic drugs need to be improved for an effective pharmacokinetic impact in the body.

Pharmaceutical nanotechnology offers integrative solutions to the drawbacks of chemotherapeutic agents mostly administered via the parenteral route. In nanomedicine, nanoparticles are promising delivery systems to facilitate oral administration. Among nanoparticulate carriers, nanocapsules differ from other nanoparticles with their oily core rendering improved solubility to lipophilic drugs,

better physical stability, and improved bioavailability and efficacy of anticancer drugs with bioavailability problems.

One of the chemotherapeutic agents used often in cancer treatment is docetaxel (DOC). Docetaxel is used for a variety of tumors as a single agent or in combination with other chemotherapeutic agents according to the needs related with the type and stage of cancer. It is known to be highly cytotoxic and has a very low aqueous solubility due to its hydrophobic nature. Furthermore, docetaxel's stability problem in physiological conditions and probable side effects after intravenous administration are other issues that should be overcome. All these characteristic specifications make docetaxel a good model drug candidate to be encapsulated in a nanomedicine for improved safety and efficacy in cancer therapy.

In this study, the preparation and *in vitro* & *in vivo* evaluation of oral biodegradable poly(ϵ -caprolactone) (PCL) based core-shell nanocapsule formulations with anionic or cationic coating were aimed. By these designed formulations, the problems arising from physicochemical properties of docetaxel itself and bioavailability problems associated with oral chemotherapy were intended to be solved.

2. GENERAL INFORMATION

2.1. Chemotherapy

Cancer treatment is mainly depending on single or combined application of chemotherapy, radiotherapy and surgery. Chemotherapy is the keystone and most common treatment in cancer therapy. However, the chemotherapeutic agents are cytotoxic in a non-selective manner and act by killing rapidly dividing cells. During this process they harm other healthy rapidly growing cells such as bone marrow, cells lining the GI system and hair follicles, because these chemotherapeutic drugs are not specific to tumor cells.

Chemotherapy depends on the circulation dynamics of drugs in blood. Intravenous administration of a chemotherapeutic agent causes a sudden rise of drug concentration in circulation and the second dose is given at a point just as the efficacy of first one drops under minimum effective dose. The main idea here is the concentration of drug to remain between the toxic level and the minimum effective concentration.

There exists a broad range of chemotherapeutic agents used in cancer treatment according to the type of cancer and the stage of progression of disease. The most commonly driven drugs aim to directly disrupt the nucleus of cancer cell, interfere with the cell cycle by affecting the synthesis or breakdown of mitotic spindles and inhibit the synthesis of building blocks of new DNA strand (Figure 2.1) [1].

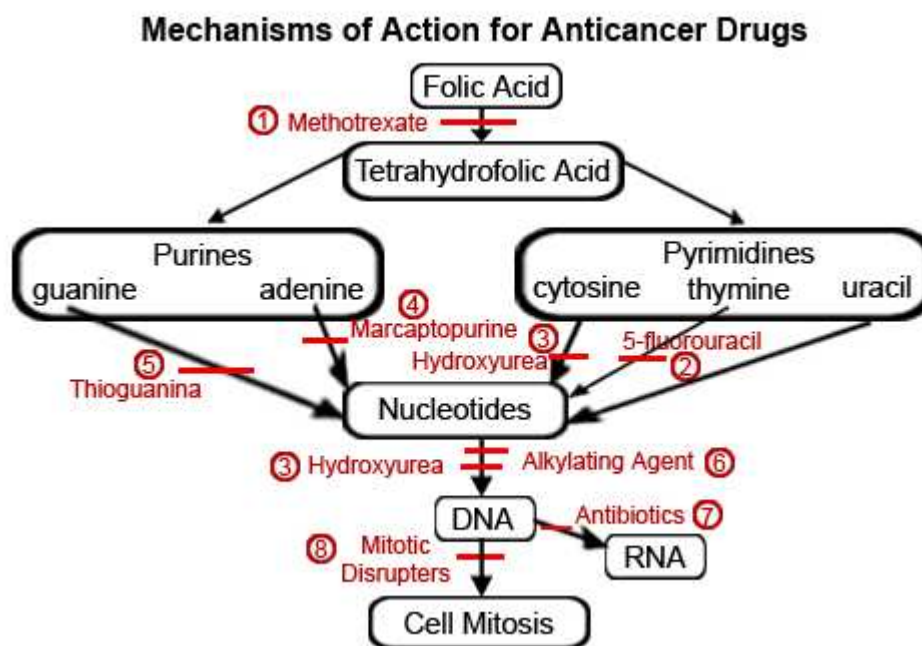


Figure 2.1. Action modes of chemotherapeutic agents [1]

Benefits, advantages and challenges of oral chemotherapy

Cancer patients dream about a treatment of any kind of cancer “at home” since they are obliged to receive conventional chemotherapy at a medical center from professionals. This idea is a key step of self-medication of patients themselves improving the quality of their lives. Oral chemotherapy can make it possible and radically change clinical practices of chemotherapy. It is especially important and necessary for elderly cancer patients at further stages or who cannot endure harsh treatments [2, 3].

First of all, oral chemotherapy has non-invasive characteristics that result in better patient compliance. As a matter of fact, it is considered as more cost-effective than intravenous administration of drugs in terms of time and labor. Moreover, oral chemotherapy offers prolonged and stable systemic exposure to drugs [4]. However, oral bioavailability is strongly affected by the physicochemical properties of drugs used and the permeability of intestine and absorption potential through the GI system. According to Biopharmaceutics Classification System (BCS) (Table 2.1), drugs are classified into 4 groups considering the absorption potential through intestinal epithelium and the solubility of the drugs. BCS is a scientific framework for drug absorption studies defined by Amidon *et al.* in 1995. In

principle, the intestinal permeability against orally administered drug molecules and the aqueous solubility of drugs were accepted as main parameters in the process of intestinal absorption [5, 6].

Table 2.1. The Biopharmaceutics Classification System (BCS) [7]

	High Solubility	Low Solubility
High Permeability	Class 1 High solubility High permeability	Class 2 Low solubility High permeability
Low Permeability	Class 3 High solubility Low permeability	Class 4 Low solubility Low permeability

There are many orally administered anticancer drugs studied in scientific literature mostly in the form of conventional dosage forms such as tablets, capsules or oral emulsions. Two of the cytotoxic agents that are nanoencapsulated for oral administration were taxane derivatives, paclitaxel and docetaxel [8]. The use of polymeric nanoparticulate systems is important for enhancing the bioavailability for poorly soluble anticancer agents in oral chemotherapy by increasing the solubility of the drug due to the large surface area of the nanoparticles. There is a wide range of solutions in nano-scale encapsulation to solve bioavailability issues and other problems such as stability, solubility/re-crystallization, toxicity, effectiveness and targeting to specific sites at the GI system. Using a biocompatible, effective and suitable polymeric material, different strategies for coating and surface functionalization are utilized for an optimum drug encapsulation and release as well as intended biological interaction properties. Hence, the oral bioavailability of anticancer drugs that are known to be P-glycoprotein (P-gp) substrates, specifically taxanes having poor absorption profile can be altered and improved by using polymeric colloidal particles in the nanoscale [9, 10].

GI tract is a very complicated and long muscular tube extending from the mouth to the anus. Its complexity depends mainly on the extensive release of hormones

and enzymes during food digestion, and also the physicochemical environmental change due to digestion and absorption process. The movement of muscles known as peristalsis enables the nutrients or water to move from the beginning of GI system, the oesophagus, towards the intestines where a large portion of absorption process takes place. The absorption function is carried out by intestinal epithelia via small spurs covering the surface of whole epithelium called villi. These structures increase the surface area extensively and cause intensive absorption. Villi are covered with absorptive enterocyte cells and mucus secreting goblet cells. This region is known as Peyer's patch and covered with M cells (Figure 2.2). M cells are important for drug delivery process since they are covered with less mucus [2, 11] and allow entering blood circulation through lymphatic system by absorption of oral nanoparticles [12].

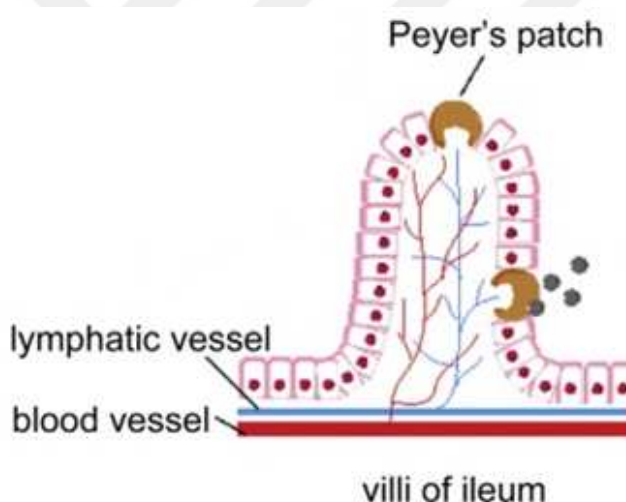


Figure 2.2. Schematic representation of Peyer's patches on villi of ileum [12]

The design of the GI system also acts as a barrier for unwanted molecules such as toxins, pathogens and even the therapeutic agents already foreign for the body. The nanoparticulate systems also face these GI barriers. The main problems with these barriers are the drug and dosage form instability in environments having a wide range of pH, low drug solubility causing poor bioavailability and low penetration through the mucus layer preventing or decreasing effective absorption [11].

The acidity of GI system differs within a very broad range of pH values. Inside the mouth, the pH is around 6.5 to 7.5 due to presence of saliva. All foods orally taken are exposed to saliva for up to 1 minute. Then, in gastric mucosa there is a pH gradient from 1 to 7. The upper part of stomach has a pH gradient of 4-7 and the lower part of it has a pH of 1-4. The emptying time of upper stomach and lower stomach are 30-60 minutes and 1-3 hours, respectively. The acidity changes according to the interactions with nutrients causing release of acid into stomach. This low pH is required for break down and digestion of foods into small pieces during whole stomach emptying. Further in the GI tract, the duodenum has a pH range of 7-8.5 for the next 30-60 minutes against the orally taken substances. Then, the small intestine and the large intestine both have pH range of 4-7 and intestinal emptying time is 1 to 5 hours and 10 hours to several days, respectively [13]. As can be seen, the pH values inside GI tract changes from acidic towards alkaline (Figure 2.3). pH and also ionic strength are crucial factors for ensuring the stability and the integrity of oral drug carriers.

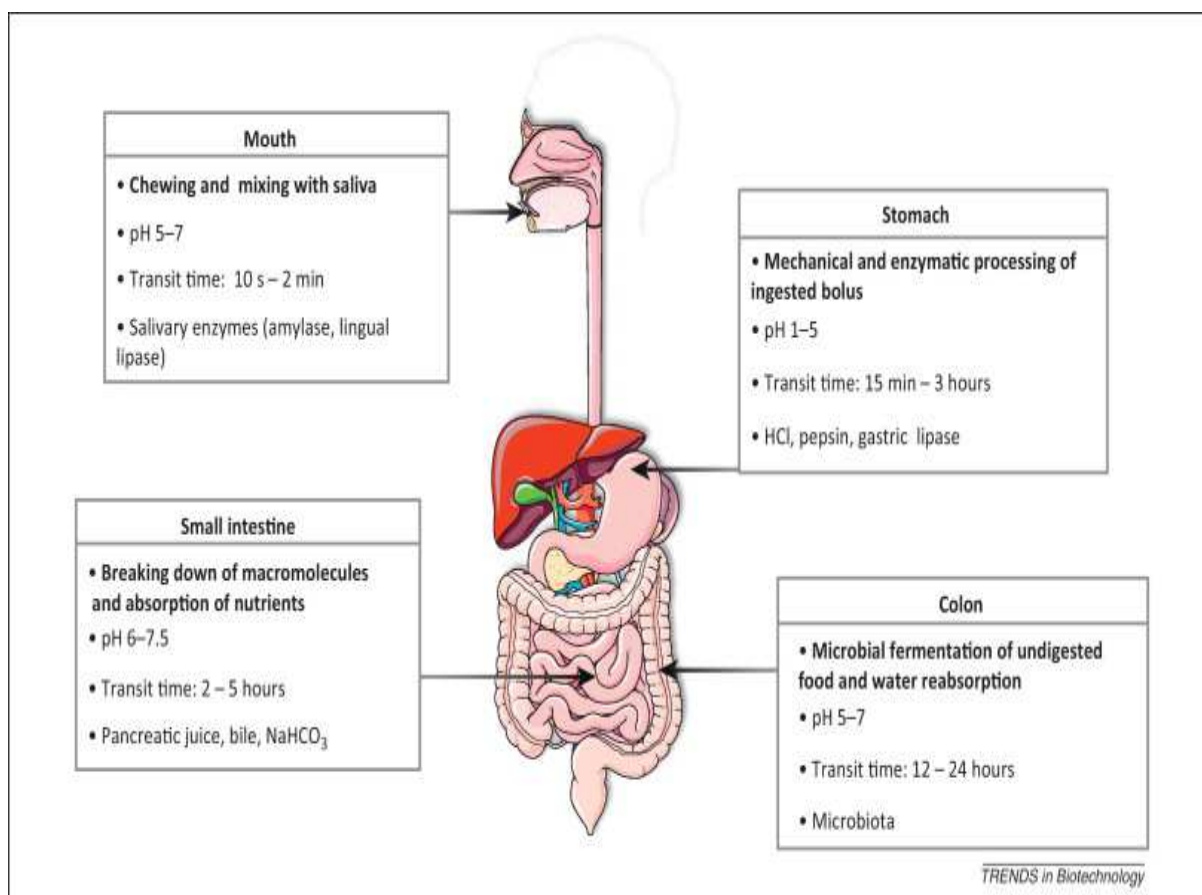


Figure 2.3. Regional specifications of human GI tract [14]

Beside acidity, enzymes and other digestive substances are also other problems for the stability of oral drug delivery systems. Starting from the beginning of GI tract, the whole cavity contains several digestive substances such as amylases, pepsin, hydrochloric acid, bile salts, lipases and peptidases. Chemical and colloidal stability of oral drugs and carriers gain more importance against these enzymatic and chemical conditions in the GI tract.

Other barriers in GI canal affecting the absorption process are mucus and the GI epithelial lining itself. Mucus is primarily composed of water (95%), and also contains lipids, salts, a small amount of proteins, which act defensively such as lysozyme, immunoglobulins and growth factors [15]. However, the main protein component of mucin is the glycoprotein that crosslinks together, forming viscoelastic gel properties adhesively and reversibly. The viscoelasticity of mucus is vital for protective and lubricant features. Mucosal surfaces remove the drug molecules from body by either mucus clearance mechanisms or systemic

absorption. Nanoparticulate systems having high penetrating ability into mucus secretions can avoid this rapid clearance and sustain local drug doses over time [16]. This ability is mimicking the viruses which can penetrate through the mucus and diffuse into the epithelium due to being as small in size as can fit into mucus mesh spacing and non-mucoadhesive surface [17]. Hence, to stay on the line between mucoadhesion and mucus penetration is highly important for an operative oral drug delivery.

Beside the mucus barrier, the epithelial cells are also critical for drug absorption from or with oral nanocarriers. Particle absorption involves both paracellular and transcellular ways. It is admitted that paracellular route is not suitable for transportation of nanocarriers through the intestinal barrier since the junctions prevent passage of molecules larger than several nanometer in size. However, several compounds such as polycarbophil, chitosan (CS) and its derivatives were found to open tight junctions, allow paracellular diffusion and enhance permeation [17-19]. There are two types of nanoparticle internalization pathways: passive physical penetration due to small size and active endocytosis [20]. The internalization route mainly depends on physicochemical characteristics of nanoparticle and the cell type.

Major challenges for oral drug delivery are known to be hepatic first pass metabolism, and P-gp efflux system. After absorption of drug molecules from the intestinal epithelium, they enter into the hepatic portal system before they reach the systemic circulation. Drug molecules are carried to the liver through portal vein and the liver metabolizes many of them. However, sometimes small amount of active drug is effluxed from the liver to the body circulation. This phenomenon is known as first-pass hepatic metabolism (Figure 2.4). This first pass greatly reduces the oral bioavailability of the absorbed drug.

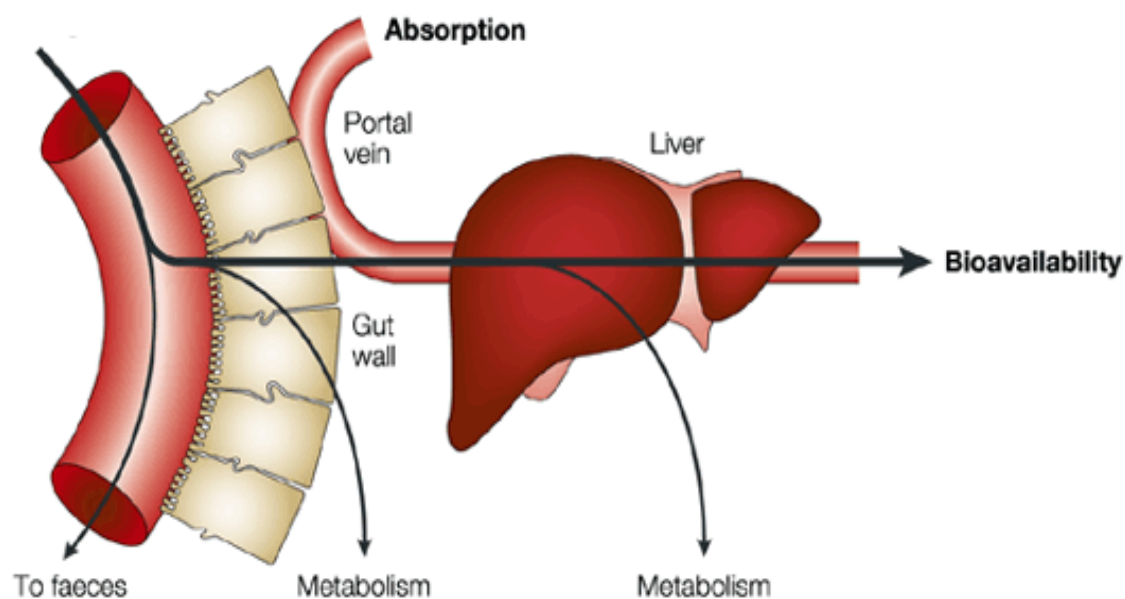


Figure 2.4. Schematic illustration of first-pass hepatic metabolism [21]

Many clinically substantial drug molecules undergo extensive first pass hepatic effect following absorption. The major therapeutic implication of first-pass hepatic metabolism to obtain equivalent plasma concentration is the requirement of a much higher oral dose than intravenous dose [22] which creates another obstacle in formulation development.

Beside first-pass effect, the poor oral bioavailability is also related with affinity to the multidrug efflux pump P-gp [23].

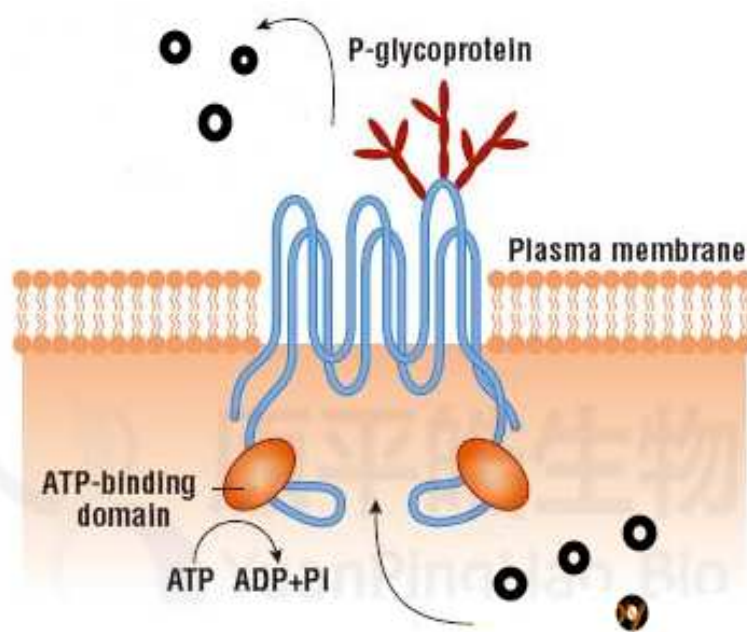


Figure 2.5. Schematic illustration of P-gp drug efflux [24]

P-gp is a membrane associated protein belonging to *ATP-binding cassette* (ABC) superfamily and a member of multi drug resistance (MDR) sub-family. It is ATP-dependent and requires ATP to function for drug efflux (Figure 2.5) [17]. Many anti-cancer drug molecules are the substrates of P-gp efflux pump resulting in poor bioavailability of orally taken drugs.

Their unique extremely small size of nanoparticles and surface characteristics due to appropriate surface coating provide them the ability to circumvent the P-gp efflux from intestinal epithelium or cytochrome P450 elimination in the first-pass and to protect the drug from damaging factors in the GI passage. Thus, the permeability of drug molecules through the intestinal barrier increases [23, 25].

2.2. Nanotechnology Concept

The word “nano” means “dwarf” in ancient Greek. The concept of a “nanometer” is not new, it was first proposed during the first decade of the 20th century by a successful chemist, Richard Zsigmondy, the winner of the 1925 Nobel Prize in chemistry, who used the term “nanometer” for characterizing the particle size [26]. The term of nano in science and the use of nanoscale initially came from a physicist, Richard P. Feynman, many years later in 1959. Nanotechnology is

defined as “science, engineering, and technology carried out at the nanometer-length scale (between 1 to 100 nanometers)” by National Nanotechnology Initiative (NNI) [27]. According to US Food and Drug Administration (FDA), a nanotechnological material or end product is defined as “a material that is engineered to have at least one external dimension, or an internal or surface structure in a size range of 1-100 nm, or a material that is engineered to exhibit properties or phenomena, including physical or chemical properties or biological effects, that are attributable to its dimension(s), even if up to 1000 nm” [28].

Nanotechnology has become an exciting and promising field that is helping to lead critical global problems to find remarkable solutions in a very broad range from medical to environmental topics. It is in everyday practices today and impacts human life to a large extent with potential and diverse benefits of products in the nanoscale [26, 29]. Nanotechnology is the engineering of materials; particles, coatings and devices at nano-scale that influences material properties such as electrostatic forces and surface tension to acquire the desired effect [30]. Reduction of size to the nanometer range also changes physicochemical and physiological properties of drug delivery systems in the field of nanomedicine. Nanomaterials have mainly a high surface to volume ratio and different characteristics from their bulk materials as a unique feature, suggesting a wide potential to use in clinical field for therapeutics and disease diagnostics [29].

Nanotechnology mainly emerges in three areas in medicine. Among them, the emerging one is nanoparticulate systems which have a wide range of application area due to their extraordinary chemical, electrical and optical characteristics, high stability and biocompatibility, controllable biodegradation, morphology, size and surface functionalization properties. The second topic is the miniaturization of known devices for the use of medicine. The last but not the least one is the control of surface characteristics in atomic-scale [29, 31].

2.2.1. Nanomedicine

Nanotechnology is a nascent topic appearing in emerging therapeutics and medical diagnostics, especially on personalized and targeted medicine. New areas of nanotechnology include immunotherapy, imaging/diagnostics, stem cell therapies, tumor characterization and targeted delivery of drugs [32]. In

healthcare, there exists tremendous progress on nanotherapies and diagnostics in laboratory. Non-toxic and multifunctional smart nanosystems are being improved for a broad variety of biomedical applications in the fields of diagnostics, imaging, therapy and drug delivery. Nanotechnology has a pivotal role in providing novel therapeutics mainly specific to cancer with maximum therapeutic outcome and minimum side effect [29, 33].

Nanoparticulate drug delivery systems are characterized by the capability of targeted, controlled and sustained delivery. Drugs in nanocarriers are protected from enzymatic degradation and pH destabilization. The biocompatibility and desired biodistribution potential of nanocarriers are the challenging key factors in design among other important factors such as non-toxicity, effective drug loading capacity, proper targeting and release ability [34].

However, aside from the benefits of nanotechnology in healthcare, there is a critical issue on potential health and environmental risks about extensive exposure of humans to nanoparticles. These risks breed other scientific disciplines such as nanotoxicology [35]. Although toxic properties could be partially eliminated by the selection of materials used in production of nanomaterials, other factors originating from the nature of being nanosized are the primary concern.

2.2.2. Nanocarriers

Nanocarriers are colloidal systems in nano-size and are capable of transporting many small agents or molecules that can be defined as indirect targeted therapy (Figure 2.6).

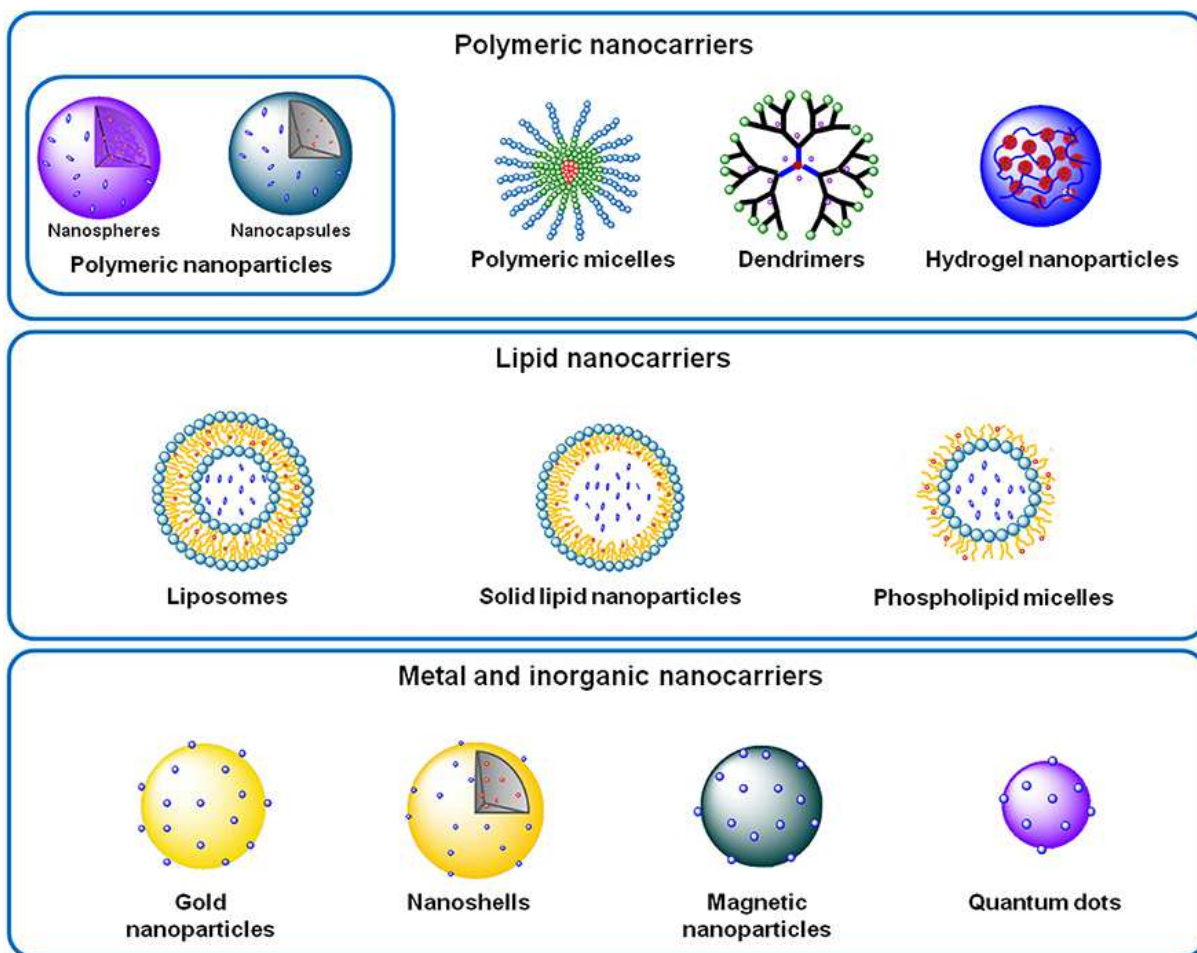


Figure 2.6. Several examples of nanocarriers [36]

Among the various carrier systems, nano-sized systems are more reliable in transportation of agents due to phagocytic elimination in circulation by their “stealth” characteristics. In addition because of their nano-size, the enhanced permeability and retention (EPR) effect that is defined as increased permeability of new vessels generated due to angiogenesis around tumor tissue together with the poor lymphatic drainage results in the passive accumulation of nanocarriers in tumor tissues [8] which is the basis of nanoparticle targeting to tumors as well as inflamed tissues. The EPR effect is illustrated in Figure 2.7. Hence, the chemotherapeutics inside the nanocarriers can be transported directly to tumoral tissues by the help of EPR effect and passive targeting.

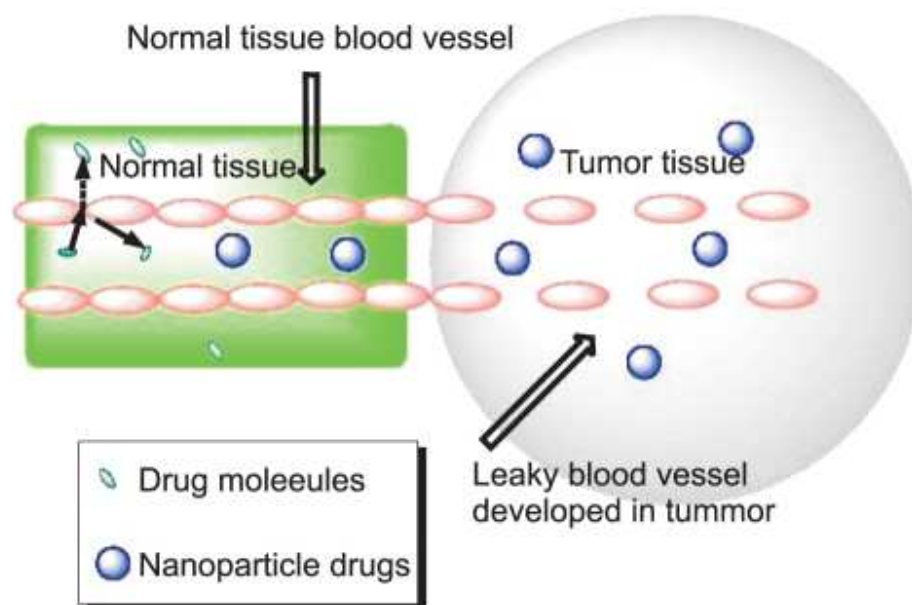


Figure 2.7. EPR effect [37]

It is also possible to realize active targeting by conjugating different molecules onto the surface of nanocarriers. For this purpose, monoclonal antibodies, some peptides which play important roles in biochemical pathways or charged molecules can be used to modify the surface of nanocarriers leading to active targeting. However, there are only a few clinical trials in progress to date on actively targeted nanomedicines and only one antibody-targeted nanoparticle (Zevalin[®]) is in use with FDA approval [8, 38-40].

Nanocarriers are a very large family of delivery systems including nanoparticles, micelles, nanoshells, liposomes, nanotubes, nanostructures, dendrimers and quantum dots. One of the main types of nanocarrier systems is nanoparticles. The particulate nanocarrier systems are studied intensively in literature because of their additional advantages over other carrier systems, such as high stability and better protection of conjugated agent from environment [8]. Nanoparticles have unique chemical and physical properties which can be modified according to the basic target. The tunable features include mainly nanoparticle size, surface characteristics and shape. They provide reduced dose of drugs, increased

efficiency and bioavailability, tissue specific targeting and reduced toxicity and secondary effects [41].

So far, the nanoparticles FDA approved or the ones currently in clinical trials are based on polymers or liposomes [37]. Nanoparticles are mostly constituted of polymers due to their many known advantages such as biocompatibility, capability of chemical modification to tune drug loading and release properties and biodistribution and safety. Amphiphilic core-shell (polymeric micelles), hyperbranched macromolecules (dendrimers), or capsules-particles (polymeric nanoparticles) can be listed as different structures of polymeric nanoparticulate systems [8].

Polymeric micelles are intensively studied form of nanoparticulate systems. Usually, the inner part of the micelle construct is self-assembled by hydrophobic blocks and the outer micelle shell is formed by hydrophilic blocks. This hydrophilic shell protects the cargo inside whereas, the inner part functions as a reservoir for the poorly soluble hydrophobic agents [42]. Dendrimers are highly hyperbranched macromolecules, which is starting from a central molecule and can form bonds with at least three other molecules to form sequentially added a number of generations of monomers (Figure 2.8). They can bind with drug molecule via encapsulation, electrostatic interactions or covalent conjugations. They have many internal cavities and surface functional groups within their structure. Although they offer high drug loading capacity because of their unique structure, they still have safety and toxicity problems [42, 43].

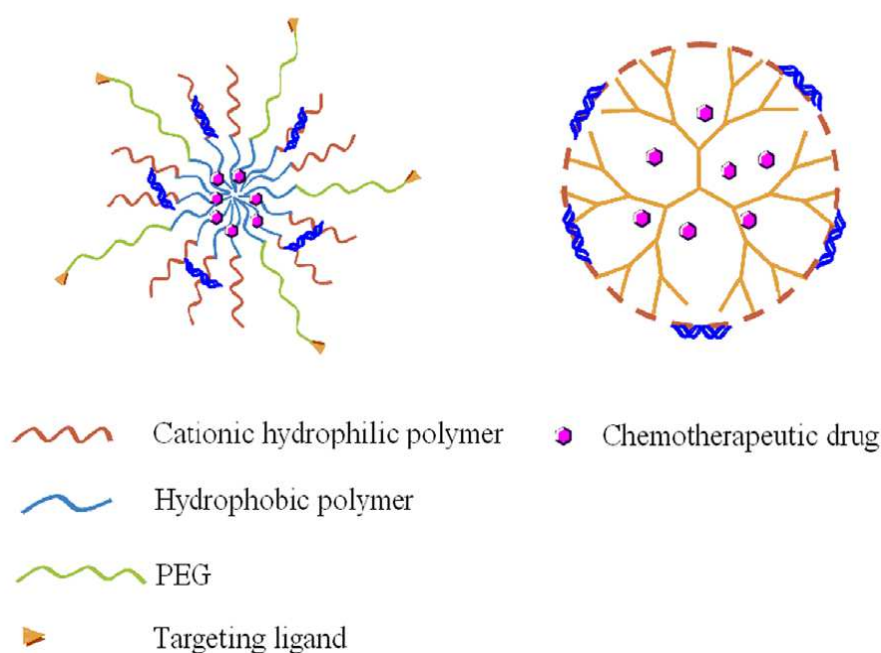


Figure 2.8. Illustration of micelle and dendrimer systems [42].

Polymeric nanoparticles are biodegradable colloidal systems in spherical form. They are solid carriers and comprised of natural, synthetic or semi-synthetic polymers. They range from 10-1000 nm in diameter. The polymeric nanoparticles appear in either nanosphere or nanocapsule form, which differs in structural organization and composition. The drug is either dispersed in homogenous polymer matrix system, which is called nanosphere or entrapped within a core cavity composed of oil surrounded by a single polymeric membrane, which is called nanocapsule [44, 45]. In nanocapsules the drug can be dissolved in the oily core or adsorbed onto the polymeric membrane according to the physicochemical properties of drug, whereas in nanospheres the drug is either retained or adsorbed on the surface of particle or entrapped within the polymer matrix [46].

2.2.3. Nanocapsules

Polymeric nanoparticles are generally defined as carrier systems which are of size smaller than 1 μm . Nanocapsules or nanospheres are named according to the composition of the particle formed. Nanospheres are comprised of a matricial organization of polymer chains whereas nanocapsules contain an oil core leading

to a vesicle or membrane-type structure [47].

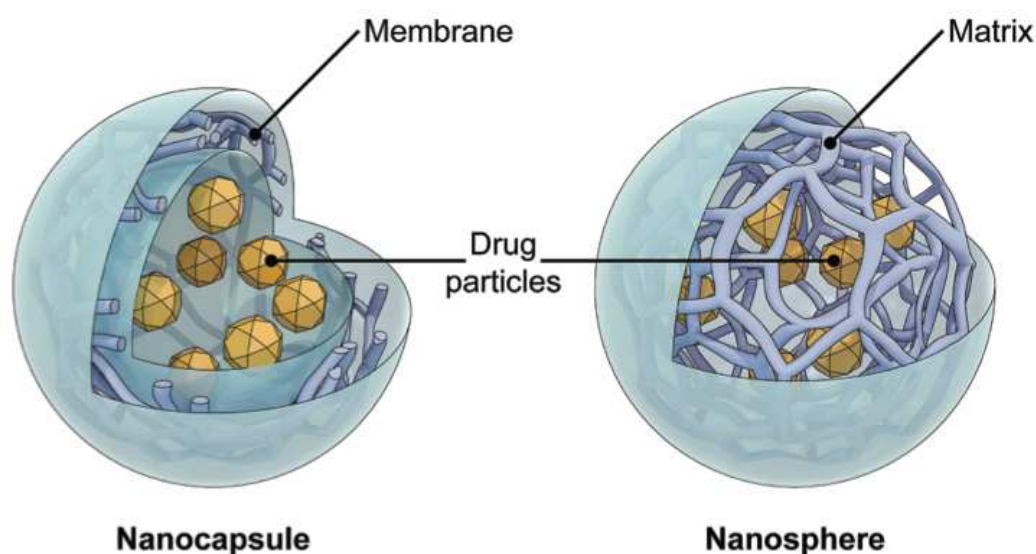


Figure 2.9. Nanocapsule vs nanosphere [32]

Nanocapsules are colloidal-sized vesicular drug delivery systems made up of an oily or an aqueous reservoir core surrounded by a polymer membrane or coating as illustrated in Figure 2.9. If the core is composed of an oily liquid, then the vesicular structure is referred as “nanocapsule”. These types of vesicles have ability to easily encapsulate hydrophobic drugs. Typically the polymers used for the formation of nanocapsules include polyester homopolymers such as well-known PLA, PLGA and PCL. Alternatively, if the core is an aqueous phase and the structure in which the surrounding polymer is a bilayer, it is named as “polymersome” (Figure 2.10). They are very similar to liposomes and suitable for encapsulation of hydrophilic drugs, but differ in external bilayer which is composed of amphiphilic copolymers such as polyethylene glycol-*block*-polybutadiene (PEG-*b*-PBD) [48, 49].

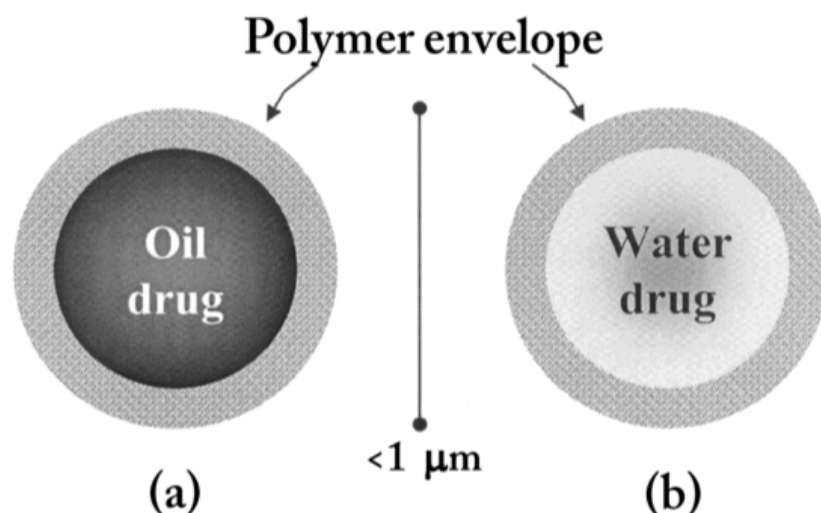


Figure 2.10. Schematical illustration of a) nanocapsule and b) polymersome [49]

The drug encapsulation mechanism can be considered in three categories which can be classified as; 1) dispersed through the matrix, 2) dissolved within or 3) adsorbed on the nanoparticles. These mechanisms may take place simultaneously in nanoparticles. In nanospheres, the drug is dispersed within the polymer homogenously whereas in nanocapsules the drug is confined to the core cavity [47, 49]. The drug release in a biological media depends on diffusion through the polymer membrane, desorption of adsorbed drug molecule, polymer matrix erosion or the combination of all these factors [50, 51]. However in nanocapsules, drug release profile is predominantly governed by oil-water partition coefficient of the encapsulated drug.

Nanocapsules have many advantages over conventional drugs and their applications. They primarily offer controlled and sustained release, improved bioavailability of drugs, drug selectivity and effectiveness, protection, targeting, improved *in vitro* and *in vivo* stability and reduced toxicity [52, 53]. All those advantages encourage the potential practices of nanocapsules in various fields such as agriculture, genetic engineering, cosmetic products, wastewater treatment and field of biomedicine. Some potential use of nanocapsules are given in Table 2.2 [52].

Table 2.2. Potential application fields of nanocapsules [52]

Application	Drug	Mode of Preparation
Agrochemicals	Abamectin nanocapsules Cypermethrin nanocapsules Pyrethrium nanocapsules	Emulsion polymerization Microemulsion polymerization Microemulsion polymerization
Anti-inflammatory drugs	Diclofenac sodium Indomethacin loaded nanocapsules	Sol-gel method Interfacial polymerization
Antiseptics	Monodisperse polymer nanocapsules	Precipitation
Cosmetics	Hinokitiol-loaded PCL nanocapsules	Emulsion-diffusion
Diabetes	Insulin-loaded PIBCA nanocapsules	Interfacial polymerization
Nanocapsules for cancer	Artemisinin Camptothecin and doxorubicin Indomethacin-loaded PIBCA nanocapsules	Nanoencapsulation Sol-gel method Interfacial polymerization
	Lipid nanocapsules loaded with Rhenium-188 Cisplatin	Phase-inversion Repeated freezing and thawing of a concentrated solution of cisplatin in the presence of negatively charged phospholipids
Nanocapsules for topical use	Chlorhexidine	Interfacial polymerization

2.3. Nanotechnologically formulated drug delivery systems for cancer therapy and marketed products

Nanotechnology is a newly emerging cutting edge technology including fascinating methods and applications for scientific advancements especially in medical areas. Nanotechnologically produced materials have impact in our everyday life, providing important benefits and characteristics to novel products which are the heart of this exponentially growing technology [54]. The drug industry is nowadays, focused on the production of simple molecules with exact specified structures and properties, which possess characteristics and impacts of complex molecules. They are called non-biological complex drugs (NBCDs). Nanoscale materials fulfill this gap in biomedical field by obtaining novel perspectives for research and development in pharmaceutical sciences and industry [55]. Today, cancer is a

leading cause of death world-wide. Over the last few decades, nano-scale medicine has been a reality with a wide range of functional applications and offers hope in cancer cure [56].

The important advantages of nanotechnology caused the emergence of a new field called “Nanooncology”, which is the cancer science incorporating nanotechnology. The main research area of Nanooncology is mostly the nanoparticles engineered in the range of nanotechnology to design a wide variety of newly produced chemotherapeutic and diagnostic agents due to unique fashion and tunable properties of nanomaterials [57]. Some examples of nanoparticular studies are given below in Table 2.3.

Table 2.3. Selected strategies for cancer treatment using nanocapsules [57]

Strategy	Therapeutic active agent	Indication/Result
Antitumor effect	Camptothecin Doxorubicin salt	High <i>in vitro</i> and <i>in vivo</i> antitumor activity [58]
Overcoming multidrug resistance	Paclitaxel	Enhanced cell growth inhibition a resistant ovarian cancer cell line [59]
Antitumor effect	Paclitaxel	Preferential accumulation at tumor site, Suppression of tumor growth [60]
Antiangiogenesis and antitumor effect	Paclitaxel Combretastatin A4	Efficient cellular uptake, Long circulation in body fluid and accumulation in tumor [61]
Antitumor effect	Docetaxel	Anti-proliferation of breast cancer cells [62]

Stake of nanotechnology in global market for health care is expected to rise up to 196.02 billion USD by the year 2020. The combination of nanotechnology and medicine has a huge potential for pharmaceutical industry. In addition, it is announced that nanotechnology applications dominate over total clinical oncology market revenue accounting for 35% in 2013 [63]. The National Cancer Institute (NCI) expends approximately 150 million USD annually on research activities and

efforts in nanomedicine [64]. As the field of nanotechnology grows, new regulations and rules should be required.

At this point, the governmental bodies have some definitions and regulations for production and application of nanotechnology. There are guidance documents published by FDA about regulations, characterization and validation procedures as well as nano concept, nano-scale definition and industrial guidelines on nanotechnological drugs and biologics [28, 65].

Among a wide variety of nanoparticulated systems, a small portion of nanotherapeutic delivery vehicles has received approval by FDA for the clinical use in the treatment of cancer events. It is critically important that the biodistribution of colloidal nanoparticles and targeting directly into desired site in body should completely be managed. Furthermore, targeted nanotherapy seems to be widely accepted due to its potential benefits, however, only a few of nanosystems are validated clinically [38, 66]. Other systems are currently in various pre-clinical and clinical developmental stages. The nanotechnologically manufactured drug delivery systems, which are validated by FDA and/or EMA, and also in phase-state nanotechnologically formulated systems are given in Table 2.4 below.

Table 2.4. FDA, EMA approved and in phase state nanotechnologically formulated drug delivery systems [38, 40, 63, 66-68]

Product	Therapeutic active agent	Route/form	Technology	FDA approval	Indication
Abelcet®	Amphotericin B	i.v.	Liposome	FDA-1995	Invasive fungal infections
Abraxane®	Paclitaxel	i.v.	Albumin based NP	FDA-2005, EMA-2008	Breast cancer, Various cancer types
Adagen®	Adenosine deaminase	i.m.	PEGylated nanoformulation	FDA-1990	Severe combined immunodeficiency disease
AmBisome®	Amphotericin B	i.v.	Liposome	FDA-1997, EMA-1990	Fungal infections
Amphotec®	Amphotericin B	i.v.	Liposome	FDA-1996	Invasive aspergillosis when amphotericin B is not recommended
Avinza®	Morphine sulfate	oral	Nanocrystal	FDA-2002	Moderate/severe pain

Cimzia®	Certolizumab pegol	s.c.	PEGylated antibody	FDA-2008	Crohn's disease, rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis
Copaxone®	Glatiramer acetate	s.c.	Polymeric nanoformulation	FDA-1996	Multiple sclerosis
Curosurf®	Poractant alfa	i.t.	Liposome	FDA-1999	Respiratory Distress Syndrome (RDS) in premature infants
DaunoXome®	Daunorubicin citrate	i.v.	Liposome	FDA-1996	HIV-related Kaposi's sarcoma
Definity®	Perflutren	i.v.	Liposome	FDA-2001	Contrast agent
DepoCyt®	Cytarabine	i.v.	Liposome	FDA-1999, EMA-2001	Leukemia Glioblastoma
DepoDur®	Morphine sulfate	epidural route	Liposome	FDA-2004	Chronic pain
Diprivan®	Propofol	i.v.	Surfactant-based nanoformulation	FDA-1989	Anesthetic
Doxil®/Caelyx®	Doxorubicin hydrochloride	i.v.	PEGylated liposome	FDA-1995, EMA-1996	Breast cancer, Ovarian cancer, Multiple myeloma, HIV-related Kaposi's sarcoma
Elestrin®	Estradiol	topical	Surfactant-based nanoformulation	FDA-2006	Hot flashes during menopause FDA
Eligard®	Leuprolide acetate	i.m/s.c.	Polymeric nanoformulation	FDA-2002	Advanced prostate cancer
Emend®	Aprepitant	oral	Nanocrystal	FDA-2003, EMA-2003	Emesis, antiemetic for chemotherapy patients
Estrasorb™	Estradiol hemihydrate	topical	Surfactant-based nanoformulation	FDA-2003	Reduction of vasomotor symptoms during menopause
Feraheme®	Ferumoxytol	i.v.	Metal nanoformulation	FDA-2009	Treatment of iron deficiency anemia in adults with chronic kidney disease
Ferrlecit®	Sodium ferric gluconate complex	i.v.	Metal nanoformulation	FDA-1999	Iron deficiency anemia
Focalin XR®	Dexmethylphenidate hydrochloride	oral	Nanocrystal	FDA-2005	Attention deficit hyperactivity disorder
Fosrenol®	Lanthanum carbonate	oral	Metal nanoformulation	FDA-2004	End stage renal disease

Fungizone®	Amphotericin B	i.v.	Surfactant nanoformulation	FDA-1966	Systemic fungal infections
Invega®	Paliperidon	oral	Nanocrystal	FDA-2006, EMA-2007	Schizophrenia
Kadcyla®	Ado-Trastuzumab Emtansine	i.v.	Protein-drug conjugate	FDA-2013	Breast cancer
Macugen®	Pegaptanib sodium	intravitreal	PEGylated anti-VEGF aptamer	FDA-2004, EMA-2006	Neovascular age-related macular degeneration
Marqibo®	Vincristine sulfate	i.v.	Liposome	FDA-2012	Philadelphia chromosome and acute lymphoblastic leukemia
Megace ES®	Megestrol acetate	oral	Nanocrystal	FDA-2005	Anorexia, cachexia, breast and endometrial cancer
Mepact®	Mifamurtide	i.v.	Liposome	EMA-2009	Osteosarcoma
Mircera®	Methoxy polyethylene glycol-epoetin beta	i.v./s.c.	PEGylated epoetin beta	FDA-2007, EMA-2007	Anemia associated with chronic renal failure
Myocet®	Doxorubicin	i.v.	Liposome	EMA-2000	Breast cancer
Naprelan®	Naproxene sodium	oral	Nanocrystal	FDA-1996	Rheumatoid arthritis and osteoarthritis, gout
Neulasta®	Filgrastim	s.c.	PEGylated nanoformulation	FDA-2002, EMA-2002	Febrile neutropenia
Oncaspar®	L-asparaginase	i.v./i.m.	PEGylated nanoformulation	FDA-1994	Lymphoblastic leukemia
Ontak®	Denileukin diftitox	i.v.	Protein-drug conjugate	FDA-1999	Persistent or recurrent cutaneous T-cell lymphoma
Pegasys®	Peginterferon alfa-2a	s.c.	Polymer-protein conjugate	FDA-2002, EMA-2002	Chronic hepatitis B and C virus infection
Pegintron®	Peginterferon alfa-2a	s.c.	Polymer-protein conjugate	FDA-2001, EMA-2000	Hepatitis C in patients with compensated liver disease
Rapamune®	Sirolimus	oral	Nanocrystal	FDA-2002, EMA-2001	Immunosuppressant
Renagel®	Sevelamer hydrochloride	oral	Polymeric nanoformulation	FDA-2000, EMA-2000	Hyperphosphatemia in patients with chronic kidney disease on dialysis
Ritalin LA®	Methylphenidate hydrochloride	oral	Nanocrystal	FDA-2002	Attention deficit hyperactivity disorder

Somavert®	Pegvisomant	s.c.	PEGylated human growth hormone receptor agonist	FDA-2003, EMA-2002	Acromegaly
Survanta®	Beractant	i.t.	Liposome	FDA-1991	Neonatal respiratory distress in premature infants
Tricor®	Fenofibrate	oral	Nanocrystal	FDA-2004	Dislipidemias
Triglide®	Fenofibrate	oral	Nanocrystal	FDA-2005	Dislipidemias
Venofer®	Iron sucrose (iron (III)-hydroxide sucrose complex)	i.v.	Metal nanoformulation	FDA-2000	Iron deficiency
Verelan PM®	Verapamil	oral	Multiparticulate system	FDA-1998	Hypertension
Verelan®	Verapamil	oral	Multiparticulate system	FDA-1990	Hypertension
Visudyne®	Verteporfin	i.v.	Liposome	FDA-2000	Photodynamic therapy
Zevalin®	Ibritumomab tiuxetan	i.v.	Antibody-targeted nanoparticle	FDA-2002, EMA-2004	Follicular lymphoma
Zyprexa®	Olanzapine	oral	Nanocrystal	FDA-2009	Schizophrenia
INVEGA TRINZA™	Paliperidone palmitate	i.m.	Nanocrystal	May, 2015	Schizophrenia
Thermodox®	Doxorubicin	i.v.	PEGylated liposome	Phase III	Liver cancer, Breast cancer
Genexol-PM®	Paclitaxel	i.v.	PEG-PLA NP	Phase II	Breast cancer, Lung cancer, Ovarian cancer
Opaxio™	Paclitaxel	i.v.	PGA NP	Phase III	Lung cancer, Ovarian cancer
CRLX101	Camptothecin	i.v.	PEG cyclodextrin NP	Phase II	Non-small-cell lung cancer
NC-6004	Cisplatin	i.v.	PEG-PGA	Phase II	Pancreatic cancer

2.4. Preparation Techniques

Mainly there are six different and well-known methods for the preparation of nanocapsule. They can be listed as;

- 1- nanoprecipitation,
- 2- emulsion-diffusion,

- 3- double emulsification,
- 4- emulsion-coacervation,
- 5- polymer coating and
- 6- layer-by-layer techniques as given in Figure 2.11 [69].

Among these, the most common method is nanoprecipitation which is also called interfacial deposition of preformed polymers. The nanoprecipitation technique includes a drug solution in a water-miscible organic solvent containing an oil which is miscible with the solvent and an aqueous phase containing a hydrophilic surfactant. The organic phase is added into aqueous phase rapidly but in a dropwise fashion under moderate agitation to disperse and diffuse the solvent in aqueous phase. Hence, polymer forms aggregetages around the oil droplet forming a vesicular shape having an oily core [48].

Nanocapsule Formation

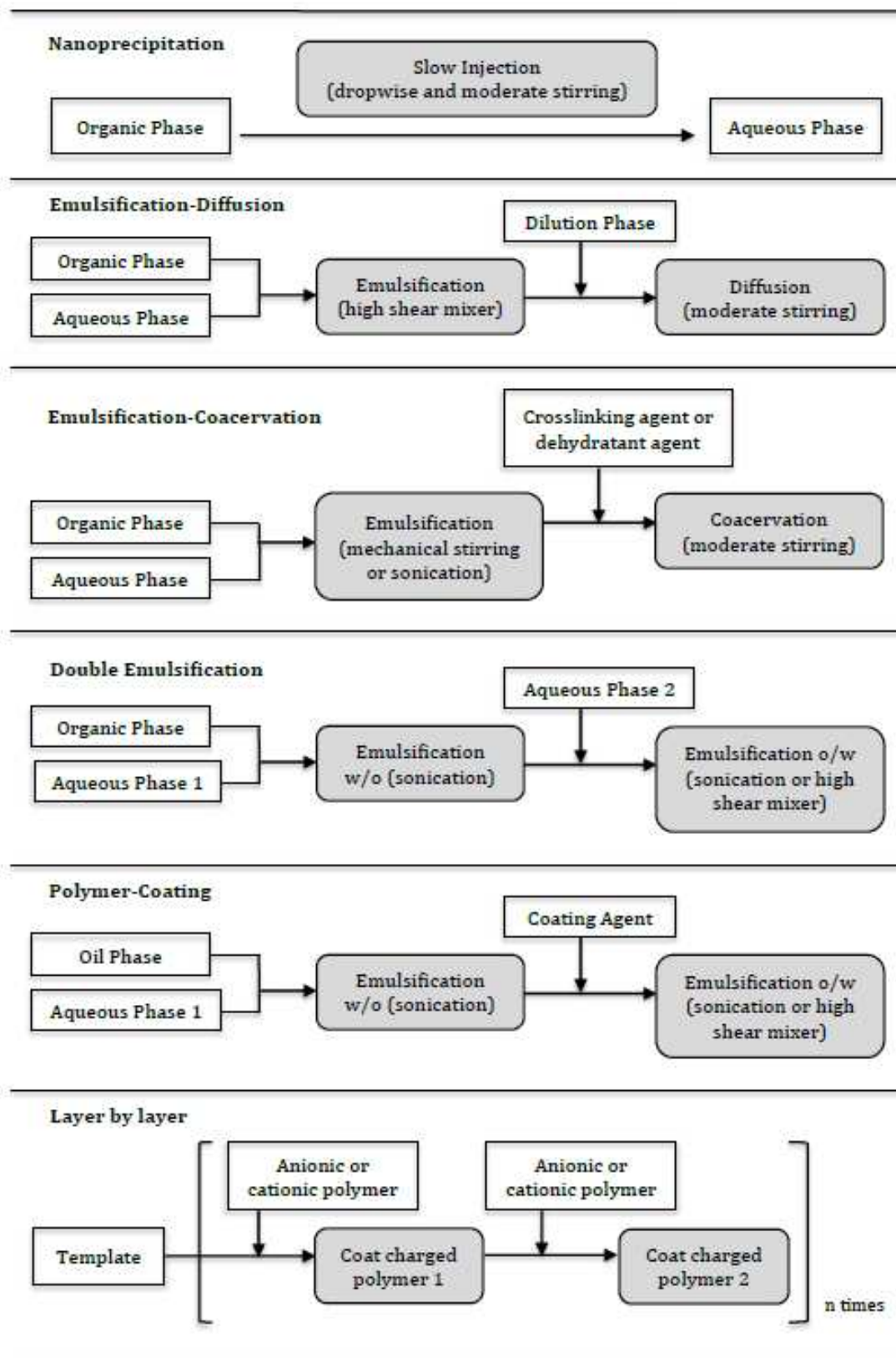


Figure 2.11. Nanocapsule preparation methods [69]

In most of the studies in literature, it is observed that one of the methods depicted above is used for the preparation of nanocapsules. The method selection is an important factor for the final nanocapsule specifications. According to the aim of researchers, nanocapsule preparation techniques and specific variables in these techniques can be modified slightly to reach the desired final product. The general trends of researchers on method selection for nanocapsule preparation are given in Figure 2.12. As it can be clearly seen, nanoprecipitation method is widely used in nanocapsule researches due to its simplicity, rapidity, reproducibility and also providing high yield of product [70].

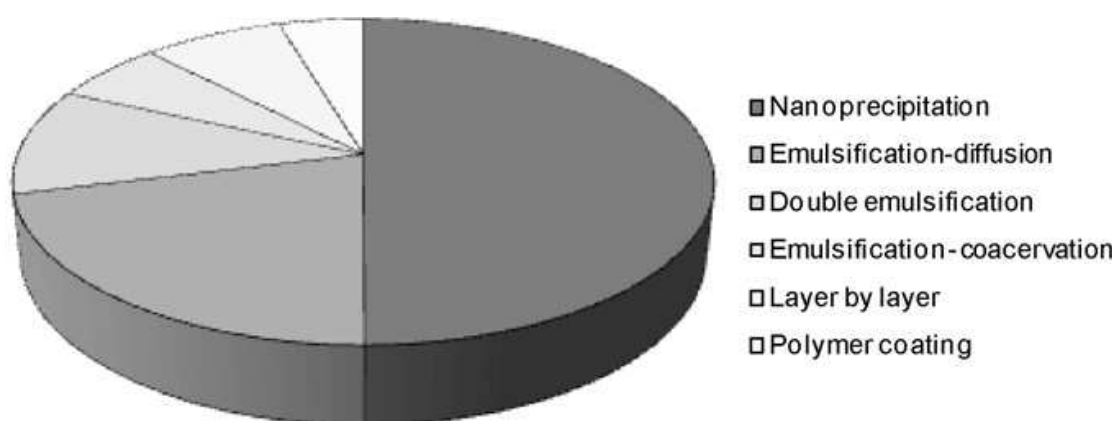


Figure 2.12. Trends on method selection in nanocapsule researches [69]

Nanoprecipitation is an easy method performed by a rapid dropwise mixing of an organic phase containing the solvent, polymer, oil and drug into an aqueous phase containing water, surfactant and other excipients such as surface modification materials [71]. This method is also known as solvent displacement method. The first inventor of this method, Fessi et al. investigated the mechanism of particle formation between two liquid phases having different properties at the interface in 1989 [72].

The basic principle of nanoprecipitation method is the interfacial deposition of polymer during displacement from hydrophobic organic solution to a water-miscible semipolar solvent. Concisely, during this interfacial turbulence, surface tension decreases while surface area increases, two phases flow into each other

via diffusion and small droplets of hydrophobic phase occur [73]. This phenomenon is also known as Marangoni effect which is shown in Figure 2.13.

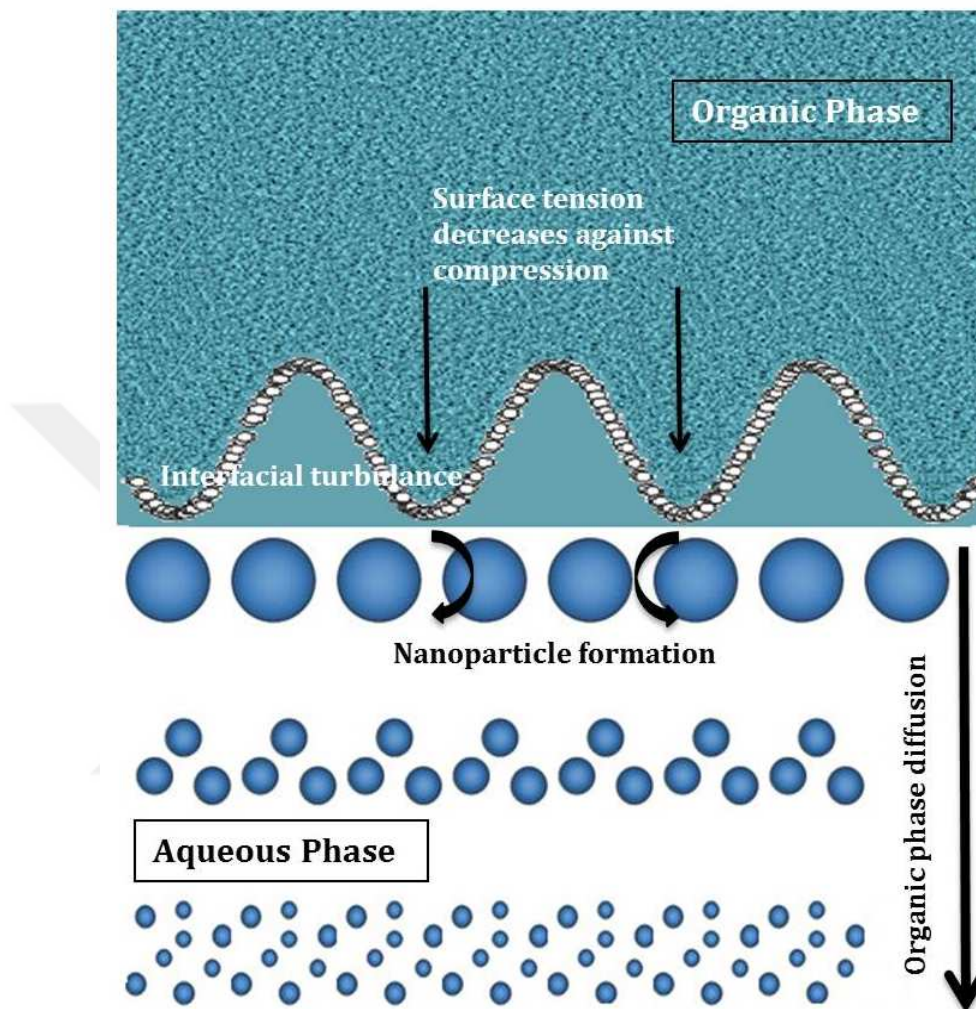


Figure 2.13. Schematical representation of Marangoni phenomenon

The flow at the interface is responsible for the turbulence occurred at the interface of two unequilibrated phases, diffusion and the possible chemical reaction, rates of heat and mass transfer [74]. The Marangoni transport between liquids is driven by the significant interfacial tension gradient occurring during the mass transfer and producing surfactant adsorption or desorption at the interface [75]. The adsorption of surfactant decreases the surface energy and consequently the surface tension which is defined as the surface energy responsible for the unit area. Furthermore,

surface exerts resistance to compression and shear forces and displays elastic properties. These compression and resistive forces cause a decrease in surface area occupied by monolayer, because the surface concentration of surface active agent increases. As a consequence, surface tension decreases leading to surface tension gradient between compressed and uncompressed regions, which is known as Marangoni effect [76] depicted in Figure 2.13.

The success of nanoprecipitation technique and the final physicochemical properties of nanocapsule depend on many parameters during preparation. The type and concentration of components, the ratio of organic phase to aqueous phase, organic phase injection rate, agitation rate during the addition of organic phase into non-solvent phase, miscibility of organic solvent and aqueous phase and the nature of interaction between polymer and solvent are key parameters affecting the final product in formulation step [70, 73]. Although a surfactant is not essential to form nanocapsules, the nanocapsule suspension can be protected from flocculation by the addition of surfactant [73].

Nanoprecipitation method includes a polymer which can be synthetic, semi-synthetic or natural. The basic polymers or polysaccharides for the nanocapsule formulation in nanoprecipitation process are poly(ϵ -caprolactone), polylactic acid, poly(lactide-co-glycolide), cyclodextrins and chitosan. The solvent should have a high solubility in water and be easily removable by evaporation. Hence, the most frequently used solvent in this method is acetone, but also other solvents can be mainly listed as ethanol, methylene chloride, hexane or dioxane. The surface active agent in the process protects the nanoparticles from aggregation and helps stabilization especially during long storage time periods [70].

Nanoprecipitation method mainly consists of nucleation, growth and aggregation steps. The rate of those steps affects the size of nanoparticles formed spontaneously. Ideally, method conditions should persist for a high nucleation and low and stable growth rate because a uniform size distribution can be obtained with a stable growth stage [77]. After each successful nanocapsule formation period, solvent should be eliminated under vacuum or moderate magnetic agitation. Then, nanocapsules are recovered by centrifugation and stabilized by lyophilization or spray-drying [69].

2.5. Characterization Techniques

Manufactured small solid particles are utilized broadly in everyday practices. Their physical, chemical and optical properties can be tailored by nanotechnological techniques according to main production purpose [78]. Nanoparticles differ from their bulk material counterparts via their physiochemical properties. For instance size, shape, composition, purity, solubility and surface characteristics are critically relevant to particular interactions with environment and other substances. The physiological behavior of nanoparticles has impact on therapeutic efficacy and diagnostic accuracy of final products. It is important to realize how the different properties of nanoparticles affect *in vivo* behavior and distribution of them. The behavior of nanoparticles may provide superiority in medical applications such as improvement of efficacy, reduction of toxic and other side effects and stability in biological media [79].

Although nanoparticles are considered as a new sort of analyte for the analytical sciences with their chemical (composition, mass and concentration) and physical (size, shape, etc.) information, they are far more complex materials than they are thought to be. Primarily, they have more complex chemical structure with their core and coating portions and this will also affects their physical properties (size, shape, agglomeration, etc.) [80]. Furthermore, the nanoparticle type (capsule or sphere), active release characteristics, encapsulating efficiency, nanodispersion stability, potential degradation products of particles and pharmacological performance behaviors must also be considered since all these information are closely attached to the composition, texture, formation, fate and toxicity of nanoparticles [69]. The characterization of nanoparticles includes the cross-and interdisciplinary relationship of chemical, physical, mechanical, optical, surface, *in vitro* and *in vivo* research subjects.

In order to explore the whole potential of nanoparticles, characterization has primary importance on nanomaterial production. It is also very important to assess the safety and toxicity parameters of fabricated nanoparticles.

Although there is no FDA protocol developed for any of diagnostic or therapeutic use of nanoparticles, there are so many applications for the evaluation of manufactured nanoparticle properties with their advantages and disadvantages including dimensions, shape, surface characteristics, stability, sensitivity,

composition, resolution and purity [79, 81]. Characterization methods include mainly state-of-the-art techniques such as optical spectroscopy, surface scanning and light scattering applications, size and zeta potential measurements; as well as methods such as chromatography, fluorescence measurements, cell culture and release studies [79, 82]. However, there is no standardized guideline for detection and characterization of nanoparticles.

Nanoparticle characterization is particularly focused on mean size and size distribution, shape, porosity, surface area, zeta potential and aggregation to ensure the quality of the nanomedicine [79, 83]. Electron microscope is one of the most reliable and powerful technique used for the visualization of nanoparticles for determination of size, shape and aggregation state [84]. Electron microscopy techniques such as scanning electron microscopy (SEM), transmission electron microscopy (TEM) and atomic force microscopy (AFM) are suitable for nanoparticle characterization in submicron scale [85]. The evaluation of electron microscope techniques is based on the images resulting from the interaction of transmitted electrons with a single sample [86]. Since, shape and morphology have critical importance on performance and fate of nanoparticles [87], the electron microscopy techniques are widely used in visualization of nanoparticle morphology and topography [84, 85]. An electron beam makes it possible to visualize the particle's core by transmitting through an ultra thin specimen, which is the main principle of TEM. Moreover, in SEM, particle's surface topography can be detected by the gathered information from the electron signals generated by interaction of electrons with the specimen [88]. The advantages and disadvantages of three electron microscopy techniques are given briefly in Table 2.5.

Table 2.5. Advantages and disadvantages of electron microscopy techniques [87].

Technique	Advantages	Disadvantages	Ease of access and availability
TEM	High resolution Two-dimensional images	Staining agent required Sample preparation causes damage Sample has to be electron transparent Uses high pressure vacuum	Very expensive technique Extensive sample preparation Requires dedicated personnel Analysis is often outsourced
SEM	Surface morphology and 3D picture of the nanoparticles	Drying process causes damage Requires coating of the surface Uses high pressure vacuum	Very expensive technique Extensive sample preparation Requires dedicated personnel Analysis is often outsourced
AFM	3D topographical view No coating required Ambient conditions maintained Integrity of sample is preserved	Dry immobilized sample required Small scan area and low scan depth Slow scan rate can lead to a thermal drift	Relatively inexpensive Relatively newer Extensively used due to ease and cost effectiveness

Moreover, light scattering techniques, especially dynamic light scattering (DLS) applications are often used for size determination of dry or dispersed nanostructures in aqueous suspensions [86]. Moreover, particle size distribution is also a vital factor in controlling the surface/volume ratio affecting chemical reactivity of nanoparticles and needs to be investigated [89]. In DLS method, the light is directed onto the experimental suspension [88]. The Brownian motion of nanoparticles is measured by detecting time-dependent fluctuation intensity caused by interferences and correlating these movements with an equivalent hydrodynamic diameter [80, 87, 89].

Furthermore, zeta potential demonstrates the surface charge of nanoparticles in dispersed state. The zeta potential is of importance for the *in vivo* fate of particles. It affects the dynamic interaction of nanoparticles with their surrounding because

zeta potential mainly depends on the strength and valency of ions in nanoparticle suspension. Zeta potential measurement originates from the electrophoretic mobility of nanoparticles suspended in the medium and generally measured by laser Doppler electrophoresis. If the zeta potential is more positive than +30 mV or more negative than -30 mV, the colloidal dispersion is said to have an optimum stability [90].

Additionally, the ability of nanoparticles to release the drug inside is important for the therapeutic outcome of the formulation. A drug can be released by desorption from the surface which the drug is bound or adsorbed on, diffusion from the matrix or release due to the erosion of polymer or a combination of these release mechanisms can be actualized [90]. The release mechanism of nanoparticles can be studied by various methods such as dialysis membrane technique and sampling and separation. In dialysis method, sink conditions should be provided for a continuous diffusion across the membrane. It is a quick and simple method for drug release investigations and also has no invasive and harsh separation processes on nanoparticles [90, 91].

Furthermore, nanoparticle stability is also vital for the contained cargo inside the particle. An orally designed nanoparticle is threatened initially by acidity and mucus layer from the beginning of its travel within the GI tract as a drug. Degradation and elimination are main problems for oral formulations beside other factors such as aggregation and drug loss. Premature drug release and unwanted biodistribution profile of drug are results of instability of nanoparticles, resulting altered efficacy. Hence, stability is one of the essential components for the success of nanoparticle formulation [90]. Stability in oral formulations begins in mouth with acidity. Low acidity is a degradation factor for nanoparticles. The whole through GI system differs in acidity levels. Since an oral formulation is aimed to carry its cargo inside towards the circulation, it should resist acidity variations in GI canal. Stability can be investigated by mimicking the acidity of microenvironment of passage. Beside, mucus layer is another barrier for retention and elimination of particles as stated in Section 2.1. Test set up can be constituted by testing the interaction between the particles and the mucus which is called turbidity analysis. The adsorption level and mucoadhesive tendency can be determined by size alteration and aggregation degree as well as zeta potential variations [92].

The many other parameters in nanoparticle characterization can be tested on cell lines, such as activity, toxicity and permeability. Cell lines are commercially available to test such factors. The controllable variables can be modified according to the aim of study. Most of cell line techniques are easy, rapid and operative in the course of practice.

2.6. Docetaxel

Docetaxel is known as a well-established semi-synthetic antineoplastic chemotherapeutic agent used clinically in many cancer types. It is a member of taxoid family of drugs originally developed from the yew tree and known with its generic name; Taxotere®.

Historically developmental steps of Docetaxel dates back to 1970's. Taxol is isolated from the outer shell of *Taxus brevifolia* grown in USA in 1971. It was registered by FDA in 1992 for ovarium cancer patients and in 1994 for breast cancer patients. The active agent was called paclitaxel and the product was called TAXOL®. Thereupon, in 1985, an analogue of taxol was synthesized and named as taxotere. Taxotere was authorized as anticancer agent in 1996. The active agent was called docetaxel and the drug form was called TAXOTERE® [93].

Docetaxel with its IUPAC name "1,7β,10β-trihydroxy-9-oxo-5β,20-epoxytax-11-ene-2α,4,13α-triyl 4-acetate 2-benzoate 13-[(2R,3S)-3-[(tert-butoxycarbonyl)amino]-2-hydroxy-3-phenylpropanoate]" has an exact molecular mass of 807.87 g/mol and chemical formula of C₄₃H₅₃NO₁₄. Docetaxel has a melting point of 186-192 °C. It is insoluble in water but soluble in ethanol and acetone and give peak at 230, 275, 283 nm in UV. The structural formula of docetaxel is given in Figure 2.14.

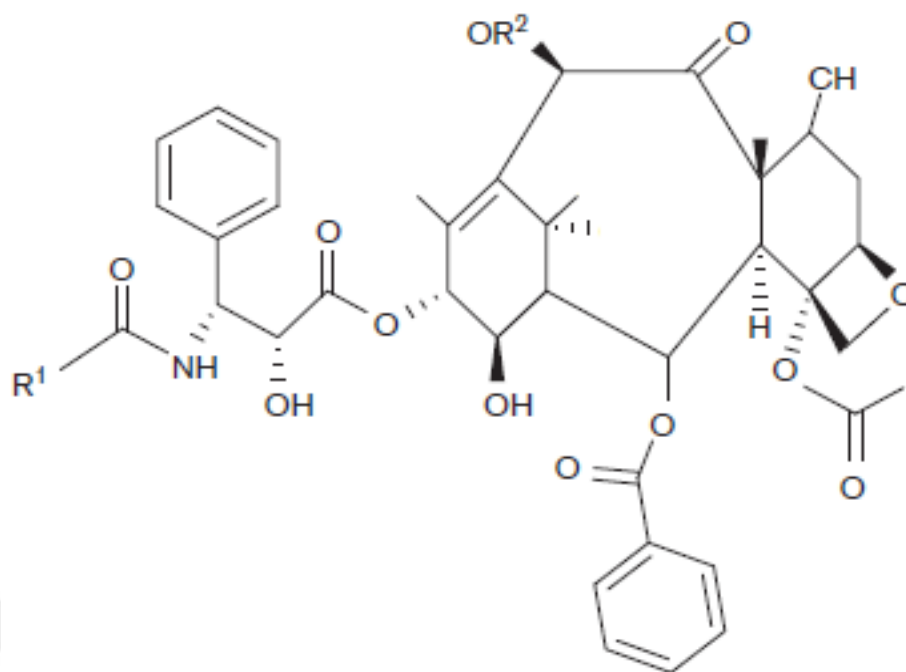


Figure 2.14. The structural formula of docetaxel [94]

Docetaxel has low water solubility and poor absorption potential as an oral drug which falls into BCS Class II group of pharmaceuticals in the BCS classification. The primary factor for showing diminished oral bioavailability of docetaxel is having low solubility in water. Furthermore, high affinity to P-gp which functions as a multidrug efflux pump, and elimination by the hepatic first pass metabolism are other pivotal reasons of poor oral bioavailability [23, 95]. The affinity of P-gp limits the bioavailability of orally administered toxoids and leads to direct excretion into the gut lumen [95]. Furthermore extensive cytochrome P450 3A4 (CYP3A4) metabolism is another important factor for poor bioavailability and efficacy of docetaxel. CYP 450 is abundantly found in liver and gut wall and mediates high first-pass effect [95, 96]. Docetaxel elimination is actualized by extensive hepatic metabolism and biliary excretion. It is eliminated from body predominantly in the feces. Docetaxel undergoes hydroxylation of tert-butyl group from M2 primary alcohol metabolite which is driven by CYP3A4 and CYP3A5 metabolism [97, 98].

Docetaxel is a mitotic inhibitor and stabilizes the mitotic spindles by preventing

tubulin depolymerization during cell division [99, 100]. Exactly, docetaxel binds specifically to the beta-tubulin subunit of microtubules and blocks the disassembly of the microtubule proteins resulting the formation of aberrant microtubule structures [94, 101]. This action blocks the progression of mitosis and prevents cancer cell growth and results in subsequent cell death.

In cancer chemotherapy docetaxel is used as a single agent or in combination with other agents. The drug is approved for the treatment of breast cancer in any stage with various drug combinations [102, 103], prostate cancer [104, 105], advanced gastric cancer [106, 107] and head and neck cancer [108, 109]. Docetaxel is internalized faster than paclitaxel to the cell, stays inside longer to exert effect on mitotic spindle. Because of these properties, docetaxel was realized being more potent than paclitaxel in *in vivo* and *in vitro* antitumor activity studies [93, 97].

However, taxoids such as paclitaxel and docetaxel have some limitations including systemic toxicity due to non-selective cytotoxic action. This affect restricts the administration route and dose levels leading to incomplete tumor recruitment [110]. They also show various undesirable side effects. For instance, docetaxel is commercially available as Taxotere® and Duopafei®, and these drugs contain high concentration of surface active agent as excipient which causes many side effects such as neutropenia, diarrhea, mucositis, fluid retention, hypersensitivity and neurotoxicity [95, 97, 111].

Application of Docetaxel in Nanoformulations

There are many studies working on drug delivery systems containing docetaxel [112, 113]. However, they are mostly in *in vitro* laboratory developmental level. The clinical translation of these nanosystems is strictly dependent to a synergistic combination of several features such as physicochemical characteristics of polymer, excipients and drug molecule, the combinational properties of the whole system including *in vivo* release capability of the formulation, bioavailability potential and probable toxic effects [66, 114, 115]. To develop controlled release nanoparticles, high-throughput nanoparticle formulation and characterization technologies are used to achieve the optimization of nanocarrier systems. Beside all those laboratory works, a nanoparticulate docetaxel carrier is under development. BIND-014 is the first targeted and having controlled release

properties PEGylated docetaxel-loaded polymeric nanoparticle developed for chemotherapy, of which the clinical trials are conducted and continued in phase II [66, 115]. The bioavailability studies revealed that the encapsulated docetaxel is delivered selectively to prostate cancer cells and exerts its effects on tumor effectively [116, 117]. Figure 2.15 represents an illustration of BIND-014 molecule.

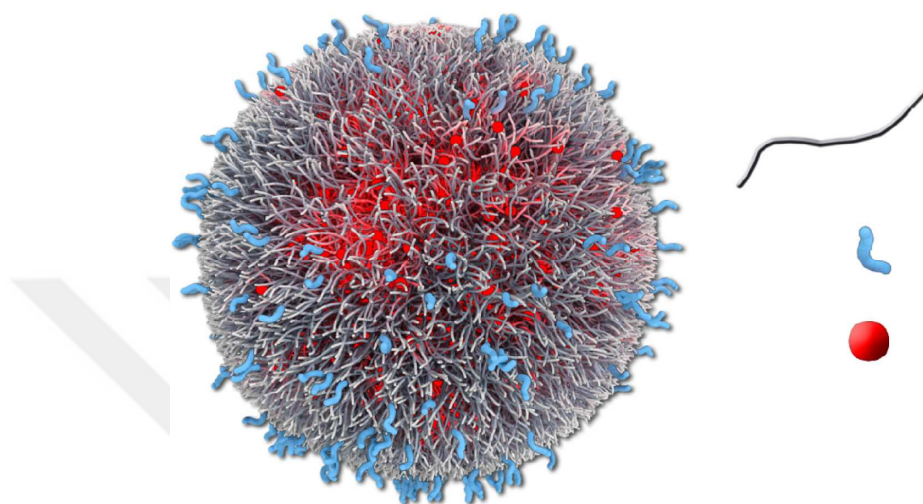


Figure 2.15. Graphical illustration of BIND-014 molecule containing hydrophobic polymeric core in which a semisynthetic taxane (docetaxel) is encapsulated and hydrophilic surface modification decorated with targeting ligands [66].
 — polymer; — ligand; ● docetaxel

Other polymer-based nanoformulations for delivery of docetaxel are represented in Table 2.6.

Table 2.6. Representative docetaxel nanoformulations compiled by Zhang et al [113]

Formulation	Polymer	Technology	Loading method
TPGS _{2k} micelles	Vitamin E TPGS 2k	Micelle	Encapsulation
Nanoxel-PM™	mPEG-PDLLA	Micelle	Encapsulation
DOC-HGC nanoparticles	Cholanic acid-modified glycol	Nanoparticle	Encapsulation

DOC-loaded HA-CE/P85-based nanoparticles	HA-CE and Pluronic	Nanoparticle	Encapsulation
DOC-HPMA conjugates	HPMA copolymer	Conjugate	Conjugation
DOC-P80-PPI dendrimers	Poly(propyleneimine)	Dendrimer	Encapsulation

2.7. Poly(ϵ -caprolactone)

Polycaprolactone is a biodegradable aliphatic semi-crystalline polyester with a chemical formula of $(C_6H_{10}O_2)_n$. It has a very low glass transition point (T_g : $\sim -61^\circ\text{C}$) and low melting point ranging between $\sim 59-65^\circ\text{C}$ depending on crystalline nature of PCL. The number average molecular weight of PCL differs between 10.000 and 42.500. It is regularly prepared by ring opening polymerization of ϵ -caprolactone using metal alkoxides as catalyst [118, 119]. The formation reaction of PCL is shown in Figure 2.16 below.

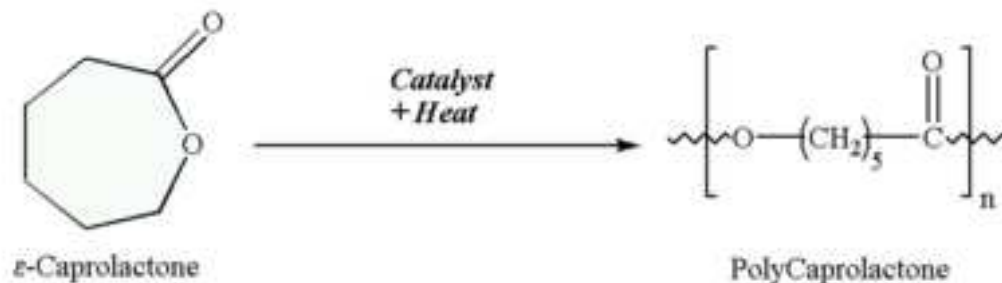


Figure 2.16. Formation reaction of Poly- ϵ -caprolactone [120]

PCL is non-toxic, broadly miscible with water, mechanically compatible with many polymers and has good adhesion to a variety of substrates. Beside its semi-crystalline and hydrophobic structure, it has good solubility properties. The crystallinity of PCL decreases while molecular weight increases. All these characteristics of PCL make it a good candidate for biomedical applications [121]. PCL has good solubility in many solvents such as chloroform, dichloromethane, benzene and toluene at room temperature. However, its low solubility is also an

utilizable property especially in pharmaceutical sciences. Acetone, ethyl acetate, acetonitrile and dimethylformamide are some of the solvents in which PCL has low solubility. Moreover, it is insoluble in alcohol and diethyl ether [118, 122].

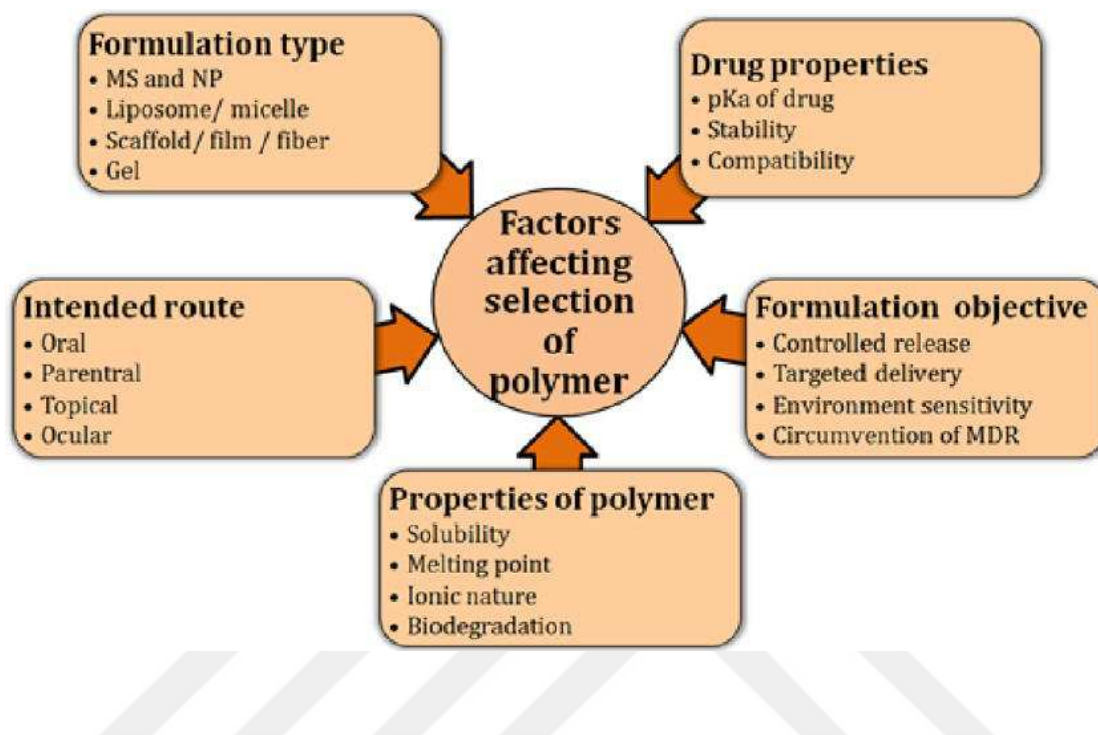


Figure 2.17. Polymer selection parameters [123]

There are many factors affecting the selection of polymer for preparation of drug delivery system as summarized in Figure 2.17. Different formulations require optimization of polymeric design to obtain effective drug delivery system. The intended design varies according to the objectives and the physicochemical properties of main cargo, which is the drug, by manipulation of production conditions. Hence, polymer selection is the first most important step for a successful drug delivery vehicle [123]. The biodegradable property of PLC was first identified in 1973. PCL can be used alone as well as blended with different polymers to improve resistance against stress crack and adhesion property. Additionally, PCL has a slow degradation rate making it appropriate for long-term delivery as well as the whole other well-known characteristics offer promise for being a suitable constituent of controlled drug delivery vehicle [118]. The degradation mechanism of PCL directly changes the release profile of drugs

encapsulated or incorporated. The hydrophobic drugs are mostly distributed throughout the polymer matrix or in the core in case of the polymeric structure is a capsule, whereas the hydrophilic drugs tend to appear mostly near the surface of particle in adsorbed state [124]. Hence, the properties of PCL are adequate to satisfy the desired release profile of formulations currently used in drug delivery.

PCL has been tested in almost all novel drug delivery systems and applications due to its tailorable physicochemical properties. Specifically, its biodegradable and biocompatible nature makes PCL more attractive especially for controlled drug delivery studies and tissue engineering applications in a wide range of area including medical and dentistry in various formulation types shown in Figure 2.18 [124].

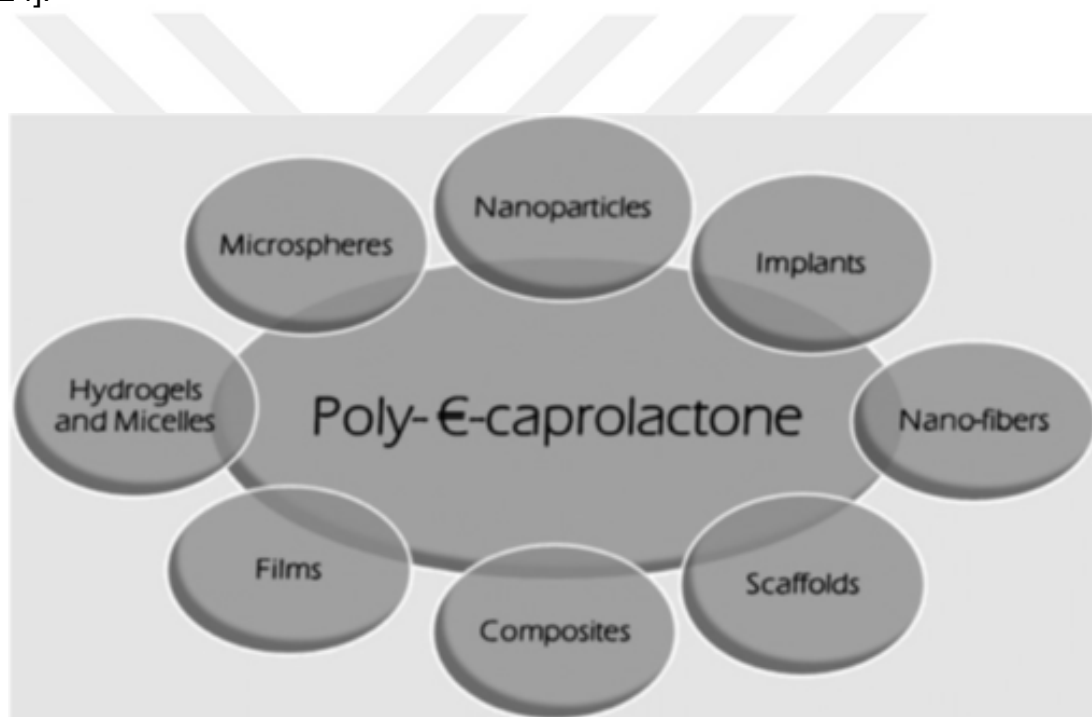


Figure 2.18. Various formulation types of PCL [124]

2.8. Polyethylene Glycol

Polyethylene glycol (PEG) is a neutral hydrophilic synthetic polyether which has so many superior physicochemical characteristics. PEG is a water soluble molecule, non-toxic and has favorable protein resistance and immunogenic properties [125]. It is also known as polyethylene oxide (PEO) and polyoxyethylene (POE) according to its molecular weight. The polymers having a molecular weight smaller

than 100.000 ($M_w < 100.000$) are usually called PEGs. They are amphiphilic and soluble in water as well as many organic solvents such as acetone, chloroform, ethanol and toluene. Additionally, low molecular weight PEGs ($M_w < 1000$) are viscous, clear and colorless liquids whereas higher molecular weight PEGs are more waxy and have white solid structure. PEG 400 is also a low molecular weight polymer widely used in many pharmaceutical formulations due to its low toxicity. It is soluble in water, acetone and alcohol, like other low molecular weight PEGs, and also slightly soluble in aliphatic hydrocarbons [126, 127]. PEG is commonly expressed as given in Figure 2.19.

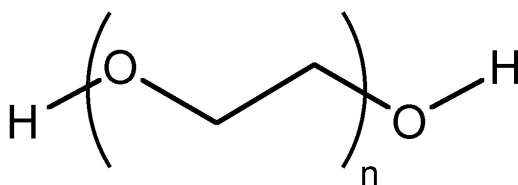


Figure 2.19. The chemical formula of polyethylene glycol

PEG molecule is one of a small number of synthetic polymer approved by FDA to use as excipient or use as a carrier in varying pharmaceutical formulations and other areas such as food, personal care products, cosmetics and biomedical applications [125, 126].

PEG is generally used to increase solubility and resistance to proteolysis, reduce immunogenicity and modulate pharmacokinetics of drugs. There are many studies on PEG conjugates with anticancer drugs such as paclitaxel, camptothecin and doxorubicin [128, 129]. Modification with PEG is useful for prolonging the half-life of drug molecules in circulation, increasing water solubility, reducing antibody recognition and self-aggregation [130]. PEG chains surround the particle surface and create a highly hydrated steric barrier around, which blocks the adhesion of opsonins and stabilizes the molecular assemblies against uptake by mononuclear phagocytic system (MPS). By that way, it also increases the stability of nanoparticles in GI fluid for oral drug carriers [48, 131, 132]. The main purpose of

surface modification is the organization of surface properties chemically or physically to improve biocompatibility by retaining the key bulk properties of material [133]. PEGylation is a term which meets the modification of surfaces with PEG due to its sophisticated properties. PEGylation is a very effective approach to overcome the drawbacks in oral delivery. With PEG modification, the drug carriers can be avoided from mucin entrapment by effectively minimizing the adhesive interactions between nanoparticles and mucus, thereby allowing the rapid penetration of nanoparticles through openings between mucin mesh fibers [16, 134]. Also, bioavailability potential of small molecules and protein drugs are shown to be enhanced after PEGylation process. However, PEGylated carriers are investigated mostly in parenteral applications. It remains unclear as to the real mechanism of oral BCS II drug nanocarrier processing in GI passage [135].

2.9. Chitosan

Chitosan is a natural, cationic, linear polysaccharide made up of D-glucosamine and N-acetyl-D-glucosamine units forming a copolymer together by the glycosidic linkage of β -(1-4). This polysaccharide has also non-toxic properties as well as having good biocompatibility and biodegradability character. It can form complexes easily with negatively charged polymers due to its cationic nature [136]. Chitosan has been used as permeation enhancer in the course of absorption of hydrophilic macromolecules. It adheres to the mucosal surfaces and interacts with tight junctions located between epithelial cells. Transiently opening of tight junctions allows the hydrophilic molecules to be absorbed and flowed into circulation [137, 138]. Its excellent multidimensional properties make chitosan attractive in many areas of biomedicine. Being non-toxic, hydrophilic, biocompatible, biodegradable and having many reactive functional groups and special mucoadhesive properties provide advantage to chitosan over other molecules. Chitosan has also good antimicrobial, adhesion, coagulation ability and immunostimulating activity. Especially the primary amine groups in chitosan structure dedicate special characteristics and make it convenient to use in many areas especially pharmaceuticals. It can form specific interactions with cell receptors and glycoproteins, serve as an anchor to conjugate therapeutics, targeting ligands and other molecules such as imaging agents. Therefore, it is used widely in drug

delivery applications. However, low aqueous solubility of chitosan at neutral pH is a limiting factor for application areas, especially in drug delivery [125, 139, 140].

Chitosan is produced by partial deacetylation of neutral chitin, one of the most abundant polysaccharides, which is isolated from crustacean shells [141, 142]. Chitin is a hard, inelastic and nitrogenous natural polysaccharide, which was first identified in 1884. Chitosan has intramolecular hydrogen bonding which leads to a rigid crystalline structure. Both polymers (chitin and chitosan) are renewable polymers [143]. The structural formula of chitin and chitosan is given in Figure 2.20.

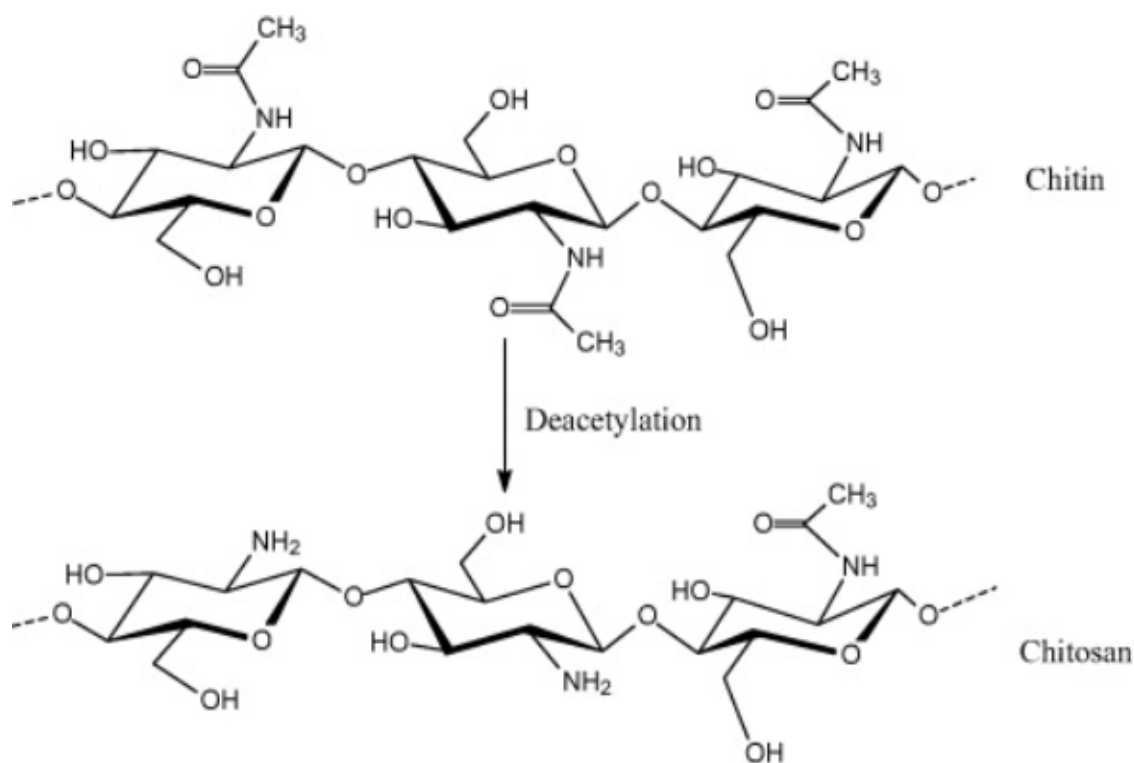


Figure 2.20. Chemical structural formula of chitin and chitosan [143]

Chitosan does not lead to allergic reactions and rejection since it breaks down into completely absorbed harmless degradation outputs, which is a result of its biocompatible nature [140]. The toxicity of chitosan depends on both the molecular weight and degree of acetylation. However, it is accepted that the toxicity of chitosan is negligible [141].

The oral bioavailability of specific agents has shown to be improved through the use of nanoparticles [138, 144-146]. The proposed mechanism of improved bioavailability depend on the surface area interaction between nanocarrier and the epithelium. The absorption potential is strongly affected by the polymer composition of nanocarriers. The modification of nanocarrier surface is an easy way to modulate the interaction with absorptive epithelium [147]. Chitosan is a quite promising polysaccharide for oral drug delivery vehicles due to its exclusive properties. The use of mucoadhesive chitosan has to be proven to enhance the drug absorption potential. Furthermore, chitosan is also used as a carrier in delivery of hydrophobic cancer drugs [148, 149]. Principal properties of chitosan related with biomedical application fields are given below in Table 2.7.

Table 2.7. Principal properties of chitosan related with biomedical applications [148]

Potential Biomedical Applications	Principal Characteristics
Surgical sutures Dental implants Artificial skin Rebuilding of bone Corneal contact lenses Sustained release Encapsulating material	Biocompatible Biodegradable Renewable Film forming Hydrating agents Non-toxic, biological tolerance Hydrolyzed by lysosome Wound healing properties Antibacterial, antifungal

Since coating of nanocarriers with chitosan enhances the biocompatibility, biodegradability and biological activities in cellular level (such as antitumor, antifungal, antibacterial, antithrombogenic etc.), the use of chitosan in drug delivery vehicle formulations possesses great application potential in many areas especially in biomedical, biotechnology and pharmaceuticals [150, 151].

3. MATERIALS

3.1. Equipments

Equipments & Specifications	Supplier
Thermostated Shaker Bath	Memmert WNE22, Germany
Precision Balance (d=0.01/0.1 mg)	Mettler Toledo XS 105 dual range, USA
Balance (max 200 g with readability 0.1 mg)	Shimadzu AUX220, Japan
Multipoint Magnetic Stirrer	Variomag Multipoint HP, Germany
Magnetic Stirrer with Hot Plate	IKA-Werke RCT-5 Power, Germany
Rotavapor	IKA RV06-ML, Germany
Vacuum pump for rotavapor	V-700 Buchi, Switzerland
Vacuum pump controller for rotavapor	V-850 Buchi, Switzerland
Freeze-dryer	Heto PowerDry PL 3000, Denmark
Micropipette (20-200 μ L, 100-1000 μ L)	Eppendorf, Germany - Eco Pipette by CAPP
Dynamic Light Scattering	Malvern Zetasizer Nano ZS, England
pH meter	Sartorius PP-20, Germany
Centrifuge (max.15000 rpm, test tube)	Hettich Universal 320, Germany
Centrifuge (max.18000 rpm, eppendorf)	Hettich EBA 21, Germany
Scanning Electron Microscope (SEM)	QUANTA 400F Field Emission SEM, USA
Ultra Pure Water System	Millipore Milli-Q Systems, USA
Tissue Homogenizer	OMNI International Sonic Ruptor 400, USA

High Performance Liquid Chromatography	HP Agilent 1100 Series, Germany
High Performance Liquid Chromatography Fluorescence detector	Shimadzu Prominence Fluorescence Detector RF-20A, Japan
HPLC C-18 reverse phase column (5 µm, 4.6 mm/250 mm)	LiChrospher®100 RP-18, Germany
UV- Spectrophotometer	Shimadzu UV-1800, Japan
Cell culture flasks	Grenier Bio-one, Germany
Cell Culture inserts (Thincert™ 12 well-plate, pore size 1 µm, area 1.13 cm ²)	Grenier Bio-one, Germany
Microplate, 96 well	Grenier Bio-one, Germany
Microplate reader	Molecular Device Versa Max, USA
Polarizing light microscope	Leica, Germany
TEER Reader	Millicell®-ERS, Millipore, USA
Vortex	IKA-MS1 Minishaker, Brasil
Ultrasonic bath	Advantage Lab AL04-12, Sweden
Laminar flow cabin Class 2B	Biolab BHG 2004, Turkey
Deep freezer (-80°C)	Heraeus Kendro HFU 586 Basic, Germany
Deep freezer (-20°C)	Beko, Turkey
Refrigerator (+4°C)	Arçelik, Turkey
Nitrogen gas (purity 99.99%)	Linde, Turkey
Incubator (170 liter)	SANYO MCO-18AIC, Japan
Incubator (49 liter)	SANYO MCO-5AC, Japan

3.2. Chemicals

Chemicals & Properties	Supplier
Poly-ε-Caprolactone (MW: 14,000 - 65,000 - 80,000 Da)	Sigma&Aldrich, USA
Docetaxel (purity ≥97%)	Sigma&Aldrich, China
Acetone (HPLC Grade)	Merck, Germany
Ethanol (HPLC Grade)	Sigma&Aldrich, Germany
Acetonitrile (HPLC Grade)	Sigma &Aldrich, Israel
Dimethylsulfoxide, DMSO	Sigma&Aldrich, UK
Dichloromethane (HPLC Grade)	Sigma&Aldrich, USA
Chitosan (Protasan™ UP G-113, MW <200 kDa, deacetylation degree, 75–90%)	Novamatrix /FMC Biopolymer, Norway
Polyethylene glycol 400 (PEG400)	Fluka, Belgium
Disodium hydrogen phosphate, Na ₂ HPO ₄	Merck, Germany
Potassium dihydrogen phosphate, KH ₂ PO ₄	Riedel-de Haën, Germany
Potassium chloride, KCl	Horasan Kimya, Turkey
Sodium chloride, NaCl	Riedel-de Haën, Germany
Sodium Hydroxide, NaOH	Merck, Germany
Sodium sulfide, Na ₂ S	Riedel-de Haën, Germany
Tween® 80	Sigma&Aldrich, UK
Span® 80	Sigma&Aldrich, USA
Miglyol 812N	Condea Chemie, Germany
Nile Red	Sigma, UK
Hydrochloric Acid 36-38%, HCl	J.T. Baker, Netherland
Sulfuric acid 95-98%, H ₂ SO ₄	Merck, Germany
Hank's Balanced Salt Solution (HBSS)	Biochrom AG, Germany

Penicilin/Streptomycin (1000 U/1000 µg/mL)	Sigma, USA
Thiazolyl Blue Tetrazolium Bromide	Sigma, USA
Trypane Blue	Sigma, USA
L-Glutamine	Sigma, USA
Dulbecco's modification of Eagle's medium (DMEM)	Biochrom AG, Germany
Dialysis Tubing Cellulose Membrane (avg. flat width 25 mm, 1.0 in., typical MW cut-off 14.000 Da)	Sigma&Aldrich, Germany

3.3. Biological Materials

Biological Materials	Supplier
Fetal Bovine Serum	Sigma, Germany
Trypsin-EDTA	Sigma, Germany
Mucin from Porcine Stomach Type II	Sigma&Aldrich, USA
Human Caucasian colon adenocarcinoma cell line (Caco-2)	American Type Culture Collection (ATCC), USA
Human breast cancer cell line (MCF-7)	American Type Culture Collection (ATCC), USA
Mouse fibroblast cell line (L929)	American Type Culture Collection (ATCC), USA
Swiss albino male mice (5-6 weeks, 20-28 gr)	H.U. Faculty of Medicine, Department of Pharmacology, Animal Lab

4. METHOD

4.1. Analytical Method Validation

Analytical method validation is the procedure of verification of an analytical technique to demonstrate the suitability for the intended purpose [65]. An analytical method explains all the steps necessary to perform an analysis. The objective of analytical measurements is to get reliable, accurate and consistent data in which a validated analytical method has critical role to achieve this goal [152, 153]. In practice, an experimental study can be designed with simultaneously considered appropriate validation characteristics including linearity, accuracy, precision, sensitivity and specificity. Those characteristics together provide an overall knowledge of capability of the analytical method [154]. The explanations for validation characteristics are given below in details.

All detections of docetaxel in this thesis, analytical method validation and *in vitro* quantification studies were performed by HP Agilent 1100 High Performance Liquid Chromatography (HPLC) reverse phase system. HPLC conditions are given below in Table 4.1:

Table 4.1. HPLC conditions

Stationary Phase	LiChrospher®100 RP-18 (5 µm) 4.6 mm/250 mm
Mobile Phase	Acetonitrile:Water (ACN:H ₂ O 50:50 v/v)
Detector	Diode Array Detector (DAD)
Injection Volume	50 µL
Flow rate	1 mL/min
Column temperature	25°C
Wavelength	230 nm
Time of analysis	15 min
Retention Time	12.474 min

4.1.1. Linearity

The linearity of an analytical procedure is defined as *“its ability to obtain test results which are directly proportional to the concentration of analyte in the sample within a given range”* according to The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines [152].

The linearity can be evaluated by visual inspection of data (plot of signals as a function of analyte concentration or content) and if there is a linear relationship, it should be demonstrated by an appropriate statistical method such as regression analysis or least squares. Linearity is established by a series of at least 5 concentrations. The response is directly proportional to the analyte concentrations [153, 154]. The resultant linear regression equation should be in the form of “ $y=ax+b$ ”, where “ a ” is the slope and “ b ” is the y-intercept. The correlation coefficient (R^2) is an indicator of the correlation strength between the signals (y-axis) and the analyte concentration (x-axis). The value of correlation coefficient is closer to “1” when a strong linear correlation between variables in regression analysis.

Briefly, the linearity of analytical method was evaluated by constructing a calibration curve with eight concentration points (1, 5, 10, 25, 50, 100, 150, 200 $\mu\text{g/mL}$). The curve was obtained by plotting known concentration values against peak area obtained from HPLC. The regression equation and correlation coefficient was evaluated for the linearity.

Construction of Calibration Curve for Docetaxel

Construction of calibration curve is the key factor of method validation. For this reason, drawing a calibration curve is the most important step of the analytical method. In order to do this, a stock solution of Docetaxel was prepared at a concentration of 200 $\mu\text{g/mL}$ (w/v) by dissolving 2 mg of docetaxel in 10 mL of acetonitrile. By using this stock solution, a series of 8 different dilution concentrations were prepared in a range of 1 - 200 $\mu\text{g/mL}$. All dilutions were performed with acetonitrile. The measurement of docetaxel was performed at 230 nm. The mobile phase was acetonitrile:water (50:50 v/v). The flow rate was set at 1.0 mL/min. The values of peak were noted and areas under curve (AUC) from

HPLC analysis were used to plot calibration curve (Table 4.2) and the equation of that curve was then maintained.

Table 4.2. Dilution concentrations of docetaxel and values of AUC

Concentration ($\mu\text{g/mL}$)	AUC
0	0,00
1	15,70
5	83,70
10	155,90
25	624,60
50	1306,40
100	2842,90
150	4443,20
200	5958,10

4.1.2. Accuracy

Accuracy term defines the closeness of conformity between a value found and the accepted reference value. Accuracy can be obtained by comparing the test results with the results of reference method or measured value of a sample with known concentrations with true value. Since, accuracy parameter measures the effectiveness of sample preparation, extreme care should be taken to repeat the actual preparation as closely as possible.

The accuracy of analytical method was evaluated by preparing three concentration points as low, mean and high (5, 50 and 150 $\mu\text{g/mL}$) in the range of calibration. Six batches were prepared freshly for each concentration point. Percent recovery (R%) and coefficient of variation (CV) were calculated for each data for evaluation of accuracy.

4.1.3. Precision

The precision of an analytical procedure defines the closeness of conformity between a series of measurements obtained from replicative sampling of the same sample under normal assay conditions. The precision parameter can be

summarized as degree of scatter. The precision is usually expressed as the variance, standard deviation or coefficient of variation of sequential measurements.

Precision is considered in three measurements; repeatability, reproducibility and intermediate precision.

Repeatability

Repeatability refers to the precision under the same operating conditions over a short period of time. It was evaluated by preparing a solution at 100 µg/mL concentration and measuring sequentially 6 times under the same conditions. The peak data was used to evaluate mean, standard deviation (SD) and coefficient of variation (CV), which were used to determine assay precision.

Reproducibility

Reproducibility refers to the precision between laboratories. It was evaluated by preparing six batches of solution at 100 µg/mL concentration from the same stock solution and measuring under the same conditions. The peak data was used to evaluate mean, standard deviation (SD) and coefficient of variation, which were used to determine assay precision.

Intermediate Precision

Intermediate precision refers to within-laboratories variations such as different days, different analysts and different equipment. It was evaluated by preparing three batches of solution at 100 µg/mL concentration at sequential three days and measuring under the same conditions. The peak data was used to evaluate mean, standard deviation (SD) and coefficient of variation (CV), which were used to determine assay precision.

4.1.4. Sensitivity

The detection limit of an analytical procedure is the minimum amount of analyte in a sample which can be detected, but not necessarily quantitated. However, the quantitation limit is the minimum amount of analyte in a sample which can be quantified with proper precision and accuracy under defined experimental

conditions. 5 µg/mL was selected as the minimum concentration for the sensitivity parameter. At this concentration point, the linear regions were defined and according to signal to noise ratio, LOD and LOQ values were determined. The signal to noise ratio was accepted 3:1 as detection limit and 10:1 as minimum detectable quantification limit.

4.1.5. Specificity/Selectivity

Specificity or selectivity is the ability to distinguish the analyte response in the presence of other components in the medium. Specificity of analytical method was evaluated by obtaining chromatograms of other excipients used during preparation of nanocapsules and any possible interference was investigated. The chromatograms of PCL, CS and PEG were obtained and according to the response, retention time of each of them was evaluated under the same conditions.

4.1.6. Stability

Stability parameter compares the test results obtained from the stored sample against the freshly prepared sample. It is a measure of bias in test results generated throughout a predefined time period using a single sample.

The short-term stability was evaluated by preparing a solution at 100 µg/mL concentration and measured at sequential three days and at defined time points (0., 24. and 48. h) under the same conditions. The peak data was used to evaluate mean, standard deviation (SD) and coefficient of variation (CV), which was used to determine assay precision

4.2. Nanoprecipitation Method

In the course of preparation of nanocapsules, the nanoprecipitation method was used. Nanoprecipitation is a spontaneous process based mainly on the deposition of polymer between interfacial portion of organic and aqueous phases, and spontaneous formation of nanoparticles while diffusion of organic phase into aqueous phase. In systems containing soluble surfactant, the surface tension gradients are created between compressed and uncompressed areas [76]. This liquid surface motion is called Marangoni effect.

Nanoprecipitation is an easy method having several easy steps. In this method generally the polymer is dissolved in a suitable volatile solvent, and the active agent is dispersed in or dissolved in this polymeric solution. The resultant solution or dispersion is then rapidly mixed with an aqueous continuous phase that contains a surfactant to form discrete small droplets. Nanocapsule formation is sudden and spontaneous at the time of mixing. After organic solvent is evaporated at the water/air interface nanocapsules can be recovered by simple centrifugation. Particle formation mechanism is crucial for size distribution and morphology, which in turn determines the delivery system behaviour during encapsulation and release. This method will be explained in detail in the relevant sections.

4.3. Pre-Formulation Studies

Pre-formulation parameters were studied to determine the effects of formulation parameters (polymer concentration, polymer molecular weight, type of surfactant, surfactant concentration, amount of oil, ratio of organic phase to aqueous phase and stirring rate) on particle characteristics such as particle size (mean diameter), polydispersity index (PDI) and zeta potential. For this purpose, preparation conditions were altered during preparation of empty nanocapsules. First, all variables, which can be used during optimization of formulation, were derived from the literature related to nanocapsule formation [72, 155-160] Working parameters and variables are given below in Table 4.3.

Table 4.3. The parameters belonging to pre-formulation study based on literature

Formulation Parameters	Variables			
Polymer concentration (w/v)	0.05%	0.1%	0.5%	1%
Polymer Mw (g/mol)	14000	65000	80000	-
Type of surfactant	Tween® 80 (hydrophilic)	Span® 80 (lipophilic)	-	-
Surfactant concentration (v/v)	0.1%	0.5%	1%	-
Oil Concentration (v/v)	0.3%	1%	3%	-
OP:AP (v/v)	1:1	1:2	1:4	-
Stirring rate (rpm)	350	700	-	-

4.3.1. Optimization of PCL Molecular Weight and Concentration

For defining the effects of polymer molecular weight, poly- ϵ -caprolactone having different molecular weights; 14000 Da, 65000 Da and 80000 Da, were investigated. By using these three types of polymer, three different batches of nanocapsule formulations were prepared and further studied.

In addition, for optimization of polymer concentration, different concentrations of polymer (0.05%, 0.1%, 0.5%, 1% w/v) were used. Nanocapsule formulations were prepared by using nanoprecipitation method. Briefly, PCL and 50 μ L oil (Miglyol 812N) were dissolved in 5 mL acetone in different concentrations of polymer ranging from 0.05% to 1% forming the organic phase. This organic solution was added rapidly into freshly prepared aqueous phase containing 10 mL of ultrapure water and 50 μ L surfactant (Tween® 80) with a dropwise fashion under magnetic stirring. After nanocapsules were hardened, organic solvent in solution was evaporated under vacuum at 60°C for 20 min. Nanocapsule dispersion was centrifuged at 6000 rpm at room temperature for 15 min to obtain hardened

nanocapsules. All prepared batches were investigated for particle size, polydispersity index and zeta potential to obtain desired specifications.

4.3.2. Optimization of Surfactant Type and Concentration

For defining the type of surfactant, two different surfactants were chosen with different characteristics; Tween® 80 as hydrophilic and Span® 80 as lipophilic surfactant. Simply, Tweens are ethoxylated Spans, they are hydrophilic in nature and are soluble or dispersible in water. The solubility of Tweens in aqueous solutions increases with the degree of ethoxylation. Due to the fact that Spans and Tweens are nonionic surfactants; they offer many advantages over ionic surfactants including increased stability, formulating flexibility and wider compatibility [161]. Both surfactants were used in nanocapsule formulation steps in different batches.

For optimization of surfactant concentration, different concentrations of surfactant (0.1%, 0.5%, 1% w/v) were used. Nanocapsule formulations were prepared by using nanoprecipitation method. Briefly, 5 mg PCL and 50 µL oil (Miglyol 812N) were added in 5 mL acetone forming the organic phase. This organic solution was added in a dropwise fashion into freshly prepared aqueous phase containing 10 mL of ultrapure water and surfactant (Tween® 80) in different concentrations ranging from 0.1% to 1% under magnetic stirring. After nanocapsules were hardened, organic solvent in solution was evaporated under vacuum at 60°C for 20 min. Nanocapsule dispersion was centrifuged at 6000 rpm at room temperature for 15 min to obtain hardened nanocapsules. All prepared batches were investigated for particle size, polydispersity index and zeta potential to obtain desired final specifications.

4.3.3. Optimization of Oil Concentration

For preparation of nanocapsule formulations, Miglyol 812N was selected as oil since its neutral, stable, biocompatible, non-toxic characteristics and liquid state at room temperature. Miglyol 812N is an oily liquid having many properties and application areas such as penetration enhancer, drug carrier, solvent, oral, dermal or intravenous applications [162].

For optimization of oil concentration, three different percent concentrations of oil (0.3, 1 and 3 % v/v) were selected. Nanocapsule formulations were prepared by using nanoprecipitation method. Briefly, 5 mg PCL and different concentrations of an oil (Miglyol 812N) were added in 5 mL acetone forming the organic phase. This organic solution was added in a dropwise fashion into freshly prepared aqueous phase containing 10 mL of ultrapure water and 50 μ L surfactant (Tween® 80) under magnetic stirring. After nanocapsules were hardened, organic solvent in solution was evaporated under vacuum at 60°C for 20 min. Nanocapsule dispersion was centrifuged at 6000 rpm at room temperature for 15 min to obtain hardened nanocapsules. All prepared batches were investigated for particle size, polydispersity index and zeta potential to obtain desired specifications.

4.3.4. Optimization of the Ratio of Organic Phase to Aqueous Phase Volume

For optimization of the ratio of organic phase volume to aqueous phase volume, three different ratios (1:1, 1:2, 1:4) were prepared. Nanocapsule formulations were prepared by using nanoprecipitation method. Briefly, 5 mg PCL and different concentration of oil (Miglyol 812N) was added in 5 mL acetone forming the organic phase. This organic solution was added in a dropwise fashion into different amount of freshly prepared aqueous phase (5 mL, 10 mL and 20 mL) containing ultrapure water and 0.5% (v/v) surfactant (Tween® 80) under magnetic stirring. After nanocapsules were hardened, organic solvent in solution was evaporated under vacuum at 60°C for 20 min. Nanocapsule dispersion was centrifuged at 6000 rpm at room temperature for 15 min to obtain hardened nanocapsules. All prepared batches were investigated for particle size, polydispersity index and zeta potential to obtain desired specifications.

4.3.5. Optimization of Stirring Rate

For optimization of stirring rate during nanocapsule formation step, slow (350 rpm) and moderate (700 rpm) were selected. Nanocapsule formulations were prepared by using nanoprecipitation method. Briefly, 5 mg PCL and different concentration of oil (Miglyol 812N) was added in 5 mL acetone forming the organic phase. This organic solution was added in a dropwise fashion into freshly prepared aqueous phase containing 10 mL of ultrapure water and 50 μ L surfactant (Tween® 80)

under slow and moderate magnetic stirring in different batches. After nanocapsules were hardened, organic solvent in solution was evaporated under vacuum at 60⁰C for 20 min. Nanocapsule dispersion was centrifuged at 6000 rpm at room temperature for 15 min to obtain hardened nanocapsules. All prepared batches were investigated for particle size, polydispersity index and zeta potential to obtain desired specifications.

4.3.6. Optimization of Surface Modification

Foreign materials and pathogens are defeated by the humoral immune system acting as self/non-self mechanism. Once that foreign material identified by opsonins, it unites with macrophages. Phagocytosis mechanism is then initiated by phagocytic cells in the reticuloendothelial system (RES) of the liver and spleen. This phenomenon is known as opsonization. The fate of engulfed material is intracellular digestion. The ones that undigested particles terminate in the liver and spleen and after all excreted from the body [163, 164].

It has been declared that nanometer size is not a determining factor for phagocytosis. Rather the rate and extent of removal from the circulation is determined by the physicochemical characteristics of the materials used. It was shown that macrophages recognize nanoparticles according to the number of opsonins attached to the surface. Large, hydrophobic or charged materials are eliminated from circulation quickly by the phagocytic mechanism and are digested in the liver, kidney or spleen [163, 165].

Precisely at this point, surface modification of nanoparticles allows being stealth against RES due to giving ability to change surface charge and being more like other blood constituents. In literature, it has been demonstrated that neutrally charged particles opsonized less than charged particles [166]. Moreover, zwitterionic molecules bearing positive and negative charges both on the same surfaces show great potential to have a long-circulating half-life [167]. In this thesis, it was decided to modify the surface of nanocapsules with PEG or alternatively with chitosan (CS).

Phagocytosis is shown to be higher for hydrophobic particles in comparison with hydrophilic particles in *in vitro* and *in vivo* studies. Nanoparticles were also retained by blood proteins according to the surface chemistry, surface net charge

and ligand binding. For the elimination of attractive forces between hydrophobic substances and circulatory proteins, modification of the hydrophobic surface with suitable materials would be a convenient way [163, 168]. Polyethylene glycol (PEG) is a hydrophilic molecule, inert having no net charge, biocompatible, biodegradable and irresponsible to plasma proteins [163]. Qingguo Xu et al. (2015) demonstrated that PEG suggests the nanoparticles to stay stealth in circulation and leads to the rapid diffusion into mucus after densely coating of the surface as shown in Figure 4.1 [169].

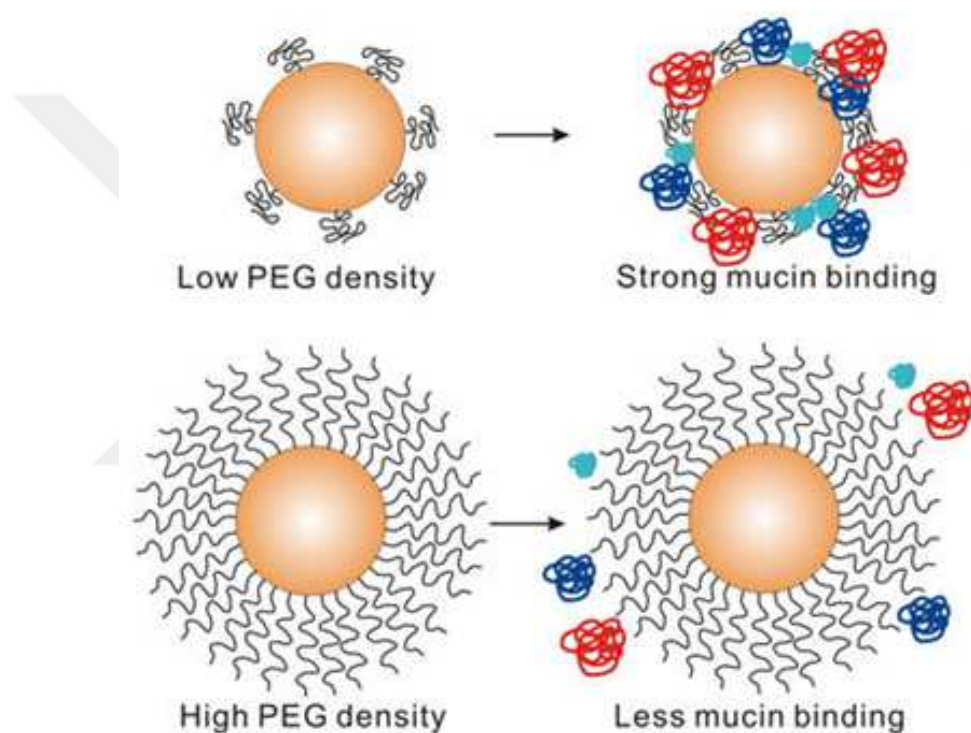


Figure 4.1. Demonstration of mucus interaction degree with PEG modification [169].

The surface chemical modification of nanoparticles with PEG reduces the adsorption potential of circulatory proteins over the surface of particle, prevents platelet adhesion and thrombolytic mechanism by steric repulsion forces [170, 171].

Chitosan is a hydrophilic cationic polymer, having high biodegradability, biocompatibility and low toxicity. Its mucoadhesive properties allow even the large

molecules to penetrate through mucosal surfaces by overcoming the mucociliary clearance. Thus, the residential time of nanoparticles in circulation would increase [170, 172, 173].

Chitosan is a molecule that facilitate the molecular uptake through the cell membrane and its absorption enhancing potential improves the bioavailability of active molecules inside the nanoparticles [174]. CS and its derivatives are known to be safe and effective absorption enhancers for mucosal transport. This effect is actualized by the opening of intercellular tight junctions causing the paracellular transport of active agents through mucosal surfaces [175].

Surface modification could be made simultaneously during spontaneous formation of nanocapsules. In nanoprecipitation method, surface modification was also spontaneous and could be obtained by adding modification material to the aqueous phase or organic phase according to the chemical composition of material.

For this purpose, four different concentrations of PEG (1%, 2% 5%, and 10%, v/v) were added into the aqueous phase prior to the addition of organic phase for the formation of nanocapsules spontaneously. Likewise, CS batches were prepared by adding CS to aqueous phase at four different concentrations (0.01%, 0.025%, 0.05% and 0.1% w/v) at the same way (Table 4.4).

Table 4.4. The parameters for surface modification

	Variables			
PEG (v/v)	%1	%2	%5	%10
CS (w/v)	0.01%	0.025%	0.05%	0.1%

4.4. Formulation Studies

Pre-formulation studies were performed to obtain convenient conditions and the optimal values of variables in formulations. According to these results gathered from pre-formulation studies, a preparation technique of anionic and cationic DOC-

loaded PCL nanocapsules was constructed. The main values of variables are listed below in Table 4.5.

Table 4.5. Optimal parameters of formulations determined through pre-formulation studies

Variables	Values
Polymer concentration (w/v)	0.1%
Polymer Mw	65000 Da
Surfactant type	Tween® 80 (hydrophilic)
Surfactant concentration (w/v)	0.5%
Oil concentration (w/v)	1%
OP:AP	1:2
Stirring rate	700 rpm (moderate)
PEG (v/v)	%5
CS (w/v)	0.025%

Principally, 5 mg of PCL was dissolved in 5 mL of heated acetone in the presence of 50 μ L of Miglyol 812N. Then, 0.5 mg docetaxel was added and stirred until it dissolved. Synchronously, 10 mL of ultrapure water containing 50 μ L of Tween® 80 was prepared. Into this aqueous phase, 500 μ L of PEG or 2.5 mg of CS was added to obtain a modified surface of nanocapsules. Afterwards, 5 mL of organic phase was rapidly added into 10 mL of aqueous phase in a dropwise fashion under moderate magnetic stirring at 700 rpm. Spontaneous nanocapsule formation is shown in Figure 4.2.



Figure 4.2. Spontaneous formation of nanocapsules with nanoprecipitation

After 20 min of stirring the nanocapsules were hardened and the organic solvent was evaporated at 60⁰C and 150 rpm for 20 min under vacuum. Nanocapsules in dispersion form were derived at a final volume of 10 mL of water. Finally, the prepared nanocapsules were recovered by centrifugation at 6000 rpm for 15 min at room temperature and freeze dried for further use.

4.5. Characterization of Nanocapsules

4.5.1. Particle Size Analysis and Size Distribution

The average size and polydispersity index (Pdl) of blank and docetaxel-loaded various nanocapsule formulations (anionic and cationic) were determined by a particle size analyzer (MALVERN Zetasizer Nano ZS Series, UK) at room temperature at a scattering angle of 173⁰ using disposable capillary cells. All measurements were performed in triplicate.

4.5.2. Zeta Potential

The surface charge (mV) of blank and docetaxel-loaded various nanocapsule formulations (anionic and cationic) were measured by a particle size analyzer (MALVERN Zetasizer Nano ZS Series, UK) at room temperature at an angle of 12⁰ using disposable capillary cells. All measurements were performed in triplicate.

4.5.3. Morphological Imaging

The morphology and surface properties were investigated by Scanning Electron Microscopy (SEM - QUANTA 400F Field Emission SEM, resolution is 1.2 nm, USA). SEM micrographs were obtained after coating with 100 Å gold-palladium alloy under vacuum at 5 to 20 kV. Imaging studies were purchased from Central Laboratory in Middle East Technical University.

4.5.4. Short-Term Physical Stability

Short-term physical stability was fixed as any change that could occur in mean diameter and size distribution of nanocapsules after drug loading during 2 weeks storage time. For investigating short-term stability of DOC-loaded nanocapsules, formulations were prepared as described before. All formulations were incubated at 4°C during defined time period. At specific time points (0., 6. and 14. days), size and size distribution measurements were performed by a particle size analyzer at room temperature in triplicate.

4.5.5. Drug Encapsulation Efficiency

Drug encapsulation efficiency of the prepared nanocapsules were quantified by previously validated HPLC method at a wavelength of $\lambda = 230$ nm for docetaxel.

Briefly, nanocapsules prepared by previously mentioned method were collected and excess amount of polymer and unbound drug were separated from the nanocapsules by centrifugation at 6000 rpm for 15 min. Then the supernatant containing nanocapsules in dispersion form was freeze-dried for 48 h at freeze dryer (Heto PowerDry PL 3000, Denmark). Afterwards, freeze-dried nanocapsules in powder form were weighed and dissolved in 300 µl of DCM 2700 µl of acetonitrile mixture (totally 3 ml) for complete resolution. DCM was evaporated under nitrogen gas. The sample was then analyzed by HPLC for docetaxel content. Area under curve (AUC) was determined for each formulation batch by a validated HPLC method and peak area was used to calculate the amount of drug loaded.

Drug encapsulation efficiency (Eqn1) and loading efficiency (Eqn2) were expressed as associated drug in terms of percent (%) loading and calculated by using the following formulas:

$$\text{Associated Drug (\%)} = \frac{\text{Experimental drug loading } (\mu\text{g})}{\text{Theoretical drug used initially } (\mu\text{g})} \times 100 \quad \text{Eqn 1}$$

$$\text{Entrapment Efficiency (\%)} = \frac{\text{Calculated drug quantity } (\mu\text{g})}{\text{Polymer quantity } (\mu\text{g})} \times 100 \quad \text{Eqn 2}$$

4.6. Physical Stability of Nanocapsules in Simulated Gastrointestinal Fluids

Since it was aimed to develop an oral nanocapsule formulation, physical stability is an important factor while GI emptying [176]. The biological media could affect nanocapsule structure and all other properties such as nanoparticle net charge and adhesivity. Because of this reason, nanocapsules had to protect their stability while passing through the GI tract until they reached to intestine to be absorbed. The stability of nanocapsules ensured the stability of drug inside providing accurate release kinetics. For this purpose, simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared and all experimental batches were investigated for particle size, polydispersity index and zeta potential to evaluate the stability of nanocapsule formulations in GI conditions.

Briefly, simulated gastric fluid was prepared by dissolving 100 mg of NaCl in 50 mL deionized water containing 0.35 mL concentrated HCl. Afterwards, 9 mL of SGF (pH 1.2) and 1 mL nanocapsule dispersion were mixed and then incubated while gastric emptying for 2 h (Figure 4.3). Simulated intestinal fluid was prepared by dissolving 680.5 mg monobasic potassium phosphate (KH_2PO_4) and 89.6 mg of NaOH in 100 mL deionized water (dH_2O). In the case of SIF (pH 6.8), 9 mL of SIF and 1 mL nanocapsule dispersion were mixed and the incubated for 6 h, during

intestinal passage. All batches were investigated for particle size, polydispersity index and zeta potential in triplicate.

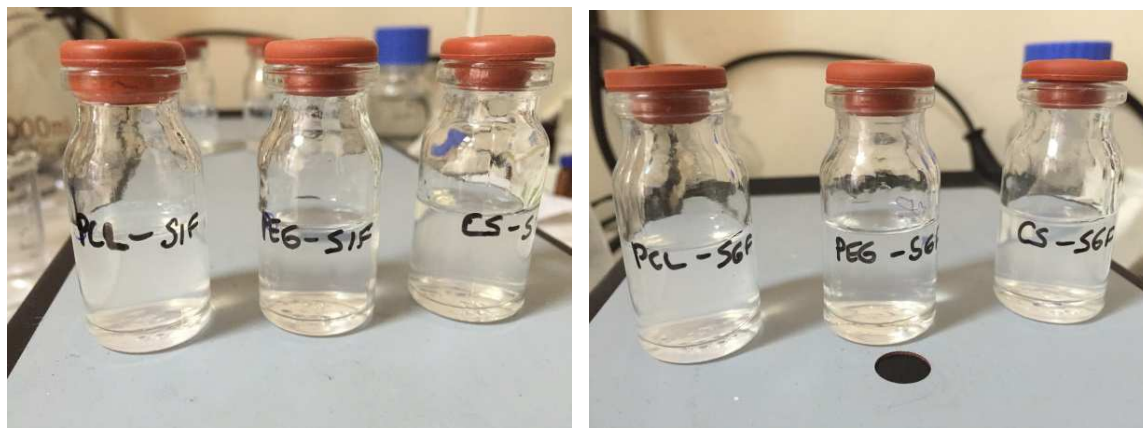


Figure 4.3. Incubation of nanocapsule formulations in a) simulated intestinal fluid and for 6 h, b) simulated gastric fluid for 2 h

4.7. Interaction with Mucus

4.7.1. Turbidimetric Measurement

While oral nanocapsule formulation is passing through the GI tract, nanocapsules will interact with the walls of GI canal which is covered with mucus. Since the main protein constituent of mucus is mucin, the mucoadhesion actualized between mucin and the nanocapsules. In order to investigate the interaction between mucin and nanocapsules, and to determine the potential differences on physical characteristics of nanocapsules, turbidity showing the interaction between mucin and nanocapsules was measured by UV spectrophotometer (Shimadzu UV-1800, Japan) at 650 nm as defined in the literature [3, 92].

For this purpose, mucin dispersion was prepared by dissolving 0.08% (w/v) mucin powder in ultrapure water and stirred for at least 6 h to complete blending. Then, excess and residue mucin was discarded by centrifugation at 7500 rpm for 20 min. Mucin and nanocapsule dispersions were blended at a ratio of 1:2 and vortexed for 1 min. All batches of samples were prepared by the same way and turbidity was measured by UV spectrophotometry at 650 nm at specific time points (0, 30, 60 min). Mucin dispersion itself was used as reference. The measurements were performed in triplicate.

4.7.2. Characterization of Nanocapsules Final Properties in Mucus

Potential differences on physical characteristics of nanocapsules caused by the interaction between mucin and nanocapsules were defined as the differences on size, Pdl and zeta potential. For determining the physical changes in nanocapsule features, the mucin dispersion was prepared by dissolving 0.08% (w/v) mucin powder in ultrapure water and stirred for at least 6 h for complete blending. Then, excess and residue mucin was discarded by centrifugation at 7500 rpm for 20 min. Mucin and nanocapsule dispersions were blended at a ratio of 1:2 and vortexed for 1 min. All batches of samples were prepared by the same method and size, Pdl and zeta potential were measured by a particle size analyzer (MALVERN Zetasizer Nano ZS Series, UK) at specific time points (0, 30, 60 min) at an angle of 12° and room temperature. The measurements were performed in triplicate.

4.8. In Vitro Drug Release

In vitro release of docetaxel was performed in phosphate buffered saline (PBS, pH 7.4) solution through a dialysis membrane (typical Mw cut-off 14000 Da) under sink conditions at 37°C.

2 mL of various drug-loaded nanocapsule formulations (PCL, PEG-PCL, CS-PCL; in dispersion form) were placed in a dialysis bag. The different batches of formulations in dialysis membrane were placed in 50 mL of freshly prepared phosphate buffered saline solution (pH 7.4) containing 0.1 % v/v Tween® 80. Batches were placed in a water bath with horizontal shaker at 100 rpm and 37 °C. At specific time points (0.5, 1, 1.5, 2, 4, 6, 24, 48, 72 h), 1 mL of sample was taken and replaced by fresh release medium. All samples were analysed by a validated HPLC method. The cumulative release percentage at each time point was calculated and then release profile was obtained. The experiments were performed in triplicate.

As an indicative of the fate of an oral nanocapsule formulation, *in vitro* release at pH 1.2 (gastric conditions) was also investigated. Simulated gastric fluid was prepared same as indicated in Section 4.6 for mimicking the gastric conditions at pH1.2 as release medium. Batches and experimental conditions were designed same as indicated above. At specific time points (0.5, 1, 1.5, 2, 4 h), 1 mL of

sample was replaced by fresh release medium and HPLC analysis was performed. The cumulative release percentage at each time point was calculated and then release profile was obtained. The experiments were performed in triplicate.

4.9. Cell Culture Studies

4.9.1. Cytotoxicity

The *in vitro* reliability of nanocapsule formulations were performed with L929 mouse fibroblast cell line, which was a standard method for cytotoxic determination defined in American Pharmacopoeia (USP). L929 mouse fibroblast cell line was obtained from the American Type Culture Collection, USA. The cytotoxicity of empty nanocapsules was determined by inhibition of cell growth. Cytotoxicity study was performed in Hacettepe University, Faculty of Pharmacy, Department of Pharmaceutical Technology Cell Culture Lab.

After thawing, L929 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 100 units/ml of penicillin and 100 µg/mL of streptomycin in 25 cm² flasks and incubated at 37°C and 5% CO₂. Confluent cell monolayers in exponentially growing phase were used in cytotoxicity experiments. Cells were seeded in a 96 well-plate at a density of 5x10³ cells/100 µL per well in DMEM and incubated for 24 h under the conditions of 95% air, 5% CO₂ at 90% relative humidity at 37°C. Then, the medium was replaced by empty nanocapsule formulations (PCL, PEG-PCL, CS-PCL) beginning from the first well and serially diluted to obtain different concentrations of nanocapsule solutions (1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128) (Figure 4.4). The sham-operated batch was non-treated. Cells were incubated for 48 h. The cell viability was determined by MTT cytotoxicity assay.



Figure 4.4. a) Dilutions and b) application of diluted nanocapsule formulations to L929 cells

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide; thiazolyl blue) is a water soluble tetrazolium salt which active cells convert to an insoluble purple formazan by cleavage of the tetrazolium ring by dehydrogenase enzymes. This water insoluble formazan is solubilized and measured spectrophotometrically yielding absorbance as a function of concentration of converted dye. An increase or decrease in cell number results in a change in the amount of formazan formed, indicating the degree of cytotoxicity caused by the test material [177].

After incubation for 48 h, 25 μL of MTT solution was added to each well and incubated at 37°C for another 4 h in CO_2 incubator (SANYO MCO-18AIC, Japan). Following, MTT solution was replaced with 200 μL of DMSO and the absorbance of the samples were measured by a microplate reader at 450 nm, 490 nm and 570 nm. All experiments were performed in triplicates.

The cytotoxic effect of blank nanocapsules was expressed in terms of percent (%) cell viability and calculated by using the following formula:

$$\text{Cell Viability (\%)} = \frac{\text{Mean absorbance of treated cells}}{\text{Mean absorbance of non - treated cells}} \times 100 \quad \text{Eqn 3}$$

4.9.2. Anticancer Efficacy

The *in vitro* efficacy study of DOC-loaded nanocapsule formulations were performed with MCF-7 human breast carcinoma cell line. MCF-7 human breast carcinoma cell line was obtained from the American Type Culture Collection, USA. Anticancer efficacy of docetaxel-loaded nanocapsule formulations was determined by inhibition of cell growth. Anticancer efficacy study was performed in Hacettepe University, Faculty of Pharmacy, Department of Pharmaceutical Technology Cell Culture Lab.

Primarily, MCF-7 cells were cultured in DMEM containing 10% FBS, 100 units/ml of penicillin and 100 µg/mL of streptomycin in 25cm² flasks and incubated at 37⁰C and 5% CO₂. Confluent cell monolayers in exponentially growing phase were used in experiments. Cells were seeded in a 96 well-plate at a density of 1x10⁴ cells/100 µL per well in DMEM and incubated for 24 h under the conditions of 95% air, 5% CO₂ at 90% relative humidity at 37⁰C for the attachment of cells to plate surface. Then, the medium was replaced by DOC-loaded nanocapsule formulations (PCL, PEG-PCL, CS-PCL) beginning from the first well and serially diluted with DMEM to obtain different concentrations of nanocapsule solutions (1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128). In comparison with nanocapsule formulations, an equivalent dose of docetaxel solution (0.81 mg/mL) in 1% DMSO was used as negative control and added to cell culture at dilution rates of (1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128). 1% DMSO in the same dilution rates was also used as control for docetaxel solution. The sham-operated batch was non-treated. Cells were incubated for 72 h. The cell viability was determined by MTT cytotoxicity assay (Figure 4.5).

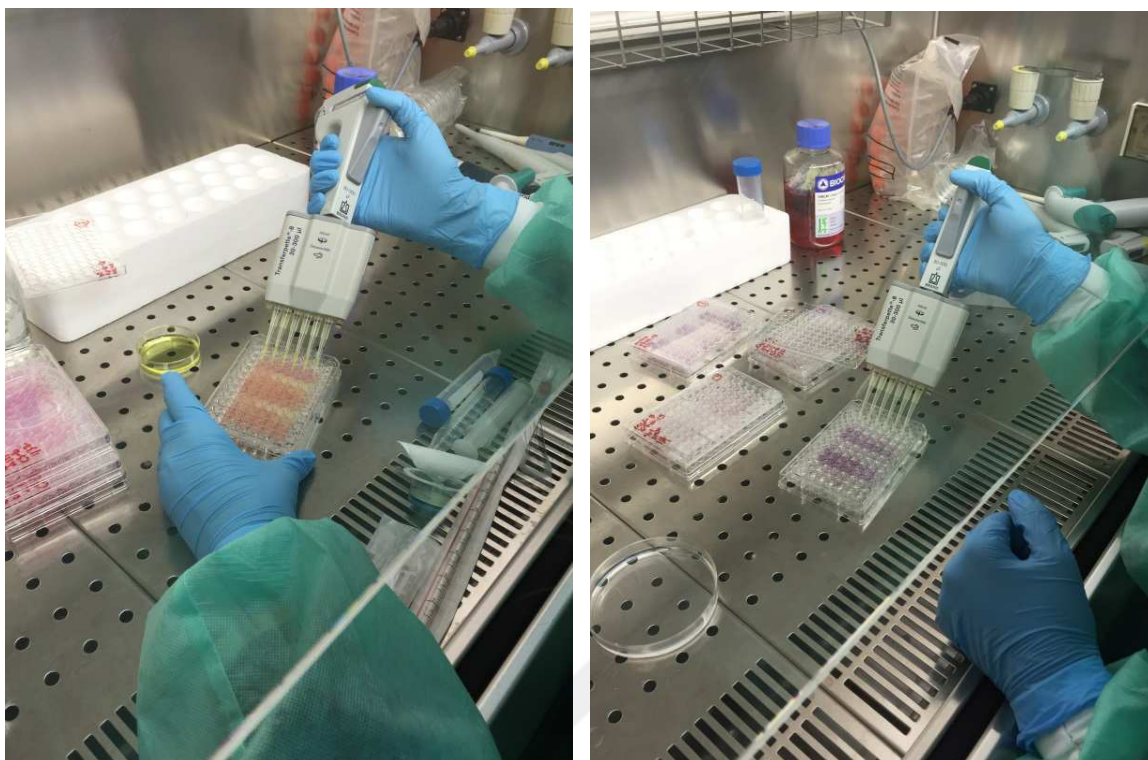


Figure 4.5. Treatment of MCF-7 cells with MTT solution before ELISA measurement

After incubation, 25 μL of MTT solution was added to each well and incubated at 37°C for another 4 h in CO_2 incubator. Following, MTT solution was replaced with 200 μL of DMSO and the absorbance of the samples were measured by a microplate reader at 450 nm, 490 nm and 570 nm. All experiments were performed in triplicates.

The anticancer efficacy of three nanocapsule formulations was expressed in terms of percent (%) cell viability and calculated by using the formula given above in Eqn3..

4.9.3. Permeability Through Caco-2 Monolayer

The Caco-2 cell monolayer system is a well-known *in vitro* cell line and a standart model for human intestinal absorption for evaluating permeability of drug candidates [178]. Caco-2 Human Caucasian colon adenocarcinoma cell line was obtained from the American Type Culture Collection, USA. Anticancer efficacy study was performed in Hacettepe University, Faculty of Pharmacy, Department of

Pharmaceutical Technology Cell Culture Lab.

Caco-2 cells were cultured on Thincerts™ (polycarbonate membrane filters, transparent, pore size 1 µm, area 1.13 cm², Greiner bio-one, Germany, 12 well plates were used) at a density of 1.2×10^5 cell/well. For every other day through the next 21 days, the culture medium was added to the apical side of filter so as to 500 µl and to the basolateral side as to 1 mL (Figure 4.6). Culture flasks were incubated under the conditions of 95% air, 5% CO₂ at 90% relative humidity at 37°C.



Figure 4.6. Treatment of Caco-2 cells through 21 days

After cells were cultivated, they were harvested with the transport buffer containing Hank's balanced salt solution (HBSS) containing 10 mM of n-(2-hydroxyethyl) piperazine-n-(2-ethanesulfonic acid) (HEPES) at pH 7.4 and transepithelial electrical resistance (TEER) was measured by TEER reader (Millicell®-ERS-2 system, Millipore Corp., Bedford, MA). TEER values were used to determine the integrity of the cell monolayers. All the TEER values were found over 800 Ω.cm². The concentration of docetaxel inside each formulation was set to 50 µg/mL by

diluting with HBSS. In comparison with DOC-loaded nanocapsule formulations (PCL, PEG-PCL, CS-PCL), an equivalent dose of docetaxel was dissolved in 1.5% DMSO (v/v) and diluted with HBSS as negative control. 500 µl of each formulation and docetaxel solution were applied to the apical side and 1 mL of HBSS to the basolateral side of membrane filter. The cells were incubated for 3 h at 37°C in a horizontal shaker at 30 rpm. After 3 h, samples obtained from basolateral side of filter were analysed by HPLC. The control batch was non-treated with either formulation or docetaxel solution and negative control batch contained no cell. All sham-operated batches were incubated with HBSS on both sides of membrane filter. All experiments were performed in triplicates.

Apparent permeability coefficient (P_{app}) was calculated according to following formula and expressed in centimeters per second:

$$P_{app} = \frac{dq}{dt} \times \frac{1}{A C_0} = \frac{V_{basolateral} \times C_{basolateral}}{A C_0 t} \quad \text{Eqn 4}$$

where;

dq/dt is the permeability rate which is the amount of drug filtered through the membrane in unit time,

C_0 is the initial drug concentration applied to the apical side,

A is the surface area of the membrane,

t is the unit time in seconds.

4.10. *In Vivo* Studies

In vivo studies were performed with fluorescent labeled nanocapsule formulations. The luminescence was measured with fluorescent HPLC at 25 °C at an emission wavelength of $\lambda_{em} = 630$ nm and at an excitation wavelength of $\lambda_{ex} = 559$ nm. The fluorescent marker in *in vivo* studies was Nile Red (NR) that was highly fluorescent and hydrophobic dye used in many chemical and biological systems [179, 180].

In vivo studies were performed with Swiss albino male mice (20-28 g and 5-6 weeks). The animals used in this study were a kind gift of Prof. Dr. Alper İskit from H.U. Faculty of Medicine, Department of Pharmacology. All animal experiments were operated with regard to the internationally accepted principles of H.U. Ethical Commission on laboratory animal use and care. The ethical permission date and number is 15/12/2016 – 2015/93-03.

For *in vivo* studies, PCL and CS-PCL nanocapsule formulations, which give better *in vitro* results, were included. The animals used were deprived of food for 12 h prior to treatment and operation.

4.10.1. Construction of Calibration Curve for Nile Red

For the purpose of calibration curve construction, a stock solution at a concentration of 0.1 mg/mL (w/v) was prepared. Briefly, 1 mg of a fluorescent dye (nile red) was dissolved in 10 mL ethanol and diluted to obtain desired concentration. By using this stock solution, a series of seven different dilution concentrations were prepared in a range of 0.001-0.1 µg/mL (v/v). All dilutions were performed with ethanol. The fluorescence measurements were performed with Shimadzu Prominence HPLC fluorescence detector. The fluorescence of Nile Red was measured at an emission wavelength of 630 nm and at an excitation wavelength of 559 nm under isocratic conditions. The mobile phase was ethanol:water (95:5 v/v). The flow rate was set at 1.0 mL/min.

The values of fluorescent intensity were noted and the concentration values of NR solutions were used to plot calibration curve (Table 4.6) and the equation of that curve was then maintained.

Table 4.6. Dilution rates of Nile Red and values of fluorescent intensity for plotting the calibration curve

Concentration (µg/ml)	Fluorescent Intensity
0	0
0.001	2.214
0.002	4.207
0.005	5.855
0.01	8.254
0.02	10.075
0.05	11.932
0.1	14.249

4.10.2. Preparation of Nile Red Loaded Nanocapsules

Primarily, for the preparation of Nile Red loaded nanocapsules, a stock NR solution at a concentration of 0.1 mg/mL (w/v) was prepared by dissolving 0.5 mg of a NR in 5 mL acetone.

Following, 3 mg of PCL was dissolved in a mix of 300 µL of NR solution and 2700 µL of acetone in the presence of 30 µL of Miglyol 812N on hot plate. Synchronously, 6 ml of ultrapure water containing 30 µL of Tween® 80 was prepared. Then, 3 ml of organic phase was rapidly added into 6 mL of aqueous phase in a dropwise fashion under moderate magnetic stirring at 700 rpm. After 20 min of stirring the nanocapsules were hardened and the organic solvent was evaporated at 60°C and 150 rpm for 20 min under vacuum. Nanocapsules in dispersion form were derived at a final volume of 6 ml of water. Nanocapsules were recovered by centrifugation at 6000 rpm for 15 min at room temperature and freeze dried for further use.

4.10.3. Encapsulation Efficiency of Nile Red

Previously prepared and freeze-dried NR-loaded nanocapsules weighed and dissolved in 300 µl of DCM and 2700 µl of acetonitrile mixture (totally 3 ml) for complete resolution. DCM was evaporated under nitrogen gas. The peak area was determined by HPLC method with fluorescence detector and used to calculate the

amount of drug loaded. Drug encapsulation efficiency was expressed as associated drug in terms of percent (%) loading and calculated by using the formula (Eqn1) given at Section 4.5.5.

4.10.4. Recovery of Nile Red from GI Tissues

Prior to *in vivo* studies, the intestinal uptake of NR loaded nanocapsules was evaluated by calculating the recovery of NR from intestinal tissue. The recovery calculation was designed on stomach and intestinal segments of Swiss albino male mice.

Primarily, three mice were sacrificed to get stomachs and intestines as shown in Figure 4.7. Every intestine was divided into 8 segments and stomach into 2 pieces. Every two pieces of intestine and one piece of stomach from an animal were bundled for each one of the formulation groups. Freshly prepared NR-loaded nanocapsule formulations were directly administered on intestinal segments as 10 μ L and stomach segments as 20 μ L.



Figure 4.7. a) Recovery group of mice and b) stomach and intestine derived from a mouse

Afterwards, all tissues were added twice as much tissue weights of PBS solution and homogenized with tissue homogenizator (OMNI International Sonic Ruptor 400, USA) at least 4-5 minutes on ice until all tissue was completely homogenized as shown in Figure 4.8.



Figure 4.8. Homogenization of stomach and intestinal segments of experimental groups on ice

The homogenates were then centrifuged for 10 min. at 13000 rpm twice and the supernatant was collected. 50 μ L of each sample was diluted with 10 μ L DCM and 440 μ L DMSO (totally 500 μ L) for intestinal samples and 10 μ L DCM and 190 μ L DMSO (totally 250 μ L) for stomach samples. The fluorescence intensity of each sample was measured with fluorescent HPLC at 25 $^{\circ}$ C at an emission wavelength of $\lambda_{em} = 630$ nm and at an excitation wavelength of $\lambda_{ex} = 559$ nm. The peak area was used to calculate the percentage recovery of NR from intestinal and stomach tissues in order to be used as correction factor in calculations for accurate intestinal uptake in *in vivo* experiment.

4.10.5. Nanocapsule Uptake and Gastrointestinal Absorption Study

Uptake studies were performed on 3 mice for every formulation group. 12 animals were divided into 4 groups for each of PCL, PCL-NR, CS, CS-NR formulation groups, which refer to PCL nanocapsules, NR-loaded PCL nanocapsules, CS-PCL nanocapsules and NR-loaded CS-PCL nanocapsules respectively. The freshly prepared nanocapsule formulations are shown in Figure 4.9.



Figure 4.9. Preparation of empty and NR-loaded nanocapsule formulations for *in vivo* experiments

Every group of mice was administered with 100 μ L of nanocapsule formulation with oral gavage as represented in Figure 4.10. After 30 min incubation time, animals were sacrificed to get stomachs and intestines. Every intestine was divided into 4 segments; stomachs and all pieces of intestine were then weighed. Afterwards, all tissues were added twice as much tissue weights of PBS solution and homogenized with tissue homogenizator at least 6-7 minutes on ice until all tissue was completely homogenized.



Figure 4.10. Application of PCL and CS-PCL nanocapsule formulations to experimental group of mice via oral gavage a) empty nanocapsules b) NR-loaded nanocapsules

The homogenates were then centrifuged for 10 min. at 13000 rpm twice and the supernatant was collected. 50 μL of each sample was diluted with 10 μL DCM and 440 μL DMSO (totally 500 μL). The fluorescence intensity of each sample was measured with HPLC at 25 $^{\circ}\text{C}$ at an emission wavelength of $\lambda_{\text{em}} = 630 \text{ nm}$ and at an excitation wavelength of $\lambda_{\text{ex}} = 559 \text{ nm}$. The peak area was used to calculate the amount of NR detected in stomach or intestinal tissue.

4.11. Statistics

All statistical data analysis were performed using SPSS v22.0 software package (IBM Corp., Armonk, NY, USA). The significance between batches was assessed by one sample t-test and repeated measures test. A p value of less than 0.05 ($p < 0.05$) was considered as evidence of a statistically significant difference [181].

5. RESULTS AND DISCUSSION

5.1. Quantification of Docetaxel with HPLC Analysis

Docetaxel quantification during in vitro experiments performed in this thesis was performed by HP Agilent 1100 High Performance Liquid Chromotography (HPLC) reverse phase system. All detections of docetaxel were performed under HPLC conditions given in Section 4.1. Chromatogram of docetaxel was shown in Figure 5.1. The retention time of docetaxel was found at 12.474 minutes at room temperature.

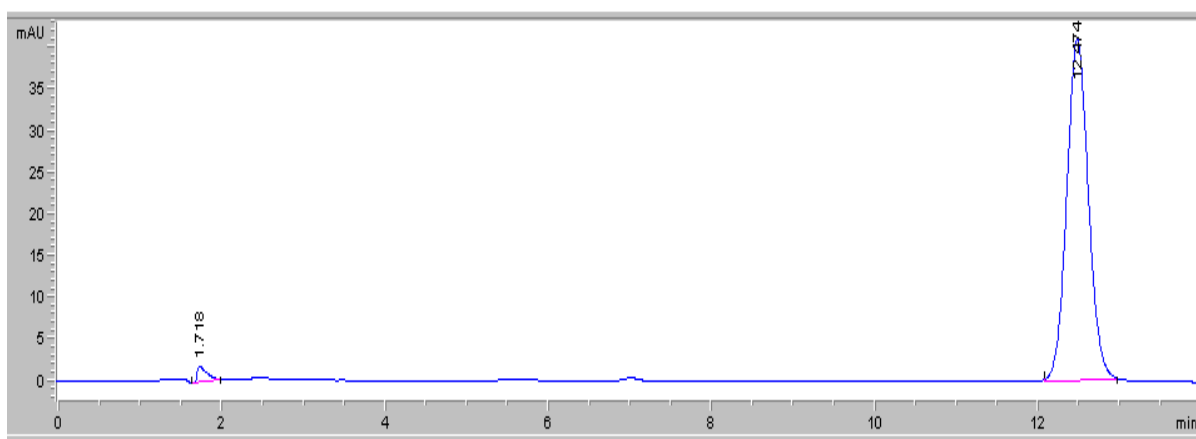


Figure 5.1. HPLC chromatogram of docetaxel in acetonitrile. Retention time was detected at ~12.5 min.

5.2. HPLC Method Validation

Method validation is the procedure for verification of an analytical technique to represent the propriety for requested purpose [65]. For an HPLC method, the appropriate validation parameters include linearity, accuracy, precision, specificity and sensitivity. These parameters together provide an overall knowledge about reliability, exactness and acceptability of results obtained by an analytical method. HPLC method used in this thesis was analytically validated by studying all above mentioned parameters which are given below in details.

5.2.1. Linearity

The linearity of the analytical method refers to the direct proportionality of test results to the analyte concentration. Therefore, the relationship between the analyte concentration and the corresponding response is necessary for reliable quantification. For an appropriate calibration model a minimum of five to eight concentration levels are required [182]. To demonstrate the linearity, a calibration curve with eight concentration points (1, 5, 10, 25, 50, 100, 150, 200 µg/ml) was constructed by dissolving fixed amount of drug in acetonitrile. According to this curve, the regression equation and correlation coefficient was evaluated.

Construction of Calibration Curve

The calibration curve of docetaxel was constructed with the dilution concentrations in a range of 1 to 200 µg/mL as given in Table 4.2 in Section 4.1.1. The AUC values from HPLC analysis were used to plot calibration curve, which was shown in Figure 5.2, and the regression equation of that curve was then maintained. According to the data derived, calibration curve slope was 30.02, intercept point was 90.011 and $R^2 = 0.99905$.

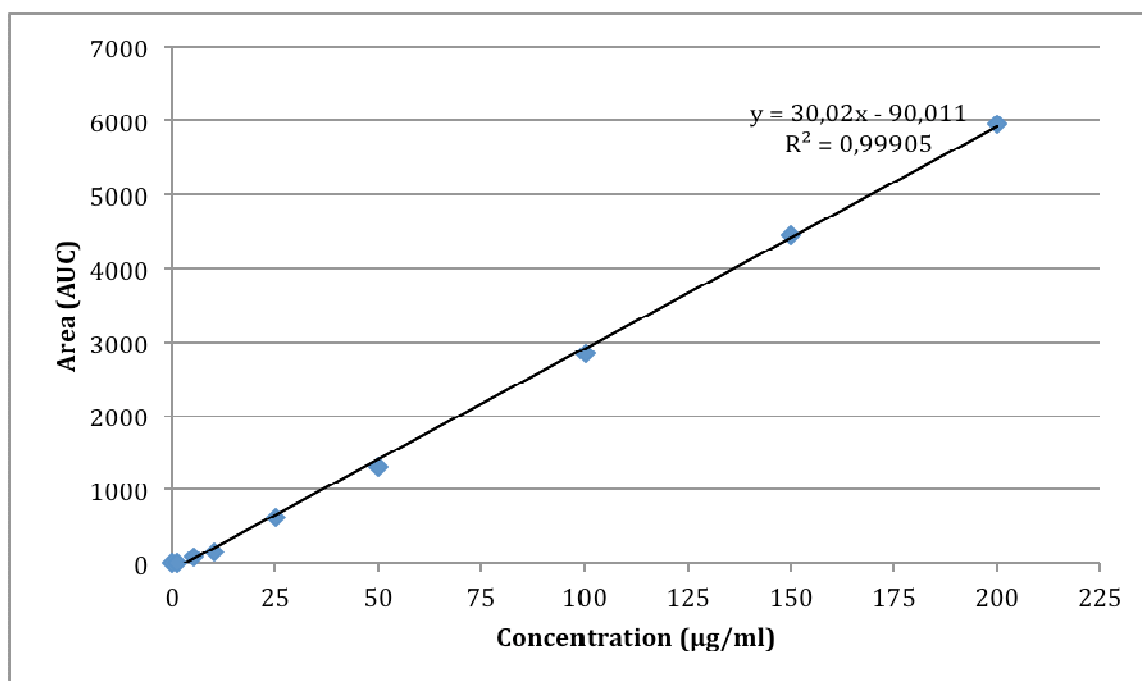


Figure 5.2. Calibration curve and regression equation of docetaxel in acetonitrile (n=3).

The correlation coefficient is admitted sufficient evidence to have a perfect linear calibration when the value is close to unity ($R^2=1$) [183]. Hence, this regression data was linear over studied range with an optimum correlation coefficient of $R^2 = 0.99905$.

5.2.2. Accuracy

Accuracy is a parameter showing coherence of outcomes to the real values. The coherence between the results and a reference value is expressed as percent deviation from reference value [182]. Accuracy of method should be studied with at least three concentrations which are close to the lower and higher levels and near the medium within the range of standard curve [184]. For the purpose of testing the accuracy of analytical method used, three different concentrations, so as to low, medium and high (5, 50 ve 150 µg/mL), were selected in the range of calibration and the six batches at a time were prepared. After HPLC analysis, concentration values were evaluated from the peak data and percent recovery (R%) were calculated for each data. Then, average percent recovery, standard deviation (SD) and coefficient of variation (CV) were computed for each of dilution point (Table 5.1).

Table 5.1. Accuracy data for docetaxel solution (n=3)

Sample \ R%	5 µg/mL	50 µg/mL	150 µg/mL
1	116.20	101.45	100.69
2	116.93	102.03	100.91
3	116.13	100.50	100.83
4	114.46	100.57	100.96
5	116.86	102.39	100.90
6	114.26	100.95	100.67
Average R%	115.81	101.32	100.83
SD	1.07	0.71	0.11
CV (%)	1.14	0.50	0.01

The real concentration (5, 50 or 150 µg/mL) of docetaxel and the calculated docetaxel concentration were compared and recovery percent of each batch were calculated. The recovery values differ in a range of 100-115%, and the variation coefficients of all three concentration points were under 2 %. According to FDA, the mean recovery should be $\pm 2\%$ [185, 186]. Hence, the accuracy values were found to be within the acceptable limits

5.2.3. Precision

Precision is a parameter defining the degree of consistency of analytical method in between sequential measurements. Precision implies the degree of dispersion of a series of separate measurements by means of standard deviation or the coefficient of variation [183]. For this purpose three basic steps; repeatability, reproducibility and intermediate precision were investigated by means of coefficient of variation. All data considering the CV values of three levels of precision parameter proved that the analytical method was precise. The detailed results were given at following sections.

Repeatability

This parameter was investigated by preparing a docetaxel solution at a concentration of 100 µg/mL and analyzing by HPLC sequentially 6 times under the same conditions. The peak data were used to evaluate coefficient of variation (CV), which was used to determine assay precision. Mean, standard deviation (SD) and coefficient of variation (CV) are given in (Table 5.2).

Table 5.2. Data of repeatability for docetaxel (n=3)

Sample	Concentration (µg/mL)
1	97.70
2	95.00
3	97.48
4	97.40
5	97.37
6	97.10
Mean	97.00
SD	0.92
CV (%)	0.84

The variation coefficient was found 0.84% which was lower than 2 %. According to the repeated measurements of the same sample, this result indicated that the analytical method was within acceptable limits of repeatability [154].

Reproducibility

This parameter was investigated by preparing six batches of docetaxel solution at 100 µg/mL concentration from the same stock solution and quantifying by HPLC under the same conditions. The peak data was used to evaluate coefficient of variation (CV), which was used to determine assay precision. Mean, standard deviation (SD) and coefficient of variation (CV) are given in (Table 5.3).

Table 5.3. Data of reproducibility for docetaxel (n=3)

Sample	Concentration (µg/mL)
1	101.09
2	100.36
3	100.88
4	99.67
5	101.84
6	102.33
Mean	101.03
SD	0.88
CV (%)	0.78

The variation coefficient was calculated as 0.78 % which was lower than 2 % indicating that the analytical method was reproducible.

Intermediate Precision

This parameter was investigated by preparing different batches of solution at 100 µg/mL concentration on three consecutive days and measuring by HPLC under the same conditions. The peak data was used to evaluate coefficient of variation (CV), which was used to determine assay precision. Mean, standard deviation (SD) and coefficient of variation (CV) are given in (Table 5.4).

Table 5.4. Data of intermediate precision for docetaxel (n=3)

Sample	Concentration (µg/mL)
1	97.70
2	99.67
3	98.90
Mean	98.76
SD	0.81
CV (%)	0.66

The variation coefficient was found as 0.66% which was lower than 2 %. This result indicated that the analytical method was precise and the difference between the data obtained on different measurement days could be ignored.

5.2.4. Sensitivity

The analytical method sensitivity was determined by the determination of both limit of detection (LOD) and limit of quantification (LOQ). At minimum concentration defined for sensitivity parameter, the linear region on peak analysis was chosen and then the signal/noise ratio was read. The minimum concentration at which signal to noise ratio was 3:1 accepted as LOD and 10:1 is accepted as LOQ, as stated in Section 4.1.4. The results were 0.38 µg/mL and 1.25 µg/mL for LOD and LOQ, respectively. These results were much lower than the selected minimum detectable concentration and thus accepted as suitable for working range and experimental design. Therefore, developed analytical HPLC method for docetaxel quantification was considered to be sensitive enough for the determination of DOC.

5.2.5. Specificity/Selectivity

A selective method should accurately distinguish and perfectly able to measure the response of analyte from all other responses in the presence of interferences [187]. Specificity of analytical method was showed by obtaining chromatograms of other excipients, which were used during preparation of nanocapsules. For this purpose, getting chromatograms and confirming the retention times of other excipients in solution were required. The chromatograms of PCL, CS and PEG were obtained by injection their solutions and reading peak and retention time of these excipients by HPLC under the same conditions. The chromatograms are given below in Figures 5.3, 5.4, 5.5.

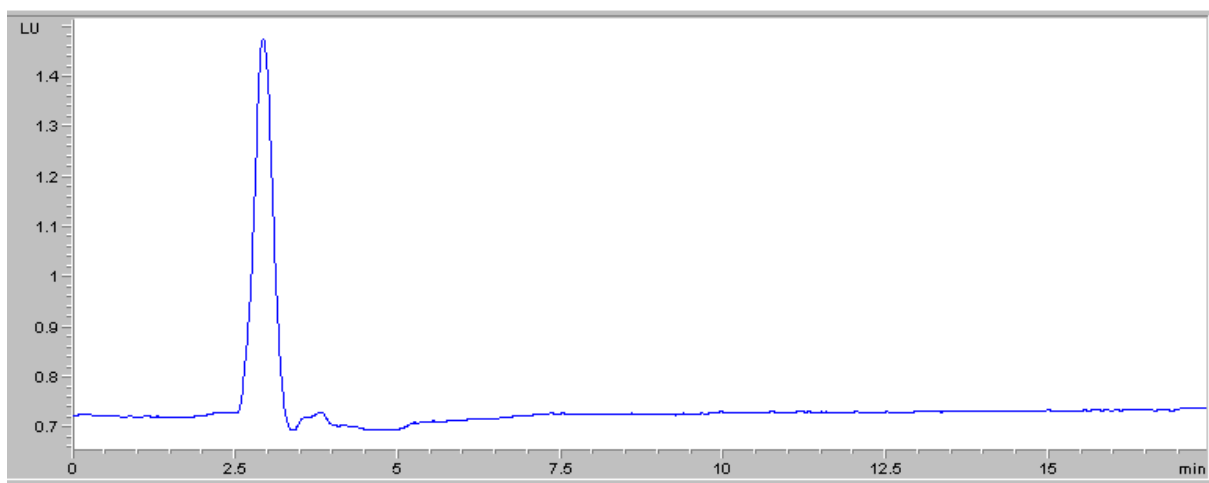


Figure 5.3. Chromatogram of polymer used in nanocapsules, PCL

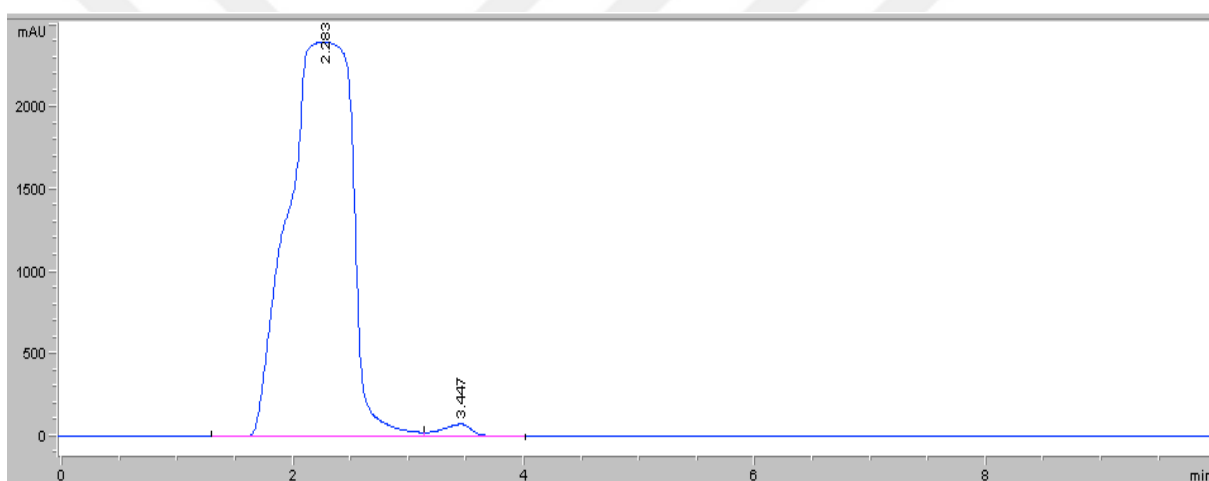


Figure 5.4. Chromatogram of coating polymer for nanocapsules, CS

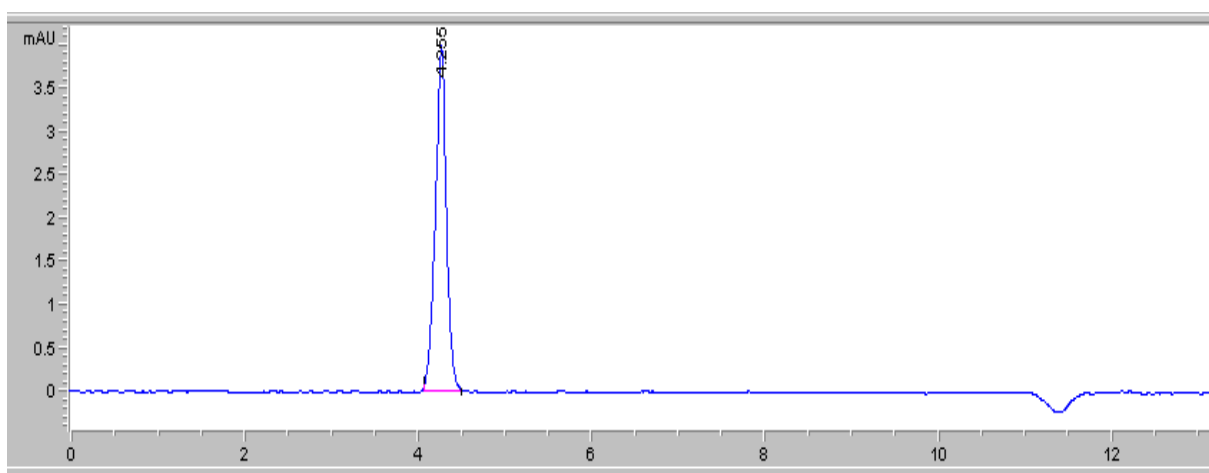


Figure 5.5. Chromatogram of coating polymer for nanocapsules, PEG

There was no overlapping or interference between retention times and the peaks derived from HPLC results of excipients in solution. This result proved that the analytical method was specific only for docetaxel within the nanocapsules studied in this thesis.

5.2.6. Stability

Stability is a parameter showing the existence of a potential difference between the results from stored and fresh samples [188]. For the purpose of justifying the stability of method, solution at 100 µg/mL concentration was prepared and measured by HPLC at three consecutive days and at defined time points (0, 24 and 48 h) under the same conditions. The peak data was used to evaluate coefficient of variation (CV), which was used to determine assay precision. Mean, standard deviation (SD) and coefficient of variation (CV) are given in (Table 5.5).

Table 5.5. Data of stability for docetaxel (n=3)

Day	Concentration (µg/mL)
1	97.70
2	98.15
3	99.03
Mean	98.30
SD	0.55
CV (%)	0.31

The variation coefficient was found as 0.31% which was lower than 2 %. Hence, the stability values were found to be within the acceptable limits and the difference between days could be ignored.

5.3. Pre-Formulation Studies

Pre-formulation studies are required to obtain optimum composition of main constituents and excipients found in a formulation and the conditions of preparation. Moreover, an optimum method for nanocapsule preparation was

constructed based on the information gathered from pre-formulation data. The effects of each factor was evaluated so as to define the primary concerns on critical nanoparticle characteristics compatible with the purpose, such as the particle size, surface charge, stability, release rate, toxicity and bioavailability. Furthermore, bioavailability of oral formulations should be assessed distinctly to endure the harsh conditions encountered during the GI passage.

In pre-formulation studies, all variables and their approximate values derived from literature are defined as given in Table 4.3 at Section 4.3, The detailed results for each variable are given in detail at following sections.

5.3.1. Optimization of PCL Molecular Weight and Concentration

The effect of polymer molecular weight on particle size and size distribution of nanocapsules is shown below in Table 5.6. The mean particle size was significantly increased by the increase of polymer molecular weight ($p=0.03<0.05$) as stated in the literature [189].

Table 5.6. Effect of polymer molecular weight on particle size and Pdl of PCL nanocapsules. Data was given as mean result $\pm$ SD of three different batches.

Polymer Mw (Da)	Mean diameter (nm)	Pdl
14.000	172.4 $\pm$ 0.8	0.099 $\pm$ 0.02
65.000	177.6 $\pm$ 1.7	0.102 $\pm$ 0.02
80.000	207.2 $\pm$ 1.8	0.089 $\pm$ 0.03

As with different polymer concentrations, the molecular weight leads to the organic solution to have higher viscosity. Organic phase disperses easier with low viscosity polymer due to reduced chain size, leading in smaller nanocapsules [190, 191]. At the same time, it is shown that higher molecular weight polymers in particular have higher affinity for adsorbing drugs due to less entropy loss. Hence, this thermodynamic relation explains why higher molecular weight polymers have better stabilization [192]. As a consequence, Mw of 65.000 Da in the medium range was selected for further studies.

The effect of polymer concentration on mean diameter and polydispersity of nanocapsules can be seen in Table 5.7. The particle size was significantly altered by the increase of polymer concentration (0.05%, 0.1%, 0.5%, 1% w/v) ($p=0.005<0.05$) as also reported in literature [193]. Polydispersity index on the other hand remained within acceptable limits and was not affected by increasing polymer percentage within the tested range.

Table 5.7. Effect of polymer concentration on particle size and Pdl of PCL (Mw: 65.000 Da) nanocapsules. Data was given as mean result $\pm$ SD of three different batches

Polymer Concentration (w/v %)	Mean diameter (nm)	Pdl
0.05	167.3 $\pm$ 0.5	0.093 $\pm$ 0.01
0.1	180.3 $\pm$ 1.4	0.096 $\pm$ 0.02
0.5	265.1 $\pm$ 2.9	0.169 $\pm$ 0.02
1	288.5 $\pm$ 2.7	0.192 $\pm$ 0.01

The main reason for the increase in size is the increase of viscosity of organic solution causing poor dispersability of polymer with the aqueous phase. Depending on this phenomenon, the coalescence in concentrated solutions triggers larger particle formation [194]. Moreover, the energy required for the homogenous distribution of particles is insufficient to recover the resistant viscous forces in aqueous phase. This would result in heterogeneous size distribution affecting the Pdl value [195, 196]. Since the average sizes in the case of both 0.5 and 1 (w/v %) are over the desired range and there was no significant difference between ($p=0.125>0.05$), Therefore, the lower polymer concentration values were considered. On the other hand, it is clearly seen that the size does not increase two fold as polymer concentration is doubled. Hence, polymer concentration is more related with capsule stability than particle size since nanocapsules have a thin polymer membranous shell maintaining the stability of both drug and the whole nanocapsule system. According to literature, higher polymer concentration yields more stable thicker shelled nanocapsules [197]. As a consequence, 0.1% of PCL concentration was selected for further studies.

5.3.2. Optimization of Surfactant Type and Concentration

The effect of type of surfactant used on particle size and size distribution of nanocapsules are given in Table 5.8. Two different types of surfactants were used during preparation namely Tween® 80 and Span® 80 representing hydrophilic and lipophilic surfactants respectively. The mean particle size significantly increased in the case of Span® 80 ($p=0<0.05$). This result was attributed to the easier adsorption of Tween® 80 on nanoparticle surface due to its strong hydrophilicity reducing the coagulation, thus forming smaller-sized nanoparticles [198]. As a consequence, Tween® 80 was chosen as surfactant for further studies.

Table 5.8. Effect of surfactant type on particle size and Pdl of PCL nanocapsules. Data was given as mean result $\pm$ SD of three different batches.

Surfactant Type	Mean diameter (nm)	Pdl
Tween® 80	172.4 $\pm$ 0.8	0.099 $\pm$ 0.02
Span® 80	240.5 $\pm$ 1.0	0.155 $\pm$ 0.16

Three different concentration (0.1%, 0.5%, 1%) of Tween® 80 were investigated to see the effect of surfactant concentration on particle size and size distribution of nanocapsules. Results are given below in Table 5.9.

Table 5.9. Effect of surfactant concentration on particle size and Pdl of PCL nanocapsules. Data was given as mean result $\pm$ SD of three different batches.

Surfactant Concentration (w/v %)	Mean diameter (nm)	Pdl
0.1	142.9 $\pm$ 0.8	0.093 $\pm$ 0.01
0.5	172.4 $\pm$ 0.8	0.099 $\pm$ 0.02
1	173.9 $\pm$ 1.3	0.092 $\pm$ 0.01

The mean particle size significantly increased as a result of increase of surfactant concentration ($p=0.04<0.05$). Similar results expressing “the higher the surfactant ratio the greater the particle size” (due to an increase in viscosity of aqueous phase leading to decrease in net shear stress and thus being dominant over

reduction of interfacial tension at OP/AP interface) support these findings on surfactant concentration [199, 200]. Moreover, at lower concentration of surfactant, the system might fail in stabilizing and also aggregation might occur [194]. It was observed in our studies that at a point between 0.5% and 1% the system was stabilized. There was no need to add more stabilizer into the system since the excess stabilizer could behave as agglomerating factor leading to flocculation easily [201]. Also higher concentrations of stabilizer might produce potential toxicity [202]. As a consequence, surfactant concentration was defined as 0.5% of polymer amount for further studies.

5.3.3. Optimization of Oil Concentration

In the structure of nanocapsules Miglyol 812N was used as oil to form the core of the oily nanocapsules. Three different percent concentration (0.3, 1 and 3 v/v %) of Miglyol 812N were investigated to see the effect of oil concentration on particle size and size distribution of nanocapsules. Results are given below in Table 5.10.

Table 5.10. Effect of oil concentration on particle size and Pdl of PCL nanocapsules. Data was given as mean result $\pm$ SD of three different batches.

Oil Concentration (w/v %)	Mean diameter (nm)	Pdl
0.3	172.4 $\pm$ 0.8	0.099 $\pm$ 0.02
1	185.4 $\pm$ 1.5	0.088 $\pm$ 0.02
3	240.5 $\pm$ 1.0	0.155 $\pm$ 0.02

The oil concentration is directly related with the solubilization of drug since the loading capacity of hydrophobic cavity, which was surrounded by a single polymeric membrane, was largely dependent on the oil. Thus, the central core is a very important reservoir for hydrophobic drugs [44]. According to results, the mean particle size increased by the oil concentration ($p=0.011<0.05$) due to increasing viscosity of organic phase [203]. Since it was closely related with the drug loading, oil concentration was defined as 1% of polymer amount for further studies, which suggested a suitable size within desired range.

5.3.4. Optimization of the Ratio of Organic Phase to Aqueous Phase Volume

The ratio of organic phase to the aqueous phase volume was investigated with three different volume ratio (1:1, 1:2 and 1:4) of two phases. The effect of different volume ratio of two phases during nanocapsule preparation was investigated in respect to particle size and size distribution of nanocapsules. Results are given below in Table 5.11.

Table 5.11. Effect of the ratio of organic phase to aqueous phase (OP:AP) on particle size and Pdl of PCL nanocapsules. Data was given as mean result $\pm$ SD of three different batches.

OP:AP	Mean diameter (nm)	Pdl
1:1	177.0 $\pm$ 1.6	0.074 $\pm$ 0.01
1:2	172.4 $\pm$ 0.8	0.099 $\pm$ 0.02
1:4	170.4 $\pm$ 3.1	0.068 $\pm$ 0.02

The mean particle size differs not significantly according to the volume ratio of two phases ($p=0.124>0.05$). The largest nanocapsules were obtained when the ratio is 1 due to the poor phase separation while spontaneous particle formation [193]. However, the mean nanocapsule diameter was close to each other for the ratios 1:2 and 1:4. Several studies performed with 1:2 OP:AP ratio suggest that decreasing organic to aqueous phase ratio results in lower encapsulation efficiency for lipophilic drugs [204, 205]. Hence, the volume ratio of 1:2 was decided as the suitable ratio for further studies within the range.

5.3.5. Optimization of Stirring Rate

The effect of stirring rate on particle size and size distribution of nanocapsules is shown in Table 5.12. Nanocapsules were prepared at two different stirring rates (350rpm, 700 rpm) while mixing organic phase to the aqueous phase. The effect of slow or moderate stirring while mixing of organic phase into the aqueous phase was investigated in respect to particle size and size distribution of nanocapsules. The mean particle size significantly decreased by the increase of stirring rate ($p=0.006<0.05$) conforming the results in literature [206, 207]. This size difference

can be explained by the droplet break-up phenomenon leading to smaller nanoparticles [208]. As a result, all nanocapsules were prepared at 700 rpm in further studies.

Table 5.12. Effect of stirring rate on particle size and Pdl of PCL nanocapsules. Data was given as mean result $\pm$ SD of three different batches.

Stirring Rate	Mean diameter (nm)	Pdl
350	209.7 $\pm$ 1.8	0.094 $\pm$ 0.02
700	185.4 $\pm$ 1.5	0.088 $\pm$ 0.02

5.3.6. Optimization of Surface Modification

Surface modification helps to operate surface properties chemically or physically to improve biocompatibility and also functionalization. Moreover, by surface coating or adsorbing, injectable nanoparticles might be gained stealth characteristics leading to effective transportation of its cargo to destination site in a safe and stable manner. In the case of PEG modification, the nanocapsule surface becomes slightly negative but close to neutral range, whereas CS causes the surface of nanocapsules to become more cationic due to different molecular charges of PEG and CS. Neutral modification of particle surface with a nonionic polymer, PEG, forms a barrier layer on particle surface leading to steric prevention and escape from RES due to lower plasma protein adsorption. Likewise, cationic modification facilitates electrostatic interaction with negatively charged cell membrane resulting in subsequent cellular internalization [209, 210]. Moreover, surface modification is an important parameter for oral delivery. Stability in GI fluids and interaction with mucus for absorption process are main concern of surface modification for oral delivery [211]. A suitable surface modification of nanoparticles together with the small size provides protection from destructive forces and P-gp efflux from intestinal epithelium or cytochrome P450 elimination in the first-pass in GI passage. Moreover, CS and its derivatives are known to be effective mucosal absorption enhancers through intestinal epithelial [175]. Hence, the bioavailability of drug molecules through intestinal epithelium increases with an appropriate surface modification [23, 25].

In this thesis, the surfaces of nanocapsules were modified by addition of PEG and CS into the solution during nanocapsule preparation as described in Section 4.3.6. The percentage of PEG and CS was defined according to similar studies in literature [212, 213]. The effect of PEG and CS concentrations after surface modification was investigated by measuring average size and size distribution of nanocapsules. The results of study are given in Table 5.13.

Table 5.13. Effect of surface modification with PEG or CS on particle size and Pdl of PCL nanocapsules. Data was given as mean result $\pm$ SD of three different batches.

Modification Parameters		Mean diameter (nm)	Pdl
PEG (v/v)	1%	146.6 $\pm$ 1.2	0.108 $\pm$ 0.02
	2%	149.5 $\pm$ 3.7	0.108 $\pm$ 0.02
	5%	172.9 $\pm$ 0.9	0.093 $\pm$ 0.03
	10%	257.2 $\pm$ 1.7	0.104 $\pm$ 0.02
CS (w/v)	0.01%	129.9 $\pm$ 1.1	0.129 $\pm$ 0.01
	0.025%	168.4 $\pm$ 2.5	0.191 $\pm$ 0.01
	0.05%	237.7 $\pm$ 1.4	0.251 $\pm$ 0.00
	0.1%	330.1 $\pm$ 3.7	0.209 $\pm$ 0.00

According to results, there was a significant increase in size when 5% PEG ($p=0.012<0.05$) and 0.025% CS ($p=0.021<0.05$) was doubled. Moreover, there was a significant size decrease below 5% PEG concentration ($p=0.006<0.05$) and 0.025% CS concentration ($p=0.016<0.05$). At this point, drug loading efficiency would be the main determining factor. The optimum size range for gathering nanoparticles with desired magnitude was intercepted exactly at above mentioned concentration points (5% PEG and 0.025% CS).

5.4. Formulation Studies

According to data obtained from pre-formulation studies, optimal conditions of nanocapsule preparation method were defined as given in Table 5.14, hence a suitable formulation and preparation technique was constructed. Those results

were used and conducted further studies in the thesis. DOC-loaded PCL, PEG-PCL and CS-PCL nanocapsules were prepared according to these specifications.

Table 5.14. Optimal parameters of formulations determined through pre-formulation studies

Variables	Values
Polymer concentration (w/v)	0.1%
Polymer Mw	65000 Da
Surfactant type	Tween® 80 (hydrophilic)
Surfactant concentration (w/v)	0.5%
Oil concentration (w/v)	1%
OP:AP	1:2
Stirring rate	700 rpm (moderate)
PEG (v/v)	%5
CS (w/v)	0.025%

5.5. Characterization of Nanocapsules

5.5.1. Particle Size Analysis and Size Distribution

The average particle size and polydispersity index of blank and docetaxel-loaded nanocapsule formulations were determined and the results are given below in Table 5.15 and Figure 5.6. According to results, the mean particle size of all DOC-loaded nanocapsule formulations fell into a size range of 180 nm to 210 nm. There was a significant difference between empty and DOC-loaded nanocapsule formulations ($p=0.004<0.05$ for all three formulations). Especially PEG and CS modified nanocapsules were bigger in size because the surface of nanocapsule was known to be adsorbed by modification material resulting the formation of an extra layer on nanocapsule. Moreover, cationic nanocapsule formulation seemed to be larger than anionic and neutral nanocapsules.

Table 5.15. Average diameter in size and polydispersity index of empty and docetaxel loaded nanocapsule formulations. Data was given as mean result $\pm$ SD of three different batches.

Formulation	Mean diameter (nm)	Pdl
PCL-NP	172.4 $\pm$ 0.8	0.099 $\pm$ 0.02
PEG-PCL-NP	172.9 $\pm$ 0.9	0.093 $\pm$ 0.03
CS-PCL-NP	168.4 $\pm$ 2.5	0.191 $\pm$ 0.01
DOC-loaded PCL-NP	184.2 $\pm$ 0.5	0.091 $\pm$ 0.0
DOC-loaded PEG-PCL-NP	200.2 $\pm$ 1.7	0.054 $\pm$ 0.03
DOC-loaded CS-PCL-NP	206.9 $\pm$ 2.9	0.252 $\pm$ 0.01

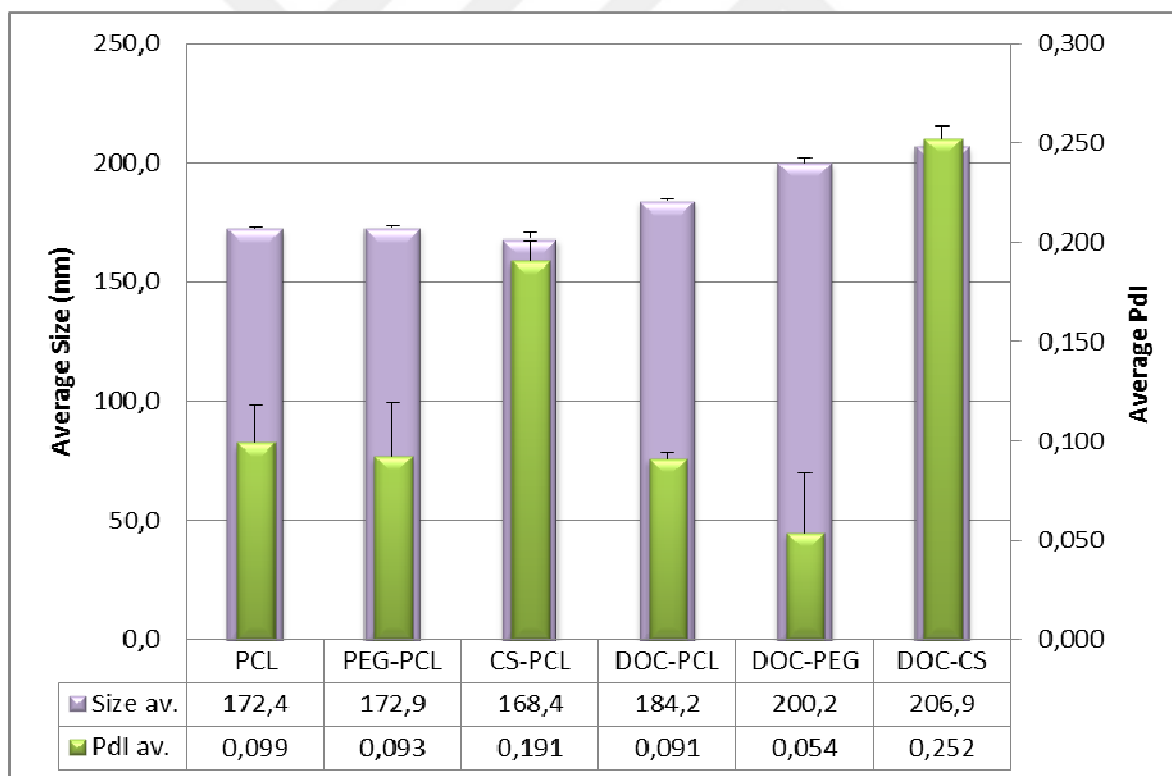


Figure 5.6. Graphical representation of average diameter in size and polydispersity index of empty and docetaxel loaded nanocapsule formulations. Data was given as mean result $\pm$ SD of three different batches.

Particle size is principally vital for the fate of oral nanoparticles. Biological barriers in body such as mucus and RES are related primarily with size and then the surface properties of the particle [11]. Particles should be small enough to diffuse through the mucus and be able to be uptaken by the intestinal cells effectively. According to literature, nanocapsules of 200-300 nm in size range were taken up by intestinal cells [11, 16]. RES is another limiting factor for size determination. After mucus barrier is overwhelmed, nanocapsules were faced with adsorbing blood proteins. Recognition of nanocapsule by opsonins leads their fate towards the end by RES. Particles can be “stealth” in circulation when their sizes are generally smaller than 400 nm [8, 137].

Furthermore, polydispersity index represents the value of variability of particle sizes in a solution. Size distribution of particles is narrow if Pdl is between 0.1 and 0.25, whereas size distribution is broader if Pdl value is greater than an average of 0.6 and is very narrow if the Pdl is smaller than 0.05 [4, 214]. The Pdl values of nanocapsule formulations were said to be in a narrow range, even if there were broader difference in size variation for CS modified particles due to adsorption of CS onto particles, which was acceptable in narrow range.

5.5.2. Zeta Potential

Zeta potential is the electrophoretic mobility measurement of particles under electrical field [215, 216]. Zeta potential is an important parameter giving information about stability or aggregation potential in a solution. A high value of zeta potential represents larger electrostatic repulsion interaction between particles enhancing suspension stability whereas low level of zeta potential indicates low repulsion forces decreasing the dispersion quality that might cause aggregation [217]. Therefore, the colloidal dispersion of nanocapsules having an absolute zeta potential of 30 mV, the stabilization could be obtained by electrical repulsion forces between particles [4]. The nanoparticles having zeta potential from this range would probably be stable due to decreased inter-particle interactions and also agglomeration rate. Opsonization is another barrier for nanoparticles, which was discussed before, related with zeta potential. Charged particles were recognized by opsonins in circulation more and readily eliminated by RES whereas neutral ones were opsonized in lower rates [218].

All nanocapsule formulations were investigated for zeta potential by a particle size analyzer (MALVERN Zetasizer Nano ZS Series, UK) at room temperature using disposable capillary cells. All measurements were performed in triplicate and results are given in Figure 5.7.

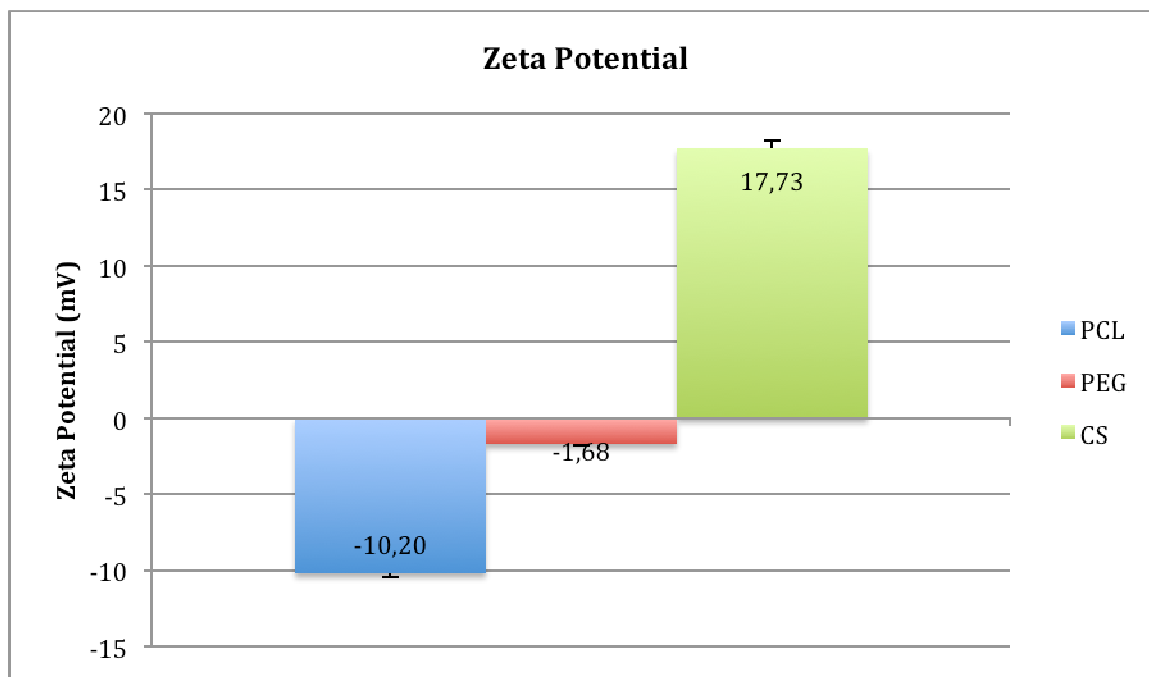


Figure 5.7. Graphical representation of zeta potential values of DOC-loaded nanocapsule formulations (PCL, PEG-PCL, CS-PCL). Data was given as mean result $\pm$ SD of three different batches.

According to results, zeta potential values were in a range of -10 and 18 mV at which nanoparticles could be accepted as stable. There was a significant difference between zeta values of all three nanocapsule formulations ($p < 0.05$). Cationic nanocapsules have clearly higher positive zeta value. The positive zeta potential value of CS-modified nanocapsules was attributed to the presence of amino groups of chitosan [23].

5.5.3. Morphological Imaging

The morphology of nanocapsules was visualized by Scanning Electron Microscopy analysis under vacuum after coating with gold-palladium alloy. SEM images of docetaxel loaded nanocapsules are given in Figure 5.8. Nanocapsules were spherical in shape and had smooth surfaces. Sizes and size distribution values

were quite correlated with the results obtained by size measurements in Section 5.5.1. The cloudy appearance of images resulted from the oily powdered form of nanocapsules which remained even after lyophilization.

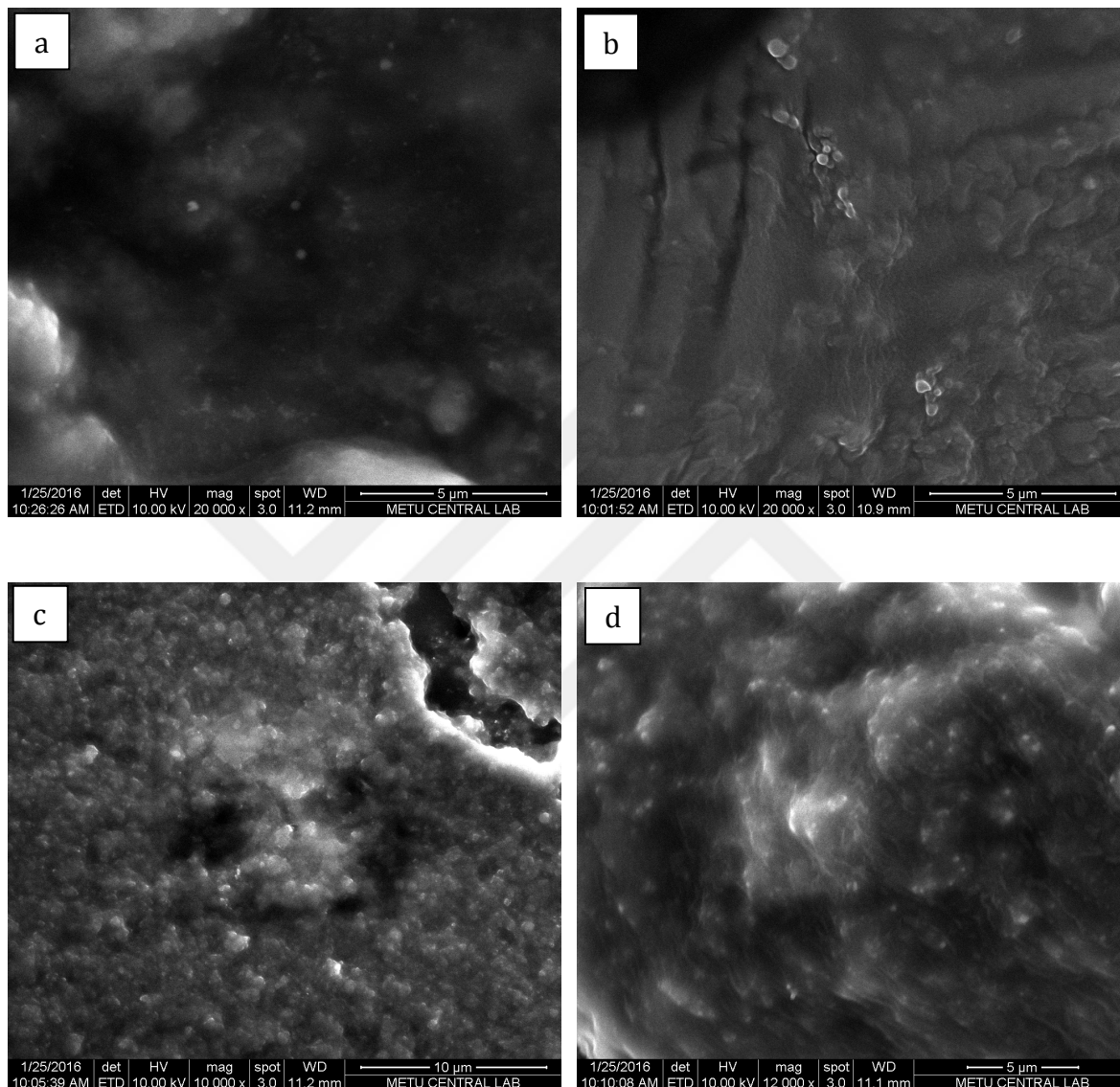


Figure 5.8. SEM images of DOC-loaded nanocapsules (mag.10000 – 20000X): a) DOC-PCL b) DOC-PEG-PCL c-d) DOC-CS-PCL

5.5.4. Short-Term Physical Stability

Physical stability was considered in terms of any change in mean diameter, size distribution and zeta potential of nanocapsules during storage for a short-time period. All DOC-loaded formulations (PCL, PEG-PCL, CS-PCL) were investigated for short-term stability by incubating at 4⁰C for further 14 days. The size and zeta

potential measurements were carried out at 0., 6. and 14. days of incubation period. The physical stability data is given in Table 5.16.

Table 5.16. Short-term physical stability values of three DOC-loaded nanocapsule formulations at 4⁰C for 14 days. Data was given as mean result $\pm$ SD of three different batches.

	Time (day)	PCL	PEG-PCL	CS-PCL
Mean diameter (nm)	0	181.8 $\pm$ 1.9	192.3 $\pm$ 0.7	203.9 $\pm$ 1.4
	6	182.8 $\pm$ 0.5	194.1 $\pm$ 1.9	199.8 $\pm$ 1.3
	14	183.2 $\pm$ 5.8	189.7 $\pm$ 1.0	191.1 $\pm$ 3.2
Pdl	0	0.077 $\pm$ 0.01	0.094 $\pm$ 0.02	0.079 $\pm$ 0.0
	6	0.075 $\pm$ 0.01	0.102 $\pm$ 0.01	0.226 $\pm$ 0.01
	14	0.143 $\pm$ 0.01	0.077 $\pm$ 0.01	0.221 $\pm$ 0.01

As can be seen from data above, there has been no significant change in visual characteristics of nanocapsule solution in short-term period. Average sizes of all nanocapsule formulations were quite coherent within themselves ($p>0.05$) and size distribution of nanocapsules was in a narrow range representing the physical stability of nanocapsule formulations upon storage.

5.5.5. Drug Encapsulation Efficiency

Drug encapsulation efficiency of nanocapsule formulations were determined to quantify the amount of drug encapsulated inside the hydrophobic core of nanocapsule formulations by a validated HPLC method as described in Section 4.1. Formulations were prepared with a known amount (0.5 mg) of hydrophobic drug (DOC). After collection of lyophilized nanocapsules, the drug quantity bound to the nanocapsule was determined and used to calculate the associated drug percentage and drug entrapment efficiency as described in Eqn1 and Eqn2 respectively. The data is given in Table 5.17.

Table 5.17. Associated drug (%) and entrapment efficiency (%) of three DOC-loaded nanocapsule formulations. Data was given as mean result $\pm$ SD of three different batches.

	Associated Drug (%)	Entrapment Efficiency (%)
PCL	44.31 $\pm$ 1.3	17.33 $\pm$ 0.6
PCL-PEG	66.51 $\pm$ 4.4	24.96 $\pm$ 2.1
PCL-CS	51.52 $\pm$ 3.0	18.29 $\pm$ 1.2

According to these results, nanocapsule formulations surface modified with either PEG or CS have higher encapsulation efficiency compared to anionic PCL nanocapsules. Furthermore, neutral nanocapsules of PCL-PEG have highest drug encapsulation potential whereas cationic ones were following them. This encapsulation efficiency difference between neutral and cationic nanocapsules might be attributed to the presence of dense chains of PEG on the surface of nanocapsules. Drug molecules are believed to be entrapped within the oil core of capsule but also bind onto densely adsorbed PEG chains on the surface leading to higher drug loading efficiency. Still docetaxel is believed to be mostly entrapped inside hydrophobic oily core due to its solubility properties, hence cationic CS coating of nanocapsules affects associated drug amount slightly. According to results this difference between anionic and cationic formulations was significance ($p < 0.05$).

According to particle sizes of nanocapsules, our results showed that smaller particles (PCL) have lower encapsulation efficiency. This is due to small-sized nanoparticles have increased surface area, which is leading to faster diffusion of drug with a consequent decreasing in encapsulation efficiency [219, 220]. However, although PCL-CS nanocapsules were bigger in size, the encapsulation efficiency was lower than PCL-PEG nanocapsules. This result was attributed to the charges of molecules. More specifically, docetaxel is a positively charged molecule, which becomes closer in proximity with the anionic molecules when in solution. During nanocapsule formation, docetaxel has higher probability of passive encapsulation within nanoparticle. However, during complexation with

chitosan, which is a cationic molecule, encapsulation of drug will be lower due to repulsion forces between charges of drug and chitosan molecules [221].

5.6. Physical Stability of Nanocapsules in Simulated Gastrointestinal Fluids

Physical stability of the drug delivery system is the key factor during gastrointestinal transition of nanocapsules which introduces harsh external factors such as drastic pH changes, enzymes, bile salts. Maintaining the stability of both nanocapsules and entrapped drug has primary importance in biological media to be able to achieve an optimum nanocarrier after oral administration. The physical stability of nanocapsules and the fact that they maintain integrity upon contact with biorelevant media ensured the stability of therapeutic load of the nanocapsules providing accurate release kinetics and suggesting improved oral absorption. Gastrointestinal tract is a very powerful barrier for drug absorption having many constituents as well as structural and chemical differences within the tract such as abundance of digestive enzymes and presence of pH variations [222, 223]. For an effective oral delivery, this barrier should be overcome to deliver the active ingredient to the intestinal lumen for eventual absorption of the drug and passage to the circulatory system for systemic effect. Since biological fluids affect the physical stability and final properties of oral nanoparticles, *in vitro* stability testing in biorelevant gastric and intestinal fluids has importance and priority for nanoparticle characterization [17, 224].

For this purpose, simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared as described in Section 4.6 and nanoparticles were investigated to detect differences in particle size, polydispersity index and zeta potential after incubation in gastrointestinal conditions.

Table 5.18. Stability values of three DOC-loaded nanocapsule formulations in gastrointestinal fluids at room temperature. (SGF: Simulated gastric fluid, SIF: simulated intestinal fluid.) Data was given as mean result $\pm$ SD of three different batches.

	Simulated Medium	Av. Size (nm)		Pdl		Zeta Potential (mV)	
		Initial	Final	Initial	Final	Initial	Final
PCL	SGF pH 1.2	174.6 $\pm$ 5.6	159.1 $\pm$ 1.2	0.15 $\pm$ 0	0.14 $\pm$ 0	-10.0 $\pm$ 0.3	-0.7 $\pm$ 0.3
	SIF pH 6.8	174.6 $\pm$ 5.6	163.8 $\pm$ 2.5	0.15 $\pm$ 0	0.14 $\pm$ 0	-10.0 $\pm$ 0.3	-4.3 $\pm$ 0.7
PEG-PCL	SGF pH 1.2	201.6 $\pm$ 0.9	218.0 $\pm$ 1.9	0.22 $\pm$ 0	0.29 $\pm$ 0	-4.6 $\pm$ 0.3	0.9 $\pm$ 0.4
	SIF pH 6.8	201.6 $\pm$ 0.9	184.4 $\pm$ 2.5	0.22 $\pm$ 0	0.21 $\pm$ 0	-4.6 $\pm$ 0.3	0.7 $\pm$ 0.3
CS-PCL	SGF pH 1.2	222.2 $\pm$ 1.8	198.8 $\pm$ 2.7	0.19 $\pm$ 0	0.17 $\pm$ 0	15.4 $\pm$ 0.5	17.1 $\pm$ 0.9
	SIF pH 6.8	222.2 $\pm$ 1.8	204.1 $\pm$ 1.3	0.19 $\pm$ 0	0.15 $\pm$ 0	15.4 $\pm$ 0.5	16.3 $\pm$ 0.8

The results in Table 5.18 showed that anionic, neutral and cationic nanocapsules were affected in different degrees in their size and zeta values. The difference in sizes of PCL nanocapsules before and after incubation in GI fluids were not significant ($p=0.148>0.05$ for SGF, $p=0.282>0.05$ for SIF). However, for CS modified nanocapsules, the difference was significant ($p=0.006<0.05$ for SGF, $p=0.002<0.05$ for SIF), whereas for PEG modified nanocapsules the difference was significant in SGF but not significant in SIF ($p=0.024<0.05$ for SGF, $p=0.057>0.05$ for SIF). pH variations through gastrointestinal passage are one of the main concerns of this study because it is difficult to maintain the nanocapsule integrity in different pH conditions [225, 226]. The size and other parameters could fluctuate but as long as the stability and the integrity of particles were maintained as a whole, it could be said that all formulations were quite stable in simulated gastric and intestinal fluids. The integrity of nanocapsule wall was maintained by PCL matrix and modification of the surface supports wall integrity and stability in physiological fluids [227]. An immediate desorption was prevented as can be seen

from results, hence nanocapsules can be said quite stable in GI fluids. Both Figure 5.9 and Figure 5.10 represent the summary of difference between stability values of DOC-loaded nanocapsules in gastrointestinal fluids.

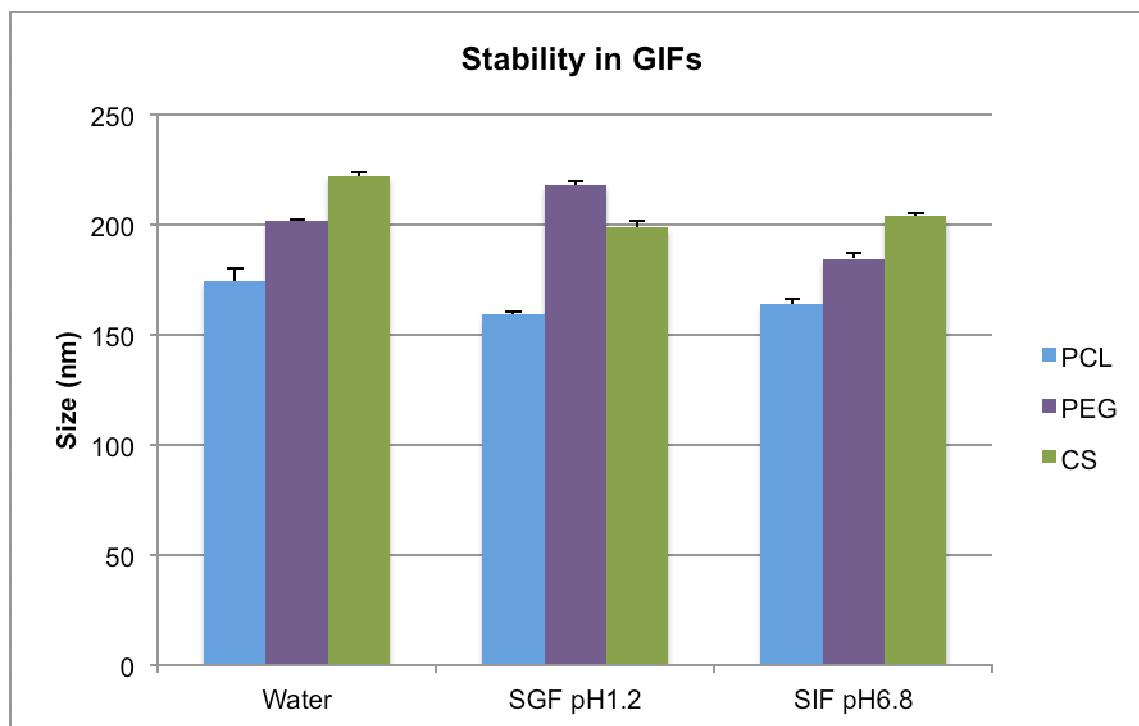


Figure 5.9. Mean diameter values of three DOC-loaded nanocapsule formulations in gastrointestinal fluids at room temperature. (SGF: Simulated gastric fluid, SIF: simulated intestinal fluid.) Data was given as mean result $\pm$ SD of three different batches.

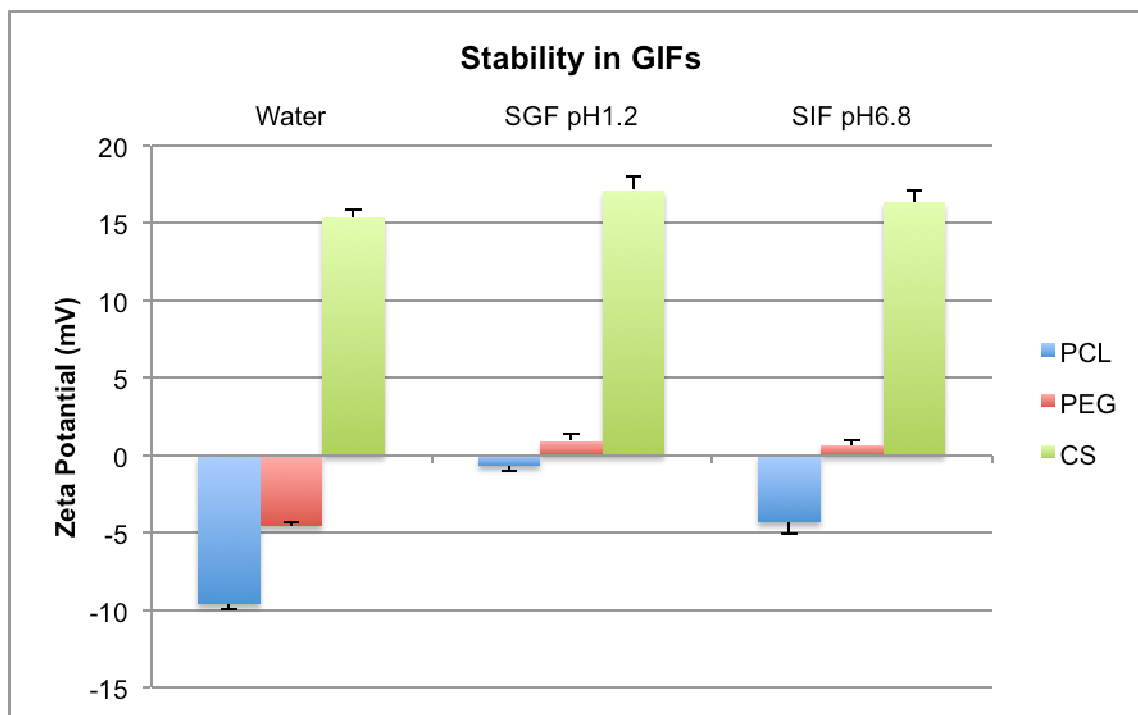


Figure 5.10. Zeta potential values of three DOC-loaded nanocapsule formulations in gastrointestinal fluids at room temperature. (SGF: Simulated gastric fluid, SIF: simulated intestinal fluid.) Data was given as mean result $\pm$ SD of three different batches.

5.7. Interaction with Mucus

Once the nanoparticles pass through the mouth, oesophagus, stomach, they reach absorption site which is the intestinal lumen. This lumen has a thick viscous hydrogel barrier consisting of mucin and glycosaminoglycan chains called mucus-gel layer as described previously. Therefore, the second barrier after the harsh pH and enzymatic conditions of the GI tract that the nanoparticles need to overcome is the mucus barrier to obtain oral absorption of the drug from the intestinal lumen. In order to elucidate the interaction of the nanocapsule formulations with mucus, several studies have been performed to determine the optimum formulation for mucosal drug delivery from the intestine.

5.7.1. Turbidimetric Measurement

Although nanocapsules have been studied for their extensive properties including prolonged residence time in both gastrointestinal tract and body circulation, mucus layer aligning the GI tract behaves like a trap for orally administered nanocapsules. This dilemma is related with viscous and adhesive mucus layer

throughout the GI passage and most drug carrying nanoparticles are readily eliminated by mucus activity through the passage when they come in contact with intestinal epithelial membrane. The presence of inter-molecular interactions such as electrostatic and hydrophobic interactions or hydrogen bonding and nanoparticle, coupled with high degree of physical hindrance between mucus can severely retard penetration [228]. The bioavailability of drug is provided only when nanoparticles are saved and be able to cross the mucus barrier to reach circulation. Hence, nanoparticles are expected to interact with mucus layer first, then pass through mucus and move into circulation. Interaction between mucin, which is main constituent of mucus, and nanoparticles can be provided by different surface modification techniques [222, 229]. Surface modification with PEG or CS was investigated by turbidity measurements with or without mucin as described in Section 4.7.1 to determine the interaction between mucin and nanocapsules of different properties.

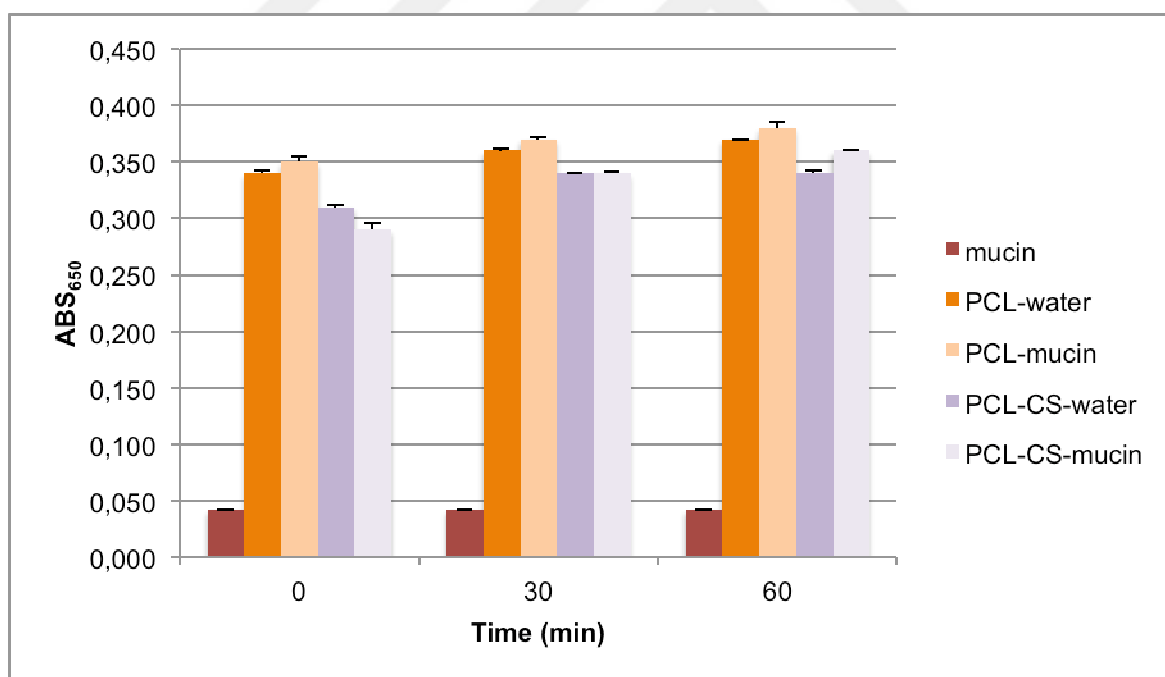


Figure 5.11. Absorbance values of CS-modified nanocapsule formulation in comparison to non-modified nanocapsules at 650 nm after mucin interaction as a function of time. Data was given as mean result $\pm$ SD of three different batches.

Mucin itself was used as reference and which gives an absorbance less than 0.05 which was very low. According to Figure 5.11, the interaction of PCL nanocapsules with mucin was almost stable in time. However, CS interaction increased by the time significantly ($p<0.05$), representing CS modified nanocapsules have higher tendency to form transient bonds as time passing compared to anionic nanocapsules due to chitosan coating. The mucoadhesive properties of CS is a result of the bonding between thiol groups of CS and cysteine-rich subdomains of glycoproteins in mucus [230].

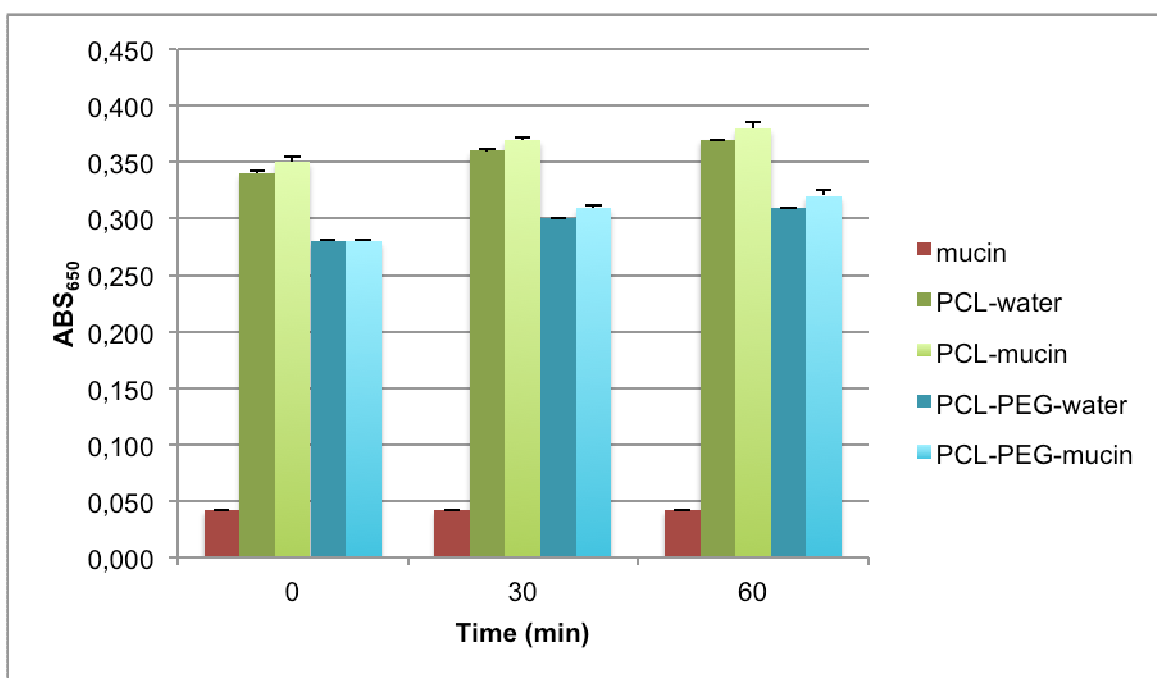


Figure 5.12. Absorbance values of PEG-modified nanocapsule formulation in comparison to non-modified nanocapsules at 650 nm after mucin interaction as a function of time. Data was given as mean result $\pm$ SD of three different batches.

Figure 5.12 represents the interaction potential of nanocapsules after PEG modification. PEG modified nanocapsules gave higher absorbance value in time when mucin was added into nanocapsule solution. The absorbance increase on the other hand was found to be slight, which might result from the hydrophilic nature of PEG and net neutral surface charge of nanoparticles due to mucoadhesivity of PEG molecule which helps in binding to intestinal mucin networks enhancing the penetration through mucin structure [225, 231]. PCL showed no net difference in absorbance values in time however. On the contrary,

surface modified nanocapsules with PEG did not increase in turbidimetry in time in the presence of mucin. Therefore it can be said that PEG and CS chains on the surface of nanocapsules interact slowly with mucin within time. CS modification showed higher absorbance difference than PEG modification, which can be attributed to the cationic character of the CS coated nanocapsules facilitating interaction with mucin [222, 228].

5.7.2. Characterization of Nanocapsule Final Properties in Mucus

During intestinal emptying, the interaction between nanocapsules and the mucus layer covering intestinal epithelial membrane leads to potential differences on physical characteristics of nanocapsules. These possible variations were investigated through changes in mean diameter, size distribution/polydispersity and zeta potential of nanocapsule formulations. Experimental set up was previously defined in Section 4.7.2. Table 5.19 represents the particle size and size distribution values of the nanocapsule formulations before or after contact with mucus. According to these results, any significance size difference was observed for all formulations in presence of mucin after 60 min ($p=0.110>0.05$ for PCL, $p=0.135>0.05$ for PEG-PCL and $p=0.156>0.05$ for CS-PCL). Negligible fluctuations can be attributed to the micro degradation and aggregation situations in solution [232]. Beside, variation in nanoparticle size (Pdl) was found in a narrow distribution range for almost all formulation and in presence of mucin interaction. In the case of CS modification, due to structural and chemical properties of CS, Pdl values were found to be slightly higher than other formulations which is coherent with the polydispersity index results given literature [233, 234].

Table 5.19. Mean particle size and size distribution of three nanocapsule formulations before and after mucin interaction for 30 and 60 minutes. Data was given as mean result $\pm$ SD of three different batches.

	Time (min)	Av. Size (nm)	Pdl
PCL-Water	0	134.1 $\pm$ 1.3	0.175 $\pm$ 0.0
	30	135.6 $\pm$ 1.1	0.173 $\pm$ 0.0
	60	137.2 $\pm$ 1.4	0.211 $\pm$ 0.0
PCL-mucin	0	136.8 $\pm$ 2.0	0.189 $\pm$ 0.0
	30	138.8 $\pm$ 1.9	0.186 $\pm$ 0.0
	60	141.6 $\pm$ 1.7	0.174 $\pm$ 0.0
CS-PCL-Water	0	149.6 $\pm$ 1.8	0.448 $\pm$ 0.1
	30	153.7 $\pm$ 2.5	0.570 $\pm$ 0.1
	60	147.7 $\pm$ 2.1	0.334 $\pm$ 0.1
CS-PCL-mucin	0	142.1 $\pm$ 2.1	0.321 $\pm$ 0.0
	30	136.5 $\pm$ 3.1	0.302 $\pm$ 0.0
	60	143.7 $\pm$ 2.1	0.259 $\pm$ 0.0
PEG-PCL-Water	0	127.8 $\pm$ 1.6	0.229 $\pm$ 0.0
	30	130.5 $\pm$ 1.5	0.221 $\pm$ 0.0
	60	131.7 $\pm$ 1.6	0.189 $\pm$ 0.0
PEG-PCL-mucin	0	126.5 $\pm$ 2.6	0.218 $\pm$ 0.0
	30	131.9 $\pm$ 2.1	0.221 $\pm$ 0.0
	60	132.0 $\pm$ 2.2	0.206 $\pm$ 0.0

Moreover, Table 5.20 indicates the change in zeta potential values of nanocapsules in contact with mucin-negative or mucin-positive environments after 60 min. Mucin dispersion itself has a net negative charge with a zeta potential value of -8.14 ± 0.02 mV. Mucin interaction with nanocapsules was represented by the variation in surface potential values for all formulations in consistence with that of mucin. However, the potential of CS-modified nanocapsules, which have strong positive charge, decreased compared to the potential of PEG-modified and non-modified nanocapsules. This observed change in zeta potential is not significant ($p=0.14$ for non-modified NP, $p=0.05$ for PEG-modified NP, $p=0.14$ for CS-

modified NP). The strong interaction between CS and mucin is totally related with the interaction of opposite charges [23, 229, 235-237]. Moreover, the interaction was attributed to the penetration of PEG chains covering surface of nanocarrier into the mucin structure [235]. However, the minimum variation in results of PEG-modified nanocapsules could be explained by the neutral characteristics as well as the potential of maintaining the stability of nanocarrier by PEG chains [238]. Additionally, anionic nanocapsules showed no net significant difference in zeta potential ($p=0.142>0.05$). Therefore, the results of turbidity study seen in Figures 5.11 and 5.12 were well correlated with zeta potential measurements in Table 5.20.

Table 5.20. Zeta potential values of three nanocapsule formulations before and after mucin interaction. Data was given as mean result $\pm$ SD of three different batches.

	Zeta Potential (mV) Mucin (-)	Zeta Potential (mV) Mucin (+)
mucin	-8.14 $\pm$ 0.02	
PCL	-7.13 $\pm$ 0.0	-11.3 $\pm$ 0.01
CS-PCL	17.3 $\pm$ 0.0	10.94 $\pm$ 0.0
PEG-PCL	-2.32 $\pm$ 0.0	-2.70 $\pm$ 0.0

5.8. *In Vitro* Drug Release

In vitro release studies of docetaxel from nanocapsule formulations were performed in two different media; phosphate buffered saline (PBS, pH 7.4) solution and simulated gastric fluid (SGF, pH 1.2) using a dialysis method under sink conditions at 37°C as described in Section 4.8.

The cumulative release representation in PBS (pH 7.4) is given in Figure 5.13. According to this release profile, a rapid burst release was observed for all three formulations and approximately 36%, 38% and 50% of encapsulated docetaxel was released from PCL, PEG-PCL and CS-PCL nanocapsule formulations respectively within first 1 hour. The burst release was based on adsorbed docetaxel on the surface of nanocapsules. The drug incorporated to the surface released rapidly in medium at earlier time periods as stated in literature [239]. Furthermore, nanocapsule formulations were prepared at approximately 200 nm in

size rendering a large surface area. Reduction in particle size leads to larger surface area, causing high amount of initial burst release.

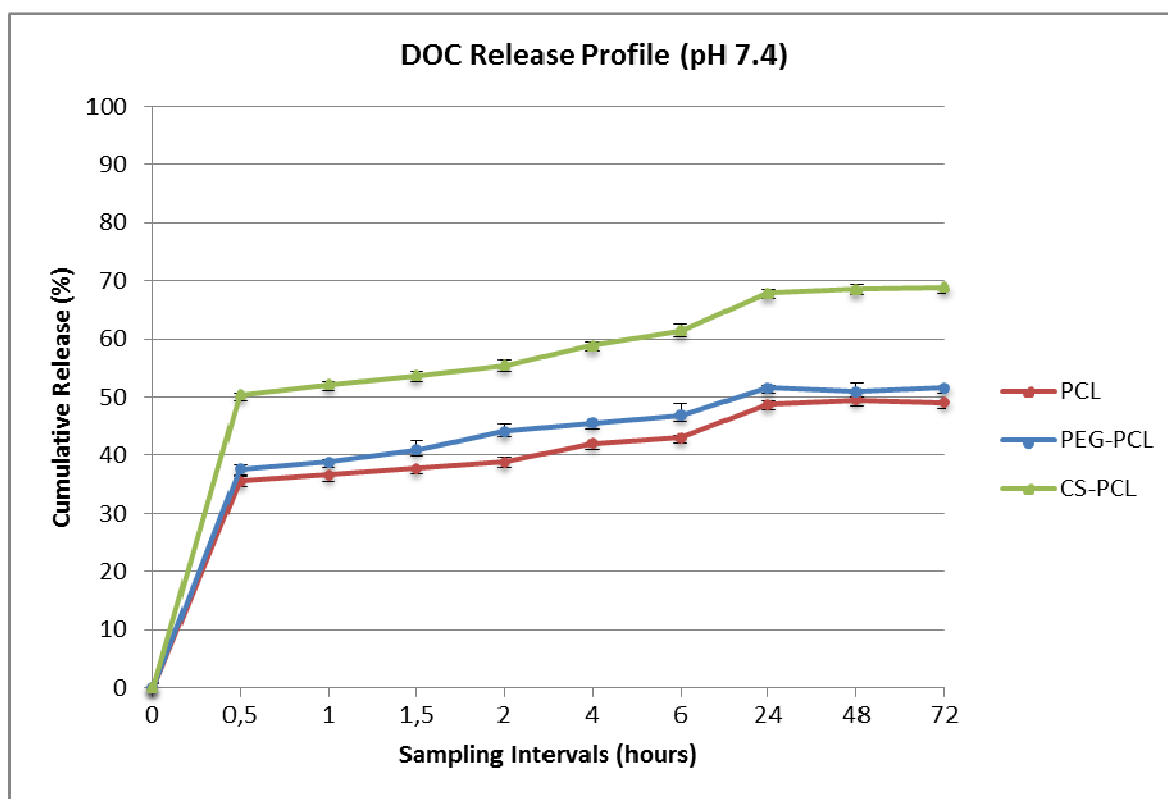


Figure 5.13. Cumulative release percentage of DOC from three nanocapsule formulations within 72 h. (Drug was released in PBS, 0.01 M, pH 7.4, with horizontal shaking at 100 rpm at 37°C.) Data was given as mean result $\pm$ SD of three different batches.

—●— PCL: non-modified NC, —●— PEG-PCL: PEG modified NC, —●— CS-PCL: CS modified NC.

Following the burst release in the first hour, a stable and sustained release observed for further 24 h. This active sustained release for 24 hours was a desirable release profile. After nanoparticle accumulation in tumor tissue, the release of active agent in a sustained manner around the tumor site provides constant drug exposure to cancerous cells and facilitated diffusion of the drug into the cancer cells as a result of concentration gradient formed within the tumor tissue.

At the end of 24 h, approximately 49%, 52% and 68% of encapsulated docetaxel was released from PCL, PEG-PCL and CS-PCL nanocapsule formulations respectively. A plateau was reached for all three formulations after 24 hours of

release, meaning very slow diffusion of docetaxel was continuing from this moment on since hydrophobic characteristics of drug retained docetaxel to stay inside the oily core of nanocapsule structure. This biphasic release behaviour might depend on firstly the release of weakly adsorbed drug onto the surface of nanocapsule structure and following a gradual release of encapsulated drug [240, 241]. Moreover, the membrane permeability in dialysis method has importance on drug release. The dialysis membrane plays a role as rate limiting factor [242].

Furthermore, cationic chitosan modified nanocapsules released docetaxel significantly faster than neutral or anionic nanocapsules. Since PEG-modified nanocapsules had higher encapsulation efficiency with large size and small surface area, the drug release rate was observed to be slow in PEG-modified nanocapsules. On the other hand, CS-modified nanocapsules also have larger size and higher docetaxel content compared to non-modified PCL nanocapsules. However, the adsorbed drug molecules on the surface of CS-modified nanocapsules releases faster causing a higher drug release.

Additionally, the oral chemotherapy of docetaxel could be effective only if the nanocarrier was able to overcome barriers in gastrointestinal passage. An oral chemotherapeutic agent should pass through the acidic environment of stomach safely. To demonstrate the stability of nanocapsule formulations in gastric conditions during gastric emptying, the release profile of nanocapsule formulations were investigated in SGF (pH 1.2). Cumulative release profile in SGF is given in Figure 5.14. According to the results, no drug release observed within first 2 hours which signifies the gastric passage time of nanocapsules as stated in literature [243].

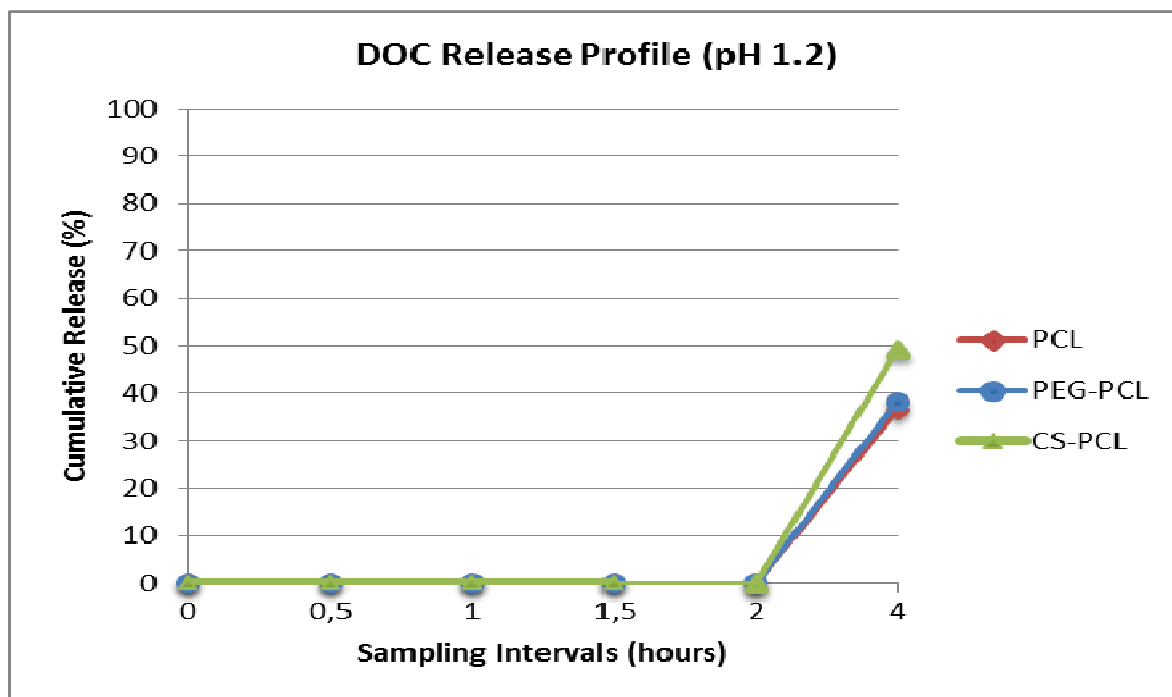


Figure 5.14. Cumulative release of DOC from three nanocapsule formulations for 4 h. (Drug was released in SGF, pH 1.2, with horizontal shaking at 100 rpm at 37°C.) Data was given as mean result $\pm$ SD of three different batches.

—◆— PCL: non-modified NC, —●— PEG-PCL: PEG modified NC, —▲— CS-PCL: CS modified NC.

5.9. Cell Culture Studies

5.9.1. Cytotoxicity

The cytotoxicity studies for blank nanocarriers were performed on L929 mouse fibroblast cell line as described in Section 4.9.1. Unloaded nanocapsule formulations (PCL, PEG-PCL, CS-PCL) were applied on L929 cell line in different dilution concentrations and incubated for 48 h. After incubation period, percent cell viability was measured by using MTT colorimetric method and evaluated according to the Eqn3 given in Section 4.9.1. With these cytotoxicity studies, the safety of three different empty nanocapsule formulations was investigated. The results are given in Figure 5.15.

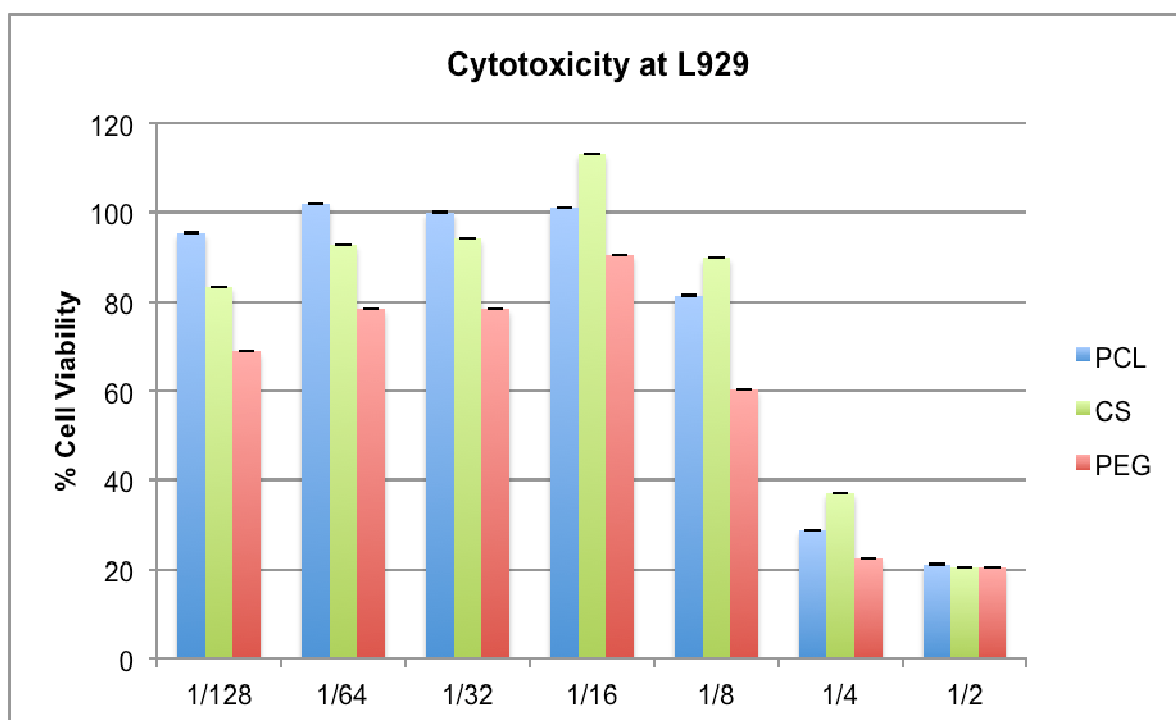


Figure 5.15. Cell viability after 48 h of incubation with blank nanocapsule formulations against L929 cell line. Data was given as mean result $\pm$ SD of triplicate study. (5×10^3 cells/100 μ l per well).

The absorbance values of control group were considered as 100% visible. Then, the cytotoxicity was determined based on relative viability of the colored cells. [244].

According to the results, it was observed that the toxicity was concentration dependent for all three formulations. As concentration increased over 1:8, all nanocapsule formulations were said to have toxic effect on cells. However, since nanoparticle toxicity was dose dependent, formulations were accepted to be in safe line in accordance to circulation volume in body as mentioned in literature [159]. Additionally, dilution ratio 1:8 and more were suggested to be safe as can be derived from Figure 5.15.

The results showed that there was no significant difference between anionic and cationic nanocapsules ($p > 0.05$) whereas there is significant difference between PEG and CS-modified nanocapsule formulations ($p < 0.05$) for each dilution point in terms of cytotoxicity. In fact, PEGylation is widely used to increase biocompatibility [245], although higher cytotoxic effect of PEG modification was clearly seen from the graph with respect to other formulations. The reason might be the small

amount of residual organic solvent in PEG formulation affecting cell growth and cell division [246]. On the contrary, CS modified nanocapsules indicated the least toxic formulation group among all experimental batches confirming the biocompatibility of chitosan as indicated in literature [247].

5.9.2. Anticancer Efficacy

The anticancer efficacy studies were performed on MCF-7 human breast cancer cell line as described in Section 4.9.2. DOC-loaded nanocapsule formulations (PCL, PEG-PCL, CS-PCL) and docetaxel solution prepared in 1% DMSO, in which contains equivalent dose of docetaxel (0.81 mg/ml), were applied on MCF-7 cell line in different dilution concentrations and incubated for 72 h. The toxic effect of DOC-loaded formulations in comparison with docetaxel solution was defined as percent cell viability and measured by using MTT colorimetric method. The calculations were performed according to the Eqn3 given in Section 4.9.1. The results are given in Figure 5.16.

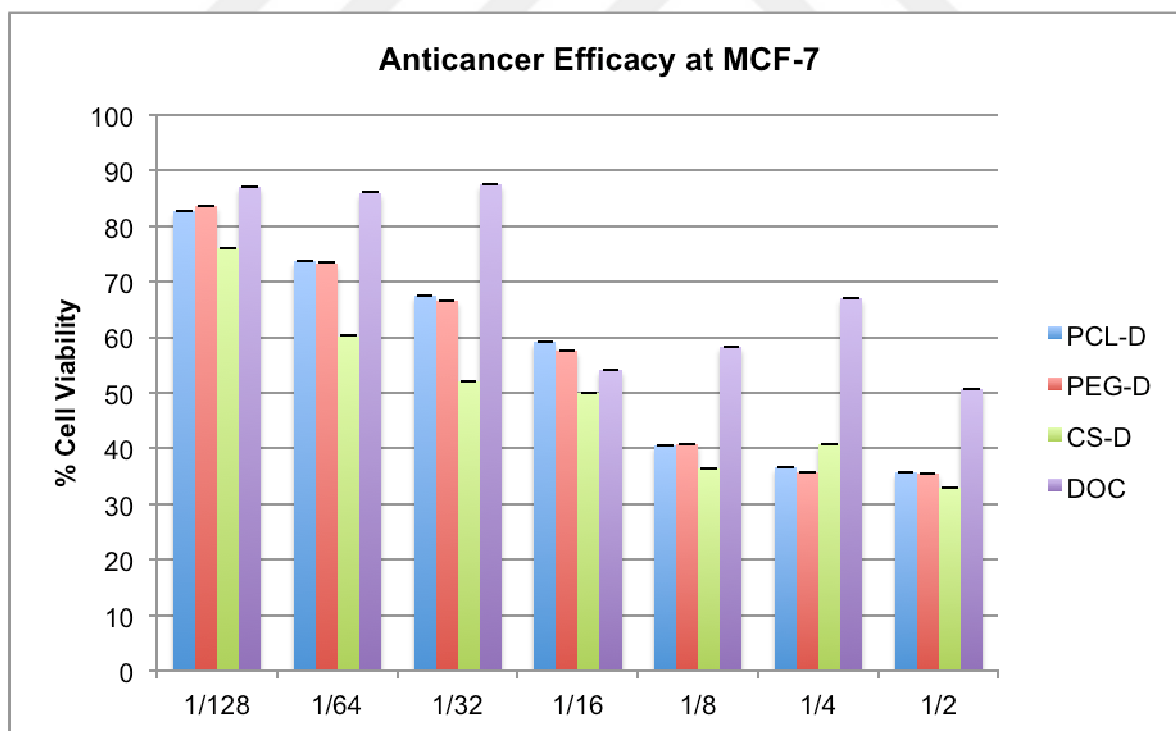


Figure 5.16. Cell viability after 72 h of incubation with DOC-loaded nanocapsules in comparison with DOC solution in DMSO. Data was given as mean result $\pm$ SD of triplicate study. (1×10^4 cells/100 μ l per well).

According to the results given in Figure 5.16, docetaxel in all three nanocapsule formulations had higher anticancer efficacy than docetaxel solution for almost each dilution point. Statistically, there was a significant difference between nanocapsule formulations and docetaxel solution itself ($p < 0.05$). Additionally, cationic chitosan modified nanocapsule formulation lead to higher cytotoxic effect than other formulations within toxicity safe line, at 1:8 dilution point and above. This increased cytotoxic effect of CS-modified nanocapsules possibly resulted from the positive surface charge ensuring stronger interaction with cell membrane. Cationic particles might lead to higher interaction with negative surface charge of cell membrane resulting higher cellular uptake rate and stronger cytotoxic effects [248]. Beside, there was not enough evidence to say that there was significant difference between formulations having non-modified surface (anionic) and PEG-modified surface (neutral) ($p > 0.05$). The overall result can be attributed to the affinity of positively charged surfaces to negatively charged cell membrane [248]. In general, neutral and anionic nanoparticles were reported to be less internalized than cationic ones [41, 249]. Chitosan is a strong positive charged material to which gave nanocapsules a positive surface facilitating internalization. Thus, CS-modified nanocapsule formulations were found to be more efficient in anticancer efficiency studies similarly in the literature [250, 251].

5.9.3. Permeability Through Caco-2 Monolayer

The permeability studies were performed on Caco-2 cell monolayer system as described in Section 4.9.3. Caco-2 cell line is widely used in gastrointestinal drug absorption studies [18, 252-256]. This monolayer system resembles the human intestinal epithelium in many respects by mimicking it. Caco-2 cells form tight junctions in between and thus allow transition of drug or carrier molecules across monolayer by paracellular movement [257, 258]. Therefore, the absorption rate of molecules through intestinal epithelium could suitably be investigated by Caco-2 cell monolayer assay. The data obtained from this protocol could easily be used to calculate apperent permeability coefficient (P_{app}), which represents the drug absorption rate of intestinal epithelium layer in human accepted as indicative of oral drug absorption.

Docetaxel-loaded nanocapsule formulations (PCL, PEG-PCL, CS-PCL) and docetaxel solution, all containing equivalent dose of docetaxel (50 µg/ml) were applied on Caco-2 monolayer as described before. The transportation across monolayer was defined as apparent permeability coefficient and the amount of transported drug measured by a validated HPLC method. The calculations were performed according to the Eqn4 given in Section 4.9.3. The permeability results are given in Figure 5.17 and percent increase in P_{app} in comparison to docetaxel solution is given in Figure 5.18.

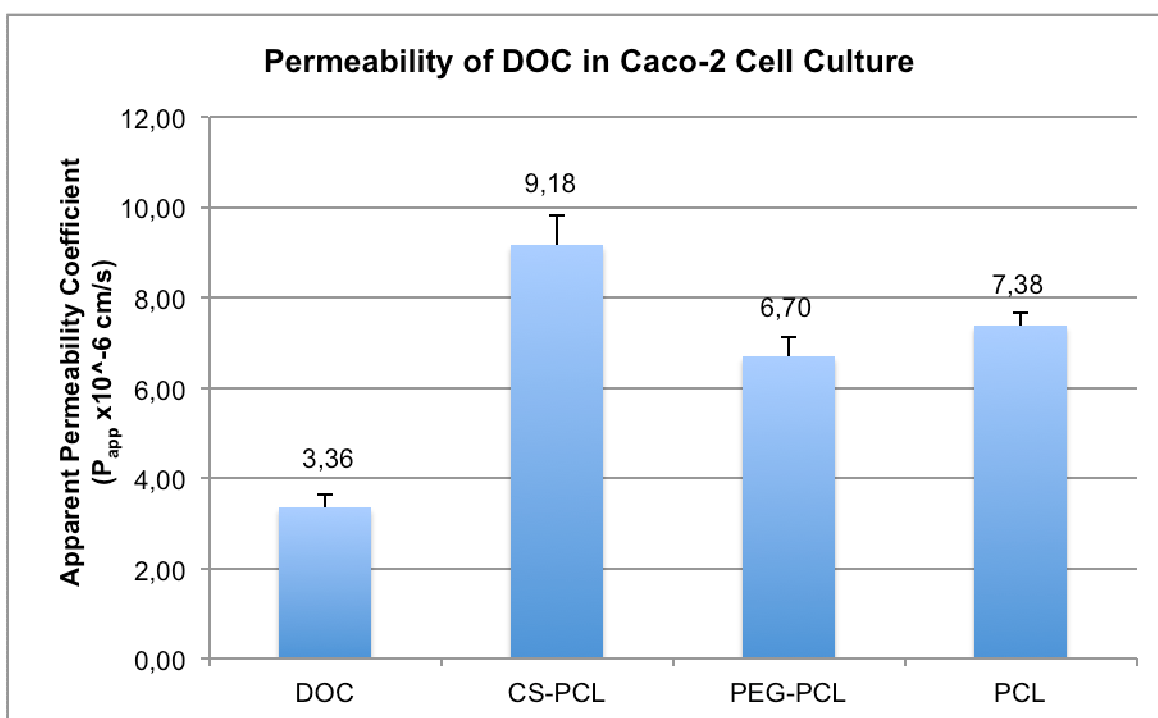


Figure 5.17. Apparent permeability coefficient of DOC loaded formulations and DOC solution. Data was given as mean result $\pm$ SD of triplicate study. ($1,2 \times 10^5$ cells/well).

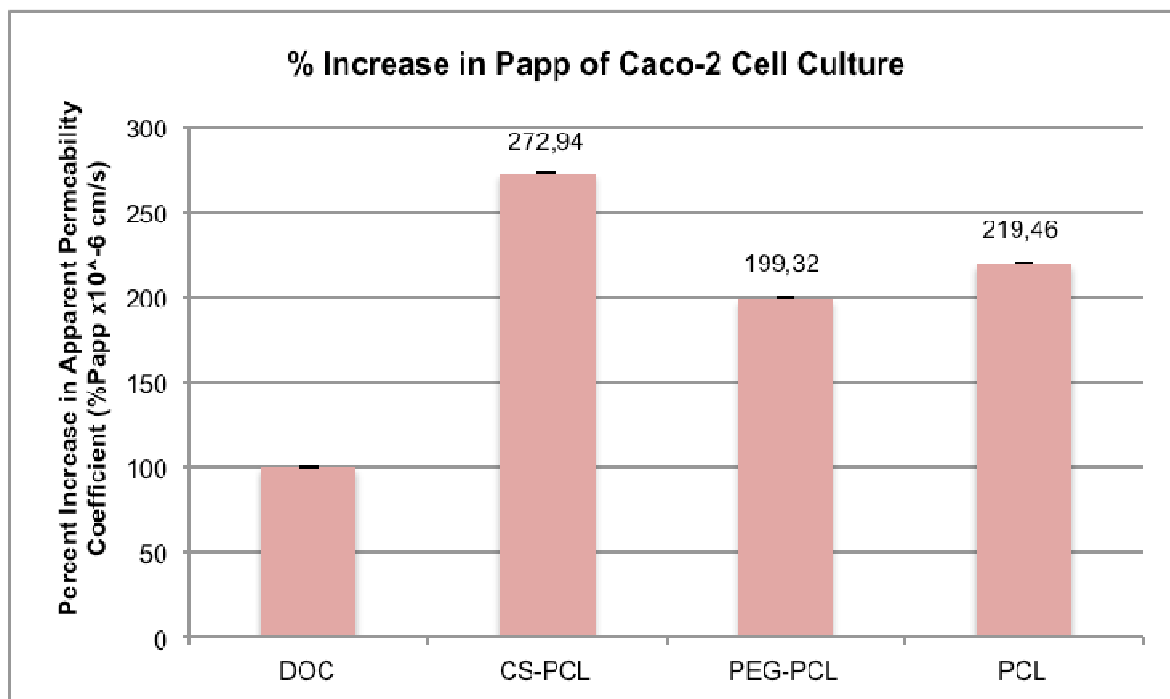


Figure 5.18. Percent increase in apparent permeability coefficient of DOC loaded-formulations in comparison with DOC solution.

The result of *in vitro* transport studies showed that there was significant difference ($p < 0.05$) between nanocapsule formulation groups and docetaxel solution. The effect of encapsulation and use of nano-technology increased the drug permeability rate through monolayer by approximately 2 to 3 fold with respect to docetaxel solution. Moreover, CS-modified nanocapsules could penetrate better than PEG-modified and non-modified nanocapsule formulations. This difference was attributed to the ability of chitosan binding to cell membrane better due to its positive charge and increasing adsorbance time at cell surface thus significantly and dose-dependently decreasing the *trans*-epithelial electrical resistance (TEER) of cell monolayers and raising the paracellular permeability [175, 258]. CS and its derivatives are already known that they are absorption enhancers, opening tight junctions and enhancing paracellular transition [259, 260]. Thus, CS modified nanocapsules were transported through cell monolayer faster. Furthermore, although dense PEG chains on the nanocapsules might lower the mucoadhesion by reducing hydrophobic and electrostatic interactions, thus minimizing permeability [261]; the property of inhibition of efflux transport of nanocarrier or

drug from the membrane by inhibition of active pumps was thought to be the main reason for increased permeability [243].

Eventually, CS-modified nanocapsules were found to be the most promising formulation in *in vitro* studies. Therefore further *in vivo* experimental design was constructed over CS-modified nanocapsules.

5.10. In Vivo Studies

In vivo studies of novel nanocapsule formulations were performed on mice and conducted with CS-PCL nanocapsule formulations which offered the most encouraging results during *in vitro* and cell culture studies. These selected formulations were loaded with fluorescent marker NR as visualization material and fluorescence characteristics were used for detection and quantification in *in vitro* uptake studies. NR is a fluorescent agent that is commonly used in cellular uptake studies [160, 262, 263]. It was preferred for further *in vivo* studies due to its hydrophobic character resembling docetaxel.

5.10.1. Construction of Calibration Curve for Nile Red

The calibration curve of NR was constructed with dilution concentrations between 0.001 and 0.1 µg/ml as given in Table 4.6 in Section 4.10.1. The fluorescent intensities of NR solutions were obtained from fluorescence measurements by HPLC. The data used to plot calibration curve shown in Figure 5.19. The regression equation of that curve was then maintained. According to data, the slope was 2.004 and intercept point was 1.9197. This regression data was linear over studied range with a correlation coefficient of $R^2 = 0.999$.

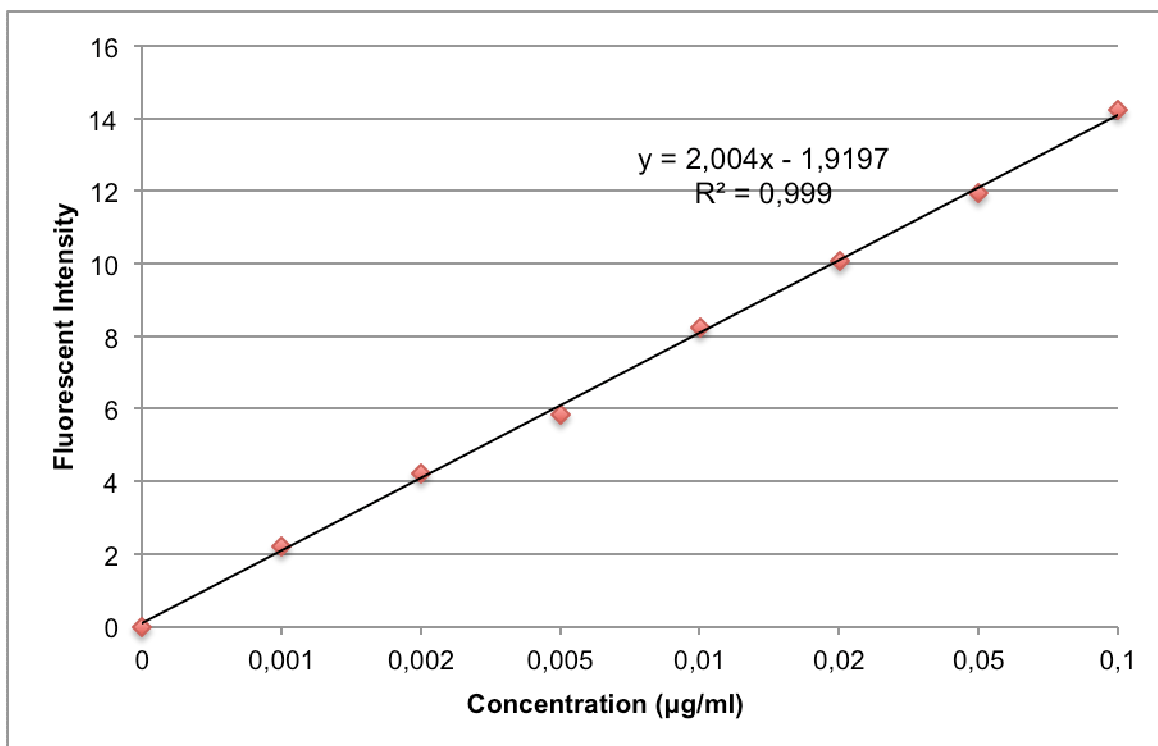


Figure 5.19. Calibration curve and regression equation of hydrophobic fluorescent dye NR dissolved in ethanol.

5.10.2. Preparation of Nile Red Loaded Nanocapsules

NR loaded nanocapsules were prepared by using the same method and same conditions as it was in nanocapsule formulation step. The protocol was defined in Section 4.10.2. All nanocapsule formulations were evaluated for particle size, polydispersity index and zeta potential by particle size analyzer (MALVERN Zetasizer Nano ZS Series, UK) at room temperature using disposable capillary cells. All measurements were performed in triplicate.

Table 5.21. Average diameter in size, polydispersity index and zeta potential of NR-loaded nanocapsule formulations. Data was given as mean result $\pm$ SD of three different batches.

Formulation	Average size (nm)	Pdl	Zeta Potential (mV)
PCL-NR	193.7 $\pm$ 1.6	0.091 $\pm$ 0.0	-16.6 $\pm$ 0.3
CS-NR	211.7 $\pm$ 0.2	0.074 $\pm$ 0.0	10.1 $\pm$ 0.3

According to results in Table 5.21, the mean particle size of NR-loaded nanocapsule formulations was about 200 nm. The suitable size range of nanocapsules to offer an effective cellular uptake in *in vivo* studies was reported as around 200-300 nm, which supports our results [11, 16]. The size distribution values were also found in acceptable narrow range and in accordance with docetaxel loaded nanocapsule formulations.

5.10.3. Encapsulation Efficiency of Nile Red

Encapsulation efficiency of NR-loaded nanocapsule formulations was determined to quantify the amount of NR encapsulated inside the hydrophobic core of nanocapsules. NR quantification was performed by fluorescence detector of HPLC for each nanocapsule formulation. Previously prepared and lyophilized NR-loaded nanocapsules were used to calculate percent associated NR (Eqn1). The data is given in Table 5.22.

Table 5.22. Encapsulation efficiency given as associated drug (%) of NR-loaded nanocapsule formulations. Data was given as mean result $\pm$ SD of three different batches.

Formulation	Associated drug (%)
PCL-NR	83.69 $\pm$ 0.6
CS-NR	87.40 $\pm$ 0.2

Encapsulation efficiency of NR-loaded nanocapsules was found quite high due to hydrophobic nature of NR, which increases its tendency to stay inside the hydrophobic core of nanocapsules [263]. Beside there was significance in results between two formulations ($p < 0.05$).

5.10.4. Recovery of Nile Red from GI Tissues

Recovery of NR is required for the accurate calculation of data gathered from *in vivo* studies by the elimination of experimental errors. There could be many factors that cannot be controlled in *in vivo* studies in opposite to presence of totally controlled experimental conditions of *in vitro* studies. In nanocapsule uptake studies, amount of NR derived from intestinal and stomach tissues could also be

affected by some other factors such as tissue specifications of different animals, different nature of intestinal segments and the rate of absorption. Recovery studies obtain a normalization factor to correct the recovered NR amount from intestinal tissue in *in vivo* experiments. Accurate calculations could be performed and exact values of intestinal uptake could be obtained only in presence of a correction factor. For this reason, a recovery study was performed prior to *in vivo* nanocapsule uptake studies as described in Section 4.10.4. The percent mean recovery values from intestine segments and stomach after direct administration of NR are shown in Figure 5.21.

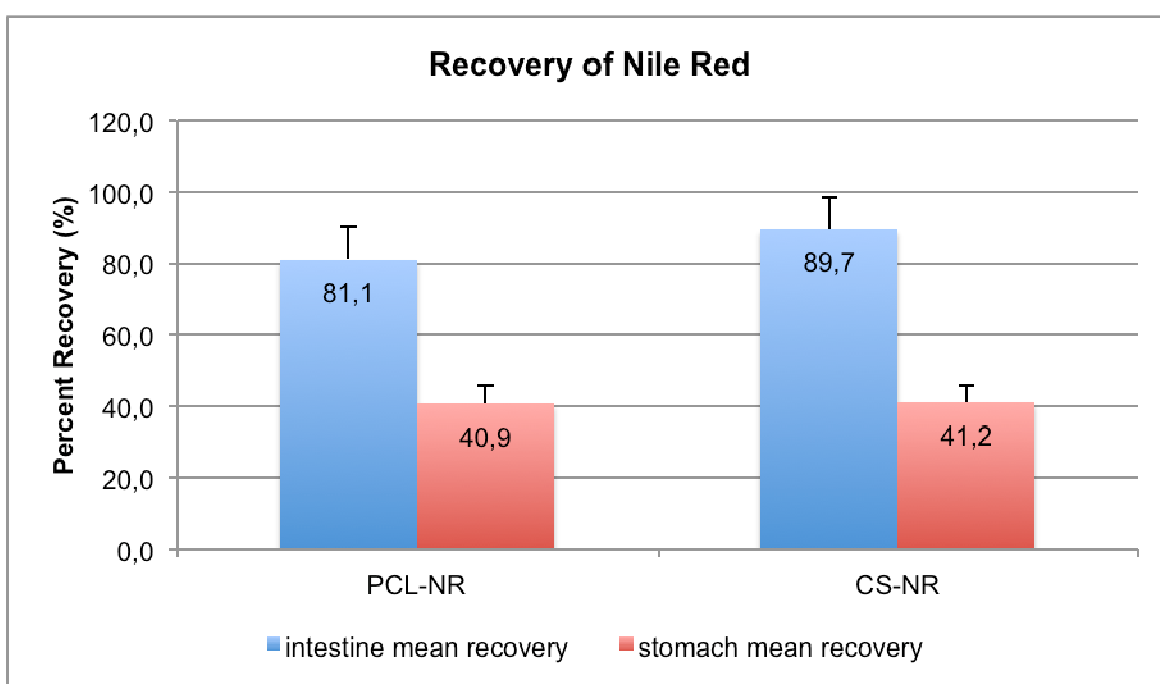


Figure 5.20. Percent mean recovery of NR from intestine segments and stomach on which NR loaded PCL and CS nanoparticles directly administered. Data was given as mean result $\pm$ SD of triplicate study.

According to recovery data, the mean NR recovery from intestine was much higher than stomach recovery. This is because the absorption is mainly realized in intestinal tissue. Moreover, there was a significant difference ($p < 0.05$) between CS modified and non-modified nanocapsule formulations. It has been known that CS acts as an absorption enhancer, modulating and increasing paracellular transition during penetration through intestinal epithelium by facilitating the contact between nanocarriers and colonic mucosa [253, 264, 265].

5.10.5. Nanocapsule Uptake and Gastrointestinal Absorption Study

The drugs or drug carriers which reached the intestine should be taken effectively before elimination to obtain an efficient bioavailability. Hence, the uptake rate and extent are important factors for an efficient and successful absorption process. The extent of nanocapsule uptake from gastrointestinal tract was evaluated on male Swiss albino mice as described in Section 4.10.5. CS-modified and non-modified nanocapsule formulations were given to pre-defined experimental animal groups via oral gavage. Intestinal and stomach tissues were treated as defined before and NR-labelled nanocapsule amount was measured by fluorescent HPLC. The exact of nanocapsule uptake amount was evaluated after the data was normalized with the correction factor found in Section 5.10.4. The percent uptake data is presented in Figure 5.22

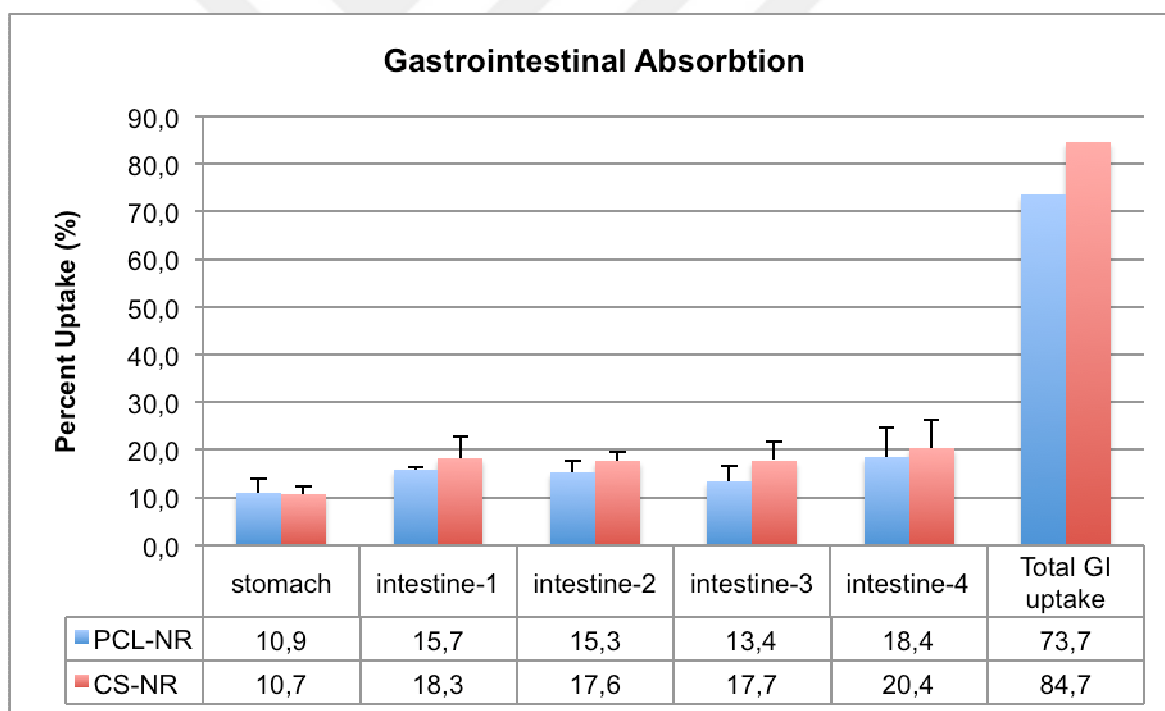


Figure 5.21. Percentage PCL and CS-PCL nanocapsule uptake in mouse stomach and intestine segments. Data was given as mean result $\pm$ SD of triplicate study.

The results revealed that a very large quantity of NR from nanocapsules (~74% and 85% for PCL and CS-PCL nanocapsules respectively) was detected in stomach and intestinal tissues totally. A small portion of nanocapsules was

detected in stomach whereas nanocapsules were mostly found in intestinal segments for both formulations. This was an expected result because intestine was the place where absorption rate was highest. The stability of nanocapsules was also proved by this experimental set up. While the nanocapsules were moving by peristalsis in gastrointestinal tract, labeled nanocapsules were mostly able to be uptaken and detected. Furthermore, it was clear that CS-modified nanocapsules had high affinity to be uptaken by intestinal tissue. There was a significant difference ($p < 0.05$) between the uptake percent of CS-modified and non-modified nanocapsules. This difference was attributed to the cationic nature of nanocapsules due to chitosan because CS known as an absorption enhancer, which helps intestinal epithelial cells to open tight junctions enhancing paracellular transition [258, 266-268]. Moreover, mucoadhesivity of CS promotes the residence time in tissue and interaction of nanocapsule with intestinal epithelium which leading to increase in uptake of CS-modified nanocapsules [18, 267].

As a conclusion, penetration of nanocarriers through intestinal epithelium and flow into circulation was still the first step of bioavailability of oral drugs but it also has a vital importance. The most striking factors affecting absorption is related to the adhesiveness and mainly depend on the intrinsic properties of nanocarrier such as size and surface properties; and also physicochemical characteristics of matrix and coating materials [269]. They affect the rate and extent of penetration as can be seen from the results of uptake studies. Fortunately, those properties can be easily manipulated to get better results.

The novelty of this thesis as a whole is that the formulation of oral polymeric docetaxel-loaded anionic or cationic PCL nanocapsules for the first time. The novel nanocapsule formulations offer promising strategy for a safe and effective oral chemotherapy considering the results of *in vitro* and *in vivo* studies.

REFERENCES

- [1] Ophardt, C. E. Virtual Chembook - Elmhurst College, <http://chemistry.elmhurst.edu/vchembook/655cancer.html> (09/11/2015)
- [2] Mei, L., Zhang, Z., Zhao, L., Huang, L., Yang, X. L., Tang, J., and Feng, S. S. Pharmaceutical nanotechnology for oral delivery of anticancer drugs, *Adv Drug Deliv Rev* 65, 880-890, **2013**
- [3] Unal, H., d'Angelo, I., Pagano, E., Borrelli, F., Izzo, A., Ungaro, F., Quaglia, F., and Bilensoy, E. Core-shell hybrid nanocapsules for oral delivery of camptothecin: formulation development, in vitro and in vivo evaluation, *J Nanopart Res* 17, **2015**
- [4] Cho, H. J., Park, J. W., Yoon, I. S., and Kim, D. D. Surface-modified solid lipid nanoparticles for oral delivery of docetaxel: enhanced intestinal absorption and lymphatic uptake, *Int J Nanomedicine* 9, 495-504, **2014**
- [5] Wolk, O., Agbaria, R., and Dahan, A. Provisional in-silico biopharmaceutics classification (BCS) to guide oral drug product development, *Drug Des Devel Ther* 8, 1563-1575, **2014**
- [6] Shah, V. P., and Amidon, G. L. G.L. Amidon, H. Lennernas, V.P. Shah, and J.R. Crison. A theoretical basis for a biopharmaceutic drug classification: the correlation of in vitro drug product dissolution and in vivo bioavailability, *Pharm Res* 12, 413-420, 1995--backstory of BCS, *Aaps J* 16, 894-898, **2014**
- [7] Shugarts, S., and Benet, L. Z. The role of transporters in the pharmacokinetics of orally administered drugs, *Pharm Res* 26, 2039-2054, **2009**
- [8] Perez-Herrero, E., and Fernandez-Medarde, A. Advanced targeted therapies in cancer: Drug nanocarriers, the future of chemotherapy, *Eur J Pharm Biopharm* 93, 52-79, **2015**
- [9] Attili-Qadri, S., Karra, N., Nemirovski, A., Schwob, O., Talmon, Y., Nassar, T., and Benita, S. Oral delivery system prolongs blood circulation of docetaxel nanocapsules via lymphatic absorption, *Proc Natl Acad Sci U S A* 110, 17498-17503, **2013**
- [10] Nipun, T. S., and Ashraful Islam, S. M. SEDDS of gliclazide: Preparation and characterization by in-vitro, ex-vivo and in-vivo techniques, *Saudi Pharm J* 22, 343-348, **2014**
- [11] Ensign, L. M., Cone, R., and Hanes, J. Oral drug delivery with polymeric nanoparticles: the gastrointestinal mucus barriers, *Adv Drug Deliv Rev* 64, 557-570, **2012**
- [12] Wang, Y. J., Chen, L. J., Tan, L. W., Zhao, Q., Luo, F., Wei, Y. Q., and Qian, Z. Y. PEG-PCL based micelle hydrogels as oral docetaxel delivery systems for breast cancer therapy, *Biomaterials* 35, 6972-6985, **2014**
- [13] University, T. S. M. The main methods of functional diagnostics of diseases in gastroenterology, http://intranet.tdmu.edu.ua/data/kafedra/internal/klinpat/classes_stud/en/meldlik/ptn/Functional%20diagnostics_4%20course/4/02.%20The%20main%20

[Omethods%20of%20diagnostics%20of%20diseases%20in%20gastroenterology.htm](#) (23/11/2015)

- [14] Guerra, A., Etienne-Mesmin, L., Livrelli, V., Denis, S., Blanquet-Diot, S., and Alric, M. Relevance and challenges in modeling human gastric and small intestinal digestion, *Trends Biotechnol* 30, 591-600, **2012**
- [15] Bansil, R., and Turner, B. S. Mucin structure, aggregation, physiological functions and biomedical applications, *Curr Opin Colloid In* 11, 164-170, **2006**
- [16] Tang, B. C., Dawson, M., Lai, S. K., Wang, Y. Y., Suk, J. S., Yang, M., Zeitlin, P., Boyle, M. P., Fu, J., and Hanes, J. Biodegradable polymer nanoparticles that rapidly penetrate the human mucus barrier, *P Natl Acad Sci USA* 106, 19268-19273, **2009**
- [17] Plapied, L., Duhem, N., des Rieux, A., and Preat, V. Fate of polymeric nanocarriers for oral drug delivery, *Curr Opin Colloid In* 16, 228-237, **2011**
- [18] Sadeghi, A. M. M., Dorkoosh, F. A., Avadi, M. R., Weinhold, M., Bayat, A., Delie, F., Gurny, R., Larijani, B., Rafiee-Tehrani, M., and Junginger, H. E. Permeation enhancer effect of chitosan and chitosan derivatives: Comparison of formulations as soluble polymers and nanoparticulate systems on insulin absorption in Caco-2 cells, *European Journal of Pharmaceutics and Biopharmaceutics* 70, 270-278, **2008**
- [19] Luessen, H. L., Rentel, C. O., Kotze, A. F., Lehr, C. M., deBoer, A. G., Verhoef, J. C., and Junginger, H. E. Mucoadhesive polymers in peroral peptide drug delivery .4. Polycarbophil and chitosan are potent enhancers of peptide transport across intestinal mucosae in vitro, *Journal of Controlled Release* 45, 15-23, **1997**
- [20] Li, Y., Zhang, X., and Cao, D. Nanoparticle hardness controls the internalization pathway for drug delivery, *Nanoscale* 7, 2758-2769, **2015**
- [21] Reviews, N. Bioavailability & First-Pass Effect, <https://canna-pet.com/first-pass-effect/> (24/12/2015)
- [22] Consequences, *Clin Pharmacokinet* 9, 1-25, **1984**
- [23] Saremi, S., Dinarvand, R., Kebriaeezadeh, A., Ostad, S. N., and Atyabi, F. Enhanced Oral Delivery of Docetaxel Using Thiolated Chitosan Nanoparticles: Preparation, In Vitro and In Vivo Studies, *Biomed Res Int*, **2013**
- [24] Sorrentino, B. R. Gene therapy to protect haematopoietic cells from cytotoxic cancer drugs, *Nature Reviews Cancer* 2, 431-441, **2002**
- [25] Joshi, G., Kumar, A., and Sawant, K. Enhanced bioavailability and intestinal uptake of Gemcitabine HCl loaded PLGA nanoparticles after oral delivery, *Eur J Pharm Sci* 60, 80-89, **2014**
- [26] Hulla, J. E., Sahu, S. C., and Hayes, A. W. Nanotechnology: History and future, *Hum Exp Toxicol* 34, 1318-1321, **2015**
- [27] Initiative, N. N. What is Nanotechnology?, <http://www.nano.gov/nanotech-101/what/definition> (22/10/2015)
- [28] Administration, U. S. F. a. D. Guidance for Industry Considering Whether an FDA-Regulated Product Involves the Application of Nanotechnology, U.S. Department of Health and Human Services Food and Drug Administration, USA.(2014)
- [29] Lim, S. J., Zahid, M. U., Le, P., Ma, L., Entenberg, D., Harney, A. S., Condeelis, J., and Smith, A. M. Brightness-equalized quantum dots, *Nat Commun* 6, 8210, **2015**

- [30] Fissell, R., and Hakim, R. M. Improving outcomes by changing hemodialysis practice patterns, *Curr Opin Nephrol Hypertens* 22, 675-680, **2013**
- [31] Fissell, W. H. What is nanotechnology?, *Adv Chronic Kidney Dis* 20, 452-453, **2013**
- [32] Suffredini, G., East, J. E., and Levy, L. M. New applications of nanotechnology for neuroimaging, *AJNR Am J Neuroradiol* 35, 1246-1253, **2014**
- [33] Adelman, J., Aschner, J., Schechter, C., Angert, R., Weiss, J., Rai, A., Berger, M., Reissman, S., Parakkattu, V., Chacko, B., Racine, A., and Southern, W. Use of Temporary Names for Newborns and Associated Risks, *Pediatrics* 136, 327-333, **2015**
- [34] Kura, A. U., Fakurazi, S., Hussein, M. Z., and Arulselvan, P. Nanotechnology in drug delivery: the need for more cell culture based studies in screening, *Chem Cent J* 8, 46, **2014**
- [35] Hulland, K. R., Chase, R. P., Caruso, B. A., Swain, R., Biswal, B., Sahoo, K. C., Panigrahi, P., and Dreibelbis, R. Sanitation, Stress, and Life Stage: A Systematic Data Collection Study among Women in Odisha, India, *PLoS One* 10, e0141883, **2015**
- [36] Conriot, J., Silva, J. M., Fernandes, J. G., Silva, L. C., Gaspar, R., Brocchini, S., Florindo, H. F., and Barata, T. S. Cancer immunotherapy: nanodelivery approaches for immune cell targeting and tracking, *Front Chem* 2, 105, **2014**
- [37] Wang, X., Wang, Y. Q., Chen, Z., and Shin, D. M. Advances of Cancer Therapy by Nanotechnology, *Cancer Res Treat* 41, 1-11, **2009**
- [38] Lammers, T., Kiessling, F., Hennink, W. E., and Storm, G. Drug targeting to tumors: principles, pitfalls and (pre-) clinical progress, *J Control Release* 161, 175-187, **2012**
- [39] Society, C. R. *Nano-Oncologicals*, Springer, **2014**
- [40] Sainz, V., Conriot, J., Matos, A. I., Peres, C., Zupancic, E., Moura, L., Silva, L. C., Florindo, H. F., and Gaspar, R. S. Regulatory aspects on nanomedicines, *Biochem Bioph Res Co* 468, 504-510, **2015**
- [41] Ji, J. T., Debora; Ruyra, Angels; Roher, Nerea. Nanodelivery Systems as New Tools for Immunostimulant or Vaccine Administration: Targeting the Fish Immune System, *Biology* 4, 664-696, **2015**
- [42] Kang, L., Gao, Z. G., Huang, W., Jin, M. J., and Wang, Q. M. Nanocarrier-mediated co-delivery of chemotherapeutic drugs and gene agents for cancer treatment, *Acta Pharm Sin B* 5, 169-175, **2015**
- [43] Gotze, I. O., Archer, A. J., and Likos, C. N. Structure, phase behavior, and inhomogeneous fluid properties of binary dendrimer mixtures, *J Chem Phys* 124, 084901, **2006**
- [44] Vrignaud, S., Benoit, J. P., and Saulnier, P. Strategies for the nanoencapsulation of hydrophilic molecules in polymer-based nanoparticles, *Biomaterials* 32, 8593-8604, **2011**
- [45] Orbach, S. A., Bonifacio, S. L., Kuzniewicz, M. W., and Glass, H. C. Lower incidence of seizure among neonates treated with therapeutic hypothermia, *J Child Neurol* 29, 1502-1507, **2014**
- [46] Bonifacio, B. V., Silva, P. B., Ramos, M. A., Negri, K. M., Bauab, T. M., and Chorilli, M. Nanotechnology-based drug delivery systems and herbal medicines: a review, *Int J Nanomedicine* 9, 1-15, **2014**

- [47] Guterres, S. S., Alves, M. P., and Pohlmann, A. R. Polymeric nanoparticles, nanospheres and nanocapsules, for cutaneous applications, *Drug Target Insights* 2, 147-157, **2007**
- [48] Letchford, K., and Burt, H. A review of the formation and classification of amphiphilic block copolymer nanoparticulate structures: micelles, nanospheres, nanocapsules and polymersomes, *Eur J Pharm Biopharm* 65, 259-269, **2007**
- [49] Couvreur, P., Barratt, G., Fattal, E., Legrand, P., and Vauthier, C. Nanocapsule technology: A review, *Crit Rev Ther Drug* 19, 99-134, **2002**
- [50] Cruz, L., Soares, L. U., Costa, T. D., Mezzalana, G., da Silveira, N. P., Guterres, S. S., and Pohlmann, A. R. Diffusion and mathematical modeling of release profiles from nanocarriers, *Int J Pharm* 313, 198-205, **2006**
- [51] Soppimath, K. S., Aminabhavi, T. M., Kulkarni, A. R., and Rudzinski, W. E. Biodegradable polymeric nanoparticles as drug delivery devices, *J Control Release* 70, 1-20, **2001**
- [52] Kothamasu, P., Kanumur, H., Ravur, N., Maddu, C., Parasuramrajam, R., and Thangavel, S. Nanocapsules: the weapons for novel drug delivery systems, *Bioimpacts* 2, 71-81, **2012**
- [53] Diaspro, A., Krol, S., Cavalleri, O., Silvano, D., and Gliozzi, A. Microscopical characterization of nanocapsules templated on ionic crystals and biological cells toward biomedical applications, *IEEE Trans Nanobioscience* 1, 110-115, **2002**
- [54] Madhwani, K. P. Safe development of nanotechnology: A global challenge, *Indian J Occup Environ Med* 17, 87-88, **2013**
- [55] Kroto, H. Nanoscience and nanotechnology in the twenty-first century, *Drug Deliv Transl Res* 3, 297-298, **2013**
- [56] Ghalandari, B., Divsalar, A., Saboury, A. A., and Parivar, K. The new insight into oral drug delivery system based on metal drugs in colon cancer therapy through beta-lactoglobulin/oxali-palladium nanocapsules, *J Photoch Photobio B* 140, 255-265, **2014**
- [57] Yurgel, V., Collares, T., and Seixas, F. Developments in the use of nanocapsules in oncology, *Braz J Med Biol Res* 46, 486-501, **2013**
- [58] Shen, Y., Jin, E., Zhang, B., Murphy, C. J., Sui, M., Zhao, J., Wang, J., Tang, J., Fan, M., Van Kirk, E., and Murdoch, W. J. Prodrugs forming high drug loading multifunctional nanocapsules for intracellular cancer drug delivery, *J Am Chem Soc* 132, 4259-4265, **2010**
- [59] Vergara, D., Bellomo, C., Zhang, X., Vergaro, V., Tinelli, A., Lorusso, V., Rinaldi, R., Lvov, Y. M., Leporatti, S., and Maffia, M. Lapatinib/Paclitaxel polyelectrolyte nanocapsules for overcoming multidrug resistance in ovarian cancer, *Nanomedicine* 8, 891-899, **2012**
- [60] Lee, J. Y., Bae, K. H., Kim, J. S., Nam, Y. S., and Park, T. G. Intracellular delivery of paclitaxel using oil-free, shell cross-linked HSA - Multi-armed PEG nanocapsules, *Biomaterials* 32, 8635-8644, **2011**
- [61] Wang, Z., and Ho, P. C. A nanocapsular combinatorial sequential drug delivery system for antiangiogenesis and anticancer activities, *Biomaterials* 31, 7115-7123, **2010**
- [62] Sanchez-Moreno, P., Boulaiz, H., Ortega-Vinuesa, J. L., Peula-Garcia, J. M., and Aranega, A. Novel drug delivery system based on docetaxel-loaded nanocapsules as a therapeutic strategy against breast cancer cells, *Int J Mol Sci* 13, 4906-4919, **2012**

- [63] Nagaich, U. Nanotechnology: The vision of 2025, *J Adv Pharm Technol Res* 5, 105-106, **2014**
- [64] Chang, E. H., Harford, J. B., Eaton, M. A., Boisseau, P. M., Dube, A., Hayeshi, R., Swai, H., and Lee, D. S. Nanomedicine: Past, present and future - A global perspective, *Biochem Biophys Res Commun* 468, 511-517, **2015**
- [65] Administration, U. S. F. a. D. Analytical Procedures and Methods Validation for Drugs and Biologics - Guidance for Industry, (Services, U. S. D. o. H. a. H., and Administration, F. a. D., Eds.), USA.(2015)
- [66] Sanna, V., Pala, N., and Sechi, M. Targeted therapy using nanotechnology: focus on cancer, *Int J Nanomed* 9, 467-483, **2014**
- [67] Tinkle, S., McNeil, S. E., Muhlebach, S., Bawa, R., Borchard, G., Barenholz, Y., Tamarkin, L., and Desai, N. Nanomedicines: addressing the scientific and regulatory gap, *Ann Ny Acad Sci* 1313, 35-56, **2014**
- [68] Johnson&Johnson U.S. FDA Approves INVEGA TRINZA™, First and Only Four-Times-A-Year Treatment for Schizophrenia, <http://www.inj.com/news/all/US-FDA-Approves-INVEGA-TRINZA-First-and-Only-Four-Times-A-Year-Treatment-for-Schizophrenia> (27/02/2016)
- [69] Mora-Huertas, C. E., Fessi, H., and Elaissari, A. Polymer-based nanocapsules for drug delivery, *Int J Pharm* 385, 113-142, **2010**
- [70] Allouche, S., Le Marec, T., Noble, F., and Marie, N. Different patterns of administration modulate propensity of methadone and buprenorphine to promote locomotor sensitization in mice, *Prog Neuropsychopharmacol Biol Psychiatry* 40, 286-291, **2013**
- [71] Khayata, N., Abdelwahed, W., Chehna, M. F., Charcosset, C., and Fessi, H. Preparation of vitamin E loaded nanocapsules by the nanoprecipitation method: From laboratory scale to large scale using a membrane contactor, *International Journal of Pharmaceutics* 423, 419-427, **2012**
- [72] Fessi, H., Puisieux, F., Devissaguet, J. P., Ammoury, N., and Benita, S. Nanocapsule Formation by Interfacial Polymer Deposition Following Solvent Displacement, *Int J Pharm* 55, R1-R4, **1989**
- [73] Rao, J. P., and Geckeler, K. E. Polymer nanoparticles: Preparation techniques and size-control parameters, *Progress in Polymer Science* 36, 887-913, **2011**
- [74] Kundan, A., Plawsky, J. L., and Wayner, P. C., Jr. Effect of capillary and marangoni forces on transport phenomena in microgravity, *Langmuir* 31, 5377-5386, **2015**
- [75] Duner, G., Kim, M., Tilton, R. D., Garoff, S., and Przybycien, T. M. Effect of polyelectrolyte-surfactant complexation on Marangoni transport at a liquid-liquid interface, *J Colloid Interface Sci* 467, 105-114, **2016**
- [76] Langevin, D., and Monroy, F. Marangoni stresses and surface compression rheology of surfactant solutions. Achievements and problems, *Adv Colloid Interface Sci* 206, 141-149, **2014**
- [77] Lince, F., Marchisio, D. L., and Barresi, A. A. Strategies to control the particle size distribution of poly-epsilon-caprolactone nanoparticles for pharmaceutical applications, *Journal of Colloid and Interface Science* 322, 505-515, **2008**
- [78] Niskanen, I., Hibino, K., and Raty, J. Immersion liquid techniques in solid particle characterization: A review, *Talanta* 149, 225-236, **2016**

- [79] Lin, P. C., Lin, S., Wang, P. C., and Sridhar, R. Techniques for physicochemical characterization of nanomaterials, *Biotechnol Adv* 32, 711-726, **2014**
- [80] Laborda, F., Bolea, E., Cepria, G., Gomez, M. T., Jimenez, M. S., Perez-Arantegui, J., and Castillo, J. R. Detection, characterization and quantification of inorganic engineered nanomaterials: A review of techniques and methodological approaches for the analysis of complex samples, *Anal Chim Acta* 904, 10-32, **2016**
- [81] Gangadoo, S., Taylor-Robinson, A., and Chapman, J. Nanoparticle and biomaterial characterisation techniques, *Mater Technol* 30, B44-B56, **2015**
- [82] Sapsford, K. E., Tyner, K. M., Dair, B. J., Deschamps, J. R., and Medintz, I. L. Analyzing nanomaterial bioconjugates: a review of current and emerging purification and characterization techniques, *Anal Chem* 83, 4453-4488, **2011**
- [83] Ringe, E. Nanocrystalline materials: recent advances in crystallographic characterization techniques, *IUCrJ* 1, 530-539, **2014**
- [84] Sadik, O. A., Du, N., Kariuki, V., Okello, V., and Bushlyar, V. Current and Emerging Technologies for the Characterization of Nanomaterials, *Acs Sustain Chem Eng* 2, 1707-1716, **2014**
- [85] Lapresta-Fernandez, A., Salinas-Castillo, A., de la Llana, S. A., Costa-Fernandez, J. M., Dominguez-Meister, S., Cecchini, R., Capitan-Vallvey, L. F., Moreno-Bondi, M. C., Marco, M. P., Sanchez-Lopez, J. C., and Anderson, I. S. A General Perspective of the Characterization and Quantification of Nanoparticles: Imaging, Spectroscopic, and Separation Techniques, *Crit Rev Solid State* 39, 423-458, **2014**
- [86] Carvalho Silva, R., Alexandre Muehlmann, L., Rodrigues Da Silva, J., de Bentes Azevedo, R., and Madeira Lucci, C. Influence of nanostructure composition on its morphometric characterization by different techniques, *Microsc Res Tech* 77, 691-696, **2014**
- [87] Kathe, N., Henriksen, B., and Chauhan, H. Physicochemical characterization techniques for solid lipid nanoparticles: principles and limitations, *Drug Dev Ind Pharm* 40, 1565-1575, **2014**
- [88] Domingos, R. F., Baalousha, M. A., Ju-Nam, Y., Reid, M. M., Tufenkji, N., Lead, J. R., Leppard, G. G., and Wilkinson, K. J. Characterizing manufactured nanoparticles in the environment: multimethod determination of particle sizes, *Environ Sci Technol* 43, 7277-7284, **2009**
- [89] Brar, S. K., and Verma, M. Measurement of nanoparticles by light-scattering techniques, *Trac-Trend Anal Chem* 30, 4-17, **2011**
- [90] Cho, E. J., Holback, H., Liu, K. C., Abouelmagd, S. A., Park, J., and Yeo, Y. Nanoparticle characterization: state of the art, challenges, and emerging technologies, *Mol Pharm* 10, 2093-2110, **2013**
- [91] de Andrade, D. F., Zuglianello, C., Pohlmann, A. R., Guterres, S. S., and Beck, R. C. Assessing the In Vitro Drug Release from Lipid-Core Nanocapsules: a New Strategy Combining Dialysis Sac and a Continuous-Flow System, *AAPS PharmSciTech* 16, 1409-1417, **2015**
- [92] Ungaro, F., d'Angelo, I., Coletta, C., d'Emmanuele di Villa Bianca, R., Sorrentino, R., Perfetto, B., Tufano, M. A., Miro, A., La Rotonda, M. I., and Quaglia, F. Dry powders based on PLGA nanoparticles for pulmonary delivery of antibiotics: modulation of encapsulation efficiency, release rate

- and lung deposition pattern by hydrophilic polymers, *J Control Release* 157, 149-159, **2012**
- [93] Erdemoğlu, N. Ş. E., Bilge. Taksan sınıfı bileşiklerin antitümör etkileri, *J. Fac. Pharm.* 29, 77-90, **2000**
- [94] Jain, V., Jain, B., Tiwari, P., Saini, J., Jain, U. K., Pandey, R. S., Kumar, M., Katare, O. P., Chandra, R., and Madan, J. Nanosolvated microtubule-modulating chemotherapeutics: a case-to-case study, *Anticancer Drugs* 24, 327-336, **2013**
- [95] Verma, P., Meher, J. G., Asthana, S., Pawar, V. K., Chaurasia, M., and Chourasia, M. K. Perspectives of nanoemulsion assisted oral delivery of docetaxel for improved chemotherapy of cancer, *Drug Deliv* 23, 479-488, **2016**
- [96] Jain, A. S., Makhija, D. T., Goel, P. N., Shah, S. M., Nikam, Y., Gude, R. P., Jagtap, A. G., and Nagarsenker, M. S. Docetaxel in cationic lipid nanocapsules for enhanced in vivo activity, *Pharm Dev Technol* 21, 76-85, **2016**
- [97] Joerger, M. Treatment regimens of classical and newer taxanes, *Cancer Chemother Pharmacol* 77, 221-233, **2016**
- [98] Gerritsen-van Schieveen, P., Royer, B., Therapeutic drug monitoring group of the French Society of, P., and Therapeutics. Level of evidence for therapeutic drug monitoring of taxanes, *Fundam Clin Pharmacol* 25, 414-424, **2011**
- [99] Zhu, D., Tao, W., Zhang, H., Liu, G., Wang, T., Zhang, L., Zeng, X., and Mei, L. Docetaxel (DTX)-loaded polydopamine-modified TPGS-PLA nanoparticles as a targeted drug delivery system for the treatment of liver cancer, *Acta Biomater* 30, 144-154, **2016**
- [100] Kim, Y. S., Lee, S. I., Park, S. H., Park, S., Hwang, I. G., Lee, S. C., Sun, J. M., Lee, J., and Lim, H. Y. A Phase II Study of Weekly Docetaxel as Second-Line Chemotherapy in Patients With Metastatic Urothelial Carcinoma, *Clin Genitourin Cancer* 14, 76-81, **2016**
- [101] Kavoi, B. M., Plendl, J., Makanya, A. N., Ochieng, S., and Kiama, S. G. Effects of anticancer drug docetaxel on the structure and function of the rabbit olfactory mucosa, *Tissue Cell* 46, 213-224, **2014**
- [102] Roche, H., Fumoleau, P., Spielmann, M., Canon, J. L., Delozier, T., Serin, D., Symann, M., Kerbrat, P., Soulie, P., Eichler, F., Viens, P., Monnier, A., Vindevoghel, A., Campone, M., Goudier, M. J., Bonnetterre, J., Ferrero, J. M., Martin, A. L., Geneve, J., and Asselain, B. Sequential adjuvant epirubicin-based and docetaxel chemotherapy for node-positive breast cancer patients: the FNCLCC PACS 01 Trial, *J Clin Oncol* 24, 5664-5671, **2006**
- [103] Rivera, E., Mejia, J. A., Arun, B. K., Adinin, R. B., Walters, R. S., Brewster, A., Broglio, K. R., Yin, G. S., Esmaeli, B., Hortobagyi, G. N., and Valero, V. Phase 3 study comparing the use of docetaxel on an every-3-week versus weekly schedule in the treatment of metastatic breast cancer, *Cancer* 112, 1455-1461, **2008**
- [104] Berthold, D. R., Pond, G. R., Soban, F., de Wit, R., Eisenberger, M., and Tannock, I. F. Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer: updated survival in the TAX 327 study, *J Clin Oncol* 26, 242-245, **2008**

- [105] Tannock, I. F., de Wit, R., Berry, W. R., Horti, J., Pluzanska, A., Chi, K. N., Oudard, S., Theodore, C., James, N. D., Turesson, I., Rosenthal, M. A., Eisenberger, M. A., and Investigators, T. A. X. Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer, *N Engl J Med* 351, 1502-1512, **2004**
- [106] Wang, J. W., Xu, R. H., Li, J., Bai, Y. X., Liu, T. S., Jiao, S. C., Dai, G. H., Xu, J. M., Liu, Y. P., Fan, N. F., Shu, Y. Q., Ba, Y., Ma, D., Qin, S. K., Zheng, L. Z., Chen, W. C., and Shen, L. Randomized multicenter phase III study of a modified docetaxel and cisplatin plus fluorouracil regimen compared with cisplatin and fluorouracil as first-line therapy for advanced or locally recurrent gastric cancer, *Gastric Cancer* 19, 234-244, **2016**
- [107] Van Cutsem, E., Moiseyenko, V. M., Tjulandin, S., Majlis, A., Constenla, M., Boni, C., Rodrigues, A., Fodor, M., Chao, Y., Voznyi, E., Risse, M. L., and Ajani, J. A. Phase III study of docetaxel and cisplatin plus fluorouracil compared with cisplatin and fluorouracil as first-line therapy for advanced gastric cancer: A report of the V325 study group, *Journal of Clinical Oncology* 24, 4991-4997, **2006**
- [108] Takacs-Nagy, Z., Hitre, E., Remenar, E., Oberna, F., Polgar, C., Major, T., Godeny, M., Fodor, J., and Kasler, M. Docetaxel, cisplatin and 5-fluorouracil induction chemotherapy followed by chemoradiotherapy or chemoradiotherapy alone in stage III-IV unresectable head and neck cancer: Results of a randomized phase II study, *Strahlenther Onkol* 191, 635-641, **2015**
- [109] Posner, M. R., Hershock, D. M., Blajman, C. R., Mickiewicz, E., Winquist, E., Gorbounova, V., Tjulandin, S., Shin, D. M., Cullen, K., Ervin, T. J., Murphy, B. A., Raez, L. E., Cohen, R. B., Spaulding, M., Tishler, R. B., Roth, B., Viroglia Rdel, C., Venkatesan, V., Romanov, I., Agarwala, S., Harter, K. W., Dugan, M., Cmhlak, A., Markoe, A. M., Read, P. W., Steinbrenner, L., Colevas, A. D., Norris, C. M., Jr., Haddad, R. I., and Group, T. A. X. S. Cisplatin and fluorouracil alone or with docetaxel in head and neck cancer, *N Engl J Med* 357, 1705-1715, **2007**
- [110] Ganesh, T. Improved biochemical strategies for targeted delivery of taxoids, *Bioorg Med Chem* 15, 3597-3623, **2007**
- [111] Manjappa, A. S., Goel, P. N., Vekataraju, M. P., Rajesh, K. S., Makwana, K., Ukawala, M., Nikam, Y., Gude, R. P., and Murthy, R. S. Is an alternative drug delivery system needed for docetaxel? The role of controlling epimerization in formulations and beyond, *Pharm Res* 30, 2675-2693, **2013**
- [112] Montero, A., Fossella, F., Hortobagyi, G., and Valero, V. Docetaxel for treatment of solid tumours: a systematic review of clinical data, *Lancet Oncol* 6, 229-239, **2005**
- [113] Zhang, L., and Zhang, N. How nanotechnology can enhance docetaxel therapy, *Int J Nanomed* 8, 2927-2941, **2013**
- [114] Elsabahy, M., and Wooley, K. L. Design of polymeric nanoparticles for biomedical delivery applications, *Chem Soc Rev* 41, 2545-2561, **2012**
- [115] Kamaly, N., Xiao, Z., Valencia, P. M., Radovic-Moreno, A. F., and Farokhzad, O. C. Targeted polymeric therapeutic nanoparticles: design, development and clinical translation, *Chem Soc Rev* 41, 2971-3010, **2012**
- [116] Farokhzad, O. C., Cheng, J., Teply, B. A., Sherifi, I., Jon, S., Kantoff, P. W., Richie, J. P., and Langer, R. Targeted nanoparticle-aptamer bioconjugates

- for cancer chemotherapy in vivo, *Proc Natl Acad Sci U S A* 103, 6315-6320, **2006**
- [117] Hrkach, J., Von Hoff, D., Mukkaram Ali, M., Andrianova, E., Auer, J., Campbell, T., De Witt, D., Figa, M., Figueiredo, M., Horhota, A., Low, S., McDonnell, K., Peeke, E., Retnarajan, B., Sabnis, A., Schnipper, E., Song, J. J., Song, Y. H., Summa, J., Tompsett, D., Troiano, G., Van Geen Hoven, T., Wright, J., LoRusso, P., Kantoff, P. W., Bander, N. H., Sweeney, C., Farokhzad, O. C., Langer, R., and Zale, S. Preclinical development and clinical translation of a PSMA-targeted docetaxel nanoparticle with a differentiated pharmacological profile, *Sci Transl Med* 4, 128ra139, **2012**
 - [118] Sinha, V. R., Bansal, K., Kaushik, R., Kumria, R., and Trehan, A. Poly-epsilon-caprolactone microspheres and nanospheres: an overview, *Int J Pharm* 278, 1-23, **2004**
 - [119] Abdolmohammadi, S., Siyamak, S., Ibrahim, N. A., Yunus, W. M., Rahman, M. Z., Azizi, S., and Fatehi, A. Enhancement of mechanical and thermal properties of polycaprolactone/chitosan blend by calcium carbonate nanoparticles, *Int J Mol Sci* 13, 4508-4522, **2012**
 - [120] Wikimedia Foundation, I. Polycaprolactone, <https://en.wikipedia.org/wiki/Polycaprolactone> (13/11/2015)
 - [121] Woodruff, M. A., andHutmacher, D. W. The return of a forgotten polymer- Polycaprolactone in the 21st century, *Progress in Polymer Science* 35, 1217-1256, **2010**
 - [122] Coulembier, O., Degee, P., Hedrick, J. L., and Dubois, P. From controlled ring-opening polymerization to biodegradable aliphatic polyester: Especially poly(beta-malic acid) derivatives, *Progress in Polymer Science* 31, 723-747, **2006**
 - [123] Dash, T. K., and Konkimalla, V. B. Polymeric modification and its implication in drug delivery: poly-epsilon-caprolactone (PCL) as a model polymer, *Mol Pharm* 9, 2365-2379, **2012**
 - [124] Dash, T. K., and Konkimalla, V. B. Poly-small je, Ukrainian-caprolactone based formulations for drug delivery and tissue engineering: A review, *J Control Release* 158, 15-33, **2012**
 - [125] Song, X., Wang, J., Luo, X., Xu, C., Zhu, A., Guo, R., Yan, C., and Zhu, P. Synthesis, biocompatible, and self-assembly properties of poly (ethylene glycol)/lactobionic acid-grafted chitosan, *J Biomater Sci Polym Ed* 25, 1062-1075, **2014**
 - [126] Sigma-Aldrich Poly(ethylene glycol) and Poly(ethylene oxide), <https://www.sigmaaldrich.com/materials-science/material-science-products.html?TablePage=20204110> (21/02/2016)
 - [127] Library, W. O. Polyethylene glycol, pp 247-270, The MAK Collection for Occupational Health and Safety.(2012)
 - [128] Veronese, F. M., Schiavon, O., Pasut, G., Mendichi, R., Andersson, L., Tsirk, A., Ford, J., Wu, G., Kneller, S., Davies, J., and Duncan, R. PEG-doxorubicin conjugates: influence of polymer structure on drug release, in vitro cytotoxicity, biodistribution, and antitumor activity, *Bioconjug Chem* 16, 775-784, **2005**
 - [129] Greenwald, R. B., Choe, Y. H., McGuire, J., and Conover, C. D. Effective drug delivery by PEGylated drug conjugates, *Adv Drug Deliv Rev* 55, 217-250, **2003**

- [130] Plesner, B., Fee, C. J., Westh, P., and Nielsen, A. D. Effects of PEG size on structure, function and stability of PEGylated BSA, *Eur J Pharm Biopharm* 79, 399-405, **2011**
- [131] Yang, J., Lv, J., Behl, M., Lendlein, A., Yang, D., Zhang, L., Shi, C., Guo, J., and Feng, Y. Functionalization of polycarbonate surfaces by grafting PEG and zwitterionic polymers with a multicombed structure, *Macromol Biosci* 13, 1681-1688, **2013**
- [132] Vukovic, L., Khatib, F. A., Drake, S. P., Madriaga, A., Brandenburg, K. S., Kral, P., and Onyuksel, H. Structure and dynamics of highly PEG-ylated sterically stabilized micelles in aqueous media, *J Am Chem Soc* 133, 13481-13488, **2011**
- [133] Kurella, A., and Dahotre, N. B. Review paper: Surface modification for bioimplants: The role of laser surface engineering, *Journal of Biomaterials Applications* 20, 5-50, **2005**
- [134] Wang, Y. Y., Lai, S. K., Suk, J. S., Pace, A., Cone, R., and Hanes, J. Addressing the PEG Mucoadhesivity Paradox to Engineer Nanoparticles that "Slip" through the Human Mucus Barrier, *Angew Chem Int Edit* 47, 9726-9729, **2008**
- [135] Zhang, X., Chen, G., Zhang, T., Ma, Z., and Wu, B. Effects of PEGylated lipid nanoparticles on the oral absorption of one BCS II drug: a mechanistic investigation, *Int J Nanomedicine* 9, 5503-5514, **2014**
- [136] Lertsutthiwong, P., Rojsitthisak, P., and Nimmannit, U. Preparation of turmeric oil-loaded chitosan-alginate biopolymeric nanocapsules, *Mat Sci Eng C-Bio S* 29, 856-860, **2009**
- [137] Sharma, G., Sharma, A. R., Nam, J. S., Doss, G. P., Lee, S. S., and Chakraborty, C. Nanoparticle based insulin delivery system: the next generation efficient therapy for Type 1 diabetes, *J Nanobiotechnology* 13, 74, **2015**
- [138] Liu, M., Zhang, J., Zhu, X., Shan, W., Li, L., Zhong, J., Zhang, Z., and Huang, Y. Efficient mucus permeation and tight junction opening by dissociable "mucus-inert" agent coated trimethyl chitosan nanoparticles for oral insulin delivery, *J Control Release* 222, 67-77, **2016**
- [139] Wang, K., Lin, S., Nune, K. C., and Misra, R. D. Chitosan-gelatin-based microgel for sustained drug delivery, *J Biomater Sci Polym Ed* 27, 441-453, **2016**
- [140] Agnihotri, S. A., Mallikarjuna, N. N., and Aminabhavi, T. M. Recent advances on chitosan-based micro- and nanoparticles in drug delivery, *Journal of Controlled Release* 100, 5-28, **2004**
- [141] Mao, S., Shuai, X., Unger, F., Simon, M., Bi, D., and Kissel, T. The depolymerization of chitosan: effects on physicochemical and biological properties, *Int J Pharm* 281, 45-54, **2004**
- [142] Frank, L. A., Sandri, G., D'Autilia, F., Contri, R. V., Bonferoni, M. C., Caramella, C., Frank, A. G., Pohlmann, A. R., and Guterres, S. S. Chitosan gel containing polymeric nanocapsules: a new formulation for vaginal drug delivery, *Int J Nanomedicine* 9, 3151-3161, **2014**
- [143] Rafique, A., Zia, K. M., Zuber, M., Tabasum, S., and Rehman, S. Chitosan functionalized poly(vinyl alcohol) for prospects biomedical and industrial applications: A review, *Int J Biol Macromol*, **2016**
- [144] Prego, C., Torres, D., and Alonso, M. J. The potential of chitosan for the oral administration of peptides, *Expert Opin Drug Deliv* 2, 843-854, **2005**

- [145] Chronopoulou, L., Nocca, G., Castagnola, M., Paludetti, G., Ortaggi, G., Sciubba, F., Bevilacqua, M., Lupi, A., Gambarini, G., and Palocci, C. Chitosan based nanoparticles functionalized with peptidomimetic derivatives for oral drug delivery, *N Biotechnol* 33, 23-31, **2016**
- [146] Ramalingam, P., and Ko, Y. T. Improved oral delivery of resveratrol from N-trimethyl chitosan-g-palmitic acid surface-modified solid lipid nanoparticles, *Colloids Surf B Biointerfaces* 139, 52-61, **2016**
- [147] Prego, C., Torres, D., Fernandez-Megia, E., Novoa-Carballal, R., Quinoa, E., and Alonso, M. J. Chitosan-PEG nanocapsules as new carriers for oral peptide delivery - Effect of chitosan pegylation degree, *Journal of Controlled Release* 111, 299-308, **2006**
- [148] Rinaudo, M. Chitin and chitosan: Properties and applications, *Prog Polym Sci* 31, 603-632, **2006**
- [149] Li, J., Jiang, C., Lang, X., Kong, M., Cheng, X., Liu, Y., Feng, C., and Chen, X. Multilayer sodium alginate beads with porous core containing chitosan based nanoparticles for oral delivery of anticancer drug, *Int J Biol Macromol* 85, 1-8, **2016**
- [150] Li, P. W., Wang, G., Yang, Z. M., Duan, W., Peng, Z., Kong, L. X., and Wang, Q. H. Development of drug-loaded chitosan-vanillin nanoparticles and its cytotoxicity against HT-29 cells, *Drug Deliv* 23, 30-35, **2016**
- [151] Younes, I., and Rinaudo, M. Chitin and chitosan preparation from marine sources. Structure, properties and applications, *Mar Drugs* 13, 1133-1174, **2015**
- [152] McPolin, O. *Validation of Analytical Methods for Pharmaceutical Analysis*, Validation of Analytical Methods for Pharmaceuticla Analysis, UK, **2009**
- [153] Huber, L. Validation of Analytical Methods, (Technologies, A., Ed.), Agilent Technologies, Germany.(2010)
- [154] Agency, E. M. Validation of Analytical Procedures: Text and Methodology, (Agency, E. M., Ed.).(1995)
- [155] Mosqueira, V. C., Legrand, P., Morgat, J. L., Vert, M., Mysiakine, E., Gref, R., Devissaguet, J. P., and Barratt, G. Biodistribution of long-circulating PEG-grafted nanocapsules in mice: effects of PEG chain length and density, *Pharm Res* 18, 1411-1419, **2001**
- [156] Garcia, G. M., Oliveira, L. T., Pitta Ida, R., de Lima Mdo, C., Vilela, J. M., Andrade, M. S., Abdalla, D. S., and Mosqueira, V. C. Improved nonclinical pharmacokinetics and biodistribution of a new PPAR pan-agonist and COX inhibitor in nanocapsule formulation, *J Control Release* 209, 207-218, **2015**
- [157] Moinard-Checot, D., Chevalier, Y., Briancon, S., Beney, L., and Fessi, H. Mechanism of nanocapsules formation by the emulsion-diffusion process, *J Colloid Interface Sci* 317, 458-468, **2008**
- [158] Sartori, T., Seigi Murakami, F., Pinheiro Cruz, A., and Machado de Campos, A. Development and validation of a fast RP-HPLC method for determination of methotrexate entrapment efficiency in polymeric nanocapsules, *J Chromatogr Sci* 46, 505-509, **2008**
- [159] Bilensoy, E., Gurkaynak, O., Dogan, A. L., and Hincal, A. A. Safety and efficacy of amphiphilic beta-cyclodextrin nanoparticles for paclitaxel delivery, *Int J Pharm* 347, 163-170, **2008**
- [160] Bilensoy, E., Sarisozen, C., Esendagli, G., Dogan, A. L., Aktas, Y., Sen, M., and Mungan, N. A. Intravesical cationic nanoparticles of chitosan and

- polycaprolactone for the delivery of Mitomycin C to bladder tumors, *Int J Pharm* 371, 170-176, **2009**
- [161] Croda. Span and Tween, (Care, C. H., Ed.), pp 1-6, Croda Europe Ltd, England
- [162] Sasol *Excipients for pharmaceuticals*, Germany,
- [163] nanotubes to evade opsonization and improve biodistribution, *Int J Nanomedicine* 9 Suppl 1, 85-105, **2014**
- [164] Meng, H., Xue, M., Xia, T., Ji, Z. X., Tarn, D. Y., Zink, J. I., and Nel, A. E. Use of Size and a Copolymer Design Feature To Improve the Biodistribution and the Enhanced Permeability and Retention Effect of Doxorubicin-Loaded Mesoporous Silica Nanoparticles in a Murine Xenograft Tumor Model, *Acs Nano* 5, 4131-4144, **2011**
- [165] Gao, D., Xu, H., Philbert, M. A., and Kopelman, R. Bioeliminable Nanohydrogels for Drug Delivery, *Nano Lett* 8, 3320-3324, **2008**
- [166] Owens, D. E., 3rd, and Peppas, N. A. Opsonization, biodistribution, and pharmacokinetics of polymeric nanoparticles, *Int J Pharm* 307, 93-102, **2006**
- [167] Liu, X., Li, H., Chen, Y., Jin, Q., Ren, K., and Ji, J. Mixed-charge nanoparticles for long circulation, low reticuloendothelial system clearance, and high tumor accumulation, *Adv Healthc Mater* 3, 1439-1447, **2014**
- [168] Shah, N. B., Vercellotti, G. M., White, J. G., Fegan, A., Wagner, C. R., and Bischof, J. C. Blood-nanoparticle interactions and in vivo biodistribution: impact of surface PEG and ligand properties, *Mol Pharm* 9, 2146-2155, **2012**
- [169] Xu, Q., Ensign, L. M., Boylan, N. J., Schon, A., Gong, X., Yang, J. C., Lamb, N. W., Cai, S., Yu, T., Freire, E., and Hanes, J. Impact of Surface Polyethylene Glycol (PEG) Density on Biodegradable Nanoparticle Transport in Mucus ex Vivo and Distribution in Vivo, *Acs Nano* 9, 9217-9227, **2015**
- [170] Parveen, S., and Sahoo, S. K. Long circulating chitosan/PEG blended PLGA nanoparticle for tumor drug delivery, *Eur J Pharmacol* 670, 372-383, **2011**
- [171] Amiji, M., Tailor, R., Ly, M. K., and Goreham, J. Gelatin poly(ethylene oxide) semi-interpenetrating polymer network with pH-sensitive swelling and enzyme-degradable properties for oral drug delivery, *Drug Dev Ind Pharm* 23, 575-582, **1997**
- [172] Hu, F. Q., Meng, P., Dai, Y. Q., Du, Y. Z., You, J., Wei, X. H., and Yuan, H. PEGylated chitosan-based polymer micelle as an intracellular delivery carrier for anti-tumor targeting therapy, *Eur J Pharm Biopharm* 70, 749-757, **2008**
- [173] Sawaengsak, C., Mori, Y., Yamanishi, K., Mitrevej, A., and Sinchaipanid, N. Chitosan nanoparticle encapsulated hemagglutinin-split influenza virus mucosal vaccine, *AAPS PharmSciTech* 15, 317-325, **2014**
- [174] Kashyap, P. L., Xiang, X., and Heiden, P. Chitosan nanoparticle based delivery systems for sustainable agriculture, *Int J Biol Macromol* 77, 36-51, **2015**
- [175] Nagpal, K., Singh, S. K., and Mishra, D. N. Chitosan nanoparticles: a promising system in novel drug delivery, *Chem Pharm Bull (Tokyo)* 58, 1423-1430, **2010**

- [176] Roger, E., Lagarce, F., and Benoit, J. P. The gastrointestinal stability of lipid nanocapsules, *Int J Pharmaceut* 379, 260-265, **2009**
- [177] Sigma-Aldrich. Biofiles MTT Cell Viability Applications, (Sigma-Aldrich, Ed.), p 18, Sigma
- [178] Masungi, C., Borremans, C., Willems, B., Mensch, J., Van Dijck, A., Augustijns, P., Brewster, M. E., and Noppe, M. Usefulness of a novel Caco-2 cell perfusion system. I. In vitro prediction of the absorption potential of passively diffused compounds, *J Pharm Sci* 93, 2507-2521, **2004**
- [179] Dias, L. C., Custodio, R., and Pessine, F. B. T. Investigation of the Nile Red spectra by semi-empirical calculations and spectrophotometric measurements, *Int J Quantum Chem* 106, 2624-2632, **2006**
- [180] Primavera, R., Di Francesco, M., De Cola, A., De Laurenzi, V., Paolino, D., Ciancaioni, M., Carafa, M., Celia, C., Di Ilio, C., Di Stefano, A., Fresta, M., Locatelli, M., and Di Marzio, L. HPLC-FLD and spectrofluorometer apparatus: How to best detect fluorescent probe-loaded niosomes in biological samples, *Colloids Surf B Biointerfaces* 135, 575-580, **2015**
- [181] Mandal, T. K., and Bostanian, L. A. Effect of peptide loading and surfactant concentration on the characteristics of physically crosslinked hydrogel, *Pharm Dev Technol* 5, 555-560, **2000**
- [182] Peters, F. T., Drummer, O. H., and Musshoff, F. Validation of new methods, *Forensic Sci Int* 165, 216-224, **2007**
- [183] Araujo, P. Key aspects of analytical method validation and linearity evaluation, *J Chromatogr B* 877, 2224-2234, **2009**
- [184] Shah, V. P., Midha, K. K., Findlay, J. W., Hill, H. M., Hulse, J. D., McGilveray, I. J., McKay, G., Miller, K. J., Patnaik, R. N., Powell, M. L., Tonelli, A., Viswanathan, C. T., and Yacobi, A. Bioanalytical method validation--a revisit with a decade of progress, *Pharm Res* 17, 1551-1557, **2000**
- [185] Fuster, J., Negro, S., Salama, A., Fernandez-Carballido, A., Marcianes, P., Boeva, L., and Barcia, E. HPLC-UV method development and validation for the quantification of ropinirole in new PLGA multiparticulate systems: Microspheres and nanoparticles, *Int J Pharm* 491, 310-317, **2015**
- [186] Shabir, G. A. Validation of high-performance liquid chromatography methods for pharmaceutical analysis. Understanding the differences and similarities between validation requirements of the US Food and Drug Administration, the US Pharmacopeia and the International Conference on Harmonization, *J Chromatogr A* 987, 57-66, **2003**
- [187] Taverniers, I., De Loose, M., and Van Bockstaele, E. Trends in quality in the analytical laboratory. II. Analytical method validation and quality assurance, *Trac-Trend Anal Chem* 23, 535-552, **2004**
- [188] Chen, G., Mo, L., Lin, F., Zhang, X., Liu, J., Wang, H., and Yang, C. Development, validation and application of an HPLC method for reduced vitamin C qualification in HBOCs solution, *Artif Cells Nanomed Biotechnol* 44, 456-461, **2016**
- [189] Poletto, F. S., Fiel, L. A., Donida, B., Re, M. I., Guterres, S. S., and Pohlmann, A. R. Controlling the size of poly(hydroxybutyrate-co-hydroxyvalerate) nanoparticles prepared by emulsification-diffusion technique using ethanol as surface agent, *Colloid Surface A* 324, 105-112, **2008**

- [190] Gaspar, M. M., Blanco, D., Cruz, M. E. M., and Alonso, M. J. Formulation of L-asparaginase-loaded poly(lactide-co-glycolide) nanoparticles: influence of polymer properties on enzyme loading, activity and in vitro release, *Journal of Controlled Release* 52, 53-62, **1998**
- [191] Baran, E. T., Ozer, N., and Hasirci, V. Poly(hydroxybutyrate-co-hydroxyvalerate) nanocapsules as enzyme carriers for cancer therapy: an in vitro study, *Journal of Microencapsulation* 19, 363-376, **2002**
- [192] Choi, J. Y., Park, C. H., and Lee, J. Effect of polymer molecular weight on nanocomminution of poorly soluble drug, *Drug Delivery* 15, 347-353, **2008**
- [193] Cheng, J., Teply, B. A., Sherifi, I., Sung, J., Luther, G., Gu, F. X., Levy-Nissenbaum, E., Radovic-Moreno, A. F., Langer, R., and Farokhzad, O. C. Formulation of functionalized PLGA-PEG nanoparticles for in vivo targeted drug delivery, *Biomaterials* 28, 869-876, **2007**
- [194] Mainardes, R. M., and Evangelista, R. C. PLGA nanoparticles containing praziquantel: effect of formulation variables on size distribution, *Int J Pharmaceut* 290, 137-144, **2005**
- [195] Budhian, A., Siegel, S. J., and Winey, K. I. Production of haloperidol-loaded PLGA nanoparticles for extended controlled drug release of haloperidol, *J Microencapsul* 22, 773-785, **2005**
- [196] Noori Koopaei, M., Khoshayand, M. R., Mostafavi, S. H., Amini, M., Khorramizadeh, M. R., Jeddi Tehrani, M., Atyabi, F., and Dinarvand, R. Docetaxel Loaded PEG-PLGA Nanoparticles: Optimized Drug Loading, In-vitro Cytotoxicity and In-vivo Antitumor Effect, *Iran J Pharm Res* 13, 819-833, **2014**
- [197] Cauchetier, E., Deniau, M., Fessi, H., Astier, A., and Paul, M. Atovaquone-loaded nanocapsules: influence of the nature of the polymer on their in vitro characteristics, *Int J Pharmaceut* 250, 273-281, **2003**
- [198] Li, X., Qin, Y., Liu, C., Jiang, S., Xiong, L., and Sun, Q. Size-controlled starch nanoparticles prepared by self-assembly with different green surfactant: The effect of electrostatic repulsion or steric hindrance, *Food Chem* 199, 356-363, **2016**
- [199] Gutierrez, J. M., Gonzalez, C., Maestro, A., Sole, I., Pey, C. M., and Nolla, J. Nano-emulsions: New applications and optimization of their preparation, *Curr Opin Colloid In* 13, 245-251, **2008**
- [200] Wang, H., Jia, Y., Hu, W., Jiang, H., Zhang, J., and Zhang, L. Effect of preparation conditions on the size and encapsulation properties of mPEG-PLGA nanoparticles simultaneously loaded with vincristine sulfate and curcumin, *Pharm Dev Technol* 18, 694-700, **2013**
- [201] Mu, L., and Feng, S. S. A novel controlled release formulation for the anticancer drug paclitaxel (Taxol (R)): PLGA nanoparticles containing vitamin E TPGS, *Journal of Controlled Release* 86, 33-48, **2003**
- [202] VasanthaKumar, S., Ahamed, H. N., and Saha, R. N. Nanomedicine I: In vitro and in vivo evaluation of paclitaxel loaded poly-(epsilon-caprolactone), poly (DL-lactide-co-glycolide) and poly (DL-lactic acid) matrix nanoparticles in wistar rats, *Eur J Drug Metab Pharmacokinet* 40, 137-161, **2015**
- [203] Unal, H., Ozturk, N., and Bilensoy, E. Formulation development, stability and anticancer efficacy of core-shell cyclodextrin nanocapsules for oral chemotherapy with camptothecin, *Beilstein Journal of Organic Chemistry* 11, 204-212, **2015**

- [204] Kshirsagar, S. J., Bhalekar, M. R., Patel, J. N., Mohapatra, S. K., and Shewale, N. S. Preparation and characterization of nanocapsules for colon-targeted drug delivery system, *Pharm Dev Technol* 17, 607-613, **2012**
- [205] Blouza, I. L., Charcosset, C., Sfar, S., and Fessi, H. Preparation and characterization of spironolactone-loaded nanocapsules for paediatric use, *Int J Pharmaceut* 325, 124-131, **2006**
- [206] Venkatesh, D. N., Baskaran, M., Karri, V. V., Mannemala, S. S., Radhakrishna, K., and Goti, S. Fabrication and in vivo evaluation of Nelfinavir loaded PLGA nanoparticles for enhancing oral bioavailability and therapeutic effect, *Saudi Pharm J* 23, 667-674, **2015**
- [207] Peroche, S., Degobert, G., Putaux, J. L., Blanchin, M. G., Fessi, H., and Parrot-Lopez, H. Synthesis and characterisation of novel nanospheres made from amphiphilic perfluoroalkylthio-beta-cyclodextrins, *Eur J Pharm Biopharm* 60, 123-131, **2005**
- [208] Galindo-Rodriguez, S. A., Puel, F., Briancon, S., Allemann, E., Doelker, E., and Fessi, H. Comparative scale-up of three methods for producing ibuprofen-loaded nanoparticles, *Eur J Pharm Sci* 25, 357-367, **2005**
- [209] Salatin, S., Maleki Dizaj, S., and Yari Khosroushahi, A. Effect of the surface modification, size, and shape on cellular uptake of nanoparticles, *Cell Biol Int* 39, 881-890, **2015**
- [210] He, C. B., Hu, Y. P., Yin, L. C., Tang, C., and Yin, C. H. Effects of particle size and surface charge on cellular uptake and biodistribution of polymeric nanoparticles, *Biomaterials* 31, 3657-3666, **2010**
- [211] Groo, A. C., Saulnier, P., Gimel, J. C., Gravier, J., Ailhas, C., Benoit, J. P., and Lagarce, F. Fate of paclitaxel lipid nanocapsules in intestinal mucus in view of their oral delivery, *Int J Nanomedicine* 8, 4291-4302, **2013**
- [212] Xu, Y. M., Du, Y. M., Huang, R. H., and Gao, L. P. Preparation and modification of N-(2-hydroxyl) propyl-3-trimethyl ammonium chitosan chloride nanoparticle as a protein carrier, *Biomaterials* 24, 5015-5022, **2003**
- [213] Spek, S., Haeuser, M., Schaefer, M. M., and Langer, K. Characterisation of PEGylated PLGA nanoparticles comparing the nanoparticle bulk to the particle surface using UV/vis spectroscopy, SEC, H-1 NMR spectroscopy, and X-ray photoelectron spectroscopy, *Appl Surf Sci* 347, 378-385, **2015**
- [214] Lobato, K. B., Paese, K., Forgearini, J. C., Guterres, S. S., Jablonski, A., and Rios Ade, O. Characterisation and stability evaluation of bixin nanocapsules, *Food Chem* 141, 3906-3912, **2013**
- [215] Eloy, J. O., Petrilli, R., Topan, J. F., Antonio, H. M., Barcellos, J. P., Chesca, D. L., Serafini, L. N., Tiezzi, D. G., Lee, R. J., and Marchetti, J. M. Co-loaded paclitaxel/rapamycin liposomes: Development, characterization and in vitro and in vivo evaluation for breast cancer therapy, *Colloids Surf B Biointerfaces* 141, 74-82, **2016**
- [216] Leroy, P., Tournassat, C., Bernard, O., Devau, N., and Azaroual, M. The electrophoretic mobility of montmorillonite. Zeta potential and surface conductivity effects, *J Colloid Interface Sci* 451, 21-39, **2015**
- [217] Sabuncu, A. C., Grubbs, J., Qian, S., Abdel-Fattah, T. M., Stacey, M. W., and Beskok, A. Probing nanoparticle interactions in cell culture media, *Colloids Surf B Biointerfaces* 95, 96-102, **2012**
- [218] Nie, S. Understanding and overcoming major barriers in cancer nanomedicine, *Nanomedicine (Lond)* 5, 523-528, **2010**

- [219] Yang, Y., Gao, Y. L., and Mei, X. G. Effects of formulation parameters on encapsulation efficiency and release behavior of thienorphine loaded PLGA microspheres, *Pharmaceutical Development and Technology* 18, 1169-1174, **2013**
- [220] Su, Z. X., Sun, F. Y., Shi, Y. N., Jiang, C. J., Meng, Q. F., Teng, L. R., and Li, Y. X. Effects of Formulation Parameters on Encapsulation Efficiency and Release Behavior of Risperidone Poly(D, L-lactide-co-glycolide) Microsphere, *Chem Pharm Bull* 57, 1251-1256, **2009**
- [221] Masarudin, M. J., Cutts, S. M., Evison, B. J., Phillips, D. R., and Pigram, P. J. Factors determining the stability, size distribution, and cellular accumulation of small, monodisperse chitosan nanoparticles as candidate vectors for anticancer drug delivery: application to the passive encapsulation of [(14)C]-doxorubicin, *Nanotechnol Sci Appl* 8, 67-80, **2015**
- [222] Boegh, M., and Nielsen, H. M. Mucus as a barrier to drug delivery - understanding and mimicking the barrier properties, *Basic Clin Pharmacol Toxicol* 116, 179-186, **2015**
- [223] Pridgen, E. M., Alexis, F., and Farokhzad, O. C. Polymeric nanoparticle technologies for oral drug delivery, *Clin Gastroenterol Hepatol* 12, 1605-1610, **2014**
- [224] Wilcox, M. D., Van Rooij, L. K., Chater, P. I., Pereira de Sousa, I., and Pearson, J. P. The effect of nanoparticle permeation on the bulk rheological properties of mucus from the small intestine, *Eur J Pharm Biopharm* 96, 484-487, **2015**
- [225] Desai, D., Prabhakar, N., Mamaeva, V., Karaman, D. S., Lahdeniemi, I. A., Sahlgren, C., Rosenholm, J. M., and Toivola, D. M. Targeted modulation of cell differentiation in distinct regions of the gastrointestinal tract via oral administration of differently PEG-PEI functionalized mesoporous silica nanoparticles, *Int J Nanomedicine* 11, 299-313, **2016**
- [226] D'Souza, A. A., and Devarajan, P. V. Bioenhanced oral curcumin nanoparticles: Role of carbohydrates, *Carbohydr Polym* 136, 1251-1258, **2016**
- [227] des Rieux, A., Fievez, V., Garinot, M., Schneider, Y. J., and Preat, V. Nanoparticles as potential oral delivery systems of proteins and vaccines: A mechanistic approach, *Journal of Controlled Release* 116, 1-27, **2006**
- [228] Crater, J. S., and Carrier, R. L. Barrier properties of gastrointestinal mucus to nanoparticle transport, *Macromol Biosci* 10, 1473-1483, **2010**
- [229] Svensson, O., Thuresson, K., and Arnebrant, T. Interactions between chitosan-modified particles and mucin-coated surfaces, *J Colloid Interface Sci* 325, 346-350, **2008**
- [230] Fan, B., Xing, Y., Zheng, Y., Sun, C., and Liang, G. pH-responsive thiolated chitosan nanoparticles for oral low-molecular weight heparin delivery: in vitro and in vivo evaluation, *Drug Deliv* 23, 238-247, **2016**
- [231] Aljayyousi, G., Abdulkarim, M., Griffiths, P., and Gumbleton, M. Pharmaceutical nanoparticles and the mucin biopolymer barrier, *Bioimpacts* 2, 173-174, **2012**
- [232] de Campos, A. M., Diebold, Y., Carvalho, E. L. S., Sanchez, A., and Alonso, M. J. Chitosan nanoparticles as new ocular drug delivery systems: in vitro stability, in vivo fate, and cellular toxicity, *Pharm Res-Dordr* 21, 803-810, **2004**

- [233] de Souza, T. D., Ziembowicz, F. I., Muller, D. F., Lauermann, S. C., Kloster, C. L., Santos, R. C., Lopes, L. Q., Ourique, A. F., Machado, G., and Villetti, M. A. Evaluation of photodynamic activity, photostability and in vitro drug release of zinc phthalocyanine-loaded nanocapsules, *Eur J Pharm Sci* 83, 88-98, **2016**
- [234] Groo, A. C., Mircheva, K., Bejaud, J., Ailhas, C., Panaiotov, I., Saulnier, P., Ivanova, T., and Lagarce, F. Development of 2D and 3D mucus models and their interactions with mucus-penetrating paclitaxel-loaded lipid nanocapsules, *Pharm Res* 31, 1753-1765, **2014**
- [235] Peppas, N. A., and Huang, Y. B. Nanoscale technology of mucoadhesive interactions, *Adv Drug Deliver Rev* 56, 1675-1687, **2004**
- [236] da Silva, S. B., Ferreira, D., Pintado, M., and Sarmiento, B. Chitosan-based nanoparticles for rosmarinic acid ocular delivery-In vitro tests, *Int J Biol Macromol* 84, 112-120, **2016**
- [237] Modi, J., Joshi, G., and Sawant, K. Chitosan based mucoadhesive nanoparticles of ketoconazole for bioavailability enhancement: formulation, optimization, in vitro and ex vivo evaluation, *Drug Dev Ind Pharm* 39, 540-547, **2013**
- [238] Blanchette, J., Kavimandan, N., and Peppas, N. A. Principles of transmucosal delivery of therapeutic agents, *Biomedicine & Pharmacotherapy* 58, 142-151, **2004**
- [239] Ye, Y. J., Wang, Y., Lou, K. Y., Chen, Y. Z., Chen, R., and Gao, F. The preparation, characterization, and pharmacokinetic studies of chitosan nanoparticles loaded with paclitaxel/dimethyl-beta-cyclodextrin inclusion complexes, *Int J Nanomedicine* 10, 4309-4319, **2015**
- [240] Hwang, H. Y., Kim, I. S., Kwon, I. C., and Kim, Y. H. Tumor targetability and antitumor effect of docetaxel-loaded hydrophobically modified glycol chitosan nanoparticles, *J Control Release* 128, 23-31, **2008**
- [241] Abul Kalam, M., Khan, A. A., Khan, S., Almalik, A., and Alshamsan, A. Optimizing indomethacin-loaded chitosan nanoparticle size, encapsulation, and release using Box-Behnken experimental design, *Int J Biol Macromol* 87, 329-340, **2016**
- [242] Xie, L., Beyer, S., Vogel, V., Wacker, M. G., and Mantele, W. Assessing the drug release from nanoparticles: Overcoming the shortcomings of dialysis by using novel optical techniques and a mathematical model, *Int J Pharm* 488, 108-119, **2015**
- [243] Calleja, P., Espuelas, S., Vauthier, C., Ponchel, G., and Irache, J. M. Controlled Release, Intestinal Transport, and Oral Bioavailability of Paclitaxel Can be Considerably Increased Using Suitably Tailored Pegylated Poly(Anhydride) Nanoparticles, *J Pharm Sci* 104, 2877-2886, **2015**
- [244] Konjhodzic-Prcic, A. J., Selma; Hasic-Brankovic, Lajla; Vukovic, Amra. Evaluation of Biocompatibility of Root Canal Sealers on L929 Fibroblasts with Multiscan EX Spectrophotometer, *ACTA INFORM MED* 23, 135-137, **2015**
- [245] Wysokinska, E., Cichos, J., Ziolo, E., Bednarkiewicz, A., Strzadala, L., Karbowski, M., Hreniak, D., and Kalas, W. Cytotoxic interactions of bare and coated NaGdF₄:Yb(3+):Er(3+) nanoparticles with macrophage and fibroblast cells, *Toxicol In Vitro* 32, 16-25, **2016**

- [246] Jiang, G., Sun, J., and Ding, F. PEG-g-chitosan thermosensitive hydrogel for implant drug delivery: cytotoxicity, in vivo degradation and drug release, *J Biomater Sci Polym Ed* 25, 241-256, **2014**
- [247] Jain, A., Thakur, K., Kush, P., and Jain, U. K. Docetaxel loaded chitosan nanoparticles: formulation, characterization and cytotoxicity studies, *Int J Biol Macromol* 69, 546-553, **2014**
- [248] Yu, B., Zhang, Y., Zheng, W., Fan, C., and Chen, T. Positive surface charge enhances selective cellular uptake and anticancer efficacy of selenium nanoparticles, *Inorg Chem* 51, 8956-8963, **2012**
- [249] Verma, A., and Stellacci, F. Effect of Surface Properties on Nanoparticle-Cell Interactions, *Small* 6, 12-21, **2010**
- [250] Yang, R., Shim, W. S., Cui, F. D., Cheng, G., Han, X., Jin, Q. R., Kim, D. D., Chung, S. J., and Shim, C. K. Enhanced electrostatic interaction between chitosan-modified PLGA nanoparticle and tumor, *Int J Pharmaceut* 371, 142-147, **2009**
- [251] Torrecilla, D., Lozano, M. V., Lallana, E., Neissa, J. I., Novoa-Carballal, R., Vidal, A., Fernandez-Megia, E., Torres, D., Riguera, R., Alonso, M. J., and Dominguez, F. Anti-tumor efficacy of chitosan-g-poly(ethylene glycol) nanocapsules containing docetaxel: Anti-TMEFF-2 functionalized nanocapsules vs. non-functionalized nanocapsules, *Eur J Pharm Biopharm* 83, 330-337, **2013**
- [252] Georgantzopoulou, A., Serchi, T., Cambier, S., Leclercq, C. C., Renaut, J., Shao, J., Kruszewski, M., Lentzen, E., Grysan, P., Eswara, S., Audinot, J. N., Contal, S., Ziebel, J., Guignard, C., Hoffmann, L., Murk, A. J., and Gutleb, A. C. Effects of silver nanoparticles and ions on a co-culture model for the gastrointestinal epithelium, *Part Fibre Toxicol* 13, 9, **2016**
- [253] Guo, F., Zhang, M., Gao, Y., Zhu, S., Chen, S., Liu, W., Zhong, H., and Liu, J. Modified nanoparticles with cell-penetrating peptide and amphipathic chitosan derivative for enhanced oral colon absorption of insulin: preparation and evaluation, *Drug Deliv*, 1-12, **2015**
- [254] Hu, X., Fan, W., Yu, Z., Lu, Y., Qi, J., Zhang, J., Dong, X., Zhao, W., and Wu, W. Evidence does not support absorption of intact solid lipid nanoparticles via oral delivery, *Nanoscale*, **2016**
- [255] Reix, N., Parat, A., Seyfritz, E., Van der Werf, R., Epure, V., Ebel, N., Danicher, L., Marchioni, E., Jeandidier, N., Pinget, M., Frere, Y., and Sigrist, S. In vitro uptake evaluation in Caco-2 cells and in vivo results in diabetic rats of insulin-loaded PLGA nanoparticles, *Int J Pharm* 437, 213-220, **2012**
- [256] Ghaffarian, R., and Muro, S. Models and methods to evaluate transport of drug delivery systems across cellular barriers, *J Vis Exp*, e50638, **2013**
- [257] Artursson, P., Lindmark, T., Davis, S. S., and Illum, L. Effect of Chitosan on the Permeability of Monolayers of Intestinal Epithelial-Cells (Caco-2), *Pharm Res-Dordr* 11, 1358-1361, **1994**
- [258] Prego, C., Garcia, M., Torres, D., and Alonso, M. J. Transmucosal macromolecular drug delivery, *J Control Release* 101, 151-162, **2005**
- [259] Benediktsdottir, B. E., Gudjonsson, T., Baldursson, O., and Masson, M. N-alkylation of highly quaternized chitosan derivatives affects the paracellular permeation enhancement in bronchial epithelia in vitro, *Eur J Pharm Biopharm* 86, 55-63, **2014**
- [260] Sonaje, K., Chuang, E. Y., Lin, K. J., Yen, T. C., Su, F. Y., Tseng, M. T., and Sung, H. W. Opening of epithelial tight junctions and enhancement of

- paracellular permeation by chitosan: microscopic, ultrastructural, and computed-tomographic observations, *Mol Pharm* 9, 1271-1279, **2012**
- [261] Yuan, H., Chen, C. Y., Chai, G. H., Du, Y. Z., and Hu, F. Q. Improved transport and absorption through gastrointestinal tract by PEGylated solid lipid nanoparticles, *Mol Pharm* 10, 1865-1873, **2013**
- [262] Vilos, C., Morales, F. A., Solar, P. A., Herrera, N. S., Gonzalez-Nilo, F. D., Aguayo, D. A., Mendoza, H. L., Comer, J., Bravo, M. L., Gonzalez, P. A., Kato, S., Cuello, M. A., Alonso, C., Bravo, E. J., Bustamante, E. I., Owen, G. I., and Velasquez, L. A. Paclitaxel-PHBV nanoparticles and their toxicity to endometrial and primary ovarian cancer cells, *Biomaterials* 34, 4098-4108, **2013**
- [263] Snipstad, S., Westrom, S., Morch, Y., Afadzi, M., Aslund, A. K., and de Lange Davies, C. Contact-mediated intracellular delivery of hydrophobic drugs from polymeric nanoparticles, *Cancer Nanotechnol* 5, 8, **2014**
- [264] Li, X., Guo, S., Zhu, C., Zhu, Q., Gan, Y., Rantanen, J., Rahbek, U. L., Hovgaard, L., and Yang, M. Intestinal mucosa permeability following oral insulin delivery using core shell corona nanoliposomes, *Biomaterials* 34, 9678-9687, **2013**
- [265] Lopes, M. A., Abraham, B. A., Cabral, L. M., Rodrigues, C. R., Seica, R. M., de Baptista Veiga, F. J., and Ribeiro, A. J. Intestinal absorption of insulin nanoparticles: contribution of M cells, *Nanomedicine* 10, 1139-1151, **2014**
- [266] Chuah, L. H., Roberts, C. J., Billa, N., Abdullah, S., and Rosli, R. Cellular uptake and anticancer effects of mucoadhesive curcumin-containing chitosan nanoparticles, *Colloids Surf B Biointerfaces* 116, 228-236, **2014**
- [267] Caramella, C., Ferrari, F., Bonferoni, M. C., Rossi, S., and Sandri, G. Chitosan and its derivatives as drug penetration enhancers, *J Drug Deliv Sci Tec* 20, 5-13, **2010**
- [268] Yin, L., Ding, J., He, C., Cui, L., Tang, C., and Yin, C. Drug permeability and mucoadhesion properties of thiolated trimethyl chitosan nanoparticles in oral insulin delivery, *Biomaterials* 30, 5691-5700, **2009**
- [269] Galindo-Rodriguez, S. A., Allemann, E., Fessi, H., and Doelker, E. Polymeric nanoparticles for oral delivery of drugs and vaccines: a critical evaluation of in vivo studies, *Crit Rev Ther Drug Carrier Syst* 22, 419-464, **2005**

CURRICULUM VITAE

Credentials

Name, Surname : Demet Daşkın

Place of Birth : Afyonkarahisar

Marital Status : Married

E-mail : demet.daskin@tubitak.gov.tr

Address : Mehmet Akif Ersoy dist. 328.st. 6/B-55 Yenimahalle-ANKARA

Education

High School : Yıldırım Bayezit Anatolian High School

BSc. : Biology/METU (2004)

MSc. : Biotechnology/METU (2007)

PhD. : Division of Nanotechnology and Nanomedicine/H.U. (2016)

Foreign Languages

English (advanced), German (beginner), Spanish (beginner)

Work Experience

- The Secretariat of Basic Sciences Research Grant Committee (KBAG), TUBITAK (2016 -)
- Scientific Programs Expert in Health Sciences Research Grant Committee (SBAG), TUBITAK (2006 - 2016)
- Region Specialist (Ankara), Sales & Application Specialist, Biyolojik Analiz Tibbi Cihaz Malz. Tic. ve San. A.Ş. (2005)

Areas of Experiences

Nanomedicine, Nanoparticles, Polymeric drug delivery systems, National academic R&D grants

Projects & Memberships

- AB 7. FP. EuroNanoMed, Turkey Delegate/Project Manager (2009 - 2012)
- COST TN:1302 BESTPRAC, Member of Management Committee (2014 -)

Publications

- D.Daşkın, U.Gündüz, "Preparation and Evaluation of Polymer Based Microcarriers for All-trans-Retinoic Acid" Pharmaceutical Development and Technology (2013) 18(5): 1017–1025.

Oral and Poster Presentations

- 17th International Pharmaceutical Technology Symposium (IPTS 2014) – September 2014, Antalya, Turkey (**Invited speaker**)
- 8. Ulusal Kimya Muhendisligi Kongresi (UKMK-8) – August 2008, Malatya, Turkey (**Poster Presentation**)
- 2. Ulusal Polimer Bilim ve Teknolojisi Kongresi ve Sergisi – April 2008, Şanlıurfa, Turkey (**Poster Presentation**) Best poster award
- 4th European Symposium on Biopolymers (ESBP 2007) – October 2007, Kuşadası, Turkey (**Poster Presentation**)