FORMULATION AND ANTICANCER EFFICACY EVALUATION OF POLYMERIC AND CYCLODEXTRIN NANOCAPSULES DESIGNED FOR ORAL APPLICATION

ORAL KULLANIM İÇİN TASARLANAN POLİMERİK VE SİKLODEKSTRİN NANOKAPSÜLLERİN FORMÜLASYONU VE ANTİKANSER ETKİNLİĞİNİN BELİRLENMESİ

HALE ÜNAL

PROF. DR. EREM BİLENSOY

Supervisor

Submitted to Institute of Sciences 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

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This work named "**Formulation and Anticancer Efficacy Evaluation of Polymeric and Cyclodextrin Nanocapsules Designed for Oral Application**" by **Hale Ünal** has been approved as a thesis for the Degree of **DOCTOR OF PHILOSOPHY IN DIVISION OF NANOTECHNOLOGY AND NANOMEDICINE** by the below mentioned Examining Committee Members.

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Prof. Dr. Fatma SEVİN DÜZ
Director of the Institute of
Graduate School of
Science and Engineering

To my family and my love…

ETHICS

In this thesis study, prepared in accordance with the spelling rules of Institute of Graduate School of Science and Engineering of Hacettepe University,

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- all the information and documents have been obtained in the base of the academic rules
- all audio-visual and written information and results have been presented according to the rules of scientific ethics
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- all cited studies have been fully referenced
- I did not do any distortion in the data set
- and any part of this thesis has not been presented as another thesis study at this or any other university.

15/12/2014

HALE ÜNAL

ÖZET

ORAL KULLANIM İÇİN TASARLANAN POLİMERİK VE SİKLODEKSTRİN NANOKAPSÜLLERİN FORMÜLASYONU VE ANTİKANSER ETKİNLİĞİNİN BELİRLENMESİ

Hale ÜNAL

Doktora , Nanoteknoloji ve Nanotıp Anabilim Dalı

Tez Danışmanı: Prof. Dr. Erem BİLENSOY

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Bu tezin amacı oral kemoterapide kullanılmak üzere farklı yüzey yüklerine sahip antikanser ilaç yüklü polimerik veya siklodekstrin nanokapsül formülasyonları geliştirmek ve hazırlanan nanokapsülleri in vitro ve in vivo açıdan değerlendirmektir. Model ilaç olarak seçilen Kamptotesin (CPT) birçok kanser türünde yüksek etkiye sahip bir antikanser ajan olmasına rağmen sudaki düşük çözünürlüğü ve stabilite sorunu nedeniyle tedavide etkin bir şekilde kullanılamamaktadır. Kamptotesinin bu sorunları nanoteknolojik yaklaşımlarla çözülmeye çalışılmıştır. Bu alanda membran tipi ilaç taşıyıcı sistemler olan nanokapsüllerin ilaç taşıyıcı sistemler olarak kullanılması düşünülmüş ve bu sistemlerin yapısal avantajları sebebiyle CPT'in stabilitesinin sağlanarak etkin ve güvenilir formülasyonlarının geliştirilmesi amaçlanmıştır.

Tezin ilk aşamasında CPT yüklü nanokapsül formülasyonları amfifilik siklodekstrinler (CD) ve polikaprolaktan (PCL) kullanılarak hazırlanmıştır. Katyonik yüzey yüküne sahip formülasyonların eldesi için pozitif yüklü bir polimer olan Kitosan (CS) kaplama materyali olarak kullanılmıştır. CPT yüklü anyonik ve katyonik nanokapsüller nanoçöktürme yöntemi ile hazırlanmış ve formülasyon paramatreleri incelenerek ön formülasyon çalışmaları sonucu optimum formülasyonlar seçilmiştir. Hazırlanan partiküller ortalama partikül büyüklüğü, polidispersite indeksi ve zeta potansiyel değerleri açısından in vitro karakterize edilmiştir. İlaç yüklü nanokapsüller yüzey özelliklerinin incelenmesi ve morfolojik analizleri için Taramalı Elektron Mikroskopu (SEM) ile görüntülenmiştir. Nanokapsüllere yüklenen CPT miktarının belirlenmesi

valide edilmiş bir HPLC yöntemi ile gerçekleştirilmiştir. CPT' in nanokapsüllerden in vitro salımı diyaliz membran metodu ile sink koşullarda belirlenmiştir. CPT'in taşıyıcı sisteme enkapsüle edilmesinin stabilitesi üzerine etkilerini araştırmak için valide edilmiş bir HPLC metodu kullanılmıştır. Bununla birlikte, oral uygulanan taşıyıcı sistemlerin gastrointestinal (GI) kanal boyunca stabilitelerinin incelenmesi, simüle edilmiş gastrik sıvıda (SGF) ve simüle edilmiş intestinal sıvıda (SIF) gerçekleştirilmiştir.

Tezin ikinci aşamasında öncelikle oral absorpsiyon için önemli bir basamak olan mukusun nanopartiküllerle etkileşim çalışmaları gerçekleştirilmiştir. İtalya'da Napoli Federico II Üniversitesi'nde gerçekleştirilen çalışmalarda ilk olarak müsin-partikül etkileşimi türbidimetrik analizle aydınlatılmıştır. Ayrıca nanokapsüllerin mukusla etkileşimi mukus varlığında ve yokluğunda partikül büyüklüğü, polidispersite indeksi ve zeta potansiyel değerleri açısından karşılaştırmalı olarak incelenmiştir. Ardından CPT yüklü nanokapsüllerin yapay mukustan geçişi incelenip ilacın yapay mukustan geçişi kantitatif olarak belirlenmiştir.

Nanokapsüllerin etkinlik, güvenilirlik ve permeabilite özellikleri farklı hücre hatlarında çalışılarak aydınlatılmaya çalışılmıştır. Boş nanokapsüllerin sitotoksisitesi ile ilgili çalışma L929 hücre hattında gerçekleştirilmiştir. CPT yüklü nanokapsüllerin antikanser etkinliği ise MCF-7 hücre hattında CPT çözeltisi ile karşılaştırmalı olarak gerçekleştirilmiştir. Bununla birlikte yapay mukus modeli ile mukustan penetrasyonu incelenen nanokapsüllerin, Caco-2 hücre hattından permeabilitesi CPT çözeltisi ile karşılaştırılmalı olarak hesaplanmıştır. Son olarak floresan madde (Nil Kırmızısı) yüklü nanokapsüllerin intestinal absorpsiyonu in vivo hayvan modelinde çalışılmıştır. Oral uygulama sonrası dişi farelerde yapılan intestinal alım çalışmaları ile nanokapsüllerie yüklü molekülün in vivo ortamda mide-barsak kanalından alımı hesaplanmıştır.

Yapılan çalışmalar sonucunda CPT yüklü anyonik ve katyonik nanokapsül formülasyonları 180-220 nm arasında ortalama partikül büyüklüğünde, düşük polidispersite indeksine sahip ve istenilen yüzey yükünde elde edilmiştir. CPT'in yüksek oranda nanokapsüllere enkapsüle edilebildiği ve nanokapsüllerden 48 saat süresince kontrollü bir şekilde salındığı gözlenmiştir. İlacın nanokapsül formülasyonları sayesinde hidrolitik stabilitesinin sağlandığı valide edilen HPLC yöntemi ile gösterilmiştir. Gastrointestinal kanal boyunca değişkenlik gösteren farklı pH koşullarını taklit edilen simüle sıvılar, nanokapsüllerin fiziksel ve kimyasal stabilitesinde partikül

büyüklüğü, PDI ve yüzey yükü açısından belirgin değişikliğe neden olmamıştır. İtalya'da gerçekleştirilen türbidimetri çalışmaları sonucu hazırlanan nanokapsüllerin müsinle etkileşimini doğrulanmıştır. Buna ek olarak musin varlığında partiküllerin çapı, polidispersitesi ve zeta potansiye değerlerinde meydana gelene değişmeler de müsin-partikül ilişkisini desteklemektedir. CPT'in yapay mukozadan penetrasyonu kaplama materyali CS sayesinde katyonik nanokapsüller ile anlamlı ölçüde artmıştır. ($p<0.05$). Caco-2 hücre hattından yapılan permeabilite çalışmaları CPT'in intestinal permeabilitesinin nanokapsüllere enkapsüle edilmesi ile çözelti haline göre 3 kat artırdığını göstermiştir. L929 hücre hattında gerçekleştirilen güvenirlik çalışmaları boş nanokapsüllerin uygulanan konsantrasyonlarda sitotoksik olmadığını kanıtlamıştır. İlaç yüklü nanokapsüllerin MCF-7 hücre hattında antikanser etkinliği serbest ilaca göre anlamlı ölçüde artmıştır ($p<0.05$). İn vivo hayvan deneylerinden elde edilen verilere göre tez kapsamında hazırlanan nanokapsüllere yüklü etkin maddenin barsaktan yüksek oranda alındığı ve katyonik nanokapsüllerin ilacın GI kanaldan hücresel alımını artırdığı gözlenmiştir.

Tez kapsamında gerçekleştirilen in vitro ve in vivo çalışmaların sonuçları göz önüne alındığında nanokapsüllerin, çözünürlük ve stabilite sorunları nedeniyle tedavide etkin bir şekilde kullanılamayan CPT 'in çözünürlüğünü artırmada, stabilitesinin sağlanıp ilacın intestinal permeabilitesinin artırılmasında ümit verici bir nanoteknolojik yaklaşım olarak kullanılabileceği öngörülmektedir. Elde edilen veriler aynı zamanda oral kemoterapide yeni ve etkili bir tedavi yönteminin gerçekleşebileceğini düşündürmektedir.

Bu tez çalışması, literatürde nanopartiküler taşıyıcı sistemler açısından rapor edilmiş fazla sayıda çalışması bulunmayan antikanser ilaç Kamptotesinin, oral kemoterapide güvenli ve etkili bir şekilde kullanılabilmesine yönelik olarak siklodekstrin ve polikaprolaktan nanokapsüllerle formüle edilmesi ve in vitro ve in vivo olarak değerlendirilmesi açısından yeni bir çalışmadır. Yine bu tez kapsamında oral uygulanan negatif ve pozitif yüklü CD nanokapsüller karşılaştırmalı olarak ilk kez incelenmiştir.

Anahtar Kelimeler: kamptotesin, kitosan, mukozal nanopartiküller, nanokapsül, oral kemoterapi, permeabilite, polikaprolakton, siklodekstrinler

ABSTRACT

FORMULATION AND ANTICANCER EFFICACY EVALUATION OF POLYMERIC AND CYCLODEXTRIN NANOCAPSULES DESIGNED FOR ORAL APPLICATION

Hale ÜNAL

Doctor of Philosophy, Department of Nanotechnology and Nanomedicine

Supervisor: Prof. Dr. Erem BİLENSOY

December, 2014, 149 Pages

The objective of this thesis was to design and *in vitro-in vivo* evaluate oral nanocapsules prepared from amphiphilic cyclodextrins (CD) or poly-ε-caprolactone (PCL) with anionic or cationic surface charge for the effective oral delivery of an anticancer agent, Camptothecin (CPT). CPT loaded anionic and cationic nanocapsules coated with Chitosan (CS) were prepared by nanoprecipitation method and characterized in terms of mean particle size, polydispersity index and zeta potential. Morphological analysis of nanocapsules was performed by Scanning Electron Microscope (SEM). The percentage of CPT incorporated in nanocapsules was measured by a previously validated HPLC method. *In vitro* release of CPT from nanocapsules was evaluated using dialysis method under sink conditions. To determine the protective effect and drug stability provided by nanocapsules, all the formulations were incubated in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). Furthermore, CPT stability when incorporated in nanocapsules was determined with a validated HPLC method in phosphate buffer solutions (PBS) with different pH values of 1.2, 6.8 and 7.4 representing the pH range of the GI tract. In order to investigate mucin-particle interactions, measurement of mucoadhesive tendency of CPT loaded nanocapsules was realized by turbidimetric method. The interaction between mucin and particles were also evaluated in terms of mean particle size, polydispersity index and zeta potential values in the presence of mucin or not. Penetration of nanocapsules through an artificial mucus model was performed according to an artificial mucus model. Cytotoxicity of blank nanocapsules were

investigated in L929 cell line. The permeability of CPT in solution form and bound to nanocapsule formulations were demonstrated across Caco-2 cell line. Anticancer efficacy of CPT loaded nanocapsules was determined against MCF-7 cell line in comparison to CPT solution. Finally, the intestinal uptake of nanocapsules was evaluated in vivo, in a mouse model with oral gavage in female CD1 mice.

Both anionic and cationic CPT loaded CD and PCL nanocapsules were in the range of 180 to 220nm with a narrow size distribution and desired zeta potential values. CPT loaded nanocapsules were found to be stable in simulated gastrointestinal media. Turbidimetric measurements confirmed the interaction between nanoparticles and mucin. Penetration of CPT through an artificial mucus gel layer was higher when incorporated in CD nanocapsules than PCL nanocapsules, coating with cationic polymer Chitosan further increased penetration. Permeation of CPT across Caco-2 cell line was found to be higher when incorporated in nanocapsules than CPT solution in DMSO. *In vivo* animal studies confirmed that the intestinal uptake of nanocapsules was significantly higher with cationic nanocapsules. Both *in vitro* and *in vivo* results suggested that CPT loaded positively charged CD nanocapsules might be an attractive and promising treatment to improve the stability and bioavailability of anticancer drug camptothecin and create a new platform for oral chemotherapy.

Keywords: camptothecin, chitosan, cyclodextrins, mucosal nanoparticles, nanocapsules, oral chemotherapy, permeability, polycaprolactone

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SYMBOLS AND ABBREVIATIONS

Symbols

μ	micro
α	alpha
β	beta
γ	gamma
λ	wavelength

Abbreviations

α CD	Alpha cyclodextrin
β CD	Beta cyclodextrin
γ CD	Gamma cyclodextrin
λ_{em}	Emission wavelength
λ_{ex}	Excitation wavelength
μg	Microgram
μl	Microliter
9-AC	9-Amino-20(S)-Camptotesin
9-NC	9-Nitro-20(S)-Camptothecin
ABS	Absorbance rate
ART	Artemisinin
BCS	Biopharmaceutical Classification System
CD	Cyclodextrin
CPT	Camptothecin
CPT-11	Irınotecan
CS	Chitosan
CV	Coefficient of variation
CXB	Celecoxib
DD	Degree of deacetylation
DLS	Dynamic light scattering
DMEM	Dulbecco's modification of Eagle's medium
DMF	Dimethylformamid

DMSO	Dimethylsulfoxide
DTPA	Diethylene triamine pentaacetic acid
EMA	European Medicines Agency
FAE	Follicle Associated Epithelium
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FPHM	First pass hepatic metabolism
GI	Gastrointestinal tract
h	Hour
HBSS	Hank’s balanced salt solution
HEPES	n-(2-hydroxyethyl) piperazine-n-(2-ethanesulfonic acid)
HPLC	High performance liquid chromatography
IUPAC	International Union of Pure and Applied Chemistry
iv	Intravenous
LbL	layer-by-layer
LOD	Limit of detection
LOQ	Limit of quantification
MDR	Multidrug resistance
mg	Milligram
min	Minute
MRP	Multidrug resistant associated protein
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
mV	Millivolt
MW	Molecular weight
NaOH	Sodium hydroxide
NC	Nanocapsule
nm	Nanometer
NNI	National Nanotechnology Initiative
NP	Nanoparticle
NR	Nile red
OD	Optical densities
PBS	Phosphate buffer saline
PCL	Poly-ε-caprolactone

PDI	Polydispersity index
PEG	Poly(ethylene glycol)
P-gp	P-glycoprotein
PLA	Poly(lactide)
PLGA	Poly(lactide-co-glicolide)
PP	Peyer's patches
R^2	Correlation coefficient
rpm	Route per minute
SD	Standard deviation
SEM	Scanning electron microscopy
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
TEAA	Triethylamine acetate buffer
TEER	Transepithelial electric resistance
TEM	Transmission electron microscopy
v	Volume
ZP	Zeta potential

1. INTRODUCTION

Cancer is one of the major fatal diseases in the world and causes abnormal growth of cells spreading to surrounding tissues in the body. Although in cancer therapy, intravenous route is more common than other administration routes, it is thought that with recent advances oral chemotherapy will be the breakthrough step in future chemotherapy. From the patient's viewpoint, oral route allows for painless self-medication and thus it is considered the most convenient route. Besides, it reduces reimbursement load in the health budget also, since it does not require therapy in medical centres. By means of oral administration, it is possible to prevent the initial rapid increase and subsequent decay of drug concentration in blood that occurs via intravenous route. However most anticancer drugs are not good candidates for oral delivery owing to their low absorption in the gastrointestinal tract (GI) and as a result exhibit low oral bioavailability. In order to develop an effective oral chemotherapy, bioavailability of anticancer drugs should be improved. On the other hand most anticancer drugs are not good candidates for oral delivery and Camptothecin, which was used in this thesis, is cited as a prime example.

Camptothecin (CPT) is a very effective anticancer agent against a wide spectrum of cancers, however, its poor aqueous solubility and pH-dependent stability problems result in diminishing of clinical efficacy for the drug. Therefore formulation approaches should be considered with the aim to design novel drug delivery systems able to improve the aqueous solubility and to protect this drug from hydrolysis in pH 7.4 in order to obtain an effective in vivo administration of CPT for cancer therapy.

Nanoparticles are promising systems in this field to facilitate oral delivery of anticancer drugs. In recent years, nanoparticles as oral drug delivery vehicles provide numerous advantages over conventional approaches in oncology. Nanoparticles are defined as submicron colloidal systems that include both nanospheres and nanocapsules. Nanocapsules are especially beneficial for oral administration of anticancer drugs e.g CPT, since nanocapsules can enhance the poor aqueous solubility owing to the oily liquid core, improve the stability of drugs and prolong residence time in GI tract due to the polymeric wall and enhance

permeability of drugs by taking the advantages of both small size of nanoparticles and mucoadhesive properties of polymers as coating materials.

Poly-ε-caprolactone (PCL) is one of the most frequently used commercial biodegradable polyesters in drug delivery applications. Due to its higher resistance to chemical hydrolysis and lack of toxicity, PCL and its derivatives are well suited for application in colloidal drug delivery.This polymer has been approved by the US FDA and known to be hydrophobic, semi-crystalline, non-toxic, biodegradable and biocompatible.

Cyclodextrins (CDs) are natural polymers produced from enzymatic degradation of starch. The most common advantages of CDs in the pharmaceutical field are to enhance the stability, solubility, and bioavailability of drug molecules. Amphiphilic CDs are derivatives of natural cyclodextrins which are chemically obtained and modified on the primary and/or secondary face. CDs have mainly been used as complexing agents to increase the aqueous solubility of poorly soluble drugs and modify release profiles and accordingly to increase their bioavailability and stability. Besides, CDs act as a permeability enhancer upon oral administration which plays an important role for drugs suffering from low intestinal permeability.

The aim of this study was to design and evaluate comparatively oral PCL and CD nanocapsules of positive or negative surface charge, intended for the oral delivery of anticancer agent CPT in order to maintain drug stability in the body and to improve its eventual bioavailability.

2. GENERAL INFORMATION

2.1. Oral Chemotherapy

Oral route is the most preferred route for administration of therapeutics. It has been reported that most patients prefer oral to intravenous administration [1]. In drug delivery market, the largest sector is the oral pharmaceutical products and there is no sign that it is decelerating in the near future [2]. Oral route offers an attractive and convenient route of drug administration. Almost 60% of prescriptions is made up of oral formulations of total global pharmaceutical sales [3]. The oral route presents several advantages in many aspects that make it the most preferred route of drug administration. From the patient's viewpoint, oral route allows for painless self-medication, less stressful due to fewer hospital visits and compared to parenteral delivery, it reduces cost, increases patient compliance, allows flexible and controlled dosing schedule and have a low risk of systemic infections [1, 4]. It is also cheaper to produce because it does not need to be manufactured under sterile conditions resulting in cost saving in terms of time and labour as well.

Historically, chemotherapy has been administered mainly intravenously (i.v) [5]. The intravenous route is the most direct one and leads to immediate and complete bioavailability and therefore, accurate dosing. However, this route could be hazardous, due to the risk of the high concentration of the drug which is destined to normal tissues. Besides, i.v. chemotherapy regimens are designed to deliver the maximal tolerated dose of cytotoxic agent to kill cancer cells in a short period of therapy followed by a period of several weeks without administration [6]. However, it is generally believed that long-term exposure to drug at modest concentrations would be more beneficial than a pulsed supply of the drug at higher concentrations [7]. Since 1990s, the oral chemotherapy has been an interesting alternative to the i.v. therapy and is expected to be more common in the near future [5, 6]. Currently molecules designed for oral chemotherapy builds up at least 25% of all new antineoplastic drugs in development [5, 8, 9]. Several agents that are already proven to be effective in a range of tumour types (e.g. capecitabine, erlotinib, gefitinib, imatinib, lapatinib, lenalidomide, sunitinib, sorafenib) and many others in development (e.g. vatalanib, satraplatin) are or will be available only as oral formulations [9].

2.1.1. Advantages & Challenges in Oral Chemotherapy

Oral chemotherapy is a key step for “therapy at home”. The patient becomes the real actor of the therapy and this home-based therapy makes the patients feel less sick helping them to face their illness better and as a result provides better patient compliance together with the improvement of patient’s quality of life [1, 6, 10]. Generally, health-care professionals are consistent with the idea that patients dislike injections, thus they are more likely to prescribe oral treatments [11] since oral chemotherapy may reduce anxiety in patients who are worried about injections [9].

Since anticancer drugs have narrow therapeutic windows, correct administration is crucial for both efficacy and safety. Oral dosage forms have become more important in therapy by means of reduced dosing frequency, improving both efficacy and safety thereupon [11]. Oral administration of anticancer drugs can maintain a sustained concentration of the drug providing prolonged systemic exposure with less fluctuation which may lead to decreased side effects as well as lower toxicity and improved therapeutic efficacy [7, 12]. From an economical point of view, oral therapy potentially offers considerable financial savings compared with intravenous therapy since it limits the cost of hospitalization and the infusion equipment supplies. Benefits of oral chemotherapy are summarized in Table 2.1. by means of patients, healthcare professionals and healthcare funders [6, 9].

Table 2.1. Benefits and needs of oral therapy from the perspectives of patients, healthcare professionals and funders [6, 9]

	Patients	**Healthcare professionals**	**Healthcare funders**
Benefits	No needles	Tailored/flexible dosing	Cost savings
	Reduce clinic time	Staffing savings	Staffing savings
Needs	Efffective education	Efficacy equivalent to i.v. options	Reimbursement reform in some countries
	Simple schedule	High and predictable bioavailabiltiy	

Despite the advantages of oral chemotherapy, orally administered drugs face many difficulties during oral absorption owing to their physicochemical properties and

biobarriers. According to the statistics, more than 60% of all anticancer drugs are available for clinical therapy in oral dosage form, however very few of them are actually put into use in clinic owing to limited oral bioavailability. As shown in Figure 2.1., multiple barriers that limit the effectiveness of oral chemotherapy can be divided into three main groups: [13, 14]

1) pyhicochemical properties of drugs,

2) physiological barriers in Gastrointestinal (GI) tract and

3) biochemical barriers in GI tract

The pyhsicochemical properties of anticancer agents can be counted as the innate limiting factors wheras physiological and biochemical factors as external.

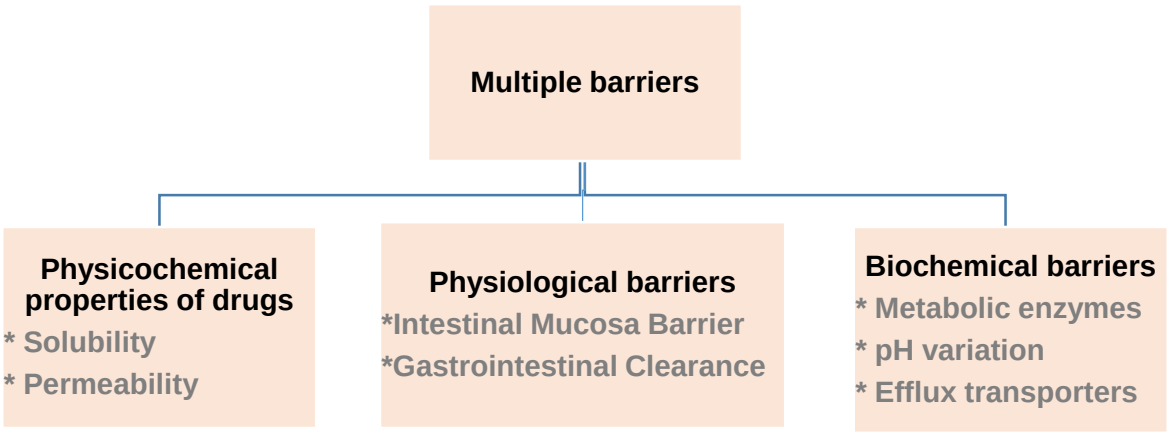


Figure 2.1. Multipler barriers limiting oral chemotherapy.

2.1.1.1. Effect of Physicochemical properties of drugs

Pharmaceutical scientists have mainly focused on ways to improve the bioavailability of therapeutic agents for an efficient oral drug delivery. Oral bioavailability is strongly affected by drug physicochemical properties and limited oral absorption potential of anticancer drugs can be generally attributed to poor solubility, limited permeability-limited or both [13]. Therefore solubility and permeability are the two important parameters for oral bioavailability of anticancer agents.

Biopharmaceutical Classification System (BCS) gives a definition for drugs in four categories depending on their solubility and their permeability [1]. BCS class I drugs (e.g. verapamil, diltiazem, metoprolol) with good solubility and high permeability can

be easily absorbed in GI tract. For BCS class II drugs (e.g danazol, ketoconazole, nifedipine) with low solubility and high permeability, solubility enhancement is necessary whereas for Class III drugs (e.g cimetidine, acyclovir, captopril) with high solubility and poor permeability, permeability enhancement is the primary factor to achieve effective oral delivery. For the Class IV drugs (e.g paclitaxel and docetaxel) with poor solubility and poor permeability, both of the factors are limiting factors for oral absorption and results in poor biovailability (Figure 2.2.). From this classification, it can be seen that, except for class I drugs, all pharmaceuticals will be faced with bioavailability issues due to their poor dissolution rate, their poor permeability or both.

Therefore several strategies should be taken into account to overcome these limitations [4]. For non-cytotoxic drugs the oral route is most commonly used, however most of the anticancer drugs such as Taxoids (paclitaxel, docetaxel) belong to the Class IV of BCS and have poor oral bioavailability (less than 10%) as a result of poor solubility, low absorption rate in GI tract and partially high affinity for multidrug efflux pump P-glycoprotein (P-gp) leading to poor intestinal permeability [15, 16]. Furthermore, >65% of anticancer drugs can be used as oral dosage form but very few of them are actually used which could be attributed to the limited oral bioavailability due to poor physicochemical properties and efflux mechanisms.

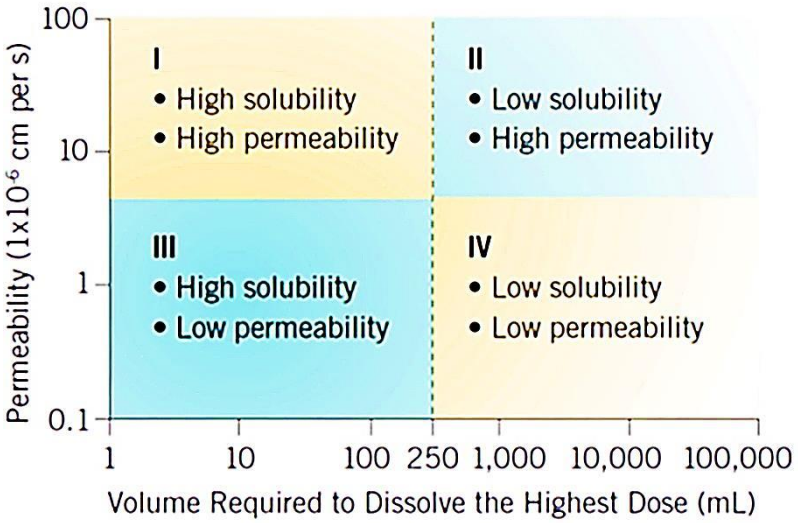


Figure 2.2. Biopharmaceutical Classification System (BCS) [16].

2.1.1.2. Physiological barriers: Role of mucus in the gastrointestinal tract

GI tract is an extremely complicated system in terms of physical structure and chemical constituents (Figure 2.3.). Physiologically GI tract serve as a complex environment taking charge in digesting and absorbing nutrients, water and vitamins from food. Morever it is also designed as a barrier to prevent the entry of pathogens, toxins and undigested molecules [17]. In other words, the GI epithelia form a barrier between the body system and the lumenal environment, which limits the entry of potentially hostile substances, as well as therapeutic agents [18]. The human intestinal epithelium is highly absorptive and is composed of villi that increase the total absorptive surface area in the gastrointestinal (GI) tract to 300–400 m^2. Enterocytes (absorptive) and goblet cells (mucus secreting) cover the villi, which are interspersed with Follicle Associated Epithelium (FAE). These lymphoid regions, Peyer's patches, are covered with M cells specialized for antigen sampling. M cells are significant for drug delivery, since they are relatively less protected by mucus and have a high transcytotic capacity [19].

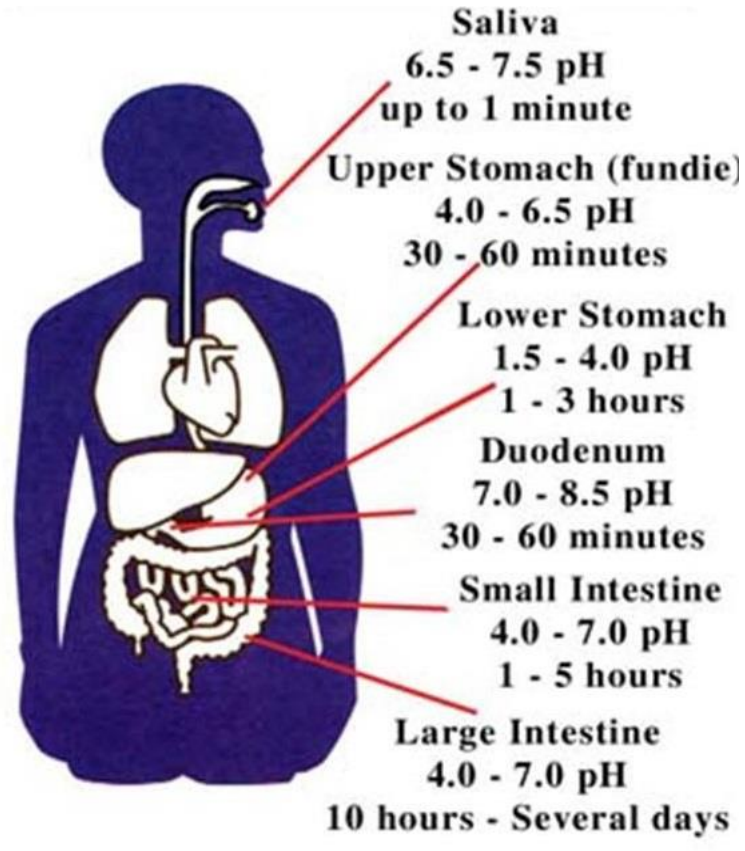


Figure 2.3. The human GI tract with pH range chart [20].

For particulates to be absorbed, two barriers exist; the mucus gel layer and the mucosa consecutively. Mucus is a viscoelastic gel layer serving to exclude pathogens and other dangerous materials from the underlying cells allowing the diffusion of only nutrients, proteins and essential molecules and prevents the tissue from dehydration. Mucosal surfaces protect all major portals of our body such as nose, lung, intestine, gall bladder, urinary bladder and reproductive tract. Furthermore mucus sticks to most particles, preventing their penetration to the epithelial surface [21-23] and therefore it is known to be the primary defense mechanism of mucosal tissues in order to efficiently trap and remove nano- and micro-sized objects, viruses and bacteria as well [24].

Mucus is mainly made up of water (95% w/w) and mucin (about 5% w/v) as well as small amounts of proteins, lipids, DNA and electrolytes [25, 26].These various components work together to build up a heterogeneous environment for particulate transport [27]. Mucins are large, highly glycosylated proteins secreted by the goblet cells and responsible for the rheological properties of mucus and disulfide bonds between non-glycosylated regions of the proteins in addition to numerous non-covalent bonds constitute the highly entangled network of mucin fibers. These crosslinked and entangled mucin fibers [27] are large molecules coated with proteoglycans [26]. It is negatively charged due to the presence of sialic acid and sulfated monosaccharides in the sugar chains.

Mucus pH exhibits a great variability depending on the mucosal surface which means lung and nasal mucus are in general pH neutral and eye mucus is slightly basic with pH ~ 7.8, whereas gastric mucus is exposed to a wide range of pH from ~1, ~2 to ~7 [27]. In addition to that, the thickness of the mucus also varies for different mucosal surfaces and for different diseases since when an epithelium undergoes malignant transformation, the mucin genes can undergo aberrant expression causing reduced production of structually different and unexpected mucin such as cervical cancer [28]. In the human GI tract, the mucus layer is the thickest in stomach and colon but exibits significant variation though depending on digestive activity [19].

The viscosity of mucus affects the diffuson barrier and the viscoelastic properties of GI mucus are essential for its protective and defense effect and lubricating properties as well. Consequently, mucus of the GI-tract constitutes an important

barrier to oral delivery of therapeutics and it is one of the many obstacles for successful oral delivery and is acknowledged as a rate-limiting step to drug absorption in general.

2.1.1.3. Biochemical barriers

The biochemical barrier is mainly composed of efflux transporters, drug metabolic enyzmes and surfactants and variable pH along the GI tract [13, 14]. One of the major barriers is the wide pH range and harsh acidic environment of the GI tract, which includes the stomach and the intestines [29].

Upon oral administration materials encounter the physicochemical environment of the GI tract. The complex digestive system of the GI tract is designed to absorb many nutrients as possible in a safe, effective and selective way. However, materials administered orally face the very harsh environment of stomach which is strongly acidic [30]. The pH along the GI tract changes from acidic to alkaline presenting a challenge for the stability of the drugs and integrity of drug loaded carriers. [13]. The pH of the stomach ranges from 1.0 to 2.5 [31, 32], it rises to 6.6–7.5 from the proximal end to the ileum of the small intestine, decreases to 6.4 at the ceacum, and then rises again to 7.0 from the right to the left colon. Hence, this pH variation in the GI tract makes it difficult to maintain particle integrity throughout the GI tract and, therefore, stability of administered materials over a wide pH range is necessary.[33]

Besides this pH variation, the contents of gastric fluids present an additional obstacle for oral delivery due to abundance of enzymes. Specifically, the stomach contains hydrochloric acid and pepsin, which degrade proteins wheras the duodenum of the small intestine contains bile salts and degradative enzymes including amylase, trypsin, and lipase [9], which degrade starch, proteins, and fats respectively. The small intestine contains pancreatic juices comprising pancreatin, trypsin, lipase, peptidases and maltase [29]. The GI tract presents a unique microenvironment of enzymes and ionic strength, which impact both the chemical and colloidal stability of administered particulates.

The gastro-intestinal metabolism, also referred as luminal metabolism is performed by digestive enzymes secreted by pancreas such as amylase, lipase, and peptidases and from the bacterial flora present especially in the lower part of the

gastrointestinal tract. The intracellular metabolism in the gut is principally carried out by extrahepatic microsomal enzymes present within the cytoplasm on the endoplasmic reticulum. Cytochrome P450 3A family, especially CYP 3A4, are known as phase I metabolizing enzymes. Among all of the human CYP 450 homologues, CYP 3A4 plays an extremely important role overall metabolism of drugs. They are present in the enterocytes which causes to the metabolism of the drug substances at the gastrointestinal wall [34, 35]. Drug metabolizing enzymes in the liver and gut mucosa are present to limit the systemic exposure of foreign molecules that have been absorbed from the gastrointestinal tract. Any foreign molecule that is absorbed from the G.I. lumen travels across G.I. mucosa, capillary beds of small and large intestine, liver via portal circulation and is then transported to the rest of the body organs. Only exception to this is the molecules that are absorbed into the lymphatic system or distal rectum which effectively bypass the liver [36]. Once the drug gets absorbed from the gastrointestinal tract, it enters into the entero-hepatic portal vein and reaches to liver, where a fraction of absorbed dose is metabolized, being referred as First Pass Hepatic Metabolism (FPHM). As depicted in Figure 2.4.

Liver is the hub of various enzymes and is referred as “metabolic clearing house”. This first pass metabolism is considered as major contributor for low oral bioavailability of many drugs [14].

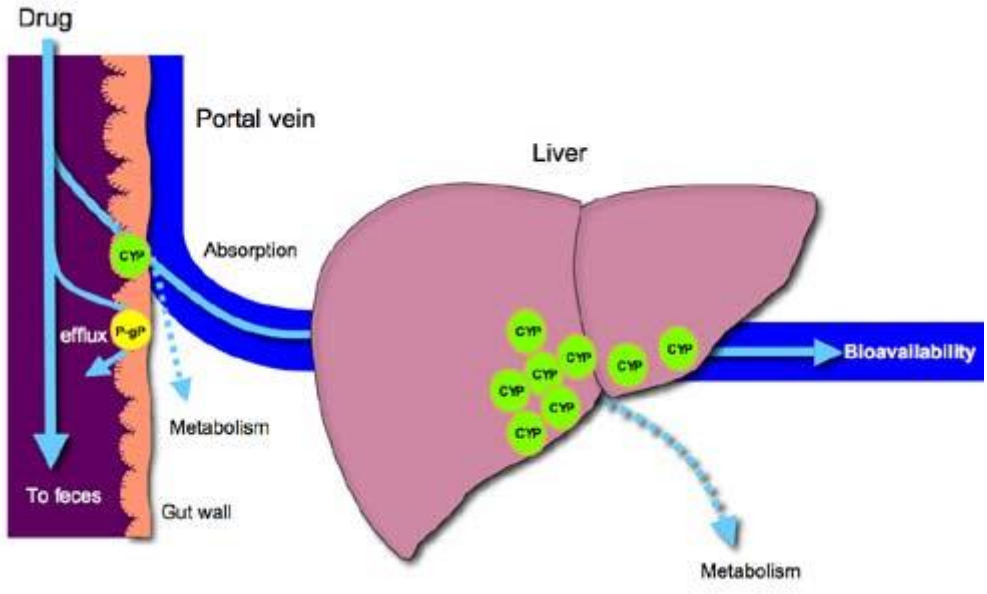


Figure 2.4. First Pass Hepatic Metabolism [37]

One of the other key factors limiting the oral bioavailability is P-glycoprotein (P-gp) efflux pump. Transmembrane efflux of drugs is defined as the expulsion of the drug molecules across the cellular membrane from the cells via a clinically significant systematic transportation system such as P-gp, breast cancer resistant protein (BCRP), cytoplasmic transport, multidrug resistant associated protein (MRP) assisted pathways, etc.

P-gp, encoded by the multidrug resistance-1 (MDR1) gene, localized in enterocytes, is one of the significant efflux transporters leading to the excretion of drug back in to the intestinal lumen [38]. It is extensively distributed in intestinal epithelia, hepatocytes, kidneys, various glands and capillary endothelial cells comprising blood–brain and blood–testes barriers. This is a membrane associated protein belonging to the superfamily of ATP binding cassette (ABC). It mainly operates at three major locations, luminal (apical) membrane enterocytes: the drug is limited by entering in the body; canalicular membrane of hepatocytes: increased elimination into bile and urine; and, sensitive tissues such as brain, lymphocytes, testis, and fetal circulation: limiting the drug penetration. Most of the anticancer drugs are the substrates for P-gp efflux pump resulting in the limitation of oral delivery [14].

2.2. Nanoparticulate drug delivery systems

Nanotechnology derived from the Greek word “nano” meaning dwarf [39] is a key step in drug delivery systems [31]. According to the NNI (National Nanotechnology Initiative) definition, nanotechnology is science, engineering, and technology managed at the nanoscale where unique, size dependent properties in physicochemical phenomena enable novel applications (from 1 to 100 nanometers). Similarly EMA (European Medicine Agency) gives the definitions for nanotechnology as “the production and application of structures, devices and systems by controlling the shape and size of materials at nanometre scale. “

At this size, the substance's physical, chemical and biological properties are different from what they were at the micrometer and larger scales [40].

Nanotechnology involves both:

1) The deliberate manipulation of matter by certain chemical and/or physical processes (referred to as “bottom-up” production) to create materials with specific properties that are not displayed in their larger forms.

2) The use of manufacturing processes such as milling or grinding (called “top-down” production) to produce nanosized particles. These particles may or may not have properties different from those of the bulk material from which they are developed.

Nanotechnology is a multidisciplinary field and can be applied to almost all the other science fields, such as chemistry, biology, physics, mathematics, engineering, material science, pharmaceutical science etc. Nanotechnology focuses on the understanding and control of matter at the nanoscale, including application with specific top-down or bottom-up techniques and engineering of nanoscale materials, systems and devices [41]. Furthermore, nanotechnology has enabled development of entirely new materials and devices that can be exploited in each of these and countless other applications.The idea of nanotechnology started with a talk entitled “There’s Plenty of Room at the Bottom” by physicist Richard Feynman on December 29, 1959 [42].

Nanomedicine is an important area in nanotechnology which refers to highly specific medical intervention at the molecular scale for diagnosis, prevention and treatment of diseases [43]. The application of nanotechnology to discipline of medicine is called, nanomedicine [44]. The term nanomedicine refers to any application of nanomaterials for medical purposes ranging from therapeutic to diagnostic applications [41]. The use of nanoscale materials for diagnosis, monitoring, control, preventetion and treatment of disease [44]. Nanomedicine lies at the intersection of many disciplines including biology, chemistry, physics, bioengineering, pharmaceutical science and clinical medicine. Although the application of nanotechnology to medicine appears to be a relatively recent trend, the basic nanotechnology approaches for medical application date back several decades. The first example of liposomes were described in 1965, the first long circulating stealth polymeric nanoparticle was described in 1994 [45]; the first quantum dot

bioconjugate was described in 1998 [46] and the first nanowire nanosenser dates back to 2001.

Nanomedicine has the inevitable potential and capacity for both providing advanced therapeutic options to patients, and anticipating commercial potential to pharmaceutical industries. According to Business Cominication Company (BCC) research market forecasting, the global nanomedicine market reached $63.8 billion in 2010 and $72.8 billion in 2011. At this rate, it is expected to increase to $130.9 billion by 2016 [47]. The potential of nanomedicine that positively impacts healthcare at many levels includes; detection of molecular changes responsible for disease diagnosis and imaging, drug delivery and therapy and multi-functional systems for combined therapeutic and diagnostic applications. Over the last decade, lots of devices and systems at the nanoscale have been developed for the diagnosis, imaging, and therapy of diseases. In the last decade nanotechnology and nanofabrication have significantly impacted the field of drug delivery [39].

The applications of nanotechnology in various disciplines and specifically in healthcare are becoming increasingly common and the process of replacing traditional medicines has already begun. Thus, although efficient drug delivery is one of the most prominent problems faced by the biotechnological and pharmaceutical industries, nanotechnology can promote the innovative utilization of the myriad existing drugs produced by these industries. The emergence of nanotechnology is likely to have a significant impact on the drug-delivery sector and nanoparticles (NPs) are at the leading edge, with many potential applications in clinical medicine and research. [48]

Nanoparticulate drug delivery systems used in medicine are becoming increasingly sophisticated. They are a large subject area of nanomedicine and include polymeric nanoparticles, nanoshells, micelles, liposomes, dendrimers, nucleic acid based nanoconstructs, magnetic nanoparticles, silicon oxide nanoparticles, and quantum dots. In general nanoparticles are submicron sized, colloidal particles, with two or more dimensions [49]. The unique properties of nanoparticles together with high surface: volume ratio and the possibility of modulating their properties, make them powerful tools in nanomedicine [50] .

Although the definition of NNI for nanoparticles is in the range of 1-100nm, for most pharmaceutical applications, nanoparticles are defined as having a size up to 1,000 nm [51].

Advantages of polymeric nanoparticles can be summarized below [52] :

- ✓ Increases the stability of any volatile pharmaceutical agents.
- ✓ A significant improvement over traditional treatments in terms of efficiency and effectiveness.
- ✓ A favorable blood half-life and physiological behavior with minimal off-target effects.
- ✓ Effective clearance from the human organism, and minimal or no toxicity to healthy tissues in living organisms.
- ✓ Easily and cheaply fabricated in large quantities by a multitude of methods

Nanoparticle-based drug delivery provides many advantages, such as enhancing drug-therapeutic efficiency and pharmacological properties. For example, nanoparticles improve the solubility of poorly water-soluble drugs, modify pharmacokinetics, increase drug half-life by reducing immunogenicity, increase specificity towards the target cell or tissue (therefore reducing side effects), improve bioavailability, diminish drug metabolism and enable a more controllable release of therapeutic agents [39, 53].

2.2.1. Nanocapsules

A great variety of nanoparticles have been presented in the literature with different sizes, surface properties, with polymer coatings imparting negative, positive, or neutral electrostatic charges; compositions, including lipids, polymers, carbon, silicon and various metals; and shapes etc [54]. In recent years, biodegradable polymeric nanoparticles have attracted considerable attention as potential drug delivery devices in view of their applications of drugs to treat a particular disease [55]. The polymeric nanoparticles which are prepared from biocompatible and biodegradable polymers, can be named nanocapsules (reservoir-type nanodevices) or nanospheres (matrix-type nanodevices) depending on their composition. The difference between these forms lies in the morphology and structure of the particles [55-57].

Nanospheres are generally defined as matrix-type, solid, colloidal nanoparticles in which drugs are dispersed, entrapped, encapsulated, chemically bounded or adsorbed to the constituent polymer matrix [55] whereas, nanocapsules are vesicular systems in which the drug is confined to a reservoir or within a cavity consisting of an inner liquid core surrounded by a polymer membrane or coating as represented schematically in Figure 2.5. [58, 59]

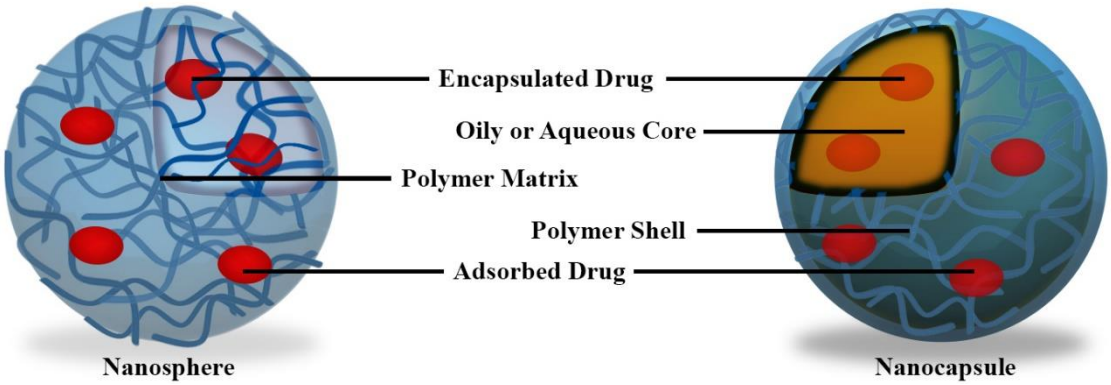


Figure 2.5. Schematic presentation of nanospheres and nanocapsules

Nanocapsules can be defined as nano-vesicular systems that exhibit a typical core-shell structure. In that case, an active substance is usually dissolved in the core but can also be adsorbed at their surface [48]. There are two variations possible, depending on the core and the structure of the surrounding polymer. Frequently, the core is an oily liquid, the surrounding wall is a layer of polymer and the vesicle is referred to as a nanocapsule. These systems have found utility in the encapsulation and delivery of hydrophobic drugs. Alternatively, if the core of the vesicle is an aqueous phase and the surrounding coating is a polymer bilayer, the particle is referred to as a polymersome. [58]

The presence of oil in the nanocapsules leads to a vesicular structure wheras its absence in nanospheres provides a matrix-type structure of the polymeric chains [57]. The nanocapsule core is composed of oil together with the lipophilic active substance. The criteria for the selection of oil are; the absence of toxicity, highest capacity to dissolve the drug and lack of risk of polymer degradation. Different capric/caprylic triglyceride types are often used because of their wide range of solubilization for active substances. Other oils such as benzyl benzoate, benzyl

alcohol, oleic acid, ethyl oleate, argan oil, sunflower seed oil and soybean oil can be also used for nanocapsule preparation [59].

Nanocapsules present the same advantages as mentioned above. Furthermore, nanocapsules have some other advantages over nanospheres that can be counted as high drug loading capacity, low polymer content, better protection against degradation factors and reduction of tissue irritation [59]. Theoretically, nanocapsules have a constant rate drug release due to the polymeric wall of the particle (zero order drug release), whereas first order drug release is typically observed with nanospheres. Nanocapsules require a lower amount of polymer for each particle and as a consequence drug loading as percentage of polymer content is higher [60]. A thicker and mechanically more resistant shell might be capable of protection of encapsulated drugs presenting a higher stability when in contact with biological fluids. Furthermore compared with liposomes, which are also nanovesicular drug delivery systems, nanocapsules are robust and stable in both liquid and powder forms.

2.2.1.1. Nanocapsule Preparation Methods

Polymers generally used for nanoparticle preperation, can be of different origins and can be classified as natural (arabic gum, gelatine), semi-synthetic (ethylcellulose, hydroxyprophilmethylcellulose phthalate or more commonly synthetic (poly-e-caprolactone (PCL), poly(lactide) (PLA) and poly(lactide-co-glicolide) (PLGA). Synthetic polymers have higher purity and better reproducibility than natural polymers [61]. Both lipophilic and hydrophilic surfactants can be used in the preparation of nanocapsules.

Nanocapsules can be prepared by different preparation methods like nanoprecipitation, emulsion–diffusion, double emulsification, emulsion-coacervation and layer-by-layer. All these preparation techniques offer their individual advantages and disadvantages for drug carrier systems as well [59].

Nanoprecipitation method

The nanoprecipitation method which is also called solvent displacement or interfacial deposition was published for the first time by Fessi *et al*. in 1989. This method is simple, rapid, economic, and requires low amounts of organic solvent yielding small particle sizes with narraw polydispercity indices. Emulsification steps

which are usually part of a nanoparticle preparation process, laborious processing conditions or special laboratory ware are not required [62, 63]. This method necessitates both solvent and non-solvent phases. Briefly the solvent phase essentially consisting of a solution in a solvent or in a mixture of solvents (i.e. ethanol, acetone, hexane, methylene chloride or dioxane) of a polymer (synthetic, semi-synthetic or naturally occurring polymer), the active substance and oil (with or without lipophilic surfactant). On the other hand, the non-solvent phase consisting of a non-solvent or a mixture of non-solvents supplemented with one or more hydrophilic surfactants. In most cases, the solvent and non-solvent phases are called organic and aqueous phases, respectively. Generally, the solvent is an organic medium, while the non-solvent is mainly water. With the nanoprecipitation method, the nanocapsules are obtained as a colloidal suspension.

The choice of two solvents suitable for the nanoprecipitation was based on the requirements of the method and on the physico-chemical characteristics of the polymer. It is reported that within this method organic solvents must be able to dissolve the polymer, must be miscible with non-solvent phases and have a low boiling point to facilitate their elimination by evaporation [64]. In the nanoprecipitation process, particle formation is spontaneous, due to the polymer precipitation in the aqueous environment. (Figure 2.6.)

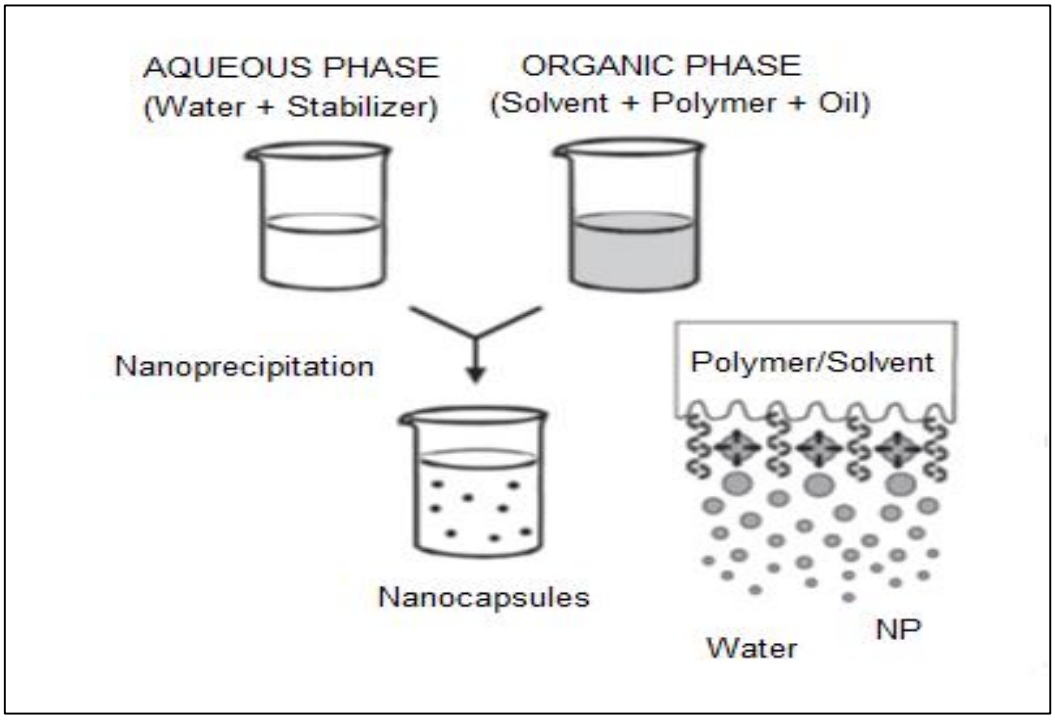


Figure 2.6. Formation of nanocapsules via nanoprecipitation method.

The Marangoni effect is considered to explain the process: Solvent flow, diffusion and surface tension at the interface of the organic solvent and the aqueous phase cause turbulences, which form small droplets containing the polymer. When a liquid with a high surface tension (aqueous phase) pulls more strongly on the surrounding liquid than one with a low surface tension (organic phase solvent) this difference between surface tensions causes interfacial turbulence (Figure 2.7.) [62]. Consequently, violent spreading is observed due to mutual miscibility between the solvents, and the solvent flows away from regions of low surface tension and the polymer tends to aggregate on the oil surface forming nanocapsules. According to this explanation, nanocapsule formation is due to polymer aggregation in stabilized emulsion droplets [64, 65].

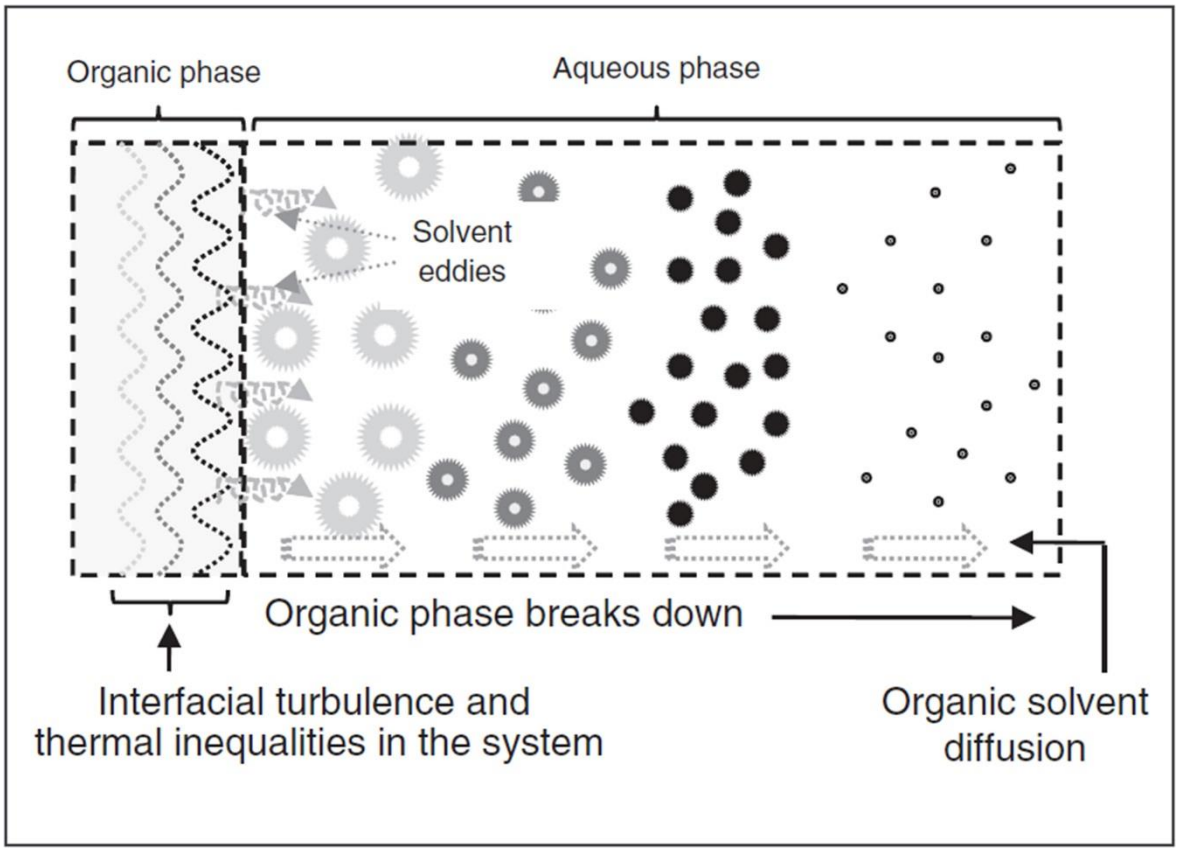


Figure 2.7. Gibbs-Marangoni Effect [62].

Emulsion-Diffusion Method

Preparation of nanocapsules by the emulsion–diffusion method was first proposed by Quintanar-Guerrero et al. [66, 67]. The experimental procedure requires three phases: Organic, aqueous and dilution. For preparation of nanocapsules using the emulsion–diffusion method, the organic phase is emulsified under vigorous agitation

in the aqueous phase. This technique is based on the initial formation of an O/W emulsion containing an oil, polymer and a drug in the organic solvent, in an aqueous solution of stabilizing agent. Finally the subsequent addition of water to the system causes the diffusion of the solvent into the external phase, resulting in nanocapsule formation. (Figure 2.8)

It has been shown that nanocapsule size is affected by the shear rate used in the emulsification process, the chemical composition of the organic phase, the polymer concentration, the oil-to-polymer ratio and the drop size of the primary emulsion [68].

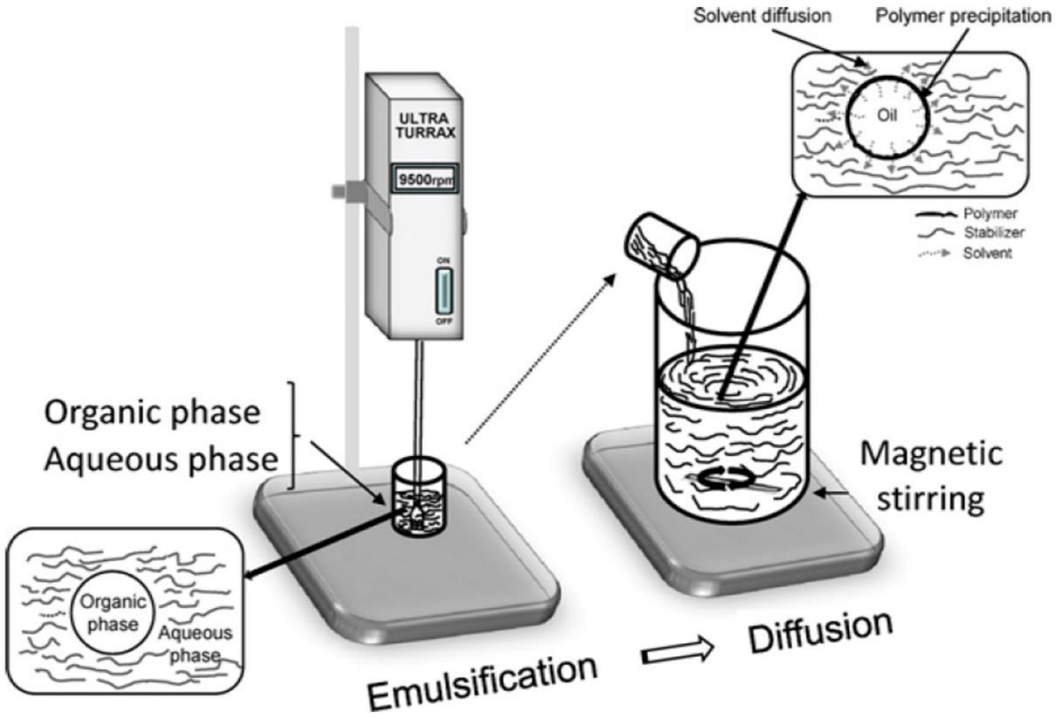


Figure 2.8. Preparation of nanocapsules via emulsion-diffusion method [59].

Double Emulsification Method

Double emulsions are complex heterodisperse systems called “emulsions of emulsions”, in which the droplet of one dispersed liquid is further dispersed in another liquid that can be classified into two major types: Water-oil-water emulsion (w/o/w) and oil-water-oil (o/w/o) emulsion [61]. For preparation of nanocapsules, the principle of double emulsion formation, specifically of the w/o/w type, is associated with the principles of both nanoprecipitation and emulsion–diffusion methods. In this case, in the primary w/o emulsion the oil is changed by an organic phase containing a solvent that is totally or partially miscible in water, the film-formed polymer and a w/o surfactant. Then the water containing a stabilizing agent is added to the system

to obtain the water in organic in water emulsion. However in this step, particle hardening is obtained through solvent diffusion and polymer precipitation. Water is frequently added to the double emulsion in order to achieve full solvent diffusion. Finally, the solvents are removed by evaporation or extraction by vacuum, leaving hardened nanocapsules in an aqueous medium (Figure 2.9). As mentioned previously, as an optional step, nanocapsule dispersion can be diluted before extraction under vacuum to ensure full solvent diffusion [69].

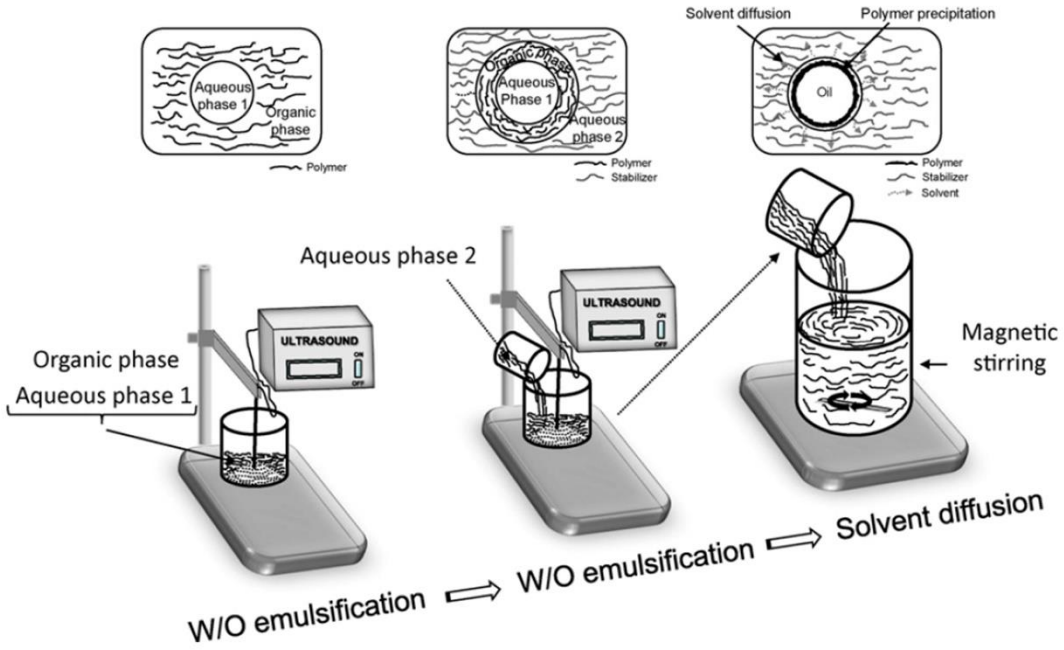


Figure 2.9. Preparation of nanocapsules with double-emulsification method. [59]

Emulsion- Coacervation Method

Nanocapsule formation by the emulsion-coacervation method uses the emulsion as a template phase and the formation of a coacervate phase that causes polymer precipitation from the continuous emulsion-phase to form a film on the template forming the nanocapsule. The procedure involves the O/W emulsification of an organic phase (oil, active substance) with an aqueous phase (water, polymer, stabilizing agent) by mechanical stirring or ultrasound. Then, a simple coacervation process is performed by using either electrolytes or a dehydration agent or by temperature modification. Finally, the coacervation process is complemented with additional crosslinked steps that make it possible to obtain a rigid nanocapsule shell structure. (Figure 2.10.) Although electrolytes, dehydration and temperature modification are frequently used to reduce polymer solvation, other factors such as

changing pH and adding other materials that are incompatible with the polymer solution can also be used [59].

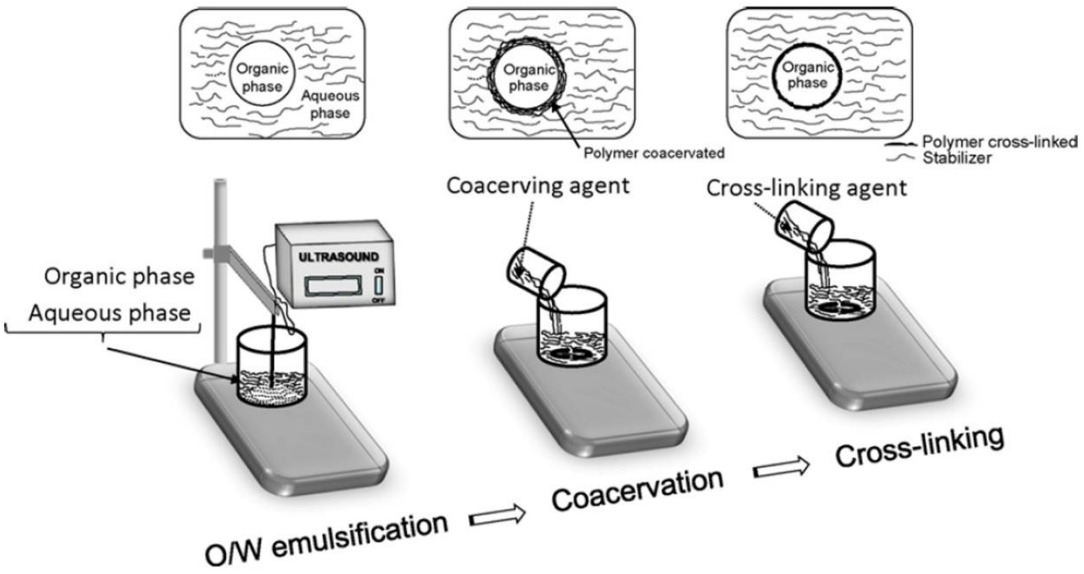


Figure 2.10. Preparation of nanocapsules with emulsion coacervation method [59].

Layer-by-layer

A layer-by-layer nanoassembly technique (LbL) is a widely used technique for the fabrication of multilayered polymeric nanocapsules based on alternate adsorption of oppositely charged materials mostly linear pollyelectrolytes, via electrostatic interactions [70, 71]. This method requires a colloidal template onto which is adsorbed a polymer layer either by incubation in the polymer solution, subsequently washed, or by decreasing polymer solubility by drop-wise addition of a miscible solvent. This procedure is then repeated with a second polymer and multiple polymer layers are deposited, one after another. However there are some difficulties for this method like formation of counterion aggregates, the separation of the remaining free polyelectrolyte from the particles prior to the next deposition cycle. In addition, another difficulty is the particle sizes obtained which are higher than 500nm. Although these particle sizes are at submicronic scale, they are obviously larger than the size commonly accepted for nanocapsules. The other limitations can be counted as the high number of assembly steps involved is quite complex and time consuming [59].

2.2.1.2. Characterization of Nanocapsules

Characterization of a nanoparticulate system is essential for understanding and prediction of the performance of the system in the body. Hence various techniques are used to characterize nanocapsule and predict their ultimate fates in the body [72]. Analytical methods such as electron microscopy, size measurements and zeta potential determinations were included in the nanocapsule characterization. These techniques allow efficient comparison across nanoparticles and facilitate the nanocapsule optimization.

Size Measurements

Particle size and size distribution are the most important characteristics of nanocapsules. They determine the in vivo distribution, biological fate, toxicity, and targeting ability of these delivery systems. In addition, they can influence drug loading, drug release. Currently, the fastest and most routine method of determining nanocapsule size is by photon-correlation spectroscopy or dynamic light scattering [73].

Dynamic light scattering (DLS) is commonly used for particle size determination. DLS measures Brownian motion of nanoparticles in suspension and relates its velocity, known as translational diffusion coefficient, to the size of nanoparticles according to the Stokes−Einstein equation. The result is reported as a mean particle size and homogenity of size distribution. The latter is expressed as polydispersity index (PDI), a dimensionless parameter calculated from a cumulant analysis of the DLS-measured intensity autocorrelation function. A PDI value from 0.1 to 0.25 indicates a narrow size distribution, and a PDI value greater than 0.5 indicates a broad distribution [74].

Morphology of Nanocapsules

Conventional light microscopy is not suitable for nanocapsule characterization due to its limited resolution, therefore electron microscopy provides an accurate assessment of the size and shape of an nanocapsules. Techniques for the characterization of nanoparticle size and morphology are scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The results obtained by photon-correlation spectroscopy are usually verified by scanning or transmission electronmicroscopy (SEM or TEM). Another imaging-based method is

the particle tracking analysis includes dark field (confocal microscopy) or fluorescence microscopy [75].

Surface Charge Measurements

Surface charge, expressed as zeta potential, critically influences the interaction of nanocapsules with the environment. The zeta potential measurement depends on the strength and valency of ions contained in the nanocapsule suspension. There are two liquid layers surrounding a nanoparticle; strongly bound inner part (Stern layer) and weakly bound outer layer. Zeta potential is commonly measured by laser Doppler electrophoresis, which evaluates electrophoretic mobility of suspended nanoparticles in the medium, thus measuring the potential at the boundary of the outer layer. Generally, particles with zeta potential more positive than +15 mV or more negative than −15 mV have colloidal stability [75].

2.2.2. Nanoparticles in Oral Chemotherapy

Nanotechnology has revolutionized the field of oral drug delivery research and over the last decades nanotechnology based formulations have received great interest. Table 2.2. enlists nanotechnology based oral formulations which are available in the market and in clinical trials [76]. As seen from Table. In oral drug delivery among various nano-structures, only nanosuspensions and spontaneously emulsifying systems have succeeded in reaching pharmaceutical market. However it is expected that there will be an accelerating expansion of the development of nanotechnology based oral formulations in the near future.

Table 2.2. List of nanotechnology based oral formulations in pharmaceutical market and in clinical trials [76].

Product	Drug	Nanotechnology	Dosage form	Indication
Rapamune®	Sirolimus	Nanosuspensions	Tablet	Immuno-suppressant
Megace ES®	Megestrol acetate	Nanosuspensions	Nanosuspension	Treatment of anorexia
Emend®	Aprepitant	Nanosuspensions	Capsule	Antiemetic
Tricor®	Fenofibrate	Nanosuspensions	Tablet	Antihyperlipidemic agent
Triglide ®	Fenofibrate	Nanosuspensions	Tablet	Antihyperlipidemic
Panzem NCD	2-Methoxy estradiol	Spontaneously emulsifying systems	Nanosuspension	Anti-angiogenic effect
Sandummine Neoral®	Cyclosporine	Spontaneously emulsifying systems	Soft gelatin capsule	Immuno-suppressant
Gengraf ®	Cyclosporine	Spontaneously emulsifying systems	Hard gelatin capsule	Immuno-suppressant
Norvir®	Ritonavir	Spontaneously emulsifying systems	Soft gelatin capsule	Anti-retroviral (anti-HIV)
Fortovase®	Saquinavir	Spontaneously emulsifying systems	Soft gelatin capsule	Anti-retroviral (anti-HIV)

Taking account of traditional practices into cancer therapy, nanomedicine is not only just one more tool but also a revolution in cancer chemotherapy. Treatment of cancer can be achieved with “personalized medicine” and nanotechnology has this potential as an effective strategy to facilitate oral delivery of anticancer drugs and the oral nanoparticulate drug delivery systems show such distinct advantages that they are able to bypass a series of biobarriers during oral absorption (Figure 2.11). Nanoparticles also protect the entrapped drug from chemical and enzymatic

degradation, prolong the systemic circulation time and control the release of drug in blood. All of these benefits can contribute to reduction of dose and dosing frequency, thereby reducing the side effects and improving the patient compliance.

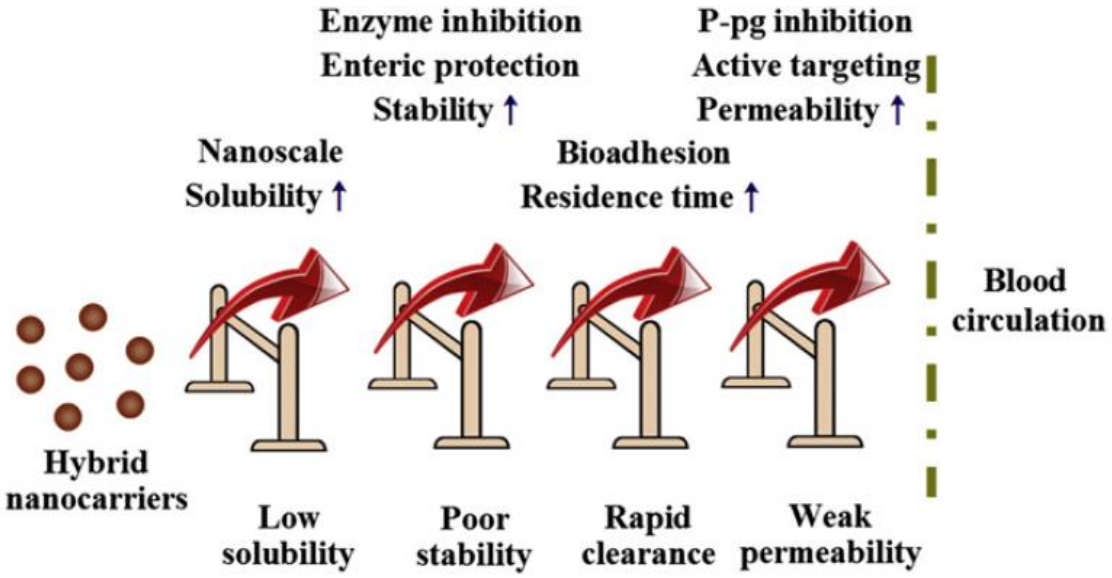


Figure 2.11. Nanoparticles for overcoming multiple barriers [13].

Among its many advantages, nanopharmaceuticals may facilitate the oral administration of drugs that are currently delivered only by injection. Nanomedicine will drastically improve a patient's quality of life by early detection and/or more efficient treatment with less drug-related side effects. Over all, expansion of these nanopharmaceuticals will improve the practice of chemotherapy and clinical outcomes in the coming years.

2.2.2.1. Overcoming multiple barriers

Nanoparticulate drug delivery systems as oral chemotherapy agents exhibit numerous advantages from the pharmaceutical perspective over conventional approaches. As it can be clearly seen from the Figure 2.12., nanotechnology holds great potential as an effective strategy to facilitate oral delivery of chemotherapeutical drugs falling under different classes (I-IV) of BCS [77].

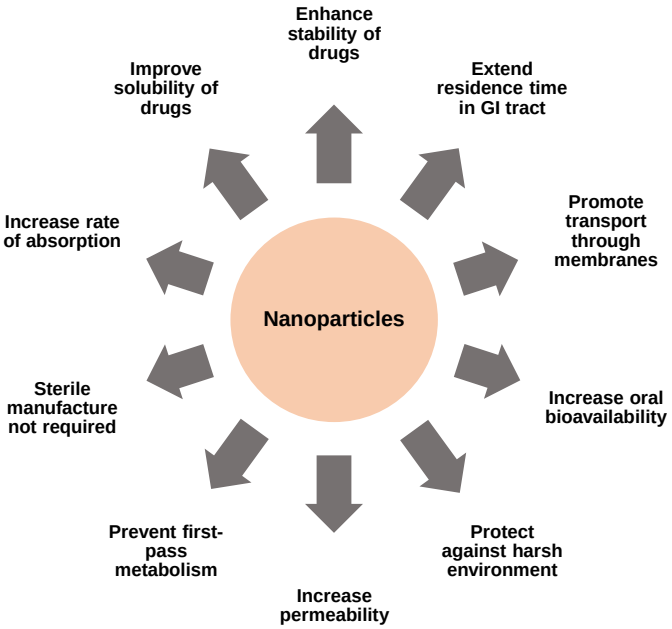


Figure 2.12. Benefits of nanoparticles in oral chemotherapy

Thus far, few nanotherapeutic (and diagnostic) materials have been approved by the US Food and Drug Administration (FDA) for clinical use, although more are currently in various stages of preclinical and clinical development. On the other hand, only a few nanomedicines are approved by FDA for use in the treatment of cancer in general and neither of them are for oral chemotherapy in particular (Table 2.3.) [41, 53].

As it was stated in section 1.1.1. orally administered drugs encounter many difficulties during absorption process, including low solubility, poor chemical stability, low permeability, harsh environment of GI tract (variable pH values, short residence time and abundant metabolic enzymes) and affinity to the drug efflux pump P-glycoprotein (P-gp). Therefore, in addition to the enhancement of solubility/ dissolution rate, three main strategies should be taken into account to improve oral bioavailability: 1) to enhance the stability of not only the incorporated molecule but also nanocarriers; 2) to prolong the residence time in GI tract and 3) to enhance the permeability of the drug and carriers through epithelium [13].

Table 2.3. Examples of non-targeted nanosystems in clinical use for anticancer therapy. [41, 53]

Name	Formulation	Active agent	Indications	Status
Doxil®/ Caelyx® (in the EU)	PEGylated liposomes	Doxorubicin	Breast cancer, ovarian cancer, multiple myeloma	Approved
Abraxane®	Albumin nanoparticles	Paclitaxel	Breast cancer	Approved
Myocet®	Non-PEGylated liposomes	Doxorubicin	Breast cancer	Approved
Daunoxome®	Non-PEGylated liposomes	Daunorubicin	Kaposi's sarcoma	Approved
Oncaspar®	PEG-L-asparaginase	Asparagine specific enzyme	Acute lymphoblastic leukemia	Approved
Onco TCS®	Non-PEGylated liposomes	Vincristine	Non-Hodgkin's lymphoma	Approved
Thermodox®	PEGylated liposomes	Doxorubicin	Liver cancer, breast cancer	Phase III
SPI-77	PEGylated liposomes	Cisplatin	Ovarian cancer	Phase II
Genexol-PM®	PEG-poly(lactic acid)	Paclitaxel	Breast cancer, lung cancer, ovarian cancer	Phase II
Opaxio™	PGA-paclitaxel	Paclitaxel	Lung cancer, ovarian cancer	Phase III
ProLindac™	HPMA	DACH-Pt	Ovarian cancer	Phase II
NC-6004	PEG-poly (glutamic acid)	Cisplatin	Pancreatic cancer	Phase II
Depocyt®	Non-PEGylated liposomes	Cytarabine	Leukemia	Phase III
CRLX101	PEG-cyclodextrin	Camptothecin	Non-small-cell lung cancer	Phase II
NL CPT	PEGylated liposomes	Irinotecan	Glioma	Phase I

HPMA: hydroxypropylmethacrylamide, DACH-Pt: diaminocyclohexane-platinum

In terms of overcoming multiple barriers, nanoparticulate systems take the advantages of improving drug solubility, drug stability and prolonging residence time as well as improving diffusion through membranes which can be listed as follows:

Improving solubility of drugs

Poor aqueous solubility and intrinsic dissolution rate (mass of the drug dissolved per time unit and area) are the major factors that influence oral delivery of many anticancer drugs. If a drug is not getting dissolved then it becoms very difficult to administer and hence shows poor bioavailability. Importance of solubity enhancement is for the absorption of drug from the site of absorption. Poor water soluble drug show poor bioavailability. Low aqueos solubility may account for problems like high intra- and inter- subcet variability, lack of dose proportionality, performance limitations, incomplete absorption, and poor bioavailability. [78]

For drug compounds trying to make their way from the lab to the market, poor solubility is often challenging. It's one of the most common reasons for a drug candidate to drop out of the development pipeline. The poor water solubility of the drug may restrict intestinal absorption, leading to low bioavailability and gastrointestinal side effects, which are common problems faced by most of the anticancer drugs. In other words, poor solubility and/or low dissolution are the rate-limiting steps for oral absorption and associated with poor oral bioavailabilty. [79].

A nanoparticle formulation approach has proven to be very useful for the improvement of poorly soluble drugs (BCS II and IV) in all stages of the drug development. Since nanoparticles have very high specific surface areas (i.e. surface area-to-mass ratios), they can be an alternative for enhancing the dissolution rate of poorly soluble drugs [80]. Large drug particles cannot adequately dissolve resulting in the inability to be absorbed whereas nanometer drug particles are rapidly dissolved during transit through the gut, thus maximizing absorption and improving bioavailability. The increase in surface area leads to an increase in the dissolution velocity according the Noyes- Whitney quation which describs the dissolution rate of th drug in a diffusion- controlled process [81].

The ability to formulate poorly-water soluble molecules as nanometer sized particles can have a dramatic effect on performance, such as enhancing bioavailability, eliminating food effects and hence improving efficacy and safety. Enhanced

dissolution rate and improvement in the solubility of a nanosized drug particle can maximize absorption and thereby bioavailability of a poorly-water soluble drug candidate. [81].

Liu et al [82] investigated the effect of nanoparticles on dissolution and bioavailability of poorly water soluble Celecoxib (CXB). According to the results stable CXB nanoparticles with markedly enhanced dissolution rate and oral bioavailability were observed due to an increased solubility with increased surface area. Similar results were obtained by the work of Gao et al [79] with poorly water soluble Lapatinib incorporated in core-shell nanoparticles. Results showed that core-shell nanoparticles effectively increased the solubility of lapatinib and improved the treatment of glioma.

Improving the stability of drugs in the GI tract

To ensure the stability of drugs and drug-loaded nanocarriers is a priority for efficient oral drug delivery since orally administered drugs or drug loaded carriers must survive transit through the chemical and enzymatic GI liquids. Several factors can influence their stability in GI tract, including physical forces (e.g. GI motility and the interference of GI contents) and chemical pressures (e.g. varying pH, surfactants, enzymes in GI fluid) [13]. Nanoparticles can protect the incorporated drug molecules from GI degradation as well as gut wall metabolism, thereby ensuring the stability of the drug before absorption. [77]. However solid-state nanoparticles (nanocrystal) and polymer based nanoparticles show better stability compared with the liquid-crystal like nanoassemblies (micelles, liposomes and lipid vesicles, etc.) [13]. Furthermore pH sensitive nanoparticles based on enteric polymer coating have a strong potential for ensuring the stability of drugs and drug loaded carriers as well. The composition of the nanocarrier will strongly affect its stability in the GI tract. Nanoparticles prepared with insoluble polymers will neither be immediately degraded nor rapidly release incorporated drugs. On the other hand, nanoparticles prepared with water-soluble polymers will be strongly influenced by the variable pH changes and/or ionic strength thereby more likely to be destabilized [1].

Furthermore metabolic enzyme inhibition can efficiently enhance the metabolic stability of drugs. The metabolism of CYP 450 enzymes poses a great threat to the stability of drug molecules. Although the physical encapsulation by nanocarriers can

to a certain degree protect drugs, it would not work with the released free drug molecules. Therefore, oral delivery of anti-cancer drugs, especially for the substrates of CYP-450 enzymes, will certainly benefit from metabolic enzyme inhibition. On the contrary of low molecule weight metabolic inhibitors, high molecule weight metabolic inhibitors (functional polymers and surfactants) e.g. PEG, Pluronic F68 and Tween-80, possess the ability to suppress the metabolism of CYP-450 enzymes and have a limited in vivo biodistribution and low-probability polymer–drug interaction. Thus, application of these polymers as nanocarrier matrices holds great potential to enhance the metabolic stability of drugs.

Qu et al [83] developed a silica based nanomatrix system for the oral absorption of peptides. This pH sensitive nanomatrix systems enabled to maintain the stability of peptides with an improvement of intestinal permeability and relative bioavailability. Similarly Hao et al [84] prepared nanocarriers using enteric Eudragit L100-55 as carrier material and omeprazole as model drug. It was found that these enteric nanoparticles showed a high physical stability and cellular uptake studies indicated that these nanosystems could be easily taken up by Caco-2 cells.

Improving drug adhesion and diffusion in the GI mucosa

Adhesion and diffusion of the drug in the mucosal environment of the GI is another problem for oral chemotherapy [31]. When nanoparticles administered orally these scenarios may take place in GI tract 1) direct transit through the GI tract and fecal elimination; 2) trapped by mucin fibers followed by mucosal clerance and fecal elimination; 3) prolonged residence time in the mucus layer and transport across the mucus mesh. Nevertheless, most of the administered particles do not have the ability to adhere and/or transit through the mucus layer, rather undergo direct transit through GI tract. This short transit time in the GI tract can be inadequate for particles to release a significant fraction of encapsulated drugs, thereby preventing in many cases the realization of a high local drug concentration over extended periods of time and as a result causing low bioavailability and poor efficacy [27].

Upon oral administration, drug loaded nanocarriers can not directly contact with GI epithelial cells due to the existence of a “mucus barrier” which forms the defence mechanism of the body. However, the association with mucus whereby a prolonged retention time provided, is of primiraly concern for oral drug delivery systems since

only in this way drug loaded nanocarriers can make full contact with intestinal epithelial cells and thereafter be transported into the lymphatic or blood circulation. [85]. Most of the orally administered particles are not retained long enough in the GI tract. Due to the small size and large dispersibility, nanoparticles have themselves certain mucoadhesion ability, however this plays only a marginal role. Thus many strategies have been developed with the intention of improving mucoadhesion. This phenomenon, known as mucoadhesion is defined as the ability of particulates to adhere the mucus layer. Mucoadhesion has been commonly employed in attempting to improve the residence time of particles in GI tract and [19].

Two primary strategies have been accepted and widely used to enhance bioadhesion of nanosystems:

1) surface characteristics can be tailored to optimize mucoadhesion

2) polymer materials with sulfhydryl groups increase the interaction with mucin. (Figure 2.13.)

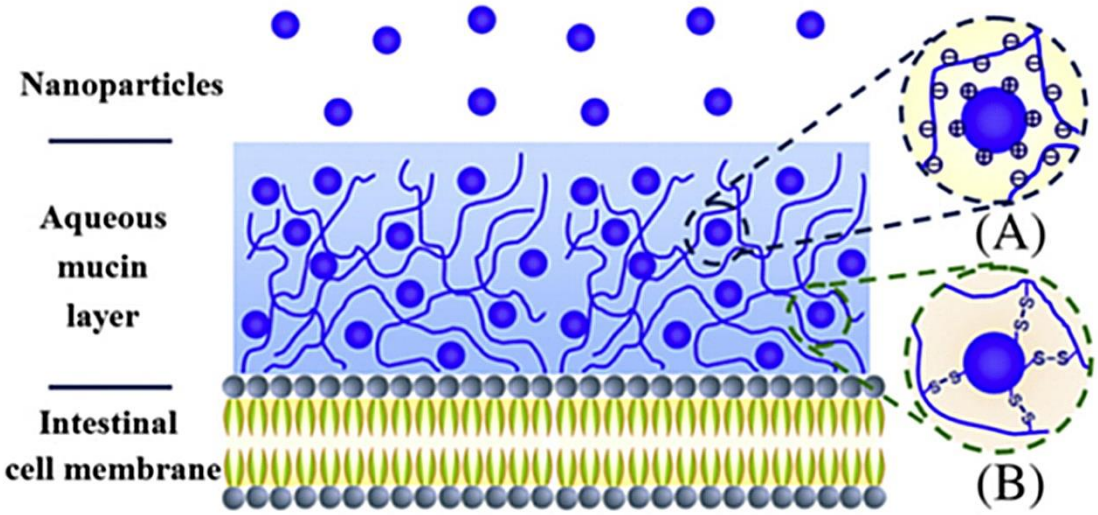


Figure 2.13. Mucin-particle interactions via; A) electrostatic forces and B) disulfide bonds. [13]

As it is stated in section 2.1.1.2., aqueous mucin layer is negatively charged and is rich in cysteine with sulphydryl groups. Strong interactions with mucus could increase retention time of particulates at mucosal surface. These interactions are

driven by hydrogen bonding, Van der Waals interactions, polymer chain interpenetration, hydrophobic forces and electrostatic /ionic interactions. Positively charged polymer materials from which nanoparticles are prepared have the ability to prolong the residence time in GI tract by electrostatic forces with mucin layer. Negatively charged intestinal mucosa due to the gloycocalix, attracts positively charged nanoparticles. Thus cationic polymers like chitosan and its derivatives, and groups binding to mucin (e.g thiol) has been widely applied in mucoadhesive carrier systems. Once the mucin barrier is overcome, the next step should be permeability across the intestinal wall [86, 87]. Orally delivered drugs must penetrate across the epithelium in order to reach the circulation system and eventually other target sites in the body [31]. In order to achieve an efficient absorption, nanoparticles need to be taken up from the GI tract with a sufficient rate and extent.

As emphasized before, mucoadhesive nanocarriers have been designed to adhere onto the mucus layer in order to improve the interaction with the mucus and prolong residence time in GI tract. However, mucoadhesive nanocarriers can also be trapped in the fiber mesh of sticky mucus and readily be swept away [19]. Therefore, in addition to the adhesiveness, the penetration capability of nanocarriers through the mucus is equally critical for oral delivery systems as depicted in Figure 2.14. [88, 89] . In order to overcome mucosal barriers, drug loaded carriers should be not only mucoadhesive to provide full contact with the mucosa and prolong retention time, but also should avoid trapping with steric inhibition by fiber mesh and mucosal clearance. It is important being able to develop of particles designed to overcome mucosal clearance mechanisms, since they must penetrate mucus at rates markedly faster than mucus renewal and clearance in order to overcome the barrier. Hence, there is a potential for development of oral nanoparticulate systems utilizing both adhesiveness and penetration.

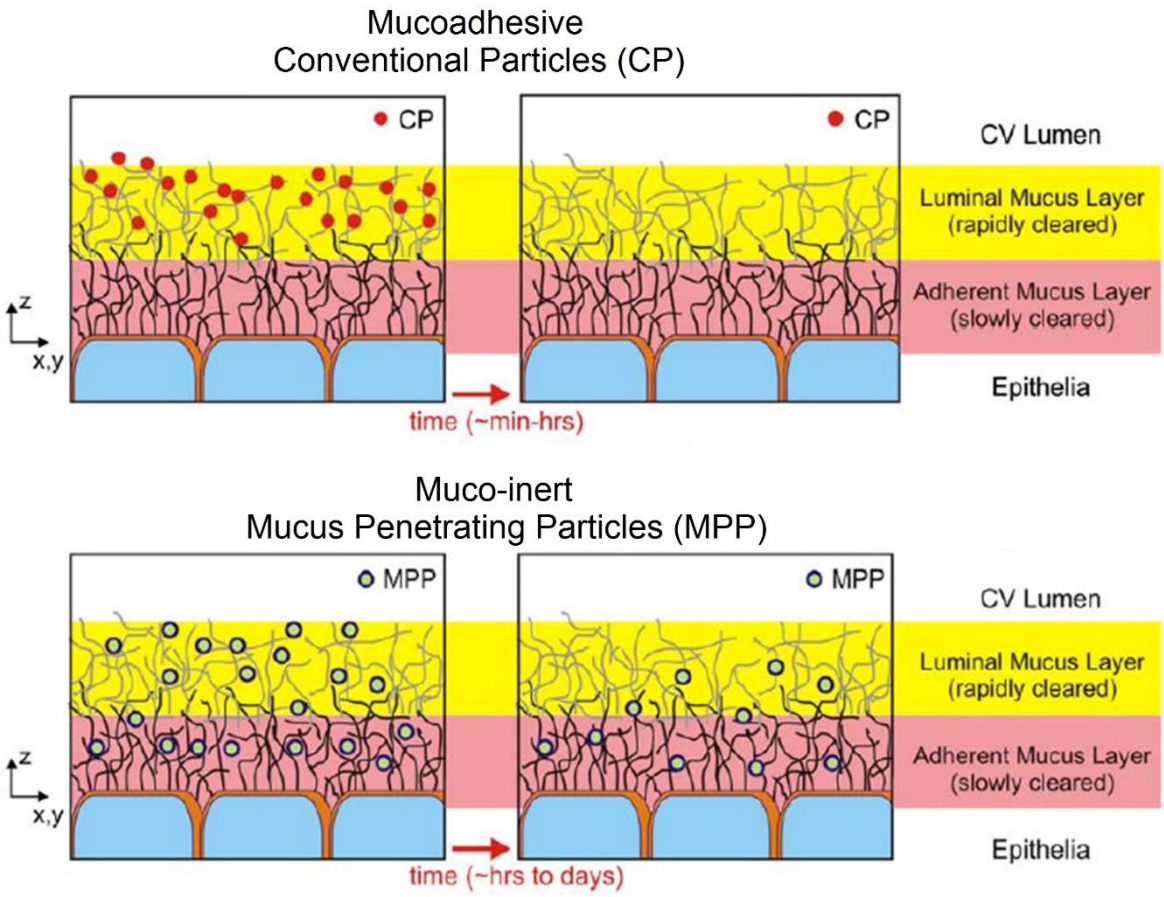


Figure 2.14. Schematic illustrating the fate of mucus-penetrating particles and conventional mucoadhesive particles administered to a mucosal surface [27].

Jiang et al [90] has investigated the effect of mucoadhesive nanoparticles prepared from thiolated chitosan for the oral delivery of paclitaxel for lung cancer. Results obtained from this work has revealed that thiolated chitosan-modified nanoparticles have significantly higher level of the cellular uptake than unmodified nanoparticles. It seems that the mucoadhesive nanoparticles can increase paclitaxel transport by opening tight junctions and bypassing the efflux pump of P-glycoprotein. Similar results have obtained with the work performed by Zhang et al [91] for oral delivery of insulin using both chitosan coated and uncoated PLGA nanoparticles. Results showed that chitoan modified nanoparticles displayed better bioadhesion and higher oral delivery efficiency of insulin than unmodified nanoparticles.

For the transport of nanoparticles across the intestinal epithelial barrier, two different pathways have been suggested, the paracellular route and the transcellular route. In transcellular pathway the absorption takes place through the intestinal epithelial

cells, enterocytes, and M cells of Peyer's patches (PP) either passively or actively (Figure 2.15.) The surface area for transcellular absorption is very high compared with paracellular absorption. Most of the reported studies have revealed that the uptake of nanoparticles take place via M cells of Peyer's patches due to high transcytotic capability. Nanoparticles with size less than 50 nm are transported paracellularly, those with sizes between 50 and 200 nm are endocytosed by enterocytes, and those between 200 nm up to 5 μm are taken up by M cells of the Peyer's patches [86].

Besides, nanoparticles take the advantages of the transport via M cells, since these cells play a role of taking up the nanoparticles from the lumen and transporting them into the lymphoid tissue by by-passing the first pass metabolism [92]

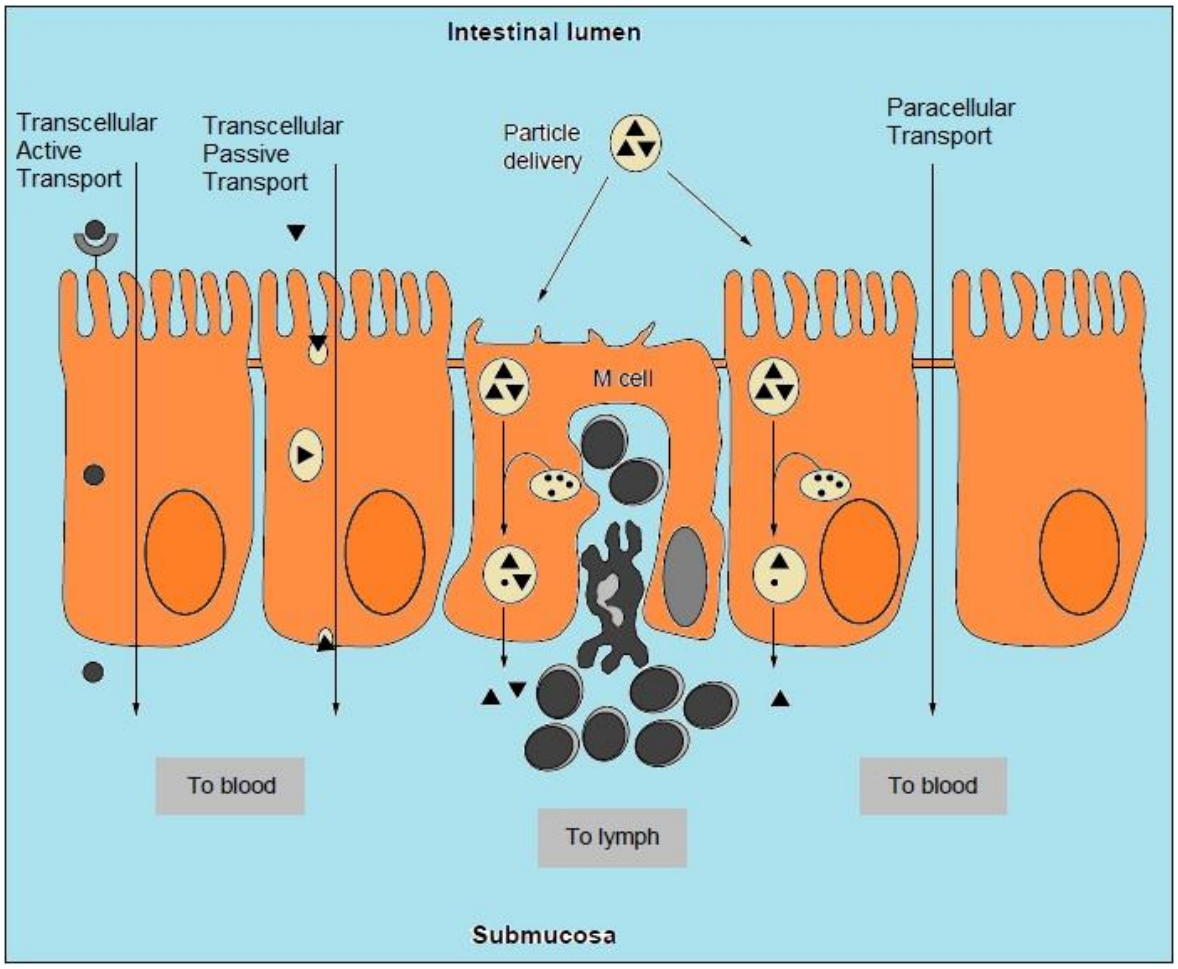


Figure 2.15. Schematic representation of transepithelial intestinal pathways: a) transcellular active transport, b) transcellular passive transport, c) paracellular transport [93].

On the other hand, the paracellular route for nanoparticle translocation is very limited because of the very small surface area of the intercellular spaces, contributing to less than 1% of the total mucosal surface area and also, tightness of the intercellular junctions (pore diameter < 10 Å). These tight junctions restrict or totally blocks molecules larger than 1 nm [77]. However, paracellular transport of drugs can be enhanced by nanoparticles prepared and/or coated with various polymers such as chitosan and poly(acrylate) which facilitate paracellular transport (Figure 2.16.) resulting in opening of tight junctions. Several nanocarriers have been reported to open tight junctions and increase paracellular transport of drugs [1].

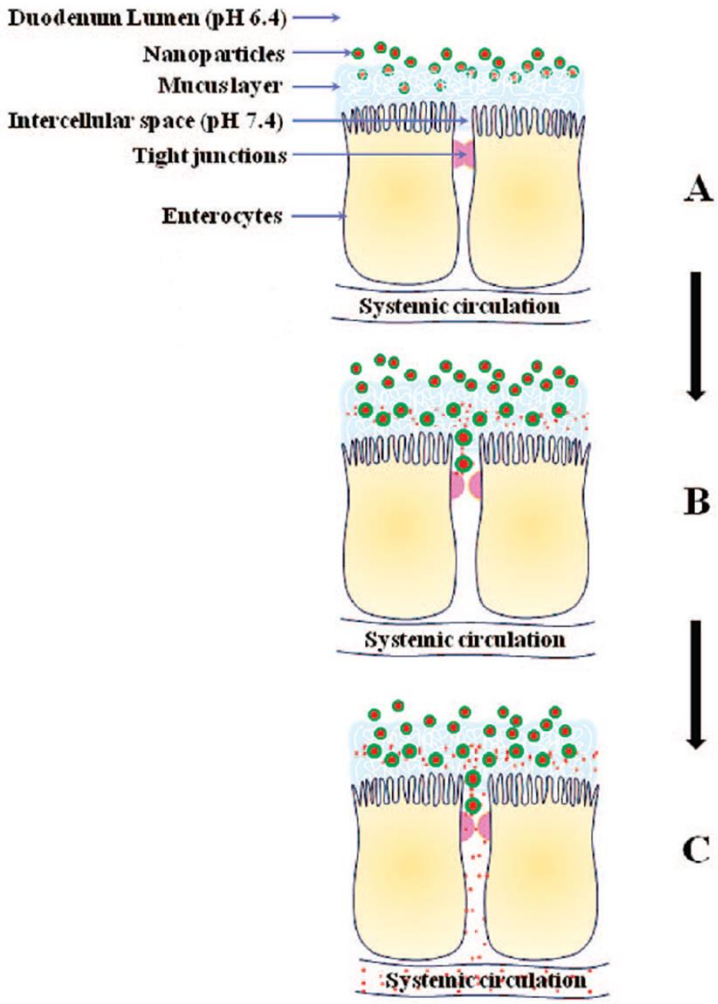


Figure 2.16. Mechanism of opening tight junctions by nanoparticles and transporting of their encapsulated drug (using duodenum as an example). A) Nanoparticles adhere to the mucus of the intestinal tract. B) Nanoparticles begin to infiltrate into the mucus C) The infiltrated nanoparticles permeate while openining the tight junctions through the opened paracellular pathway [94].

Most of the anticancer agents are available in oral dosage form for clinical use but very few of them are actually used which could be attributed to the limited oral bioavailability owing to poor physicochemical properties and efflux mechanisms. Transport of Taxanes (Paclitaxel and Docetaxel) is strongly hampered by affinity to P-gp in addition to their poor solubility and metabolism by enzymes resulting in poor oral bioavailability [31, 95, 96].

P-gp transporter impedes the permeability of drugs through physiological barriers producing limited pharmacological response. Therefore the inhibition of this efflux pump should be a common strategy to overcome low oral bioavailability [14]. Transmembrane efflux of drugs can be tackled by co-administration of various P-gp modulators or inhibitors along with the drugs. P-gp inhibitors have been broadly classified to three classes as i)first generation, ii) second generation and iii) third generation inhibitors. First-generation inhibitors act as competitive inhibitors and hence block the drug efflux e.g. verapamil and cyclosporin A. But considering their poor binding affinities (needing higher doses to achieve desired results) and pharmacokinetic interactions (due to induction/inhibition of CYP 3A enzymes) these are not clinically advisable. Second generation included those lacking pharmacological activity and were developed with the intention of high P-gp binding and inhibiting effect along with lower toxicities. However, they fail to remain inert with CYPs thereby limiting their use. Therefore the third generation came in to play and highly P-gp specific inhibitors such as elacridar, zosuquidar and tariquidar were developed but this approach is rarely used clinically owing to associated clinical complications such as suppression of immune system thereby causing long term medical complications.

Hence, the novel approach of exploiting similar properties of excipients can be sought. Some commonly used functional excipients, such as surfactants, polymers, lipids, etc. can be employed as bioavailability enhancers. Natural polymers (dextran, sodium alginate), polyethylene glycol (PEG) and its derivatives, thiolated polymers, surfactants (mainly polysorbates, pluronic block co-polymers) and cyclodextrins (methylated cyclodextrins) are well reported to inhibit the P-gp efflux. Utilizing functional excipients in improving the oral delivery of anticancer drugs is a more logical and scientific approach and nanoparticles prepared of these functional

excipients are capable of altering absorption pathways by inhibition efflux transporters and enhancing net drug transport through intestinal barrier.

Ma et al. [97] investigated the effect of Pluronic F68 on P-gp transport, and found that Pluronic F68 produced similar inhibitory effect of P-gp as verapamil, thereby increasing intestinal absorption of rifampicin. Similarly Guan et al. [98] demonstrated that Pluronic P123 and F127 have obvious inhibitory effect on the intestinal P-gp activity.

These altered absorption pathways further appreciate the improvement in oral bioavailability of anticancer drugs that are difficult to deliver [14]. Besides when drugs are encapsulated in polymeric nanoparticles, they remain mainly associated with the particles and are not likely to be substrate of the efflux pump [1].

2.3. Camptothecin

In recent years inhibitors of the enzyme topoisomerase I have proven to be one of the most promising new classes of antineoplastic agents introduced into the clinic and have a relevant impact on cancer therapy. DNA topoisomerases are nuclear enzymes that play an essential role in several DNA functions including DNA replication, transcription, recombination and repair. Topoisomerase I acts by formation of transient single-strand breaks in DNA [99]. 20(S)-Camptothecin (CPT) in this class, was first isolated from the wood and bark of *Camptotheca acuminate* (Nyssaceae), a tree native to China, in 1966. *C. acuminata* also goes by the names cancer tree and tree of life. These names are appropriate considering the anticancer properties of camptothecin. The Chinese have used *C. acuminata* in traditional medicine for thousands of years. Initial clinical trials in the 1970's did not make use of native camptothecin, but of its water soluble salt. Camptothecin is a naturally occurring cytotoxic pentacyclic alkaloid [100, 101] and shows great utility in the treatment of various cancers including primary and metastatic colon carcinoma, small cell lung carcinoma, ovarian, breast, pancreatic cancers [102-104].

2.3.1. Structure and Physicochemical Properties

Camptothecin, with the IUPAC name 4-ethyl-4-hydroxy-1H-pyrano[3',4':6,7] indolizino[1,2-b] quinoline-3,14-(4H,12H)-dione has a molecular weight of 348.35 g/mol corresponds to the formula $C_{20}H_{16}N_2O_4$. CPT is insoluble in water, soluble in DMSO and chloroform: methanol (4:1, v/v). CPT has a boiling point at 757.01 °C at

760 mm Hg and a melting degree 264-267ºC. CPT also exhibits intense blue fluorescence when exposed to UV light, which could be useful in optical experiments.

The CPT compound has a pentacyclic ring system with an asymmetrical center in ring E with 20S configuration including a five-ring backbone includes a pyrrolo[3,4-β] quinoline moiety (rings A, B, and C), a conjugated pyridone (ring D), and a six-membered lactone (ring E) with an R-hydroxyl group (Figure 2.17.) [105, 106].

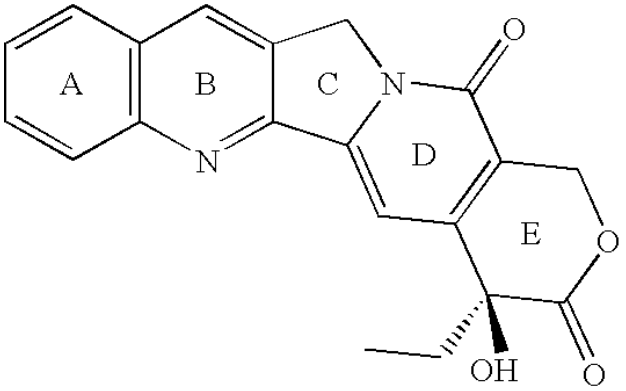


Figure 2.17. Chemical Structure of Camptothecin [106].

Camptothecin exist in two forms, a lactone and a carboxylate. All known camptothecins can undergo a pH-dependent reversible interconversion between this lactone form and a ring-opened carboxylate (or hydroxy acid) form as depicted in Figure 2.18. Active lactone form of CPT exists under pH 5 but hydrolyses rapidly into the inactive carboxylate form at physiological or alkaline pH resulting in lack of clinical activity and higher toxicity. The lactone comprises a majority at acidic pH, and the carboxylate at neutral alkaline pH. The presence of a lactone functionality in the E-ring, is essential for antitumor activity, is the only form of CPT able to diffuse across cell membranes, and exert the characteristic topoisomerase I inhibitory activity. The carboxylate form of CPT has superior water solubility but it is ~10 times less potent than lactone structure. The equilibrium solubility of the drug was found to increase with increasing temperature and decreasing pH. The enhanced solubility of the drug at very low pH is attributed to the protonation of the nitrogen atom in the ring B and the increased solvency of the highly concentrated acidic media. [107].

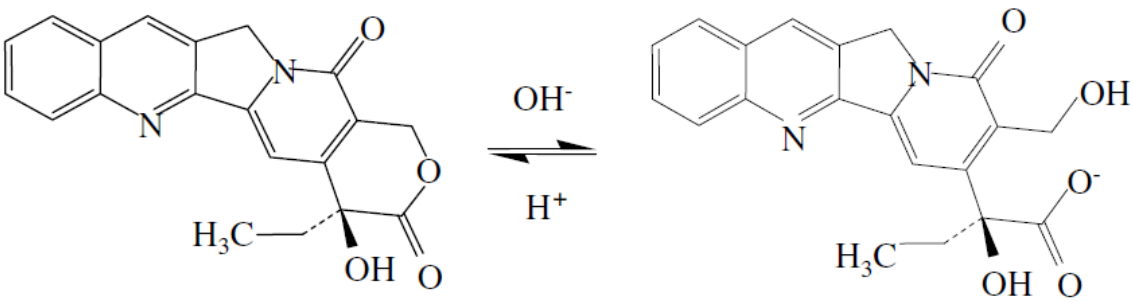


Figure 2. 18. Chemical structure of camptothecin (CPT); an active lactone form at pH below 5 and an inactive carboxylate form at basic pH.

The opening of the labile E-ring in the lactone form at physiological pH and above, which may render the drug much less active and highly toxic form carboxylate, represents a major obstacle to the clinical application of CPTs. The rapid hydrolysis of CPT is also accelerated in the presence of human serum albumin resulting in an occurrence only a few minutes after intravenous injection. This hydrolysis reaction is reversible, pH-dependent and influenced by solution composition. Although the equilibrium is potentially reversible, it tends to be transformed into inactive carboxylate form, which then is tightly bound to serum albumin and the active lactone is rapidly eliminated in the bloodstream [108, 109]. Therefore, in addition to stability problem, oral delivery of camptothecin is limited by poor and variable bioavailability attributed to low solubility, low permeability and P-gp efflux of the drug [110].

2.3.2. Mechanism of Action

The unique mode of action for this potent cytotoxic compound was found to act via inhibition of an enzyme known as DNA topoisomerase I [111]. CPT was found capable of inhibiting DNA synthesis which led to the cancer cell death arrested in the S-phase of the cell cycle [112].

The DNA topoisomerases are nuclear enzymes that reduce the torsional stress of supercoiled DNA. This action enables selected regions of DNA to become

sufficiently exposed and relaxed to facilitate essential cellular processes such as DNA replication, recombination, and transcription to occur [113].

Many attempts have been made to modify the camptothecin structure in order to reduce its toxicity and maintain or increase its activity [106]. These key findings then required drug-research efforts with the aim to identify and develop new semi-synthetic analogues with improved aqueous solubility while maintaining CPT's unique mechanism of action. Therefore many CPT analogs were synthesized, and some have been applied in clinical therapy of cancers [108].

To date, only two water-soluble CPT analogs have gained FDA approval: Topotecan (Hycamtin®) and irinotecan (Camptosar®). Both of them are now commercially available as aqueous solutions irinotecan (Camptosar®) and topotecan (Hycamtin®) which are injectable by intravenous route for human treatment. Topotecan is currently a standard second-line therapy for patients with ovarian and small cell lung cancer and Irinotecan is currently used for treatment of colon cancers [114, 115].

2.3.3. Novel approaches for Camptothecin

The rationale for reformulation of CPT or its analogues has been either to stabilize the lactone moieties or to induce sustained release combined with specific tumor targeting of these agents. Although much effort is being put into development of new analogues, the question to answer remains if drugs under development will ultimately lead to the theoretically expected higher activity and/or reduced toxicity. A number of Camptothecin analogues are currently used for clinical treatment. However, none of these analogs surpasses camptothecin in potency of action [116]. Besides, synthesis of these semisynthetic derivatives is also time consuming and expensive. CPT thus appears to be a promising drug if the barriers against its clinical use can be resolved.

In the pharmaceutical field, several advantages of drug delivery systems in the nano size range have been demonstrated, including increasing solubility, reducing side effects, prolonging pharmacological effects, and improving bioavailability. They may protect camptothecin from rapid hydrolysis of its lactone ring in plasma to the essentially inactive carboxylate form. Moreover, camptothecin requires a prolonged schedule of administration and drug targeting to expand its efficacy. Numerous

studies have focused on using certain innovative carriers for CPT such as nanospheres [105, 117-120], nanocapsules [121, 122], solid lipid nanoparticles [123-126] polymeric micelles [127-131], liposomes [132-135], dendrimers [136-138], nanocristals [139], nanoemulsions [140] and cyclodextrin complexes [141-145]. However, relatively less work has been completely and comprehensively evaluated with nanocapsules.

2.4. Cyclodextrins

Cyclodextrins (CDs) are natural polymers discovered by Villiers in 1891 and became the topic of prominent scientific interest in the late 1970s [146]. CDs are also called cyclo amyloses, cyclomaltoses or “Schardinger dextrin”s and are non-reducing in the nature [147]. Cyclodextrins are cyclic maltooligosaccharides produced from starch by the enzyme cyclodextrin glycosyltransferase (GTF) [148].

2.4.1. Structure and Physicochemical Properties

When starch is degraded by the enzyme cyclodextrin glycosyltransferase one of several turns of the amylose helix is hydrolyzed and the ends joint together to form cyclic oligosaccharide called cyclodextrin (CD) [149]. The glucose units are linked by α-1,4-bonds and the chair formation of the glucopyranose units shapes the CD molecule into a cone with secondary hydroxyl groups extending from the wider edge and the primary groups from the narrow edge [150]. The most common natural cyclodextrins are α-cyclodextrin (αCD), β-cyclodextrin (βCD) and γ-cyclodextrin (γCD) which consist of six, seven and eight glucopyranose units respectively (Figure 2.19.). However due to steric factors, cyclodextrins having fewer than six glucopyranose units cannot exist, cyclodextrins containing nine, ten, eleven, twelve and thirteen glucopyranose units, which are designated δ-, ε-, ζ-, η- and θ-CD, respectively have been reported [151]. The α-CD comprises six glucopyranose units, β-CD comprises seven such units, and γ-CD comprises eight such units [152]. Due to the chair conformation of the glucopyranose units, the CDs take the shape of a truncated cone or torus rather than a perfect cylinder [153] with a unique structure presenting a hydrophilic exterior “shell” and a hydrophobic inner cavity capable of hosting a wide range of organic guest molecules [154] Figure 2.20. [155]

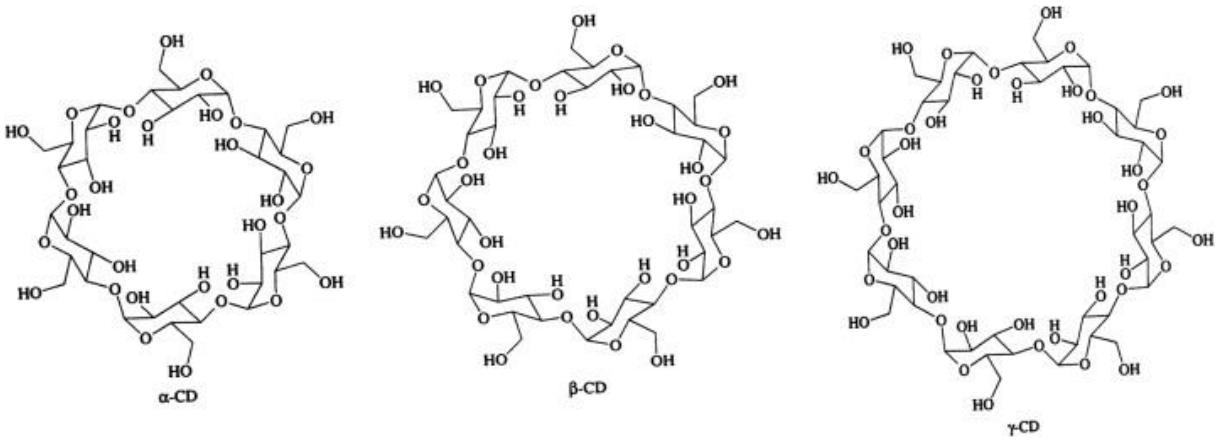


Figure 2.19. Chemical structures of α-CDs, β-CDs and γ-CDs [151].

The cavity is lined by the hydrogen atoms and the glycosidic oxygen bridges. The nonbonding electron pairs of the glycosidic oxygen bridges are directed toward the inside of the cavity producing a high electron density there and lending to it some Lewis base characteristics [156].

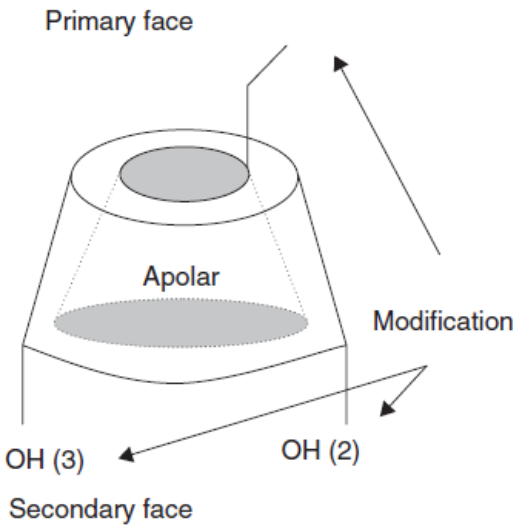


Figure 2.20. Schematic representation of the CD cone shape [155].

In the cyclodextrin molecules, the glucose units, all in classical C1 chair conformation, are linked by α-1,4 bonds. The number of glucose units determines the dimension and size of the cavity. This geometry gives the cyclodextrin the overall shape of a truncated cone with the wider side formed by the secondary 2- and 3-hydroxyl groups and the narrower side by the primary 6-hydroxyl. The cavity is lined by the hydrogen atoms and the glycosidic oxygen bridges. The nonbonding electron

pairs of the glycosidic oxygen bridges are directed toward the inside of the cavity, producing a high electron density. As a result of this special arrangement of the functional groups in the cyclodextrin molecules, the cavity is relatively hydrophobic compared to water while the external faces are hydrophilic [157]. By means of the unique structure of CDs, a guest molecule of appropriate size and shape can be incorporated into hydrophobic cavity in aqueous media.

Compared with α-CDs and β-CDs, γ-CD with a large internal cavity and high solubility exhibits more favorable properties. β-CD has the lowest water solubility in water due to the hydrogen bond. Such relatively low water solubility limits β-CD applications since the complex formation with lipophilic compounds frequently results in the precipitation of solid cyclodextrin complexes. The γ-CD molecule is a non-coplanar, flexible structure, the most soluble of three natural CDs [151, 158]. Both α- and β-cyclodextrin, unlike γ-cyclodextrin, cannot be hydrolyzed by human salivary and pancreatic amylases, instead can be fermented by the intestinal microflora [159]. The cavity size of α-CD is insufficient for many drugs and γ-CD is expensive. Furthermore the hemolytic activity of the parent CDs is reported to be in the order of β-CD>α-CD>γ-CD [160]. The ability of CDs to cause hemolysis to erythrocytes and membrane irritation is related with their ability to extract lipid membrane components such as cholesterol and phospholipids [155].

Natural cyclodextrins have the ability to maintain stability under physiological or ambient conditions, reduce or mask completely the side effects related with the included drug by masking the taste or odor of the active ingredients [161]. In the solid state natural CDs are at least as stable as sucrose or starch and can be stored for several years at room temperature withouth any detectable degradation. One of most characteristic functional properties of cyclodextrins is to form reversible, noncovalent inclusion complexes with compounds that geometrically fit inside the cavity and are less polar than water [162]. The complexes are formed when a “guest” molecule is partially or fully included inside a “host” molecule. This host-guest inclusion complex formation gives the unique nature imparted by their structure. It is known that complexation of drugs with cyclodextrins provide an improvement in its dissolution rate and consequently in oral absorption. As a result, these molecules have found a number of applications in a wide range of fields.

Despite the advantages of natural CDs, relatively low solubility in water and poor interaction with biological membranes limit their uses in pharmaceutical formulations. Besides, two of the natural cyclodextrins (α- and β-cyclodextrins) are known to be parenterally unsafe owing to nephrotoxic effects probably related with the uptake by kidney tubule cells resulting in disruption of cellular membranes, or the extraction of lipid membrane components [155]. For this reason, many cyclodextrin derivatives have been synthesised in order to achieve these goals: to alter their water solubility, reduce the nephrotoxicity and hemolysis encountered on intravenous (i.v.) administration, improve the interaction with biological membranes and provide controlled drug release profiles. These derivatives usually are produced by aminations, esterifications or etherifications of primary and secondary hydroxyl groups of the cyclodextrins [155]. Depending on the substituent, the solubility of the cyclodextrin derivatives is usually different from that of their parent cyclodextrins and also these modifications can improve solubility, stability and help control the chemical activity of guest molecules [158].

2.4.2. Amphiphilic cyclodextrins

Amphiphilic cyclodextrins have been synthesized with the intention of overcoming problems of natural cyclodextrins that limit their pharmaceutical applications. Amphiphilic CDs are obtained by modification on the primary and/or secondary face. [163]. The main reasons for the synthesis of amphiphilic cyclodextrins can be classified as follows:

i) to enhance the interaction with biological membranes,
ii) to ameliorate water solubility,
iii) to modify the rate or time of drug release.

The major advantage of amphiphilic cyclodextrins is their self-alignment or packaging properties at interfaces, which is sufficient to form nanoparticles spontaneously without the presence of a surfactant [164] along with their demonstrated ability of forming inclusion complexes with various drugs in their cavity and within the long aliphatic chains. Because of the hydrophilic character of the native cyclodextrin, preparation of amphiphilic cyclodextrins involves the modification of the primary face and/or at the secondary face with aliphatic chains of varying length (C12 to C18) and different chemical bonds [165].

Amphiphilic cyclodextrins are generally classified according to their surface charge as follows: i) non-ionic amphiphilic cyclodextrins; ii) cationic amphiphilic cyclodextrins; and iii) anionic amphiphilic cyclodextrins. Non-ionic amphiphilic cyclodextrins are obtained by grafting aliphatic chains of different length onto the primary and/or secondary face of the CD main glucopyranose unit and have different shapes according to the structures (Figure 2.21) [155]. Cationic amphiphilic CDs are obtained with an amino group as the ionic group whereas anionic amphiphilic CDs may carry sulfate group that renders anionic property to their structure. Cationic amphiphilic CDs emerge as a promising option owing to their very strong cellular interaction properties and good cellular uptake [166].

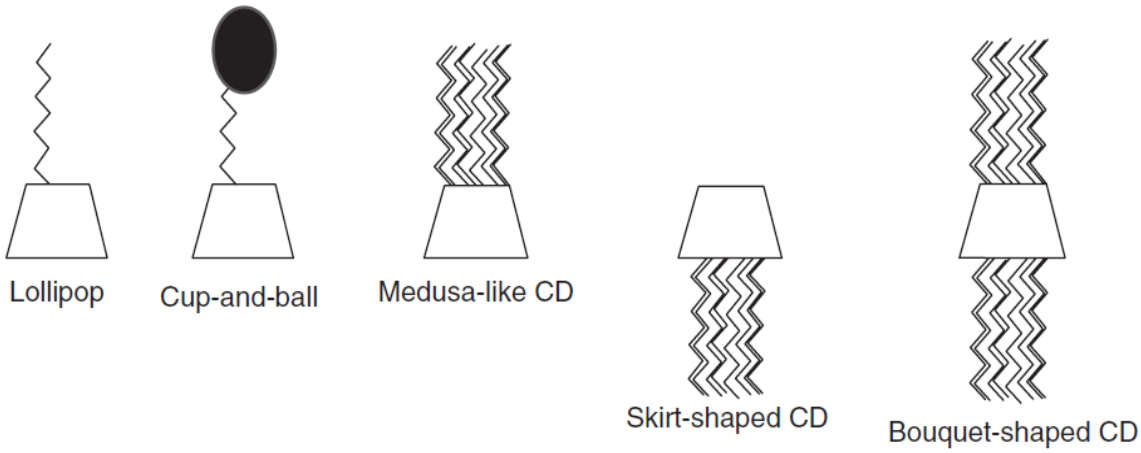


Figure 2.21 Non-ionic Amphiphilic cyclodextrins derivatives [155].

6-O-CAPRO-β-CD, (Heptakis (6-O-hexanoyl) cyclomaltoheptaose), which is the amphiphilic CD used in this thesis, is a non-ionic amphiphilic cyclodextrin derivative modified on the primary face with linear 6C fatty acids with an ester bond and was obtained by iodination of the primary hydroxyl groups and reaction with cesium hexanoate which proved to be a good nucleophile in DMF (dimethylformamid) [167].

CDs constitute very powerful tools in the design of nanoparticulate drug delivery systems. CDs have found utility with the aim of increasing the loading capacity of nanoparticles. Furthermore, new amphiphilic derivatives, cyclodextrin diesters, are capable of forming nanoparticles (either nanospheres or nanocapsules) without the necessity of any polymerization process [168].

CDs have important effects on the drug properties in formulations in terms of drug solubility and dissolution, absorption and bioavailability, safety and stability. These effects can be briefly summarized as follows;

Effect on Drug Solubility and Dissolution

CDs have been playing a very important role in formulation of poorly water-soluble drugs by improving apparent drug solubility and/or dissolution through inclusion complexation in which the guest and host molecules are in dynamic equilibrium within the complex [153, 169]. The mechanism for this solubilization is rooted in the ability of cyclodextrin to form non-covalent dynamic inclusion complexes in solution by means of interaction with poorly water-soluble drugs and drug candidates resulting in an increase in their apparent water solubility.

Klein et al [170] reported that complexing Glyburide, a drug for type 2 diabet, with hydroxybutenyl-beta-cyclodextrin, resulted in a improvement of drug solubility almost 400 fold compared with pure drug. In an other work which is demonstrated by Kakran et al [171], dissolution rate of artemisinin (ART) was found significantly faster with ART/β-CD complexes than the pure drug. Similarly, in an other study, performed by Koontz et al [172], showed that water solubility of natamycin was increased 16-fold, 73-fold, and 152-fold when formed an inclusion complex with beta-CD, gamma-CD, and HP beta-CD, respectively.

Effect on Drug Absorption and Bioavailability

CDs enhance the bioavailability of insoluble drugs by increasing the drug solubility, dissolution and/or drug permeability of insoluble, hydrophobic drugs by making the drug available at the surface of the biological barrier [173]. Cyclodextrins do not in general enhance permeability of hydrophilic drugs through lipophilic membranes, however lipophilic cyclodextrins such as methylated β-cyclodextrins are able to penetrate mucosa and enhance absorption through biological membranes. Besides, the enhancement of bioavilablity of drugs can partially be attributed to the stabilization of drug molecules at the biological membrane surface [174]. It was hypothesized that CDs enhance absorption mainly by increasing membrane permeability through complexation with membrane phospholipids and cholesterols [151].

In the study which is performed by Zhao et al [175], poly (alkyl-cyanoacrylate) nanoparticles were prepared with Hydroxypropyl-β-cyclodextrin and significantly higher relative bioavailability (211.6 %) of Flurbiprofen was obtained in male Wistar rats. Similarly Arima et al [176] has investigated the enhancing effects of CDs on solubility, dissoluliton rate and bioavailability of an anticancer agent Tacrolimus. Results revealed that heptakis (2,6-di-O-methyl)-beta-cyclodextrin increased the bioavailability of tacrolimus with low variability in the absorption after oral administration of the tacrolimus suspension to rat.

Effect on Drug Stability and Safety

CDs have been used to diminish the irritation caused by drugs. The increased drug efficacy and potency by means of reduction of the dose required for optimum therapeutic activity, may reduce drug toxicity by making the drug effective at lower doses. Morever, toxicty of poorly water soluble drugs which is related with the crystallization can be altered by inclusion complexes [174]. Furthermore CDs can improve the stability of drugs against dehydration, hydrolysis, oxidation thereby increasing the shelf life of drugs [169].

Saleh et al [177] has investigated the stability effect of heptakis; 2,6 di-O-methyl-beta-cyclodextrin (DM-beta-CD) on the stability of nitrazepam. Results obtained from this work suggested that Inclusion complexation of nitrazepam in DM-beta-CD resulted in a relatively improved stability of the drug in solution at 30 ^{0}C. Similar results were obtained from a study performed by Alvarez et al [178] in which the effect of hydroxypropyl-beta-cyclodextrin on the solubility and stability of thalidomide for clinical oral administration to be used in HIV-infected children. Results showed that complexing thalidomide with cyclodextrins resulted in an improvement of the chemical stability of drug.

2.4.3 Applications of Cyclodextrins in Drug Delivery Systems

In the pharmaceutical industry cyclodextrins have mainly be used as complexing agents to increase aqueous solubility of poorly soluble drugs and to increase their bioavailability and stability. Furthermore, cyclodextrins can be used to reduce gastrointestinal irritation, prevent drug-drug and drug-excipient interactions. Worldwide there are currently about 35 drug products of cyclodextrins available on the market as seen in Table 2.4 [153, 174].

Table 2.4. Marketed pharmaceutical products contain cyclodextrins as excipient

Drug/cyclodextrin	Trade name	Formulation	Company (Country)
α-Cyclodextrin			
Alprostadil	Caverject Dual	i.v. solution	Pfizer (Europe)
Cefotiam-hexetil HCl	Pansporin T	Tablet	Takeda (Japan)
OP-1206	Opalmon	Tablet	Ono (Japan)
PGE_1	Prostavastin	Parenteral solutions	Ono (Japan); Schwarz (Europe)
β-Cyclodextrin			
Benexate HCl	Ulgut, Lonmiel	Capsule	Teikoku (Japan); Shionogi (Japan)
Cephalosporin	Meiact	Tablet	Meiji Seika (Japan)
Cetirzine	Cetrizin	Chewing tablet	Losan Pharma (Germany)
Chlordiazepoxide	Transillium	Tablet	Gador (Argentina)
Dexamethasone	Glymesason	Tablet	Fujinaga (Japan)
Diphenhydramin and chlortheophyllin	Stada-Travel	Chewing tablet	Stada (Europe)
Deferasirox	Exjade	Tablet	Novartis (Switzerland)
Meloxicam	Mobitil	Tablet and suppository	Medical Union Pharmaceuticals (Egypt)
Nicotine	Nicorette	Sublingual tablets	Pfizer (Europe)
Nimesulide	Nimedex	Tablets	Novartis (Europe)
Nitroglycerin	Nitropen	Sublingual tablet	Nihon Kayaku (Japan)
Omeprazole	Omebeta	Tablet	Betafarm (Europe)
PGE_2	Prostarmon E	Sublingual tablet	Ono (Japan)
Piroxicam	Brexin, Flogene, Cicladon	Tablet, suppository	Chiesi (Europe); Aché (Brazil)
Tiaprofenic acid	Surgamyl	Tablet	Roussel-Maestrelli (Europe)
2-Hydroxypropyl-β-cyclodextrin			
Cisapride	Propulsid	Suppository	Janssen (Europe)
Hydrocortisone	Dexocort	Solution	Actavis (Europe)
Indomethacin	Indocid	Eye drop solution	Chauvin (Europe)

Itraconazole	Sporanox	Oral and i.v. solutions	Janssen (Europe, USA)
Mitomycin	MitoExtra, Mitozytrex	i.v. infusion	Novartis (Europe)
Sulfobutylether-β-cyclodextrin sodium salt			
Aripiprazole	Abilify	i.m. solution	Bristol-Myers Squibb (USA); Otsuka Pharm. (USA)
Maropitant	Cerenia	Parenteral solution	Pfizer Animal Health (USA)
Voriconazole	Vfend	i.v. solution	Pfizer (USA, Europe, Japan)
Ziprasidone mesylate	Geodon, Zeldox	i.m. solution	Pfizer (USA, Europe)
RandomLy methylated-β-cyclodextrin			
17-β-Estradiol	Aerodiol	Nasal Spray	Servier (Europe)
Chloramphenicol	Clorocil	Eye drop solution	Oftalder (Europe)
γ-cyclodextrin			
Sugammadex	Bridion	i.v. solution	Merck (Europe)
2-Hydroxypropyl-γ-cyclodextrin			
Diclofenac sodium salt	Voltaren	Eye drop solution	Novartis (Europe)
Tc-99 Teoboroxime	CardioTec	i.v. solution	Bracco (USA)

The information listed in this table is based partly on [153] and [174] and updated in 2011.

As it can be clearly seen from Table 2.4. most of the CDs used in the market are β-CDs since the purification of α- and γ-CDs increases considerably the cost of production [156]. CDs and their derivatives were extensively studied in pharmaceutical field with different administration routes e.g. oral, parenteral, ocular, nasal, rectal, and transdermal delivery.

Cyclodextrins in Oral Chemotherapy

As it was stated in section 2.1.1., oral chemotherapy is very challenging due to the multiple physiological barriers in the GI tract. Taking into the consideration that most of the anticancer agents have poor solubility, poor stability and poor permeability, CD nanoparticles may promote the oral bioavailability of these therapeutic agents. Owing to the numerous advantages of CDs and their derivatives in terms of the ability to increase solubility, stability, permeablity and consequently bioavailability, cyclodextrin nanoparticles could be promising for oral drug delivery of problematic drugs. Morever the inhibitory effect of CDs on the activity of P-gp and cytochrome P450 could ameloriate therapeutic effectiveness. However, there is limited work for cyclodextrin nanocapsules and even nanoparticles .Therefore evaluating nanoparticles with CD and their derivatives loaded with anticancer drug for oral delivery would be promising in this research field.

2.5. Poly-ε-Caprolactone

Polycaprolactone (PCL) was one of the earliest polymers synthesized by Carothers *et.al* in the 1930s. During the resorbable polymer boom of the 1970s and 1980s, PCL and its copolymers were used in the biomedical field especially in a number of drug delivery devices. Attention was drawn to this biopolymer owing to the numerous advantages over other biopolymers like tailorable degredation kinetics and mechanical properties, in terms of ease of shaping and manufacture [179]. Coupled with these advantages and FDA approval (PCL was already approved by the U.S. Food and Drug Administration (FDA) as a component of the contraceptive implant (Capronor®) (Injectable hydrogels of poly(3-caprolactone-co-glycolide)epoly (ethylene glycol)epoly(3-caprolactone-co-glycolide) triblock copolymer aqueous solutions), PCL is attracting research attention in biomedical field [180].

2.5.1. Physicochemical Properties

PCL is an aliphatic polyester composed of hexanoate repeat units [181] (Figure 2.22). PCL is a semi-crystalline, hydrophobic polymer with a relatively polar ester group and five non-polarmethylene groups in its repeating unit [182]. It is non-toxic, biodegredable and biocompatible. PCL is a semi-crystalline polymer having a glass transition temperature (Tg) of −60 ◦C and melting point ranging between 59 and 64

°C, dictated by the crystalline nature of PCL which enables easy formability at relatively low temperatures. Its cristalinity tends to decrease with increasing molecular weight. The number average molecular weight of PCL samples may generally vary from 3,000 to 80,000 g/mol and can be graded according to the molecular weight [179]. In this thesis poly-Ɛ-caprolactone with different molecular weights (14,000, 65,000 and 80,000 Da) was used as core polymers. The physical, thermal and mechanical properties of PCL depend on its molecular weight and its degree of crystallinity [181].

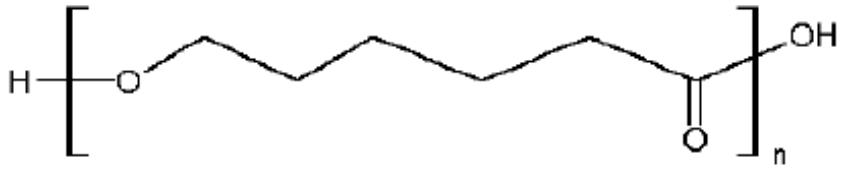


Figure 2.22. Chemical Structure of Poly-Ɛ-caprolactone [183].

PCL is prepared by the ring opening polymerization of cyclic monomer Ɛ-caprolactone [182]. Catalysts are used to catalyze the polymerization and there are also various mechanisms affect the polymerization of PCL. PCL is soluble in chloroform, dichloromethane, carbon tetrachloride, benzene, toluene, cyclohexanone and 2-nitropropane at room temperature. It has a low solubility in acetone, 2-butanone, ethyl acetate, dimethylformamide and acetonitrile and is insoluble in alcohol, petroleum ether and diethyl ether. PCL displays the rare property of being miscible with many other polymers. PCL biodegrades within several months to several years depending on the molecular weight, the degree of crystallinity of the polymer, and the conditions of degradation. While PCL can be enzymatically degraded in the environment by outdoor living organisms (bacteria and fungi), it cannot be degraded enzymatically in the body because of the lack of suitable enzymes [179, 181].

2.5.2. Applications in Drug Delivery Systems

In view of its biological, chemical and physical properties, PCL has great potential in the biomedical field [184, 185]. Very low *in vivo* degradation rate of PCL has found favor as a long-term implant delivery device. Capronor® is a commercial contraceptive PCL product that is able to deliver levonorgestrel *in vivo* for over a year and has been on the market for over 25 years [180]. PCL has low tensile

strength (~23 MPa), but very high elongation at breakage (4700%) making it a very good elastic biomaterial which allows for paramedical application, wound dressing and dentistry. [186] Besides, it is non-toxic in nature and found to be cyto-compatible with several body tissues that makes it an ideal material for scaffolds in tissue engineering [187]. PCL and PCL composites have been used as tissue engineering scaffolds for regeneration of bone, ligament, cartilage, skin, nerve, and vascular tissues [180].

Current research is being conducted into the development of micro- and nano-sized drug delivery vehicles with PCL or PCL blended/copolymerized polymers. Due to excellent biocompatibility and its ability to be fully excreted from the body once bioresorbed, PCL is suitable for controlled drug delivery. Recently several studies have been realised with PCL as drug delivery systems including microparticles, nanoparticles, micelles, hydrogels. Although numerous studies have reported PCL nanocapsules loaded with different types of drugs, limited studies for anticancer agent Camptothecin and its derivatives are performed.

2.6. Chitosan

The history of chitosan (CS) dates back to the 19th century when the deacetylated forms of the parent chitin natural polymer was discussed in 1859 [188]. Chitosan is derived from naturally occurring sources, which is the exoskeleton of insects, crustaceans and fungi. CS polymers are semi-synthetically derived aminopolysaccharides that have unique structures, multidimensional properties, highly sophisticated functionality and a wide range of applications in biomedical and other industrial areas. They have become interesting not only because they are made from an abundant renewable. resource but because they are very compatible and effective biomaterials that are used in many biomedical applications and is also a food supplement thus approved for oral intake [189].

2.6.1. Chemical Structure and Physicochemical Properties

Chitin is the second most abundant polysaccharide in the nature after cellulose and is found in the exoskeleton of crustacea, insects, and some fungi. CS is obtained by the thermochemical deacetylation of chitin in the presence of alkali [190] representing a cheap and readily available source for chitosan [191]. The treatment of chitin with an aqueous 40–45% (w/v) NaOH solution at 90–120 ^{0}C for 4–5 h

results in N-deacetylation of chitin. The conditions used for deacetylation determines the polymer molecular weight and the degree of deacetylation (DD). Chitosan (poly[b-(1-4)-2-amino-2-deoxy-D-glucopyranose]) is a cationic polysaccharide comprising Nacetyl- d-glucosamine and d-glucosamine units available in different grades depending upon the degree of acetylated moieties. It is a polycationic polymer that has one amino group and two hydroxyl groups in the repeating glucosidic residue (Figure 2.23) [192].

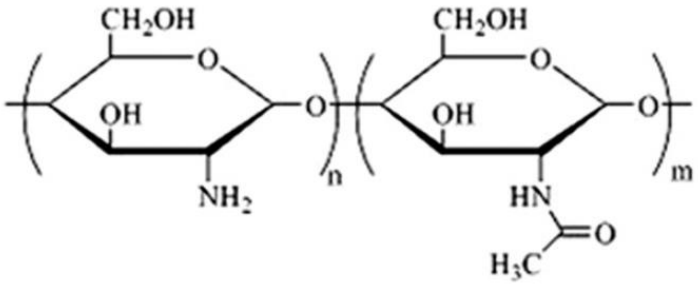


Figure 2.23. Chemical Structure of Chitosan [192].

Commercial CS is available in two grades of high and low molecular weight. Low molecular weight chitosan grade is characterized by molecular weight comprised between 20 kDa and 190 kDa with DD< 75% whereas high molecular weight chitosan grade is generally characterized by molecular weight comprised between 190 kDa and 375 kDa with DD> 75%.

At pH less than 6.5, CS is soluble in most organic acidic solutions including formic, acetic, tartaric, and citric acid. However at neutral and alkaline pH chitosan is not soluble in aqueous solution, a potential hindrance to its application to oral insulin delivery. This poor solubility can be corrected by derivatization at the amine group, rendering the polymer soluble and effective [193].

In this thesis, Chitosan glutamate (Protasan™ UP G-113, MW< 200 kDa, deacetylation degree, 75–90%) is used. The cationic polymer is a highly purified and well-characterized water-soluble chloride salt with a molecular weight of 50000-150000 g/mol range (measured as a chitosan acetate) [194].

2.6.2. Applications in Drug Delivery Systems

CS is biocompatible, non-immunogenic, non-toxic, mucoadhesive, biocompatible and biodegradable, making it an excellent choice as a component of oral drug delivery systems and making it highly attractive for also biomedical applications.[189, 195, 196]. The various applications of chitosan are mainly due to its good physicochemical properties which are summarized as follows: [195]

1. Being a natural polymer, it is considered as a safe material that has biocompatibility and biodegradability.

2. CS has a readily modifiable pH responsive solubility which allows it to respond by assembling as a thin film.

3. It improves the drug bioavailability due to its absorption enhancing effect and facilitates the drug uptake through the cell membrane.

4. CS shows mucoadhesion as it is able to open tight junctions.[197]

5. These offer a versatile route of administration, especially non-invasive routes like oral, nasal, ocular and transdermal which are the most preferable.

6. CS provides a greater flexibility in the development of a formulation as it is avilable in a wide range of molecular weight. By coupling with a suitable ligand it can be chemically modified easily.

7. Chitin has complex and size-dependent effects on innate and adaptive immune responses, which include the ability to recruit and activate innate immune cells and induce cytokine and chemokine production. Also it is believed that chitosan enhances both humoral and cell-mediated immune responses [198].

CS has many applications in tissue engineering, nerve and cartilage regeneration, wound healing, gene delivery and drug delivery [199, 200]. Chitosan-based systems are used for the delivery of proteins/peptides, growth factors, anti-inflammatory drugs, antibiotics as well as anticancer agents.

CS can act on tumor cells directly to interfere with cell metabolism, inhibit cell growth, or induce cell apoptosis. It also has an antitumor role through improving the body’s immune function. Studies suggested that chitosan had antitumor effects in vitro and in vivo, leading to good prospects for their application as a supplementary antitumor drug and drug carrier [201, 202]. However, when using CS in a delivery

system for anticancer drug, the synergistic effect of CS on the anticancer efficacy should be noted.

The prime consideration, when aiming at preparing mucoadhesive polymeric nanoparticles, is the basic material, which is supposed to be a mucoadhesive, biocompatible and biodegradable polymer. CS derivatives positively charged on their repeating units are candidate materials in this respect [203]. CS belongs to the category of these so-called mucoadhesive materials. Indeed, due to its positive charge, chitosan is able to interact with the negatively charged mucus components. This property has attracted significant attention to the use of chitosan for transmucosal drug delivery and in particular, for nasal and also for oral drug delivery [204].

It is well known that CS can enhance the permeability of different compounds through the intestinal monolayer [205]. The increase in the permeability has been generally related to a decrease in the transepithelial electric resistance (TEER), which was attributed to a partial disruption of the tight junctions. Interestingly, it is also indicated that the effect of chitosan on the Caco-2 cell monolayer is reversible and, hence, that the opening of the cellular barrier is transient. This specific behaviour makes a great difference in terms of toxicity between chitosan and the classical penetration enhancers, which are known to cause irreversible epithelial damage [204].

Morever, CS has also been used as a coating material in order to change the surface properties of colloidal drug carriers. The purpose of designing these systems was to improve the interaction of the cores with mucosal surfaces. Different types of nanosystems, oily nanodroplets, solid nanoparticles and liposomes were selected as cores for the coating process [206]. The CS coating around the carriers are expected to show a positive effect at improving the pharmacological response of drugs, which is mainly attributed to the mucoadhesive properties and absorption enhancer effect of the polymer. All these versatile capabilities of chitosan and its nanoparticles suggest that this biopolymer has a very bright future in the field of pharmaceutical nanotechnology.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Equipments

Equipments	**Supplier**
Thermostated Shaker Bath	Memmert WNE22, Germany
Precision Balance (d=0.01/0.1 mg)	Mettler Telode XS 105 dual range, ABD
Magnetic Stirrer with Hot Plate	IKA RCT Basic, Germany
Freeze-dryer (Lyophilizator)	Heto PowerDry PL 3000, Denmark
Multipoint Magnetic Stirrer	Variomag Multipoint HP, Germany
Micropipette (10-100 µL, 100-1000 µL)	Eppendorf, Germany
Dynamic Light Scattering	Malvern Zetasizer Nano ZS, England
pH meter	Sartorius PP-20, Germany
Rotavapor	IKA RV06-ML, Germany
Centrifuge (500-18000 rpm)	Hettich EBA21, Germany
Syringe (DistriTip 12.5 mL)	Gilson DITRITIPS®, France
Scanning Electron Microscope (SEM)	FEI Nova™ NanoSEM 430, ABD
Balance (max 200 g with readability 0.1 mg)	Shimadzu AUX220, Japan
Ultra Pure Water System	Simplicity 185-Milipore, ABD
Tissue Homogenizer	POLYTRON® System PT 3100, Switzerland
High Performance Liquid Chromatography (HPLC)	HP Agilent 1100 Series, Germany
HPLC Column C 18 (250x4.6mm)	Hichrom Berkshire 5 RP, UK
Fluorescence Detector (Agilent 1100 Series)	Hewlett Packard, UK
UV- Spectrophotometer	Shimadzu, Japan

Fluorescence Spectrometer	Shimadzu, Japan
Cell culture wells	Grenier Bio-one, Germany
Cell Culture inserts	Grenier Bio-one, Germany
Microplate, 96 well	Grenier Bio-one, Germany
Microplate reader	Molecular Device Versa Max, USA
Light microscope	Leica, Germany
TEER Reader	Millicell ® ERS, Millipore
Vortex	Heidolph, Germany
Incubator	SANYO MCO-18AIC, Japan

3.2.1. Chemicals

Chemicals	**Supplier**
Poly-ε-Caprolactone (MW: 14,000 - 65,000 - 80,000 Da)	Sigma & Aldrich, USA
20-S-Camptothecin	Sigma & Aldrich, USA
6-O-CAPRO β CD (MW: 1820 g/mol)	Sevilla University, Spain
Acetone (HPLC Grade)	Sigma & Aldrich, USA
Ethanol (HPLC Grade)	Sigma & Aldrich, USA
Dimethylsulfoxide	Sigma & Aldrich, Germany
Dichloromethane (HPLC Grade)	Sigma & Aldrich, USA
Disodium hydrogen phosphate	Merck & Co, Germany
Chitosan (Protasan™ UP G-113, MW <200 kDa, deacetylation degree, 75–90%)	Novamatrix /FMC Biopolymer, Norway
Potassiumdihydrogen phosphate	Riedel-de Haën, Germany
Potassium chloride	Horasan Kimya, Turkey
Sodium chloride	Riedel-de Haën, Germany
Tween® 80	Merck & Co, Germany
Miglyol 812	Condea Chemie, Germany
Triethylamine	Merck, Germany
Nile Red	Sigma &Aldrich, USA
Diethylene triamine pentaacetic acid (DTPA)	Sigma &Aldrich, USA

Acetic Acid (Glacial)	Aklar Kimya, Turkey
Sodium Hydroxide	Merck, Germany
Hank's Balanced Salt Solution (HBSS)	Grenier Bio-one, 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)	Sigma, USA
Dialysis Tubing Cellulose Membrane(avg. flat width 25mm, MW cut-off 14,000 Da)	Sigma & Aldrich, USA
Monobasic potassium phosphate	Sigma &Aldrich, Germany
Hydrochloric Acid %36-38	J.T. Baker, Netherland
Gelatin Type B (Gelatin from bovine skin)	Sigma, USA
Egg Yolk Emulsion	Sigma & Aldrich, Switzerland
Mucin from Porcine Stomach Type II	Sigma, USA
Acetonitrile	Sigma &Aldrich, USA
Sodium sulfide	Sigma &Aldrich, Germany

3.2.2. Biological Materials

Biological Materials	**Supplier**
Fetal Bovine Serum	Sigma, Germany
Trypsin-EDTA	Sigma, Germany
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
CD1 Female Mice (20-25 g)	Harlan, Italy

3.2. Method

3.2.1. *In vitro* Quantification of Camptothecin and HPLC Method Validation

In this thesis, in vitro quantification of CPT in lactone or carboxylate forms and analytical method validation were performed by HP Agilent 1100 systems, reverse phase high pressure liquid chromatography (RP-HPLC). Chromotographic conditions of the developed method are as listed:

Column: Hichrom 5 C-18 (250x4.6 mm)

Detector: Florescence Detector

Mobile phase: Triethylamine acetate buffer (TEAA) : Acetonitrile (75:25 v/v) (pH 5.5)

Injection volume: 20µL

Flow rate: 1mL/min

Wavelength: Excitation: 370 nm, Emission: 435 nm

Temperature: 25 ^{0}C

3.2.1.1. Analytical Method Validation

The principle purpose of the validation of an analytical method is to ensure that the selected analytical procedure will give reproducible and reliable results and that every future measurement in routine analysis will be sufficiently close to the unknown true value for the content of the analyte in the sample [207]. Method validation is, therefore, an essential component of the measures that a laboratory should establish to be able to produce reliable analytical data. Results from method validation can be used to judge the quality, reliability and consistency of analytical results [208]. Analytical methods need to be validated or revalidated prior to introduction into routine use whenever the conditions change for which the method has been validated (e.g., an instrument with different characteristics or samples with different characteristics), and whenever the method is changed [208].

Typical validation characteristics were studied including linearity, accuracy, precision, specificity and sensitivity and explained below in more details.

3.2.1.1.1. Construction of Calibration Curve

In order to perform the quantification as well as the calibration curve of Camptothecin, a stock solution was prepared at a concentration of 100 µg/mL (w/v)

by dissolving 1mg CPT in 10 mL dimethylsulfoxide (DMSO). Starting from this stock solution, different CPT solutions with a series of concentration ranging between 1-50 µg/mL (as of 1, 2, 5, 10, 20, 25 and 50 µg/mL) were prepared by diluting this stock solution with PBS (phosphate buffer saline) at pH 7.4. In order to obtain the lactone form and the carboxylate form of CPT, pH was adjusted to 3 and 10.5 respectively [209].

The calibration curve is obtained by fitting an appropriate equation to calibration data consisting of the measured responses to known concentrations of analyte. Hereby calibration curve was prepared according to the HPLC conditions listed in the section 3.2.1. by plotting the obtained peak areas against the CPT solutions with different concentrations and ultimately calibration data.

3.2.1.1.2. Linearity

Linearity of analytical method is the ability to obtain test results that are directly proportional to the concentration of the analyte in the sample within a given range. Linearity is determined by a series of five to six injections of five or more standards. The response should be directly proportional to the concentrations of the analytes or proportional by means of a well-defined mathematical calculation.

Linearity should be evaluated by visual inspection of a plot of signals as a function of analyte concentration or content. If there is a linear relationship, test results should be evaluated by appropriate statistical methods, e.g. by calculation of a regression line by the method of least squares. Calculation of the regression line is straightforward and the equation will have the form $y = bx + a$, where b is the slope of the line and a is the y-intercept. If x and y are linearly correlated, it is expected all the points to fall on a straight line passing through the centroid. The correlation coefficient, R, (sometimes displayed as R^2) is a measure of the strength of the degree of correlation between the y and x values indicating that the closer it is to 1, the stronger the correlation is. It s an important tool in determining the degree of linear-correlation of variables ('goodness of fit') in regression analysis.

Hereby, linerarity was demonstrated using a seven-point (1, 2, 5, 10, 20, 25 and 50 µg/mL) calibration curve. Peak area of each concentration point was plotted as a function of CPT concentrations. Linearity was observed by calculating of a regression line and correlation coefficient were then calculated.

3.2.1.1.3. Accuracy

Accuracy is the measure of how close the experimental value is to the true value. Accuracy is calculated as the percentage of recovery by the assay of the known added amount of analyte in the sample or as the difference between the mean and accepted true value together. Accuracy should be measured using a minimum of five determinations per concentration and a minimum of three concentrations in the range of expected concentrations is recommended.

Hereby accuracy is assessed by using a minimum of three concentration levels (2, 20 and 50 µg/mL) covering the specified range for both lactone and carboxylate forms. (i.e 3 concentrations and 6 replicates of each concentration). Percentage of recovery was then calculated and mean value was compared within the actual value.

3.2.1.1.4. Precision

Precision is the variability in the data from replicate determinations of the same homogeneous sample under the normal assay conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility and usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.

Repeatability expresses the precision under the same operating conditions over a short interval of time. For assessing the repeability, one of the concentrations of prepared CPT solutions (20 µg/mL) was chosen and analysed with 6 replicates time after time. Mean values, standard deviation or coefficient of variation of a series were then calculated.

Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment, etc. For assessing the inter-days, the chosen concentration (20 µg/mL) that was prepared in 3 consequtive days, was measured and mean values, standard deviation or coefficient of variation were then calculated.

Reproducibility expresses the precision between laboratories. For assessing the reproducibility, 6 samples of CPT solution which are prepared at the same concentrations (20 µg/mL) were analysed and mean values, standard deviation or coefficient of variation were then calculated.

For an HPLC assay precision methods with coefficient of variation values (CV<2 %) may be required.

3.2.1.1.5. Sensitivity

Sensitivity of the method was determined by finding the limit of detection and limit of quantitation. **Limit of detection (LOD)** is the lowest concentration of analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions. The minimum concentration (1µg/mL) at which a signal to noise ratio is 3:1 was accepted as detection limit. **Limit of Quantification (LOQ)** is the lowest concentration of analyte in a sample that can be quantified with acceptable precision and accuracy under the stated experimental conditions. The minimum concentration (1µg/mL) at which a signal to noise ratio is 10:1 was accepted as quantification limit.

3.2.1.1.6. Specificity:

Specificity is the ability of the method to accurately measure the analyte response in the presence of all different potential sample components. The specificity of analytical methods is typically assessed by examining system interference with the detection and quantification of analytes. For this reason, sample components containing formulation excipients such as PCL, CD, CS and DMSO were compared with the response of a solution containing only the analyte during the analysis.

3.2.2. Pre-Formulation Studies

Pre-formulation studies were performed to determine the effect of formulation parameters e.g polymer concentration, polymer molecular weight, oil concentration, ratio of organic to aqueous phase and amount of surfactant on nanoparticle characteristics such as mean diameter, polydispersity and zeta potential. Therefore, preparation of blank PCL and CD nanocapsules was performed by changing the variables such as polymer concentration (concentration range 0.05 % to 0.5 % w/v), oil concentration (concentration range 0.3 % to 3 % v/v), organic phase to aqueous phase volume ratio (1:1, 1:2, 1:4 v/v) with nanoprecipitation method as described previously in the section 2.1.1. These selected concentrations of formulation parameters were in accordance with the studies reported in literature [59, 67, 210]. In addition to these formulation variables, surfactant concentration (concentration range from 0.1% to 1% v/v) and polymer molecular weight (14,000, 65,000 and

80,000 Da) were also investigated as formulation parameters for the preparation of blank PCL nanocapsules. (Table 3.1)

Table 3.1. Summary of formulation parameters and variables for pre-formulation studies based on the literature [59].

Formulation Parameters	***Variables***
Polymer concentration	0.05%, 0.1%, 0.2%, 0.5% (w/v)
Oil Concentration	0.3%, 1%, 3% (v/v)
O/A volume ratio	1:1, 1:2, 1:4 (v/v)
Surfactant concentration (for PCL)	0.1%, 0.5%, 1% (v/v)
Polymer MW (for PCL)	14,000 Da, 65,000 Da, 80,000 Da

3.2.2.1. Optimization of polymer concentration and polymer molecular weight

For the optimization of the polymer concentration, different concentrations of the polymer in percentage (0.05 %, 0.1 %, 0.2 % and 0.5 % w/v) were selected [59]. Briefly, an organic solution of PCL (concentration range from % 0.05 to % 0.5 w/v) and 50 µL oil Miglyol 812 in 5mL acetone was added dropwise into 10 mL ultra-pure water containing 50 µL Tween 80 ® under moderate magnetic stirring. Organic solvent was then evaporated under vacuum at 40°C for 30 min to obtain nanocapsule dispersion at the final desired volume 10mL. Nanocapsules were recovered by centrifugation at 3500 rpm at room temperature for 15 min. The preparation of the CD nanocapsules was evaluated with the same preparation technique by omitting the surfactant in the aqueous phase as it was stated in section 4.2 and using ethanol as organic phase instead of acetone.

In addition to the polymer concentration, the effect of polymer molecular weight was also investigated. For this reason, poly-Ɛ-caprolactone polymers with different molecular weight (14,000, 65,000 and 80,000 Da) were selected as variables and blank PCL nanocapsules were prepared in the same manner.

3.2.2.2. Optimization of oil concentration

For the optimization of the oil concentration, different concentrations of oil in percentage, Miglyol 812 (0.3 %, 1 % and 3 %, v/v) were selected. Miglyol 812, a

medium-chain triglyceride, is non-toxic, biocompatible, biodegradable and liquid at room temperature [211].

Briefly, an organic solution of 5 mg PCL and Miglyol 812 (concentration range from 0.3 % to 3 % v/v) in 5mL acetone was added dropwise into 10 mL ultra-pure water containing 50 µLTween 80 ® under moderate magnetic stirring. Organic solvent was then evaporated under vacuum at 40°C for 30 min to obtain nanocapsule dispersion at the final desired volume 10mL. Nanocapsules were recovered by centrifugation at 3500 rpm at room temperature for 15 min. The preparation of the CD nanocapsules was evaluated with the same preparation technique by omitting the surfactant in the aqueous phase as it was stated in section 4.2 and using ethanol as organic phase instead of acetone.

3.2.2.3. Optimization of organic phase to aqueous phase volume ratio

The optimization of organic phase to aqueous phase volume ratio was performed by using different volumes of ultrapure water e.g 5 mL, 10 mL and 20 mL corresponding the organic to aqueous phase volume ratios 1:1, 1:2 and 1:4 respectively. Briefly, an organic solution of 5 mg PCL and 50 µL Miglyol 812 in 5mL acetone was added drop wise into different volumes of ultra-pure water (5, 10 and 20 mL) containing 0.5 % v/v Tween 80 ® under moderate magnetic stirring. Organic solvent was then evaporated under vacuum at 40°C for 30 minutes. Nanocapsules were recovered by centrifugation at 3500 rpm at room temperature for 15 min. The preparation of the CD nanocapsules was evaluated with the same preparation technique by omitting the surfactant in the aqueous phase as it was stated in section 4.2 and using ethanol as organic phase instead of acetone.

3.2.2.4. Optimization of surfactant concentration

For the optimization of the surfactant concentration, different concentrations of Tween 80 ® in percentage (0.1%, 0.5% and 1%) were selected as formulation parameters. Briefly 5 mg PCL and 50 µL oil were dissolved 5 mL acetone and added drop by drop into 10 mL ultrapure water containing 10 µL, 50 µL and 100 µL of Tween 80 ® under moderate magnetic stirring. Organic solvent was then evaporated under vacuum at 40°C for 30 minutes. Nanocapsules were recovered by centrifugation at 3500 rpm at room temperature for 15 min. This formulation parameter was only used for blank PCL nanocapsules while CD nanocapsules were

prepared without surfactant. As indicated in the section 3.2.2.4., CDs have the ability to form nanoparticles spontaneously without the presence of a surfactant. Hence, optimization of the surfactant concentration was performed only with PCL nanocapsule formulations.

3.2.3. Formulation of anionic and cationic CPT loaded nanocapsules

According to the pre-formulation results both anionic and cationic CPT loaded nanocapsules were prepared with the same method as previously stated in section 3.2.2 by adding CPT in the organic phase as summarized in Figure 3.1. Briefly 0.5 mg CPT were dissolved in the organic phase consisting of 50 µL Miglyol 812 and 5mg PCL in 5mL acetone. This organic solution was added drop wise into an aqueous phase consisting of 10 mL of ultra-pure water containing 10 µL surfactant Tween 80, under moderate magnetic stirring. Organic solvent was then evaporated under vacuum at 40°C for 30 min to obtain nanocapsule dispersion at the final desired volume (10mL). CD nanocapsules were prepared with the same method with a slight modification by using ethanol instead of acetone as organic solvent and omitting the surfactant, Tween 80 in the aqueous phase. Nanocapsules were recovered by centrifugation at 3500 rpm at room temperature for 15 min.

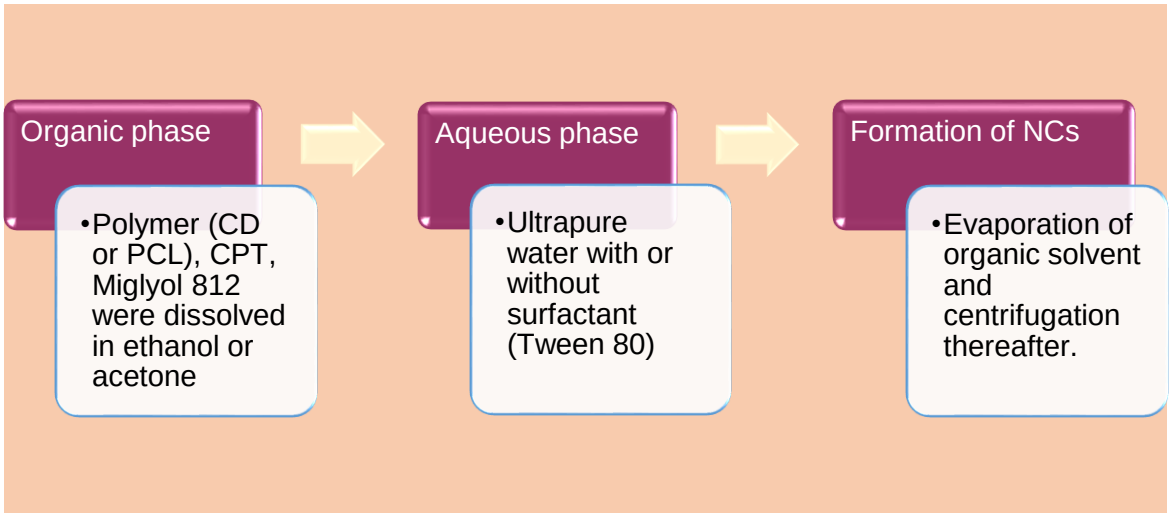


Figure 3.1. Formation of nanocapsules via nanoprecipitation method

In order to obtain positively charged nanocapsules, 0.025 % (w/v) of Chitosan (Protasan™ (UP G-113) was added to aqueous phase during preparation.

3.2.4. Physicochemical characterization of Nanocapsules

3.2.4.1. Particle Size Distribution

Mean particle size and polydispersity index (PDI) of different nanocapsule formulations were determined by dynamic light scattering (DLS) (Malvern Zetasizer Nano ZS series, UK) at a scattering angle of 173^0 at room temperature using a disposable capillary cell. Each measurement was carried out in triplicate.

3.2.4.2. Zeta Potential

In order to determine the surface charge of nanoparticles zeta potential values of nanocapsules were measured in mV with Malvern Zetasizer Nano ZS at an angle of 12^0 and at 25^0C using a disposable capillary cell. Each measurement was carried out in triplicate.

3.2.4.3. Imaging of Nanocapsules

The morphology of drug loaded nanocapsule formulations was evaluated by using SEM, Scanning Electron Microscope (FEI Nova™ Nano SEM 430, ABD). Samples were mounted on metal stubs and coated with 100 Å thick layer of Gold-Palladium alloy. Then the particles were imaged at 5 to 20 kV.

3.2.4.4. Encapsulation Efficiency of Nanocapsules

Content of CPT bound in different nanocapsules was quantified by a previously validated HPLC method with fluorescence detection at an emission wavelength (λ_{em}) of 435nm and at an excitation wavelength (λ_{ex}) of 370nm.

Briefly, after the separation of excess amount of polymers and undissolved and/or unbound drug by centrifugation at 3500 rpm for 15 min, supernatant containing CPT loaded nanocapsule suspension was freeze-dried for 24h in order to obtain drug loaded nanocapsules in powder form. Resulting powder was weighed and dissolved in 2 mL mixture of DCM and DMSO. The experimental CPT loading was quantified using the peak area of each nanocapsule formulation. Drug loading was expressed in terms of associated drug percentage and entrapment efficiency. Associated Drug (%) (1) and Entrapment Efficiency (%) (2) were calculated as follows;

$$\text{Associated Drug (\%)} = \frac{\text{Experimental Drug Loading (µg)}}{\text{Theoretical Drug Loading (µg)}} \times 100 \qquad (1)$$

$$\text{Entrapment Efficiency (\%)} = \frac{\text{Calculated Drug Quantity (µg)}}{\text{Polymer Quantity (µg)}} \times 100 \qquad (2)$$

3.2.4.5. *In vitro* release studies of Camptothecin from Nanocapsules

Release profiles of camptothecin from nanocapsule formulations were determined at 37 ^{0}C with dialysis membrane (having a molecular weight cut-off 14,000 Da) technique under sink conditions. Briefly, 2 mL of nanocapsule dispersion was put in a dialysis bag. The dialysis bag containing the nanocapsule dispersion was placed in 50 mL of phosphate buffer solution containing 0.1 % v/v Tween 80 (PBS) at pH 7.4. The system was placed in a shaking water bath at 37°C with an agitation speed of 100 rpm. At specific time periods (0.5, 1,1.5, 2, 6, 24, 48 and 72 hours), 500µL of sample was taken from the medium with micropipette and replaced by the same volume of fresh medium. The cumulative percentage of drug released for each time point was calculated as a percentage of the total drug incorporated into the nanocapsules. Furthermore, CPT release from nanocapsule formulations were also determined at PBS pH 1.2. for 2 h to mimic the *in vivo* gastric emptying indicating gastric conditions. At specific time periods (0.5, 1,1.5 and 2 h), 500µL of sample was taken from the medium with micropipette and replaced by the same volume of fresh medium. The cumulative percentage of drug released for each time point was calculated as a percentage of the total drug incorporated into the nanocapsules.

3.2.4.6. Stability studies for CPT in various media

As it was stated in section 2.3.1. CPT suffers from instability due to the the rapid hydrolysis of active lactone form under pH 5 into the inactive and more toxic carboxylate form in physiological or alkaline pH. This hydrolysis is pH-dependent and drastically reduces the effectiveness of the lactone form. Lactone form is not only essential for the inhibitory effect, but also essential for the diffusion of the drug [212]. Therefore, being able to ensure the stability of this lactone form thereby reducing and/or preventing this hydrolysis into carboxylate form in case of pH changes, is of primary concern. For this reason, CPT stability when incorporated in nanocapsules was determined with a validated HPLC method in phosphate buffer

solutions (PBS) with different pH values of 1.2, 6.8 and 7.4 representing the wide pH range of the GI tract and compared with CPT in solution form. Briefly, CPT loaded nanocapsule formulations were prepared according to the method indicated in section 3.2.3. All anionic and cationic nanocapsule formulations were diluted with PBS with different pH values (pH 1.2, pH 6.8 and pH 7.4) in order to obtain CPT concentration at 25 µg/ml mimicking the physiological conditions. For the comparison of data obtained from nanocapsules with CPT solution, 0.1 mg CPT was dissolved in 2ml DMSO and diluted with PBS to obtain CPT concentration at 25 µg/ml. After the injection of the samples to HPLC, concentrations against the obtained peak area of different formulations were then calculated at predetermined time intervals during 48h and the percentage quantity of lactone and carboxylate forms at these pH values were compared within.

3.2.4.7. Physical Stability of Nanocapsules in Simulated Gastrointestinal Fluids

The first barrier to cross after oral administration is constituted by the physicochemical environment of the gastrointestinal tract as stated in section 2.1.1. To design effective nanocapsules for oral drug delivery, their stability has to be assessed not only in stock solutions but also in fluids mimicking biologic media. Estimating the physical stability of nanoparticles in biological environments e.g GI tract, is critical for oral delivery of drugs [96] since it is essential to understand whether and how the different body environments influence nanoparticle stability, to design optimal nanoformulations, and to identify their fate upon oral administration. [213]. Therefore, ın order to determine the stability of nanocapsule formulations against harsh environment and test the protective effect of nanocapsules on the stability of encapsulated CPT, simulated gastric and simulated intestinal fluids were used as media.

For this purpose all the formulations were incubated in both simulated gastric fluid (SGF) pH 1.2 for 2 hours and simulated intestinal fluid (SIF) pH 6.8 for 6 hours. SIF was prepared by dissolving 680.5 mg monobasic potassium phosphate (KH_2PO_4) in 100mL deionized water. Then 89.6 mg of NaOH were added to adjust the pH of the media. SGF was prepared by dissolving 100 mg of NaCI in 50 mL deionize water containing 0.35 mL concentrated HCl. For each formulation; 1 mL of nanocapsule dispersion was added to 9 mL of simulated media and incubated for 2 h in the case

of SGF and for 6 h in the case of SIF. Mean particle size, PDI and zeta potential values were measured before and after incubation.

3.2.4.8. Interaction Studies with Mucus

Turbidimetric Measurement:

In order to investigate the interaction between mucin and nanocapsules, turbidity of the nanocapsule at 650 nm was measured by UV spectrophotometry with a slightly modified method described previously by Ungaro et al. [214]. Mucoadhesive characteristics of CPT loaded CD and PCL nanocapsules were determined by measuring the absorbance rate (ABS) of the samples in the presence of mucin or not. Mucin dispersion was prepared by dissolving 0.08% w/v mucin powders in ultrapure water. After stirring for 6 hours, mucin dispersion was centrifuged at 7500 rpm for 20 minutes to separate excess amount of mucin. Mucin dispersion and prepared nanocapsule dispersions were then mixed at a 1:4 volume ratio and vortexed for 1 minute. The turbidity of the samples with or without mucin was measured at 650 nm at predetermined time intervals (0, 30 and 60 minutes). The absorbance rate (ABS) of mucin dispersion itself was also measured as reference.

Turbidimetric studies were conducted under the supervision of Assoc. Prof. Fabiana Quaglia, in the University of Naples Federico II, Department of Pharmacy, Italy.

Characterization of Nanocapsules in Mucus: Particle size and Zeta Potential Measurement

Nanocapsule formulations were further tested in the presence of mucin in terms of mean particle size and zeta potential. After the measurement of mucoadhesive tendency, the zeta potential and particle diameter of NC dispersions in mucin were also assessed. CPT loaded PCL and CD nanocapsule formulations were prepared as described in the section 3.2.3. and mucin dispersion as described above. Mucin dispersion and prepared nanocapsule dispersions were then mixed at a 1:4 volume ratio and vortexed for 1 minute. Afterwards mean particle size, polydispersity index and zeta potential of the NCs were measured in the presence of mucin or not. Zeta potential of mucin dispersion was also measured as reference.

Characterization of nanocapsules in mucus was performed under the supervision of Assoc. Prof. Fabiana Quaglia, in the University of Naples Federico II, Department of Pharmacy, Italy.

3.2.4.9. Artificial Mucus Studies

Artificial mucus studies were conducted with CPT loaded nanocapsules with Spectofluorophotometer (Shimadzu, Japan) in the University of Naples Federico II, Department of Pharmacy, Italy. For this reason, a calibration curve of CPT for the Spectoflorophotometer was constructed at 25 ^{0}C at an emission wavelength (λ_{em}) of 435 nm and at an excitation wavelength (λ_{ex}) of 370 nm before the artifical mucus study.

Construction of Calibration Curve

1mg CPT was dissolved in 10 mL DMSO and diluted two times with dilution rates 1:10 and 1:100 respectively, in order to obtain a stock solution at a concentration of 0.1 µg/mL. Starting from this stock solution, different CPT standard solutions with concentration range 0.0005-0.02 µg/mL (as of 0.0005, 0.001,0.002, 0.005, 0.01, and 0.02 µg/mL) were prepared by diluting this stock solution with DMSO at a dilution rates 1:200, 1:100, 1:50, 1:20, 1:10 and 1:5 v/v respectively. Calibration curve was prepared by plotting the obtained florescence intensity against to the CPT solutions with different concentrations and ultimately calibration equation and correlation coefficient were calculated.

Penetration through Artificial Mucus

Penetration of CPT loaded CD and PCL nanocapsules through an artificial mucus model was studied as described previously by Ungaro et al. [214] with a slight modification. An artificial mucus layer was prepared and placed upon a gelatin layer as summarized in Figure 3.2. Briefly, the gelatin solution was prepared by dissolving % 10 (w/v) gelatin powder (gelatin from bovine skin) in 10 mL ultrapure hot water (60^0 C). 1 mL of this gelatin solution was poured into a 24-well plate and hardened at room temperature. On the other part, artificial mucus layer was prepared by dissolving 250 mg of mucin, 0.295 mg DTPA, 250 mg NaCl, 110 mg KCl and 250 µL of sterile egg yolk emulsion in 50 mL of ultrapure water (Figure 3.3).

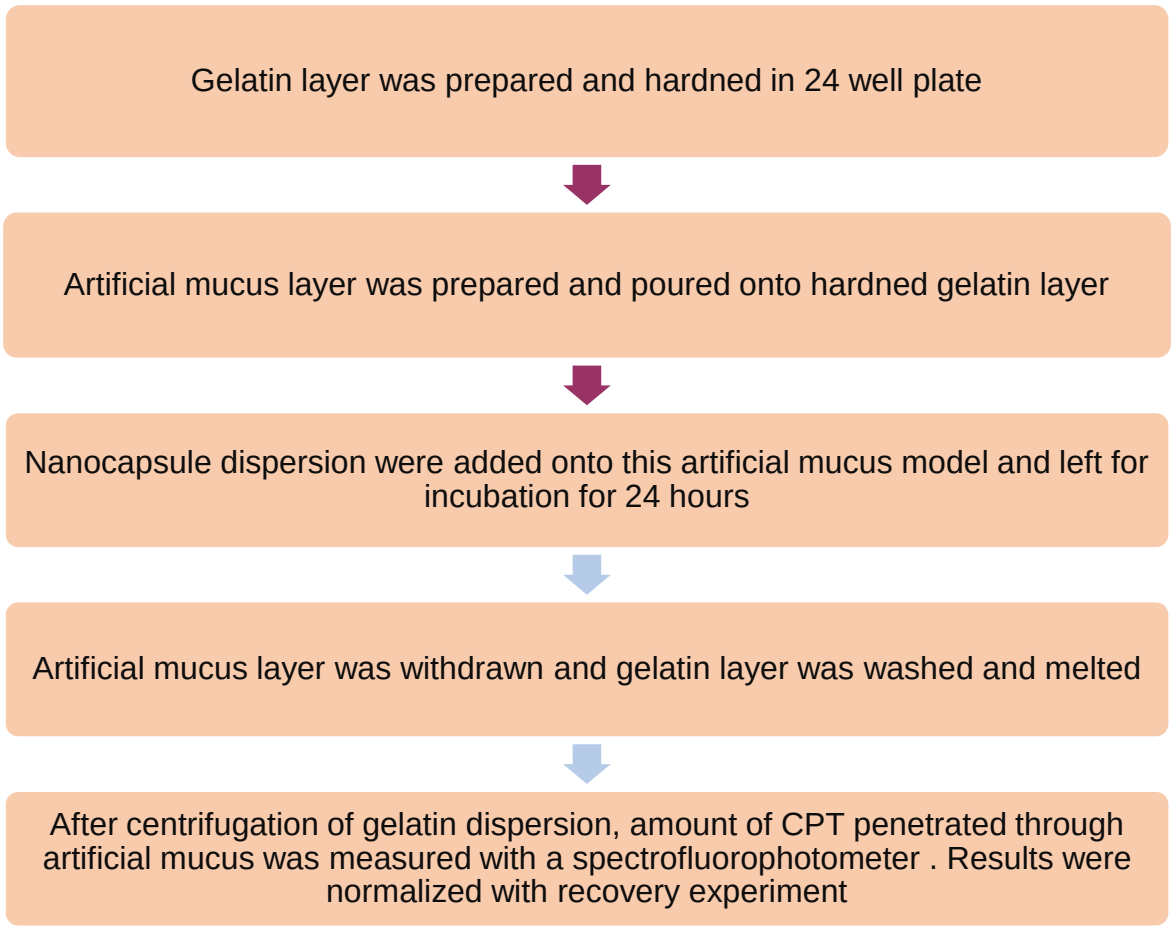


Figure 3.2. Schematic representation of artificial mucus experiment.

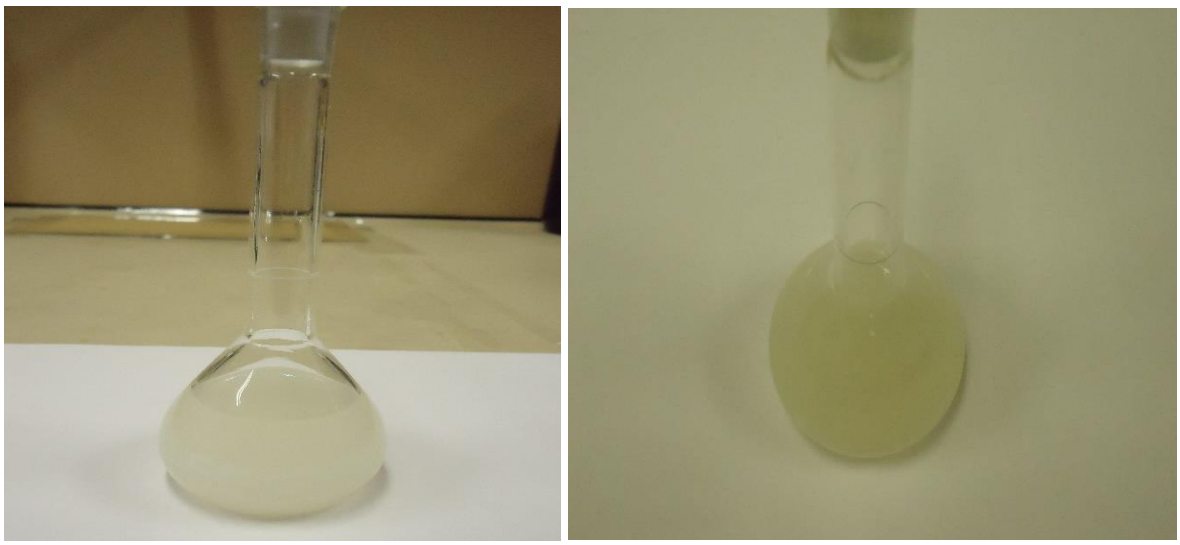


Figure 3.3. Visual representation of the preparation of artificial mucus dispersion.

1 mL of freshly prepared artificial mucus was placed onto the hardened gelatin layer. In order to evaluate the penetration of CPT loaded nanocapsules, 500 µL of NC dispersion were added onto this artificial mucus model and left for incubation for 24 hours (Figure 3.4) .

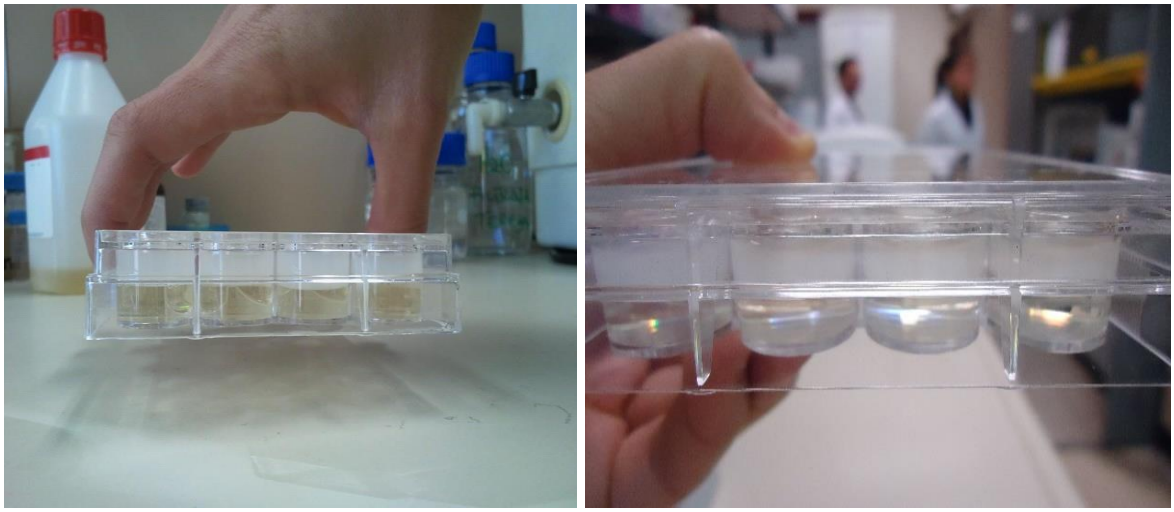


Figure 3.4. Images of experimental apparatus for penetration through artificial mucus.

After 24 h, artificial mucus layer was withdrawn. Gelatin layer was washed with ultrapure water and warmed at 60^0 C. Gelatin dispersion was then centrifuged at 7500 rpm for 20 minutes. Supernatant was taken and amount of CPT penetrated through artificial mucus was measured with a spectrofluorophotometer at an emission wavelength (λ_{em}) of 435 nm and at an excitation wavelength (λ_{ex}) of 370 nm. In order to calculate the real amount of CPT, a recovery calculation was evaluated. For this recovery calculation, briefy 250 µL of a CPT solution in DMSO (at the same concentration as in NC dispersions) was directly added onto the hardened gelatin layer and left for incubation for 24 h. After 24 h, CPT was extracted according to the procedure described above. Recovery of CPT was then calculated and results were normalized to account for recovery and reported as percentage of CPT penetrated through artificial mucus according to the formula;

$$\% \text{ Penetrated CPT} = \frac{\text{Amount of CPT determined in gelatin plates (µg)}}{\text{Amount of CPT in NCs (µg)}} \times 100 \quad (3)$$

3.2.5.Cell Culture Studies

Cell culture studies were performed in Cell Culture Laboratory of Pharmaceutical Technology Department at Faculty of Pharmacy in Hacettepe University.

3.2.5.1. Cytotoxicity Assay

The cytotoxicity of blank CD nanocapsules were determined on L929 mouse fibroblast cells with MTT assay. L929 (American Type Culture Collection, USA) was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), penicillin (100 units/mL) and streptomycin (100µg/mL) in 25 cm^2 culture flasks. L929 fibroblast cells were seeded at a density of $5x10^3$ cells/well in 100µL DMEM. The cultures were incubated at 37^0C in an incubator humidified with 5% CO_2. After 24 hours, cell culture medium was replaced with fresh medium containing blank nanocapsule formulations with different dilution ratios (1:8, 1:16, 1:32, 1:64 and 1:128). After 48h of incubation, 25 µL of MTT solution in PBS (5 mg/mL) were added to each well and were incubated further for 4 h. MTT was aspirated off and in order to dissolve formazan crystals, 200 µL of DMSO was added onto it. Optical densities (OD) were determined by a microplate reader at 450nm. Cells incubated in culture medium alone served as a control for cell viability (nontreated wells). Cell viability (%) was calculated according to the formula;

$$\text{Cell viability \%} = \frac{\text{Optical density of treated wells}}{\text{Optical density of nontreated cells}} \times 100 \qquad (4)$$

3.2.5.2. Anticancer Efficacy

The anticancer efficacy of CPT loaded nanocapsules were determined against MCF-7 cell line in comparision to CPT solution with MTT assay. MCF-7, human breast adenocarcinoma cell lines were obtained from the American Type Culture Collection, USA. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/mL) and streptomycin (100µg/mL). The cultures were maintained at 37^0C in a humidified 5% CO_2 incubator. MCF-7 cells were seeded in 96- well plates at a density of 1×10^4/100µL per well and incubated for 24 hours to allow the cell attachment. The cells were incubated with CPT loaded anionic and cationic nanocapsule

formulations in comparision with free CPT (CPT in DMSO) for 72 h. The incubation time is depending on both the doubling time of MCF-7 cell line and release characteristics of the CPT from formulations. All the formulations were diluted with DMEM (dilution rate 1:16) at an equivalent dose of Camptothecin at 3,125 µg/mL. At determined time, formulations were replaced with 25µL of MTT solution and cells were then incubated for an additional 4 h. MTT was aspirated off and in order to dissolve formazan crystals, 200µL DMSO were added onto it. Optical density (OD) was measured at 450nm using a microplate reader. Untreated cells (cells incubated with only culture medium) were taken as control with 100% cell viability. Experiments were performed in triplicate and results are expressed as mean ±SD.

3.2.5.3. Permeability through Caco-2 cell monolayer

Intestinal absorption is a formidable barrier that restricts the oral bioavailability of many potential new drugs. To address the need for in vitro assays of intestinal permeability of potential new drugs as part of the drug development process, a variety of approaches has been developed and among these approaches, the Caco-2 cell monolayer assay of intestinal permeability has emerged as one of the standard in vitro tools [215].

The Caco-2 cell monolayer system has become a standard model for human intestinal absorption of xenobiotic compounds that is more accurate than physical models such as artificial membranes, more convenient and accurate than in vitro models such as everted gut sacs and less expensive than in vivo models [215]. Hereby, in vitro transport studies were carried out accross the human adenocarcinoma Caco-2 cell line, which mimics the gastrointestinal barrier for oral chemotherapy [216, 217].

Caco-2 cells with a passage number of 30–35, were cultured on polycarbonate membrane filters (Thincerts ™ transparent, pore size 1 µm, area 1,13 cm^2) in 12-well plates (Greiner bio-one, Germany) at a seeding density of 6 × 10^4 cells in each well. The culture medium was added to both the apical (500 µL) and basolateral (1m L) of filter insert and was replaced every other day during 20 days. The cells were left at 37°C in an incubator under atmosphere of 95% air and 5% CO_2 at 90% relative humidity. Prior to the experiments, cell monolayers were replaced with transport buffer containing Hank's balanced salt solution (HBSS) buffered with

10 mM n-(2-hydroxyethyl) piperazine-n-(2-ethanesulfonic acid) (HEPES) at pH 7.4 and the trans epithelial electrical resistance (TEER) of monolayers was measured by using a Millicell®-ERS-2 system (Millipore Corporation, Bedford, MA) and found to be between 200-300 Ω cm^2. The transport of CD nanocapsule formulations (CD NCs and Chitosan coated CD NCs) and camptothecin solution in DMSO were studied from the apical to basolateral direction in Caco-2 cells. All the test solutions were diluted with HBSS in order to evaluate CPT concentration at 25 µg/mL both in NPs and in solution form. Each experiment was started by adding 0.5 mL of test solution to the apical side and 1 mL of HBSS to the basolateral side of the Transwell inserts. The cells were then incubated at 37° C and 30 rpm for 4 hours. Samples were taken from the basolateral side and analyzed with a validated HPLC method. Apparent permeability coefficient (P_{app}) which is expressed in centimeters per second was calculated according to the formula;

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{A\,Co} \qquad (5)$$

where dQ/dt is the permeability rate, Co is the initial concentration on the apical side and A is the surface area of the polycarbonate membrane.

3.2.6. *In vivo* Studies

In vivo studies were performed under the supervision of Assoc. Prof. Fabiana Quaglia and Assoc. Prof. Francesca Borrelli in the University of Naples Federico II, Department of Pharmacy, Italy. In vivo studies were conducted with CD and CS-CD nanocapsule formulations which gave the most promising results in in vitro and cell culture studies. In this part of the study, Nile Red was used as fluorescent dye. Nile Red (NR) is a hydrophobic, highly fluorescent dye that has been extensively applied to examine the structure, dynamics and environment in biological and microheterogeneous systems. In apolar solvents it fluoresces with a high quantum yield in and at reasonably long wavelengths. Morever, once incorporated into the particles, NR do not leach and its color and fluorescence remains stable for long periods of time. [218, 219].

3.2.6.1. Construction of Calibration Curve for Nile Red

In vivo studies were performed with fluorescently labeled (NR loaded) nanocapsules with Spectofluorophotometer (Shimadzu, Japan). Therefore, initially a calibration curve of NR for the Spectoflorophotometer was constructed at 25 ^{0}C at an emission wavelength (λ_{em}) of 590nm and at an excitation wavelength (λ_{ex}) of 546nm before *in vivo* uptake studies.

1mg NR was dissolved in 10 mL DMSO and diluted two times with dilution rates 1:10 and 1:100 respectively, in order to obtain a stock solution at a concentration of 0.1 µg/mL. Starting from this stock solution, different NR standard solutions with a series of concentration 0.0005-0.05 µg/mL (as of 0.0005, 0.001, 0.002, 0.005, 0.01, 0.02 and 0.05 µg/mL) were prepared by diluting this stock solution with DMSO at a dilution rates 1:200, 1:100, 1:50, 1:20, 1:10, 1:5 and 1:2 v/v espectively. Calibration curve was prepared by plotting the obtained florescence intensity against to the NR solutions with different concentrations and ultimately calibration equation and correlation coefficient were calculated.

3.2.6.2. Preparation of Nile Red loaded Nanocapsules

For the preparation of anionic NR loaded CD nanocapsules, a stock solution of NR in ethanol was prepared by dissolving 1 mg NR in 10 mL ethanol. From this stock solution, 100µL was withdrawn and added to an organic phase including 900 µL ethanol, 1 mg CD and 10 µL Miglyol. This organic phase was added dropwise to an aqueous phase (2mL) under moderate magnetic stirring. Organic solvent was then evaporated under vacuum at 40°C for 5 minutes. Nanocapsules were recovered by centrifugation at 3500 rpm at room temperature for 15 min. In order to obtain cationic NR loaded nanocapsules % 0.025 w/v Protasan™ was added to the aqueous phase.

Content of Nile Red in the nanocapsules was quantified by florescence spectrophotometry at 25 ^{0}C at an emission wavelength (λ_{em}) of 590nm and at an excitation wavelength (λ_{ex}) of 546nm. Briefly, after the separation of unwanted materials by centrifugation, supernatant containing NR loaded nanocapsule suspension was freeze-dried for 24h in order to obtain NR loaded nanocapsules in powder form. Resulting powder was weighed and dissolved in 1mL of DCM and diluted with DMSO at a dilution rate of 1:100. The experimental NR loading was

quantified using the florescence intensity of each nanocapsule formulation. Associated Drug (%) was then calculated according to the formula (1) as reported previously in section 3.2.4.4.

3.2.6.3. Animals

CD1 female mice (20-25 g) were purchased from Harlan Italy, S. Pietro al Natisone UD and were maintained under controlled conditions of temperature (23 ± 2 °C) and humidity (50 ± 10%) ad libitum until used. All animals were deprived of food 12 h prior to experimentation but had free access to water. All experiments on animals were conducted in accordance with the internationally accepted principles for laboratory animal use and care- Italian D.L. no. 116 of 27 January 1992 and associated guidelines in the European communities Council Directive of 24 November 1986 (86/609/ECC).

3.2.6.4. Recovery Experiment

In order to be able to determine the intestinal uptake of NR loaded fluorescent nanocapsules, a recovery calculation should be performed initially. Recovery calculation depends on the measurement of NR content recovered from the GI tissues. For this reason, before particle uptake studies, recovery experiment was conducted in CD1 mice with CD and CS-CD nanocapsules recovery experiment was evaluated by direct administration of these blank and NR loaded anionic and cationic NCs onto the intestinal tissues. Briefly, 2 female CD1 mice were sacrified with dislocation. The intestines were cut into 8 segments as seen in Figures 3.5. and Figure 3.6.

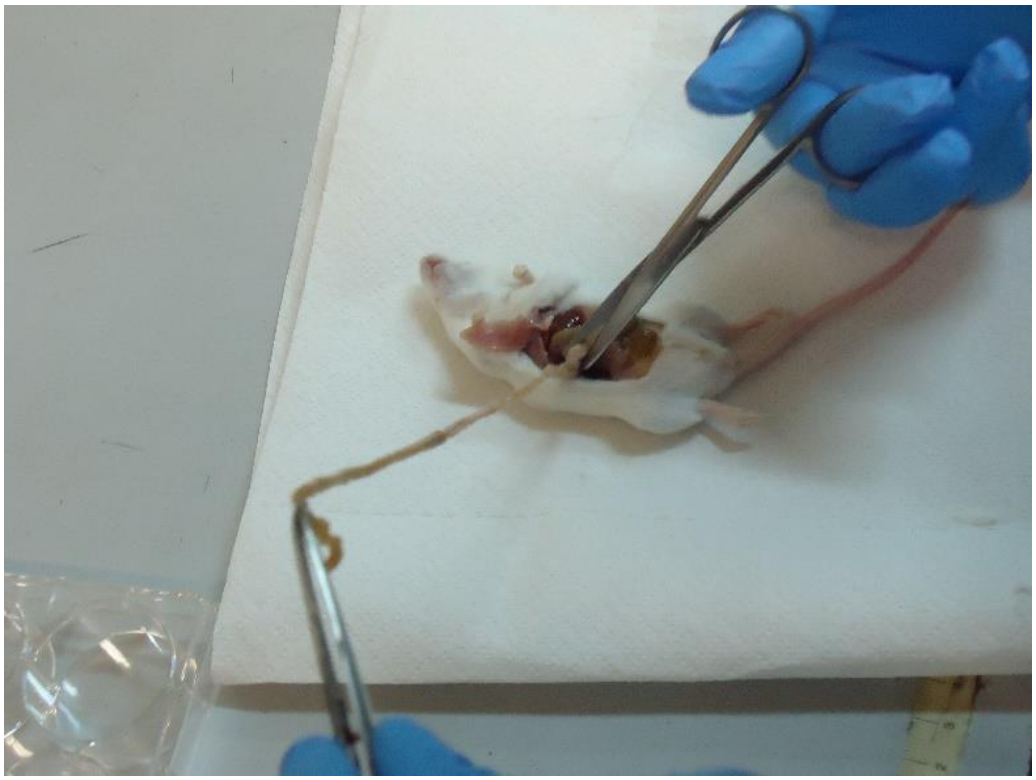


Figure 3.5. Representation of in vivo GI uptake experiment

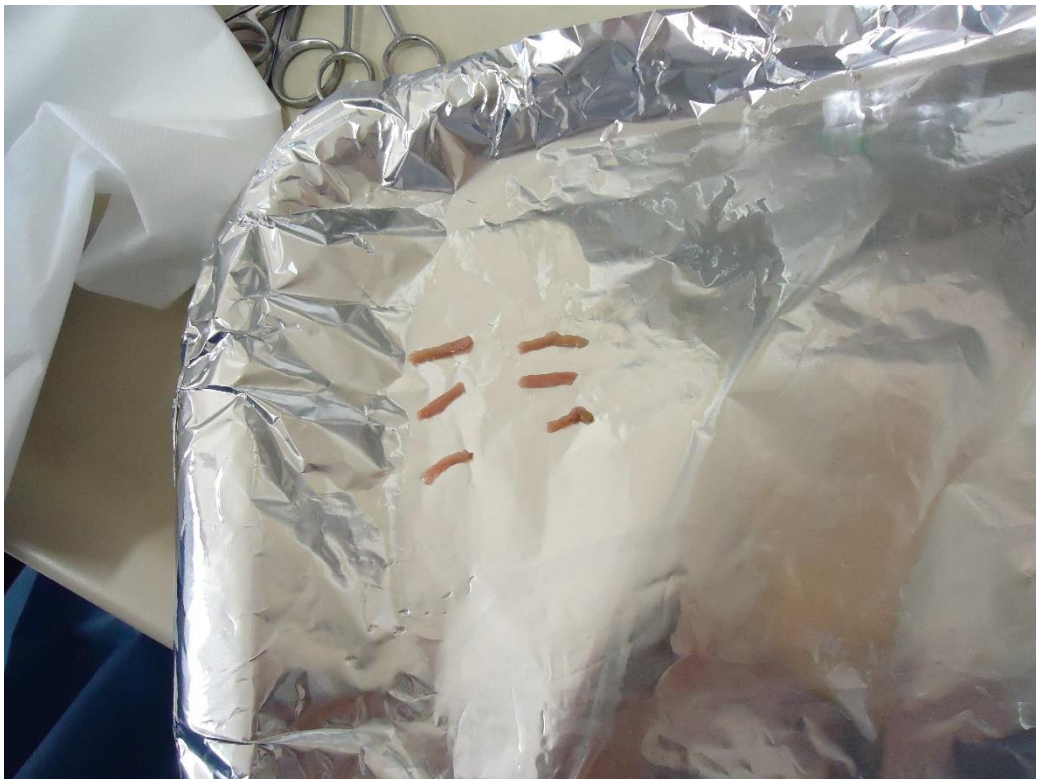


Figure 3.6. Representation of in vivo GI uptake experiment: Image for the instestinal segments of mice.

Every stomach was divided into 2 pieces including another stomach from another animal. (there is one more stomach from another animal). From each animal the cut two pieces of the intestine segments were weighed and directly administered with either 10 µL of blank or 10 µL of NR loaded Nanocapsule dispersions. Afterall is said to be every two pieces of an animal intestine and stomachs had an administration of the same NCs solution. Upon the administration, all the samples were placed in a lysis buffer solution in a volume of 2 fold of the tissue weighs. These tissues were then homogenized on ice 10 seconds 3 times. After the homogenization of the tissues, the tissue homogenats were centrifuged for 10 minutes 2 times at 13,000 rpm. Supernatants were withdrawn and 10 µL from each sample was taken. Each 10 µL sample was then added to a mixture of 10 µL DCM and 980 µL of DMSO at a final volume of 1mL. To detect the fluorescence intensity in the tissue homogenates, spectofluorophotometer was used at an emission wavelength (λ_{em}) of 590 nm and excitation wavelength (λ_{ex}) of 546 nm by measuring the florescence intensity of the samples. Results are calculated as percentage recovery according to the calibration curve of NR which were already constructed as reported in section 3.2.6.1.

3.2.6.5. Nanoparticle Uptake Studies

After the recovery experiments, nanoparticle uptake studies were performed with CD1 female mice. Animals were divided in four groups with 3 mice per group based on the administration of different nanoparticle dispersions. Group 1 was treated with NR loaded CD nanocapsule dispersion. Group 2 was treated with NR loaded CS-CD nanocapsule dispersion. Group 3 was treated with blank CD nanocapsule dispersion and group 4 was treated with blank CS-CD nanocapsule dispersion as control groups. All the formulations were administered at a concentration of 100µL with an oral gavage. After 30 minutes, animals were killed via cervical dislocation and their small intestine and stomach were collected. The intestines were cut into 4 pieces and each segment was weighed. All the intestinal segments and stomachs were placed into lysis buffer solution and homogenized on ice 3 times x 10 seconds and 4 times x 10 seconds for the intestines and for the stomachs respectively. The resulting homogenats were then centrifuged for 2 times x10 minutes at 13,000 rpm. Supernatant was taken and diluted 100 times before analysis with a spectofluorophotometer at an emission wavelength (λ_{em}) of 590 nm and excitation

wavelength (λ_{ex}) of 546 nm to detect fluorescence in the tissue homogenates (Figure 3.7). Results are normalized with the percentage recovery in reference to the recovery experiment as reported in section 3.2.6.4.

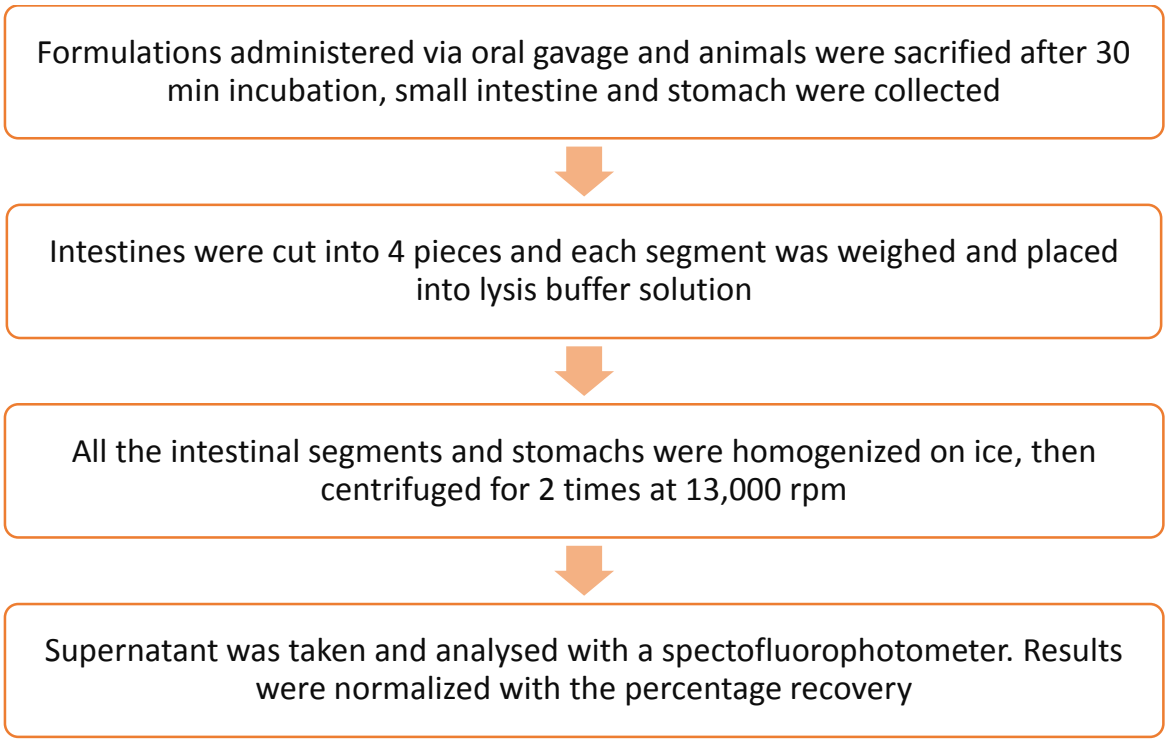


Figure 3.7. Schematic representation of nanoparticle uptake experiment.

3.2.7. Statistical Analysis

All statistical analysis were performed using SPSS v 22.0 (IBM Corp., Armonk, NY, USA). USA). The significance of differences was assessed by Student's *t*-test. *P* value less than 0.05 ($p<0.05$) was considered to denote a statistically significant difference.

4. RESULTS AND DISCUSSION

4.1. *In vitro* Quantification of Camptothecin and HPLC Method Validation

Quantitative determination of CPT was performed under the conditions described in section 3.2.1. Representative chromatograms for the two forms of CPT, namely actone and carboxylate forms are given in Figure 4.1. and Figure 4.2. respectively. Under the chromatographic conditions used for the analysis of CPT, the retention time for lactone form was 19.7 minute wheras for carboxylate form was 7.2 minute.

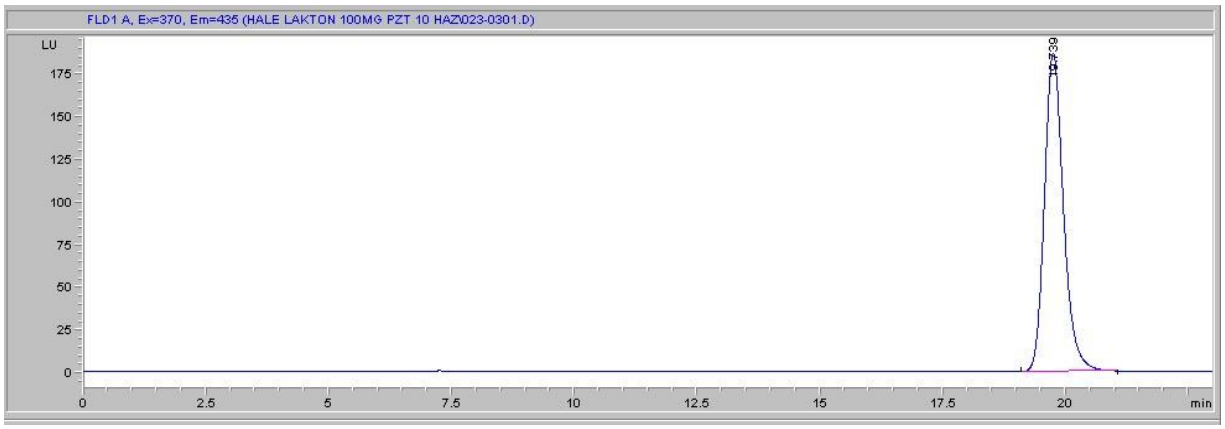


Figure 4.1. HPLC Chromatogram of active Lactone form of CPT.(Retention time 19.7 min)

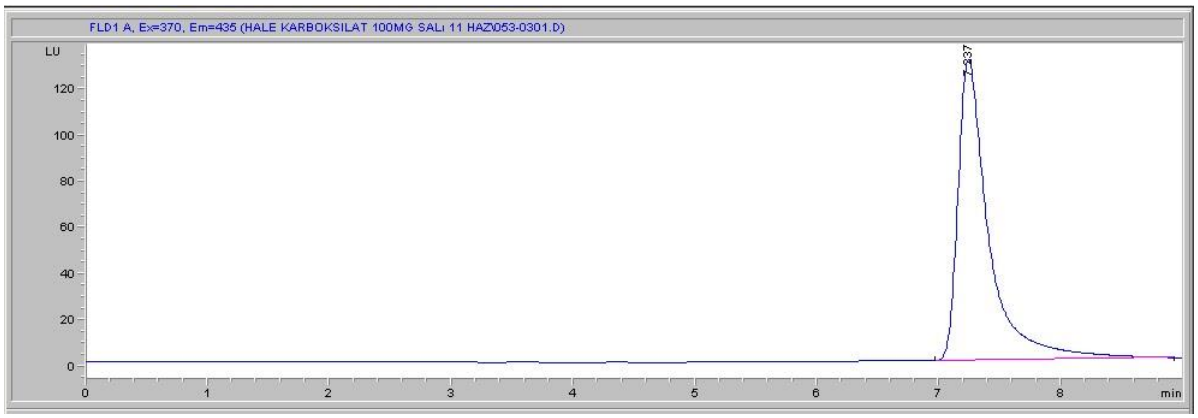


Figure 4.2. HPLC Chromatogram of inactive Carboxylate form of CPT. (Retention time 7.2 min.)

4.1.1. Construction of Calibration Curve

CPT calibration curve was constructed according to the method described in the Section 3.2.1.1.1.The calibration curve is given in Figure 4.3. and Figure 4.4. and

the slope of this curve derived from different series were calculated along with intercept and correlation coefficient as can be seen in Table 4.1.

Table 4.1. Linear regression data from HPLC analysis for Lactone and Carboxylate forms of CPT.

Parameter	Active Lactone form	Inactive Carboxylate form
Concentration Range	1-50 µgl/mL	1-50 µgl/mL
Slope	922.58	508.83
Intercept	+ 370.96	-77.435
Correlation Coefficient	0.9994	0.9996

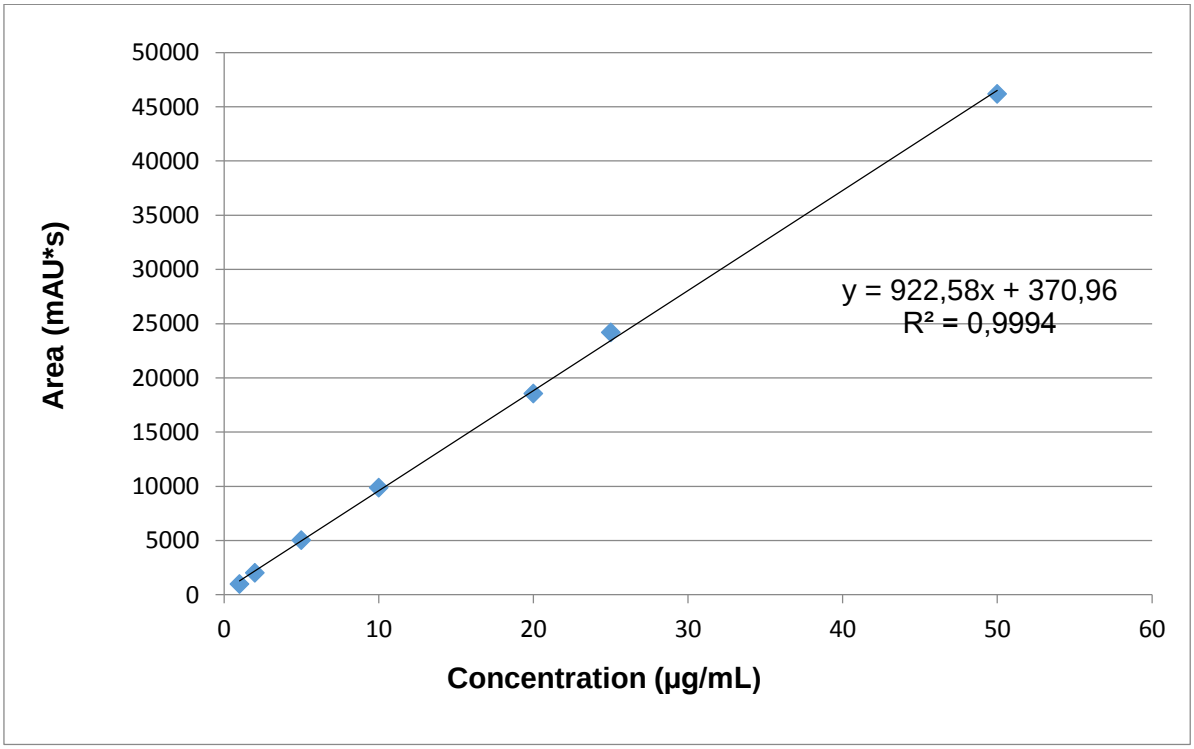


Figure 4.3. Calibration Curve of Lactone form of CPT and regression equation

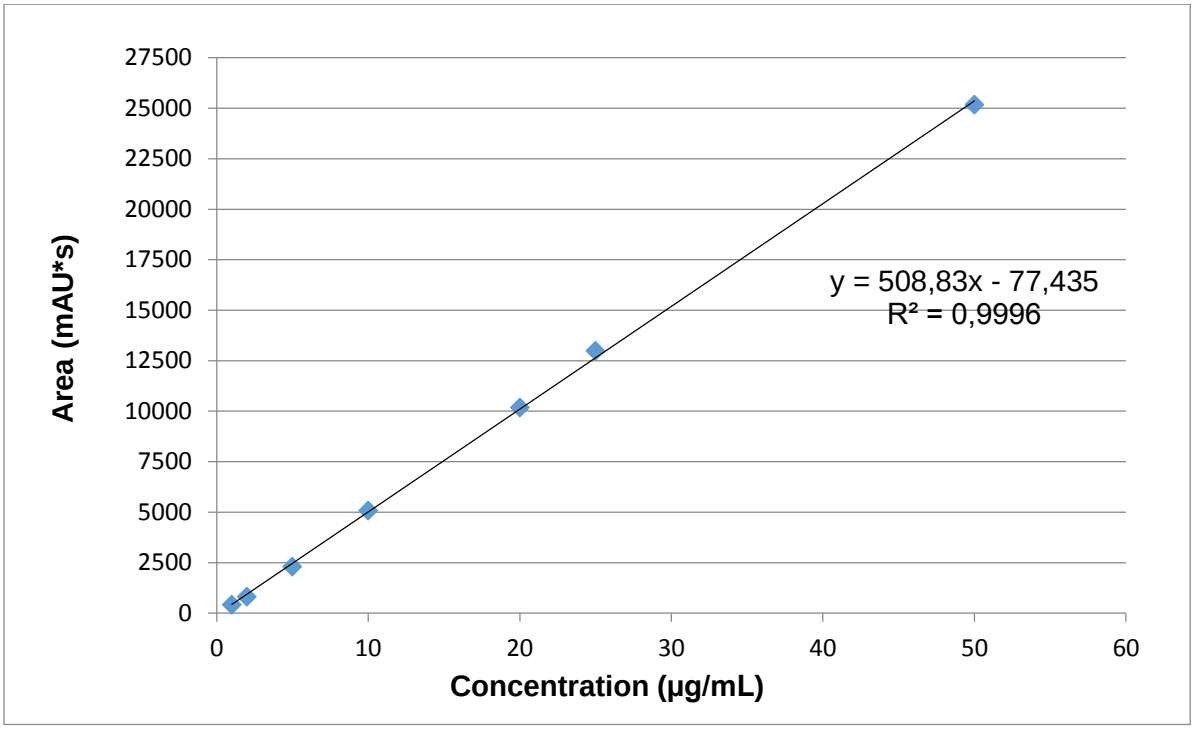


Figure 4.4. Calibration Curve of Carboxylate form of CPT and regression equation

4.1.2. Linearity

Linearity was evaluated in the range from 1 to 50 µg/µL standard solutions of CPT as described in the section 3.2.1.1.2. Linearity of the assay procedure was determined by calculation of a regression line. Concentrations of CPT standard solutions were calculated from the resulting peak heights and the regression equation of the calibration curve [220]. Linear relation between CPT concentration and peak area was supported by the regression analysis from Table 4.1. The calibration curves constructed for both lactone and carboxylate forms show that the analytical method was linear from 1 to 50 µg/mL. Both the lactone and carboxylate forms of CPT standard solutions displayed good linearity in this tested range. Coefficient also proves the linearity. A linearity correlation coefficient above 0.999 is acceptable for HPLC methods of major component analysis. The square correlation coefficient (R^2) was found to be 0.9994 and 0.9996 for lactone and carboxylate forms respectively which are both found to very close to 1 representing absolute linearity [221].

4.1.3. Accuracy

Accuracy is defined as the agreement between the found value and the true, independently determined, concentration value [222]. As described in the section

3.2.1.1.3., accuracy is determined by replicate analysis of samples containing known amounts (2, 20 and 50 µg/mL) of CPT covering the specified range and representing low, medium and high concentrations in that specific range. The accuracy of the method was evaluated considering the recovery.

Based on the calculated CPT concentrations and the known composition of CPT, the percent recovery of CPT was calculated. Considering each point concentration of lactone form, the average recovery was found within the range 99–103%, (Table 4.2) whereas for the carboxylate form was found within the range 98-102%. (Table 4.3). For each point on the calibration curves for both lactone and carboxylate forms of CPT, the concentrations back-calculated from the equation of the regression analysis were within acceptable limits [221].

Table 4.2. Accuracy data for Lactone form of CPT

Samples	2 µg/mL Recovery (%)	20 µg/mL Recovery (%)	50 µg/mL Recovery (%)
1	99.15	101.85	103.45
2	98.25	102.83	98.98
3	98.72	101.32	100.18
4	98.04	102.91	100.64
5	100.13	105.10	101.58
6	98.86	102.52	100.98
Mean	98.85	102.74	100.87
Standard Deviation	0.74	1.30	1.37
CV	0.75	1.26	1.35

Table 4.3. Accuracy data for Carboxylate form of CPT

Samples	2 µg/mL Recovery (%)	20 µg/mL Recovery (%)	50 µg/mL Recovery (%)
1	101.65	101.52	98.34
2	99.55	99.85	98.30
3	98.14	101,91	96.84
4	98.77	102,60	97.68
5	98.33	103.23	98.12
6	100.2	100,25	98.34
Mean	99.42	101.55	97.93
Standard Deviation	1.34	1.30	0.59
CV	1.35	1.28	0.60

4.1.4. Precision

As stated in section 3.2.1.1.4. precision of an analytical procedure expresses the closeness of agreement between a series of measurements and was measured as repeatability, reproducibility and intermediate precision. The precision of an analytical method is usually expressed as coefficient of variation of a series of measurements [223].

Repeatability, which is also termed intra-assay precision, was determined by assaying six replicates of a CPT concentration (20 µg/mL) in a single run. Assay precision was calculated as Coefficient of Variation (CV) expressed as a percentage of the mean observed concentrations and the observed data were given in Table 4.4. and 4.5. for lactone and carboxylate forms respectively.

Table 4.4. Data of repeatability for lactone form of CPT.

Sample	**Calculated Concentration (µg/mL)**
1	19.25
2	19.24
3	19.26
4	19.33
5	19.24
6	19.32
Mean	19.27
SD	0.03
CV	0.15

Table 4.5. Data of repeatability for carboxylate form of CPT.

Sample	**Calculated Concentration (µg/mL)**
1	19.42
2	19.59
3	20.04
4	19.77
5	19.79
6	19.42
Mean	19.67
SD	0.22
CV	1.11

According to the results for both lactone and carboxylate forms, coefficient of variation (CV) values were found to be 0.15 and 1.11 respectively. The intraday relative standard deviation values were < 2% indicating the analytical method was repeatable [224].

Intermediate precision was studied by running the whole method on three different days. Each day, equivalent concentrations of CPT standard solutions (20 µg/mL) were prepared and analysed. Assay precision was calculated as coefficient of variation (CV) and the observed data were given in Table 4.6. and 4.7. for lactone and carboxylate forms respectively.

Table 4.6. Data of intermediate precision for lactone form of CPT

Days	**Calculated Concentrations (µg/mL)**
1.	20.44
2.	21.07
3.	21.01
Mean	20.84
SD	0.34
CV	1.63

Table 4.7. Data of intermediate precision for carboxylate form of CPT

Days	**Calculated Concentrations (µg/mL)**
1.	20.98
2.	21.03
3.	20.79
Mean	20.93
SD	0.12
CV	0.57

According to the results for both lactone and carboxylate forms, coefficient of variation (CV) values were found to be 1.63< 2 % and 0.57< 2 % respectively, indicating the analytical method was precise.

Reproducibility was determined by the assay of 6 seperate CPT solution prepared at the same concentrations (20 µg/mL). Assay precision was calculated as coefficient of variation and the observed data were given in Table 4.8. and 4.9. for lactone and carboxylate forms respectively.

Table 4.8. Data of reproducibility for lactone form of CPT

Sample	**Calculated Concentration (µg/mL)**
1	20.04
2	20.26
3	20.12
4	19.52
5	19.83
6	19.78
Mean	19.92
SD	0.27
CV	1.35

Table 4.9. Data of reproducibility for carboxylate form of CPT

Sample	**Obtained Concentration (µg/mL)**
1	21.09
2	21.44
3	21.17
4	21.40
5	21.59
6	21.76
Mean	21.40
SD	0.25
CV	1.17

According to the results for both lactone and carboxylate forms, CV values were found to be 1.34 < 2% and 1.17 < 2% respectively, indicating the analytical method was reproducible.

Considering the CV values in terms of repeatability, intermediate precision, and reproducibility were found lower than 2% for both CPT forms, lactone and carboxylate indicating the precision of this analytical method.

4.1.5. Sensitivity

Sensitivity of the method was determined by finding the limit of detection and limit of quantification at a minimum concentration as stated in section 3.2.1.1.5. **Limit of detection (LOD)** at which a signal to noise ratio is 3:1 was found to be 0.22 µg/mL for the lactone form and 0.78 µg/mL for carboxylate form. **Limit of Quantification (LOQ**) at which a signal to noise ratio is 10:1 was found to be 0.15 µg/mL for lactone form and 0.36 µg/mL for carboxylate form. In the set up of our experiment, both LOQ and LOD values were accepted as suitable for our purpose and working range indicating this analytical method as sensitive.

4.1.6. Specificity

Specificity of the analytical method was proved by comparing chromatograms and retention times obtained in the standard solutions of CPT with the used excipients obtained from blank [225]. Chromatograms obtained after the injection of PCL, CD, CS and DMSO seperately, are given in Figure 4.5 to Figure 4.8. According to data derived from HPLC analysis, no interference was observed to the CPT peak (both in lactone and carboxylate forms) by other formulation components. This method has been shown to be specific as none of the excipients interfered with the analytes of interest.

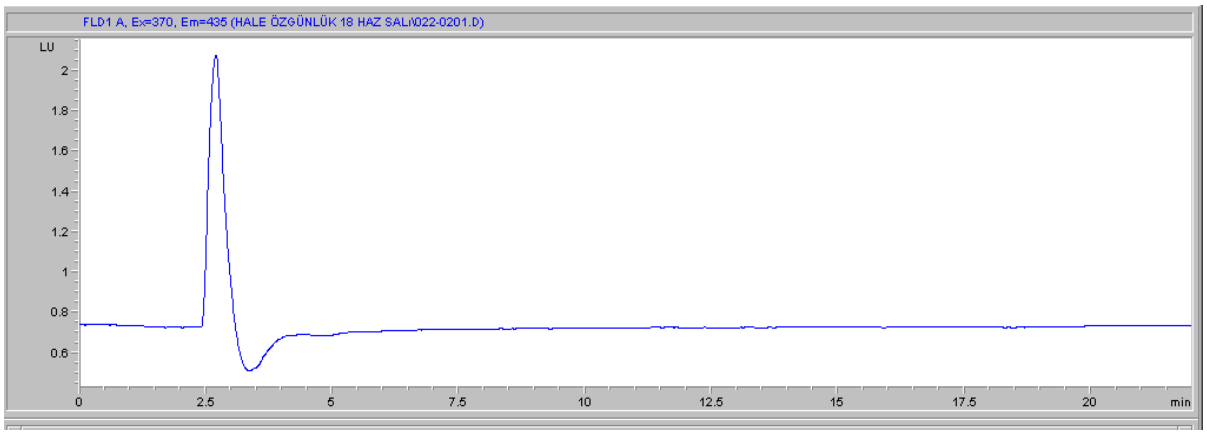


Figure 4.5. HPLC chromatogram of formulation excipient (CS).

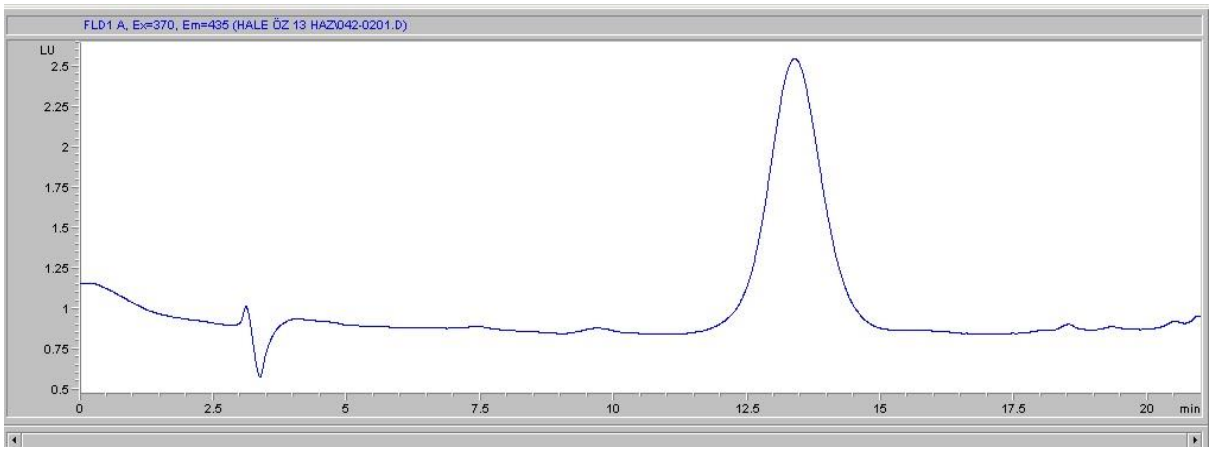


Figure 4.6. HPLC chromatogram of the formulation excipient (CD)

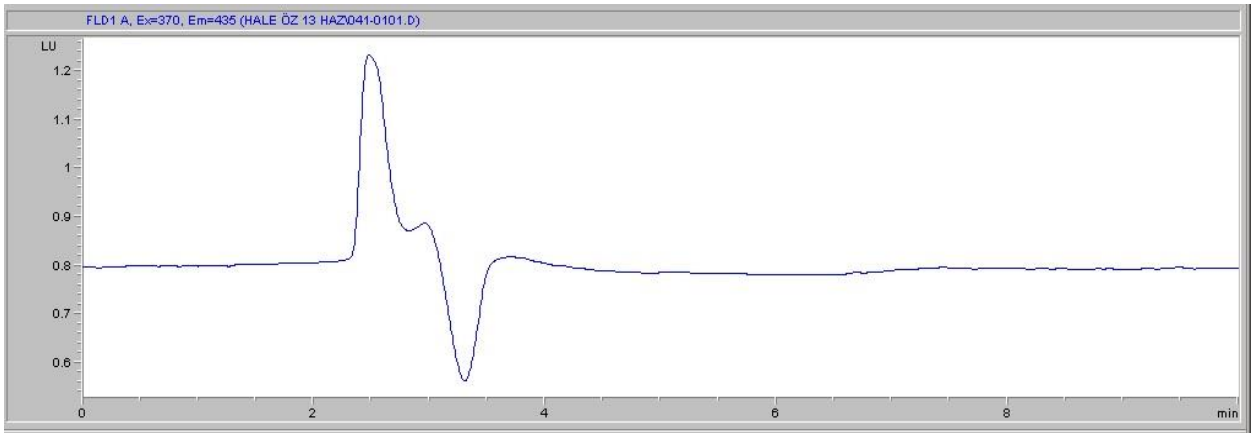


Figure 4.7. HPLC chromatogram of the formulation excipient (DMSO)

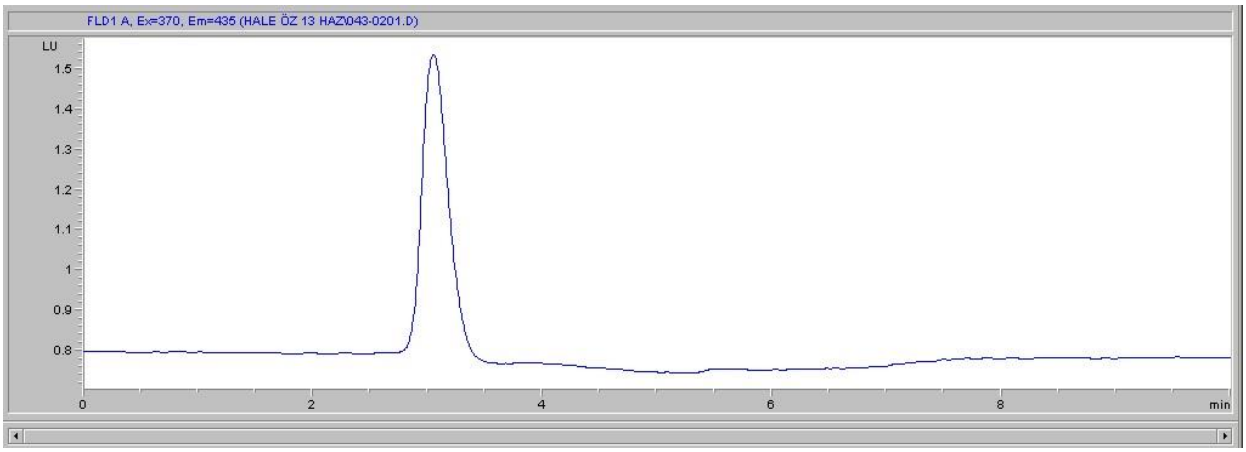


Figure 4.8. HPLC chromatogram of the formulation excipient (PCL)

The rate of equilibration between the two components (lactone and carboxylate form) of CPT is slow enough to permit their separation by reverse-phase HPLC [226]. This reverse- phase HPLC method was validated for linearity, accuracy, precision, sensitivity and specificity. Considering the results, the validated HPLC method was found to be specific, linear, precise and accurate with low limits of detection and quantification and can be successfully employed for the assay of CPT. Results were in accord with the studies in literature [209, 227] indicating this HPLC analytical method is proved to be a valid method for the quantitative determination of CPT in the aimed formulation development study.

4.2. Pre-formulation studies

Pre-formulation studies were evaluated in order to attain the ultimate nanoparticle formulations. Herein mean particle size has a critical importance as selection criteria for nanoparticle characteristics since mean particle size affects both in vitro profiles and also in vivo fate of nanoparticles in terms of stability, drug release profile and encapsulation efficiency. Furthermore mean particle size has also an important influence on the interaction with membranes, cellular uptake, absorption rates and biodistribution in the body as well as toxicity. Taking into consideration that absorption rate and permeability are of primary concerns for oral bioavailability, mean particle size should be critically assessed. On the other hand, in addition to the mean particle size, stability and solubilization issues which are also related with the formulation parameters [228] should also be considered for drugs suffering from poor aqueous solubility and instability leading to bioavailability problems. Therefore

formulation parameters affecting mean particle size are of primary concern and should be optimized according to these discussions.

4.2.1. Optimization of polymer concentration and the polymer molecular weight

The effect of the concentration of polymers (both CD and PCL) on particle size and PDI is given in Table 4.10. and 4.11. According to the data, it is clearly seen that particle size increases linearly with polymer concentration which can be attributed to higher organic solution viscosity [229]. In the solvent displacement method, the viscosity of the organic phase is highly dependent on polymer concentration even at the lowest values [62].

Table 4.10. Effect of polymer concentration on particle size and PDI of CD nanocapsules. Data represents the mean results±SD values of three different batches.

Polymer concentration (% w/v)	Particle Size(nm)	PDI±SD
0.05	154.2 ± 3.2	0.10 ± 0.04
0.1	161.5 ± 3.7	0.11 ± 0.09
0.2	268.4 ± 4.0	0.19 ± 0.10
0.5	347.6 ± 5.6	0.32 ± 0.19

Table 4.11. Effect of polymer concentration on particle size and PDI of PCL nanocapsules. Data represents the mean results±SD values of three different batches.

Polymer concentration (% w/v)	Particle Size(nm)	PDI±SD
0.05	167.3 ± 2.8	0.09 ± 0.03
0.1	171.5 ± 1.7	0.13 ± 0.10
0.2	293.4 ± 3.1	0.18 ± 0.13
0.5	369.6 ± 3.4	0.32 ± 0.17

Although the smallest particle size was obtained with 0.05 % w/v concentration of polymer, there is no significant difference between the concentrations of 0.05 % w/v and 0.1 % w/v (P>0.05) for both CD and PCL nanocapsules. Even if the polymer concentration increases two fold (from 0.05 % w/v to 0.1 % w/v,) mean particle size does not increase by two fold either. Herein providing stability might be an important

factor at selecting the polymer concentration since polymeric shell has a key position for protection of encapsulated molecules and maintaining the stability. CD nanocapsules were prepared without a stabilizing agent, e.g. surfactant, which means drug stability is mostly yielded by the polymeric wall. PDI values indicated monodisperse distribution of particles and a higher polydispersity at increasing polymer concentration. It has been reported that a higher polymer concentration in the organic phase led to an increase in shell thickness of the nanocapsules obtained [63]. Therefore polymer concentration that is probably related with the polymeric wall thickness, is an important parameter in regard to predicting the thickness of this nanocapsule shell and for the stability thereby. Hence for further studies 0.1 % w/v concentration of polymer was retained.

In addition to the effect of polymer concentration, influence of polymer molecular weight was also investigated. Different types of PCL with molecular weight of 14,000, 65,000 and 80,000 Da were used as formulation parameters as indicated in Section 3.2.2.1. As it can be shown in Table 4.12. we observed that the larger sized nanoparticles were obtained with the higher molecular weight polymer, due to higher organic solution viscosity [230].

Table 4.12. Effect of polymer MW on particle size and PDI of PCL nanocapsules. Data represents the mean results±SD values of three different batches.

Polymer MW (Da)	Mean Particle Size(nm)	PDI±SD
14,000	170.3 ± 1.2	0.08 ± 0.04
65,000	181.5 ± 2.1	0.12 ± 0.09
80,000	218.4 ± 2.7	0.14 ± 0.11

Table 4.12. shows that the mean particle size significantly increased with the increase of MW of PLC ($P<0.05$) which was in accordance with the result reported by Budhian et al. [231]. This phenomenon was probably resulted from the increase of the viscosity of internal phase, thereby decreasing the net shear stress and increasing the particle size [232]. In particular polymers of higher molecular weight have less entropy loss related to their freedom of motion which results in a higher affinity to the drug sruface i.e stronger adsorption and slower desorption. According to the thermodynamic predicton, polymers of higher molecular weights provide

better stabilization. Polymers of relatively low molecular weights have a redispersion advantage [233]. Therefore PCL with polymer molecular weight 65,000 was retained for further studies.

4.2.2. Optimization of oil concentration

This pre-formulation study was performed to select optimum oil concentration with the goal of not only minimizing the particle size but also maximizing the capacity of the nanoparticle to dissolve the drug which is directly affected by the oily core of the nanocapsules. Therefore particle size should be in an optimum range which enables particles to diffuse and permeate through the biological membranes, but also should provide the maximum ability for encapsulation of drugs and sustained release. In nanocapsules the oily core is essential for the solubilization and encapsulation of hydrophobic drugs, however an increase in the concentration of oil, results in an increase of nanoparticle size. Concentration of oil directly relates to the particle size of nanocapsules which is probably due to the increase in the viscosity of organic phase [234]. Miglyol 812, is a medium chain caprylic and capric fatty acid triglyceride and has desired characteristics; it is as non-toxic, biocompatible and biodegradable. [211]. Therefore, Miglyol 812 was selected for the preparation of oily core of nanocapsules.

As shown in Table 4.13. and 4.14., an increase in the concentration of oil resulted in an increase of nanoparticle size. This effect was attributed to the increase of the viscosity of the organic phase, since the higher the oil concentration is, the more viscous the organic phase becomes. As far as CD nanocapsules are concerned for all the concentrations tested there is a significant difference ($P<0.05$) whereas for PCL nanocapsules there is no significant difference between the concentrations 0.3 % v/v and 1 % v/v ($P>0.05$) in terms of mean particle size. Therefore, the criteria for selecting oil concentration should be with the intention of maximizing drug encapsulation [31]. For this reason 1% v/v oil concentration which was expected to pose higher encapsulation effect than %0.3 v/v, was retained for further studies.

Table 4.13. Effect of oil concentration on particle size and PDI of CD nanocapsules. Data represents the mean results±SD values of three different batches.

Oil concentration (% v/v)	Particle Size(nm)	PDI±SD
0.3	173.2 ± 2.4	0.07 ± 0.02
1.0	182.5 ± 1.7	0.10 ± 0.04
3.0	296.0 ± 3.5	0.24 ± 0.10

Table 4.14. Effect of oil concentration on particle size and PDI of PCL nanocapsules. Data represents the mean results±SD values of three different batches.

Oil concentration (% v/v)	Particle Size(nm)	PDI±SD
0.3	174.2 ± 1.1	0.09 ± 0.04
1.0	178.5 ± 2.7	0.12 ± 0.07
3.0	312.1 ± 2.5	0.31 ± 0.12

4.2.3. Optimization of organic phase to aqueous phase volume ratio

The effect of increased aqueous phase volume was determined by investigating the particle size of nanocapsules using organic to aqueous phase volume ratio in a range of 1:1, 1:2 and 1:4 as performed in the literature [59]. Table 4.15. and 4.16.show that the mean particle size is unchanged with the organic to aqueous phase volume ratio 1:2 and 1:4 which means in terms of particle size there is no significance difference between these ratios (p>0.05). On the other hand, the highest value of particle size was obtained with a lower volume of water 5 mL indicating that at the organic to aqueous phase ratio 1:1 a large increase in particle size was observed presumably due to the poor phase separation [210]. According to the studies in literature, several studies were performed by using the ratio of 1:2 O/A for nanoprecipitation method. As it was also indicated by Simões et al [235], Kshirsagar et al [236] and Limayem et al [230], decreasing the O/A ratio, in other words increasing the volume of the aqueous phase, results in lower encapsulation efficiency especially for lipophilic drugs. Therefore 1:2 organic to aqueous phase volume ratio was selected for further studies.

Table 4.15. Effect of organic to aqueous phase ratio (O/A) on CD nanocapsules' particle size and PDI.Data represents the mean results±SD values of three different batches.

O/A	Particle Size(nm)	PDI±SD
1:1	341.2 ± 4.7	0.31 ± 0.14
1:2	182.5 ± 1.6	0.09 ± 0.01
1:4	178.3 ± 2.5	0.07 ± 0.02

Table 4.16. Effect of organic to aqueous phase ratio (O/A) on PCL nanocapsules' particle size and PDI.Data represents the mean results±SD values of three different batches

O/A	Particle Size(nm)	PDI±SD
1:1	371.4 ± 3.3	0.37 ± 0.13
1:2	201.3 ± 2.7	0.12 ± 0.06
1:4	196.6 ± 2.5	0.10 ± 0.04

4.2.4. Optimization of surfactant concentration

Since Tween 80 ® could be used as a surfactant in the NCs' formulation process, it was important to investigate the effect of Tween 80 ® concentration (0.1 %, 0.5% and 1% v/v) on the particle mean diameter and PDI. According to the results stated in Table 4.17., smallest particle size was obtained with the concentration of 0.5 % v/v of surfactant. From the %0.5 v/v data, an increase or decrease in the surfactant amount produced an increase in nanocapsule mean size. It can be concluded that a lower concentration of stabilizer than 0.5% v/v was not sufficient to prevent coalescence during the nanocapsule formation. In other words, an insufficient amount of emulsifier would fail in stabilizing all the nanoparticles and thus some of them would tend to aggregate [237]. Adding more surfactant led to more stable nanocapsule with less coalescence and lower mean size, as shown in Table 4.17. Above this value, the mean diameter of the nanocapsules increased again. Smilar results were reported by Khayata et al [238] and Guhagarkar et al [239]. Therefore 0.5 % v/v surfactant was retained for further experiments.

Table 4.17. Effect of surfactant concentration on PCL nanocapsules' particle size and PDI.Data represents the mean results±SD values of three different batches

Surfactant Concentration (% v/v)	Particle Size(nm)	PDI±SD
0.1	219.2 ± 2.2	0.22 ± 0.12
0.5	203.5 ± 1.4	0.11 ± 0.05
1	228.6 ± 2.1	0.14 ± 0.09

4.3. Formulation of anionic and cationic CPT loaded nanocapsules

According to the overall formulation development data, 0.1 % w/v polymer, 1 % v/v oil and 1:2 organic to aqueous phase volume ratio were selected as parameters for CPT loaded PCL and CD nanocapsule preparation. In addition to these formulation parameters, polymer molecular weight was selected as 65,000 Da and the concentration of surfactant as 0.5 % v/v for the preparation of PCL nanocapsules

4.4. Physicochemical characterization of Nanocapsule formulations

4.4.1. Mean particle Size

Mean particle size and polydispersity index of anionic and cationic nanocapsule formulations are presented in Table 4.18. As it can be clearly seen from the results both anionic and cationic CPT loaded nanocapsules were in the range of 180 to 220nm with a narrow size distribution. Particle size is a critical determinant of the biological fate of orally delivered nanoparticles and should be small enough to be able to promote diffusion through the mucus and uptake by intestinal cells [1]. All nanocapsule formulations showed a particle diameter ranging up to 200 nm, suitable to obtain an effective intracellular uptake [240]. The PDI is an approximation of the broadness of the particle size distribution [241]. PDI values ranging from 0.1 to 0.25 indicate a narrow size distribution while greater than 0.5 related to a broad distribution [242]. Herein, a low PDI was shown for all the formulations (<0.25) indicating monodisperse homogenous nanocapsules. Coating with CS resulted in an increased particle size because the surface of the nanocapsule was believed to be surrounded by the adsorbed CS molecules [243].

Table 4.18. Mean diameter and Polydispersity index (PDI) values of CPT loaded nanocapsule formulations. Data represents the mean results±SD values of three different batches.

Nanocapsule Formulations	Mean Particle Size (nm)	PDI
PCL	191.3 ±3.12	0.09±0.04
CS-PCL	218.1 ±5.21	0.22±0.08
CD	187.5 ±5.46	0.11±0.05
CS-CD	204.7 ±6.05	0.14±0.05

4.4.2. Zeta Potential

Zeta potential is the potential difference between the dispersion medium and the stationary layer of fluid attached to the dispersed particle. [244, 245]. On the other hand, surface charge density is the amount of electric charge per unit area of space, indicating that surface charge and zeta potential are not the same but they are related.

Zeta potential is another important parameter that is related to nanoparticle stability or aggregation behavior in a dispersion, and can have significant implications on product performance since zeta potential can be used to predict the monodispersity (or agglomeration) of particles. Moreover determination of zeta potential is important in terms of nanoparticle-nanoparticle interactions and nanoparticle-cell interactions as well [246]. Considering the necessity of the interaction between particles and mucus for oral delivery, surface charge is another important factor which drives the interaction with the mucus layer and thereby the uptake from GI tract.

A higher level of zeta potential results in greater electrostatic repulsion forces between the particles. This repulsion leads to greater separation distances between particles in the suspension, reducing aggregation/flocculation caused by Van der Waals interactions [247]. On the other hand, zeta potential values neither highly negative nor highly positive, perhaps between -15 mV and +15mV would be appropriate whereas low zeta potential (<5mV) can lead to agglomeration. Zeta potential is one of the factors which are related with the opsonization rate of nanoparticles. Opsonization is the process by which a foreign organism or particle

becomes covered with nonspecific proteins, thereby making it more visible to phagocytic cells (e.g., monocytes, macrophages, neutrophils and dendritic cells) [248]. Neutrally charged particles have a much lower opsonization rate than highly charged particles. [249].

In this study, the ZP values were assessed by determining the particle electrophoretic mobility using the Malvern Nanosizer [250]. Surface charge of anionic and cationic nanocapsules were demonstrated between -11 mV and +18 mV. (Figure 4.9). Taking into consideration that zeta potential values obtained from this work were neither highly positive nor highly negative, indicating that near these neutrally charged nanoparticles may be protected from opsonization.

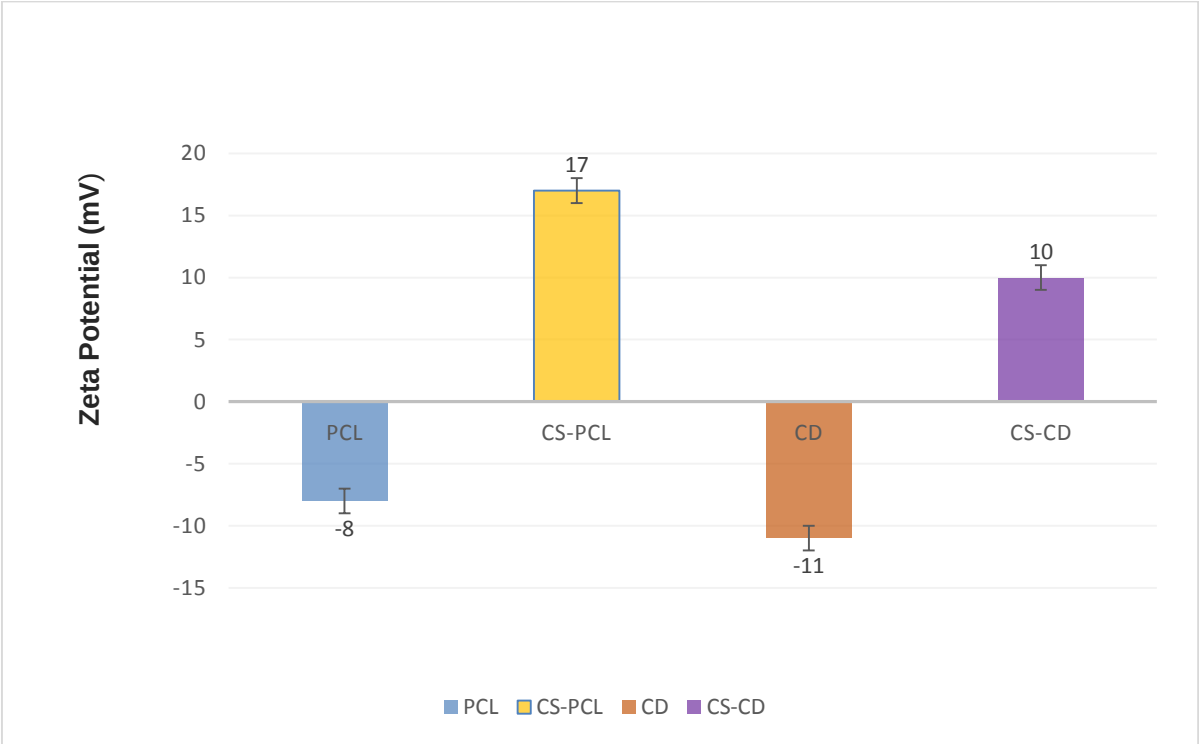


Figure 4.9. Zeta Potential values of CPT loaded PCL, CS-PCL, CD and CS-CD nanocapsules. Data represents the mean results±SD values of three different batches.

The zeta potential of chitosan-coated nanocapsules were significantly higher than that of the non-coated NCs and indicated cationic nature for the nanocapsules due to protonation of the amino group. ($p<0.05$). Chitosan coating was found to be effective in imparting a positive charge to nanocapsules with anionic cores. Thus, it

is assumed that adsorption of CS on the nanocapsule surface will cause a modification of the nanocapsule zeta potential as well as mean particle size.

4.4.3. Imaging of Nanocapsules

Morphology of CPT loaded nanocapsules were evaluated as indicated in Section 2.2.1.2. with Scanning Electron Microscopy. SEM imaging is also a tool for justifying the results obtained from Zetasizer based on DLS measurement. Figure 4.10. shows typical SEM images of NCs with spherical shapes having smooth surfaces. It can be clearly seen that SEM photographs confirm that particle size was in a good correlation with the results obtained by DLS in the Section 4.4.1.

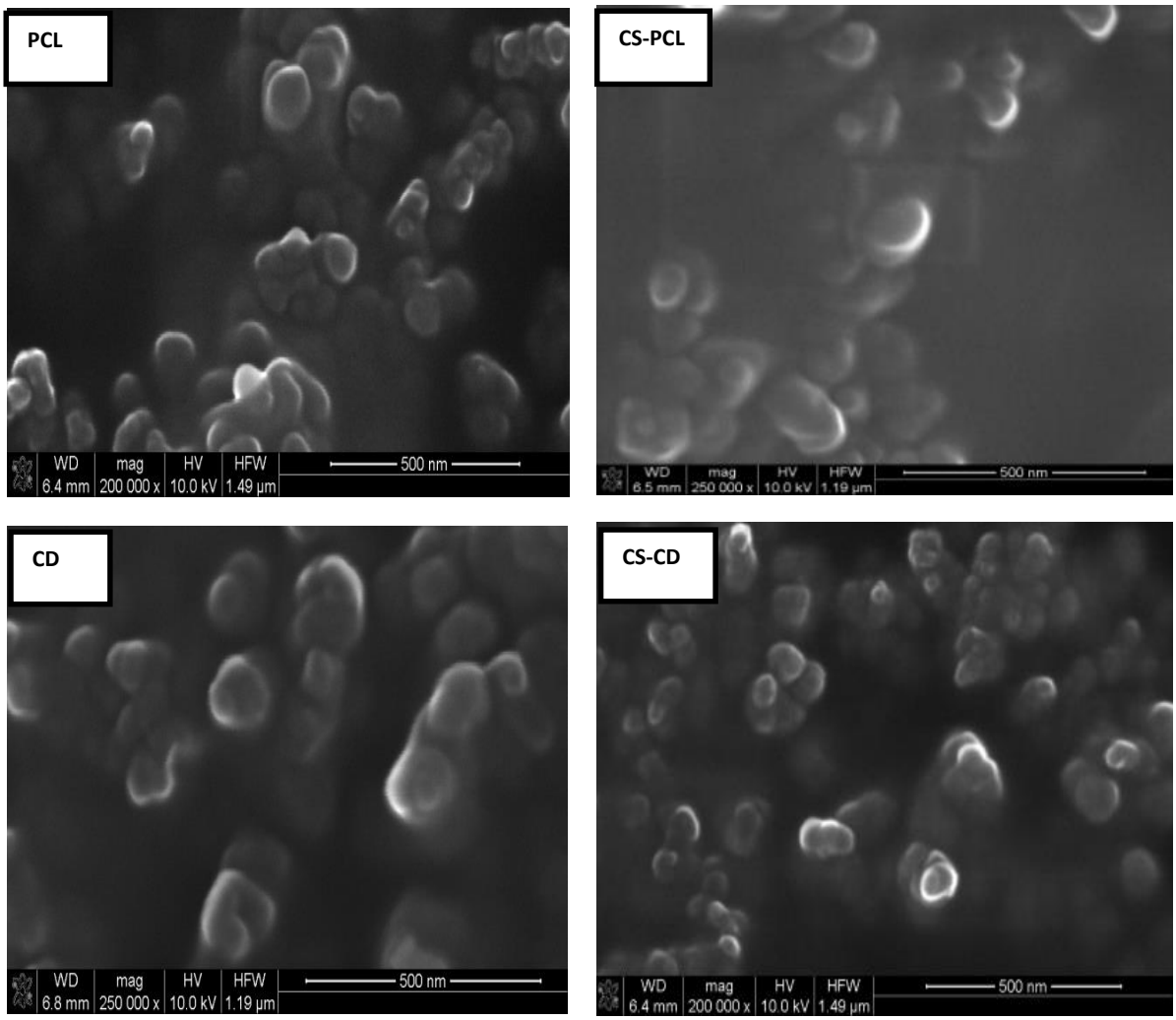


Figure 4.10. Scanning electron microphotographs of PCL, CS-PCL, CD and CS-CD nanocapsules.

4.4.4.Encapsulation Efficiency of Nanocapsules

The ability of nanocapsule formulations of different surface charge to entrap CPT was evaluated using Camptothecin (10% w/w of polymers) as a model drug. As it can be seen from the results in Table 4.19., chitosan-coated cationic nanocapsules have slightly higher association efficiency compared with that of corresponing anionic nanocapsules. It can be expected that coating nanocapsules with CS will not result in a significant increase in drug loading since the lipophilic drug CPT will be largely encapsulated in the oily core of the nanocapsules with a smaller quantity adsorbed on the nanocapsules surface.

CD nanocapsules have higher encapsulation efficiency compared with PCL nanocapsules. This may be attributed to the structure of the amphiphilic cyclodextrin 6OCAPRO. In fact PCL is a hydrophobic polymer and thereby has the ability to increase loading capacity of lipophilic drugs, however, amphiphilic cyclodextrins have a hydrophobic cavity and aliphatic chains which enable drugs to interact with both the CD cavity and the aliphatic chains as well.

Table 4.19. Associated drug (%) and Entrapment efficiency (%) of CPT loaded nanocapsules. Data represents the mean results±SD values of three different batches

Formulations	Associated Drug (%)	Entrapment Efficiency (%)
PCL	39 ± 2	40 ± 1
CS-PCL	46 ± 4	51± 2
CD	47 ± 3	68 ± 1
CS-CD	51 ± 3	73 ± 1

4.4.5. *In vitro* release studies of Camptothecin from Nanocapsules

In vitro release of CPT from both cationic and anionic PCL and CD nanocapsules was performed by dialysis in phosphate buffer saline (pH 7.4). Figure 4.11. shows the cumulative percentages of Camptothecin released from nanocapsules as a function of time. Release profiles indicate that in the first 2 hours approximately 25% of CPT was released from the CD nanocapsules and 30% of CPT from PCL

nanocapsules. The initial first release was possibly accounted for an amount of CPT adsorbed on the nanocapsules surface which may facilitate drug release from nanocapsules. Complete release of encapsulated drug was found within a period of 48 h with a slower rate. (Figure 4.12) This may be attributed to the high affinity of CPT to the oily core of nanocapsules and its slow diffusion into the aqueous media. These results suggest favorable release behavior for an effective local cancer treatment since nanocapsules may accumulate within the tumor tissue and release the drug in a sustained manner over time.

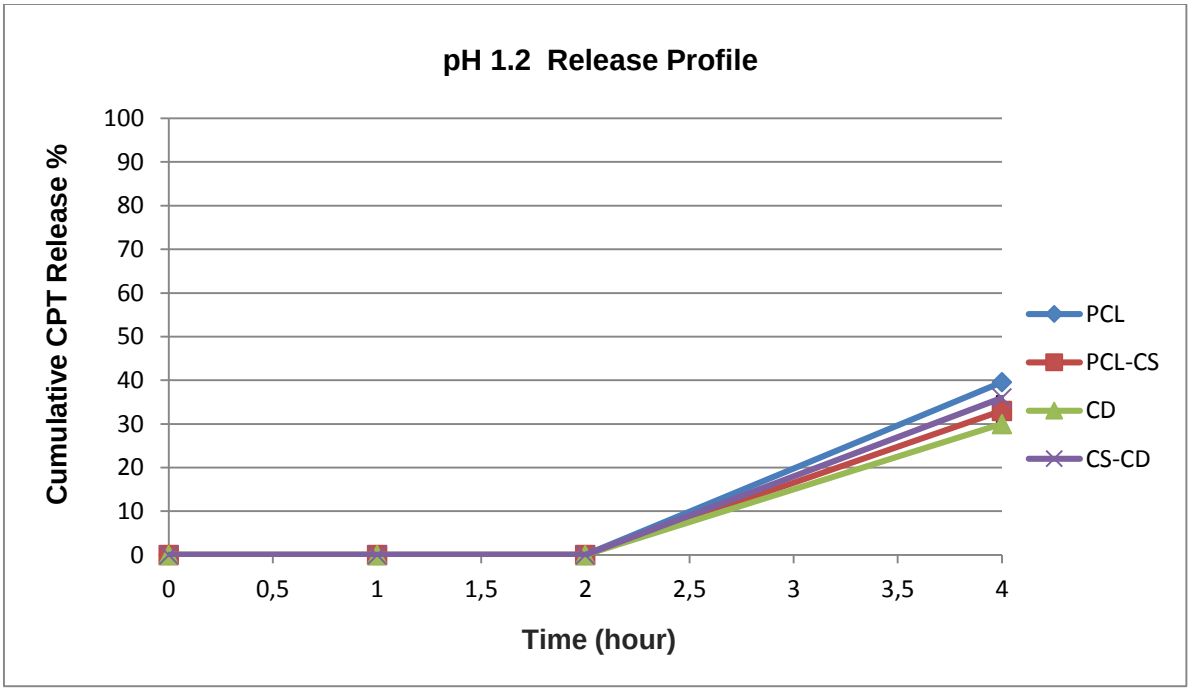


Figure 4.11. In vitro release profiles of CPT from anionic and cationic nanocapsules in pH 1.2 PBS for 4 h. Data represents the mean results ± SD values of three different batches.

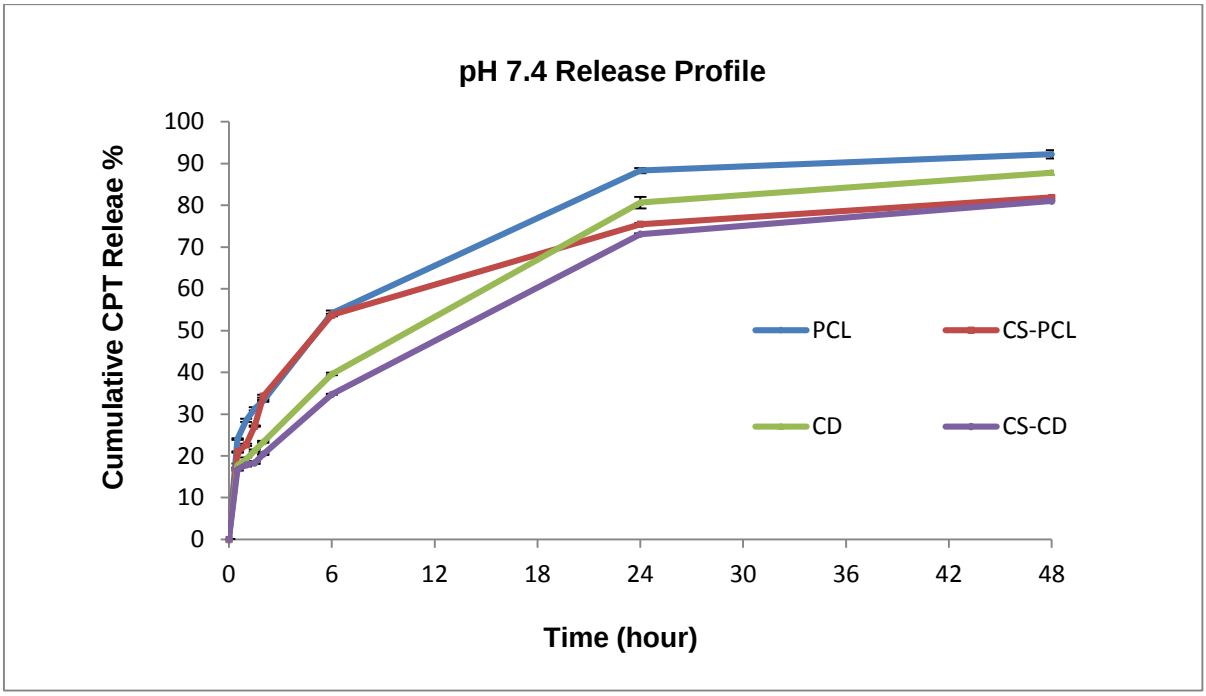


Figure 4.12. In vitro release profiles of CPT from anionic and cationic nanocapsules in pH 7.4 PBS for 48 h. Data represents the mean results ± SD values of three different batches.

In addition, anionic nanocapsules released CPT slightly faster than chitosan-coated cationic nanocapsules. These results might be due to the smaller particle size and lower drug contents of anionic nanoparticles compared with the chitosan-coated nanocapsules. Cationic nanocapsules have larger particle size and higher drug content [251, 252]. Thus drug release rate from nanocapsules could be slower than that of small size nanocapsules due to the increased surface area with reduced particle size in analogy to matrix-type nanoparticles [59].

4.4.6. Stability studies for CPT in various media

The failure of CPT in clinical trials is mostly attributed to its stability problem as described in section 2.3.1. Although CPT has a very potent inhibitory effect, antitumor efficacy has not been successfully used in cancer therapy due to lactone ring instability. It is reported that at physiological pH active lactone form rapidly hydrolyses into pharmacologically inactive carboxylate form, which leads to unpredictable and severe toxicity [103, 122]. The stable lactone form is not only critical for CPT's anticancer efficacy, but also it is responsible for the diffusibility through membranes since ring opening carboxylate form exhibits poor diffusibility than lactone form [253]. In addition, the carboxylate form binds to human serum albumin and hence inhibits its own cellular uptake [212].

In order to be able to reduce and/or prevent this hydrolysis of biologically active lactone form into carboxylate form and therefore maintain the stability of CPT, a stability-dependent analysis was evaluated by HPLC and results were calculated as percentage quantity of lactone and carboxylate forms at different pH values and as described in the Section 3.2.4.6

At pH 1.2, all the formulations (PCL, CS-PCL, CD, CS-CD nanocapsules and CPT solution) exhibited a lactone quantity of higher than 90% which can be attributed to the acidic pH conditions. Considering the lactone-carboxylate equilibrium these results are not suprising since at acidic pH, CPT exists in lactone form. (Table 4.20). As indicated above, at slightly basic conditions i.e pH 6.8, in case of CPT solution, active lactone form begins to hydrolyse into carboxylate form rapidly indicating that more than 60% of lactone form was turned into carboxylate form. However, it can be clearly seen from Table 4.20 that active lactone form of CPT when incorporated in nanocapsules, was preserved indicating a slight decrease less than 10% of its content during 48 h for all the formulations.

At physiological pH, the active lactone form of CPT in nanocapsules was preserved at least for 12 h. (Figure 4.13 to 4.16). In 24 h, approximately 30% of lactone form was hydrolyes into carboxylate form for anionic nanocapsules, whereas only 15% of lactone was turned into carboxylate form for cationic nanocapsules. Even after 48h, more than 60% of lactone form was preserved in the nanocapsules. On the other hand, by CPT solution, more than 75% of active lactone was already turned into the carboxylate form after 6 h indicating the rapid hydolysis. After 48 h, only 15% of CPT was found as lactone form in DMSO solution.

Table 4.20. CPT lactone form quantity (%) of different CPT formulations after 48h.

Formulations	**Lactone Form (%)**		
	pH 1.2	**pH 6.8**	**pH 7.4**
CPT in DMSO	96.2± 2	37.4±1	15.3±2
PCL	95.3±1	90.2±2	64.7±2
CS-PCL	98.1±2	92.4±2	80.6±3
CD	97.2±3	92.8±4	73.4±4
CS-CD	98.7±2	93.1±2	85.9±2

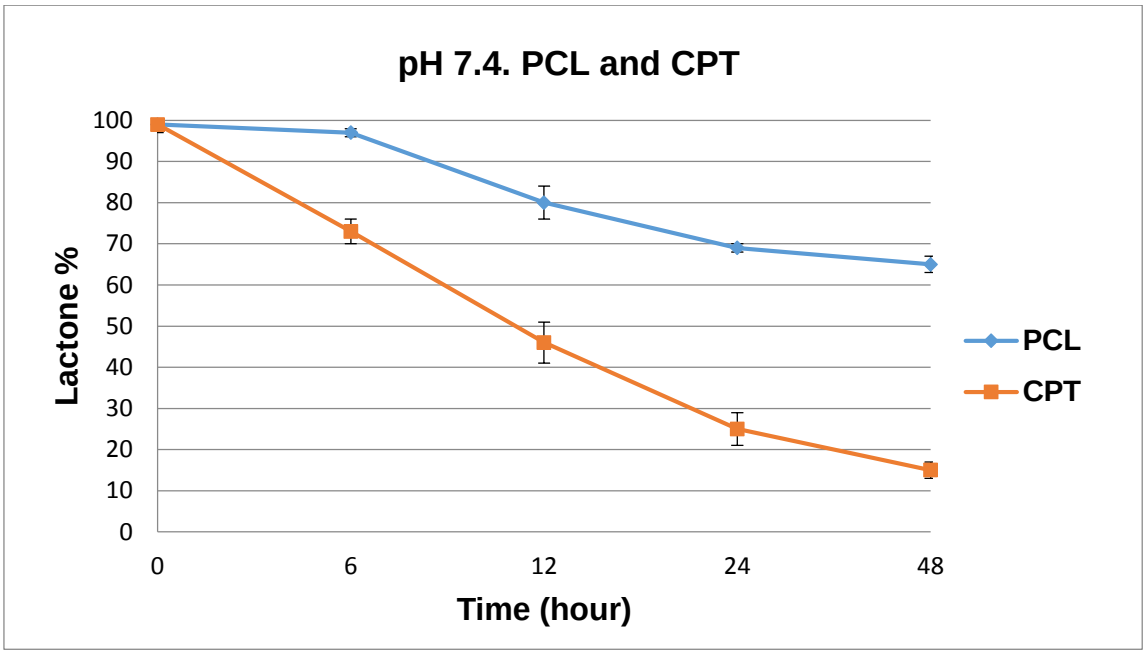


Figure 4.13. Lactone % of CPT both in PCL nanocapsules and in solution form at pH 7.4. Data represents the mean results ±SD (n=3)

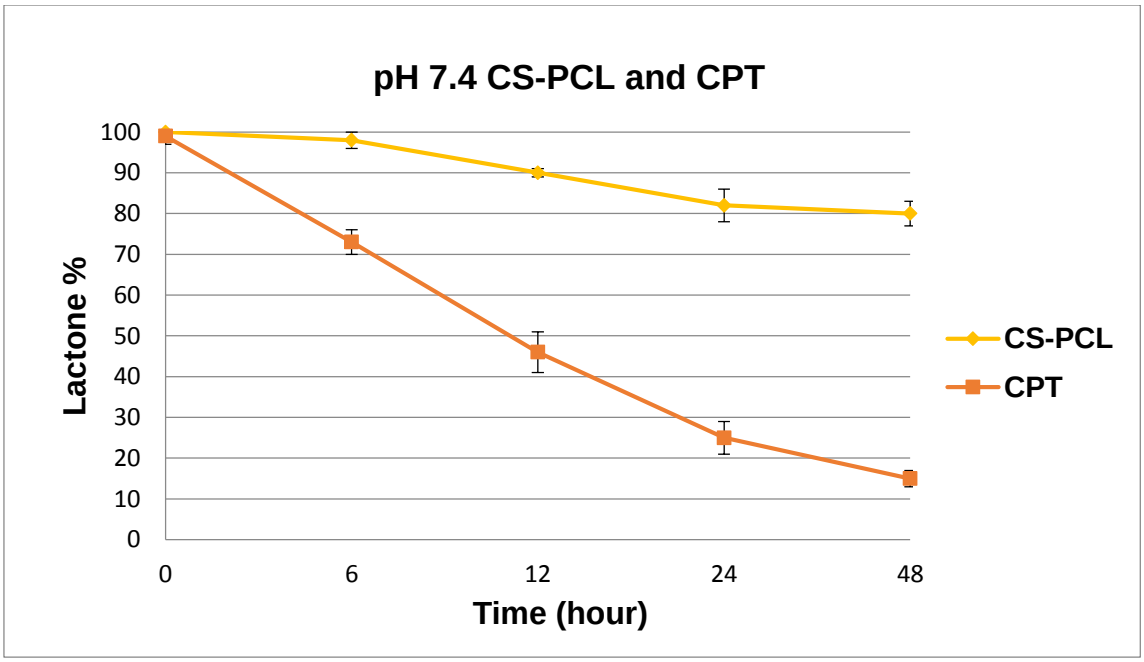


Figure 4.14. Lactone % of CPT both in CS-PCL nanocapsules and in solution form at pH 7.4. Data represents the mean results ±SD (n=3)

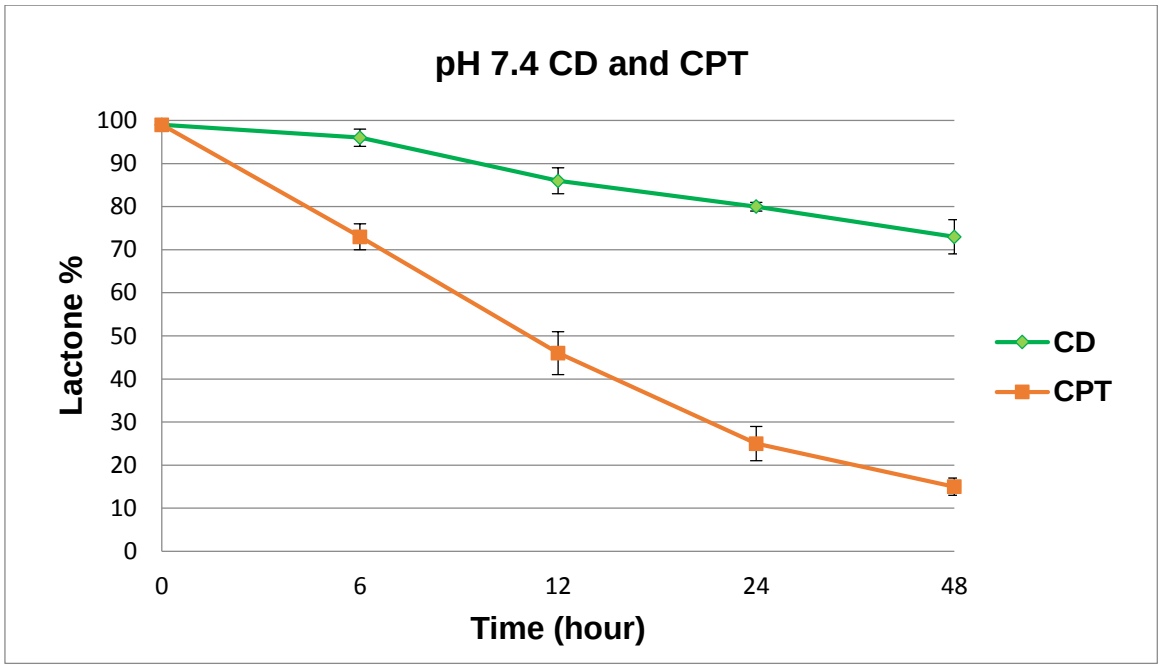


Figure 4.15. Lactone % of CPT both in CD nanocapsules and in solution form at pH 7.4. Data represents the mean results ±SD (n=3)

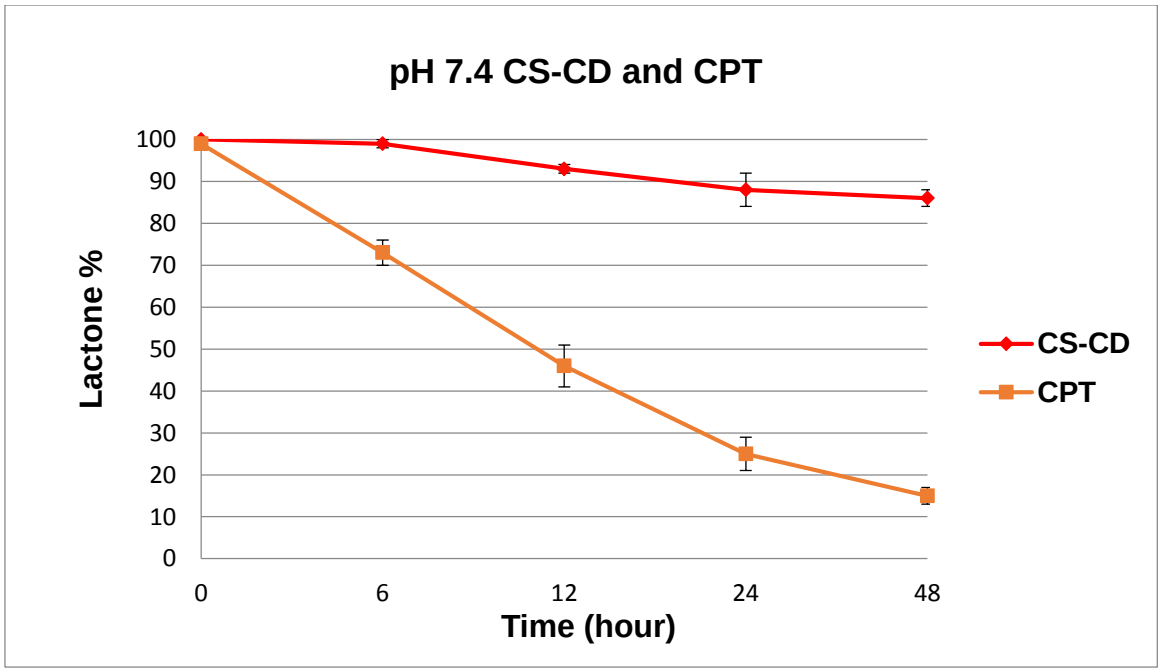


Figure 4.16. Lactone % of CPT in CS-CD nanocapsules and in solution form at pH 7.4. Data represents the mean results ±SD (n=3)

According to the results, it can be clearly seen that at physiological pH, the active lactone form of CPT was preserved owing to the incorporation of CPT into the nanocapsules. Considering the structure of nanocapsules, the polymeric wall which

provides a protection of active molecules against degradation and/or hydrolysis and the inner oily core which confines the drug molecules, improved together stability of lactone form. Similar results were observed by Lvov et al [122] and Çırpanlı et al. [254]. Encapsulation of drug molecule into nanocapsules enables the preservation of active lactone form in the inner core without degradation.

Taking into consideration that the decreasing of the cell membrane binding, membrane diffusibility, and ineffectiveness of anticancer potency related with the carboxylate form, these findings obtained from stability indicating HPLC analysis were found important since the rapid hydrolysis of active lactone form was reduced by means of nanocapsules.

4.4.7. Physical Stability of Nanocapsules in Simulated Gastrointestinal Fluids

Being able to maintain the stability of both the entrapped drug and drug-loaded nanocarriers is of primary importance for efficient oral drug delivery. Nanoparticles should remain stable in the GI tract while releasing the drug at the appropriate site and at appropriate rate [255]. Gastrointestinal tract acts as an obstacle for oral drug delivery due to its structure and chemical constituents. Extensive pH variation, drug metabolic enzymes in GI tract and liver, surfactants in GI tract (e.g bile salts) constitute biochemical barriers for an effective oral delivery. Since biological fluids influence the stability of particles, in vitro stability testing in gastric and intestinal fluids are a priority to investigate [1]. In order to check the GI stability of nanocapsules, formulations were incubated with simulated gastrointestinal media and the changes in mean particle size, polydispersity index and zeta potential values before and after incubation were measured. The effects of simulated gastric fluid SGF and simulated intestinal fluids on physical stability of CPT loaded nanocapsules are shown in Table 4.21.

Table 4.21. Stability of various CPT loaded nanocapsules in SGF; Simulated Gastric Fluid and SIF; Simulated Intestinal Fluid. Data represents the mean results ±SD (n=3)

	Simulated GI Media	Mean diameter (nm)		PDI		Zeta Potential (mV)	
		Initial	Final	Initial	Final	Initial	Final
PCL	SGF pH 1.2	208.6±2.1	214.4±2.2	0.04	0.14	-8.3±0.9	-2.6±0.2
	SIF pH 6.8	208.6±2.1	230.7±3.1	0.04	0.11	-8.3±0.9	-3.8±0.4
CS-PCL	SGF pH 1.2	220.4±1.3	239.3±3.2	0.21	0.14	+6.2±1.1	+14.6±1.3
	SIF pH 6.8	220.4±1.3	244.9±2.6	0.21	0.18	+6.2±1.1	+12.1±1.6
CD	SGF pH 1.2	187.3±2.4	189.4±2.4	0.09	0.12	-10.4±0.9	-5.9±1.1
	SIF pH 6.8	187.3±2.4	199.7±2.7	0.09	0.17	-10.4±0.9	-7.6±1.0
CS-CD	SGF pH 1.2	197.8±2.1	189.9±2.4	0.10	0.12	+17.1±2.1	+19.2±2.5
	SIF pH 6.8	197.8±2.1	203.7±1.9	0.10	0.11	+17.1±2.1	+18.4±2.6

According to the results it can be seen that both anionic and cationic nanocapsules were found to be stable in simulated gastric and simulated intestinal fluids. Table 4.21. also indicates that the slightest changes in size, PDI and zeta potential were observed with CS-CD. ($p>0.05$). Although, the pH variation in the GI tract makes it difficult to maintain particle integrity throughout the GI tract, nanocapsules hold this potential to provide particle stability and integrity as well against harsh environment. As seen from the results, by means of nanocapsules an immediate desorption of particles could be prevented in both gastrointestinal fluids, due to the polymeric wall of nanocapsules surrounding the encapsulated drug. This represents an important finding as nanocapsules must remain stable in gastrointestinal fluids until they reach absorption site [256]. Hence stability of CPT was provided by the protective effect of nanocapsules and physically stable nanoparticles would increase the consequential efficacy of CPT due to increased residence time in GI tract as well as better uptake and absorption of the drug [257].

4.4.8. Interaction with Mucus

Prolonged residence time in GI tract is one of important factors in oral drug delivery in order to maintain drug-loaded nanocarriers in full contact with the intestinal epithelial membrane and then allow drug absorption by the GI tissues. Most of the orally administered particles are not retained and are eliminated fastly through the GI tract [13]. Nanocarriers are expected to adhere to the mucus and to cross the mucus layer thereafter. Interaction between particle and mucin influences nanoparticle retention time at the mucosal surface in GI tract. In order to increase nanoparticle interactions with the intestinal mucosa, its surface can be suitably modified by adsorption or chemical grafting of hydrophilic (e.g. PEG) or mucoadhesive (e.g. chitosan) macromolecules [197]. Mucoadhesion has been commonly employed in the attempt to improve residence time of particles [19]. Mucoadhesive nanocarriers have been designed to present strong interaction with the mucus and thus to prolong residence time of the nanocarriers on the mucosal surface. Nanoparticle surface charge plays an important role in driving interaction with mucus since negatively charged intestinal mucosa attracts positively charged nanoparticles and the strong interactions with mucus could increase retention at mucosal surface. In this way nanoparticles decorated with cationic polymers like chitosan and its derivatives could increase residence time in the GI tract [258].

Turbidimetric Measurement:

Mucoadhesion studies were performed by measuring the turbidity at 650 nm of nanocapsule dispersions in the presence or not of mucin [214]. As shown in Figure 4.17 and 4.18 absorbance (ABS) of the reference mucin dispersions was very low (being within the range 0.010–0.015). For aqueous dispersions of all the nanocapsule formulations, at each time-point significantly higher values of ABS were observed due to light scattering. In the presence of chitosan-coated cationic nanoparticles, ABS significantly increased after nanoparticle addition to mucin (P<0.05). Results suggest that CS coated cationic nanocapsules have a higher tendency to interact with mucin as compared with anionic nanocapsules which can be attributed to chitosan coating [13]. Stability of CPT was provided by the protective effect of nanocapsules and physically stable nanoparticles would increase the consequential efficacy of CPT due to increased residence time in GI tract as well as better uptake and absorption of the drug [7].

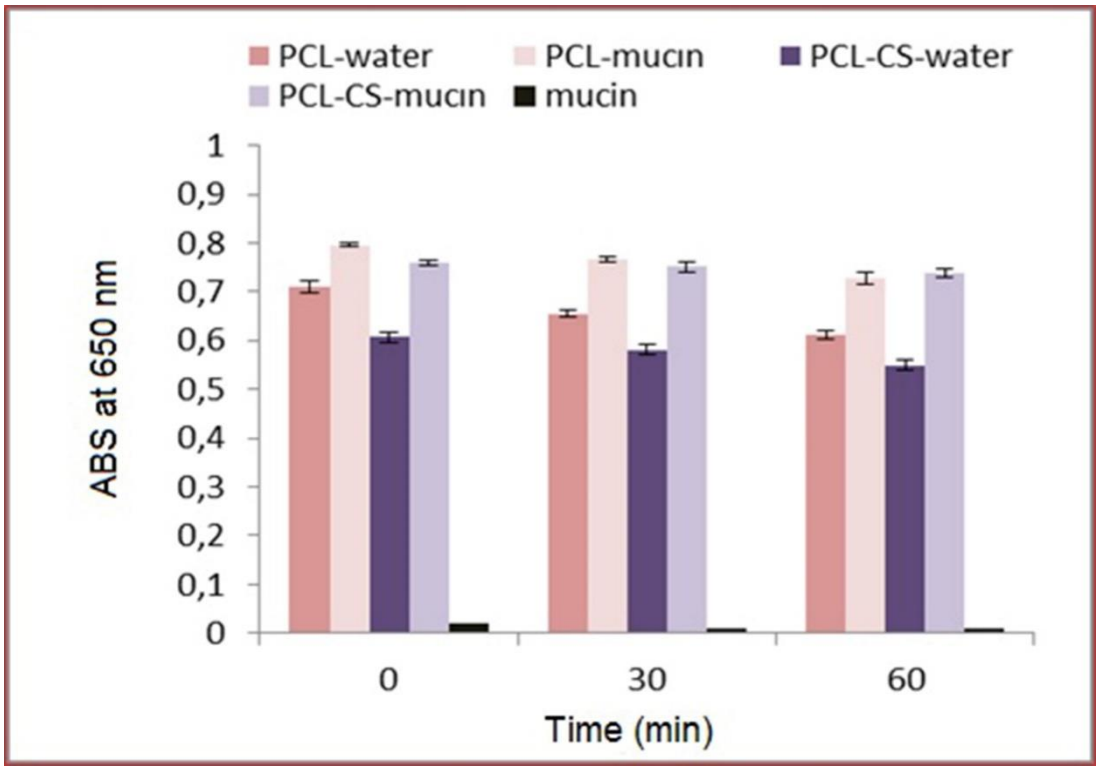


Figure 4.17. Absorbance (ABS) at 650 nm of PCL formulations either in water or in mucin dispersion as a function of time. Data represents the mean results ±SD (n=3)

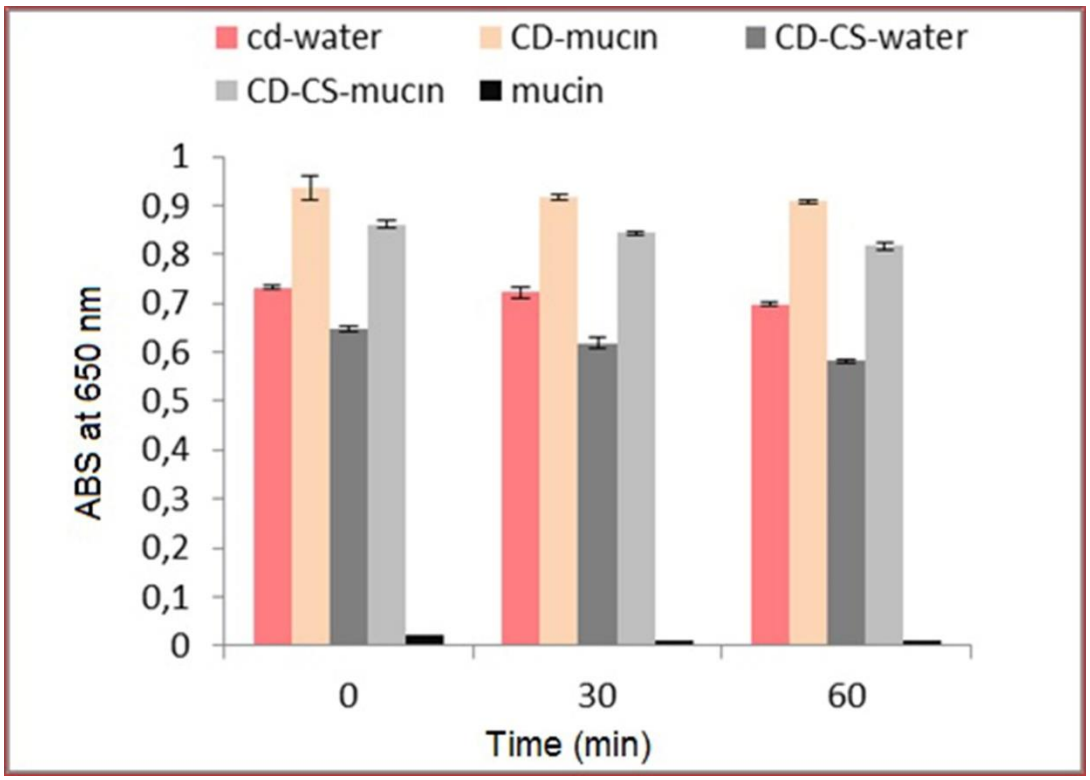


Figure 4.18. Absorbance (ABS) at 650 nm of CD formulations either in water or in mucin dispersion as a function of time. Data represents the mean results ±SD (n=3)

Characterization of Nanocapsules in Mucus: Particle size and Zeta Potential Measurement

The interaction between nanocapsules and mucin dispersion was then assessed by means of chacterization of particles in the presence of mucin in terms of mean particle size and, zeta potential values as described in the section 3.2.4.8.

Table 4.22. shows the zeta potential values of nanocapsules in mucin dispersion and mucin dispersion itself as reference. As seen from Table 4.22, mucin dispersion has a negative surface charge having a zeta potential value of -9.27 mV. The strongly positive zeta potential values of cationic PCL and CD nanocapsules (+20.9 mV and +11.5mV, respectively) was inverted to +14.6 mV and +7.9 mV, respectively by the addition of formulations into mucin dispersion. This significantly increase in the zeta potential values ($p<0.05$) of particles in the presence of mucin shows that there is an interaction between the cationic nanocapsules and negatively charged mucin. [214, 259]. On the other hand, for the anionic nanocapsules the negative zeta potential value did not significantly differ in the presence of mucin ($p>0.05$)

Table 4.22. Mean particle size, PDI and zeta potential values of formulations in the presence of mucin or not. Data represents the mean results ±SD (n=3)

Nanocapsule Formulations	Zeta Potential (mV) (nm)	Zeta Potential (mV) Mucin (+)
Mucin	-9,27 ± 1.23	-
PCL	-8,13 ± 1.34	-10,20 ± 1.29
CS-PCL	+20,9 ± 1.74	+14,62 ± 1.88
CD	-12,2 ± 1.09	-14,17 ± 1.56
CS-CD	+11,5 ± 1.87	+7,13 ± 1.67

The particle size determination of formulations in the presence of mucin was well correlated with zeta potential values indicating the mucin-particle interaction between the nanocapsules and musin dispersion. As seen in Table 4.23, mean particle size was changed after 60 min of incubation with mucin by all the formulations. On the other hand, the reduction in particle size was significant only with cationic nanocapsules ($p<0.05$). It was promising that no aggregation of nanocapsules were observed both in contact with SGF and SIF or mucin as other

researchers came across with uncoated nanoparticles [214]. Chitosan coating was beneficial in maintaining nanocapsule integrity. Slight reductin in size observed only for CS coated nanocapsules can be attributed to the minör degredation of CS dependent on deacetylation degree [260].

Table 4. 23. Mean particle size of nanocapsules in the presence of mucin or not. Data represent the mean results ±SD values of three different batches

Time(min)	0	30	60		0	30	60
Mean particle size (nm)					**PDI**		
PCL	205,7±2	204,4±1	204,9±3		0,082	0,085	0,093
M-PCL	202,1±1	200,3±2	197,1±2		0,076	0,083	0,081
CS-PCL	241,6±3	243,1±4	243,2±4		0,231	0,217	0,205
M-CS-PCL	232,2±3	228,3±2	224,7±3		0,169	0,147	0,123
CD	200,3±1	199,4±2	201,5±2		0,101	0,096	0,103
M-CD	194,3±2	192,5±4	188,9±1		0,092	0,089	0,940
CS-CD	224,1±4	221,3±2	223,5±3		0,201	0,193	0,197
M-CS-CD	208,3±2	201,4±3	193,4±2		0,167	0,159	0,164

4.4.9. Artificial Mucus Studies

Construction of Calibration Curve

CPT calibration curve with was constructed according to the method described in the Section 3.2.4.9 with spectrofluorophotometer.The calibaration curve is obtained by plotting the fluorescence intensity versus CPT concentration as seed in Figure 4.19. The slope from 6 different series were calculated along with intercept and correlation coefficient as it can be seen in Table 4.24.

Table 4.24. Linear regression data from spectroflorometric analysis for CPT

Parameter	Data
Concentration Range	0.005-0.02 µgl/mL
Slope	37285
Intercept	+ 6.2517
Correlation Coefficient	0.9993

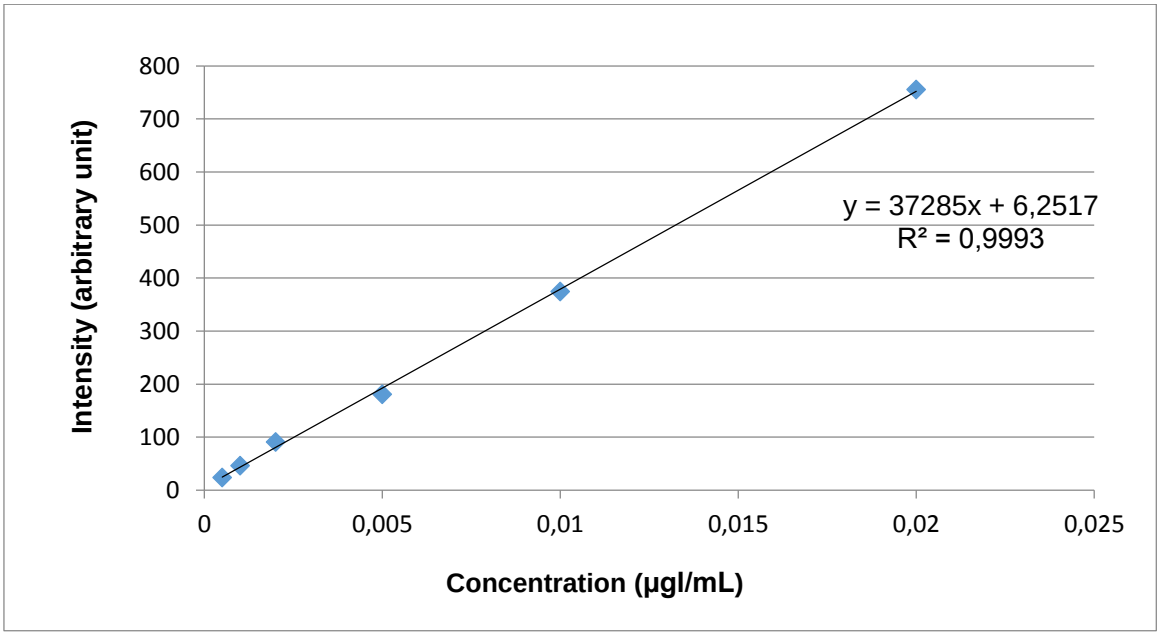


Figure 4.19. Calibration Curve and regression equation from Spectroflorometric assay for CPT.

The calibration curve shows that the method was linear in the range of 0.0051-0.02 µg/mL. The square correlation coefficient (R^2), which also proves the linearity, was found to be 0.9993 which is very close to 1 indicating absolute linearity.

Penetration through Artificial Mucus

When drugs or drug loaded nanoparticles reach the intestinal epithelial cells, they must penetrate through an aqueous mucus layer. Behaviour of nanoparticles in the GI tract is influenced by their mucoadhesive properties; adhesion of nanoparticles to the mucosa enhances absorption of the associated drug, thus increasing bioavailability [261]. However, mucoadhesive nanocarriers can also be trapped in the sticky mucus and then be swept away. Therefore, in addition to the importance of adhesiveness of nanocarriers, penetration capability through mucosa is a key factor [13].

In order to calculate amount of the CPT penetrated through the mucus, studies in a model of mucus covered epithelium were performed. The results obtained from the spectrofluorometric analysis led us to perform a quantitative analysis of the different nanocapsule formulations. As shown in Figure 4.20. penetration of CPT through artificial mucus was found to be significantly higher for CD nanocapsules than PCL nanocapsules (p<0.05) which may be attributed to cyclodextrin characteristics since

it is well known that cyclodextrins can enhance the permeation of poorly soluble drugs through biological membranes [262]. Indeed, nanocapsules are penetrating inside the mucus layer and are also releasing CPT. The overall result is that CS-coated nanocapsules give the higher amount of CPT able to reach the gelatin layer.

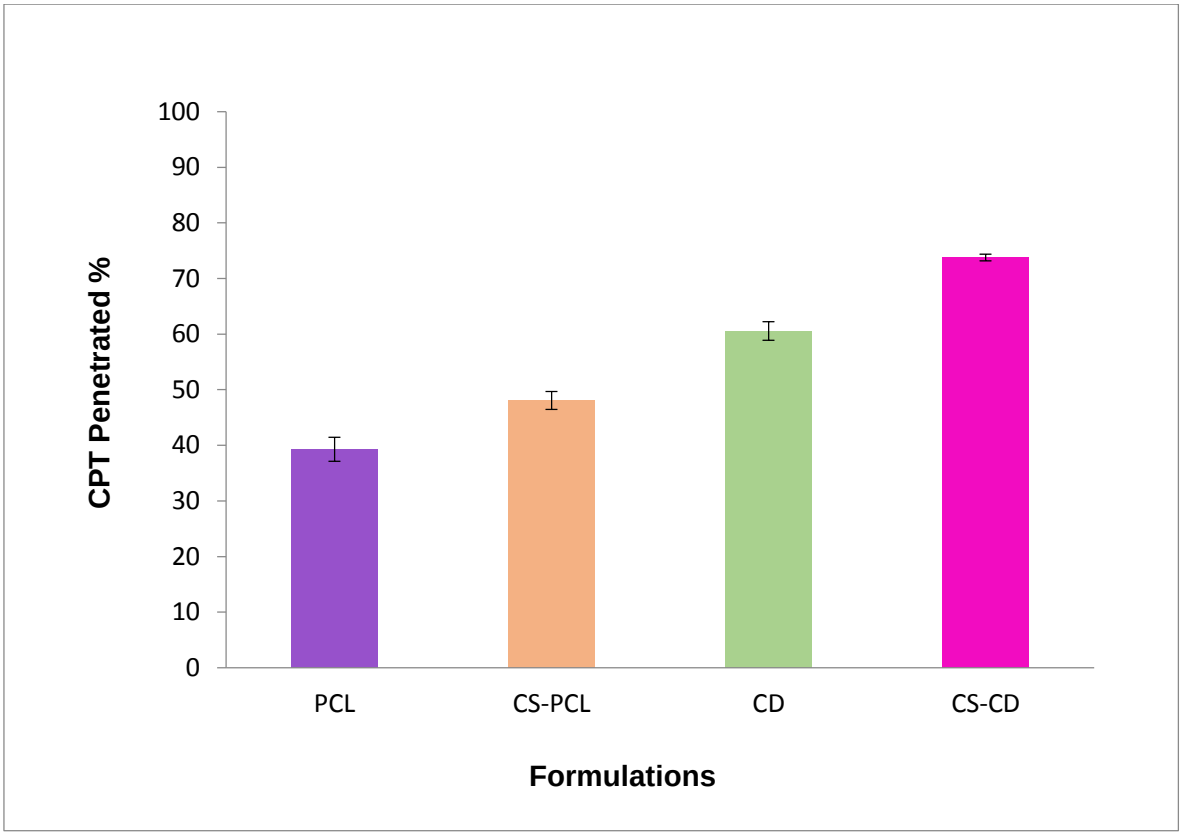


Figure 4.20. Amount of CPT penetrated through a mucus covered gelatin layer from different nanocapsule formulations after 24 h. Data represent the mean results ± SD values of three different batches.

A combination of surface charge and particle diameter plays the major role in affinity for penetration through membranes. Results with chitosan coated nanocapsules showed the highest fluorescent intensity, in agreement with the enhanced mucoadhesion properties reported for CS coated nanosystems [263]. The lower fluorescent response observed with the anionic PCL nanocapsules appeared to be related to the negative surface charge of these nanocapsules [264].

It is well known that surface chemistry of the particle has the influence on its transport through the mucus [265]. In order to penetrate mucus, nanoparticles must

be small enough to be able to diffuse through the mucus and mucoadhesive to prolong retention time and contact with mucosa. Furthermore, nanoparticles must avoid rapid mucus clearance and steric inhibition by the fibre mesh of mucin as well [266]. In conclusion, a balance between mucoadhesion and mucus penetration should be taken into account for an efficient oral delivery [27].

It has been reported that CD can be considered as ideal penetration enhancers since they can improve drug penetration through the membranes without affecting biological barriers. Biological membranes are lipophilic and only molecules with an optimal lipophilic/hydrophilic balance can penetrate through them. On the other hand, passive diffusion occurs with high drug concentration outside the cells generating the driving force for drug entry inside cells. Therefore CD acts as "penetration enhancer" by keeping the drug in the hydrophobic cavity and by increasing drug availability at the surface of the biological barrier resulting in the enhancement of drug permeation [262, 267].

4.5. Cell Culture Studies

In vivo studies were performed with CD and CS-CD nanocapsule formulations which gave the most promising results in in vitro studies.

4.5.1. Cytoxicity assay

L929 cell line is the recommended cell line by the USP to test cytotoxicity of polymeric systems [268] since it has the advantages of easy to culture and subculture in vitro, breed rapidly and easy to store, it was applied as cell lines of cytotoxicity evaluation for many materials [269]. Hence, L929 cell line was used in this study to investigate whether the toxicity of nanocapsules is associated with the polymer material itself or not. Cytotoxicity of blank anionic and cationic CD nanocapsules was evaluated against L929 cell line with MTT assay. Figure 4.21. shows the cell viability of L929 mouse fibroblast cells after 48 hours of incubation with unloaded CD nanocapsules at dilution rates 1:8, 1:16, 1:32, 1:64 and 1:128. According to the results, no significant difference was observed between anionic and cationic CD nanocapsules in terms of cell viability values for all the concentrations tested (p>0.05). It is also seen that toxicity of blank nanocapsules were concentration dependent and from the dilution rate 1:16 v/v (concentration is 3.125 µg/mL) on, blank nanocapsules were suggested to be safe. Furthermore,

considering the dilution nanoparticles undergo in the body, the toxicity profile is dose-dependent and considered safe in the light of studies that are designed in the same way in literature [254, 270].

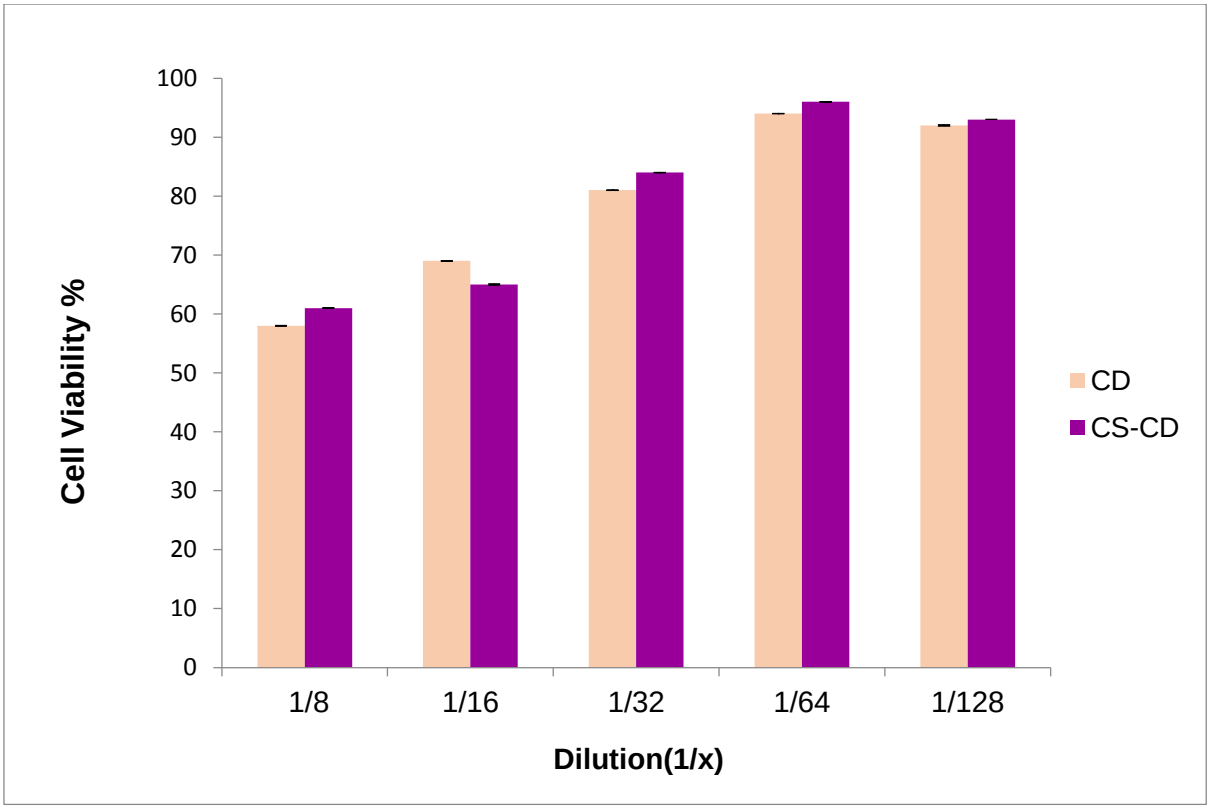


Figure 4.21. Cell viability of blank CD and CS-CD nanocapsules against L929 cell line after 48 h incubation. Data represents the mean results ±SD (n=3) (5 x10^3 cells/100 µL per well)

4.5.2. Anticancer Efficacy

Anticancer efficacy of CPT loaded anionic and cationic CD nanocapsules and free CPT was evaluated as described in section 3.2.5.2. Figure 4.22. shows the viability of MCF-7 cancer cells after 72 hours of incubation with the formulations diluted with DMSO at a dilution rate 1:16. Cytotoxic effect of both anionic and cationic nanocapsule formulations has investigated in comparison with CPT solution (CPT in DMSO) at a concentration of 3.125µg/mL. Cell viability is expressed as the ratio between the amount of formazan produced by cells treated with different formulations (CD and CS-CD nanocapsule dispersions) and non-treated (control) cells.

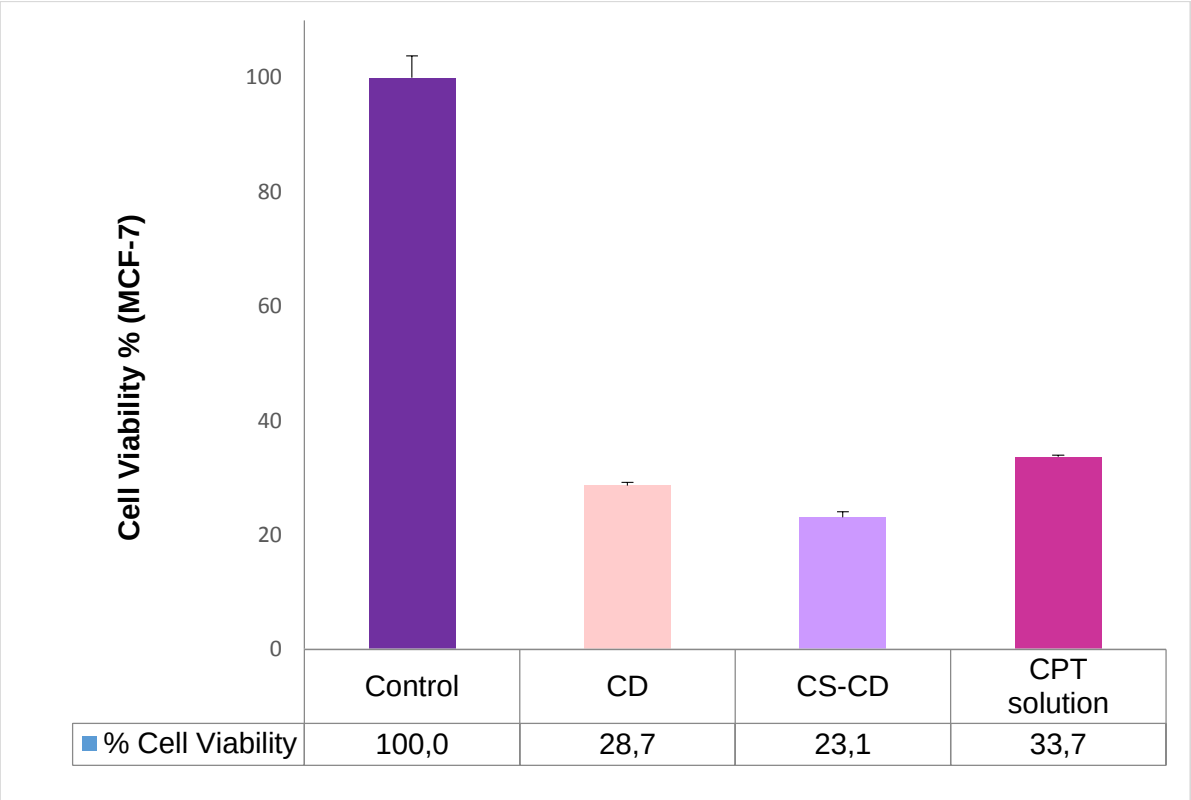


Figure 4.22. Viability of MCF-7 cells treated with CPT loaded CD and CD nanocapsules in comparison with CPT in solution form (DMSO). Data represents the mean results ±SD (n=3). (1x10^4 cells/100 µL per well)

Results indicate that both of the CD nanocarrier systems caused higher cancer cell death than CPT in DMSO resulting in a significant difference between the formulations and free CPT (p<0.05). Furthermore chitosan coated cationic nanocapsules showed significantly higher cytotoxic effect compared with the anionic nanocapsules (p<0.05). This increasing cytotoxic effect can be attributed to the surface charge of coating material, Chitosan [263]. CS coated cationic NCs exhibited a stronger affinity for the negatively charged cell membrane [271]. CS can increase the chance of cellular uptake of nanoparticles by improving the residence time at the cellular surface due to the electrostatic interaction between the cell membrane and nanoparticle since positively charged CS has the ability to interact with negatively charged surface membrane of cells via electrostatic forces [272].

4.5.3. Caco-2 cell monolayer permeability assay

In vitro transport studies were carried out accross the human adenocarcinoma Caco-2 cell line, which is a widely used model for intestinal drug absorption and widely employed to mimic GI barrier for oral chemotherapy [1]. Caco-2 cell line is a

valuable model for studying gastrointestinal drug absorption. When cultured as a monolayer, Caco-2 cells differentiate to form tight junctions between cells to serve as a model of paracellular movement of compounds across the monolayer. In addition, Caco-2 cells express transporter proteins, efflux proteins, and Phase II conjugation enzymes to model a variety of transcellular pathways as well as metabolic transformation of test substances. In many respects, the Caco-2 cell monolayer mimics the human intestinal epithelium [215]. An improved permeability might be a first step in predicting blood concentration of the drug with appropriate pharmaceutical effect [132] and rates of absorption (determined as apparent permeability coefficients) could be determined by means of Caco-2 cell assay. Hereby, the permeation of CPT both in solution form and in nanocapsule formulations across the Caco-2 cell monolayer is reported in Figure 4.23. as apparent permeability coefficient. Results indicate that in terms of permeability coefficients, there is a significant difference between the formulations and free CPT (CPT in DMSO) (p<0.05). Encapsulation of CPT in nanocapsules increased CPT transport 3 fold compared with free CPT.

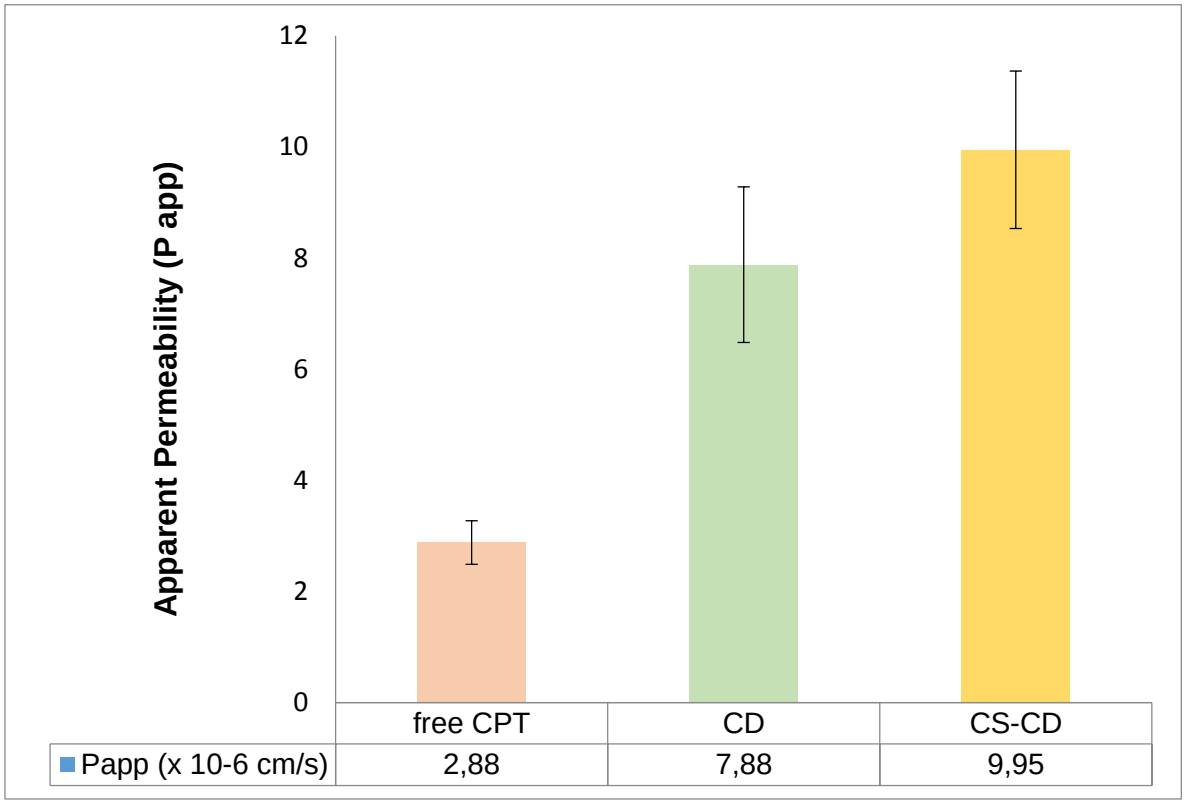


Figure 4.23. Apparent permeability coefficient (P_{aap}) of different CPT formulations: CPT in DMSO solution, CPT loaded CD and CS-CD nanocapsules. Data represents the mean results ±SD (n=3)

Even though particle size plays a critical role for the penetration through membranes, the reason for the lower permeability coefficient that free CPT displayed can be attributed to the lactone-carboxylate equilibrium of CPT. It is known that CPT exists in two forms depending on the different pH values. Active lactone form of CPT rapidly hydrolyses into inactive carboxylate form at neutral or physiological pH. As it was indicated in section 3.2.5.3. for the Caco-2 permeability assay, all the formulations were diluted with HBSS solution at pH 7.4. At this pH, it is known that active lactone form of free CPT was rapidly turned into inactive carboxylate form and it is reported that active lactone form is essential for the diffusion of the drug through membranes and inactive carboxylate form shows poor diffusibility when compared to lactone form [212]. On the other hand, nanocapsules have the ability to maintain the stability of CPT at this pH by reducing rapid hydrolysis and probability of the occurrence of carboxylate form as reported in the section 4.4.6. Hence encapsulation of CPT in nanocapsules results in an enhancement of the lactone form rate thereby improving permeability. In addition to this stability effect, CDs as absorption enhancers play also an important role in the improvement of CPT permeability when formulated with nanocapsules.

Although the differences between anionic and cationic formulations are not significant (p>0.05), CS coated cationic nanocapsules were able to better penetrate into cells than anionic nanocapsules probably due to higher cellular adhesion and increasing residence time at cell surface provided by CS molecules [273].This finding confirms the electrostatic interactions between positively charged CS amino groups and the negatively charged cell membrane. Therefore, the penetration of CS coated cationic CD NCs was greater than the anionic CD NCs. [274, 275].

4.6. In vivo studies

Intestinal permeability of potential new drugs is a primary concern as part of the drug development process and a variety of in vitro assays for determination of intestinal absorption of pharmaceutical compounds has been developed such as everted gut sacs, Caco-2 cell monolayer, intestinal mucosa/using chamber, isolated membrane vesicles. However, in vitro activity determined from in these in vitro assays do not guarantee in vivo efficacy. Therefore, in vivo studies were performed with an animal model on mice. In vivo studies were conducted with CD and CS-CD nanocapsule formulations which gave the most promising results in in vitro and cell culture

studies. In order to study cellular uptake of nanoparticles in vivo, the use of fluorescently or radioactively labeled nanoparticles is the most common experimental approach found in the literature [276]. For this reason Nile Red (NR) was used as fluorescent dye for in vivo uptake studies. NR is a hydrophobic, highly fluorescent dye that has been extensively applied for intracellular detection and quantification of particles in biological environment [277]. NR is highly soluble in the lipids indicating the facility of incorporation of dye into nanocapsules' oily core nanocapsules and it does not interact with any tissue constituent except by solution [278]. Therefore in this part of thesis, CD and CS-CD nanocapsules were formulated with NR for *in vivo* studies and NR loaded anionic and cationic CD nanocapsule formulations were evaluated in terms of in vitro characterization and intestinal uptake.

4.6.1. Construction of Calibration Curve for Nile Red

Nile Red calibration curve was constucted according to the method described in the Section 3.2.6.1. with spectrofluorophotometer. The calibration curve of fluorescence intensity versus its corresponding Nile red concentration was shown in Figure 4.24. The slope from 6 different series were calculated along with intercept and correlation coefficient as it can be seen in Table 4.25.

Table 4.25. Linear regression data obtained from Spectroflorophotomer analysis for Nile Red.

Parameter	Data
Concentration Range	0.005-0.05 µgl/mL
Slope	17571
Intercept	+ 19.508
Correlation Coefficient	0.9992

The calibration curve shows that the method was linear in the range of 0.005-0.05 µg/mL. The square correlation coefficient (R^2), which also proves the linearity, was found to be 0.9992 very close to 1 representing absolute linearity.

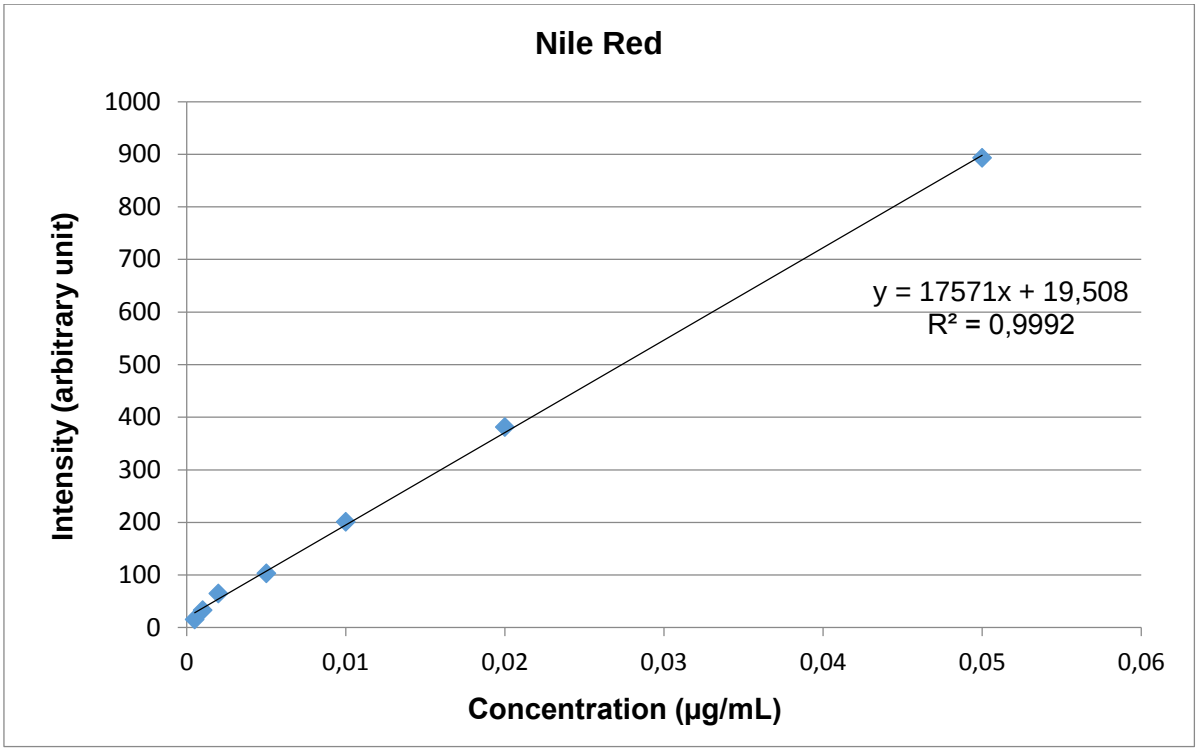


Figure 4.24. Calibration Curve and regression equation from Spectroflorometer analysis for Nile Red.

4.6.2. Preparation of Nile Red loaded Nanocapsules

To determine nanoparticle uptake by the GI tissues, fluorescent labelled (NR loaded) anionic and cationic CD nanocapsules were prepared and characterized in terms of mean particle size, PDI and zeta potential values as described in the section. The experimental NR loading was quantified using the florescence intensity of each nanocapsule formulation. Associated Drug (%) was then calculated according to the formula (1) and results were summarized in Table 4.26.

Table 4.26. Mean diameter, Polydispersity index (PDI), Zeta potential (mV) and Entrapment efficiency (%) of NR loaded nanocapsules. Data represents the mean results ± SD values of three different batches.

Formulations	Diameter (nm)	PDI	Zeta Potential (mV)	Associated Drug (%)
CD	190.6 ±5.2	0.05	-8.3 ±0.9	78.9± 2.3
CS-CD	197.8 ±3.4	0.09	+8.4 ±1.2	83.7 ±1.7

As presented in Table 4.26. both anionic and cationic NR loaded nanocapsules have a particle size below 200 nm which is suitable to obtain an effective intracellular uptake [279]. Both of the formulations showed a narrow size distribution indicating low PDI index values (<0.1) Surface charge of anionic and cationic nanocapsules were demonstrated as -8.34 mV and +8.48 mV respectively. Encapsulation efficiency of nanocapsules were quite high for both anionic and cationic formulations.

4.6.3. Recovery Experiment

The recovery experiment is performed to estimate proportional systematic error and is important in respect to determination of the accuracy of the method. [280]Recovery experiment enabled to calculate real amount of nanoparticle uptake in percentage accurately and in this thesis, it was carried out for the normalization of the results obtained by nanoparticle uptake studies. For this reason, prior to the in vivo particle uptake study, a recovery calculation was performed initially as described in the section 3.2.6.4 by calculating the NR content recovered from the GI tissues. Table 4.27. represents the results as percentage recovery (%) upon direct administration of formulations on different intestine tissues and stomach.

Table 4.27. Recovery (%) of NR upon direct administration on different intestine segments and stomach.

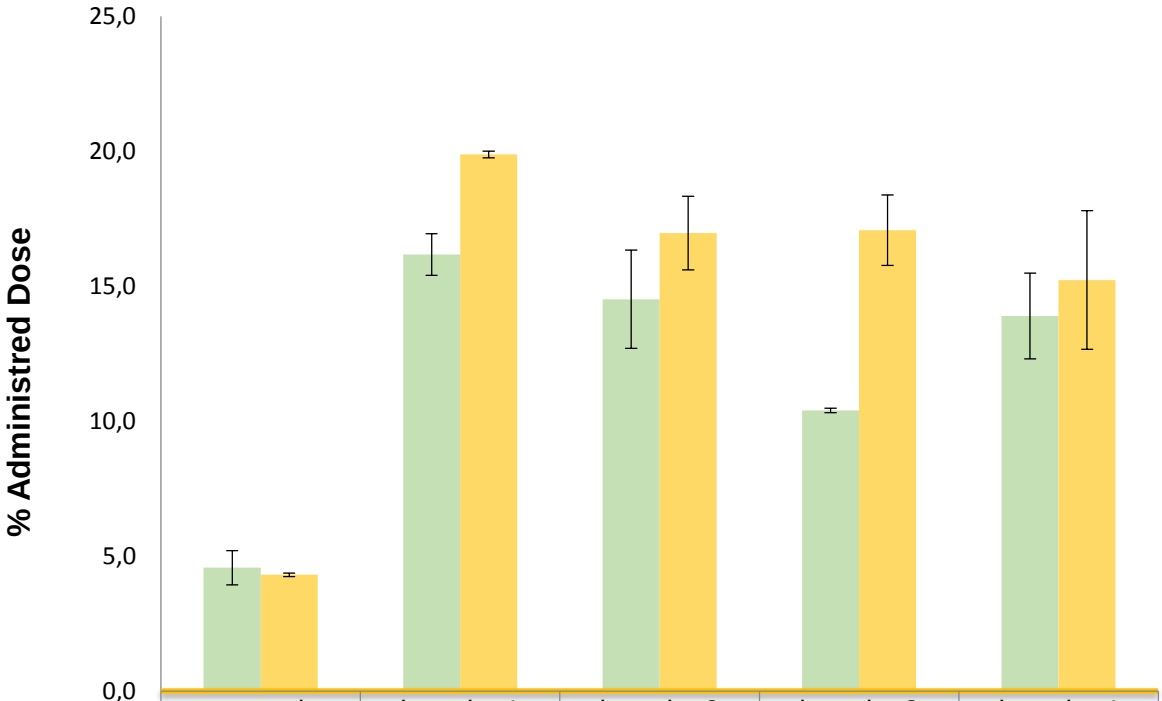
Formulations	Mean Recovery (%) Intestine	Mean Recovery (%) Stomach
CD	82.9 ±3.8	34.9±7.6
CS-CD	94.2±8.2	32.0±1.5

As it is shown in Table 4.27. average recovery from intestines was found 82.9% and 94.2% for anionic and cationic formulations respectively. On the other hand, from the stomach average recovery was found 34.9% and 32.0 % for anionic and cationic formulations respectively. It can be clearly seen that mean percentage recovery is quite high by intestine tissues whereas quite low by stomachs. This is quite understandable since intestine tissues play the major role in the absorption of particulates. Recovery of NR from intestines was significantly higher with cationic

nanocapsules ($p<0.05$). However in case of stomach, there is no significant difference between the formulations of anionic and cationic nanocapsules ($p>0.05$). In addition to the NR loaded nanocapsules, blank nanocapsules were also evaluated for the interfrence. According to the results blank nanocapsules exhibited no interference with NR loaded nanocapsules.

4.6.4. Nanoparticle Uptake Studies

In order to achieve an efficient absorption, nanoparticles must be taken up from the GI tract with a sufficient rate and extent. Anionic and cationic CD nanoparticle uptake by the GI tract region of mice was evaluated after oral gavage. Results are obtained after the normalization of data with the percentage recovery calculated as the section 3.2.6.5. Figure 4.25. shows the percentage uptake of anionic and cationic CD nanocapsules by stomach and intestine segments.



	stomach	intestine1	intestine2	intestine3	intestine4
CD	4,6	16,2	14,5	10,4	13,9
CS-CD	4,3	19,9	17,0	17,1	15,2

Figure 4.25. Percentage uptake of CD nanocapsules and CS-CD nanocapsules in mice stomach and intestine segments. Values are in mean ± SD (n=3).

From these data it is evident that most of the particles were detected in the intestine whereas only small amounts of the particles were observed in the stomach. Around

55% of CD nanocapsules and 69 % of CS coated CD nanocapsules were found in the intestines. On the contrary, only 4.6% of the administered dose of CD nanocapsules and 4.3% of CS-CD nanocapsules were taken up in stomach. A total of 59.6 % was detected for uncoated CD nanocapsule whereas increased to 73.6% by coating with CS and giving the NCs a positive charge. These results are promising since en effective oral delivery should allow the passage of drugs through the stomach in a protective structure and then release them later in the GI tract to allow their absorption. The results also indicate that intestinal uptake was significantly higher with CS coated cationic nanocapsules than anionic nanocapsules ($p<0.05$) which may be attributed to the coating material Chitosan, a known penetration enhancer and mucoadhesive polymer [252, 281-283].

Nanoparticle uptake is believed to be closely related to the following factors :

- ✓ the small size which allow them to be easily transported through barriers, the mucus in the mucosal epithelium by passive or active transport [284],
- ✓ structure of nanoparticles which improves penetration through and into cells, delivering drugs without suffering extracellular degradation [285],
- ✓ the bioadhesion of the nanoparticles which contributes to the increase in the residence time of the drug on the tissue, leading to an enhancement of the substance penetration [286],
- ✓ controlled release property provided by nanoparticles, which can prolong the release thereby increase the contact time of the drug with the mucosa. Therefore, the nanoencapsulation of a hydrophobic active ingredient, especially in positively charged nanocapsules, could increase the penetration through mucosa, improving the drug's efficacy. Moreover, an increase in penetration could also enable a reduction of dose. [285]

Briefly as for particle uptake by phagocytic cells, physicochemical properties of particles have been described to play a crucial role in their interaction with epithelial barriers. Size and surface charge being the more readily accessible parameters for characterizing particles, have been shown to be essential regarding their interaction with cells, since a smaller particle size is often correlated with a greater extent of uptake [287].

5. CONCLUSION

This thesis is focused on a multidisciplinary study of a novel drug delivery system to produce nanocapsules for pharmaceutical application. The system under investigation comprises PCL and CD as core polymers and CPT as a model drug.

Both PCL and CD were successfully used in nanocapsule production, main parameters were investigated and evaluated. Conclusions obtained in the light of data from this thesis are as follows:

1. All nanocapsule formulations were observed with appropriate mean particle size (<220nm) , PDI (<0.2) and zeta potential values.
2. Validated HPLC method for the assay of active and inactive forms of CPT was found to be linear, precise, accurate, specific with low limits of detection and quantification for CPT. This analytical method was proved to be valid for the quantitative determination of CPT in both lactone and carboxylate forms.
3. Encapculation efficiency of CPT were very high in both the systems. The encapsulation efficiency of CD nanocapsules were higher than PCL nanocapsules and this loading property could further be improved by coating with cationic polymer CS.
4. Drug release experiments highlighted a sustained release of CPT from nanocapsules within a period of 2 days, due to the great affinity of the lipophilic drug to the inner core. CPT was not released in gastric conditions but liberated in intestinal media.
5. Nanocapsule formulations could maintain their stability and integrity in simulated gastric and intestinal fluids during incubation time.
6. CD nanocapsules displayed significantly higher penetration than PCL nanocapsules through artifical mucus model and penetration was found higher with positively charged nanocapsules than negatively charged nanocapsules. ($p<0.05$)
7. Compared with CPT in solution form, all the formulations could maintain the active lactone of CPT in physiological conditions up to 48 h preventing hydrolyis of active lactone form into inactive carboxylate form. Therefore nanocapsules and the encapsulated drug were stable under GI conditions.

8. Turbidity studies showed that there is a strong interaction between positively charged nanocapsules and mucin. These results were found in accordance with characterization results of nanocapsules when incubated in mucus and further supported the time-dependent mucoadhesive characteristics of the nanocapsules.
9. Blank CD nanocapsules displayed no cytotoxic effect against L929 mouse fibroblast cells.
10. Anticancer efficacy of CPT bound to anionic or cationic CD nanocapsules were found to be significantly higher than CPT solution. ($p<0.05$)
11. In vitro transport studies across Caco-2 cell line showed that apparent permeability coefficient of anionic and cationic CD nanocapsules were significantly higher than CPT in solution form. ($p<0.05$). CS coating resulted in higher intestinal permeabiltiy.
12. In vivo studies on mice indicated that nanoparticle uptake by intestinal tissues were significantly higher with positively charged nanocapsules ($p<0.05$). Total GI uptake of active ingredient was increased from 59% to 73% by CS coating

The novelty of this work is that Camptothecin loaded amphiphilic CD and PCL nanocapsules were developed and evaluated for oral chemotherapy for the first time. The results obtained from this thesis suggest that CD nanocapsules offer a new strategy for the development of a safe and effective oral chemotherapy. The drug loading and cellular interaction of the CD nancapsules can easily be modulated by coating with a positively charged biocompatible material with penetration enhancer properties such as chitosan. Thus, CPT loaded CD nanocapsules can be effective in improving the oral bioavailability and decreasing the dosing frequency, thereby minimizing the dose dependent adverse effects and maximizing the patients' compliance.

Considering the advantages of oral chemotherapy, oral nanoparticulate drug delivery systems show such distinct advantages that they are able to bypass a series of biobarriers during oral absorption. Oral nanoparticulate delivery of anticancer agents has shown promising applications in simplifying the therapeutic procedures, prolonging survival time for patients and improving the cancer patient's life quality.

REFERENCES

[1] L. Plapied, N. Duhem, A. des Rieux, and V. Preat, "Fate of polymeric nanocarriers for oral drug delivery," *Current Opinion in Colloid & Interface Science,* vol. 16, pp. 228-237, Jun **2011.**

[2] D. B. K. P. S Kumar, Chiranjib, Margret. Chandira, K. K.Tripathi, "Innovations in Sustained Release Drug Delivery System and Its Market Opportunities," *Journal of Chemistry Pharmaceutical Research,* vol. 2, pp. 349-360, **2010.**

[3] http://www.espicom.com/advanced-oral-parenteral-drug-delivery-technologies.html (October, **2014**)

[4] G. Gaucher, P. Satturwar, M. C. Jones, A. Furtos, and J. C. Leroux, "Polymeric micelles for oral drug delivery," *European Journal of Pharmaceutics and Biopharmaceutics,* vol. 76, pp. 147-158, Oct **2010**.

[5] D. A. McCue, L. K. Lohr, and A. M. Pick, "Improving Adherence to Oral Cancer Therapy in Clinical Practice," *Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy,* vol. 34, pp. 481-494, **2014**.

[6] S. Mazzaferro, K. Bouchemal, and G. Ponchel, "Oral delivery of anticancer drugs I: general considerations," *Drug Discovery Today,* vol. 18, pp. 25-34, Jan **2013**.

[7] D. R. Kalaria, G. Sharma, V. Beniwal, and M. N. V. R. Kumar, "Design of Biodegradable Nanoparticles for Oral Delivery of Doxorubicin: In vivo Pharmacokinetics and Toxicity Studies in Rats," *Pharmaceutical Research,* vol. 26, pp. 492-501, Mar **2009**.

[8] F. Bassan, F. Peter, B. Houbre, M. J. Brennstuhl, M. Costantini, E. Speyer*, et al.*, "Adherence to oral antineoplastic agents by cancer patients: definition and literature review," *European Journal of Cancer Care,* vol. 23, pp. 22-35, Jan **2014**.

[9] M. Findlay, G. von Minckwitz, and A. Wardley, "Effective oral chemotherapy for breast cancer: pillars of strength," *Annals of Oncology,* vol. 19, pp. 212-22, Feb **2008**.

[10] L. Bromberg, "Polymeric micelles in oral chemotherapy," *Journal of Controlled Release,* vol. 128, pp. 99-112, Jun 4 **2008**.

[11] L. Fallowfield, L. Atkins, S. Catt, A. Cox, C. Coxon, C. Langridge*, et al.*, "Patients' preference for administration of endocrine treatments by injection or tablets: results from a study of women with breast cancer," *Annals of Oncology,* vol. 17, pp. 205-210, Feb **2006**.

[12] H. J. Cho, J. W. Park, I. S. Yoon, and D. D. Kim, "Surface-modified solid lipid nanoparticles for oral delivery of docetaxel: enhanced intestinal absorption and lymphatic uptake," *International Journal of Nanomedicine,* vol. 9, pp. 495-504, **2014**.

[13] C. Luo, J. Sun, Y. Q. Du, and Z. G. He, "Emerging integrated nanohybrid drug delivery systems to facilitate the intravenous-to-oral switch in cancer chemotherapy," *Journal of Controlled Release,* vol. 176, pp. 94-103, Feb 28 **2014**.

[14] K. Thanki, R. P. Gangwal, A. T. Sangamwar, and S. Jain, "Oral delivery of anticancer drugs: Challenges and opportunities," *Journal of Controlled Release,* vol. 170, pp. 15-40, Aug 28 **2013**.

[15] H. B. Chen, Y. Zheng, G. Tian, Y. Tian, X. W. Zeng, G. Liu*, et al.*, "Oral Delivery of DMAB-Modified Docetaxel-Loaded PLGA-TPGS Nanoparticles for Cancer Chemotherapy," *Nanoscale Research Letters,* vol. 6, **2011**.
[16] J. M. M. Terwogt, J. H. M. Schellens, W. W. T. Huinink, and J. H. Beijnen, "Clinical pharmacology of anticancer agents in relation to formulations and administration routes," *Cancer Treatment Reviews,* vol. 25, pp. 83-101, Apr **1999**.
[17] J. R. Turner, "Intestinal mucosal barrier function in health and disease," *Nature Reviews Immunology,* vol. 9, pp. 799-809, Nov **2009**.
[18] X. W. Zhang and W. Wu, "Ligand-mediated active targeting for enhanced oral absorption," *Drug Discovery Today,* vol. 19, pp. 898-904, Jul **2014**.
[19] L. M. Ensign, R. Cone, and J. Hanes, "Oral drug delivery with polymeric nanoparticles: The gastrointestinal mucus barriers," *Advanced Drug Delivery Reviews,* vol. 64, pp. 557-570, May 1 **2012**.
[20] Available: http://intranet.tdmu.edu.ua/data/kafedra/internal/klinpat/classes_stud/en/med/lik/ptn/Functional%20diagnostics_4%20course/4/02.%20The%20main%20methods%20of%20diagnostics%20of%20diseases%20in%20gastroenterology.htm (November, **2014**).
[21] R. A. Cone, "Barrier properties of mucus," *Advanced Drug Delivery Reviews,* vol. 61, pp. 75-85, Feb 27 **2009**.
[22] J. S. Crater and R. L. Carrier, "Barrier Properties of Gastrointestinal Mucus to Nanoparticle Transport," *Macromolecular Bioscience,* vol. 10, pp. 1473-1483, Dec 8 **2010**.
[23] L. M. Lichtenberger, "The Hydrophobic Barrier Properties of Gastrointestinal Mucus," *Annual Review of Physiology,* vol. 57, pp. 565-583, **1995**.
[24] G. Y. Chen and T. S. Stappenbeck, "Mucus, It Is Not Just a Static Barrier," *Science Signaling,* vol. 7, Apr 29 **2014**.
[25] Y. Cu and W. M. Saltzman, "Mathematical modeling of molecular diffusion through mucus," *Advanced Drug Delivery Reviews,* vol. 61, pp. 101-114, Feb 27 **2009.**
[26] M. Boegh, S. G. Baldursdottir, A. Mullertz, and H. M. Nielsen, "Property profiling of biosimilar mucus in a novel mucus-containing in vitro model for assessment of intestinal drug absorption," *European Journal of Pharmaceutics and Biopharmaceutics,* vol. 87, pp. 227-235, Jul **2014**.
[27] S. K. Lai, Y. Y. Wang, and J. Hanes, "Mucus-penetrating nanoparticles for drug and gene delivery to mucosal tissues," *Advanced Drug Delivery Reviews,* vol. 61, pp. 158-171, Feb 27 **2009**.
[28] S. Guven, C. Kart, E. S. Guvendag Guven, and G. S. Gunalp, "The underlying cause of cervical cancer in oral contraceptive users may be related to cervical mucus changes," *Medical Hypotheses,* vol. 69, pp. 550-2, **2007**.
[29] J. M. Gamboa and K. W. Leong, "In vitro and in vivo models for the study of oral delivery of nanoparticles," *Advanced Drug Delivery Reviews,* vol. 65, pp. 800-810, Jun 15 **2013**.
[30] R. J. Mrsny, "Oral drug delivery research in Europe," *Journal of Controlled Release,* vol. 161, pp. 247-53, Jul 20 2012.
[31] L. Mei, Z. P. Zhang, L. Y. Zhao, L. Q. Huang, X. L. Yang, J. T. Tang*, et al.*, "Pharmaceutical nanotechnology for oral delivery of anticancer drugs," *Advanced Drug Delivery Reviews,* vol. 65, pp. 880-890, Jun 15 **2013**.

[32] X. Q. Wang and Q. Zhang, "pH-sensitive polymeric nanoparticles to improve oral bioavailability of peptide/protein drugs and poorly water-soluble drugs," *European Journal of Pharmaceutics and Biopharmaceutics,* vol. 82, pp. 219-229, Oct **2012**.

[33] M. J. O'Neill, L. Bourre, S. Melgar, and C. M. O'Driscoll, "Intestinal delivery of non-viral gene therapeutics: physiological barriers and preclinical models," *Drug Discovery Today,* vol. 16, pp. 203-218, Mar **2011**.

[34] G. Branden, T. Sjogren, V. Schnecke, and Y. F. Xue, "Structure-based ligand design to overcome CYP inhibition in drug discovery projects," *Drug Discovery Today,* vol. 19, pp. 905-911, Jul **2014.**

[35] A. E. van Herwaarden, R. A. B. van Waterschoot, and A. H. Schinkel, "How important is intestinal cytochrome P450 3A metabolism?," *Trends in Pharmacological Sciences,* vol. 30, pp. 223-227, May **2009**.

[36] Y. N. Gavhane and A. V. Yadav, "Loss of orally administered drugs in GI tract," *Saudi Pharmaceutical Journal,* vol. 20, pp. 331-344, Oct **2012**.

[37] http://www.doctorfungus.org/thedrugs/antif_interaction.php (November, **2014**).

[38] T. Bansal, N. Akhtar, M. Jaggi, R. K. Khar, and S. Talegaonkar, "Novel formulation approaches for optimising delivery of anticancer drugs based on P-glycoprotein modulation," *Drug Discovery Today,* vol. 14, pp. 1067-74, Nov **2009**.

[39] D. F. Emerich and C. G. Thanos, "The pinpoint promise of nanoparticle-based drug delivery and molecular diagnosis," *Biomolecular Engineering,* vol. 23, pp. 171-184, Sep **2006.**

[40] http://www.nano.gov/nanotech-101/special (October, **2014**)

[41] S. Tinkle, S. E. McNeil, S. Muhlebach, R. Bawa, G. Borchard, Y. Barenholz*, et al.*, "Nanomedicines: addressing the scientific and regulatory gap," *Annals Reports,* vol. 1313, pp. 35-56, **2014.**

[42] http://www.nano.gov/nanotech-101/what/definition (November, **2014**)

[43] C. Moorthi, R. Manavalan, and K. Kathiresan, "Nanotherapeutics to Overcome Conventional Cancer Chemotherapy Limitations," *Journal of Pharmacy and Pharmaceutical Sciences,* vol. 14, pp. 67-77, **2011**.

[44] O. C. Farokhzad and R. Langer, "Nanomedicine: Developing smarter therapeutic and diagnostic modalities," *Advanced Drug Delivery Reviews,* vol. 58, pp. 1456-1459, Dec 1 **2006.**

[45] R. Gref, Y. Minamitake, M. T. Peracchia, V. Trubetskoy, V. Torchilin, and R. Langer, "Biodegradable Long-Circulating Polymeric Nanospheres," *Science,* vol. 263, pp. 1600-1603, Mar 18 **1994.**

[46] M. Bruchez, M. Moronne, P. Gin, S. Weiss, and A. P. Alivisatos, "Semiconductor nanocrystals as fluorescent biological labels," *Science,* vol. 281, pp. 2013-2016, Sep 25 **1998**.

[47] T. N. L. Lai, A. Ting, T. Yang and S. Bai, "Nanomedicine: Economic Prospect and Public Safety," *Journal of Developing Drugs,* vol. 2, pp. 1-2, **2013**.

[48] S. Parveen, R. Misra, and S. K. Sahoo, "Nanoparticles: a boon to drug delivery, therapeutics, diagnostics and imaging," *Nanomedicine-Nanotechnology Biology and Medicine,* vol. 8, pp. 147-166, Feb **2012**.

[49] S. K. Sahoo and V. Labhasetwar, "Nanotech approaches to delivery and imaging drug," *Drug Discovery Today,* vol. 8, pp. 1112-1120, Dec 15 **2003**.

[50] N. Sanvicens and M. P. Marco, "Multifunctional nanoparticles--properties and prospects for their use in human medicine," *Trends Biotechnology,* vol. 26, pp. 425-33, Aug **2008**.
[51] http://www.uspharmacist.com/content/s/197/c/33020/ (October **2014**)
[52] V. M. a. Y. Chen, "Nanoparticles – A Review," *Tropical Journal of Pharmaceutical Research,* vol. 5, pp. 561-573, June **2006**.
[53] V. Sanna, N. Pala, and M. Sechi, "Targeted therapy using nanotechnology: focus on cancer," *International Journal of Nanomedicine,* vol. 9, pp. 467-483, **2014.**
[54] P. Decuzzi, B. Schrefler, and W. K. Liu, "Nanomedicine," *Computational Mechanics,* vol. 53, pp. 401-402, Mar **2014.**
[55] K. S. Soppimath, T. M. Aminabhavi, A. R. Kulkarni, and W. E. Rudzinski, "Biodegradable polymeric nanoparticles as drug delivery devices," *Journal of Controlled Release,* vol. 70, pp. 1-20, Jan 29 **2001.**
[56] P. Couvreur, "Nanoparticles in drug delivery: Past, present and future," *Advanced Drug Delivery Reviews,* vol. 65, pp. 21-23, Jan **2013.**
[57] S. S. Guterres, M. P. Alves, and A. R. Pohlmann, "Polymeric nanoparticles, nanospheres and nanocapsules, for cutaneous applications," *Drug Target Insights,* vol. 2, pp. 147-57, **2007.**
[58] K. Letchford and H. Burt, "A review of the formation and classification of amphiphilic block copolymer nanoparticulate structures: micelles, nanospheres, nanocapsules and polymersomes," *European Journal of Pharmaceutics and Biopharmaceutics,* vol. 65, pp. 259-269, Mar **2007.**
[59] C. E. Mora-Huertas, H. Fessi, and A. Elaissari, "Polymer-based nanocapsules for drug delivery," *International Journal of Pharmaceutics,* vol. 385, pp. 113-142, Jan 29 **2010.**
[60] C. Mayer, "Nanocapsules as drug delivery systems," *International Journal of Artificial Organs,* vol. 28, pp. 1163-1171, Nov **2005**.
[61] S. Khoee and M. Yaghoobian, "An investigation into the role of surfactants in controlling particle size of polymeric nanocapsules containing penicillin-G in double emulsion," *European Journal of Medicinal Chemistry,* vol. 44, pp. 2392-2399, Jun **2009**.
[62] C. E. Mora-Huertas, H. Fessi, and A. Elaissari, "Influence of process and formulation parameters on the formation of submicron particles by solvent displacement and emulsification-diffusion methods critical comparison," *Advanced Colloid Interface Science,* vol. 163, pp. 90-122, Apr 14 **2011.**
[63] E. Cauchetier, M. Deniau, H. Fessi, A. Astier, and M. Paul, "Atovaquone-loaded nanocapsules: influence of the nature of the polymer on their in vitro characteristics," *International Journal of Pharmaceutics,* vol. 250, pp. 273-281, Jan 2 **2003**.
[64] P. Legrand, S. Lesieur, A. Bochot, R. Gref, W. Raatjes, G. Barratt*, et al.*, "Influence of polymer behaviour in organic solution on the production of polylactide nanoparticles by nanoprecipitation," *International Journal of Pharmaceutics,* vol. 344, pp. 33-43, Nov 1 **2007.**
[65] M. R. Kulterer, M. Reischl, V. E. Reichel, S. Hribernik, M. Wu, S. Kostler*, et al.*, "Nanoprecipitation of cellulose acetate using solvent/nonsolvent mixtures as dispersive media," *Colloids and Surfaces a-Physicochemical and Engineering Aspects,* vol. 375, pp. 23-29, Feb 5 **2011.**
[66] D. Quintanar-Guerrero, E. Allemann, E. Doelker, and H. Fessi, "Preparation and characterization of nanocapsules from preformed polymers by a new

process based on emulsification-diffusion technique," *Pharmaceutical Research,* vol. 15, pp. 1056-62, Jul **1998.**

[67] D. Moinard-Checot, Y. Chevalier, S. Briancon, L. Beney, and H. Fessi, "Mechanism of nanocapsules formation by the emulsion-diffusion process," *Journal of Colloid Interface Science,* vol. 317, pp. 458-68, Jan 15 **2008.**

[68] C. E. Mora-Huertas, O. Garrigues, H. Fessi, and A. Elaissari, "Nanocapsules prepared via nanoprecipitation and emulsification-diffusion methods: comparative study," *European Journal of Pharmaceutics and Biopharmacutics,* vol. 80, pp. 235-9, Jan **2012**.

[69] U. Bilati, E. Allemann, and E. Doelker, "Sonication parameters for the preparation of biodegradable nanocapsules of controlled size by the double emulsion method," *Pharmaceutical Development and Technology,* vol. 8, pp. 1-9, **2003**.

[70] H. Ai and J. Gao, "Size-controlled polyelectrolyte nanocapsules via layer-by-layer self-assembly," *Journal of Materials Science,* vol. 39, pp. 1429-1432, Feb 15 **2004.**

[71] A. Agarwal, Y. Lvov, R. Sawant, and V. Torchilin, "Stable nanocolloids of poorly soluble drugs with high drug content prepared using the combination of sonication and layer-by-layer technology," *Journal of Controlled Release,* vol. 128, pp. 255-60, Jun 24 **2008.**

[72] E. J. Cho, H. Holback, K. C. Liu, S. A. Abouelmagd, J. Park, and Y. Yeo, "Nanoparticle Characterization: State of the Art, Challenges, and Emerging Technologies," *Molecular Pharmacology,* vol. 10, pp. 2093-2110, Jun **2013**.

[73] R. Singh and J. W. Lillard, "Nanoparticle-based targeted drug delivery," *Experimental and Molecular Pathology,* vol. 86, pp. 215-223, Jun **2009**.

[74] X. Y. Lu, D. C. Wu, Z. J. Li, and G. Q. Chen, "Polymer nanoparticles," *Progress in Molecular Biology and Translational Science,* vol. 104, pp. 299-323, **2011.**

[75] E. J. Cho, H. Holback, K. C. Liu, S. A. Abouelmagd, J. Park, and Y. Yeo, "Nanoparticle characterization: state of the art, challenges, and emerging technologies," *Molecular Pharmacology,* vol. 10, pp. 2093-110, Jun 3 **2013**.

[76] "Overcoming poor oral bioavailability using nanoparticle formulations - opportunities and limitations," *Drug Discovery Today Technology,* vol. 9, pp. e71-e174, Summer **2012.**

[77] C. N. Grama, D. D. Ankola, and M. N. V. R. Kumar, "Poly(lactide-co-glycolide) nanoparticles for peroral delivery of bioactives," *Current Opinion in Colloid & Interface Science,* vol. 16, pp. 238-245, Jun **2011.**

[78] A. M. AS. Chahal, SJ Kshirsagar, MR Bhalekar, A. Dikpati, and P. Gawli, "Amorphous Nanoparticles for Solubility Enhancement," *Journal of Advanced Pharmaceutical Sciences,* vol. 2, pp. 167-178, **2012.**

[79] H. Gao, Y. Wang, C. Chen, J. Chen, Y. Wei, S. Cao*, et al.*, "Incorporation of lapatinib into core-shell nanoparticles improves both the solubility and anti-glioma effects of the drug," *Int J Pharm,* vol. 461, pp. 478-88, Jan 30 **2014.**

[80] H. K. Chan and P. C. Kwok, "Production methods for nanodrug particles using the bottom-up approach," *Advanced Drug Delivery Reviews,* vol. 63, pp. 406-16, May 30 **2011**.

[81] E. Merisko-Liversidge and G. G. Liversidge, "Nanosizing for oral and parenteral drug delivery: a perspective on formulating poorly-water soluble compounds using wet media milling technology," *Advanced Drug Delivery Reviews,* vol. 63, pp. 427-40, May 30 **2011.**

[82] Y. Liu, C. Sun, Y. Hao, T. Jiang, L. Zheng, and S. Wang, "Mechanism of dissolution enhancement and bioavailability of poorly water soluble celecoxib by preparing stable amorphous nanoparticles," *J Pharm Pharm Sci,* vol. 13, pp. 589-606, **2010.**

[83] W. Qu, Y. Li, L. Hovgaard, S. Li, W. Dai, J. Wang*, et al.*, "A silica-based pH-sensitive nanomatrix system improves the oral absorption and efficacy of incretin hormone glucagon-like peptide-1," *International Journal of Nanomedicine,* vol. 7, pp. 4983-94, **2012.**

[84] S. Hao, B. Wang, Y. Wang, L. Zhu, B. Wang, and T. Guo, "Preparation of Eudragit L 100-55 enteric nanoparticles by a novel emulsion diffusion method," *Colloids Surface Biointerfaces,* vol. 108, pp. 127-33, Aug 1 **2013**.

[85] J. S. L. Kou, Y. Zhai, Z. He "The endocytosis and intracellular fate of nanomedicines: Implication for rational design," *Asian Journal of Pharmaceutical Sciences* vol. 8, pp. 1-10, **2013.**

[86] I. J. Hidalgo, "Assessing the absorption of new pharmaceuticals," *Curr Top Med Chem,* vol. 1, pp. 385-401, Nov **2001**.

[87] M. R. Rekha and C. P. Sharma, "Oral delivery of therapeutic protein/peptide for diabetes--future perspectives," *Int J Pharm,* vol. 440, pp. 48-62, Jan 2 **2013**.

[88] S. Barua and S. Mitragotri, "Challenges associated with Penetration of Nanoparticles across Cell and Tissue Barriers: A Review of Current Status and Future Prospects," *Nano Today,* vol. 9, pp. 223-243, Apr 1 **2014**.

[89] J. das Neves, M. F. Bahia, M. M. Amiji, and B. Sarmento, "Mucoadhesive nanomedicines: characterization and modulation of mucoadhesion at the nanoscale," *Expert Opinion on Drug Delivery,* vol. 8, pp. 1085-1104, Aug **2011.**

[90] L. Jiang, X. Li, L. Liu, and Q. Zhang, "Thiolated chitosan-modified PLA-PCL-TPGS nanoparticles for oral chemotherapy of lung cancer," *Nanoscale Res Lett,* vol. 8, p. 66, **2013.**

[91] X. Zhang, M. Sun, A. Zheng, D. Cao, Y. Bi, and J. Sun, "Preparation and characterization of insulin-loaded bioadhesive PLGA nanoparticles for oral administration," *European Journal of Pharmaceutics,* vol. 45, pp. 632-8, Apr 11 **2012.**

[92] Y. Yun, Y. W. Cho, and K. Park, "Nanoparticles for oral delivery: Targeted nanoparticles with peptidic ligands for oral protein delivery," *Advanced Drug Delivery Reviews,* vol. 65, pp. 822-832, Jun 15 **2013.**

[93] A. Fasano, "Novel approaches for oral delivery of macromolecules," *Journal of Pharmaceutical Sciences,* vol. 87, pp. 1351-1356, Nov **1998**.

[94] F. L. Mi, Y. Y. Wu, Y. H. Lin, K. Sonaje, Y. C. Ho, C. T. Chen*, et al.*, "Oral delivery of peptide drugs using nanoparticles self-assembled by poly(gamma-glutamic acid) and a chitosan derivative functionalized by trimethylation," *Bioconjugate Chemistry,* vol. 19, pp. 1248-1255, Jun **2008**.

[95] X. Wang, Y. Chen, F. Z. Dahmani, L. Yin, J. Zhou, and J. Yao, "Amphiphilic carboxymethyl chitosan-quercetin conjugate with P-gp inhibitory properties for oral delivery of paclitaxel," *Biomaterials,* vol. 35, pp. 7654-65, Aug **2014.**

[96] E. Roger, F. Lagarce, and J. P. Benoit, "The gastrointestinal stability of lipid nanocapsules," *International Journal of Pharmaceutics,* vol. 379, pp. 260-265, Sep 11 **2009.**

[97] L. Ma, Y. Wei, Y. Zhou, X. Ma, and X. Wu, "Effects of Pluronic F68 and Labrasol on the intestinal absorption and pharmacokinetics of rifampicin in rats," *Arch Pharm Res,* vol. 34, pp. 1939-43, Nov **2011.**

[98] Y. Guan, J. Huang, L. Zuo, J. Xu, L. Si, J. Qiu*, et al.*, "Effect of pluronic P123 and F127 block copolymer on P-glycoprotein transport and CYP3A metabolism," *Arch Pharm Res,* vol. 34, pp. 1719-28, Oct **2011**.

[99] F. Zunino, S. Dallavalle, D. Laccabue, G. Beretta, L. Merlini, and G. Pratesi, "Current status and perspectives in the development of camptothecins," *Current Pharmaceutical Design,* vol. 8, pp. 2505-2520, **2002**.

[100] B. T. Ramesha, H. K. Suma, U. Senthilkumar, V. Priti, G. Ravikanth, R. Vasudeva*, et al.*, "New plant sources of the anti-cancer alkaloid, camptothecine from the Icacinaceae taxa, India," *Phytomedicine,* vol. 20, pp. 521-527, Apr 15 **2013**.

[101] S. Gaur, Y. Wang, L. Kretzner, L. Chen, T. Yen, X. Wu*, et al.*, "Pharmacodynamic and pharmacogenomic study of the nanoparticle conjugate of camptothecin CRLX101 for the treatment of cancer," *Nanomedicine,* vol. 10, pp. 1477-86, Oct **2014.**

[102] N. Subramanian, N. Sundaraganesan, S. Sudha, V. Aroulmoji, G. D. Sockalingam, and M. Bergamin, "Experimental and theoretical investigation of the molecular and electronic structure of anticancer drug camptothecin," *Spectrochimica Acta Part a-Molecular and Biomolecular Spectroscopy,* vol. 78, pp. 1058-1067, Mar **2011.**

[103] K. H. Min, K. Park, Y. S. Kim, S. M. Bae, S. Lee, H. G. Jo*, et al.*, "Hydrophobically modified glycol chitosan nanoparticles-encapsulated camptothecin enhance the drug stability and tumor targeting in cancer therapy," *J Control Release,* vol. 127, pp. 208-18, May 8 **2008**.

[104] T. Amna, N. A. M. Barakat, M. S. Hassan, M.-S. Khil, and H. Y. Kim, "Camptothecin loaded poly(ε-caprolactone)nanofibers via one-step electrospinning and their cytotoxicity impact," *Colloids and Surfaces A: Physicochemical and Engineering Aspects,* vol. 431, pp. 1-8, **2013.**

[105] L. Zhang, Y. Hu, X. Jiang, C. Yang, W. Lu, and Y. H. Yang, "Camptothecin derivative-loaded poly(caprolactone-co-lactide)-b-PEG-b-poly(caprolactone-co-lactide) nanoparticles and their biodistribution in mice," *J Control Release,* vol. 96, pp. 135-48, Apr 16 **2004.**

[106] Z. Cao, N. Harris, A. Kozielski, D. Vardeman, J. S. Stehlin, and B. Giovanella, "Alkyl esters of camptothecin and 9-nitrocamptothecin: synthesis, in vitro pharmacokinetics, toxicity, and antitumor activity," *J Med Chem,* vol. 41, pp. 31-7, Jan 1 **1998**.

[107] P. Opanasopit, T. Ngawhirunpat, A. Chaidedgumjorn, T. Rojanarata, A. Apirakaramwong, S. Phongying*, et al.*, "Incorporation of camptothecin into N-phthaloyl chitosan-g-mPEG self-assembly micellar system," *Eur J Pharm Biopharm,* vol. 64, pp. 269-76, Nov **2006.**

[108] Q. Q. Huang, L. Wang, and W. Lu, "Evolution in medicinal chemistry of E-ring-modified Camptothecin analogs as anticancer agents," *European Journal of Medicinal Chemistry,* vol. 63, pp. 746-757, May **2013.**

[109] R. Garcia-Carbonero and J. G. Supko, "Current perspectives on the clinical experience, pharmacology, and continued development of the camptothecins," *Clin Cancer Res,* vol. 8, pp. 641-61, Mar **2002.**

[110] K. H. Min, K. Park, Y. S. Kim, S. M. Bae, S. Lee, H. G. Jo*, et al.*, "Hydrophobically modified glycol chitosan nanoparticles-encapsulated

camptothecin enhance the drug stability and tumor targeting in cancer therapy," *Journal of Controlled Release,* vol. 127, pp. 208-218, May 8 **2008**.
[111] Q. Y. Li, Y. G. Zu, R. Z. Shi, and L. P. Yao, "Review Camptothecin: Current Perspectives," *Current Medicinal Chemistry,* vol. 13, pp. 2021-2039, Jul **2006.**
[112] W. L. Lee, J. Y. Shiau, and L. F. Shyur, "Taxol, Camptothecin and Beyond for Cancer Therapy," *Recent Trends in Medicinal Plants Research,* vol. 62, pp. 133-178, **2012.**
[113] R. Garcia-Carbonero and J. G. Supko, "Current perspectives on the clinical experience, pharmacology, and continued development of the camptothecins," *Clinical Cancer Research,* vol. 8, pp. 641-661, Mar **2002**.
[114] A. Y. Chen, P. M. Chen, and Y. J. Chen, "DNA topoisomerase I drugs and radiotherapy for lung cancer," *J Thorac Dis,* vol. 4, pp. 390-7, Aug **2012**.
[115] V. Bala, S. Rao, B. J. Boyd, and C. A. Prestidge, "Prodrug and nanomedicine approaches for the delivery of the camptothecin analogue SN38," *J Control Release,* vol. 172, pp. 48-61, Nov 28 **2013**.
[116] Z. R. Huang, S. C. Hua, Y. L. Yang, and J. Y. Fang, "Development and evaluation of lipid nanoparticles for camptothecin delivery: a comparison of solid lipid nanoparticles, nanostructured lipid carriers, and lipid emulsion," *Acta Pharmacol Sin,* vol. 29, pp. 1094-102, Sep **2008**.
[117] R. Kunii, H. Onishi, and Y. Machida, "Preparation and antitumor characteristics of PLA/(PEG-PPG-PEG) nanoparticles loaded with camptothecin," *Eur J Pharm Biopharm,* vol. 67, pp. 9-17, Aug **2007.**
[118] Y. Cirpanli, E. Allard, C. Passirani, E. Bilensoy, L. Lemaire, S. Calis*, et al.*, "Antitumoral activity of camptothecin-loaded nanoparticles in 9L rat glioma model," *Int J Pharm,* vol. 403, pp. 201-6, Jan 17 **2011.**
[119] J. Liu, Z. Jiang, S. Zhang, and W. M. Saltzman, "Poly(omega-pentadecalactone-co-butylene-co-succinate) nanoparticles as biodegradable carriers for camptothecin delivery," *Biomaterials,* vol. 30, pp. 5707-19, Oct **2009**.
[120] L. Zhang, M. Yang, Q. Wang, Y. Li, R. Guo, X. Jiang*, et al.*, "10-Hydroxycamptothecin loaded nanoparticles: preparation and antitumor activity in mice," *Journal of Control Release,* vol. 119, pp. 153-62, Jun 4 **2007.**
[121] G. Loch-Neckel, D. Nemen, A. C. Puhl, D. Fernandes, M. A. Stimamiglio, M. Alvarez Silva*, et al.*, "Stealth and non-stealth nanocapsules containing camptothecin: in-vitro and in-vivo activity on B16-F10 melanoma," *J Pharm Pharmacol,* vol. 59, pp. 1359-64, Oct **2007.**
[122] G. Parekh, P. Pattekari, C. Joshi, T. Shutava, M. DeCoster, T. Levchenko*, et al.*, "Layer-by-layer nanoencapsulation of camptothecin with improved activity," *International Journal of Pharmacutics,* vol. 465, pp. 218-27, Apr 25 **2014.**
[123] S. M. Martins, T. Wendling, V. M. Goncalves, B. Sarmento, and D. C. Ferreira, "Development and validation of a simple reversed-phase HPLC method for the determination of camptothecin in animal organs following administration in solid lipid nanoparticles," *Journal of Chromatography B,* vol. 880, pp. 100-7, Jan 1 **2012.**
[124] S. Martins, I. Tho, I. Reimold, G. Fricker, E. Souto, D. Ferreira*, et al.*, "Brain delivery of camptothecin by means of solid lipid nanoparticles: formulation design, in vitro and in vivo studies," *International Journal of Pharmaceutics,* vol. 439, pp. 49-62, Dec 15 **2012**.

[125] D. Cheng, X. Liu, J. Wu, and W. Yu, "Controlled release of methylene blue from polyelectrolyte multilayer films," *Journal of Controlled Release,* vol. 172, pp. e26-e27, **2013**.
[126] S. C. Yang and J. B. Zhu, "Preparation and characterization of camptothecin solid lipid nanoparticles," *Drug Dev Ind Pharm,* vol. 28, pp. 265-74, Mar **2002**.
[127] T. Ngawhirunpat, N. Wonglertnirant, P. Opanasopit, U. Ruktanonchai, R. Yoksan, K. Wasanasuk*, et al.*, "Incorporation methods for cholic acid chitosan-g-mPEG self-assembly micellar system containing camptothecin," *Colloids Surf B Biointerfaces,* vol. 74, pp. 253-9, Nov 1 **2009.**
[128] M. Watanabe, K. Kawano, M. Yokoyama, P. Opanasopit, T. Okano, and Y. Maitani, "Preparation of camptothecin-loaded polymeric micelles and evaluation of their incorporation and circulation stability," *International Journal of Pharmaceutics,* vol. 308, pp. 183-9, Feb 3 **2006**.
[129] Y.-C. Chang and I. M. Chu, "Methoxy poly(ethylene glycol)-b-poly(valerolactone) diblock polymeric micelles for enhanced encapsulation and protection of camptothecin," *European Polymer Journal,* vol. 44, pp. 3922-3930, **2008.**
[130] K. Kawano, M. Watanabe, T. Yamamoto, M. Yokoyama, P. Opanasopit, T. Okano*, et al.*, "Enhanced antitumor effect of camptothecin loaded in long-circulating polymeric micelles," *Journal of Controlled Release,* vol. 112, pp. 329-32, May 30 **2006**.
[131] R. Barreiro-Iglesias, L. Bromberg, M. Temchenko, T. A. Hatton, A. Concheiro, and C. Alvarez-Lorenzo, "Solubilization and stabilization of camptothecin in micellar solutions of pluronic-g-poly(acrylic acid) copolymers," *Journal of Controlled Release,* vol. 97, pp. 537-49, Jul 7 **2004.**
[132] J. A. Zhang, T. Xuan, M. Parmar, L. Ma, S. Ugwu, S. Ali*, et al.*, "Development and characterization of a novel liposome-based formulation of SN-38," *International Journal of Pharmaceutics,* vol. 270, pp. 93-107, **2004**.
[133] M. Watanabe, K. Kawano, K. Toma, Y. Hattori, and Y. Maitani, "In vivo antitumor activity of camptothecin incorporated in liposomes formulated with an artificial lipid and human serum albumin," *Journal of Controlled Release,* vol. 127, pp. 231-8, May 8 **2008**.
[134] Y. Hattori, L. Shi, W. Ding, K. Koga, K. Kawano, M. Hakoshima*, et al.*, "Novel irinotecan-loaded liposome using phytic acid with high therapeutic efficacy for colon tumors," *Journal Control Release,* vol. 136, pp. 30-7, May 21 **2009**.
[135] Y. Li, R. Liu, J. Yang, G. Ma, Z. Zhang, and X. Zhang, "Dual sensitive and temporally controlled camptothecin prodrug liposomes codelivery of siRNA for high efficiency tumor therapy," *Biomaterials,* vol. 35, pp. 9731-45, Dec **2014**.
[136] M. T. Morgan, Y. Nakanishi, D. J. Kroll, A. P. Griset, M. A. Carnahan, M. Wathier*, et al.*, "Dendrimer-encapsulated camptothecins: increased solubility, cellular uptake, and cellular retention affords enhanced anticancer activity in vitro," *Cancer Research,* vol. 66, pp. 11913-21, Dec 15 **2006.**
[137] D. S. Goldberg, N. Vijayalakshmi, P. W. Swaan, and H. Ghandehari, "G3.5 PAMAM dendrimers enhance transepithelial transport of SN38 while minimizing gastrointestinal toxicity," *Journal of Controlled Release,* vol. 150, pp. 318-25, Mar 30 **2011.**
[138] S. Sadekar, G. Thiagarajan, K. Bartlett, D. Hubbard, A. Ray, L. D. McGill*, et al.*, "Poly(amido amine) dendrimers as absorption enhancers for oral delivery

of camptothecin," *International Journal of Pharmaceuticals,* vol. 456, pp. 175-85, Nov 1 **2013.**
[139] H. Zhang, C. P. Hollis, Q. Zhang, and T. Li, "Preparation and antitumor study of camptothecin nanocrystals," *International Journal of Pharmaceuticals,* vol. 415, pp. 293-300, Aug 30 **2011.**
[140] J.-Y. Fang, C.-F. Hung, S.-C. Hua, and T.-L. Hwang, "Acoustically active perfluorocarbon nanoemulsions as drug delivery carriers for camptothecin: Drug release and cytotoxicity against cancer cells," *Ultrasonics,* vol. 49, pp. 39-46, **2009**.
[141] Y. Cirpanli, E. Bilensoy, A. L. Dogan, and S. Calis, "Development of polymeric and cyclodextrin nanoparticles for camptothecin delivery," *Journal of Controlled Release,* vol. 148, pp. e21-3, Nov 20 **2010.**
[142] J. Kang, V. Kumar, D. Yang, P. R. Chowdhury, and R. J. Hohl, "Cyclodextrin complexation: influence on the solubility, stability, and cytotoxicity of camptothecin, an antineoplastic agent," *European Journal of Pharmaceutical Science,* vol. 15, pp. 163-70, Mar **2002.**
[143] S. Swaminathan, L. Pastero, L. Serpe, F. Trotta, P. Vavia, D. Aquilano*, et al.*, "Cyclodextrin-based nanosponges encapsulating camptothecin: Physicochemical characterization, stability and cytotoxicity," *European Journal of Pharmaceutics and Biopharmaceutics,* vol. 74, pp. 193-201, Feb **2010.**
[144] C. Manaspon, P. Nittayacharn, K. Vejjasilpa, C. Fongsuk, and N. Nasongkla, "SN-38:beta-cyclodextrin inclusion complex for in situ solidifying injectable polymer implants," *Engineering in Medicine and Biological Society,* vol. 2011, pp. 3241-4, **2011**.
[145] F. Du, H. J. Meng, K. Xu, Y. Y. Xu, P. Luo, Y. Luo*, et al.*, "CPT loaded nanoparticles based on beta-cyclodextrin-grafted poly(ethylene glycol)/poly (L-glutamic acid) diblock copolymer and their inclusion complexes with CPT," *Colloids and Surfaces B-Biointerfaces,* vol. 113, pp. 230-236, Jan 1 **2014.**
[146] J. Szejtli, "Introduction and General Overview of Cyclodextrin Chemistry," *Chemistry Reviews,* vol. 98, pp. 1743-1754, Jul 30 **1998.**
[147] N. Zafar, H. Fessi, and A. Elaissari, "Cyclodextrin containing biodegradable particles: from preparation to drug delivery applications," *International Journal of Pharmaceutics,* vol. 461, pp. 351-66, Jan 30 **2014**.
[148] L. M. Hamilton, C. T. Kelly, and W. M. Fogarty, "Review: cyclodextrins and their interaction with amylolytic enzymes," *Enzyme and Microbial Technology,* vol. 26, pp. 561-567, May **2000**.
[149] R. E. Turnes and S. Bahar, "Production of cyclomaltodextrin glucanotransferase from an alkalophilic Bacillus sp. in rice and corn flakes," *Acta Cient Venez,* vol. 47, pp. 133-7, **1996.**
[150] M. Messner, S. V. Kurkov, P. Jansook, and T. Loftsson, "Self-assembled cyclodextrin aggregates and nanoparticles," *International Journal of Pharmaceutics,* vol. 387, pp. 199-208, Mar 15 **2010.**
[151] W. F. Lai, "Cyclodextrins in non-viral gene delivery," *Biomaterials,* vol. 35, pp. 401-411, Jan **2014.**
[152] E. Pinho, M. Grootveld, G. Soares, and M. Henriques, "Cyclodextrins as encapsulation agents for plant bioactive compounds," *Carbohydrate Polymymers,* vol. 101, pp. 121-35, Jan 30 **2014.**
[153] M. E. Brewster and T. Loftsson, "Cyclodextrins as pharmaceutical solubilizers," *Advanced Drug Deliv Reviews,* vol. 59, pp. 645-66, Jul 30 **2007.**

[154] L. Escuder-Gilabert, Y. Martin-Biosca, M. J. Medina-Hernandez, and S. Sagrado, "Cyclodextrins in capillary electrophoresis: Recent developments and new trends," *Journal of Chromatography A,* vol. 1357, pp. 2-23, Aug 29 **2014.**
[155] E. Bilensoy and A. A. Hincal, "Recent advances and future directions in amphiphilic cyclodextrin nanoparticles," *Expert Opinion on Drug Delivery,* vol. 6, pp. 1161-1173, Nov **2009**.
[156] G. Astray, C. Gonzalez-Barreiro, J. C. Mejuto, R. Rial-Otero, and J. Simal-Gandara, "A review on the use of cyclodextrins in foods," *Food Hydrocolloids,* vol. 23, pp. 1631-1640, Oct **2009.**
[157] S. Li and W. C. Purdy, "Cyclodextrins and Their Applications in Analytical-Chemistry," *Chemical Reviews,* vol. 92, pp. 1457-1470, Sep-Oct **1992.**
[158] E. M. M. Del Valle, "Cyclodextrins and their uses: a review," *Process Biochemistry,* vol. 39, pp. 1033-1046, May 31 **2004.**
[159] S. V. Kurkov and T. Loftsson, "Cyclodextrins," *International Journal of Pharmaceutics,* vol. 453, pp. 167-80, Aug 30 **2013.**
[160] T. Irie and K. Uekama, "Cyclodextrins in peptide and protein delivery," *Advanced Drug Delivery Reviews,* vol. 36, pp. 101-123, Mar 1 **1999.**
[161] E. Bilensoy, L. Dogan, M. Sen, and A. Hincal, "Complexation behavior of antiestrogen drug tamoxifen citrate with natural and modified beta-cyclodextrins," *Journal of Inclusion Phenomena and Macrocyclic Chemistry,* vol. 57, pp. 651-655, Apr **2007.**
[162] L. Szente and J. Szeman, "Cyclodextrins in analytical chemistry: host-guest type molecular recognition," *Analytical Chemistry,* vol. 85, pp. 8024-30, **2013**.
[163] E. Memisoglu, A. Bochot, M. Ozalp, M. Sen, D. Duchene, and A. A. Hincal, "Direct formation of nanospheres from amphiphilic beta-cyclodextrin inclusion complexes," *Pharmaceutical Research,* vol. 20, pp. 117-25, **2003**.
[164] E. Memisoglu-Bilensoy, I. Vural, A. Bochot, J. M. Renoir, D. Duchene, and A. A. Hincal, "Tamoxifen citrate loaded amphiphilic beta-cyclodextrin nanoparticles: in vitro characterization and cytotoxicity," *Journal of Controlled Release,* vol. 104, pp. 489-96, Jun 2 **2005.**
[165] E. Bilensoy, O. Gurkaynak, A. L. Dogan, and A. A. Hincal, "Safety and efficacy of amphiphilic beta-cyclodextrin nanoparticles for paclitaxel delivery," *International Journal of Pharmaceutics,* vol. 347, pp. 163-70, Jan 22 **2008.**
[166] E. Bilensoy, "Cationic nanoparticles for cancer therapy," *Expert Opinion Drug Delivery,* vol. 7, pp. 795-809, Jul **2010.**
[167] E. Memisoglu, A. Bochot, M. Sen, D. Charon, D. Duchene, and A. A. Hincal, "Amphiphilic beta-cyclodextrins modified on the primary face: Synthesis, characterization, and evaluation of their potential as novel excipients in the preparation of nanocapsules," *Journal of Pharmaceutical Sciences,* vol. 91, pp. 1214-1224, May **2002**.
[168] D. Duchene, G. Ponchel, and D. Wouessidjewe, "Cyclodextrins in targeting. Application to nanoparticles," *Advanced Drug Delivery Reviews,* vol. 36, pp. 29-40, Mar 1 **1999.**
[169] T. Loftsson and M. E. Brewster, "Pharmaceutical applications of cyclodextrins. 1. Drug solubilization and stabilization," *Journal of Pharmaceutical Science,* vol. 85, pp. 1017-25, Oct **1996**.
[170] S. Klein, M. F. Wempe, T. Zoeller, N. L. Buchanan, J. L. Lambert, M. G. Ramsey*, et al.*, "Improving glyburide solubility and dissolution by

complexation with hydroxybutenyl-beta-cyclodextrin," *Journal of Pharmcy and Pharmacology,* vol. 61, pp. 23-30, Jan **2009.**
[171] M. Kakran, N. G. Sahoo, L. Li, and Z. Judeh, "Dissolution Enhancement of Artemisinin with beta-Cyclodextrin," *Chemical & Pharmaceutical Bulletin,* vol. 59, pp. 646-652, May **2011.**
[172] J. L. Koontz and J. E. Marcy, "Formation of natamycin: Cyclodextrin inclusion complexes and their characterization," *Journal of Agricultural and Food Chemistry,* vol. 51, pp. 7106-7110, Nov 19 **2003.**
[173] P. S. Martins, R. Ochoa, A. M. Pimenta, L. A. Ferreira, A. L. Melo, J. B. da Silva*, et al.*, "Mode of action of beta-cyclodextrin as an absorption enhancer of the water-soluble drug meglumine antimoniate," *International Journal of Pharmceutics,* vol. 325, pp. 39-47, Nov 15 **2006.**
[174] T. Loftsson, P. Jarho, M. Masson, and T. Jarvinen, "Cyclodextrins in drug delivery," *Expert Opinion Drug Delivery,* vol. 2, pp. 335-51, Mar **2005.**
[175] X. Y. Zhao, W. Li, Q. H. Luo, and X. R. Zhang, "Enhanced bioavailability of orally administered flurbiprofen by combined use of hydroxypropyl-cyclodextrin and poly(alkyl-cyanoacrylate) nanoparticles," *European Journal of Drug Metabolism and Pharmacokinetics,* vol. 39, pp. 61-67, Mar **2014.**
[176] H. Arima, K. Yunomae, K. Miyake, T. Irie, F. Hirayama, and K. Uekama, "Comparative studies of the enhancing effects of cyclodextrins on the solubility and oral bioavailability of tacrolimus in rats," *Journal of Pharmaceutical Sciences,* vol. 90, pp. 690-701, Jun **2001.**
[177] S. I. Saleh, A. A. Rahman, A. E. Aboutaleb, Y. Nakai, and M. O. Ahmed, "Effect of dimethyl-beta-cyclodextrin on nitrazepam stability," *Journal of Pharmaceutics Belg,* vol. 48, pp. 383-8, Sep-Oct **1993.**
[178] C. Alvarez, J. Calero, J. C. Menendez, S. Torrado, and J. J. Torrado, "Effects of hydroxypropyl-beta-cyclodextrin on the chemical stability and the aqueous solubility of thalidomide enantiomers," *Pharmazie,* vol. 63, pp. 511-3, Jul **2008.**
[179] M. A. Woodruff and D. W. Hutmacher, "The return of a forgotten polymer—Polycaprolactone in the 21st century," *Progress in Polymer Science,* vol. 35, pp. 1217-1256, **2010.**
[180] B. D. Ulery, L. S. Nair, and C. T. Laurencin, "Biomedical Applications of Biodegradable Polymers," *Journal of Polymer Science Part B: Polymer Physics,* vol. 49, pp. 832-864, Jun 15 **2011.**
[181] M. Labet and W. Thielemans, "Synthesis of polycaprolactone: a review," *Chemical Society Reviews,* vol. 38, pp. 3484-504, Dec **2009.**
[182] X. Wei, C. Gong, M. Gou, S. Fu, Q. Guo, S. Shi*, et al.*, "Biodegradable poly(epsilon-caprolactone)-poly(ethylene glycol) copolymers as drug delivery system," *International Journal of Pharmaceutics,* vol. 381, pp. 1-18, **2009**
[183] A. Mahapatro and D. K. Singh, "Biodegradable nanoparticles are excellent vehicle for site directed in-vivo delivery of drugs and vaccines," *Journal of Nanobiotechnology,* vol. 9, p. 55, **2011.**
[184] L. Pan, X. Pei, R. He, Q. Wan, and J. Wang, "Multiwall carbon nanotubes/polycaprolactone composites for bone tissue engineering application," *Colloids Surf B Biointerfaces,* vol. 93, pp. 226-34, May 1 **2012.**
[185] M. R. Guilherme, M. R. Mauricio, E. T. Tenório-Neto, M. H. Kunita, L. Cardozo-Filho, T. S. P. Cellet*, et al.*, "Polycaprolactone nanoparticles containing encapsulated progesterone prepared using a scCO2 emulsion drying technique," *Materials Letters,* vol. 124, pp. 197-200, **2014.**

[186] A. A. Amis, A. M. Bull, C. M. Gupte, I. Hijazi, A. Race, and J. R. Robinson, "Biomechanics of the PCL and related structures: posterolateral, posteromedial and meniscofemoral ligaments," *Knee Surg Sports Traumatol Arthrosc,* vol. 11, pp. 271-81, Sep **2003.**

[187] T. K. Dash and V. B. Konkimalla, "Poly-small je, Ukrainian-caprolactone based formulations for drug delivery and tissue engineering: A review," *Journal of Controlled Release,* vol. 158, pp. 15-33, Feb 28 **2012.**

[188] M. Dash, F. Chiellini, R. M. Ottenbrite, and E. Chiellini, "Chitosan—A versatile semi-synthetic polymer in biomedical applications," *Progress in Polymer Science,* vol. 36, pp. 981-1014, **2011.**

[189] A. Anitha, S. Sowmya, P. T. S. Kumar, S. Deepthi, K. P. Chennazhi, H. Ehrlich*, et al.*, "Chitin and chitosan in selected biomedical applications," *Progress in Polymer Science,* vol. 39, pp. 1644-1667, **2014.**

[190] D. Pooja, D. J. Babu Bikkina, H. Kulhari, N. Nikhila, S. Chinde, Y. M. Raghavendra*, et al.*, "Fabrication, characterization and bioevaluation of silibinin loaded chitosan nanoparticles," *International Journal of Biological Macromolecules,* vol. 69, pp. 267-73, Aug **2014.**

[191] S.-H. Lee, B. Ryu, J.-Y. Je, and S.-K. Kim, "Diethylaminoethyl chitosan induces apoptosis in HeLa cells via activation of caspase-3 and p53 expression," *Carbohydrate Polymers,* vol. 84, pp. 571-578, **2011.**

[192] M. Rinaudo, "Chitin and chitosan: Properties and applications," *Progress in Polymer Science,* vol. 31, pp. 603-632, **2006.**

[193] P. Mukhopadhyay, R. Mishra, D. Rana, and P. P. Kundu, "Strategies for effective oral insulin delivery with modified chitosan nanoparticles: A review," *Progress in Polymer Science,* vol. 37, pp. 1457-1475, **2012.**

[194] F. Mwale, M. Iordanova, C. N. Demers, T. Steffen, P. Roughley, and J. Antoniou, "Biological evaluation of chitosan salts cross-linked to genipin as a cell scaffold for disk tissue engineering," *Tissue Engineering,* vol. 11, pp. 130-140, Jan **2005.**

[195] K. Nagpal, S. K. Singh, and D. N. Mishra, "Chitosan nanoparticles: a promising system in novel drug delivery," *Chemical and Pharmaceutical Bulletin (Tokyo),* vol. 58, pp. 1423-30, Nov **2010.**

[196] K. Songsurang, P. Suvannasara, C. Phurat, S. Puthong, K. Siraleartmukul, and N. Muangsin, "Enhanced anti-topoisomerase II activity by mucoadhesive 4-CBS-chitosan/poly (lactic acid) nanoparticles," *Carbohydrate Polymers,* vol. 98, pp. 1335-42, Nov 6 **2013.**

[197] N. Erdogar, A. B. Iskit, H. Eroglu, M. F. Sargon, N. A. Mungan, and E. Bilensoy, "Cationic core-shell nanoparticles for intravesical chemotherapy in tumor-induced rat model: safety and efficacy," *International Journal of Pharmaceutics,* vol. 471, pp. 1-9, Aug 25 **2014.**

[198] X. Li, M. Min, N. Du, Y. Gu, T. Hode, M. Naylor*, et al.*, "Chitin, chitosan, and glycated chitosan regulate immune responses: the novel adjuvants for cancer vaccine," *Clinical Development Immun,* vol. 2013, p. 387023, **2013**

[199] N. S. Rejinold, M. Muthunarayanan, K. Muthuchelian, K. P. Chennazhi, S. V. Nair, and R. Jayakumar, "Saponin-loaded chitosan nanoparticles and their cytotoxicity to cancer cell lines in vitro," *Carbohydrate Polymers,* vol. 84, pp. 407-416, **2011.**

[200] A. Anitha, N. Deepa, K. P. Chennazhi, S. V. Nair, H. Tamura, and R. Jayakumar, "Development of mucoadhesive thiolated chitosan nanoparticles

for biomedical applications," *Carbohydrate Polymers,* vol. 83, pp. 66-73, **2011.**

[201] J. J. Wang, Z. W. Zeng, R. Z. Xiao, T. Xie, G. L. Zhou, X. R. Zhan*, et al.*, "Recent advances of chitosan nanoparticles as drug carriers," *International Journal of Nanomedicine,* vol. 6, pp. 765-74, **2011.**

[202] Y. Maeda and Y. Kimura, "Antitumor effects of various low-molecular-weight chitosans are due to increased natural killer activity of intestinal intraepithelial lymphocytes in sarcoma 180-bearing mice," *Journal of Nutrition,* vol. 134, pp. 945-950, Apr **2004**.

[203] Y. Zambito, F. Felice, A. Fabiano, R. Di Stefano, and G. Di Colo, "Mucoadhesive nanoparticles made of thiolated quaternary chitosan crosslinked with hyaluronan," *Carbohydrate Polymers,* vol. 92, pp. 33-9, Jan 30 **2013**.

[204] C. Prego, D. Torres, and M. J. Alonso, "The potential of chitosan for the oral administration of peptides," *Expert Opinion Drug Delivery,* vol. 2, pp. 843-54, Sep **2005.**

[205] F. A. Moghaddam, F. Atyabi, and R. Dinarvand, "Preparation and in vitro evaluation of mucoadhesion and permeation enhancement of thiolated chitosan-pHEMA core-shell nanoparticles," *Nanomedicine,* vol. 5, pp. 208-15, Jun **2009**.

[206] S. A. Agnihotri, N. N. Mallikarjuna, and T. M. Aminabhavi, "Recent advances on chitosan-based micro- and nanoparticles in drug delivery," *Journal of Controlled Release,* vol. 100, pp. 5-28, Nov 5 **2004.**

[207] "Validation of Liquid Chromatographic Methods," in *Liquid Chromatography*, ed, pp. 57-73.

[208] M. Rambla-Alegre, J. Esteve-Romero, and S. Carda-Broch, "Is it really necessary to validate an analytical method or not? That is the question," *Journal of Chromatography A,* vol. 1232, pp. 101-109, Apr 6 **2012.**

[209] D. L. Warner and T. G. Burke, "Simple and versatile high-performance liquid chromatographic method for the simultaneous quantitation of the lactone and carboxylate forms of camptothecin anticancer drugs," *Journal of Chromatography B,* vol. 691, pp. 161-171, Mar 28 **1997.**

[210] J. Cheng, B. A. Teply, I. Sherifi, J. Sung, G. Luther, F. X. Gu*, et al.*, "Formulation of functionalized PLGA-PEG nanoparticles for in vivo targeted drug delivery," *Biomaterials,* vol. 28, pp. 869-76, Feb **2007**.

[211] X. W. Dong, C. A. Mattingly, M. Tseng, M. Cho, V. R. Adams, and R. J. Mumper, "Development of new lipid-based paclitaxel nanoparticles using sequential simplex optimization," *European Journal of Pharmaceutics and Biopharmaceutics,* vol. 72, pp. 9-17, May **2009**.

[212] N. K. Thakral, A. R. Ray, D. Bar-Shalom, A. H. Eriksson, and D. K. Majumdar, "Soluplus-Solubilized Citrated Camptothecin-A Potential Drug Delivery Strategy in Colon Cancer," *Aaps Pharmscitech,* vol. 13, pp. 59-66, Mar **2012.**

[213] S. Lazzari, D. Moscatelli, F. Codari, M. Salmona, M. Morbidelli, and L. Diomede, "Colloidal stability of polymeric nanoparticles in biological fluids," *Journal of Nanoparticle Research,* vol. 14, Jun **2012.**

[214] F. Ungaro, I. d'Angelo, C. Coletta, R. d'Emmanuele di Villa Bianca, R. Sorrentino, B. Perfetto*, et al.*, "Dry powders based on PLGA nanoparticles for pulmonary delivery of antibiotics: modulation of encapsulation efficiency, release rate and lung deposition pattern by hydrophilic polymers," *Journal of Controlled Release,* vol. 157, pp. 149-59, Jan 10 **2012.**

[215] R. B. van Breemen and Y. Li, "Caco-2 cell permeability assays to measure drug absorption," *Expert Opinion Drug Metabolism Toxicology,* vol. 1, pp. 175-85, Aug **2005.**

[216] B. Press, "Optimization of the Caco-2 permeability assay to screen drug compounds for intestinal absorption and efflux," *Methods in Molecular Biology,* vol. 763, pp. 139-54, **2011.**

[217] G. Joshi, A. Kumar, and K. Sawant, "Enhanced bioavailability and intestinal uptake of Gemcitabine HCl loaded PLGA nanoparticles after oral delivery," *European Journal of Pharmaceutical Sciences,* vol. 60, pp. 80-89, Aug 18 **2014**.

[218] A. Cser, K. Nagy, and L. Biczok, "Fluorescence lifetime of Nile Red as a probe for the hydrogen bonding strength with its microenvironment," *Chemical Physics Letters,* vol. 360, pp. 473-478, Jul 17 **2002.**

[219] A. K. Dutta, K. Kamada, and K. Ohta, "Spectroscopic studies of nile red in organic solvents and polymers," *Journal of Photochemistry and Photobiology a-Chemistry,* vol. 93, pp. 57-64, Jan 4 **1996**.

[220] T. S. Owens, H. Dodds, K. Fricke, S. K. Hanna, and K. R. Crews, "High-performance liquid chromatographic assay with fluorescence detection for the simultaneous measurement of carboxylate and lactone forms of irinotecan and three metabolites in human plasma," *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences,* vol. 788, pp. 65-74, May 5 **2003.**

[221] X. X. Yang, Z. P. Hu, S. Y. Chan, B. C. Goh, W. Duan, E. Chan*, et al.*, "Simultaneous determination of the lactone and carboxylate forms of irinotecan (CPT-11) and its active metabolite SN-38 by high-performance liquid chromatography: Application to plasma pharmacokinetic studies in the rat," *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences,* vol. 821, pp. 221-228, Jul 25 **2005.**

[222] M. P. Bartolomeo and F. Maisano, "Validation of a reversed-phase HPLC method for quantitative amino acid analysis," *Journal of Biomolecular Technology,* vol. 17, pp. 131-7, Apr **2006.**

[223] N. Kapoor, S. Khandavilli, and R. Panchagnula, "Simultaneous determination of lamivudine, stavudine and nevirapine in antiretroviral fixed dose combinations by high performance liquid chromatography," *Analytica Chimica Acta,* vol. 570, pp. 41-45, Jun 7 **2006.**

[224] A. Granada, F. S. Murakami, T. Sartori, E. Lemos-Senna, and M. A. Silva, "Development and validation of an HPLC method to quantify camptothecin in polymeric nanocapsule suspensions," *Journal of AOAC International,* vol. 91, pp. 551-6, May-Jun **2008.**

[225] M. Sarkar, S. Khandavilli, and R. Panchagnula, "Development and validation of RP-HPLC and ultraviolet spectrophotometric methods of analysis for the quantitative estimation of antiretroviral drugs in pharmaceutical dosage forms," *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences,* vol. 830, pp. 349-354, Jan 18 **2006.**

[226] J. G. Supko and L. Malspeis, "A Reversed-Phase Hplc Method for Determining Camptothecin in Plasma with Specificity for the Intact Lactone Form of the Drug," *Journal of Liquid Chromatography,* vol. 14, pp. 1779-1803, **1991**.

[227] S. M. Martins, T. Wendling, V. M. F. Goncalves, B. Sarmento, and D. C. Ferreira, "Development and validation of a simple reversed-phase HPLC

method for the determination of camptothecin in animal organs following administration in solid lipid nanoparticles," *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences,* vol. 880, pp. 100-107, Jan 1 **2012**.

[228] N. F. de Melo, R. Grillo, V. A. Guilherme, D. R. de Araujo, E. de Paula, A. H. Rosa*, et al.*, "Poly(lactide-co-glycolide) nanocapsules containing benzocaine: influence of the composition of the oily nucleus on physico-chemical properties and anesthetic activity," *Pharmaceutical Research,* vol. 28, pp. 1984-94, Aug **2011**.

[229] F. V. Leimann, L. Cardozo, C. Sayer, and P. H. H. Araujo, "poly(3-hydroxybutyrate-co-3-hydroxyvalerate) nanoparticles prepared by a miniemulsion/solvent evaporation technique. effect of phbv molar mass and concentration," *Brazilian Journal of Chemical Engineering,* vol. 30, pp. 369-377, Apr-Jun **2013.**

[230] I. Limayem Blouza, C. Charcosset, S. Sfar, and H. Fessi, "Preparation and characterization of spironolactone-loaded nanocapsules for paediatric use," *International Journal of Pharmaceutics,* vol. 325, pp. 124-31, Nov 15 **2006.**

[231] A. Budhian, S. J. Siegel, and K. I. Winey, "Haloperidol-loaded PLGA nanoparticles: Systematic study of particle size and drug content," *International Journal of Pharmaceutics,* vol. 336, pp. 367-375, May 24 **2007**.

[232] X. R. Song, Y. Zhao, W. B. Wu, Y. Q. Bi, Z. Cai, Q. H. Chen*, et al.*, "PLGA nanoparticles simultaneously loaded with vincristine sulfate and verapamil hydrochloride: Systematic study of particle size and drug entrapment efficiency," *International Journal of Pharmaceutics,* vol. 350, pp. 320-329, Feb 28 **2008.**

[233] J. Y. Choi, C. H. Park, and J. Lee, "Effect of polymer molecular weight on nanocomminution of poorly soluble drug," *Drug Delivery,* vol. 15, pp. 347-53, Jun **2008.**

[234] D. Moinard-Checot, Y. Chevalier, S. Briancon, L. Beney, and H. Fessi, "Mechanism of nanocapsules formation by the emulsion-diffusion process," *Journal of Colloid and Interface Science,* vol. 317, pp. 458-468, Jan 15 **2008**.

[235] C. Fonseca, S. Simoes, and R. Gaspar, "Paclitaxel-loaded PLGA nanoparticles: preparation, physicochemical characterization and in vitro anti-tumoral activity," *J Control Release,* vol. 83, pp. 273-286, Oct 4 **2002.**

[236] S. J. Kshirsagar, M. R. Bhalekar, J. N. Patel, S. K. Mohapatra, and N. S. Shewale, "Preparation and characterization of nanocapsules for colon-targeted drug delivery system," *Pharmaceutical Development and Technology,* vol. 17, pp. 607-13, Sep-Oct **2012**.

[237] R. M. Mainardes and R. C. Evangelista, "PLGA nanoparticles containing praziquantel: effect of formulation variables on size distribution," *International Journal of Pharmaceutics,* vol. 290, pp. 137-44, Feb 16 **2005.**

[238] N. Khayata, W. Abdelwahed, M. F. Chehna, C. Charcosset, and H. Fessi, "Preparation of vitamin E loaded nanocapsules by the nanoprecipitation method: from laboratory scale to large scale using a membrane contactor," *International Journal of Pharmaceutics,* vol. 423, pp. 419-27, Feb 28 **2012.**

[239] S. A. Guhagarkar, V. C. Malshe, and P. V. Devarajan, "Nanoparticles of Polyethylene Sebacate: A New Biodegradable Polymer," *Aaps Pharmscitech,* vol. 10, pp. 935-942, Sep **2009.**

[240] K. J. Cho, X. Wang, S. M. Nie, Z. Chen, and D. M. Shin, "Therapeutic nanoparticles for drug delivery in cancer," *Clinical Cancer Research,* vol. 14, pp. 1310-1316, Mar 1 **2008**.
[241] R. Tantra, P. Schulze, and P. Quincey, "Effect of nanoparticle concentration on zeta-potential measurement results and reproducibility," *Particuology,* vol. 8, pp. 279-285, Jun **2010**.
[242] K. B. D. Lobato, K. Paese, J. C. Forgearini, S. S. Guterres, A. Jablonski, and A. D. Rios, "Characterisation and stability evaluation of bixin nanocapsules," *Food Chemistry,* vol. 141, pp. 3906-3912, Dec 15 **2013.**
[243] K. Tahara, T. Sakai, H. Yamamoto, H. Takeuchi, N. Hirashima, and Y. Kawashima, "Improved cellular uptake of chitosan-modified PLGA nanospheres by A549 cells," *International Journal of Pharmaceutics,* vol. 382, pp. 198-204, Dec 1 **2009**.
[244] K. Thadkala, P. K. Nanam, B. Rambabu, C. Sailu, and J. Aukunuru, "Preparation and characterization of amorphous ezetimibe nanosuspensions intended for enhancement of oral bioavailability," *International Journal of Pharmaceutics Investigation,* vol. 4, pp. 131-7, Jul **2014.**
[245] W. W. Wilson, M. M. Wade, S. C. Holman, and F. R. Champlin, "Status of methods for assessing bacterial cell surface charge properties based on zeta potential measurements," *Journal of Microbiological Methods,* vol. 43, pp. 153-164, Jan **2001**.
[246] Y. Zhang, M. Yang, N. G. Portney, D. X. Cui, G. Budak, E. Ozbay*, et al.*, "Zeta potential: a surface electrical characteristic to probe the interaction of nanoparticles with normal and cancer human breast epithelial cells," *Biomedical Microdevices,* vol. 10, pp. 321-328, Apr **2008**.
[247] A. C. Sabuncu, J. Grubbs, S. Z. Qian, T. M. Abdel-Fattah, M. W. Stacey, and A. Beskok, "Probing nanoparticle interactions in cell culture media," *Colloids and Surfaces B-Biointerfaces,* vol. 95, pp. 96-102, Jun 15 **2012**.
[248] S. M. Nie, "Understanding and overcoming major barriers in cancer nanomedicine," *Nanomedicine,* vol. 5, pp. 523-528, Jun **2010.**
[249] M. Roser, D. Fischer, and T. Kissel, "Surface-modified biodegradable albumin nano- and microspheres. II: effect of surface charges on in vitro phagocytosis and biodistribution in rats," *European Journal of Pharmaceutics and Biopharmaceutics,* vol. 46, pp. 255-263, Nov **1998.**
[250] V. Teeranachaideekul, P. Boonme, E. B. Souto, R. H. Muller, and V. B. Junyaprasert, "Influence of oil content on physicochemical properties and skin distribution of Nile red-loaded NLC," *Journal of Controlled Release,* vol. 128, pp. 134-141, Jun 4 **2008.**
[251] S. Abbasi, A. Paul, W. Shao, and S. Prakash, "Cationic albumin nanoparticles for enhanced drug delivery to treat breast cancer: preparation and in vitro assessment," *Journal of Drug Delivery,* vol. 2012, p. 686108, **2012.**
[252] Y. Pan, Y. J. Li, P. Gao, P. T. Ding, H. Xu, and J. M. Zheng, "[Enhancement of gastrointestinal absorption of chitosan-coated insulin-loaded poly (lactic-co-glycolic acid) nanoparticles]," *Yao Xue Xue Bao,* vol. 38, pp. 467-70, Jun **2003**.
[253] P. Opanasopit, M. Yokoyama, M. Watanabe, K. Kawano, Y. Maitani, and T. Okano, "Influence of serum and albumins from different species on stability of camptothecin-loaded micelles," *Journal of Controlled Release,* vol. 104, pp. 313-21, May 18 **2005.**

[254] Y. Cirpanli, E. Bilensoy, A. L. Dogan, and S. Calis, "Comparative evaluation of polymeric and amphiphilic cyclodextrin nanoparticles for effective camptothecin delivery," *European Journal of Pharmaceutics and Biopharmaceutics,* vol. 73, pp. 82-89, Sep **2009.**

[255] A. C. Groo, P. Saulnier, J. C. Gimel, J. Gravier, C. Ailhas, J. P. Benoit*, et al.*, "Fate of paclitaxel lipid nanocapsules in intestinal mucus in view of their oral delivery," *International Journal of Nanomedicine,* vol. 8, pp. 4291-4302, **2013**.

[256] A. des Rieux, V. Fievez, M. Garinot, Y. J. Schneider, and V. Preat, "Nanoparticles as potential oral delivery systems of proteins and vaccines: A mechanistic approach," *Journal of Controlled Release,* vol. 116, pp. 1-27, Nov 10 **2006.**

[257] O. Borges, G. Borchard, J. C. Verhoef, A. de Sousa, and H. E. Junginger, "Preparation of coated nanoparticles for a new mucosal vaccine delivery system,"*Journal of Pharmaceutics,* vol. 299, pp. 155-66, Aug 11 **2005**.

[258] K. Bowman and K. W. Leong, "Chitosan nanoparticles for oral drug and gene delivery," *International Journal of Nanomedicine,* vol. 1, pp. 117-128, **2006**.

[259] L. Mazzarino, L. Coche-Guerente, P. Labbe, E. Lemos-Senna, and R. Borsali, "On the Mucoadhesive Properties of Chitosan-Coated Polycaprolactone Nanoparticles Loaded with Curcumin Using Quartz Crystal Microbalance with Dissipation Monitoring," *Journal of Biomedical Nanotechnology,* vol. 10, pp. 787-794, May **2014**.

[260] Y. D. Angela M. de Campos, Edison L. S. Carvalho, Alejandro Sánchez, and Maria José Alonso, "Chitosan Nanoparticles as New Ocular Drug Delivery Systems: in Vitro Stability, in Vivo Fate, and Cellular Toxicity," vol. 21, pp. 803-810, **2004.**

[261] S. Gelperina, K. Kisich, M. D. Iseman, and L. Heifets, "The potential advantages of nanoparticle drug delivery systems in chemotherapy of tuberculosis," *American Journal of Respiratory Critical Care Medicine,* vol. 172, pp. 1487-90, Dec 15 **2005.**

[262] M. Masson, T. Loftsson, G. Masson, and E. Stefansson, "Cyclodextrins as permeation enhancers: some theoretical evaluations and in vitro testing," *Journal of Controlled Release,* vol. 59, pp. 107-118, May 1 **1999**.

[263] M. V. Lozano, D. Torrecilla, D. Torres, A. Vidal, F. Dominguez, and M. J. Alonso, "Highly efficient system to deliver taxanes into tumor cells: Docetaxel-loaded chitosan oligomer colloidal carriers," *Biomacromolecules,* vol. 9, pp. 2186-2193, Aug **2008**.

[264] P. Sanchez-Moreno, J. L. Ortega-Vinuesa, A. Martin-Rodriguez, H. Boulaiz, J. A. Marchal-Corrales, and J. M. Peula-Garcia, "Characterization of Different Functionalized Lipidic Nanocapsules as Potential Drug Carriers," *International Journal of Molecular Sciences,* vol. 13, pp. 2405-2424, Feb **2012.**

[265] K. Maisel, L. Ensign, M. Reddy, R. Cone, and J. Hanes, "Effect of surface chemistry on nanoparticle interaction with gastrointestinal mucus and distribution in the gastrointestinal tract following oral and rectal administration in the mouse," *Journal of Controlled Release,* vol. 197, pp. 48-57, **2015.**

[266] A. Beloqui, M. Á. Solinís, A. d. Rieux, V. Préat, and A. Rodríguez-Gascón, "Dextran–protamine coated nanostructured lipid carriers as mucus-penetrating nanoparticles for lipophilic drugs," *International Journal of Pharmaceutics,* vol. 468, pp. 105-111, **2014.**

[267] R. L. Carrier, L. A. Miller, and M. Ahmed, "The utility of cyclodextrins for enhancing oral bioavailability," *Journal of Controlled Release,* vol. 123, pp. 78-99, Nov 6 **2007.**
[268] S. R. Mao, X. T. Shuai, F. Unger, M. Simon, D. Z. Bi, and T. Kissel, "The depolymerization of chitosan: effects on physicochemical and biological properties," *International Journal of Pharmaceutics,* vol. 281, pp. 45-54, Aug 20 **2004.**
[269] X. R. Liu, M. Tang, T. Zhang, Y. Y. Hu, S. S. Zhang, L. Kong*, et al.*, "Determination of a Threshold Dose to Reduce or Eliminate CdTe-Induced Toxicity in L929 Cells by Controlling the Exposure Dose," *Plos One,* vol. 8, Apr 5 **2013**.
[270] E. Bilensoy, O. Gurkaynak, A. L. Dogan, and A. A. Hincal, "Safety and efficacy of amphiphilic beta-cyclodextrin nanoparticles for paclitaxel delivery," *International Journal of Pharmaceutics,* vol. 347, pp. 163-170, Jan 22 **2008.**
[271] C. B. He, Y. P. Hu, L. C. Yin, C. Tang, and C. H. Yin, "Effects of particle size and surface charge on cellular uptake and biodistribution of polymeric nanoparticles," *Biomaterials,* vol. 31, pp. 3657-3666, May **2010.**
[272] R. Yang, W. S. Shim, F. D. Cui, G. Cheng, X. Han, Q. R. Jin*, et al.*, "Enhanced electrostatic interaction between chitosan-modified PLGA nanoparticle and tumor," *International Journal of Pharmaceutics,* vol. 371, pp. 142-147, Apr 17 **2009.**
[273] A. Hafner, J. Lovric, D. Voinovich, and J. Filipovic-Grcic, "Melatonin-loaded lecithin/chitosan nanoparticles: Physicochemical characterisation and permeability through Caco-2 cell monolayers," *International Journal of Pharmaceutics,* vol. 381, pp. 205-213, Nov 3 **2009.**
[274] Z. G. Yue, W. Wei, P. P. Lv, H. Yue, L. Y. Wang, Z. G. Su*, et al.*, "Surface charge affects cellular uptake and intracellular trafficking of chitosan-based nanoparticles," *Biomacromolecules,* vol. 12, pp. 2440-6, Jul 11 **2011**.
[275] S. Y. Choi, S. H. Jang, J. Park, S. Jeong, J. H. Park, K. S. Ock*, et al.*, "Cellular uptake and cytotoxicity of positively charged chitosan gold nanoparticles in human lung adenocarcinoma cells," *Journal of Nanoparticle Research,* vol. 14, Dec **2012.**
[276] K. Y. Win and S. S. Feng, "Effects of particle size and surface coating on cellular uptake of polymeric nanoparticles for oral delivery of anticancer drugs," *Biomaterials,* vol. 26, pp. 2713-2722, May **2005.**
[277] I. R. Sitepu, L. Ignatia, A. K. Franz, D. M. Wong, S. A. Faulina, M. Tsui*, et al.*, "An improved high-throughput Nile red fluorescence assay for estimating intracellular lipids in a variety of yeast species," *Journal of Microbiological Methods,* vol. 91, pp. 321-328, Nov **2012.**
[278] G. Genicot, J. L. M. R. Leroy, A. Van Soom, and I. Donnay, "The use of a fluorescent dye, Nile red, to evaluate the lipid content of single mammalian oocytes," *Theriogenology,* vol. 63, pp. 1181-1194, Mar 1 **2005.**
[279] V. Sanna, A. M. Roggio, A. M. Posadino, A. Cossu, S. Marceddu, A. Mariani*, et al.*, "Novel docetaxel-loaded nanoparticles based on poly(lactide-co-caprolactone) and poly(lactide-co-glycolide-co-caprolactone) for prostate cancer treatment: formulation, characterization, and cytotoxicity studies," *Nanoscale Research Letters,* vol. 6, p. 260, **2011.**
[280] P. Jonsson, M. P. Jonsson, J. O. Tegenfeldt, and F. Hook, "A Method Improving the Accuracy of Fluorescence Recovery after Photobleaching Analysis," *Biophysical Journal,* vol. 95, pp. 5334-5348, Dec 1 **2008.**

[281] A. M. M. Sadeghi, F. A. Dorkoosh, M. R. Avadi, M. Weinhold, A. Bayat, F. Delie*, et al.*, "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,* vol. 70, pp. 270-278, Sep **2008.**

[282] J. Takishima, H. Onishi, and Y. Machida, "Prolonged intestinal absorption of cephradine with chitosan-coated ethylcellulose microparticles in rats," *Biological and Pharmaceutical Bulletin,* vol. 25, pp. 1498-502, Nov **2002.**

[283] C. Caramella, F. Ferrari, M. C. Bonferoni, S. Rossi, and G. Sandri, "Chitosan and its derivatives as drug penetration enhancers," *Journal of Drug Delivery Science and Technology,* vol. 20, pp. 5-13, Jan-Feb **2010.**

[284] V. P. Torchilin, "Drug targeting," *European Journal of Pharmaceutical Sciences,* vol. 11 Suppl 2, pp. S81-91, Oct **2000.**

[285] L. A. Frank, G. Sandri, F. D'Autilia, R. V. Contri, M. C. Bonferoni, C. Caramella*, et al.*, "Chitosan gel containing polymeric nanocapsules: a new formulation for vaginal drug delivery," *International Journal of Nanomedicine,* vol. 9, pp. 3151-61, **2014**.

[286] R. V. Contri, T. Katzer, A. F. Ourique, A. L. da Silva, R. C. Beck, A. R. Pohlmann*, et al.*, "Combined effect of polymeric nanocapsules and chitosan hydrogel on the increase of capsaicinoids adhesion to the skin surface," *Journal of Biomedical Nanotechnology,* vol. 10, pp. 820-30, May **2014.**

[287] M. Gaumet, R. Gurny, and F. Delie, "Localization and quantification of biodegradable particles in an intestinal cell model: The influence of particle size," *European Journal of Pharmaceutical Sciences,* vol. 36, pp. 465-473, Mar 2 **2009.**

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2. Formulation development, stability and anticancer efficacy of core-shell cyclodextrin nanocapsules for oral chemotherapy with camptothecin, Beilstein Journal of Organic Chemistry, Submitted, July 2014 (Under Review)

Oral and Poster Presentations

1. 17th International Pharmaceutical Technology Symposium: IPTS 2014, September 2014, Antalya, Turkey (Poster presentation)

2. 10th Nanoscience and Nanotechnology Conference (NANOTR-10), June 2014, İstanbul, Turkey (Oral presentation)

3. 3rd European Conference on Cyclodextrins, October 2013, Antalya, Turkey (Poster Presentation)

4. 2nd International Summer School on Cyclodextrins 2013, May 2013, Giardini Naxos (ME), Italy

5. 9th Central European Symposium on Pharmaceutical Technology: CESPT 2012, September 2012, Dubrovnik, Croatia (Poster presentation)

6. 16th International Pharmaceutical Technology Symposium: IPTS 2012, September 2012, Antalya, Turkey (Poster presentation)

7. 8th Nanoscience and Nanotechnology Conference (NANOTR-8), June 2012, Ankara, Turkey (Poster presentation)

ANNEXES

1. 2nd Open Forum on Pharmaceutics and Biopharmaceutics TUFTAD-CAPSUGEL, April 2012, İstanbul, Turkey
2. CRS Nordic Chapter Meeting, June 2012, Reykjavic, Iceland **(Poster presentation)**
3. 8th Nanoscience and Nanotechnology Conference (NANOTR-8), June 2012, Ankara, Turkey **(Poster presentation)**
4. 16th International Pharmaceutical Technology Symposium: IPTS 2012, September 2012, Antalya, Turkey **(Poster presentation)**
5. 9th Central European Symposium on Pharmaceutical Technology: CESPT 2012, September 2012, Dubrovnik, Croatia **(Poster presentation)**
6. 3rd European Conference on Cyclodextrins, October 2013, Antalya, Turkey **(Poster Presentation)**
7. 2nd International Summer School on Cyclodextrins 2013, May 2013, Giardini Naxos (ME), Italy
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Formulation and in vitro characterization of oral polycaprolactone nanocapsules intended for breast cancer therapy

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Nanocapsules are vesicular systems in which a drug is confined in a cavity consisting of an inner liquid core surrounded by a polymeric membrane[1]. Due to their high loading capacity for lipophilic drugs and decreased toxicity with targeting properties, nanocapsules have potential application in cancer therapy. The aim of this study was to design oral nanocapsules for poorly soluble anticancer drugs using polycaprolactone nanocapsules. For this reason, nanoprecipitation method was investigated with changing variables such as the amount of surfactant, ratio of organic to aqueous phase and molecular weight of polymer.

The preparation of nanocapsules by nanoprecipitation method was performed with 5mg PCL (MW 14.000, 65.000 and 80.000) and 50 µl Miglyol 812 dissolved in 5 ml aceton, injected to an aqueous phase (5ml,10 ml,20ml) containg hydrophilic surfactant Polysorbate 80 (%0,1, %0,5 and %1)(v/v) under magnetic stirring. Organic solvent was then evaporated under vacuum.

Mean particle size, polydispersity index and zeta potential of nanocapsules were measured by using Malvern Zetasizer with Low Angle Laser Light Diffraction technique. Mean particle size, polydispersity index and zeta potential of nanocapsules were reported to be 214nm, -10,5mV and 0,16 for the formulation containing %0,1(v/v) surfactant, 183nm, -6,62mV and 0,11 for the formulation containing PCL MW 14,000 and 224nm, -15V and 0,101 for the formulation with organic to aqueous phase ratio 1:2 respectively. According to this study, optimum formulation is yielded with PCL MW 14,000, the ratio of organic phase to aqueous solution 1:2(v/v) and %0,1(v/v) hydrophilic surfactant by using nanoprecipitaton method. In conclusion, preparation variables such as concentration of stabilizer, molecular weight of polymer, ratio between aqueous and organic phases proved to be important factors for formulation of nanocapsules with appropriate in vitro properties.

References

1. C.E. Mora-Huertas et al. Polymer-based nanocapsules for drug delivery. Int J Pharm 2010; 385:113–142

[Abstract:0506][PP-270][D-1 Targeting Delivery System]

Chitosan coated Oral Polycaprolactone Nanocapsules for Cancer Therapy: Effect of Formulation Parameters on Particle Size

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Nanocapsules are vesicular systems in which a drug is confined in a cavity consisting of an inner liquid core surrounded by a polymeric membrane. Due to their high loading capacity for lipophilic drugs and decreased toxicity with targeting properties, nanocapsules have potential application in cancer therapy. The advantages of using nanoparticles for drug delivery are a result of two main basic properties: small size and use of biodegradable materials. Nanoparticles, because of their small size, can extravasate through the endothelium in inflammatory sites, epithelium (e.g., intestinal tract and liver), tumors, or penetrate microcapillaries. Positively charged colloidal drug carriers have shown interesting properties with respect to the negatively charged systems: they have improved stability in the presence of biological cations and their interaction with negatively charged biological membranes is facilitated. In this sense, it is believed that positively charged drug carriers will be favorable due to their facilitated interaction with epithelia and improved capacity for the transport of drugs.

The aim of this study is to design oral cationic nanocapsules for poorly soluble anticancer drugs and investigate formulation parameters on particle size.For this reason, nanoprecipitation method was investigated with changing formulation parameters such as the amount of surfactant, concentration of oil, ratio of organic to aqueous phase and molecular weight and concentration of polymer.

The preparation of nanocapsules by nanoprecipitation method was performed with the polymer,Polycaprolactone (concentration varies from %0,05-%0,5) and oil, Miglyol 812 (concentration varies from % 0,3-%1) dissolved in organic solvent (aceton), injected to an aqueous phase containing hydrophilic surfactant, Polysorbate80 (concentration varies from % 0,1-% 1) under magnetic stirring. Finally organic solvent was evaporated under vacuum. For each formulation, the amount of surfactant, concentration of oil, ratio of organic to aqueous phase and molecular weight and concentration of polymer were investigated by changing these variables. The optimal formulation was selected and then coated with positively charged polymer, Chitosan to obtain cationic nanocapsules.

Mean particle size, polydispersity index and zeta potential of nanocapsules were measured by using Malvern Zetasizer with Low Angle Laser Light Diffraction technique. Mean particle size, polydispersity index and zeta potential of nanocapsules were reported to be 214nm, -10,5mV and 0,16 for the formulation containing %0,5(v/v) surfactant; 173nm, -7,87mV and 0,087 for the formulation containing %0,3(v/v) oil; 215nm, - 5,62 mV and 0,122 for the formulation containing %0,1 (w/v) polymer; 183nm, -6,62mV and 0,11 for the formulation containing PCL MW 14,000 and 224nm, -15V and 0,101 for the formulation with organic to aqueous phase ratio 1:2 respectively.

According to characterization results, optimum formulation parameters were selected that's PCL concentration %0,1,(w/v), oil concentration %0,3 (v/v), PCL MW 65,000 (g/mol), ratio of organic phase to aqueous solution 1:2(v/v) and hydrophilic surfactant concentration %0,5(v/v) in order to coat nanocapsules with %0,05 (w/v) Chitosan. Finally, the optimal chitosan coated nanocapsule formulation is yielded with mean particle size 281 nm, polydispersite index 0,251 and zeta potential +20,1 mV respectively. It was also found that prepared nanocapsules are stable in solution for three months under natural conditions.

Keywords: cancer, nanocapsule, polycaprolactone


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Formulation and *in Vitro* Characterization of Oral Polycaprolactone Nanocapsules in Breast Cancer Therapy

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Introduction

Nanocapsules are vesicular systems in which a drug is confined in a cavity consisting of an inner liquid core surrounded by a polymeric membrane(1). Due to their high loading capacity for lipophilic drugs and decreased toxicity with targeting properties, nanocapsules have potential application in cancer therapy. The advantages of using nanoparticles for drug delivery are a result of two main basic properties: small size and use of biodegradable materials. Nanoparticles, because of their small size, can extravasate through the endothelium in inflammatory sites, epithelium (e.g., intestinal tract and liver), tumors, or penetrate microcapillaries.(2).

The aim of this study was to design oral nanocapsules for poorly soluble anticancer drugs using polycaprolactone nanocapsules. Positively charged colloidal drug carriers have shown interesting properties with respect to the negatively charged systems: they have improved stability in the presence of biological cations and their interaction with negatively charged biological membranes is facilitated. in this sense, it is believed that positively charged drug carriers will be favorable due to their facilitated interaction with epithelia and improved capacity for the transport of drugs (3).The aim of this study is to design oral cationic nanocapsules for poorly soluble anticancer drugs and investigate formulation parameters on particle size. for this reason, nanoprecipitation method was investigated with formulation parameters such as surfactant concentration, oil volume, ratio of organic to aqueous phase and molecular weight and concentration of polymer.

.

Materials and Methods

Materials

The polymer, Polycaprolactone (MW:14kDa, MW:65kDa, MW: 80kDa) was purchased from Aldrich, the oil, Miglyol 812(Capric/Caprilic Triglyceride) was purchased from Condea Chemie GmbH, surfactant Polysorbate 80 was purchased from Merck and Chitosan (Protosan ® G113, MW<200 kDa, deacetylation degree 75-90%) was purchased from FMC Biopolymers, Norway.

Preparation of blank nanocapsules

The preparation of blank nanocapsules was performed with nanoprecipitation method. Briefly, the polymer,Polycaprolactone (concentration varies from %0,05-%0,5) and oil, Miglyol 812 (concentration varies from % 0,3-%1) dissolved in organic solvent (aceton), injected to an aqueous phase containing hydrophilic surfactant, Polysorbate80 (concentration varies from % 0,1-% 1) under magnetic stirring. Finally organic solvent was evaporated under vacuum. for each formulation, the amount of surfactant, concentration of oil, ratio of organic to aqueous phase and molecular weight and concentration of polymer were investigated by changing these variables. The optimal formulation was selected and then coated with positively charged polymer, Chitosan to obtain cationic nanocapsules.

Characterization of blank nanocapsules

Particle size distributions (mean diameter (nm), polydispersity index) and zeta potential (mV) of nanocapsules were determined by using Malvern Zetasizer Nano-ZS (Malvern Instruments, Malvern UK). Measurement were realized in triplicate at 12° angle at 25° C.

Results and Discussion

Mean particle size, zeta potential and polydispersity index of blank polycaprolactone nanocapsules were reported to be respectively 214nm, -10,5mV and 0,16 for the formulation containing %0,5(v/v) surfactant; 173nm, -7,87mV and

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Preparation and characterization of oral PCL nanocapsules loaded with anticancer drug exemestane

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KEYWORDS

nanocapsule; oral; exemestane; breast cancer; polycaprolactone

INTRODUCTION

Exemestane is a potent third generation steroidal aromatase inactivator used in the treatment of advanced breast cancer in postmenopausal women [1]. It is practically insoluble in water (0.08 mg/mL), has low oral bioavailability and high hydrophobicity classified as a class IV drug in the Biopharmaceutics Classification System (BCS) [2]. Nanocapsules are vesicular systems in which a drug is confined in a cavity consisting of an inner liquid core surrounded by a polymeric membrane [3]. Due to their high loading capacity for lipophilic drugs and decreased toxicity with targeting properties, nanocapsules have potential application in cancer therapy. The aim of this study was to design oral nanocapsules for poorly soluble anticancer drug exemestane. For this reason, nanoprecipitation method was investigated with changing variables such as the amount of surfactant, ratio of organic to aqueous phase and molecular weight of polymer. The preparation of nanocapsules was performed with the organic phase containing polymer (PCL), oil (Miglyol 812), drug (exemestane) and acetone, injected to an aqueous phase containing hydrophilic surfactant (Polysorbate 80) under magnetic stirring. Organic solvent was then evaporated under vacuum. For each formulation, the amount of surfactant, ratio of organic to aqueous phase and molecular weight of polymer were investigated by changing these variables.

RESULTS & DISCUSSION

In vitro characteristics of nanocapsules were then investigated. Mean particle size, polydispersity index and zeta potential of blank nanocapsules were reported to be 214 nm, 0.16 and -10.5 mV for the formulation containing 0.1% (V/V) surfactant, 183 nm, 0.11 and -6.62 mV for the formulation containing PCL MW 14,000 and 224 nm, 0.101 and -15 mV for the formulation with organic to aqueous phase 1:2, respectively. According to this study, optimum formulation is yielded with PCL MW 14,000, the ratio of organic phase to aqueous solution 1:2 (V/V) and 0.1% (V/V) hydrophilic surfactant by using nanoprecipitation method. In conclusion, poorly soluble anticancer drugs can be easily encapsulated in nanocapsules for targeted delivery and thus more effective in cancer therapy rather than conventional methods.

REFERENCES

[1] Jukanti, R. et al. (2011) Enhanced bioavailability of exemestane via proliposomes based transdermal delivery. J Pharm Sci 100: 3208-3222. [2] Singh, A.K. et al. (2008) Exemestane loaded self-microemulsifying drug delivery system (SMEDDS): Development and optimization. AAPS PharmSciTech 9 (2). [3] Mora-Huertas, C.E. et al. (2010) Polymer-based nanocapsules for drug delivery. Int J Pharm 385: 113-142.


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ORAL CYCLODEXTRIN NANOCAPSULES FOR CAMPTOTHECIN DELIVERY: FORMULATION DEVELOPMENT AND IN VITRO EVALUATION

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Nanoparticulate drug delivery systems emerge as promising delivery systems destined to solve the problems in chemotherapy. Most of the anticancer drugs developed recently are very poorly soluble in water and therefore nanocapsules can be effective carriers for these lipophilic drugs with their oily core, solubilizing the drug for eventual absorption. Although intravenous infusion for chemotherapy is more common, it is believed that oral chemotherapy is a milestone in cancer treatment. In terms of patient compliance, oral chemotherapy is a practical, self-applied, painless therapy. The fact that it does not require application in a health center with professional personnel reduces the reimbursement cost to the health budget.

The aim of this study was to design oral cyclodextrin nanocapsules loaded with anticancer agent Camptothecin maintaining drug stability in the body and improving its eventual bioavailability. For this reason, amphiphilic cyclodextrin permodified on the primary face (6-O-CAPRO-ß-CD) was used as core polymer. Nanocapules prepared from 6OCAPRO were coated with cationic polymer Chitosan to improve cellular uptake and interaction with biological membranes through positive surface charge. Nanocapsules were characterized for their in vitro properties followed by cell culture studies evaluating anticancer efficacy and permeability in comparison to camptothecin in solution form. Morphologies of nanocapsules were determined by scanning electron microscopy(SEM). Concentration of Camptothecin entrapped in nanocapsules was determined by reverse phase HPLC and the drug encapsulation efficiency (EE) was then calculated. In vitro camptothecin release study was performed with dialysis bag method under sink conditions.

According to pre-formulation results; mean particle size of both anionic and cationic CPT loaded nanocapsules was between 187nm and 204 nm. Their polydispersity index values were under 0,4 and zeta potential values vary from -31,4 mV to +18,3 mV. 20 % of CPT was released from nanocapsules in first 2 hours followed by a slower release profile of the drug for over 72 hours. Characterization results suggested that camptothecin loaded 6-O-CAPRO-ß-CD nanocapsules with appropriate formulation parameters might be employed as a potential approach for oral drug delivery for anticancer drugs such as Camptothecin with stability and bioavailability problems.

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Keywords: amphiphilic cyclodextrin, camptothecin, core-shell, nanocapsule, oral

References:

Duchêne D., Nemati F., Puisieux F., ,Skiba M., Wouessidjewe D., Spontaneous formation of drug-containing amphiphilic β-cyclodextrin nanocapsules, International Journal of Pharmaceutics, 145, 241-245

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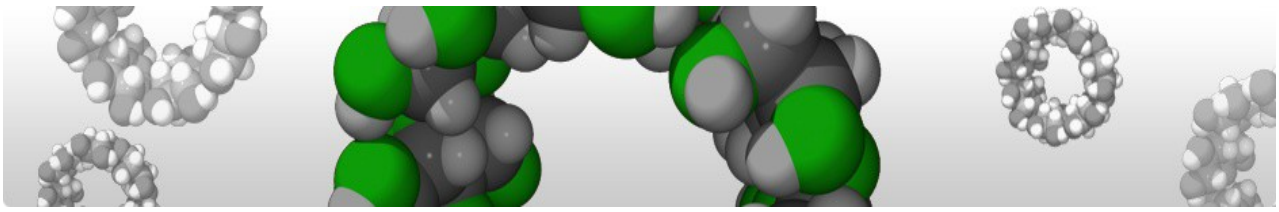

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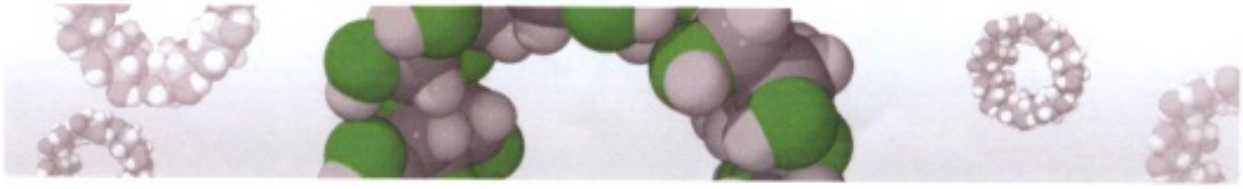

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Certificate of attendance

We herein certify that Ural Hele ..... attended the Second International Summer School on Cyclodextrins 2013, that took place in Giardini Naxos (ME) -Italy- from May 8 to May 9, 2013.

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YEDITEPE UNIVERSITY

Dear Hale Unal,

It is our pleasure to invite you to present your research at the 10th Nanoscience and Nanotechnology Conference of Turkey (NanoTR10) which will be held on June 17-21, 2014, in Istanbul, Turkey. The details of your contrubituion as viewed by our scientific committee is summarized as follows:

Presentation Type: Oral

Abstract ID: 547

Title of abstract: Core-shell hybrid nanocapsules for oral delivery of Camptothecin: Formulation development, in vitro and in vivo evaluation

As we believe that your valuable contribution will enhance the scientific athmosphere of the conference we are looking forward to see you in Istanbul for NanoTR10.

Sincerely yours,

Prof. Dr. Mustafa Çulha
Conference Chairman

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COMPARATIVE IN VITRO AND IN VIVO EVALUATION OF CAMPTOTHECIN LOADED ORAL NANOCAPSULES

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INTRODUCTION

Oral chemotherapy is a key step for chemotherapy at home. It is patient friendly, less stressful due to the less hospital visits and painless, easy for self-medication. Compared to parenteral delivery it is also cheaper to produce because it does not need to be manufactured under sterile conditions resulting in cost saving in terms of time and labour as well.(1) Camptothecin is a very effective anticancer agent against a wide spectrum of cancers such as colon, breast, ovarian, lung cancers in in vitro cell culture studies (2). However it's poor aqueous solubility and pH-dependent stability problem results in the diminishing of clinical efficacy for the drug (3). The objective of this study was to design and in vitro-in vivo evaluate oral nanocapsules prepared from amphiphilic cyclodextrins (CD) and poly-ε-caprolactone (PCL) with anionic or catinonic surface charge for the effective oral delivery of model drug Camptothecin (CPT).

MATERIALS AND METHODS

Materials

6-OCAPRO-ß-CD (MW: 1820 g/mol) has been synthesized, purified and characterized. 20 (S)-(+)-Camptothecin (95 % HPLC powder, MW:348.35 g/mol) was purchased from Sigma&Aldrich. Protasan™ (UP G-113) was purchased from Novamatrix. Poly-ε-caprolactone (PCL) (Mn: 65,000 Da) was purchased from Aldrich (St. Louis, MO, USA). All other chemicals used were of analytical grade and obtained from Sigma & Aldrich.

Preparation and in vitro characterization of Camptothecin loaded Nanocapsules

Both anionic and cationic PCL and CD nanocapsules were prepared by a modified nanoprecipitation method. Mean particle size and polydispesity index (PDI) of the CPT nanocapsules were determined by dynamic light scattering (DLS) (Malvern Zetasizer Nano ZS series, UK) and zeta potential of nanocapsules was measured in mV with Malvern Zetasizer Nano ZS. The morphology of nanocapsules was determined by using Scanning Electron Microscope (FEI Nova™ Nano SEM 430, ABD). Content of CPT in the nanocapsules was quantified by a previously validated HPLC method and experimental CPT loading was quantified using the peak area of each nanocapsule formulation.

Anticancer efficacy of CPT loaded Nanocapsules against MCF-7 cell line

The anticancer efficacy of CPT loaded nanocapsules was determined against MCF-7 cell line in comparision to CPT solution with MTT assay. The cells were incubated with CPT loaded 6OCAPRO and chitosan coated CPT loaded 6OCAPRO nanocapsules and CPT in DMSO solution for 72 h. Optical density (OD) was measured at 560 nm using a microplate reader.

In vitro transport studies across Caco-2 cell line

The transport of cyclodextrin nanoparticle formulations (6OCAPRO and CS-6OCAPRO) and CPT solution in DMSO were studied on Caco-2 cell line from the apical to basolateral direction in Caco-2 cells for 4 hours. Samples were taken from the basolateral side and analyzed with a validated HPLC method. Apparent permeability coefficient (Papp) was calculated.

In vivo Nanoparticle Uptake Studies

CD1 female mice (20-25 g) were grouped which based on the administration of different fluorescently labelled nanocapsule dispersions with an oral gavage. After 30 minutes, animals were killed via cervical dislocation and their intestines and stomachs were collected. The entire tissue sections were cut into 4 pieces and were placed into lysis buffer solution and homogenized on ice before analysis with Spectofluorophotometer at an emission wavelength (λ_{em}) of 590nm and excitation wavelength (λ_{ex}) of 546nm to detect fluorescence in the tissue homogenates.

RESULTS AND DISCUSSION

Mean particle size, polydispersity index zeta potential values and associated drug (%) of anionic and cationic nanocapsules formulations are presented in Table 1.