pH RESPONSIVE NANO CARRIERS FOR ANTI CANCER DRUG DELIVERY

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ABSTRACT

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In the recent years, development of various organic and inorganic nano-sized systems has gained great interests especially for cancer diagnosis and treatment and intense researches are carried out in this area. Regarding to the recent trends for drug delivery system design, the novel approaches for drug carriers are mainly based on development of smart and nano-size drug carriers which are targeted to cancer cells. Hence, for an effective tumor-targeted delivery device, besides its chemical structure further criteria such as detection of tumor site and sensitivity to the higher temperature and lower pH of the tumor compare to rest of the body gains importance. The aim of this study is to design and prepare polysebacic anhydride (PSA) based nanocapsules (NCs) loaded with Doxorubicin (DOX) which is an anti cancer drug. In order to obtain an intelligent delivery system, drug-loaded nanocapsules were coated with pH sensitive poly (L-histidine). PSA nano-carriers were firstly loaded with DOX and then in order to introduce pH sensitivity, they were coated with poly (L-histidine). PLH-coated NCs were modified with polyethylene glycol (PEG) to prevent their macrophage uptake. Drug release profile from this system was examined in two different buffer solutions prepared as acidic (pH 4) and physiological (pH 7.4) media. The physical and chemical properties of the nano particles were characterized by Fourier transform infrared spectroscopy (FTIR), dynamic light scattering (DLS), ultraviolet and visible absorption spectroscopy (UV-VIS), and scanning electron microscopy (SEM). In vitro studies of the prepared nanocapsules were performed on MDA-MB-231 breast cancer cells by using WST Kit 8 cell viability test. In order to obtained results, pH sensitive nanocapsules with size 230 nm exhibited cellular uptake and promising intracellular release of drug.

Keywords: Polysebacic anhydride, Poly (L-histidine), pH responsive, Nanocapsule, Drug carrier, Doxorubicin

ÖZ

pH DUYARLI ANTI KANSER İLAC TAŞIYICI NANOSİSTEMLER

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Son yıllarda, özellikle kanser hastalığında teşhis ve tedavi amaçlı kullanılmak üzere, nano boyutta çeşitli organik ve inorganik sistemlerin geliştirilmesi büyük önem kazanmıştır ve bu konuda yoğun araştırmalar yapılmaktadır. İlaç taşıyıcı sistemlerindeki son gelişimlerini göz önüne alırsak, özgün ilaç taşıyıcı yaklaşımlar, akıllı nano-boyutlu taşıyıcıların kanser hücrelerine hedeflenmesi yönündedir. Bu nedenle, etkin tümör-hedefli sistem için, taşıyıcının kimyasal yapısının yanı sıra, tümör tespit edebilme, vücüdün diğer kısımlarına göre tümörün daha yüksek olan sıcaklık ve daha düşük olan pH duyarlılığı gibi diğer kriterler de önem taşımaktadır. Bu çalışmanın amacı, Doksorubisin (DOX) kanser ilacı yüklü polisebasik anhidrit (PSA) nanokapsüllerin tasarlanması ve hazırlanmasıdır. Akıllı ve pH duyarlı bir nano ilaç taşıyıcı sistem elde etmek amacıyla ilaç yüklü kapsüllerin poli (L-histidin) (PLH), pH duyarlı molekül ile kaplanmasıdır. PSA nano taşıyıcılara önce DOX ile yüklenmiş ve sonra pH duyarlılığı için nano taşıyıcılar poli (L-histidin) ile kaplanmış. PLH ile kaplanmış nanokapsüllerin makrofagositozunu engellemek için kapsüller polietilen glikol (PEG) ile modife edilmiştir. Bu sistemlerden ilaç salım profili, asidik (pH 4) ve fizyolojik (pH 7.4) olarak hazırlanan iki farklı pH tampon çözelti ortamında incelenmiştir. Nanokapsüllerin fiziksel ve kimyasal özellikleri, Fourier dönüşümlü kızılötesi spektroskopisi (FTIR), dinamik ışık saçılım spektrometresi (DLS), ultraviyole ve görünür ışık absorpsiyon spektroskopisi (UV-VIS), ve taramalı elektron mikroskobu (SEM) ile karakterize edilmiştir. Hazırlanan nanokapsüllerin In vitro çalışmaları MDA-MB-231 meme kanser hücrelerin üzerinde WST Kit 8 hücre canlılık test ile yapılmıştır. Elde edilen sonuçlara göre, 230 nm boyutundakı pH duyarlı nanokapsüller hücre içine alınabilmek ve umut veren intraselüler ilaç salım özelliklerine sahiptir.

Anahtar Sözcükler: Polisebasik anhidrit, Poli (L-histidin), pH duyarlılık, Nanokapsül, İlaç taşıyıcı, Doksorubisin

To my husband for his endless patience and encouragement

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LIST OF ABBREVATIONS

AFM	Atomic force microscopy
ATCC	American Type Culture Collection
CNS	Central Nervous System
DAPI	4',6-diamidino-2-phenylindole
DCM	Dichloromethane
DPPC	Dipalmitoyl phosphatidylcholine
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
DLS	Dynamic Light Scattering
EA	Ethyl Acetate
EE	Encapsulation Efficiency
EPR	Enhanced Permeability and Retention
FDA	Food and Drug Administration
FTIR	Fourier Transform Infrared Spectroscopy
GPC	Gel Permeation Chromatography
HIV	Human Immunodeficiency Virus
H-NMR	Proton Nuclear Magnetic Resonance
HPV	Human Papillomavirus
HTLV	Human T-cell Lymphoma Virus
LC	Loading Capacity
MW	Molecular Weight
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltertrazolium bromide
NCs	Nanocapsules
PBS	Phosphate Buffered Saline
PDI	Poly Dispersity Index
PEG	Polyethylene Glycol
PFA	Paraformaldehyde
PLH	Poly (L-histidine)
PLL	Poly L-lysine
PSA	Polysebacic Anhydride
PVA	Polyvinyl Alcohol
PVP	Polyvinyl Pyrrolidone
RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute
SEM	Scanning Electron Microscopy
T_t	Transition temperature
TPA	12-O-tetradecanoyl-phorbol-13-acetate
T80	Tween 80
UV-VIS	Ultraviolet and Visible Absorption Spectroscopy
WHO	World Health Organization
XPS	X-ray photoelectron spectroscopy

CHAPTER 1

INTRODUCTION

The pharmacological response obtained from a medicine is extremely dependent on its concentration in the blood as well as its activity in the action site. In traditional drug administration via parenteral roots, pharmaceutical agent is distributed in whole of the body by blood circulation. The cases where notable amount of drug is eliminated by liver and kidneys cause reduction in the concentration of drug in plasma (Hagenbuch et al., 2010). Usually higher concentration of drug usually is applied to compensate the eliminated amount of drug. There is no doubt that, higher concentration leads unwanted side effects especially in therapies with cytotoxic agents such as chemotherapy drugs applied in cancer treatment. There are various studies to find a way to circumvent this problem and achieve better therapeutic effects. Drug delivery is a promising method to increase the bioavailability of a drug in the body. It generally is investigated for different purposes such as designing sustained release, hiding of biochemical agents from macrophages and targeting of medicines to specific site (Uhrich et al., 1999).

1.1 Drug Delivery

The goal of drug delivery systems is to maximize the therapeutic effect of the drug and minimize the related adverse effects. Drug carriers not only influence pharmacokinetic of the drug, but also improve its biodistribution in the body (Uhrich et al., 1999). The ideal drug delivery system possesses a wide range of properties such as biocompatibility, ease of fabrication, low cost and high loading capacity. Controlled release systems have been designed to achieve effective drug concentration for long period of time. In drug administration via traditional formulation ways, the drug level in blood exhibits the profile as shown in Figure 1.1-a. In these types of oral or injection administrations, the drug level in blood rises after each administration and then decreases until the next dose. In this case, the concentration of drug in the blood may go over the toxic level subsequently after drug administration, and reduces to below of effective dose during the time.

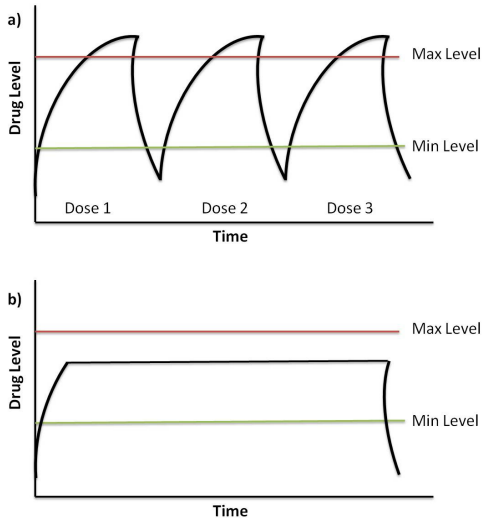


Figure 1-1 Drug levels in the blood plasma. (a) traditional drug dosing, (b) controlled-delivery dosing.

In order to prevent the concentration to go to toxic level, the applied dose can be kept low, but in this case the number of applications should be high. Getting an oral dose or injection in every two or three hours is not suitable. Therefore, controlled drug delivery systems have been designed for long-term administrations. In this manner, the drug concentration in the blood is kept constant in effective dose which is between minimum and maximum levels as it is shown in Figure 1-1-b (Park et al., 1992). Various delivery systems and formulations, either particles or devices have been produced aiming an effective drug delivery. In order to achieve a controlled release, drug can be entrapped in a matrix (Figure 1-2-a), encapsulated within a thin polymeric membrane (Figure 1-2-b) or directly bonded to polymer chains (Figure 1-2-c) (Zamboni, 2005; Jain, 2005).

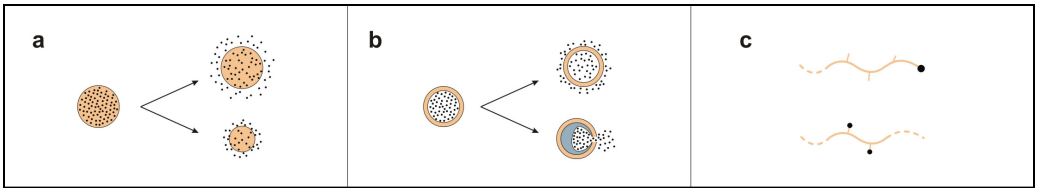


Figure 1-2 Interaction and location of drug in delivery systems. a) restrain in a matrix, b) encapsulation in a membrane, c) attachment to a polymer chain.

Polymeric carriers either natural or synthetic are known as promising materials for preparation of controlled and targeting delivery systems. These systems are able to release of the drug over an extended period of time or at a specific moment and at the specific region. The polymers applied for drug delivery devices are categorized into two part; biodegradable and non-biodegradable. Biodegradable polymers widely investigated in delivery vehicles are polylactide (Amjadi et al., 2012), polyglycolide and its derivations (Garvin et al., 2005), polyacrylate (Skwarczynski et al., 2010) and polycaprolactone (Aishwarya et al., 2008). In the case of non-biodegradable polymers, polyacrylics (Qiu et al., 2001), polystyrene (Canal et al., 2012), and polyesters (Sairam et al., 2007) can be mentioned as commonly used polymers. These polymers were studied in the preparation of different shape and size of delivery systems such as macro-size devices (Elman et al., 2009), film form systems (Noel et al., 2008), micro and nano-size particles (Majeti et al., 2000).
In the last few decades significant attempts have been done to micronization of drug delivery vehicles. Recently various types of submicron drug delivery systems have been developed due to their unique advantages such as relatively high intracellular uptake, high efficiency in targeting to objective tissue and not requiring any injury during the application.

1.1.1 Nano-size drug delivery systems

The theory of polymeric nanoparticles is base on design a delivery vesicle with higher biodistribution in the body. Polymer based nanoparticles have been developed to deliver various agents such as peptides as large molecules (Ma et al., 2002), chemotherapeutic agents as small molecules (Bertin et al., 2005; Mo et al., 2005), and genes as biological agents (Huang et al., 2005). Submicron particles demonstrate numerous benefits in compare to micro-size particles. For instance they exhibit relatively high intracellular uptake over microparticles. It was observed that nanoparticles with fine size about 100 nm have been up taken by Caco-2 cells 2.5 and 6 fold higher in compared to particles larger than 1 µm and 10 µm respectively (Desai et al., 1997). In another publication it has been reported that for gold nanoparticles with diameter between 14 nm and 100 nm, particles with 50 nm diameters are taken up by the HeLa cells more efficiently in compare to others (Chithrani et al., 2006).
Nanoparticles provide opportunity to administer of poorly water soluble drugs in aqueous body medium. As an illustration bioavailability of paclitaxel, a hydrophobic anti cancer drug, has been developed by incorporation into poly (lactic-co-glycolic acid) nanoparticles (Mo et al., 2005). It has been also observed that peptide drugs isolated in nanoparticles exhibit more resistance to enzymatic or chemical degradation during the administration (Ma et al., 2005), which results prolonged availability in the body (Luo et al., 2006).

Nano-scale delivery vehicles may be classified to some main categories due to their different properties. However, the major classification is related to the particle shapes coming from preparation process. They may be prepared as a capsule or sphere. Nanospheres are solid matrices containing molecules adsorbed at the surface of sphere or entrapped within the particle (Figure 1-2-a) (Vauthier et al., 2000). Nanocapsules are a class of submicron particles composed of a core either liquid or solid surrounded by a polymeric solid wall (Figure 1-2-b) (Sílvia et al., 2010).
The main advantage of nano-size delivery vehicles is their possibility to target the active agent to the proper tissue after intravenous administration. This is very important in the case of therapy with cytotoxic agents such as anti-cancer drugs. Chemotherapy agents using in cancer treatment exhibit serious side effects coming from their non selectivity actions. Therefore, the advanced approach in drug delivery is concentrated on the design of intelligent delivery systems for targeting a biochemical or chemical active agent to specific site (Langer et al., 1998). By this way drug would be accumulated around the objective tissue and would be prevented to distribute in the rest of the body parts. Targeting of chemotherapeutic drugs directly to tumor site leads more effect, and as a result, administration of small amount of drug will have promising effect to improve the patient's life and its quality. Therefore targeting of active agents in nano-size delivery systems has a very important place in cancer treatment.

1.2 Cancer

Cancer is a disease characterized by uncontrolled and fast growth of abnormal cells. According to statistical data reported in a global cancer statistic report, due to the count of cancer deaths reported annually by the World Health Organization (WHO), 12.7 million cancers have been diagnosed and 7.6 million cancer patients died in worldwide only in 2008 (Jemal et al., 2011).
There are various internal and external factors causing to cancer. Tobacco, diet, chemicals, radiation and infectious organisms can be mentioned as important external parameters causing cancer. For instance carcinogenic-termed chemical compounds cause DNA alteration which is a key factor for generation of cancer. The cells usually efforts to repair and correct the defected altering, but if they cannot repair the changes on DNA, it will be left uncorrected leading to convert normal cells to cancerous cells (Lodish et al., 2000). In a similar way, ionizing radiations such as x-rays and ultraviolet radiation result cancer. The efficiency of ionizing radiation to generate cancer in human can be observed recently by enhancing in skin cancer (melanoma) in case of long-term and unprotected exposing to the sunlight (Lodish et al., 2000).
Organisms initiating infectious diseases have been known as important parameters for inducing cancer. Cervical cancer, which is second generalized cancer in worldwide is mostly resulted by Human papillomavirus (HPV) (Schiffman et al., 2007). Helicobacter pylori is a microaerophilic bacterium which has been found in the stomach of patients with chronic gastritis. Researchers have linked the presence of Helicobacter pylori bacteria to the formulation of gastric cancer. This claim is based on emerge of carcinogenic region due to sustained infection or inflammation of the gastric mucosa related to this bacteria (Masamune et al., 1999). Virus owning potential to cause cancer is called "oncovirus". Viruses causing to Hepatitis B and C (Human T-cell lymphoma virus), (HTLV) and Human Immunodeficiency Virus (HIV) are known as important reasons for liver cancer, lymphoma and leukemia respectively (Fredricks et al., 1996).
Hormones, inherited mutations and immune circumstances have been explored as internal key factors to start cancer generation. Women who are exposure to early menstruating or late menopause have more produced estrogen during their lifetimes. It has been reported that this group is highly at risk of breast cancer. For this reason, the clinical trial therapy by estrogen plus progestin has been stopped in recent years due to high risk of breast cancer (Chlebowski et al., 2009). Sometimes it has been observed that combination of both internal and external factors accelerate cancer entity progress (Anand et al., 2008).

1.3 Cancer treatment

The type of the applied cancer treatment is usually selected due to the kind and stage of cancer. There are various conventional cancer treatment methods applied in the hospitals. Chemotherapy is mainly used to reduce the size of the tumor or to make it disappear. As an advanced treatment, surgery has been used to remove either the tumor or responding organ and then clean the location. High-energy radiation also has been investigated to shrink the tumor and to kill the cancer cells in a certain location. Between these methods chemotherapy is the main treatment to eliminate the cancer cells

which is often used in combination of other cancer treatments such as radiation after the application of chemotherapy (Bonetti et al., 2006).

1.3.1 Chemotherapeutics and responding side effects

Chemotherapy is a common aspect of cancer treatment by using mostly cytotoxic anti-cancer agents. Anticancer (antineoplastic) agents act on proliferation of cancerous cells via;

- destroy of DNA or block of DNA replication
- inhibition of nucleic acid synthesis
- inhibition of specific protein synthesis
- intervening to hormone balance

Mitomycin C is a type of antibiotic acting as DNA crosslinker and destructs DNA duplication. It is mostly used to treat upper gastro-intestinal and breast cancers. Prolonged administration of Mitomycin may cause to permanent bone-marrow damage. 6-mercaptopurine, methotrexate and 5-fluorouracil are antimetabolites applied as anticancer drug preventing nucleic acid synthesis. 5-fluorouracil is used in treatment of different cancers such as pancreatic, colon, rectum, head and neck cancers. As its side effect, it mainly damages to central nervous system (CNS). Paclitaxel and epipodophylotoxins have effects on protein synthesis. Cells treated by paclitaxel have been exposed to mitotic spindle assembly defects and chromosome segregation (Bharadwaj et al., 2004). Adrenal corticosteroids, estrogens and tamoxifen influence on tumor size by intervening with hormone balance. Tamoxifen is an anti estrogen substance used for hormone receptor-positive breast cancer seen in both pre and post-menopausal women (Jordan et al., 1993).
Doxorubicin (adriamycin) has been administered for various types of cancers such as ovaries, lung, breast, stomach and myeloma. It inhibits cell proliferation by inhibition of RNA synthesis. However it represents serious side effects. The main side effect of doxorubicin has been determined as cardiotoxicity causing to limit its clinical application (Chen et al., 2011).
Non selectivity of chemotherapeutic agents leads to kill cancerous cell as well as normal cells especially those cells replacing fast like as hair, skin and blood cells. Anticancer drugs also affect cells of vital organs such as liver, heart and kidney. Because of these respects, overcoming to side effects of chemotherapy agents have been main concern of cancer researchers.

1.3.2 Problems and solutions in chemotherapy delivery systems

Clinical application of chemotherapeutic agents is hampered due to some barriers such as their serious side effects, solubility, macrophage uptake and multidrug resistance. Solubility of anticancer drugs is a critical factor in their investigation either by intravenously injection or orally administration. Most of the anticancer drugs exhibit poor water solubility causing to low therapeutic effects. There are various challenges to improve solubility of hydrophobic anticancer drugs. Badjatya et al. have prepared solid dispersion paclitaxel using polyethylene glycol and Poloxamer 407. Their prepared solid particles exhibiting 14-fold enhanced dissolving compared to free drug (Badjatya et al., 2011). In another study paclitaxel has been loaded in nanogels prepared from Pluronic F127. Prepared nano-size micelles showed promising activity to increase solubility of paclitaxel by internalization of micelles into cancerous cells (Li et al., 2010).
Although investigation of chemotherapeutic agents via nano-size carriers enhances the stability and cellular uptake of drug, its application is limited due to immune system response of body. This phenomenon involves uptaking and phagocyte of particles by macrophages (Lacasse et al., 1998). Macrophages are responsible to detect, capture and digest of foreign materials passing through the blood and transfer them to liver (Soma et al., 2000). This process results accumulation of drug in unspecific site leading to serious damages of the liver. In addition, this natural response of living body causes to reduce biodistribution and availability of drugs in the blood. Plenty of researches have been reported to overcome to protect particles from the immune system. It has been observed that modification of particle by polyethylene glycol (PEG) chains can reduce their macrophage uptake (van Vlerken et al., 2007; Kanaras et al., 2002).
The other negative side of chemotherapy is resistance developed by most of cancer cells to fail the action of drug which termed as multidrug resistance. In most of solid tumors there are different population of cells, partly are responsive to drug and others are resistance. Anticancer agents can only kill cells which are sensitive to drug. Hence drug resistance cells remain alive and continue to grow faster than killing process. Drug resistance of cancer cells has been linked to molecular pumps presented in cancer cell membrane ejecting the cytotoxic compound from the cell (Gillet et al., 2010).

To enhance the activity of chemotherapeutic agents they are objected to internalize to cancer cells following to target to tumor site. Targeting of chemotherapeutic agent as a combination system is a primary strategy to fulfill internalization of delivery vehicle and intracellular release of cancer treatment agent.

1.4 Targeting delivery

During last decades, many challenges have been established to improve cancer diagnosis and treatment by targeting delivery. It was firstly considered by Paul Ehrlich hundred years ago for treatment of syphilis which is a sexually transmitted infection coming from spirochete bacterium. His theory termed as "magic bullet" represented that chemicals can be designed to bind to and also kill the specific microbes or cancer cells (Torchilin et al., 2010). Hence, as concept of chemotherapy, Salvarsan (Arsphenamine), the effective clinical treatment for syphilis, was discovered in his laboratory.

The main purpose of targeting delivery is improving the therapeutic index as well as minimizing the side effects of drugs which is more important for cytotoxic medicines such as cancer treatment agents. The nonselective toxicity of anticancer drugs has limited their clinical application near to their maximum tolerated dose. For all chemotherapy cases, the success of treatment depends on the pharmaceutic agent's ability to target and to kill the cancer cells as well as fewer damage to healthy cells (Brannon-Peppas et al., 2004). Targeting-delivery carriers have been merged to overcome to lack of selectivity of cytotoxic anticancer drugs. Targeted carriers enhance the accumulation of drug in tumor site and inhibit drug distribution in whole body. By this way more pharmaceutical effect is achieved via using less dose of drug.

In literature plenty of targeting vehicles, either active or passive, has been utilized and reported for delivery of anti-cancer agents. In active targeting drug delivery system is modified with some smart molecules such as antibodies, proteins and ligands which are able to find cancerous cells, recognize tumor-specific or tumor-associated antigens and bind to related receptors (Figure 1-3). Hence, to design this system, first we should know the tumor type, location and properties. For instance doxorubicin loaded liposomes linked to monoclonal nucleosome antibody (mAb2C5) has been studied for targeting of doxorubicin to different solid tumors (Lukyanov et al., 2004). Folic acid also is a common linker having its own receptors on surface of most cancerous cells. There is plenty of folated targeting systems reported for delivery of different anti cancer agents (Kukowska-Latallo et al., 2005; Kim et al., 2011; Zhao et al., 2008; Guaragna et al., 2012).

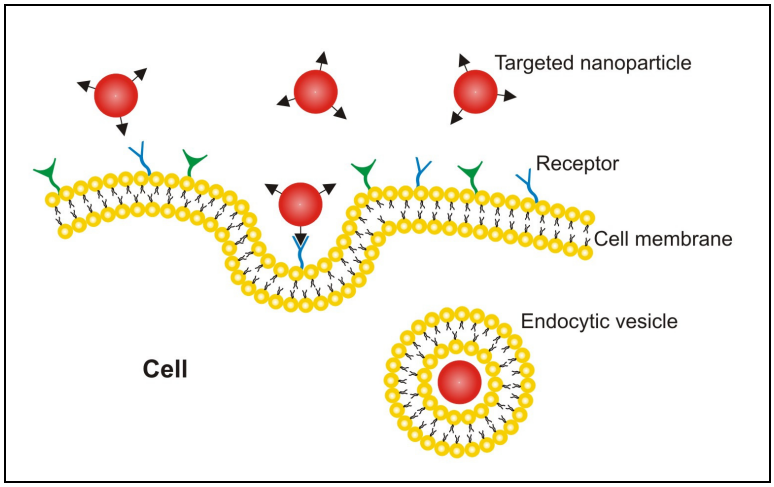


Figure 1-3 Active targeting of drugs.

In passive targeting, stimuli-responsive delivery systems are developed in order to show their bioactivity against properties or physiological changes occurring in objective site (Torchilin et al.,

2010). This physical targeting is presented by a complex drug delivery vehicle that can circulate in the body and target itself to proper tissue by responding abnormal conditions of body like variations in pH or temperature. It is known that, the pH of the blood is 7.4 but in the tumor area it shifts to acidic region around 4-5. On the other hand, fast growing of the cells in the tumor area shifts the temperature from normal body temperature of 37 ºC to about 41 ºC. By this way, chemotherapeutic compound can be entrapped or linked to a system which is designed responsive to theses variations and sent to objective tumor.

1.4.1 Stimuli responsive delivery systems

Stimuli response is a natural reflex of some materials when they present under certain conditions. This strategy has been used to design intelligent delivery systems that are able to respond to either internal physiological changes such as temperature, acidity and porosity or extrinsic conditions such as application of magnetic field, light, heat and ultra sound (Jian et al., 2009).

It is known that temperature-sensitive polymers and polypeptides display low critical solution temperature transition. They are water soluble below their transition temperature (Tt) and they exhibit an aggregation causing to water insolubility above their Tt (Figure 1-4). By this property various thermo-responsive systems have been emerged for application between body temperature (37°C) and the temperature approved for clinical hyperthermia (42°C) (Papahadjopoulos et al., 1991). It has been reported that lipids exhibiting low gel-to-liquid phase transition temperature are marked as good candidates to prepare liposome based delivery vehicles. For instance, dipalmitoyl phosphatidylcholine (DPPC) mainly used as thermosensitive lipid presents a safe gel-to-liquid phase transition temperature (41°C) (Zhu et al., 2012). It has been observed that liposomes prepared by DPPC and cholesterol release 80% of their loaded drug (methotrexate) during 30 min while by increasing the temperature from 37 to 41°C only 40% of drug is released within 24 h (Zhu et al., 2009).

Polyacrylamide is another common thermo-sensitive polymer has been used for delivery of different therapy agents. Thermally responsive poly(N-isopropyl acrylamide-co-acrylamide), P(NIPA-co-AAm), has been investigated for cancer treatment. In this research P(NIPA-co-AAm) based nanoparticles containing 5-fluorouracil were prepared and examined on S180 tumor cells (Jian et al., 2009). Hyperthermia antitumor efficiency of the prepared particles was studied on Kunming mice. Responding condition (43ºC) was achieved by using a temperature-controlled water sack. Obtained results demonstrated that hyperthermia treatment by using P(NIPA-co-AAm) based nanoparticles improved accumulation of particles in tumor site. Same copolymer also has been applied for delivery of rhodamine to ovarian tumors in athymic mice. In this work copolymer was injected to tumors heated up to 40-42ºC. According to the results, it was observed that accumulation of injected thermo-responsive copolymer at heated tumors was approximately two fold more than tumors with body temperature (Meyer et al., 2001).

Although thermo-responsive systems have been designed due to local hyperthermia in specific cells such as inflammatory or tumor cells, the actual temperature of these cells is still not mostly different than normal cells. Hence, to achieve a real thermo-sensitive system an external heat source coming from water bath (Zhu et al., 2009), high-intensity ultra sound waves (Smet et al., 2011) or light (Fu et al., 2002) is commended (Figure 1-4).

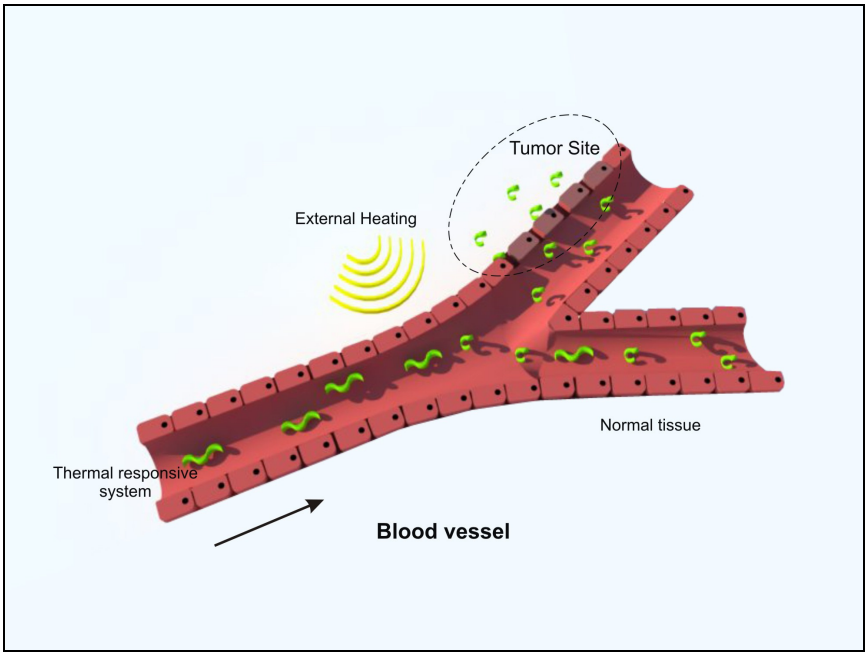


Figure 1-4 Thermal sensitive delivery systems responding to external heating.

Considering to rapid development of tumor vasculature by abnormal and poorly controlled angiogenesis leads a porous-wall vessels with pore size between 200 nm to 2 μm, and an average of 400 nm have been observed in most of solid tumors. This property causes to remain of macromolecules and lipids in the tumor site for a long time (Torchilin et al., 2010). This phenomenon has been characterized and termed the tumor-selective enhanced permeability and retention (EPR) effect of macromolecules and lipid particles (Figure 1-5).

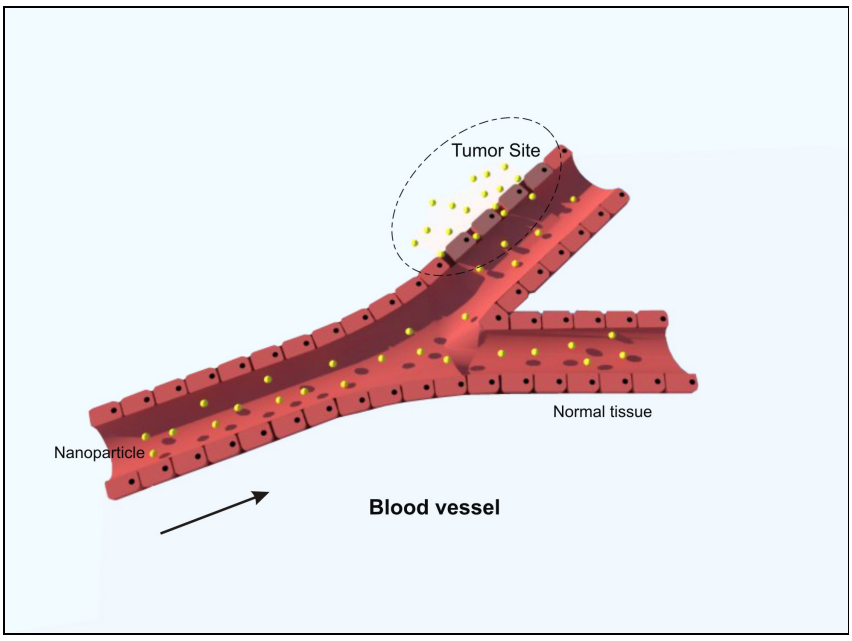


Figure 1-5 The enhanced permeability and retention effect (EPR).

The EPR effect is now regarded as a ‘‘gold standard’’ in the design of nano-sized anticancer agent delivery systems. There are many researches regarding to preparation of nano and micro-size particles investigated for different types of therapy (Hasirci et al., 2007). Some of them have been approved by United State Food and Drug Adminstration (FDA) and used in drug delivery systems and shown in Table 1.1 (Betancourt et al., 2007).

Table 1-1 Nanoparticles in market (approved by FDA, Betancourt et al., 2007).

Compound	Comercial Name	Nanocarrier	Indication
Daunorubicin	DaunoXome	Liposomes	Kaposi’s Sarcoma
Doxorubicin	Myocet	Liposomes	Breast & Ovarian cancer
Doxorubicin	Doxil/Caelyx	PEG/Liposome	Breast & Ovarian cancer
Paclitaxel	Abraxane	Albumine nanoparticles	Breast cancer

1.5 pH-responsive delivery

pH sensitive delivery systems are other category of stimulus responsive carriers. The strategy used to design these formulations is application of materials responding to pH altering in the body.
Based upon to measured pH value of most solid tumors in different patients, it was observed that pH of tumor site, dependent on tumor growth rate, is shifted to acidic region as 4-6 related to extremes amount of metabolite like as lactic acid and CO2 while normal blood pH remains constant at 7.4 (Helmlinger et al. 2002; Rofstad et al., 2006). This pathophysiology of tumors has been considered as an ideal trigger to delivery of anti cancer agents in tumor area via pH sensitive delivery systems. The strategy used to architect of these systems is investigation of components which are susceptible to pH altering. For instance macromolecules containing a bioactive function can incur to some conformational changes. Peptide molecule is a case in point for this approach. Peptide molecules exhibit different conformation in different pH. For instance 36-aa peptide, derived from the bacteriorhodopsin, is in soluble state at physiological pH while in acidic pH it transforms to an α-helix (Figure 1-6) leading to across the membrane and cell uptaking (Andreev et al., 2007; Janzso et al., 2011).

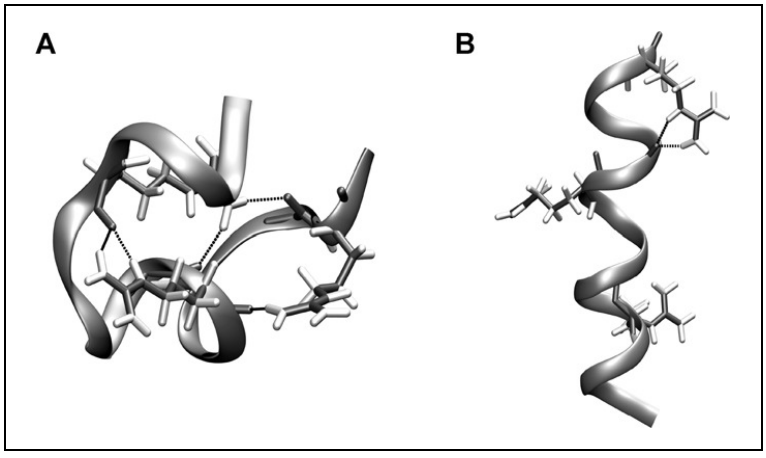


Figure 1-6 Structural conformations of polypeptides: a) a water-soluble state at physiological pH, b) α-helical conformation in acidic media.

Polyethylenimine is other example of pH sensitive molecules. This smart component has attracted researcher's attention in last decades specially for targeting delivery of siRNA in treatment of prostate cancer (Xue et al., 2001). Branched polyethylenimine has been utilized as a cationic polymer to design gene delivery vehicle due to its exhibited buffering efficiency (Zhu et al., 2010). This buffering potential arises from primary, secondary and tertiary amine groups, with different pKa values, presenting in branched polyethylenimine as it is shown in Figure 1-7. The buffering capacity and their highly positive charge in acidic medium have made them as a proton sponge for endosomal release of their encapsulated contents (Boussif et al., 1995).

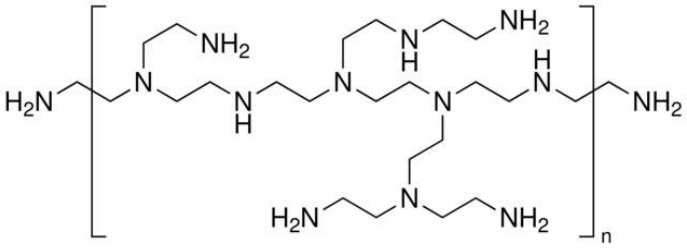


Figure 1-7 Chemical structure of branched polyethylenimine.

Poly (L-histidine) (PLH), a kind of polyamino acid, exhibits high potential to cell membrane fusion after protonation of the imidazole groups in acid medium (pH below 6). The imidazole side chain of histidine (Figure 1-8) has a pKa of approximately 6.0, and overall the amino acid has a pKa of 6.5. This means that, below a pH of 6, the imidazole ring is mostly protonated leading to hydrolysis of polymer. Therefore poly (L-histidine) is known to have an endosomal membrane disruption activity.

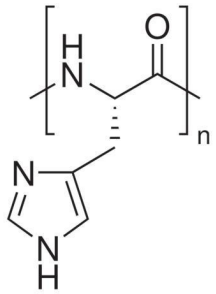


Figure 1-8 Structure of poly (L-histidine) (PLH).

There are various researches focused on application of poly (L-histidine) (PLH) for either drug or gene delivery. Bello et al. have used a polyplexes complex systems based on polylysine substituted with L-histidine to study the DNA delivery. It was indicated that early protonation of imidazol group presented in PLH highly influences the transfection efficiency of polyplexes (Bello et al., 2001). In another study a graft copolymer of poly (L-histidine) and poly L-lysine (PLH-co-PLL) has been used for plasmid DNA delivery. It was mentioned that polyplex particles with size range 117-306 nm have been prepared by electrostatic interactions with DNA and their transfection efficiency on 293T cells were compared with blank particles based on poly L-lysine (PLL). The results demonstrated that independent on the particle size, PLH-co-PLL exhibited higher transfection efficiency in compare to PLL (Benns et al., 2000).

Lee et al. have reported the pH-dependent stability as well as critical micelle concentration of poly (L-histidine) based nanoparticles. They have synthesized poly (L-histidine) via ring opening

polymerization and coupled by poly ethylene glycol (PEG). Micelles in ~ 114 nm diameter have been prepared in dimethyl sulfoxide to examine their stability in buffers with different pH. It was observed that the micelles produced at basic pH at 8.0 were mostly destabilized at lower pH at 7.4 (Lee-a et al., 2003). The same group also has prepared blank and folate conjugated pH-sensitive micelles based on poly (L-histidine), poly ethylene glycol and poly L-lactic acid block copolymers for delivery of doxorubicin (DOX). The pH-dependent DOX release was studied in buffers at different pH. It was observed that micelles showed higher released amount of DOX when pH reduced from 8.0 to 6.8 (Lee-b et al., 2003). This group has reported that DOX accumulation in solid tumor via delivery of this drug by nanoparticles prepared of poly (L-histidine), poly ethylene glycol and poly L-lactic acid block copolymers conjugated by folate is almost 20 folds of non-conjugated particles (Lee et al., 2005).

It is known that the main trouble of solid tumors is their drug-resistant property. To overcome to this problem investigation of higher concentration of drug is usually advised. Kim et al. fabricated pH sensitive delivery systems which exhibited early endosomal lysis. In this complex system they used micelles composed of folated poly (histidine-co-phenylalanine)-b-poly ethylene glycol and poly L-lactic acid for endosomal delivery of high concentration of DOX. The cytotoxicity and endosomal lysis ability of the prepared pH sensitive micelles were analyzed on DOX-resistant ovarian carcinoma cells. Results showed that high-dose DOX loaded micelles display active internalization leading to endosomal release and accumulation of high amount of DOX inside the cells (Kim et al., 2008).

Although poly (L-histidine) is a promising pH sensitive compound, some limitation such as difficulty in blocking of imidazole group, controlling the molecular weight of polymer during the synthesis and its low solubility in organic solvents have limited the production and investigation of this pH sensitive polymer. However still there are many studies which are going to overcome these limitations by either making copolymers or combining with other molecules to design pH responsive drug carrier systems.

1.6 Polyanhydrides

Pharmaceutical drug delivery systems, either synthetic or natural, are expected to be biodegradable, have small particle size, possess high loading capacity, demonstrate prolonged circulation and accumulate in objective sites when applied into the body. The most commonly used polymers in drug delivery systems are determined as polyanhydrides (Guhangarkar et al., 2010), polyesters (Yilgor et al., 2010; Tong et al., 2011), polyamino acids (Yang et al., 2008), polyorthoesters (Heller et al., 2002) and polyphosphazenes (Zheng et al., 2009) for the preparation of nano carriers for delivery of cancer therapy agents. Among these, polyanhydrides have attracted researcher's attention due to their controllable surface erosion (Figure 1-9) coming from hydrolytic instability of anhydride linkages (Figure 1-10) making their degradation via a controlled manner (Domb et al., 1988).

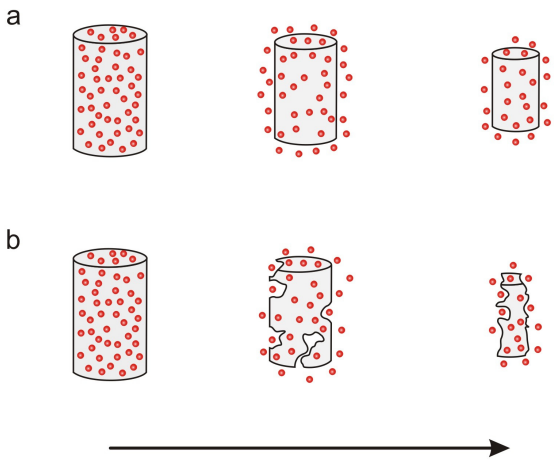


Figure 1-9 Erosion of devices. a) surface erosion, b) bulk erosion.

Degradation rate and surface erosion of a polyanhydride are mostly dependent on the type of monomer and its composition. Polyanhydrides containing aromatic groups in their backbone exhibit slower degradation and drug release rate compare to aliphatic groups containing ones. In the case of aliphatic polyanhydrides degradation rate can be controlled by changing the number of CH2 groups (Domb et al., 1987). Polysebacic anhydride (PSA) is an aliphatic biopolymer with a good performance for application in drug delivery systems.

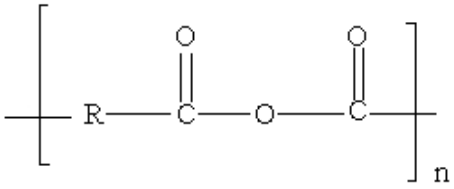


Figure 1-10 Anhydride linkage. R can be either aliphatic or aromatic.

1.6.1 Polysebacic anhydride

Polysebacic anhydride (PSA), with chemical structure shown in Figure 1-11, is a promising material for drug delivery since it has biocompatibility, can exhibit controlled erosion starting from the surface, can degrade to non-toxic metabolites, can be easily obtained from natural sources and has low cost. PSA was approved by US Food and Drug Administration (FDA) in 1990 to be used in humans for the delivery of chemotherapeutic molecules in brain cancer treatment (Brem et al., 1996). PSA is generally synthesized via condensation polymerization (Hasirci et al., 2011), of sebacic acid leading to conversion of carboxylic acid groups to anhydride linkages (Figure 1-11). Although high molecular weight PSA is usually achieved via application of higher polymerization temperature or longer polymerization time (Leong et al., 1985), investigation of some kinds of heterogenic coordination catalysts such as barium and calcium oxides has been reported to obtain PSA with molecular weight up to 245000 Da (Domb et al., 1987).

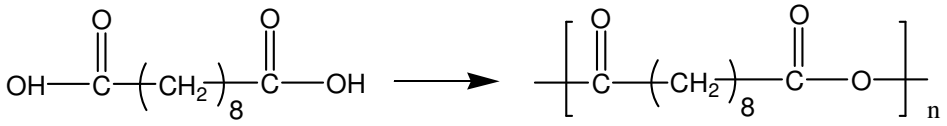
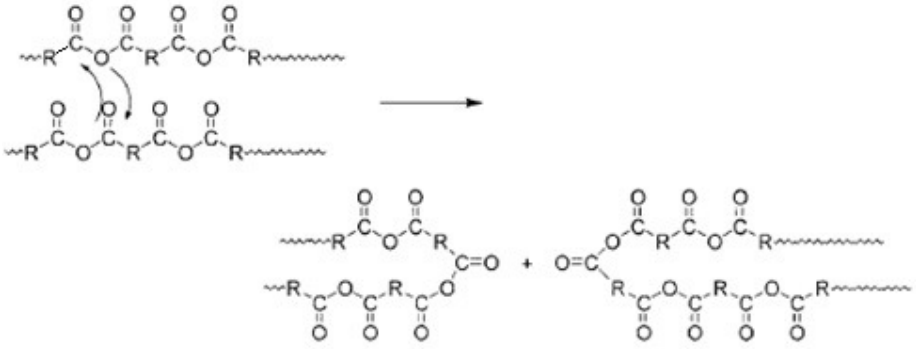


Figure 1-11 Polymerization of polysebacic acid.

Degradation of aliphatic polyanhydrides has been determined in solid state as well as in solution. This claim has been approved by degradation of both bulk and solution states of PSA. Results demonstrated that a significant decrease in molecular weight was observed in solution state in compare to the bulk. This phenomenon is attributed to different degradation pathways of polyanhydrides. Obtained results demonstrated that PSA not only can degraded via hydrolysis and conversion of hydrophilic anhydride linkage to carboxylic acid, but also it may depolymerize due to inter and intramolecular interactions (Figure 1-12).

a. Self-depolymerization via chain reaction



b. Hydrolysis into carboxylic acid

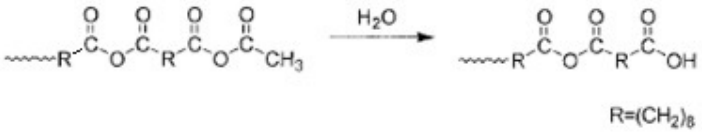


Figure 1-12 Degradation manners of PSA. a) depolymerization by chain reaction, b) hydrolysis to carboxylic acid.

In another study, it has been observed that depolymerization of PSA with molecular weight of 137000 Da to smaller cyclic molecules with molecular weight of 13500 Da is highly probable when it is in chloroform solution (Domb et al., 1989). In the same research, the influence of storing temperature on the degradation rate of PSA has been studied. It was shown that the molecular weight of solid-state PSA under dry argon condition changes from 137800 Da to 65800 at 37°C, to 78000 at 21°C, to 96600 at 0°C and to 118400 at -10°C.

It was evidenced that the degradation rate of this aliphatic polyanhydride can improved by cooperation with different polymers either via copolymerization or blending. For instance the stability of PSA against humidity has been improved by copolymerization of sebacic anhydride with aromatic groups containing compounds such as 1,3-bis(p-carboxyphenoxy)propane and 1,3-bis(p-carboxyphenoxy) hexane (Ron et al., 1991). It was shown that poly (lactic acid) has potential to delay the surface erosion of polyanhydrides. For instance degradation of films prepared by blending of PSA with poly (lactic acid) has been carried out at pH 12.4 in NaOH (aq) solution and monitored by atomic force microscopy (AFM) and X-ray photoelectron spectroscopy (XPS) (Davies et al., 1996). According to the obtained results, as the PLA amount increased the degradation of the films delayed significantly. This phenomenon comes from slow surface degrading of poly (lactic acid) leading to retard the erosion of blended films.

In addition to composition, molecular weight and storing temperature, there are various parameters that affect the degradation rate of PSA. Santos et al. examined the effect of pH on degradation of PSA based microspheres with size range between 600–850 µm. In this work the degradation of polyanhydride based microspheres was carried out at 37ºC in citrate buffer (pH 4.2), phosphate buffer (pH 7.4) and Tris buffer (pH 8.8). In predetermined times degradation has been followed by using Fourier-transform infrared (FTIR), X-ray diffraction, gel permeation chromatography (GPC) and differential scanning calorimetry (DSC). It was reported that degradation of PSA is highly dependent on pH where basic pH significantly accelerated the degradation of microspheres. Results obtained in this study showed that there was also little difference between degradation in neutral and acidic buffers (Santos et al., 1999).

The kind of degradation medium also has been reported as a key parameter imposes the degradation and drug release rates of PSA based delivery systems. For instance release of gentamicin in water was carried out via a faster manner compare to its release in phosphate buffer medium. This phenomenon is related to higher osmotic force in pure water medium causing to faster penetration of water into the device matrix (Stephens et al., 2000). The molecular weight of polymer is the most important factor influencing the degradation profile of PSA. It has been reported that there is a correlation between molecular weight of polymer and its overall degradation time. Emanuele et al. have analyzed degradation of PSA with various molecular weights from 10,000 up to 60,000 Da. It was shown that degradation of PSA was delayed by using high molecular weight polymer (Emanuele et al., 1992). In

addition to all mentioned parameters affecting the depolymerization and hydrolysis rate of PSA based delivery systems, shape and size of the particles also influence degradation behavior. For instance faster degradation of PSA and faster drug release rate was obtained for PSA prepared in film form compared to disc-shaped devices. It was also reported that micronization of PSA based particles to micro and nano size enhances the drug release rate (Cristescu et al., 2007; Wu et al., 2000).

The nontoxicity and biocompatibility of PSA and its degradation products have been proved by implanting this polymer in rabbit's corneas (Leong et al., 1986). The response of host tissue has been no inflammatory reactions during 6-week implantation time. The same result has been observed even by investigation of high dosage of PSA such as 2400 mg per kg rat implanted in rabbit's corneas for 8 weeks (Laurencin et al., 1990). The biocompatibility of implant and its related degradation products was studied by analysis of hematological parameters, tissue histology and monitoring of blood clinical chemistry.

In the literature there are various compositions of PSA with different shapes such as disc, cylinder, film, micro and nanoparticles that have been investigated for delivery of medicines (Leong et al., 1985; Xu et al., 2001; Davies et al., 1996; Gao et al., 1998; Fu et al., 2001). Liang et al. have reported a composition of PSA which exhibited higher degradation rate as well as faster drug release. In this study poly (ester anhydride) copolymer based on PSA and PEG has been synthesized to get lower melting temperature and less crystallinity degree. These obtained properties accelerated the degradation and drug release rate of PSA composition (Liang et al., 2013).

All mentioned properties have made PSA a promising polymer for delivery of various pharmaceutical agents which is summarized in Table 1-2.

Table 1-2 Various pharmaceutical agents delivered by different compositions of poly sebacic acid

Delivery agent	PSA composition	Reference
Insulin	Poly (carboxyphenoxy propane-co-sebacic acid) Poly (fumaric acid-co-sebacic acid)	Mathiowitz et al., 1988 Furtado et al., 2006
Bupivacaine	Poly (erucic acid-co- sebacic acid)	Domb et al., 1993
Somatotropin	Poly (carboxyphenoxy propane-co-sebacic acid) and Poly (carboxyphenoxy hexane-co-sebacic acid)	Ron et al., 1993
Methotrexate	Poly (erucic acid-co- sebacic acid)	Domb et al., 1994
Dicumarol	Poly(fumaric-co-sebacic)	Chickering et al., 1996
Carmustine	Poly (carboxyphenoxy propane-co-sebacic acid)	Domb et al., 1999
Gentamicin	Poly (erucic acid-co- sebacic acid) Poly (ricinoleic acid-co-sebacic acid) Poly (carboxyphenoxy propane-co-sebacic acid)	Stephens et al., 2000 Krasko et al., 2007 Cristescu et al., 2011
Dextran	Poly (ethylene glycol-co-SA) Poly (ethylene glycol-co-sebacic acid) Poly (carboxyphenoxy hexane-co-sebacic acid)	Qiu et al., 2001 Fiegel et al., 2004 Ulery et al., 2009
Ciprofloxcin	Poly (dimeracid-co-sebacic acid)	Xu et al., 2001
Phthalocyanine	Poly(phthalocyanine-co-sebacic acid)	Fu et al., 2002
Triamicinolon	Poly (fatty acids-co-sebacic acid) Poly(bile acid-co-sebacic acid)	Karasko et al., 2002 Krasko et al., 2003 (b)
Cis-platin	Poly (ricinoleic acid-co-sebacic acid)	Krasko et al., 2003 (a)
Calcein	Poly (fumaric acid-co-sebacic acids-co-ethylene glycol)	Najafi et al., 2003
Rhodamine, Piroxicam	Poly (sebacic anhydride)	Berkland et al., 2004
Paclitaxel	Poly (ricinoleic acid-co-sebacic acid)	Shikanov et al., 2004 Totiger et al., 2012
Ofloxacin	Polysebacic anhydride-blend-poly lactic acid	Chen et al., 2007
Indomethacine	Poly sebacic anhydride	Gong et al., 2007
Nifedipine	Poly (pluronic-co- sebacic acid)	Shelke et al., 2007
Etoposide	Poly (ethylene glycol-co-sebacic acid)	Tang et al., 2009
Tamsulosin	Poly (ricinoleic acid-co-sebacic acid)	Havivi et al., 2009
Doxorubicin	Poly (ethylene sebacate) Poly (ethyne glycol-co-carboxyphenoxy propane-co-sebacic acid)	Guhagarkar et al., 2010 Zhao et al., 2010
Levofloxacin	Composite of PSA and graphene oxide	Gao et al., 2011
Tamoxifen	Poly (ricinoleic acid-co-sebacic acid)	Hiremath et al., 2012
5-fluorouracil	Poly (ethylene glycol-co-sebacic acid)	Zhang et al., 2012

1.7 Aim of this study

Chemotherapeutic agents used in cancer treatment leads to death of cancer patients regarding to their adverse effects. Chemotherapy consequence for patients treated by these highly toxic drugs could be significantly improved if drug could be applied in less concentration and more therapeutic efficiency. Targeted delivery not only enhances efficacy coming from effective drug concentration in the target cells, but also it exhibits relatively high reduction of the adverse effects.
The main goal of this study is to prepare novel pegylated PSA based nanocapsules with pH responsive property and containing doxorubicin as an anticancer agent as it is shown in Figure 1-13.

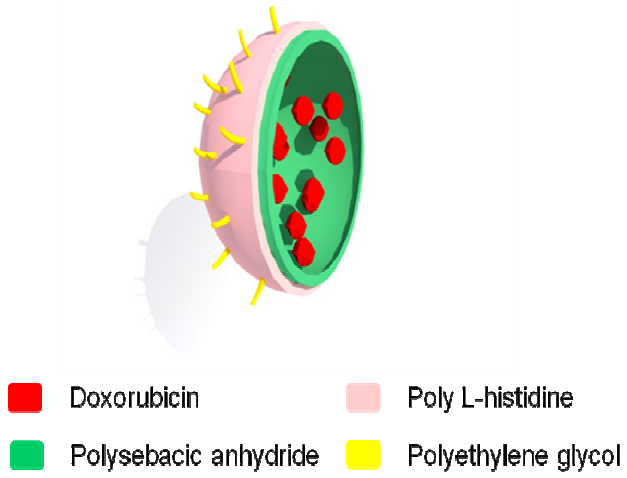


Figure 1-13 Schematic presentation of pH responsive DOX loaded nanocapsules.

This drug carrying vehicle would be targeted to tumor side as well as cancer cells. This strategy causes to enhance chemotherapeutic efficiency of doxorubicin and reduce its serious responding adverse effects. In this research doxorubicin was encapsulated into polysebacic anhydride nanocapsules. Polysebacic anhydride with promising properties described in previous parts was synthesized from its monomer, sebacic acid, and applied as a base matrix to form capsules. Synthesized polysebacic anhydride was characterized by different methods. Chemical structure of the synthesized polymer was examined with H-NMR and FTIR and the molecular weight was determined by GPC. The degradation behavior of the synthesized polysebacic anhydride and the drug release rate from prepared nanocapsules were determined in different conditions. With this object, degradation of polysebacic anhydride was characterized in acidic (pH= 4.0) and physiological (pH= 7.4) buffers. Doxorubicin loaded nanocapsules were prepared in the range of 200-400 nm. In order to find the optimum size and properties, different parameters such as organic solvent, surfactant type and concentration were applied during the nanocapsules preparation process. The size and shape of the prepared nanoparticles were characterized by dynamic light scattering (DLS), and scanning electron microscopy (SEM).
Different properties of DOX loaded nanocapsules such as loading efficiency; drug release rate, size and size distribution were analyzed to select the best preparation parameters. Further experiments were continued with nanocapsules exhibiting suitable properties for drug delivery.
The most important liability of this project is pH responsibility of the prepared nano-carriers leading to release of doxorubicin in tumor site. Poly (L-histidine) coat was used as an outer shell of nanocapsules to demonstrate this responsibility. In addition, due to high protonation capacity of PLH in acidic medium, it exhibited relatively high internalization into cancer cells. By this way it is expected that most of the applied drug would release inside the cell. PLH coating of nanocapsules was characterized by FTIR. To have an ideal nano-size delivery system planned to be used by

injection in vivo conditions, the prepared pH responsive nanocapsules were also modified by coating with polyethylene glycol (PEG).

The pH responsibility of the prepared nanocapsules was examined in vitro conditions. For this purpose, doxorubicin release studies were performed in acidic (pH= 4.0) and basic (pH= 7.4) buffers. Antitumor efficiency of formulated pH responsive and DOX loaded nanocapsules was examined on MDA-MB-231 human breast cancer cells. The obtained results were compared with the controls obtained by applying free DOX in same conditions and concentrations. The cytotoxicity of the synthesized polymer and formulated nanocapsules was also studied by investigation of drug free pH responsive nanocapsules in parallel to other groups. The cell viability and cytotoxicity test were performed by using WST Kit-8 assay. The intracellular release of DOX from the prepared nanocapsules was explored by using confocal microscopy.

The internalization of the designed nanoparticular system into MDA-MB-231 cells was proved by labeling of polysebacic anhydride with coumarin 6 fluorescence dye. The cellular uptake of nanocapsules prepared from coumarin 6-labeled polysebacic anhydride was also analyzed by confocal microscopy. The influence of pegylation was monitored by applying of THP-1 human macrophage cell line. The macrophage uptake of both pegylated and non-pegylated nanocapsules was examined by confocal microscopy. The real action of designed pH responsive nanocapsules could be observed in vivo applications on rats or rabbits.

CHAPTER 2

EXPERIMENTAL

2.1 Materials

All chemicals used during this research have been mentioned in Table 2-1.

Table 2-1 Materials used in this study

Chemicals	Company
Acetic anhydride	Sigma Aldrich (Gillinham-UK)
Chloroform	Sigma Aldrich (Gillinham-UK).
Coumarin-6	Sigma Aldrich (St. Louis, MO-USA)
Cell counting kit-8	Sigma Aldrich (Steinheim-Germany)
Dichloromethane	Sigma Aldrich (Gillinham-UK)
Doxorubicin	Sandoz, (Istanbul-Turkey)
Ethyl acetate	Sigma Aldrich (Gillinham-UK)
Ethyl ether	Sigma Aldrich (Gillinham-UK)
Hydrochloric acid	R&D Systems (CA-USA)
MTS CellTiter 96®	Promega (Madison-USA)
Paraformaldehyde	Sigma Aldrich (Steinheim-Germany)
Petroleum ether	Sigma Aldrich (Gillinham-UK)
Polyethylene glycol monomethyl ether (5000)	Sigma Aldrich (Gillinham-UK)
Poly (L-histidine) (5000-25000)	Sigma Aldrich (Gillinham-UK)
Polyvinyl alcohol (13000-23000)	Sigma Aldrich (Gillinham-UK)
Polyvinyl pyrrolidone (8000)	Across, (Geel-Belgium)
RPMI	Lonza (Verviers-Belgium)
Sebacic acid	Fluka (Gillinham-UK)
Sodium hydroxide	J.T. Baker (Holland, Netherland)
Trypsin	Lonza (Verviers-Belgium)
Tween 80	Across (Geel-Belgium)

2.2 Polysebacic anhydride synthesis

As a first step of this study, polysebacic anhydride was prepared by a method similar to that described by Shen with some modifications (Shen et al., 2001). Sebacic anhydride was synthesized by dehydration of sebacic acid. Briefly, sebacic acid (6 mg) and acetic anhydride (58 mL) were put in three-neck flask (Figure 2-1). Mixture was refluxed for 30 min at 140ºC under dry nitrogen gas sweep. During the reaction acetic anhydride was converted to acetic acid by removing water molecules from sebacic acid.

Produced acetic acid was removed by rotary evaporation at 40ºC. Prepared sebacic anhydride was dissolved in 50 mL chloroform. Non-reacted sebacic acid was removed via precipitation of sebacic anhydride from chloroform solution in 200 mL of 1:1 mixture of anhydrous petroleum ether and diethyl ether. Polysebacic anhydride with two different molecular weights were prepared via condensation polymerization by curing of 10 mg of pure sebacic anhydride prepolymer at 180ºC for 1.5 and 3 h.

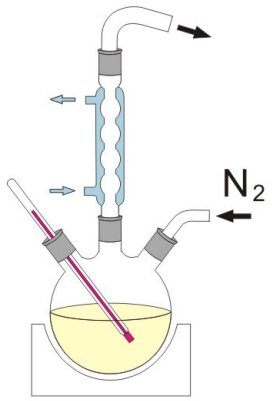


Figure 2-1 Synthesis of sebacic anhydride prepolymer.

The obtained polymers were purified by dissolving in 50 mL chloroform and re-precipitating in dry petroleum ether. To prevent hydrolysis and degradation during the storage, prepared polymers desiccated under nitrogen atmosphere and kept in freezer (-20ºC).

2.3 Polysebacic anhydride characterization

The chemical structure of polymer was characterized by H-NMR (950 US2, Bruker, Bremen, Germany) by dissolving polymer in deuterium chloroform. The chemical structure of synthesized polysebacic anhydride was also analyzed and compared with sebacic acid by using FTIR (Spotlight 65, Perkin Elmer, MO, USA).
Molecular weights of synthesized polymers were determined by gel permeation chromatography (GPC) (PL-GPC 220, CA, USA). The analysis was performed at 30ºC in tetrahydrofuran with flow rate of 1 mL/min. In GPC experiments polystyrene was used as standard polymer.

2.4 Polysebacic anhydride degradation

The degradation profile of polysebacic anhydride was performed in acidic (pH=4.0) and physiological (pH=7.4) pH buffers and followed by FTIR in different durations. In this study, 5 mg of polysebacic anhydride was put in vials containing 10 mL of phosphate buffered saline (PBS) 0.01 M with pH 4.0 and 7.4. Samples were incubated at 37ºC up to 10 days. In predetermined times, remaining samples were collected by centrifuge, dried in freeze dry and degradation was analyzed by FTIR.

2.5 Preparation of DOX loaded nanocapsules

Doxorubicin (DOX) loaded nanocapsules (NCs) were prepared via using modified double emulsion protocol which has been described in literature and shown in Figure 2-2 (Liu et al., 2010; Ashjari et al., 2012).

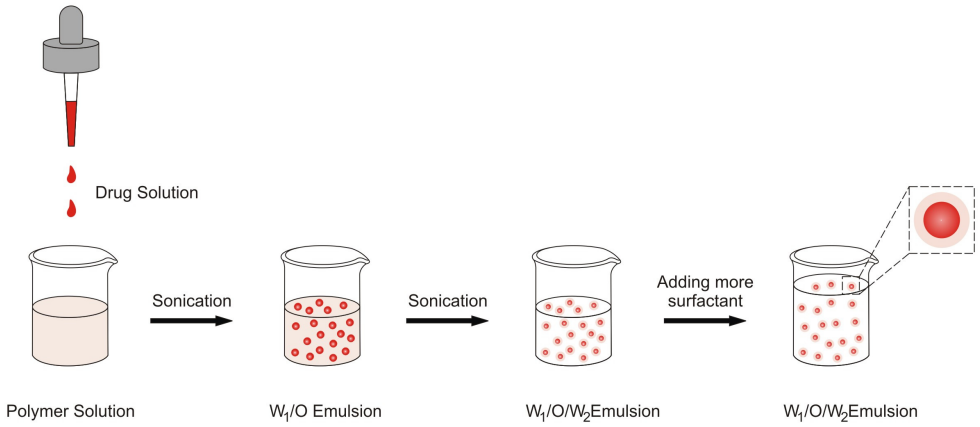


Figure 2-2 Schematic presentation of DOX loaded nanocapsule preparation via double emulsion technique.

For this purpose polysebacic anhydride (50 mg) was dissolved in 5 mL organic solvents which have been mentioned in Table 2-2. To get w1/o emulsion, aqueous solution of DOX (2 mg/mL) was added to the organic phase and sonicated for 20 seconds by probe sonicator in 60 W power output. Obtained homogen emulsion was subsequently added to 15 mL aqueous solution of surfactant and sonicated again for 20 seconds. Prepared w1/o/w2 emulsion was added to 75 mL of 1% surfactant solution and stirred at room temperature for evaporation of organic solvent and precipitation of DOX loaded NCs. Following the complete evaporation of organic phase, solidified particles were collected by centrifuge with 14000 rpm at 4ºC and washed two times with 1 mL double distilled water. To examine the effect of types of solvent and surfactant as well as concentration of surfactant, various experiments were carried out (Table 2-2). The main aim was to obtain nanoparticles with proper size of 200-400 nm which required for effective EPR effect in tumor site (Torchilin et al., 2010).

Table 2-2 Different solvent, surfactant and surfactant concentration used in nanocapsules preparation

Sample	Parameters
EA-PVA-4	NCs prepared by using ethyl acetate (EA) as solvent and polyvinyl alcohol 4% as surfactant
D-PVA-4	NCs prepared by using dichloromethane (DCM) as solvent and polyvinyl alcohol 4% as surfactant
D-PVA-1	NCs prepared by using dichloromethane (DCM) as solvent and polyvinyl alcohol 1% as surfactant
D-PVP-4	NCs prepared by using dichloromethane (DCM) as solvent and polyvinyl pyrrolidone 4% as surfactant
D-PVP-1	NCs prepared by using dichloromethane (DCM) as solvent and polyvinyl pyrrolidone 1% as surfactant
D-T80-4	NCs prepared by using dichloromethane (DCM) as solvent and tween 80 4% as surfactant
D-T80-1	NCs prepared by using dichloromethane (DCM) as solvent and tween 80 1% as surfactant

Blank nanocapsules, without DOX, were also prepared via the same manner. The only difference was using the same amount of distilled water instead of DOX solution to get w1/o emulsion. Fluorescence-labeled nanoparticles were prepared in similar way by adding coumarin-6 dye to organic phase of polysebacic anhydride. For this purpose, 20 µL of coumarin-6 stock solution (20 µg coumarin-6 in 1 mL DCM) was added to organic phase. Prepared NCs were dried by lyophilization in freeze drier for further studies.

2.6 Characterization of DOX loaded nanocapsules

The yields of the prepared nanocapsules (NCs) containing doxorubicin (DOX) were calculated by using the mass of NCs obtained per mass of polymer used initially. The morphology of NCs was analyzed by scanning electron microscopy (SEM, QUANTA 400, Oregon, USA). Size and size distributions were obtained by using particle size analyzer (Malvern Mastersizer, Worcestershire, UK).

2.7 Preparation of pH responsive nanocapsules

Both control NCs and DOX loaded NCs were made pH responsive by coating them with poly (L-histidine) (PLH) electrostatistically. Before the coating process, zeta potential of both DOX loaded NCs and pure PLH were measured by zeta sizer equipment (Malvern Mastersizer, Worcestershire, UK) in dilute acidic medium with pH~6. To check the calibration of instrument, zeta potential transfer standard sample (-68 mV ± 6.8 mV) was measured firstly. To perform the coating, PLH was dissolved in acidic aqueous medium. For this purpose HCl 0.01 M was added drop wise to 10 mL double distilled water containing PLH until getting pH around 6. DOX loaded nanocapsules were put into solution and stirred. To precipitate of PLH on the surface of NCs, NaOH (0.01 M) was added drop wise to the solution to increase the pH upto ~8. Coated nanocapsules were collected by centrifuge with 10000 rpm at 4ºC and dried in freeze dry. The level of coating was examined by analysis of PLH coated NCs (PLH-DOX-NCs) by FTIR. In this analysis, FTIR spectra of poly (L-histidine) coated NCs was compared with the FTIR spectra of DOX loaded NCs and pure PLH as control groups.

2.8 Pegylation of pH responsive nanocapsules

Transport performance of the prepared pH responsive delivery vehicles, PLH-DOX-NCs, was improved to prevent their macrophage uptakes. For this purpose, the surface of pH responsive NCs was again modified by polyethylene glycol (PEG) with molecular weight of 5000 Da to produce pegylated and PLH coated NCs (PEG-PLH-DOX-NCs). To fulfill this purpose NCs were stirred in 5 mL cold (~4 ºC) and 3% aqueous solution of polyethylene glycol monomethyl ether for 10 min. Then the particles were collected by centrifugation with 10000 rpm for 15 min, freeze dried and characterized by FTIR. The effect of PEG coating on size and morphology of NCs was examined by DLS and SEM analysis.

2.9 Drug loading capacity and encapsulation efficiency

In order to assess the drug concentration in nanocapsules, UV absorption measurements were performed. DOX concentration in supernatants was analyzed by UV spectrophotometer (1420 Wallac Victor, Turku, Finland) and calculated via calibration curve with R2= 0.992 prepared previously from different concentration of DOX in phosphate buffered saline (PBS, 0.01 M, pH=7.4) (Figure A-1, appendix A).

Percent of Encapsulation Efficiency for DOX was obtained using equation given below.

$$EE\ \% = [W_{(1)}\text{-}W_{(2)}/W_{(1)}] \times 100$$

where;
EE% is encapsulation efficiency, W(1) is amount of initial DOX, W(2) is amount of DOX in supernatant at the end of loading process and W(1) - W(2) is amount of DOX in nanocapsules.

Percent Loading Capacity of NCs was calculated by using equation given below.

$$LC\ \% = [W_{(DOX)}/W_{(NC)}] \times 100$$

In this equation $W_{(DOX)}$ is the amount of DOX in nanocapsules, and $W_{(NC)}$ is the mass of nanocapsules.

2.10 In situ drug release study

2.10.1 DOX release profile of nanocapsules prepared by PVP, PVA and T80

Release profiles of DOX from the PSA based NCs prepared by using PVP (D-PVP-4, D-PVP-1), PVA (D-PVA-4, D-PVA-1, EA-PVA-4) and T80 (D-T80-4, D-T80-1) were studied in PBS (0.01 M in pH 7.4). For this purpose, 5 mg nanocapsules of each group was resuspended in 0.5 mL double distilled water, transferred into seamless cellulose dialysis tubes (MW 12400) immersed immediately in vials containing 4.5 mL PBS (0.01 M in pH 7.4). The release studies were performed at 37°C in shaking incubator. Experiments were done as triplicates. In predetermined periods, 0.5 mL aliquots were drawn out from the medium and replaced with fresh PBS. The absorbance values of the drawn aliquots were obtained at 490 nm in UV spectrophotometer. The amount of released DOX was determined by running the calibration curve prepared previously by different concentration of DOX in PBS (Appendix A). The cumulative percentages of the released drug were plotted versus time. The obtained data was also examined by curve fitting to Higuchi drug release model.

2.10.2 DOX release profile of pH responsive NCs (PEG-PLH-DOX-NCs)

Release behavior of pegylated pH responsive nanocapsules was studied in PBS buffered solution (0.01 M) with two different pH; where one is acidic pH 4.0 and the other is physiological pH 7.4. For this purpose, 5 mg of coated nanocapsules was resuspended in 0.5 mL double distilled water and transferred into dialysis bag. The release studies were achieved in 4.5 mL responding buffer in shaking incubator at 37ºC. To provide sink condition 0.5 mL aliquots were removed from the release medium at certain times for UV absorbance measurements while equal amount of fresh PBS solutions were added into the media. The released amount of DOX was calculated by using calibration curve prepared previously which was based on the absorbance intensity of DOX at 490 nm. In the assessment of drug release behavior, the cumulative amount of the released drug was calculated, and the percentages of released drug from nanocapsules were plotted versus time. All experiments were carried out in triplicates.

2.11 In vitro cell culture study

2.11.1 Cell uptake and cytotoxicity of DOX loaded nanocapsules

The cytotoxicity of the prepared nanocapsules prepared by using PVP (D-PVP-4), PVA (D-PVA-4) and T80 (D-T80-4) was examined by using MDA-MB-231 human breast cancer cells. Cells were purchased from ATCC and cultured in RPMI 1640 cell culture medium (Lonza, Verviers, Belgium) containing 10% fetal bovine serum and 1% penicillin-streptomycin. Cells were grown by incubation in T75 flask at 37ºC in humidified atmosphere with 5% CO2. After getting enough confluence, cells were split by Trypsin with concentration of 200 mg/L. Harvested cells were seeded in 96-well plate (5000 cells per well) in four groups; control cells (contains only cells), blank NCs (NCs prepared without DOX), free DOX and DOX loaded NCs prepared by using PVP, PVA and T80 4%. To reduce general evaporation during the incubation, cell-seeded wells were surrounded with wells containing 200 µL of culture medium. The related cell seeding pattern has been shown in Figure 2-3. The cell-free background was also performed in parallel to other groups.

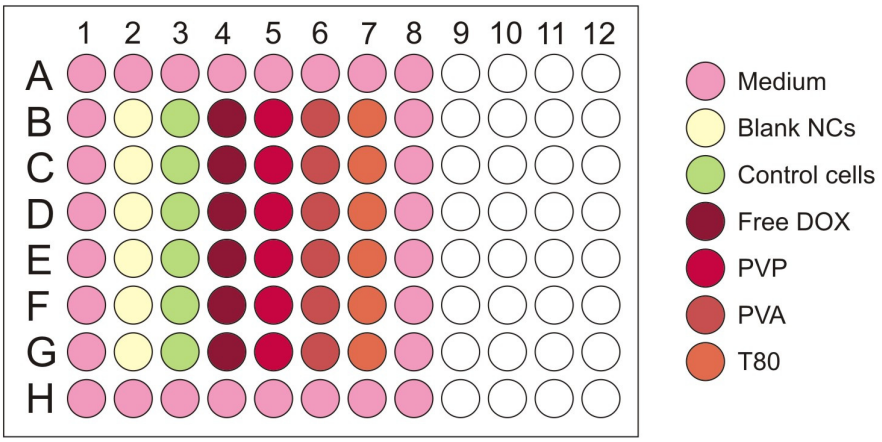


Figure 2-3 Cell seeding pattern. PVP; D-PVP-4 NCs, PVA; D-PVA-4 NCs, T80; NCs prepared by using T80.

Following to 24-hour incubation, cells were treated by 100 µL sterile PBS (0.01M) and PBS containing unloaded blank nanoparticles, 0.6 µg/mL free DOX and nanoparticles prepared via PVP, PVA and T80 having the same concentration of DOX. This concentration of DOX was selected due to tumor efficiency curve of DOX with different concentration prepared in our cell culture lab in Radium hospital (Figure A-2, Appendix A). Cell viability test was carried out after 3 days of incubation via adding 10 µL WST cell counting kit-8 (Sigma Aldrich, Oslo, Norway) to each well and incubated for 3 h. The absorbance values of the wells were measured at 490 nm by employing Wallac Victor plate reader (Turku- Finland).
Cellular uptake of NCs was performed by using fluorescence microscope (Olympus IX81, Glattbrugg, Switzerland).

2.11.2 Tumor efficiency of pH responsive nanocapsules (PEG-PLH-DOX-NCs)

2.11.2.1 MTS cell viability assay

The anti tumor activity of the prepared pH responsive NCs was examined on MDA-MB-231 breast cancer cells purchased from American Type Culture Collection (ATCC). In this study cells were cultured and seeded in 96-well plates with pattern and protocol mentioned in part 2.11.1. After 24 hours post seeding, MDA-MB-231 cells were treated by 100 µL sterile PBS (0.01 M) and PBS containing blank nanoparticles, 0.6 µg/mL and 1.2 µg/mL free DOX and PEG-PLH-DOX-NCs containing the same concentrations as free DOX sample. The effect of DOX concentration on tumor efficiency was examined by applying two different concentrations of DOX (0.6 and 1.2 µg/mL) to treat the cancerous cells. Cell viability study was performed 3 days after treatment via cell-mediate reduction by using MTS assay. In this colorimetric method tetrazolium compound, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium], is reduced biologically into colored formazan with UV absorbance of 490 nm. As the bioreduction has been evaluated by cells, the quantity of formazan product measured by UV spectroscopy is directly proportional to the number of living cells. In this study MTS assay was carried out by adding 20 µL of MTS CellTiter 96® (Promega, Madison-USA) to each well and incubated for 2 hours. The absorbance of produced formazan was measured by plate reader at 490 nm (Wallac Victor, Turku-Finland).

2.11.2.2 Cell counting Kit-8 cell viability assay

Cell counting Kit-8 was applied for the determination of cell viability in tumor cells after the application of blank NCs, free DOX and PEG-PLH-DOX-NCs. These NCs were prepared by using PVA 4% as surfactant and DCM as solvent. This cell counting kit with UV absorbance about 450 nm is more sensitive than the other colorimetric assays such as MTT or MTS.

The same cell line with the same protocol mentioned in part 2.11.1. was investigated for cell viability by using WST Kit-8 (Sigma Aldrich, Oslo Norway). Briefly, MDA-MB-231 breast cancer cells were seeded in 96-well plates, incubated for 24 hours and treated by 100 µL sterile PBS and PBS containing blank nanocapsules, free DOX and PEG-PLH-DOX-NCs with concentrations of 0.6 µg/mL and 1.2 µg/mL. After 3 days of incubation at 37ºC and 5% CO2, 10 µL of WST Kit-8 was added to each well and incubated for 2 hours. The absorbance values of the wells were obtained by UV spectroscopy (Wallac Victor plate reader, Turku, Finland) at 450 nm.

2.11.3 Microscopy study

2.11.3.1 Intracellular DOX release in cancer cells

To visualize the intracellular release of DOX from nanocapsules and their uptake by the cells confocal laser scanning microscopy was employed. To fulfill of this sense MDA-MB-231 cells (15000 cells per well) were seeded in 8-well glass slide (Lab-Tek II, NY, USA), incubated for 24 h and then treated by sterile PBS (0.01 M), PBS containing free DOX (0.6µg/mL) and PEG-PLH-DOX-NCs containing the same concentration of DOX. In the end of 3-day incubation, medium was removed and cells were rinsed two times with sterile PBS (0.067 M, pH 7.4). Fixing was evaluated by adding 400 µL of cold paraformaldehyde (PFA, 4%) to cells, incubated for 15 min at room temperature and followed by removing paraformaldehyde and rinsing with sterile PBS (0.067 M, pH 7.4). After fixing, the chamber and gasket were removed as it is shown in Figure 2-4. The cell nucleus were labeled with DAPI (4',6-diamidino-2-phenylindole) during the fixing process by adding one droplet of prolong gold antifade reagent containing DAPI (invitrogen, Oregon-USA) to each well. Fixed cells were covered by cover slips, kept at 2-8 ºC for 24 hours and then were viewed by Zeiss LSM 710 confocal microscope equipped with Plan-Apochromat 63x 1.4 NA oil immersion objective (Zeiss-Germany). Image processing and visualization were performed by using the ZEN 2011 software (Carl Zeiss, Germany).

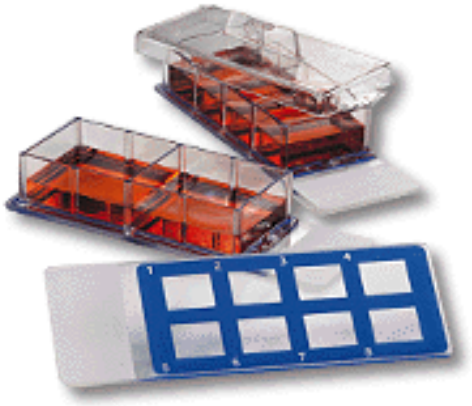


Figure 2-4 Photograph of well glass slide.

2.11.3.2 Cell uptaking of pH responsive nanocapsules

Cell uptaking of nanocapsules by cancerous cells was examined by fluorescence microscopy. In this experiment, MDA-MB-231 breast cancer cells were seeded in 8-well glass slide (15000 cells per well) and treated with sterile PBS as a control group, free DOX and Coumarin labeled PEG-PLH-DOX-NCs. Treated cells were incubated for 1 day and fixed by PFA (4%) as it has been described in part 2.11.3.1.

2.11.3.3 Macrophage uptaking of pH responsive nanocapsules

The influence of pegylation of nanocapsules on macrophage uptaking was analyzed on human acute monocytic leukemia cells (THP-1). THP-1 human acute monocyte cells were obtained as a kind gift

from Dr. Lina Prasmickaite and grown in RPMI 1640 cell culture medium (Lonza, Verviers, Belgium) containing 10% fetal bovine serum, 1% penicillin-streptomycin and 2 mM Glutamine. THP-1 cells were seeded (15000 cells per well) in 8-well glass slide and induced to differentiate into macrophages by adding 200 µL culture medium containing 10 µM TPA (12-O-tetradecanoyl-phorbol-13-acetate) and incubated at 37ºC and 5% CO2. After 48 hours macrophages were treated by replacing TPA containing medium with sterile PBS, pegylated NCs (PEG-PLH-DOX-NCs) and non-pegylated NCs (PLH-DOX-NCs) with DOX concentration of 0.6 µg/mL. Treated macrophages were incubated for 6 hours and fixed by paraformaldehyde (4%) as it has been described in part 2.11.3.1 for microscopy study.

2.11.4 In vivo study

In vivo studies were performed on female athymic nude mice. To create required tumors, MDA-MB-231 cells were injected to 6-week mice with concentration of 2.5 million cells in 100 µL cell culture medium. Injection of cells was done in mammary fat pad from both sides. Blank nanoparticles, free DOX and PEG-PLH-DOX-NCs containing the same amount of DOX were prepared in saline solution in concentration of 8 mg/kg mouse. Prepared samples were kept at -20ºC until the treatment time. Before treatment samples was defreezed and sonicated for 10 min to separate agglomerated nanoparticles and then injected to mice.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Polysebacic anhydride characterization

Polysebacic anhydride was synthesized by polycondensation polymerization described by Shen with some modifications (Shen et al., 2001). For this purpose sebacic acid was firstly converted to sebacic anhydride at 140ºC. Water produced during the dehydration of sebacic acid was removed by excess amount of acetic anhydride added to the flask. Furthermore, pure sebacic anhydride was polymerized at 180ºC under vacuum conditions. Prepared polymer was characterized by H-NMR, FTIR and GPC.
H-NMR spectrum of both sebacic acid and polysebacic anhydride were compared as it is shown in Figure 3-1. It was observed that peak related to OH groups presenting in monomer structure (3 and 14) which is observed at 10.49 ppm has been disappeared in the spectrum of the synthesized polysebacic anhydride which is related to conversion of carboxylic acid groups to anhydride.
FTIR analysis also confirmed the synthesis of polysebacic anhydride. The spectra of the both sebacic acid and polysebacic anhydride are shown in Figure 3-2. The spectrum of sebacic acid (Figure 3-2-a) shows carboxylic acid characteristic absorption bands at 1697, 1300 and 930 cm-1. The broad band presented at 3335–2500 cm-1 is due to the strong hydrogen bonding of the -OH group of free acid. While these bands were disappeared in case of polymer spectrum (Figure 3-2-b) and polysebacic anhydride characteristic absorption band has been observed at 1816-1740 cm-1. Sharp peak in the range of 1090-1030 illustrated stretching of anhydride groups (-CO-O-CO-) (Liang et al., 2012).
Peaks observed at the range of 2920 and 2870 are related to stretching of C-H bonds of CH2 groups.

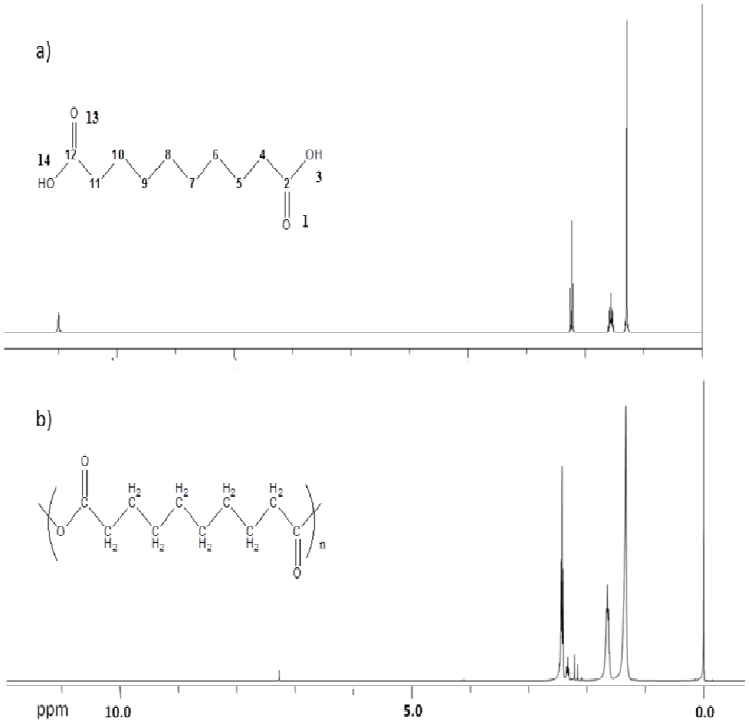


Figure 3-1 H-NMR spectra. a) sebacic acid, b) polysebacic anhydride.

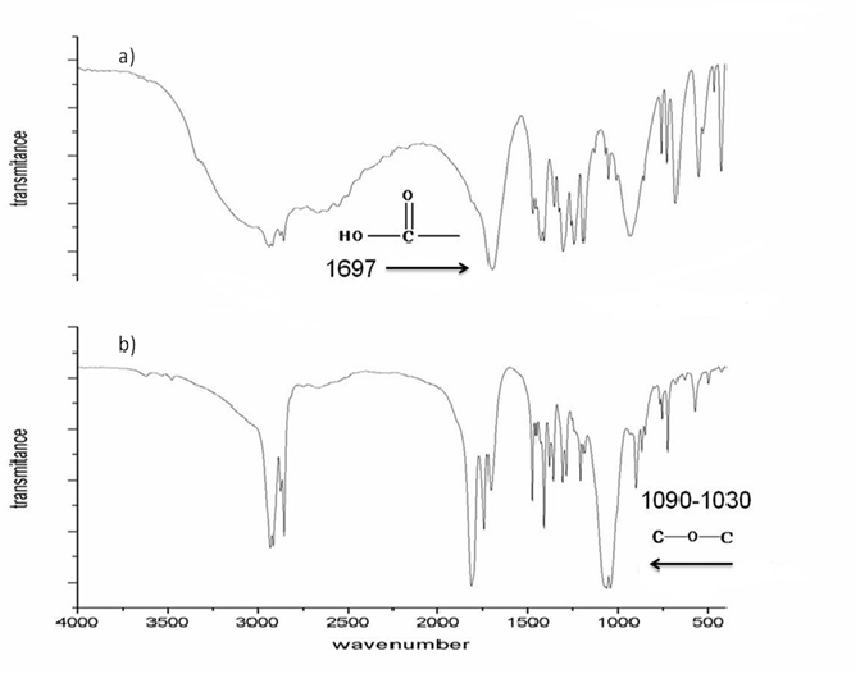


Figure 3-2 FTIR spectra. a) sebacic acid, b) polysebacic anhydride.

The molecular weights of synthesized polymers were determined by GPC. Results demonstrated 2500 and 5500 Da as molecular weights of low and high molecular weight polymers, respectively which depend on the polymerization time. The polydispersity index (PDI) of low molecular weight polysebacic anhydride has been determined as 1.30. In the case of high molecular weight, this index showed an increase to 1.98 indicating chains with different chain lengths. This difference may come from heterogeneous heating during the polymerization coming from poor heat transfer in vacuum conditions without any stirring process (Chen et al., 2003). To minimize differences in experimental results, especially in degradation and drug release studies, lower molecular weight (Mw 2500) with better PDI was used for future experiments.

3.2 Polymer degradation

In controlled drug delivery systems degradation rate and degradation behavior of the carrier play significant role in drug release profile. In this study delivery vehicles were designed to carry anticancer agent. Considering to acidic pH of tumor and neutral pH of blood, degradation studies of the prepared PSA were performed in two different pH media. The hydrolytic degradation of low molecular weight PSA was carried out in PBS (0.01 M) with pH 4.0 and 7.4. The degradations were followed by FTIR instrument after 3, 6 and 10 days of incubation in the given pH media. Converting of anhydride linkage to carboxylic acid is main feature of degradation process. Hence loosing of anhydride characteristic peaks and intensification of carboxylic acid peak in FTIR spectrum prove hydrolytically degradation of polysebacic anhydride (Fiegel et al., 2004).
FTIR spectra related to PSA degradation in pH 4.0 buffer at different times are shown in Figures 3-3 and 3-4, while the FTIR spectra related to PSA degradation at pH 7.4 are shown in Figures 3-5 and 3-6. According to the data obtained from spectra, peaks related to methyl groups appeared in 2890-2840 cm-1 are almost changeless. However band appeared in 1074 cm-1 and illustrated stretching of anhydride group exhibits a decrease during the degradation from t=0 to t=10 days. By comparing degradation spectrums in two different pH, it was revealed that PSA degradation in acidic pH (pH= 4.0) has been performed in slower manner from in neutral pH (7.4). This phenomenon is match to results reported previously (Fu et al., 2001). To focus on the behavior of peak related to vibration of carboxylic acid group appeared in wavenumber range of 2000-1000 cm-1, the spectra of degradation in pH 4 and 7.4 was expended in Figure 3-4 and Figure 3-6, respectively. As it was expected peak related to carboxylic acid appears and increases as the degradation proceeds.

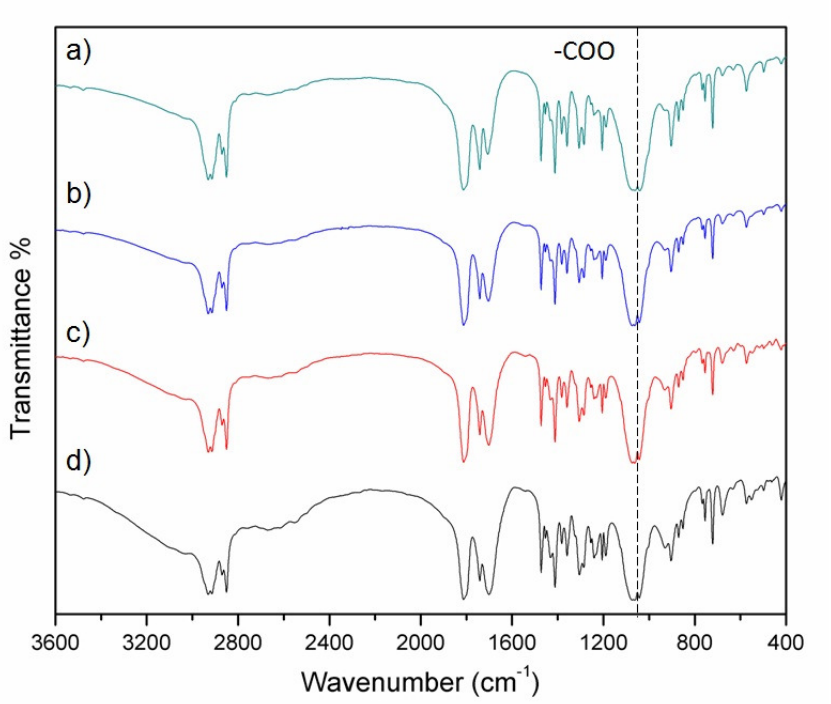


Figure 3-3 FTIR spectra related to PSA degradation at pH 4. a) t=0, b) t=3, c) t=6, d) t=10 days.

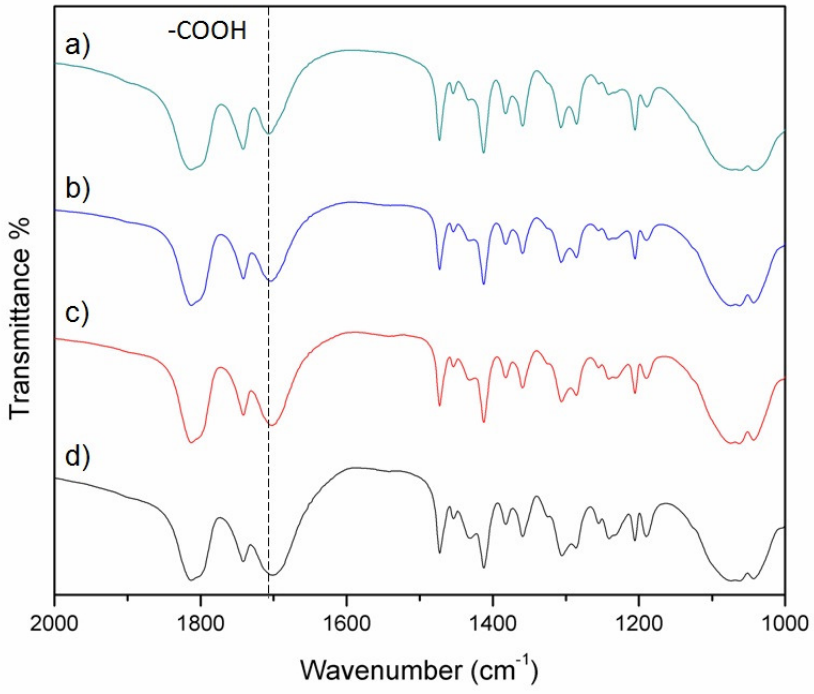


Figure 3-4 Expanded FTIR spectra related to PSA degradation at pH 4. a) t=0, b) t=3, c) t=6, d) t=10 days.

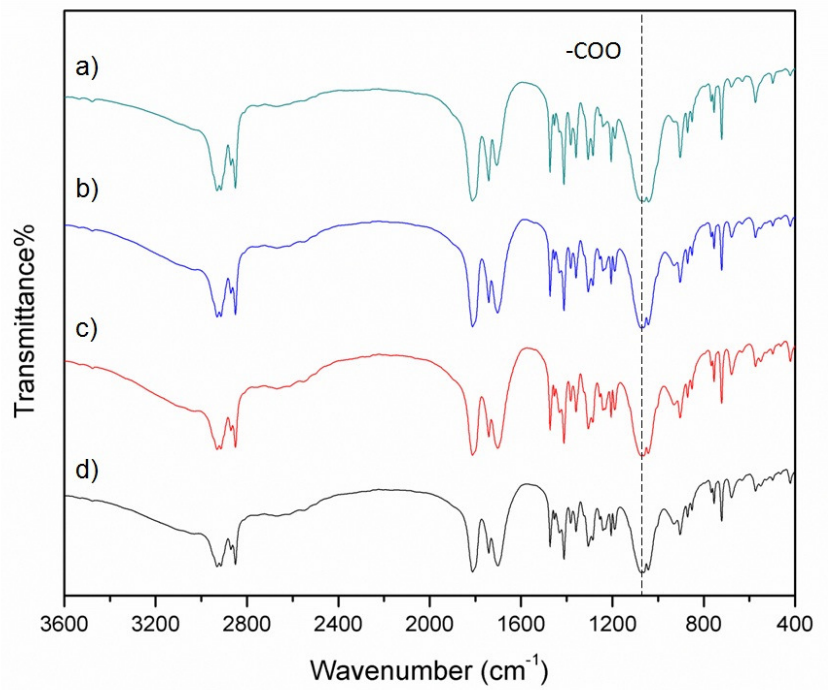


Figure 3-5 FTIR spectra related to PSA degradation at pH 7.4. a) t=0, b) t=3, c) t=6, d) t=10 days.

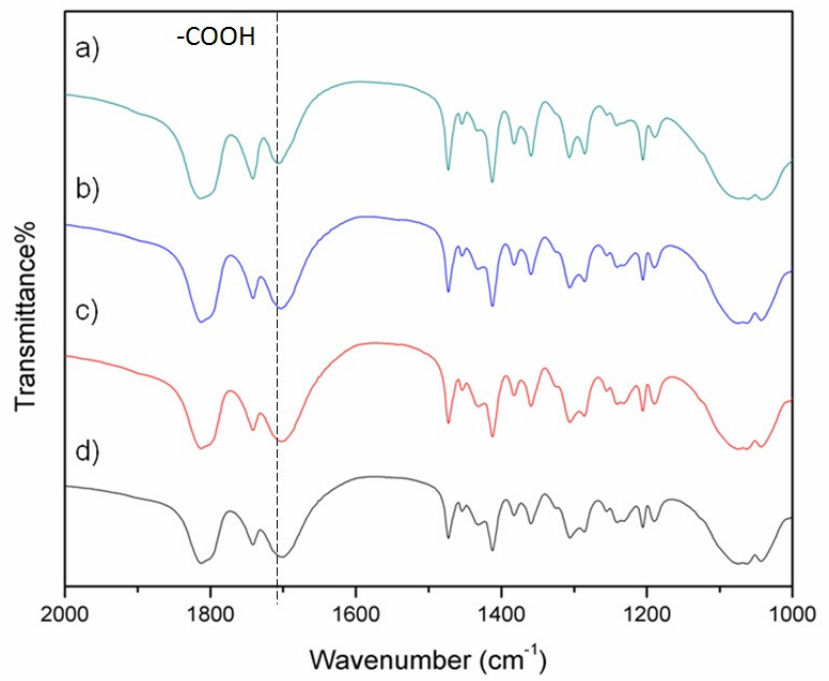


Figure 3-6 Expanded FTIR spectra related to PSA degradation at pH 7.4. a) t=0, b) t=3, c) t=6, d) t=10 days.

3.3 Characterization of DOX loaded nanocapsules

Doxorubicin loaded nanocapsules were prepared by double emulsion method. Because of the high water solubility of DOX, $w_1/o/w_2$ emulsion was performed to obtain DOX loaded PSA nanocapsules. Although the main strategy in this research is targeting via pH responsibility of nanocapsules, the EPR effect of nano-size particles has been benefited to entrap nanocapsules in tumor site (Alvarez-Lorenzo et al., 2011; Huynh et al., 2011). To exploit EPR effect in tumor targeting, nanocapsules with proper size of 200-400 nm are required (Torchilin et al., 2010). Therefore, in order to obtain nanocapsules with suitable size (200-400 nm) and with proper morphology, some key parameters of emulsion method such as type and concentration of surfactant and the type of organic solvent were altered during the nanocapsules preparation process and their effects on the nanocapsules were examined.

3.3.1 Effect of surfactant and its concentration on size and morphology of NCs

The type and concentration of the used surfactants are significantly important parameters in nanocapsule preparation. In this study polyvinyl alcohol (PVA), polyvinyl pyrrolidone (PVP) and tween 80 (T80) surfactants were used in two different concentrations as 1% and 4% for the production of PSA based NCs. The effect of surfactant type on size, production yield and DOX loading efficiency was examined by keeping the solvent type constant as dichloromethane (DCM) and altering the type and concentration of the surfactants. Obtained results shown in Table 3-1 illustrated that the particle mean size was altered from 1198 nm to 218 nm with the order of PVP>T80>PVA.
The influence of surfactant concentration on nanocapsules properties was performed by using DCM as solvent and changing each surfactant concentration from 1% to 4%. It was observed that the smaller sizes have been obtained when the concentrations were increased from 1% to 4% as shown in Table 3-1.

Table 3-1 The effect of surfactant and its concentration on the size and shape of NCs

Surfactant	Solvent	Yield (%)	Loading efficiency (%)	Size (nm)
PVA (4%)	DCM	50	48.4	218
PVA (1%)	DCM	54	75.1	503
T80 (4%)	DCM	62	59.0	709
T80 (1%)	DCM	56	77.6	807
PVP (4%)	DCM	64	82.0	834
PVP(1%)	DCM	70	89.8	1198

In the literature, the mean sizes of PSA based nanoparticles prepared via emulsion evaporation method have been reported as 334 nm (Guhagarkar et al., 2010) and >1000 nm (Hanes et al., 1998; Kipper et al., 2002; Berkland et al., 2004; Shelke et al., 2007). Obtained results in this study shows that smaller PSA nanoparticles with 218 nm size can be obtained with a significant reduction in size, by small adjustments in the preparation processes. The smallest particles were obtained for the case of using PVA as surfactant. This phenomenon can be explained by the effective adsorption of PVA on the surface of nano particles and preventing their agglomeration. On the other hand, in case of PVP larger (almost 4 times for 4% and 2 times for 1%) NCs were obtained since PVP is not as strong as PVA to prevent agglomeration of the particles. In literature it is given that PVP is a suitable surfactant to get the smallest sizes of metallic nanoparticles such as Molybdenum oxides (Reddy et al., 2009), Zinc oxide (Srivastava et al., 2012), Silver (Wang et al., 2005) and Cobalt (Shao et al., 2006) instead of polymeric nanoparticles. In our study, it was observed that the effect of T80 is moderate and it is also effective to reduce surface tension energy of PSA based nanocapsules and prevent their aglomeration.

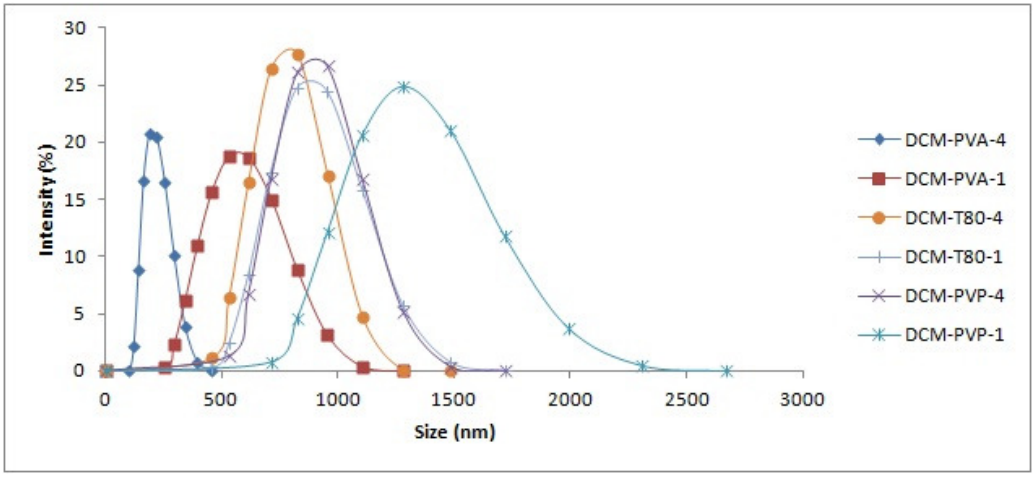


Figure 3-7 Size distribution curves of NCs prepared by using 1% and 4% concentrations of PVA, T80 and PVP.

On the other hand, the concentration of surfactant is also an effective parameter in controlling the sizes of the prepared particles. It was observed that average size of NCs became larger and size distribution became wider when the concentrations of the surfactants were decreased from 4% to 1% (Figure 3-7). This result is obvious since the presence of higher concentrations of surfactant decrease the surface energy of nanoparticles. This fact has been approved by results reported in literature related to using 1% PVA (Tang et al., 2009), 2.5% PVA (Shelke et al., 2007), 3% PVA (Pfeifer et al., 2007) and 5% PVA (Lee et al., 2008) resulting to obtain PSA based nanoparticles with mean sizes of 1800, 1000, 428 and 423 nm, respectively. As it is given, increase in the concentration of PVA caused significant decreases in the size of nanoparticles.

There are various studies related to development of loading efficiency of DOX by overcoming to migration of DOX molecules from the organic to aqueous phase which is the main cause of the high amount of non loaded drug in supernatants. Some of these challenges are cooperation of DOX with anionic polymers (Wong et al., 2004) and investigation of anionic surfactant (Chavanpatil et al., 2007) to obtain 42.5 and 49.3% encapsulation efficiency. In another study, DOX cations have been entrapped in nanoparticles prepared from polyethylene sebacate and Gantrez AN 119 (copolymer of methyl vinyl ether and maleic anhydride) by emulsion method (Guhagarkar et al., 2010). Gantrez can be hydrolyzed and produced anionic molecules when it presents in aqueous medium. This phenomenon leads the cationic DOX molecules to be captured between anionic chains of Gantrez and polyethylene sebacate leading to obtain >80% loading efficiency. In the present study, the maximum observed loading efficiency was 89.8% which was achieved in particles prepared by using PVP 1%. This high encapsulation efficiency is directly linked to the size of objective particles which are the largest size obtained in this research. It was revealed that the loading efficiency and production yield enhances by increasing in the size of NCs as it was expected.

It is known that PSA is a crystalline polymer. Therefore PSA based particles prepared by solvent evaporation method tend to yield micro or nanoparticles with fractured and porous surface and this fact was approved before (Mathiowitz et al., 1990). The surface roughness is resulted from partial degradation that takes place during micronization process (Kipper et al., 2002). SEM images presented in Figures 3-8-a and Figure 3-8-c demonstrated that against the mentioned issues related to rough surface of PSA based micro and nanoparticles, spherical-shaped particles with a smooth surface were obtained when DCM was used as solvent and PVA and T80 were used as surfactants with concentration of 4%. Figure 3-8-b shows that investigation of PVP causing to produce drug loaded polymeric matrixes which are not in spherical or capsule shape which is related to its less ability to protect the spherical shape of nanocapsules.

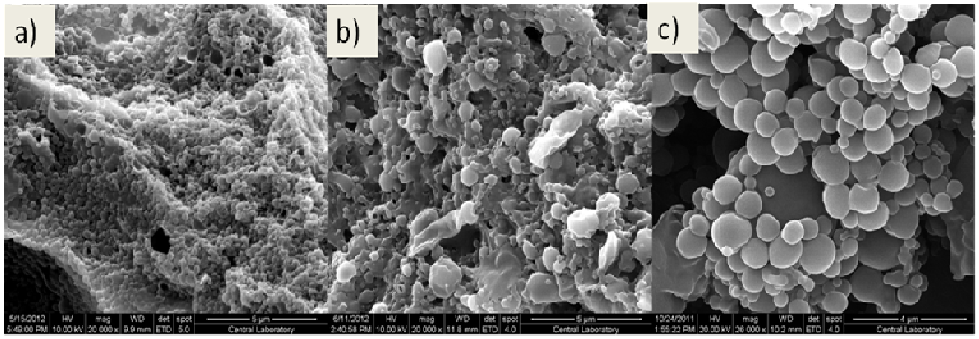


Figure 3-8 SEM micrographs of NCs prepared with DCM and different surfactants. a) PVA 4%, b) PVP 4%, c) T80 4%.

As it was expected, by increasing the surfactant concentration from 1% to 4%, the prepared NCs get fine spherical shape and show more homogenous sizes (Figure 3-9).

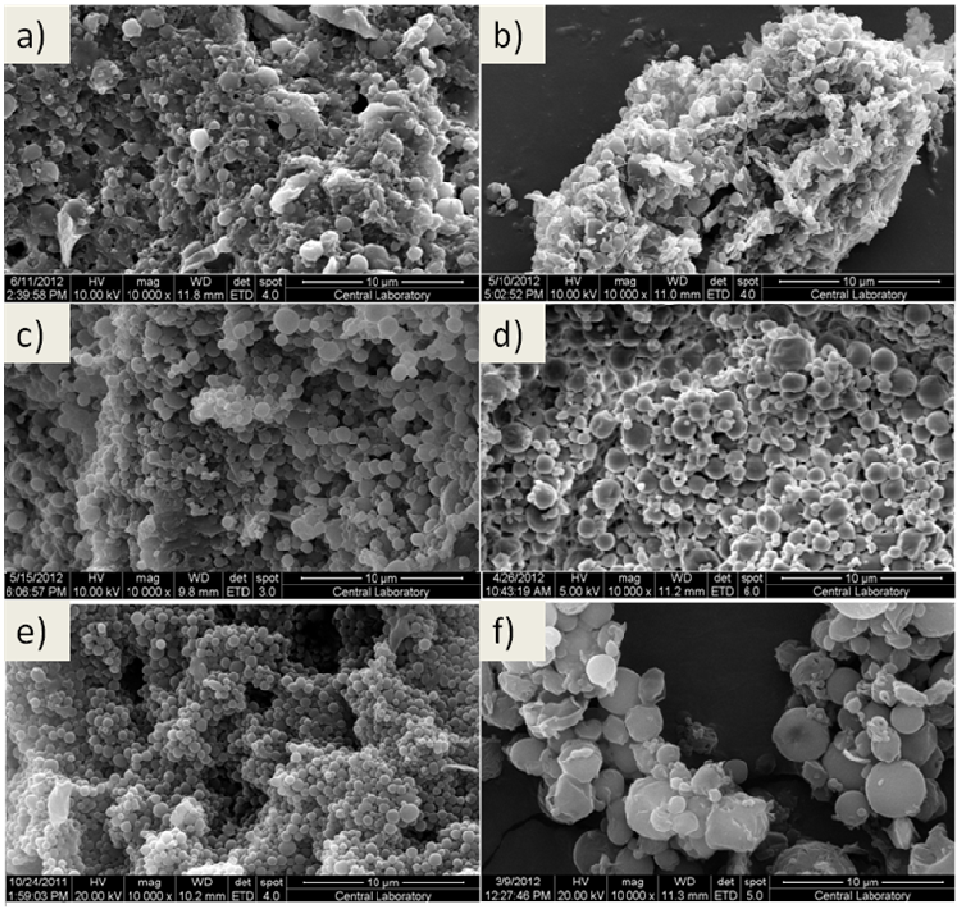


Figure 3-9 SEM micrographs of NCs prepared with different surfactants. a) D-PVP-4, b) D-PVP-1, c) D-PVA-4, d) D-PVA-1, e) D-T80-4, f) D-T80-1.

3.3.2 Effect of organic solvent on size and morphology of NCs

Type of organic solvent also has enormous effect on the solidification step of emulsion-evaporation method used in the preparation of NCs as it is shown in Table 3-2. The volatility, viscosity and miscibility of organic phase affect hardening and stability of nanoparticles (Sahana et al., 2008).

Table 3-2 The effect of solvent on the size and shape of NCs.

Solvent	Surfactant	Yield (%)	Loading efficiency (%)	Size (nm)
DCM	PVA (4%)	50	48.4	218
EA	PVA (4%)	61	85.8	496

If the solvent evaporates with difficulty having more evaporation time, this leads producing particles with larger sizes (Hiremath et al., 2012).
There are various solvents such as tetrahydrofurane (Wu et al., 2000), ethanol (Hiremath et al., 2012) and DCM (Ulery et al., 2009; Tang et al., 2009; Shelke et al., 2007) which have been used for the preparation of PSA based nanoparticles via either nano precipitation or emulsion-evaporation methods. Among them DCM is most commonly used solvent for manufacturing of anhydride based nanoparticles. The smallest size of PSA based nanoparticles prepared by using DCM and PVA reported in previous researches is 428 nm (Pfeifer et al., 2007). Considering to mentioned result, nanocapsules prepared in this study exhibit smaller and promising size (218 nm) to exploit EPR effect in passive targeting.
The type of solvent also influences the size, homogeneity and size distribution of the produced nanoparticles. It is apparent that when ethyl acetate (EA) was used as solvent, larger size and broader size distribution have been achieved compared to DCM. This can be explained with its higher water miscibility, higher viscosity and lower volatility in compare to DCM (Sahana et al., 2008). These properties cause to evaporation of EA with a slower rate in longer time, leading agglomeration of nano-sized particles leading to form larger NCs (Figure 3-10). On the other hand, size distribution peak also gets wider demonstrating more heterogeneous distribution.
As it is observed from SEM micrographs (Figure 3-11-b), by changing the solvent from DCM to EA, some of NCs consisted orifices on their surface. It may be due to miscibility of EA in water phase causing to formation of holes during the evaporation of EA during the process.

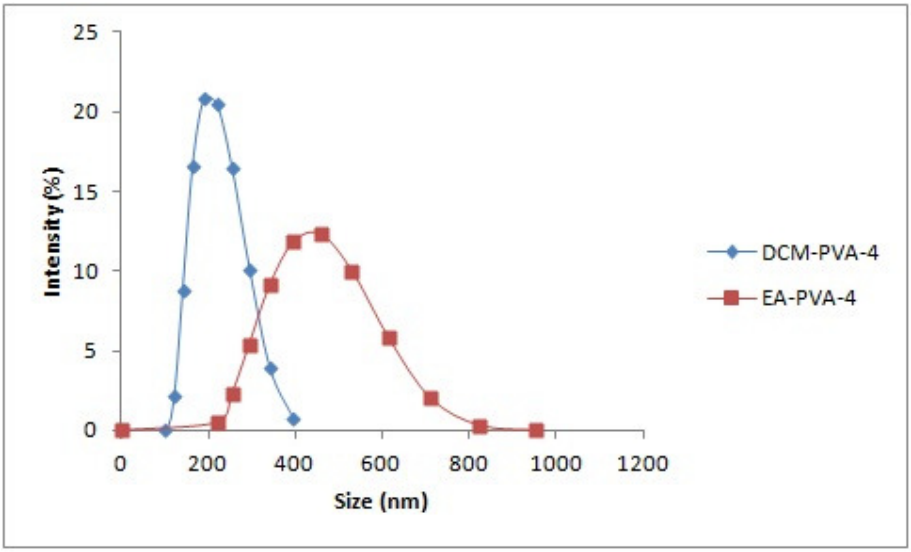


Figure 3-10 Size distribution of NCs prepared by using DCM and EA solvents.

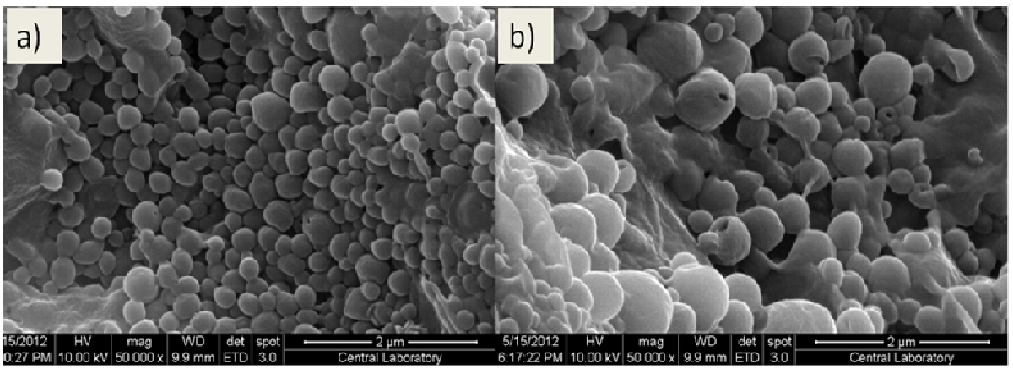


Figure 3-11 SEM micrographs of NCs prepared with different solvents. a) DCM, b) EA.

3.3.3 In situ DOX release profiles and kinetic

Controlled release drug delivery systems include delivery of a drug by a desired rate resulting to decrease side effects and dose taking frequency. Drug release profile and rate of degradation of biomaterial are important parameters in manufacturing controlled release delivery vehicles. Drug release rate from nanoparticles can be considerably changed by size, morphology and shape (Dong et al., 2009).

Drug release profiles of PSA nanoparticles were reported in literature for both hydrophobic and hydrophilic model drugs (Shen et al., 2002). It was evidenced that hydrophobic drugs distribute uniformly in the polymer matrix hence they exhibit a release profile in similar manner of monomer release. In the case of hydrophilic drugs they precipitate in the polymer matrix and consequently show a burst effect in their release profile. This scene was observed in DOX release profiles of all type of NCs prepared in this study. SEM micrographs shown in Figures 3-8 illustrated that NCs formulated using PVP have the largest size in compare to other groups. The results obtained by in situ DOX release study of NCs are displayed in Figure 3-12 and it was indicated that despite their larger sizes NCs prepared using PVP (D-PVP-1, D-PVP-4) present a faster burst release profile in compare to sustained release of DOX in case of NCs formulated via T80 (D-T80-1, D-T80-4) and PVA (D-PVA-1, D-PVA-4). This behavior is consistent to present of non spherical and drug containing polymeric matrixes in this group causing to quick and burst release of drug in the first 30 h. DOX release behavior of NCs prepared by T80 exhibit slower release rate via a sustained manner which stems from sizes of this group. Additionally there are some researches concerning to investigation of T80 as an inhibitor for corrosion of different formulations (Abdallah et al., 2003; Ramji et al., 2008; Dhanya et al., 2011). Considering to this results it was speculate that remained T80 on the surface of nanocapsules may cause to retard in polymer degradation inducing a delay in drug release from NCs.

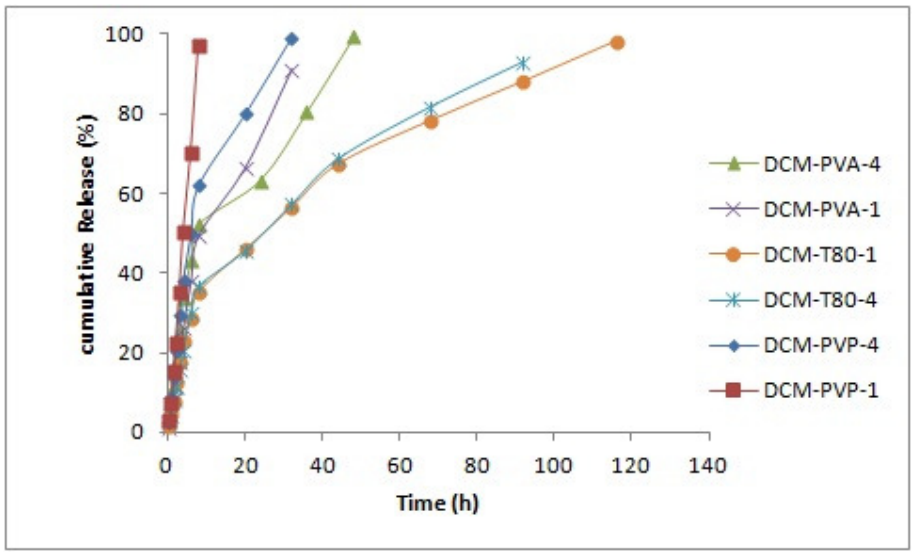


Figure 3-12 DOX release profiles of NCs.

By the study of the effect of solvent on the release rate, it was revealed that orifices presented on the surface of NCs solidified in EA (Figure 3-11-b) speed up water penetration into the inside of the nanocapsules. This phenomena influence the dissolving of hydrophilic DOX and incurring subsequently release of drug in compare to drug release from NCs formulated by DCM (Figure 3-13).

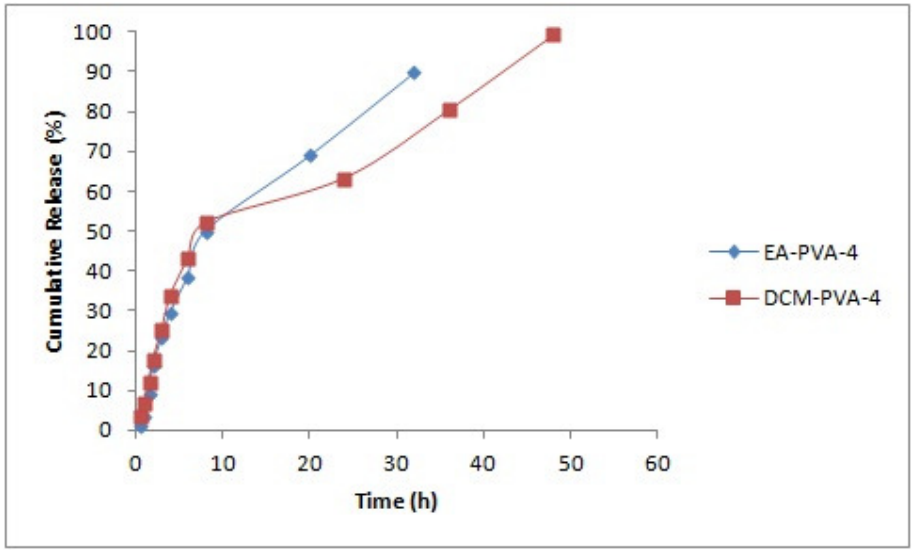


Figure 3-13 DOX release profiles of NCs prepared by using DCM, EA as solvent.

Release kinetics of all the prepared samples were studied by plotting of drug release curves due to Higuchi model. In this model cumulative drug release is proportional to square root of time as the given equation (Shoaib et al., 2006):

$$Q=K_H t1/2$$

where,
Q = Cumulative amount of released drug at time t, K_H is Higuchi constant, and t is time in hours.

Drug releases fitted to this model explain a release via diffusion manner. Figure 3-14 clarifies that approximately all NCs prepared by different surfactants and surfactant concentrations fit and follow the Higuchi release pattern. In the case of NCs emulsified by T80, the release pattern has slipped in higher percentages of cumulative release and was not compatible with the release curves of the others. This phenomenon approved this fact that T80 remained on NCs surfaces prevents the diffusion and drug release leading to delay in drug releases as it was observed in Figure 3-12. The effect of solvent on release kinetic was presented in Figure 3-15. It was observed that both curves related to drug release of NCs prepared in either DCM or EA follow Higuchi model well.

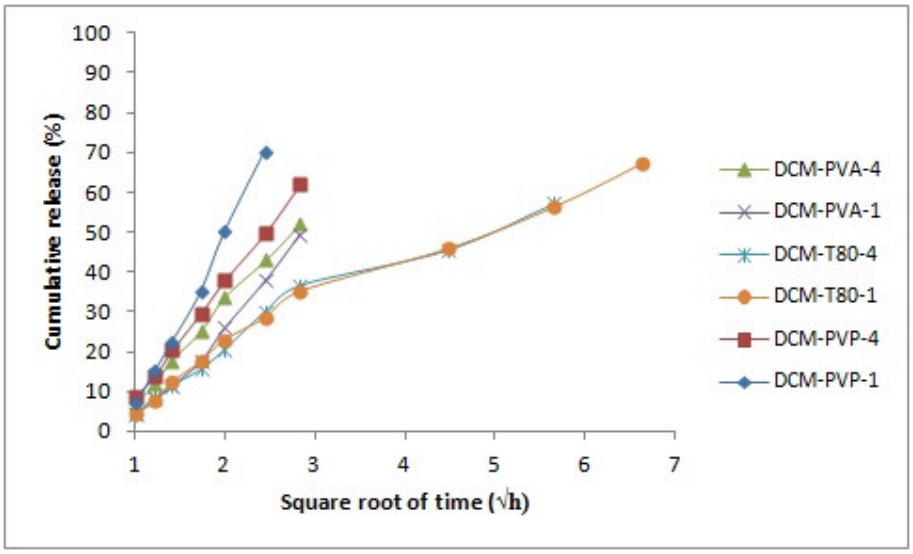


Figure 3-14 Higuchi drug release curves from the NCs prepared by different concentrations of PVA, PVP and T80.

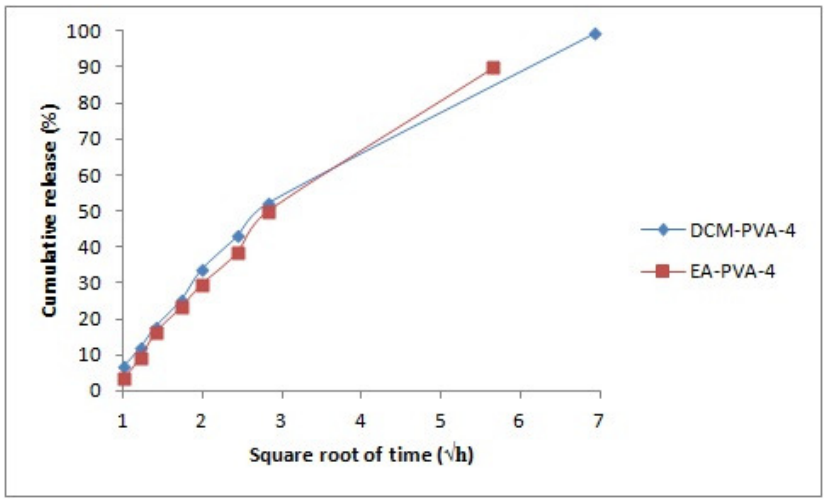


Figure 3-15 Higuchi drug release curves from the NCs prepared in DCM and EA.

3.3.4 Cellular uptake and antitumor efficiency of DOX loaded NCs

The cytotoxicity study of DOX loaded nanocapsules and free drug were performed against human breast cancer cell line MDA-MB-231 by colorimetric cell viability test. These assays are based on color shift of the culture medium via reaction of living cell metabolites with assay solution chemical (Khattak et al., 2006). In this experiment MDA-MB-231 cells were grown by protocols recommended by ATCC, seeded in 96-well plates and treated by blank unloaded nanocapsules, free DOX with concentration of 0.6 µg/mL and D-PVP-4, D-PVA-4 and D-T80-4 NCs carrying the same amount of DOX. Following to 3-day incubation cell viability tests was performed using WST cell counting kit-8. Blank nanocapsules and untreated cells were used as control groups. Viability percentage was calculated using absorbance of untreated cells as 100% survival. Results of cytotoxicity and tumor efficiency of nanocapsules is shown in Figure 3-16. According to obtained results drug-free blank nanocapsules did not exhibit any detectable cytotoxicity on MDA-MB-231 cells. This consequence was in agreement with cytotoxicity studies of PSA based formulations reported in literature (Leong et al., 1986; Laurencin et al., 1990; Shikanov et al., 2004; Zhang et al., 2012).

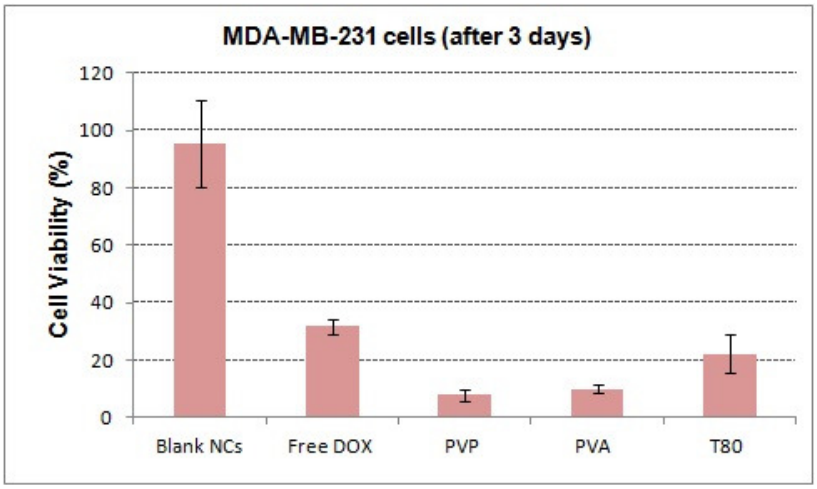


Figure 3-16 Cytotoxicity and tumor efficiency of NCs formulated by of PVP (D-PVP-4), PVA (D-PVA-4) and T80 (D-T80-4) with DOX concentration of 0.6 µg/mL.

According to survival curves in Figure 3-16 it can be observed that DOX loaded NCs formulated using PVP (D-PVP-4) and PVA (D-PVA-4) exhibit higher anticancer efficiency than the T80 (D-T80-4). This expected result arises from a mutual correlation with drug release rate from NCs. As it mentioned in part 3.3.3, almost more than 90% of loaded DOX in NCs prepared by PVP and PVA is released during 1 and 2 days, respectively (Figure 3-12). While NCs fabricated by using T80 release same amount of drug in more than 4 days. Hence to get the same antitumor activity more incubation time is required.

By comparing the antitumor efficiency of free DOX with DOX loaded NCs formulated using PVP (D-PVP-4) and PVA (D-PVA-4), it was observed that NCs display more cytotoxicity in compare to free drug. This phenomenon is attributed to cellular uptake of NCs and their intracellular drug release approved by fluorescence imaging microscopy. Regarding to fluorescence property of DOX, it was easily recognized by using TRITC filter in fluorescence microscopy as shown in Figure 3-17.

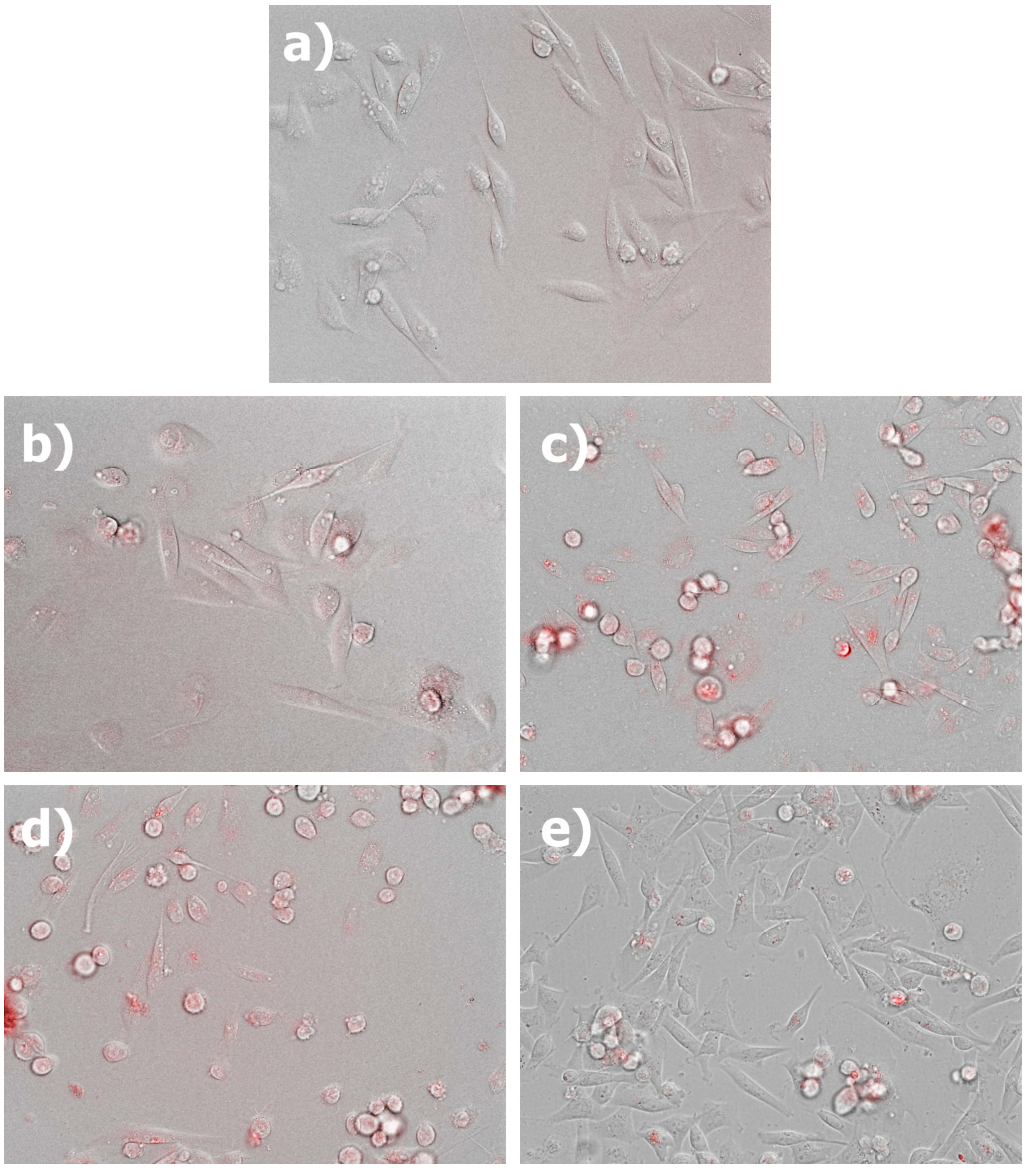


Figure 3-17 Fluorescence microscopy of cells. a) control cells, b) treated with free DOX 0.6 µg/mL, c) treated with D-PVP-4 NCs (0.6 µg/mL), d) treated with D-PVA-4 NCs (0.6 µg/mL), d) treated with D-T80-4 NCs (0.6 µg/mL).

Figure 3-17 illustrated that a large number of the NCs were internalized and distributed in cytoplasm region of the MDA-MB-231 cells, according to their nano size. This consequence indicates that NCs exhibit more effective treatment due to their significantly high internalization of DOX resulted from NCs cellular uptakes.
In defiance and compare of results obtained from characterization of NCs prepared using different solvents, surfactants and surfactant concentration it was observed that NCs formulated by PVA 4% as (Figure 3-15-d) surfactant and DCM as solvent (D-PVA-4) possess potential to investigate as an anticancer delivery systems. Consequently further parts of this research (coating with PLH and pegylation) were performed by using NCs prepared by PVA 4% as surfactant and DCM as solvent.

3.4 Characterization of pH responsive nanocapsules

3.4.1 Drug encapsulation and loading capacity

Sustained drug delivery system is usually defined as a delivery technique to carry at least two-fold dosage of pharmaceutic agents (Bankar et al., 2012; Singh et al., 2010). Hence, encapsulation efficiency and loading capacity of prepared delivery systems play an important role in sustained delivery. The advanced approach for drug-loaded and polymeric based delivery systems is towards obtaining high drug loading capacity. Water-soluble doxorubicin exhibits weak amphipathic properties (pKa 8.3) leading to stay in aqueous medium. There are various challenges related to improve DOX loading efficiency by preventing its migration from the organic to aqueous phase which is the main cause of the high amount of non loaded drug in supernatants.
For instance, in the last decades doxorubicin loading has been improved by applying a pH gradient. By this way DOX permeated to entrap inside the capsules by attraction to pre-encapsulated citrate ions (Madden et al., 1990; Li et al., 1998).
In some studies it has been reported that the loading capacity can be increased from 10 to 20 folds by creating a pH gradient such as pH 7 in outside and pH 4 in inside of the delivery systems (Ahmed et al., 2006; Choucair et al., 2005). In some of studies cooperation of DOX with anionic polymers (Wong et al., 2004) and investigation of anionic surfactant (Chavanpatil et al., 2007) have been applied to develop DOX encapsulation efficiency to 42.5 and 49.3%, respectively. In the other research, DOX cations have been entrapped in nanoparticles prepared from polyethylene sebacate and Gantrez AN 119 (copolymer of methyl vinyl ether and maleic anhydride) by emulsion method (Guhagarkar et al., 2010). Gantrez can be hydrolyzed and produced anionic molecules when it presents in aqueous medium. This phenomenon leads the cationic DOX molecules to be captured between anionic chains of Gantrez and polyethylene sebacate leading to obtain >80% loading efficiency.
In this study encapsulation efficiency and loading capacity of DOX in polysebasic anhydride base nanocapsules prepared by DCm and PVA 4% was determined as 48% and 5.30%, respectively without any modification to improve loading efficiency. Consequently, NCs fabricated in this study via double emulsion method exhibit promising content of drug encapsulation for DOX delivery.

3.4.2 pH responsibility

Poly (L-histidine) (PLH) is a kind of biodegradable polyamino acid. It involves many imidazole functional groups with pKa around 6.0 leading to exhibit buffering property in the physiological pH range. Imidazol groups can be protonated in pH below 5.8 leading to dissolve of poly (L-histidine) in dilute acids (Patchornik et al., 1957). This feature has been investigated to design different PLH based complexes as pH-sensitive delivery systems. For instance PLH with aminoethyl groups and PLH conjugated with carbohydrate have been prepared to promote delivery efficiency to mammalian cells (Asayama et al., 2004; Wang et al., 1984). PLH also has been applied either as an outer shell or in cooperation with alginate to prepare microcapsules used for protein delivery (Wang et al., 2005; Chen et al., 2011). In these researches PLH has defined as a promising compound to apply for protein delivery.
As main part of this research, stimulus response has been based on the pH responsibility of poly (L-histidine). For this purpose, DOX loaded NCs were coated by adsorbing PLH molecules on the NCs surfaces by electrostatic forces. For this purpose zeta potential of both DOX loaded NCs and PLH were analyzed in dilute acid medium regarding to create the same condition needed for coating. According to results zeta potential of +29.4 mV and -35 mV were obtained for PLH and DOX loaded nanocapsules, respectively. Due to the obtained results positively charged PLH can be adsorb on negatively charge DOX loaded NCs. To check the possibility of this hypothesis, PLH-coated NCs

(PLH-DOX-NCs) were characterized by FTIR to see absorbance peaks coming from PLH functional groups on the FTIR spectrum of NCs. Figure 3-18 presents FTIR spectra related to DOX loaded nanocapsules (a), DOX loaded nanocapsules which have been coated with PLH (PLH-DOX-NCs) (b) and pure PLH used as blank to compare the success of coating.

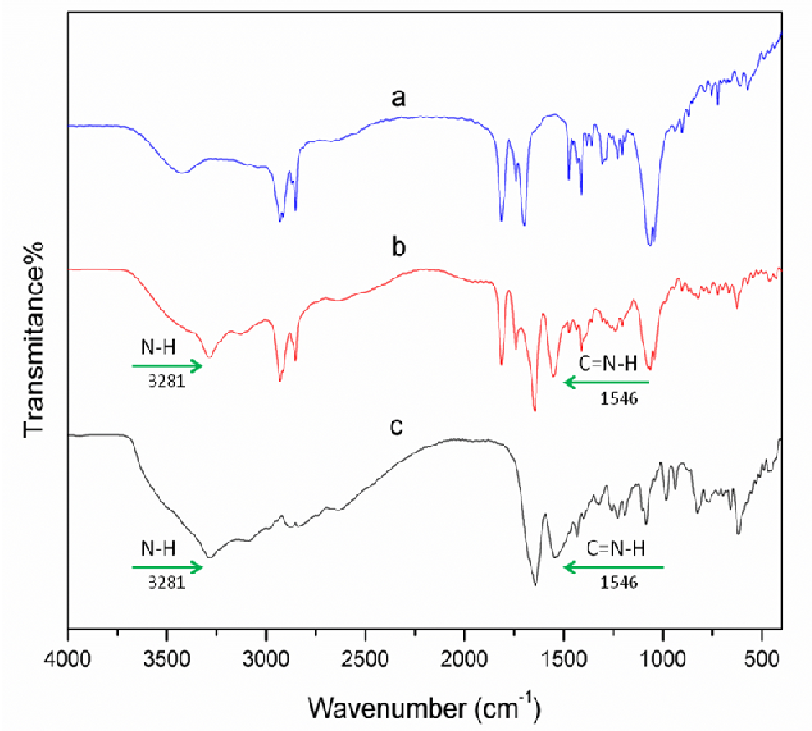


Figure 3-18 FTIR spectra. a) DOX loaded NCs, b) PLH coated and DOX loaded NCs (PLH-DOX-NCs), c) pure PLH.

By comparing these spectra, it was illustrated that spectrum of DOX loaded and PLH coated NCs (Figure 3-18-b) not only displays peaks coming from DOX loaded nanocapsules (Figure 3-18-a) but also contains absorbance peaks coming from imidazole groups presented in PLH polymer chain (Figure 3-18-c). These results show the presence of PLH on the surface of NCs; so that they gain pH responsibility from PLH.

3.4.3 Pegylation of pH responsive NCs

The macrophage uptakes of prepared pH responsive delivery vehicles were reduced by modification of nanocapsules with PEG. The modified nanocapsules were characterized by FTIR to see absorbance peaks coming from PEG functional groups on the FTIR spectrum of pH responsive NCs. Figure 3-19 presents FTIR spectra related to PEG (a), Pegylated pH responsive and DOX loaded nanocapsules (PEG-PLH-DOX-NCs) (b) and pH responsive NCs (PLH-DOX-NCs). The spectra of PEG was used as blank to compare the success of modification.

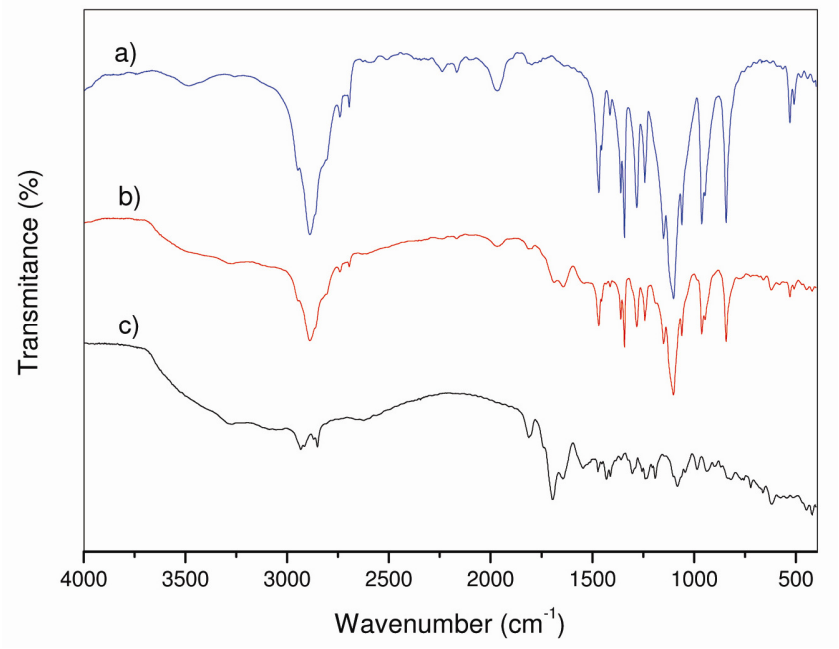


Figure 3-19 FTIR spectra. a) PEG, b) pegylated and PLH coated NCs, c) PLH coated and DOX loaded NCs.

By comparing these spectra, it was concluded that spectrum of pegylated NCs (Figure 3-19-b) displays peaks coming from pH responsive NCs (Figure 3-19-c) as well as absorbance peaks appeared in 2820 and coming from PEG functional groups (Figure 3-18-a).
The aim of this study is investigation of EPR effect for passive targeting. Hence, the final size NCs coated by PLH and modified with PEG is important. The effect of these processes on morphology and size of nanocapsules were examined by DLS and SEM. The results obtained from DLS which is shown in Figure 3-20 proved that the size of NCs is not affected significantly by coating and modification.

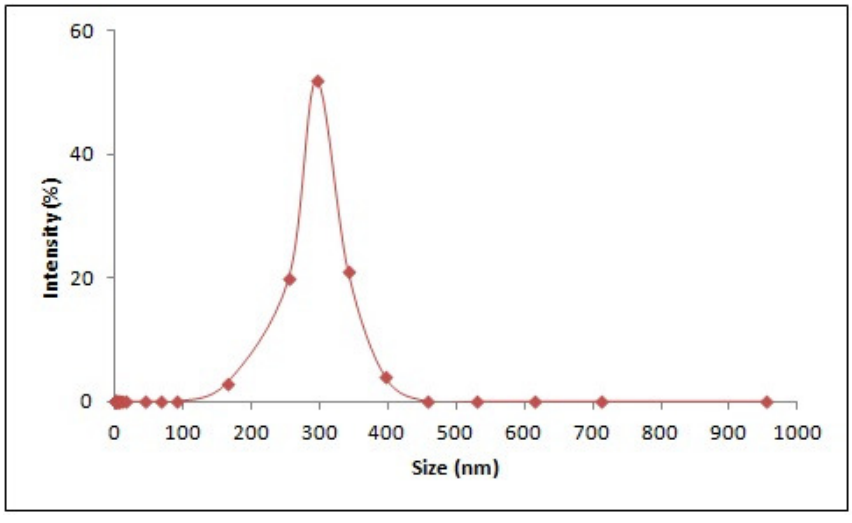


Figure 3-20 Size and size distribution of NCs after coating with PLH and modification by PEG.

The SEM micrographs shown in Figure 3-21 presents the final morphology of pegylated pH responsive NCs. It was observed that the spherical shape of NCs have been changed slightly during the coating and modification process. This phenomenon is resulted from heterogeneous coating of NCs with PLH during the precipitation of PLH on surface of NCs.

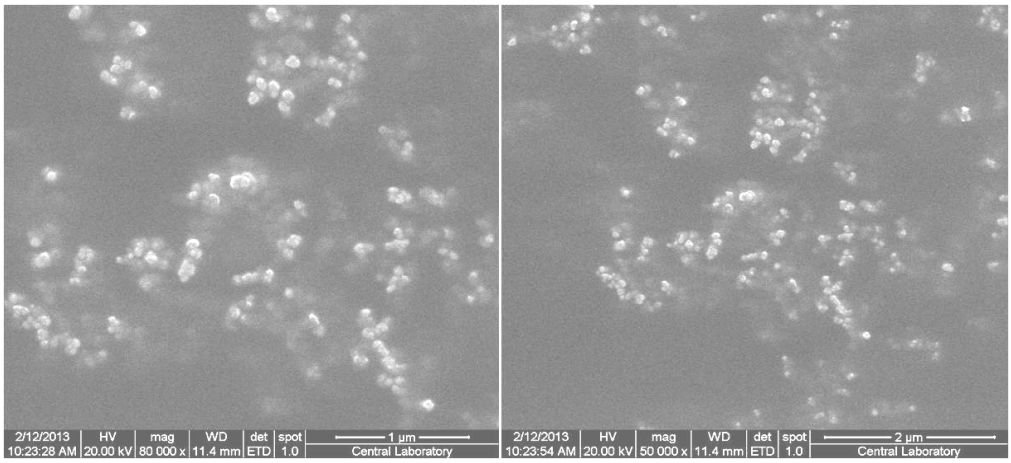


Figure 3-21 SEM micrograph of NCs after coating with PLH and modification by PEG.

3.4.4 In situ DOX release study from PEG-PLH-DOX-NCs

The in vitro release profile of the pH responsive nanocapsules (PEG-PLH-DOX-NCs) with drug loading efficiency of 48% is given in Figure 3-22. This release profile within 200 hours signifies the potential viability of the pH responsive polysebacic anhydride based nanocapsules as a device for passive targeting delivery systems. Release profiles of nanocapsules in acidic and neutral PBS demonstrated that coated nanocapsules release higher amount of drug in their both burst and sustained-release steps due to dissolving of poly (L-histidine) in acidic pH. By this way drug concentration will reach to effective dose in short time. In next step because of slow degradation of polysebacic anhydride drug will release in a sustained pattern. In the case of pH 7.4, it takes more time to achieve effective dose. To decrease the release more effectively in pH 7.4, using of high molecular weight polymers and/or double coating of nanocapsules by poly (L-histidine) can be suggested.

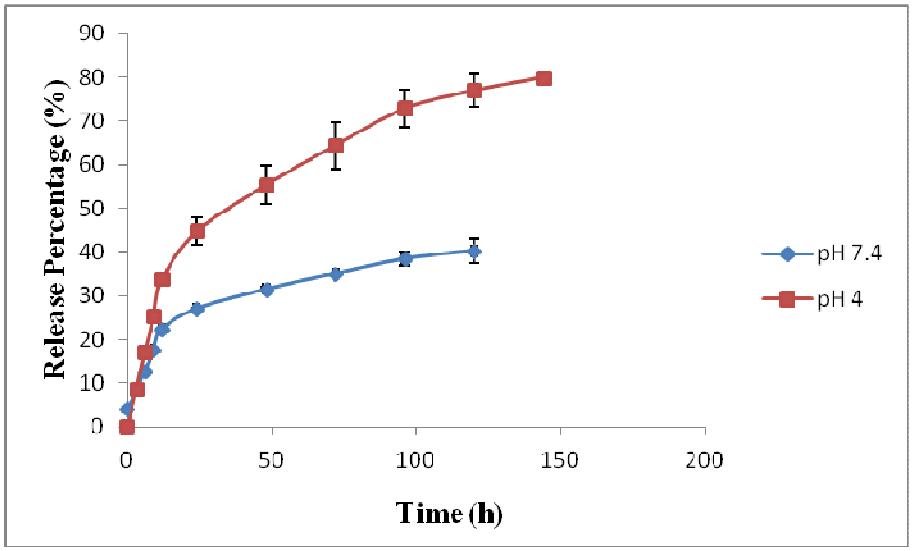


Figure 3-22 DOX release profiles of PEG-PLH-DOX-NCs at pH 4.0 and pH 7.4

3.4.5 Cell culture study of pH responsive nanocapsules

3.4.5.1 Antitumor efficiency

The antitumor efficiency of free DOX was compared with DOX encapsulated in NCs. For this purpose colorimetric cell viability tests were performed.

3.4.5.1.1 MTS cell viability assay

MTS assay is based on reduction of a tetrazolium salt by metabolites of growing cells to a colored formazan with absorbance at 490 nm (Capasso et al., 2003). Results of cytotoxicity and tumor efficiency of nanocapsules have been shown in Figure 3-20. According to obtained results drug free nanocapsules did not exhibit any detectable cytotoxicity on MDA-MB-231 cells. This consequence was in agreement with cytotoxicity studies of PSA based formulations reported in literature (Leong et al., 1986; Laurencin et al., 1990; Shikanov et al., 2004; Zhang et al., 2012). In this research, firstly all cell viability tests were performed by MTS assay. Results obtained from this calorimetric proliferation tests were not match to results expected from antitumor efficiency of both free DOX and DOX loaded NCs. In most of results more than 70% of MDA-MB-231 cancerous cells were alive after 3-day treatment by DOX with concentration of 0.6 µg/mL either in nanocapsules or even in case of free drug. Observed results were not acceptable. The experiment was repeated in different conditions by changing some parameters such as the amount of cell culture medium (from 200 and 100 µL) and the number of seeded cells (from 5000 t0 2500 cells per well) to find the source of problem. As it is shown in Figures 3-23-a and Figure 3-23-b, results illustrate the same feature with previous conditions. To get expected antitumor efficiency different concentration of free DOX (0.6 µg/mL and 1.2 µg/mL) were applied to treat MDA-MB-231 cells in 3 days and analyzed by MTS assay again. According to results shown in Figure 3-24, it was unexpectedly pointed that the absorbance values were higher indicating more living cells via applying higher concentration of DOX. This fact leads us to focus on MTS assay protocols. It was the noticed that the UV absorbance of MTS assay was 490 nm which was almost the same as the absorbance of DOX. Therefore instead of absorbance coming from the living cells, absorbance of DOX was detected accidentally. Hence to have a correct cell viability test other cell proliferation assay, WST kit-8, with UV absorbance at 450 nm was applied.

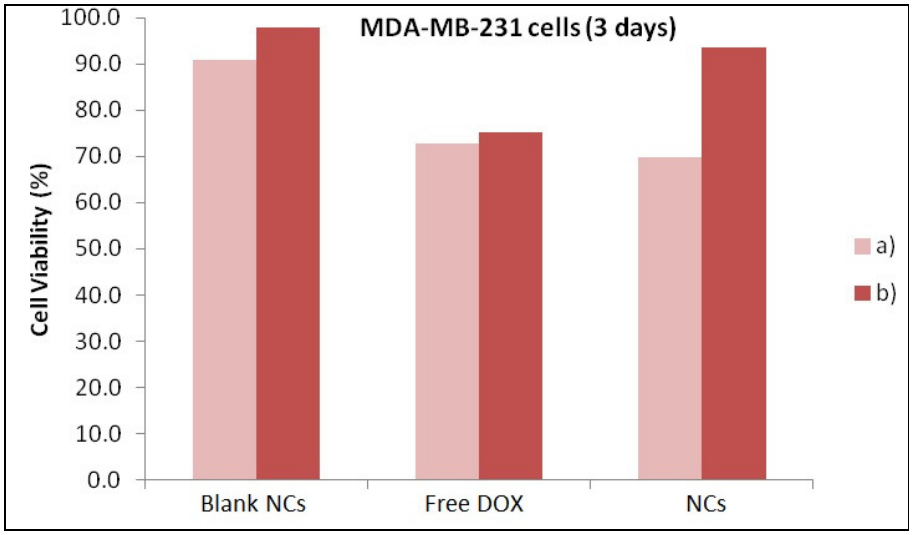


Figure 3-23 MTS cell viability assay. a) by applying less number of cells (2500 cells per well), b) by applying less amount of cell culture medium (100 µL).

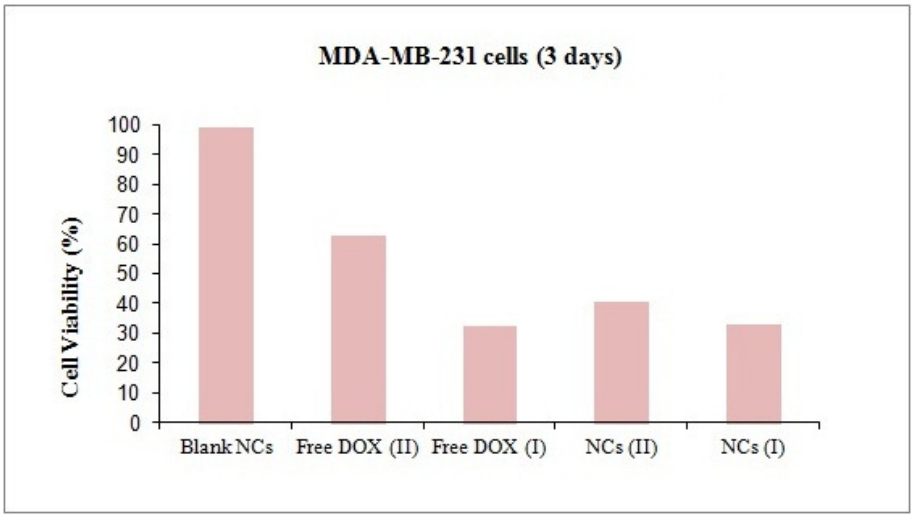


Figure 3-24 MTS cell viability assay by applying different concentration of DOX, (I): 0.6 µg/mL, (II): 1.2 µg/mL .

3.4.5.1.2 WST kit-8 cell viability assay

Antitumor activity of free DOX and PEG-PLH-DOX-NCs were analyzed by using WST cell counting kit-8. WST-8 is a kind of tetrazolium salt [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium] converting to a water soluble dye by biological reduction carried out by living cell's metabolites. In these experiments cells were seeded in 96-well plates and treated by 0.6 µg/mL free DOX, drug loaded NCs containing same amount of DOX and blank NCs. Cell viability assay was done after 3-day incubation.

In part 3.3.4 it was demonstrated that no significant cytotoxicity has been observed in MDA-MB-231 cells treated by drug-free PSA based nanocapsules which made them suitable to use as potential drug delivery systems. This expected feature is match to published studies related to cytotoxicity of PSA based components and formulations (Leong et al., 1986; Laurencin et al., 1990; Shikanov et al., 2004;

Kim et al., 2007; Shikanov et al., 2010; Zhang et al., 2012). In this part the cytotoxicity of pegylated and PLH coated blank nanocapsules (PEG-PLH-NCs) were studied and compared with literature. According to the literature no cytotoxicity effect has been observed in PLH and its derivations (Benns et al., 2000; Liu et al., 2011; Casolaro et al., 2012).
The cytotoxicity results of the pH responsive nanocapsules demonstrated that cell viability obtained from blank NCs is relatively higher than 100%. This result approves that PLH is in favor of cell proliferation.
The main action of free DOX is preventing DNA duplication and an inhibition of the topoisomerase II (Minotti et al., 2004; Hande et al., 2008). Hence treatment of cancer cells in duration more than their recycle time can guaranty the validity of the obtained results (Gautier et al., 2012). Figure 3-25 shows the cell viability of MDA-MB-231 cells exposed to DOX with concentrations of 0.6 µg/mL and 1.2 µg/mL in free and encapsulated state. Results reveal that in contrast to results obtained by MTS assay higher cytotoxicity levels were observed in cells treated with higher concentrations of DOX as expected.
By comparing the behavior of free and loaded DOX, it was seen that although same amount of DOX has been used in both cases, more antitumor activity has been observed by DOX loaded nanocapsules. This phenomenon can be linked to cellular uptake and intracellular release coming from high endosomal-lysis capacity of outer shell of NCs constituted of pH responsive property of poly (L-histidine).

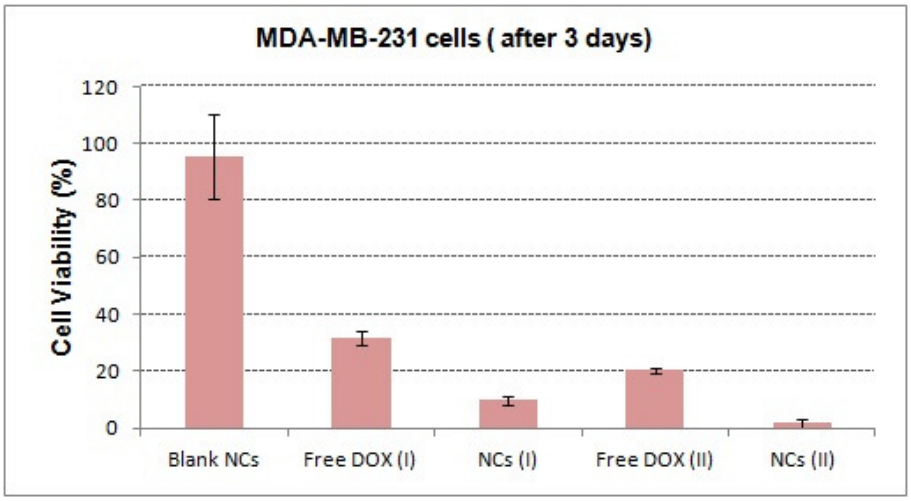


Figure 3-25 WST kit-8 cell viability assay of blank NCs (PEG-PLH-NCs), free DOX 0.6 µg/mL (I) and 1.2 µg/mL (II), PLH coated NCs (PEG-PLH-DOX-NCs) containing 0.6 µg/mL (I) and 1.2 µg/mL (II) DOX.

3.4.5.2 Intracellular release and cell uptake

Intracellular release is a promising strategy to develop the therapeutic efficacy of anticancer drugs. There are various challenges to improve the properties of DOX-loaded polymeric vehicles to enhance the intracellular concentration of DOX especially in the drug resistant cells (Kirchmeier et al., 2001; Huang et al., 2011; Gao et al., 2011). Poly (L-histidine) has been known to trigger fusion activity of cell membrane. It has been reported that PLH can be protonate in acidic medium and can induce phospholipid bi-layers by performing 16 interactions between the protonate molecules and negatively charged phospholipids (Uster et al., 1985; Wang et al., 1984).
In this study the intracellular release of DOX was performed via using a PLH shell around the DOX loaded PSA NCs. Intra cellular release was characterized by confocal microscopy. For this purpose non-treated cells, cells treated by the same concentration of free DOX and nanocapsules loaded with DOX were analyzed. To determine the location of the released drug, the nucleus was stained by using DAPI (blue) during the cell fixing process. TRITC filter responding to red fluorescence signals was

employed to recognize the region of DOX. A merge image of fluorescent photos with phase-contrast photo was obtained to better analysis. Figure 3-26 shows the resulting intracellular release of DOX from pH responsive NCs. In control cells (Figure 3-26-a) which were treated only by PBS there was no red fluorescence signal coming from DOX. In the case of cells treated by free DOX, (Figure 3-26-b) DOX primarily enters into MDA-MB-231cells and easily localizes in nuclei as it has been demonstrated previously (Suo et al., 2010; Zhang et al., 2012).
For cells treated by PLH coated NCs (PEG-PLH-DOX-NCs), significant increase has been observed for intracellular release of DOX (Figure 3-26-c). The concentrated red parts appeared in cytoplasm were attributed to uptaking of NCs by MDA-MB-231 cells.

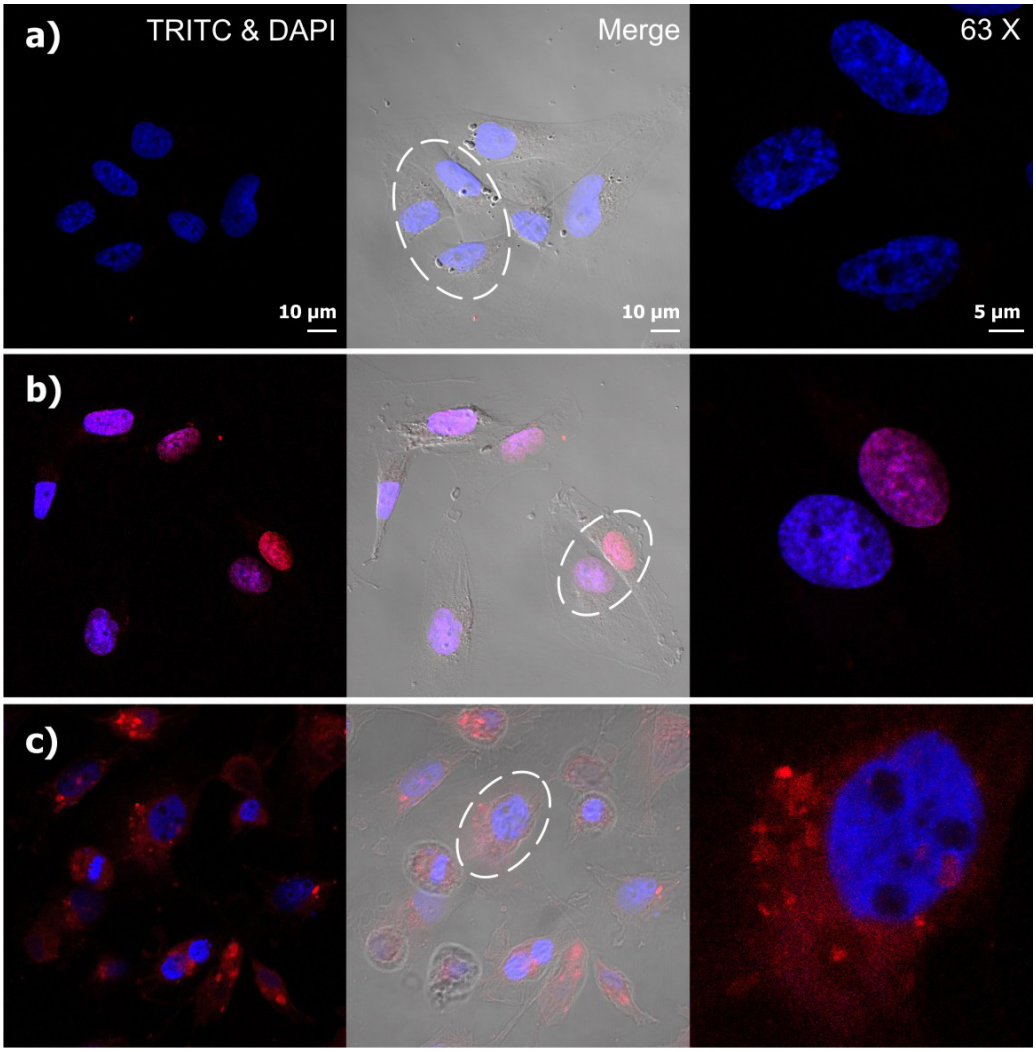


Figure 3-26 Confocal microscopy images for intracellular release of DOX. a) non-treated cells, b) cells treated by free DOX, c) cells treated by PLH coated DOX carrying NCs.

Although these results show the presence of DOX in the cell and around the nucleus, there might be other hypothesis for red parts located inside the cells. Due to high water solubility of DOX, that could be possible for DOX to release in the culture medium and then taken up by the cells as free drug. To clarify this point and to be sure that NCs were taken up into he cells, cellular internalization of NCs was visualized by using coumarin-6 green fluorescence reagent. In this experiment polysebacic anhydride was labeled by poorly water-soluble coumarin-6 before the preparation of nanocapsules.

DOX loaded NCs were prepared from coumarin-6-labeled polymer and coated by PLH and modified by PEG. Cellular uptake efficiency of labeled NCs was examined on MDA-MB-231 cells. The cells were treated with PBS and PBS containing DOX loaded and coumarin 6-labeled NCs. To prevent release of poorly water-soluble coumarin-6 in cell culture medium, cells were fixed in 1 day after treatment. Figure 3-27 shows microscope images related to MDA-MB-231 cells treated by PBS (3-27-a) and coumarin 6-labeled NCs (3-27-b-1 and 3-27-b-2).

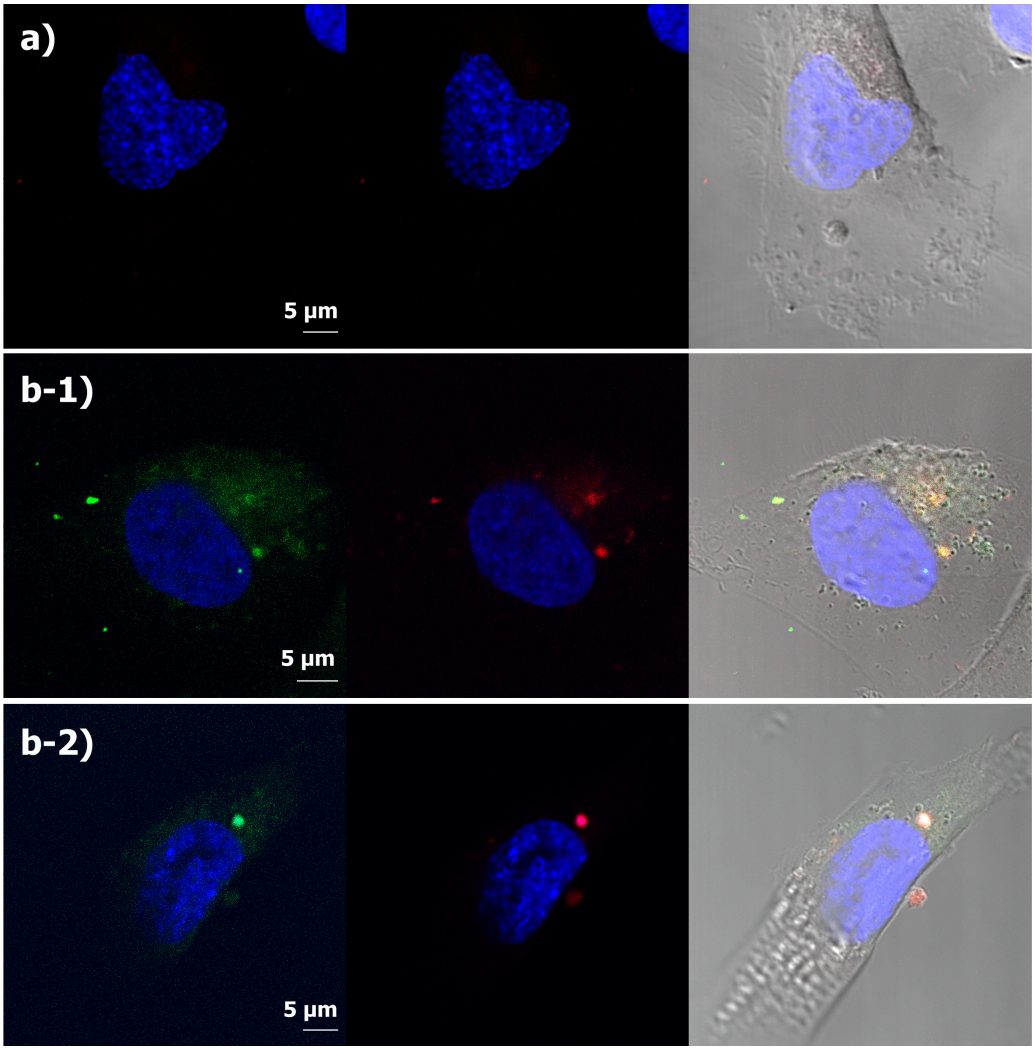


Figure 3-27 Confocal microscopy images for cellular uptake. a) cells treated with PBS, b) cells treated by PLH coated and coumarin 6-labeled NCs.

The images in the left column show images obtained by green fluorescence filter suitable for coumarin 6. The middle column is related to images obtained by TRITC red fluorescence filter responding to DOX. Images in the right column show merged images of green, red and phase contrast with DAPI filters. As it is shown in Figure 3-27-b-1 and 3-27-b-2, the green fluorescent parts can be observed after 6 hours of incubation with coumarin-6 labeled nanocapsules. This feature illustrated that formulated nanocapsules (PEG-PLH-DOX-NCs) induce potential to internalize into MDA-MB-231 cells. Results obtained in this experiment clarified that in treatment of cells by DOX loaded NCs, DOX can be taken up by MDA-MB-231 cells in encapsulated state.

3.4.5.3 Macrophage uptake

In the last decades, the in vivo applications of polymeric drug delivery systems have changed from macro-size (≥1 mm) to micro (5– 20 μm) and finally to nano (100–1000 nm) (Preis et al., 1979; Kipper et al., 2002). These nano-size particles exhibited advantages leading to bring them to fore. For instance they can be investigated without any surgery (Berkland et al., 2004), applied for multi-drug delivery (Hsu et al., 2005), and targeted to tumor site via EPR effect (Maeda et al., 2000). The negative side of these carriers limited their clinical application is their uptaking by phagocytes leading to reduce their blood circulation (Lacasse et al., 1998; Soma et al., 2000).
Pegylation (using of poly ethylene glycol) has been known as a technique to depressed elimination of nanoparticles (Harper et al., 1991; Veronese et al., 2005; Hamidi et al., 2006; Pirollo et al., 2008). PEG molecules either binded or adsorbed to surface of nanoparticles inhibit blood proteins absorption by steric hindrance and repulsion effects of PEG chains (Owens et al., 2006; Claesson et al., 1995) .
In this study the influence of pegylation on macrophage uptake was determined by using THP-1 human monocyte cells differentiated to macrophages by using 10 μM TPA (12-O-tetradecanoyl-phorbol-13-acetate) as described in literature (He et al., 2010). In this experiment PBS treated macrophages were used as control group. Confocal photomicrographs of THP-1 monocytes have been represented in Figure 3-28. It should be notified that the observed bubbles surrounding the macrophages membrane are coming from CO2 gas produced by THP-1 macrophages during the fixing process. Figure 3-28-b is corresponding to non-pegylated NCs. In this result rapidly internalize of most of non-pegylated capsules is obvious by appearance high-concentrated red parts inside the macrophages coming from loaded DOX inside the NCs. By comparing pegylated NCs (Figure 3-28-c) with non-pegylated (Figure 3-28-b), it was revealed that pegylated NCs exhibit less exposure to internalize by phagocytic pathways.

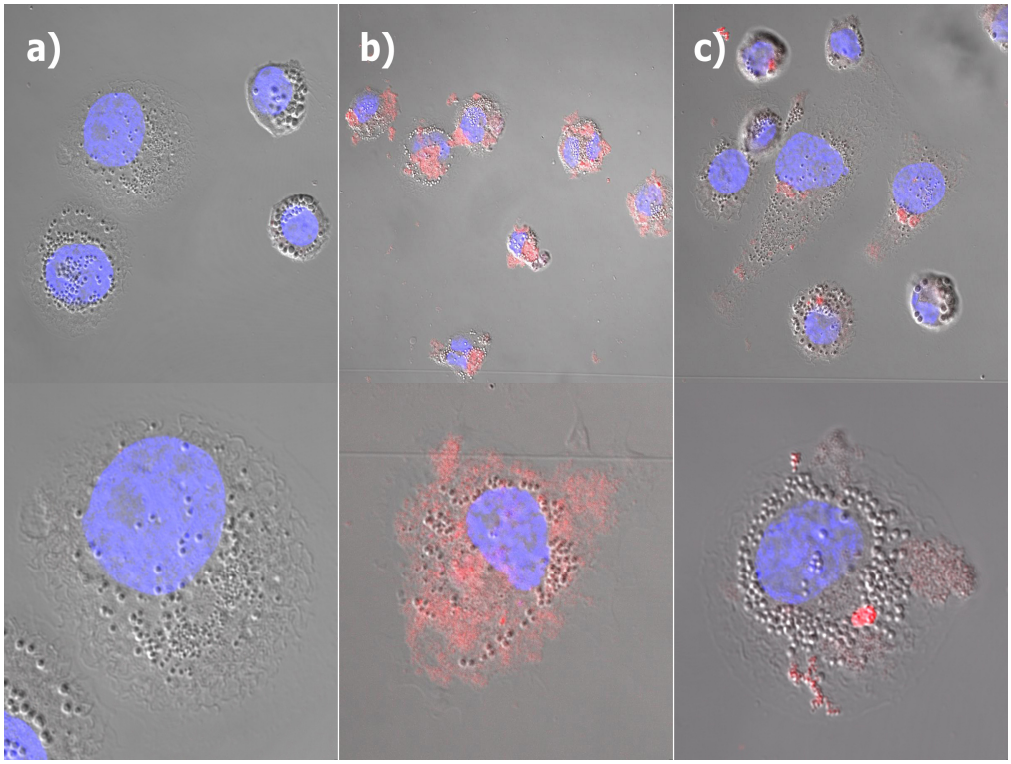


Figure 3-28 Confocal microscopy images for HTP-1 macrophage internalization in a) control cells, b) non-pegylated NCs, c) pegylated and PLH coated NCs.

It is no doubt that, due to in vitro conditions of this experiment all pegylated and non-pegylated NCs will be phagocyte by macrophages with time (Ulery et al., 2009). To get more real result macrophage internalization study was performed 6 hours after treatment of HTP-1 cells.

3.5 In vivo test of pH responsive nanocapsules

In vivo tests were aimed to carry out on nude mice. Cancer mice were treated in three groups; blank nanocapsules, free DOX and DOX loaded nanocapsules. In the case of blank nanocapsules (PEG-PLH-NCs) and free DOX no problem was observed during the treatment. While in the case of PEG-PLH-DOX-NCs loaded nanocapsules, trouble breathing was observed in injected mice. This problem attributed to agglomeration of DOX loaded nanocapsules during the freezing and storing as it was expected. Due to this agglomeration, DOX containing nanoparticles got bigger sizes and created some problems during the treatment.
To overcome to this problem fresh nanocapsules containing DOX will be prepared and in vivo test will be repeated with fresh nanocapsules.

CHAPTER 4

CONCLUSION

In light of this study, we explored a pH responsive drug delivery system based on polysebacic anhydride to target doxorubicin, as anticancer agent. This strategy causes to enhance chemotherapeutic efficiency and reduce its responding side effects. In last decades various studies reported about development of novel delivery systems to carry anticancer agents. The newest approach in drug delivery is targeting of drugs to tumor and intercellular release of cytotoxic anticancer drugs. In this research passive targeting of DOX loaded nanocapsules was performed by investigation of poly (L-histidine). It has been reported that poly (L-histidine) exhibit a promising potential to protonate resulting endosomal lysis. Cell uptake results obtained in this study proved the capacity of poly (L-histidine) to internalize into cancer cells and to carry DOX as a chemotheraputic agent.

An ideal nano-size delivery system should perform properties such as high loading capacity, proper size and size distribution and prevented macrophage uptake. Nano capsules prepared in this work own high loading capacity without any modification to develop encapsulation efficiency of water-soluble doxorubicin. The size of the prepared nanocapsules is suitable to exploit EPR effect for passive targeting into tumor site. Another important property of formulated nanocapsules was application of polysebacic anhydride as a base matrix. Polysebacic anhydride exhibits promising properties for sustained release delivery. This biodegradable polymer display surface erosion leading to release of encapsulated drug via controlled pattern.

It is no doubt that the last future of a biomedical research is swing from the bench to the bed. The cost of formulated system plays important role in its clinical application. It should be considered that monomer of polysebacic anhydride, sebacic acid, has a natural source resulting to exhibit a low cost in compare to most of synthesized polymers. All these characteristic of designed pH responsive system obtained due to results of the present study demonstrate that poly (L-histidine) coated nanocapsules based on PSA enable intracellular delivery of anticancer agents have made it as a promising delivery system for targeting doxorubicin to tumor site and for future clinical practice.

BIBLIOGRAPHY

Abdallah M, El-Etre AY. Corrosion inhibition of Nickel in sulfuric acid using tween surfactants. Portugaliae Electrochimica Acta 2003; 21: 315-26

Ahmed F, Pakunlu R I, Brannan A, Bates F, Minko T. Biodegradable polymersomes loaded with both paclitaxel and doxorubicin permeate and shrink tumors, inducing apoptosis in proportion to accumulated drug. J Control Release. 2006; 116(2): 150-8

Aishwarya S, Mahalakshmi S, Sehgal P K. Collagen-coated polycaprolactone microparticles as a controlled drug delivery system. J Microencapsul 2008; 25(5): 298-306

Alvarez-Lorenzo C, Sosnik A, Concheiro A. PEO-PPO block copolymers for passive micellar targeting and overcoming multidrug resistance in cancer therapy. Curr Drug Targets. 2011; 12(8): 1112-30

Amjadi I, Rabiee M, Hosseini MS, Mozafari M. Synthesis and characterization of doxorubicin-loaded poly(Lactide-co-glycolide) nanoparticles as a sustained-release anticancer drug delivery system. Appl Biochem Biotechnol 2012; 168(6):1434-47

Anand P, Kunnumakara A B, Sundaram C, Kuzhuvelil B. Cancer is a Preventable Disease that Requires Major Lifestyle Changes. Pharm Res 2008; 25(9): 2097–2116

Andreev O A, Dupuy A D, Segala M, Sandugu S, Serra D A, et al. Mechanism and uses of a membrane peptide that targets tumors and other acidic tissues in vivo. Proc Natl Acad Sci 2007;104(19):7893–98

Asayama S, Hamaya A, Sekine T, Kawakami H, Nagaoka S. Aminated poly(L-histidine) as new pH-sensitive DNA carrier. Nucleic Acids Symp Ser (Oxf). 2004; (48): 229-30

Ashjari M, Khoee S, Mahdavian A R. Amultiple emulsion method for loading 5-fluorouracil into a magnetite-loaded nanocapsule: a physicochemical investigation. Polym Int 2012; 61: 850–59

Badjatya J K, Bodla R B, Solanki S, KUMAR D. Enhancement of solubility of paclitaxel by solid dispersions techniques. AJPLS 2011; 1(2): 156-60

Bankar V H, Gaikwad P D, Pawar S P. Novel sustained release drug delivery systems: review. IJPRD; 2011: 3(12): 1 – 14

Bello R M, Midoux P. Histidylated Polylysine as DNA Vector: Elevation of the Imidazole Protonation and Reduced Cellular Uptake Without Change in the Polyfection Efficiency of Serum Stabilized Negative Polyplexes. Bioconjug Chem 2001; 12: 92-109

Benns J, Choi J S, Mahato R I, Park J S, Kim S W. pH-sensitive cationic polymer gene delivery vehicle. Bioconjug Chem. 2000; 11(5): 637-45

Berkland C, Kipper M J, Narasimhan B, Kim K K, Pack D W. Microsphere size, precipitation kinetics and drug distribution control drug release from biodegradable polyanhydride microspheres. J Control Release 2004; 94(1): 129-41

Bertin P A, Smith D, Nguyen S T. High-density doxorubicin-conjugated polymeric nanoparticles via ring-opening metathesis polymerization. Chem Commun (Camb) 2005; 30: 3793-5

Betancourt T, Brown B, Brannon-Peppes L. Doxorubicin-loaded PLGA nanoparticles by nanoprecipitation: preparation, characterization and in vitro evaluation. Nanomedicine (Lond) 2007; 2(2): 219-32

Bharadwaj R, Yu H. The spindle checkpoint, aneuploidy, and cancer. Oncogene 2004; 23 (11): 2016–27

Bonetti A, Zaninelli M, Durante E, Fraccon A P, Franceschi T, et al.. Multiple-target chemotherapy (LV-modulated 5-FU bolus and continuous infusion, oxaliplatin, CPT- 11) in advanced 5-FU-refractory colorectal cancer: MTD definition and efficacy evaluation. A phase I-II study. Tumori 2006; 92:389-95

Boussif O, Lezoualc'h F, Zanta M A, Mergny M D, Scherman D, et al.. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. Proc. Natl. Acad. Sci. U. S. A. 199; 92: 7297–301

Brannon-Peppas L, Blanchette J O. Nanoparticle and targeted systems for cancer therapy. Adv Drug Deliv Rev. 2004; 56(11):1649-59

Brem H, Langer R. Polymer-based drug delivery to the brain, J. Science & Medicine 1996; 3: 52-59

Canal C, Aparicio R M, Vilchez A, Esquena J, García-Celma M J. Drug delivery properties of macroporous polystyrene solid foams. J Pharm Pharm Sci. 2012; 15(1):197-207

Capasso J M, Cossío B R, Berl T, Rivard C J, Jiménez C. A colorimetric assay for determination of cell viability in algal cultures. Biomol Eng. 2003; 20(4-6): 133-8

Casolaro M, Casolaro I, Lamponi S. Stimuli-responsive hydrogels for controlled pilocarpine ocular delivery. Eur J Pharm Biopharm. 2012; 80(3): 553-61

Chavanpatil M, Khdair A, Patil Y, Handa H, Mao G U, et al.. Polymer-surfactant nanoparticles for sustained release of water-soluble drugs. J Pharm Sci. 2007; 96: 3379-89

Chen A Z, Chen M Y, Wang S B, Huang X N, Liu Y G. Poly (L-histidine)-chitosan/alginate complex microcapsule as a novel drug delivery agent. J App Polym Sci 2012; 124(5): 3728–36

Chen Y, Wan Y, Wang Y, Zhang H, Zhijun J. Anticancer efficacy enhancement and attenuation of side effects of doxorubicin with titanium dioxide nanoparticles. Int J Nanomedicine 2011; 6: 2321–26

Chen G, Zhu X, Cheng Z, Xu W, , Lu J. Controlled/''living'' radical polymerization of methyl methacrylate using AIBN as the initiator under microwave irradiation. Radiat Phys Chem 2004; 69: 129–35

Chen L, Wang H, Wang J, Chen M, , Shang L. Ofloxacin-delivery system of a polyanhydride and polylactide blend used in the treatment of bone infection. J Biomed Mater Res Part B: Appl Biomater 2007: 83B; 589-95

Chickering D, Jacob J, Mathiowitz E. Poly (fumaric-co-sebacic) microspheres as oral drug delivery systems. Biotechnol Bioeng. 1996; 52(1): 96-101

Chithrani B D, Ghazani A A, Chan W C. Determining the size and shape dependence of gold nanoparticle uptake into mammalian cells. Nano Lett. 2006; 6: 662-8

Chlebowski R T, Kuller L H, Prentice R L, Stefanick M L, Manson JE, et al. Breast cancer after use of estrogen plus progestin in postmenopausal women. N Engl J Med. 2009; 360(6): 573-87.

Choucair A, Soo P L, Eisenberg A. Active loading and tunable release of doxorubicin from block copolymer vesicles. Langmuir 2005; 21(20): 9308-13

Claesson P M, Blomberg E, Froberg J C, Nylander T, Arnebrant T. Protein interactions at solid interface. Adv Colloid Interface Sci 1995; 57: 161–227

Cristescu C, Cojanu A, Popescu S, Grigorescu C, Nastase b, et al.. Processing of poly(1,3-bis-(p-carboxyphenoxy propane)-co-(sebacic anhydride)) 20:80 (P(CPP:SA)20:80) by matrix-assisted pulsed laser evaporation for drug delivery systems. Appl Surf Sci. 2007; 254(4): 1169–73

Cristescu R, Popescu C, Socol G, Visan A, Mihailescu I N, et al.. Deposition of antibacterial of poly(1,3-bis-(p-carboxyphenoxy propane)-co-(sebacic anhydride)) 20:80/gentamicin sulfate composite coatings by MAPLE. Appl Surf Sci. 2011; 257: 5287–92

Davies M C, Shakesheff K M, Shard A G, Domb A, Roberts C J, et al.. Surface Analysis of Biodegradable Polymer Blends of Poly(sebacic anhydride) and Poly(DL-lactic acid). Macromolecules 1996; 29: 2205-12

Desai M P, Labhasetwar V, Walter E, Levy R J, Amidon G L. The mechanism of uptake of biodegradable microparticles in Caco-2 cells is size dependent. Pharm Res. 1997; 14(11): 1568-73

Dhanya K P, Santhi K, Dhanaraj S A, Sajeeth C I. Formulation and evaluation of chitosan nanospheres as a carrier for the targeting delivery of lamivudine to the brain. IJCP 2011; 2(05): 1-5

Domb A J, Langer R. Polyanhydrides. I. preparation of high molecular weight polyanhydrides, J Polym Sci Pol Chem. 1987; 25: 3373-86

Domb A J, Ron E, Langer R. Poly(anhydrides). 2. One-Step Polymerization Using Phosgene or Diphosgene as Coupling Agents, J. Macromolecules 1988; 21: 1925-29

Domb A J, Langer R. Solid-state and solution stability of poly(anhydrides) and poly (esters). Macromolecules 1989; 22: 2117-22

Domb A, Maniar M. Absorbable biopolymers derived from dimer fatty acids. J Poly Sci. 1993; 3: 1275-85

Domb A, Wartenfeld R. Methotrexate polymer implant for the treatment of head and neck cancer. Polym Adv Technol. 1994; 5(9): 577–81

Domb A J, Israel Z H, Elmalak O, Teomim D, Bentolila A. Preparation and characterization of carmustine loaded polyanhydride wafers for treating brain tumors. Pharm Res. 1999;16: 762–65

Dong J, Frethem C, Haugstad G, Hoerr R A, Foley J D. Effect of the coating morphology on the drug release from engineered drug-polymer nanocomposites. Conf Proc IEEE Eng Med Biol Soc. 2009; 2009: 6006-9

Elman N M, Ho Duc H L, Cima M J. An implantable MEMS drug delivery device for rapid delivery in ambulatory emergency care. Biomed Microdevices 2009; 11(3): 625–31

Emanuele A D, Hill J, Tamada J A, Domb A J, Langer R. Molecular weight changes in polymer erosion. Pharm Res. 1992; 9(10): 1279-83

Fredericks D N, Relman D A, Sequence-based identification of microbial pathogens: a reconsideration of koch's postulates. Clin. Microbiol. Rev. 1996; 9(1): 18-33

Fiegel J, Fu J, Hanes J. Poly(ether-anhydride) dry powder aerosols for sustained drug delivery in the lungs. J Controlled Release 2004; 96(3): 411–23

Fu J, WU C. Laser light scattering study of the degradation of poly(sebacic anhydride) nanoparticles. J Polym Sci, Part B: Polym Phys 2001; 39(6): 703-708

Fu J, Li X, Dennis K P, Wu C. Encapsulation of phthalocyanines in biodegradable poly(sebacic anhydride) nanoparticles. Langmuir 2002; 18: 3843-47

Furtado S, Abramson D, Simhkay L, Wobbekind D, Mathiowitz E. Subcutaneous delivery of insulin loaded poly(fumaric-co-sebacic anhydride) microspheres to type I diabetic rats. Eur J Pharm Biopharm. 2006; 63(2): 229-36

Gao J, Niklason L, Zhao XM, Langer R. Surface modification of polyanhydride microspheres. J Pharm Sci. 1998; 87(2): 246-8

Gao Y, Chen Y, Ji X, He X, Yin Q. Controlled intracellular release of doxorubicin in multidrug-resistant cancer cells by tuning the shell-pore sizes of mesoporous silica nanoparticles. ACS Nano. 2011; 5(12): 9788-98

Garvin K, Feschuk C. Polylactide-polyglycolide antibiotic implants, Clin Orthop Relat Res. 2005; 437:105-10

Gautier J, Munnier E, Paillard A, Hervé K, Douziech-Eyrolles L. A pharmaceutical study of doxorubicin-loaded PEGylated nanoparticles for magnetic drug targeting. Int J Pharm. 2012; 423(1): 16-25

Gillet J P, Gottesman M M. Mechanisms of multidrug resistance in cancer. Multi-drug resistance in cancer. Methods Mol Biol. 2010; 596: 47-76

Gong K, Rehman I U, Darr J A. Synthesis of poly(sebacic anhydride)-indomethacin controlled release composites via supercritical carbon dioxide assisted impregnation. Int. J. Pharm. 2007: 338; 191–97

Guaragna A, Chiaviello A, Paolella C, D'Alonzo D, Palumbo G, et al.. Synthesis and evaluation of folate-based chlorambucil delivery systems for tumor-targeted chemotherapy. Bioconjugate Chem. 2012; 23 (1): 84–96

Guhagarkar S A, Gaikwad R V, Samad A, Malshe V C, Devarajan P V. Polyethylene sebacate–doxorubicin nanoparticles for hepatic targeting. Int J Pharm. 2010; 401: 113–22

Guterres S S, Poletto F S, Colomé L M, Raffi R P, Pohlmann A R. Polymeric nanocapsules for drug delivery an overview. , Colloids in Drug Delivery, March 25, 2010 by CRC Press – 72-92

Hagenbuch B. Drug uptake systems in liver and kidney: historic perspective. Clin Pharmacol Ther. 2010; 87(1): 39-47

Hamidi M, Azadi A, Rafiei P. Pharmacokinetic consequences of pegylation. Drug Deliv. 2006; 13(6): 399-409

Hande R K. Topoisomerase II inhibitors. Update on Cancer Therapeutics 2008; 3(1): 13–26

Hanes J, Chiba M, Langer R. Degradation of porous poly(anhydride-co-imide) microspheres and implications for controlled macromolecule delivery. Biomaterials 1998; 19(1-3): 163-72

Harper G R, Davies M C, Davis S S, Tadros T F, Taylor D C, Steric stabilization of microspheres with grafted polyethylene oxide reduces phagocytosis by rat Kupffer cells in vitro. Biomaterials 1991; 12(7): 695-700

Hasirci N. Micro and Nano systems in Biomedicine and Drug delivery, in 'Nanomaterials and Nanosystems for Biomedical Applications', Editor: M. Reza Mozafari, Dordrecht, Netherlands. Springer 2007; 1-26

Hasirci V, Yilgor P, Endogan T, Eke G, Hasirci N. Polymer Fundamentals: Polymer Synthesis. Comprehensive Biomaterials, 1st Edition 2011; 1: 349-71

Havivi E, Farber S, Domb A J. Poly(sebacic acid-co-ricinoleic acid) biodegradable carrier for delivery of tamsulosin hydrochloride. Polym. Adv. Technol. 2011; 22: 114–18

He Q, Zhang J, Shi J, Zhu Z, Zhang L. The effect of PEGylation of mesoporous silica nanoparticles on nonspecific binding of serum proteins and cellular responses. Biomaterials 2010; 31(6): 1085-92

Heller J, Barr J, Ng S Y, Abdellauoi K S, Gurny R. Poly(ortho esters): synthesis, characterization, properties and uses. Adv Drug Delivery Rev. 2002; 54: 1015–39

Helmlinger G, Sckell A, Dellian M, Forbes N S, Jain R K. Acid production in glycolysis-impaired tumors provides new insights into tumor metabolism, Clin. Cancer Res. 2002; 8: 1284–91

Hiremath J G, Rudani C G, Domb A J, Suthar R V, Khamar N S. Preparation and in vitro characterization of poly(sebacic acid-co-ricinoleic acid)-based tamoxifen citrate-loaded microparticles for breast cancer. J Appl Polym Sci. 2012; 124: 4747–54

Hsu W, Lesniak M S, Tyler B, Brem H. Local delivery of interleukin-2 and adriamycin is synergistic in the treatment of experimental malignant glioma. J Neurooncol. 2005; 74(2): 135-40

Huang M, Fong C W, Khor E, Lim L Y. Transfection efficiency of chitosan vectors: effect of polymer molecular weight and degree of deacetylation. J Control Release. 2005; 106: 391-406

Huang W C, Chiang W H, Huang Y F, Lin S C, Shih Z F. Nano-scaled pH-responsive polymeric vesicles for intracellular release of doxorubicin. J Drug Target. 2011; 19(10): 944-53

Huynh N T, Morille M, Bejaud J, Legras P, Vessieres A, et al. Treatment of 9L gliosarcoma in rats by ferrociphenol-loaded lipid nanocapsules based on a passive targeting strategy via the EPR effect. Pharm Res. 2011; 28(12): 3189-98

Jain K K. Nanotechnology-based drug delivery for cancer. Technol Cancer Res Treat. 2005; 4(4): 407-16

Janzsó G, Bogár F, Hudoba L, Penke B, Rákhely G, Leitgeb B. Exploring and characterizing the folding processes of Lys- and Arg-containing Ala-based peptides: A molecular dynamics study. Comput Biol Chem. 2011; 35(4): 240-50

Jemal A, Bray F, Melissa M, Ferlay J, Ward E, et al.. Global cancer statistics. CA Cancer J Clin 2011; 61: 69–90

Jian Z , Zhiyu Q, Yueqing G. In vivo anti-tumor efficacy of docetaxel-loaded thermally responsive nanohydrogel. Nanotechnology 2009; 20: 325-33

Jordan VC. A current view of tamoxifen for the treatment and prevention of breast cancer. Br. J. Pharmacol. 1993; 110: 507-517

Kanaras A G, Kamounah F S, Schaumburg K, Kiely C J, Brust M: Thioalkylated tetraethylene glycol: a new ligand for water, soluble monolayer protected gold clusters. Chem. Commun 2002; 20: 2294–95

Khattak S F, Spatara M, Roberts L, Roberts S C. Application of colorimetric assays to assess viability, growth and metabolism of hydrogel-encapsulated cells. Biotechnol Lett. 2006; 28(17): 1361-70

Kim D, Lee E S, Oh K T, Gao Z G, Bae Y H. Doxorubicin-loaded polymeric micelle overcomes multidrug resistance of cancer by double-targeting folate receptor and early endosomal pH. Small 2008; 4: 2043–50

Kim J, Lee K W, Hefferan T E, Currier B L, Yaszemski M J. Synthesis and evaluation of novel biodegradable hydrogels based on poly(ethylene glycol) and sebacic acid as tissue engineering scaffolds. Biomacromolecules 2008; 9(1): 149-57

Kim S, Lee J. Folate-targeted drug-delivery systems prepared by nano-comminution, Drug Dev Ind Pharm. 2011; 37(2): 131-8

Kipper MJ, Shen E, Determan A, Narasimhan B. Design of an injectable system based on bioerodible polyanhydride microspheres for sustained drug delivery. Biomaterials 2002; 23(22): 4405-12

Kirchmeier M J, Ishida T, Chevrette J, Allen T M. Correlations between the rate of intracellular release of endocytosed liposomal Doxorubicin and cytotoxicity as determined by a new assay. J Liposome Res. 2001; 11(1):15-29

Krasko M Y, Shikanov A, Kumar N, Domb A J. Polyanhydrides with hydrophobic terminals. Polym. Adv. Technol. 2002: 13; 960-68

Krasko M Y (a), Ezra A, Domb A J. Lithocholic-acid-containing poly(ester–anhydride)s. Polym. Adv. Technol. 2003: 14; 832–38

Krasko M Y (b), Shikanov A, Ezra A, Domb A, J. Poly(ester anhydride)s Prepared by the Insertion of Ricinoleic Acid into Poly(sebacic acid). J. Polym. Sci., Part A: Polym. Chem. 2003: 41: 1059–69

Krasko M Y, Domb A J. Pasty injectable biodegradable polymers derived from natural acids. J Biomed Mater Res A. 2007; 83(4): 1138-45

Kukowska-Latallo J F, Candido K A, Cao Z, Nigavekar S S, Majoros I J, et al.. Nanoparticle targeting of anticancer drug improves therapeutic response in animal model of human epithelial cancer. Cancer Res. 2005; 65(12):5317-24

Lacasse F X, Filion M C, Phillips N C, Escher E, McMullen J N, et al. Influence of surface properties at biodegradable microsphere surfaces: effects on plasma protein adsorption and phagocytosis. Pharm. Res. 1998; 5(2): 312–17
Langer R, Drug delivery and targeting. Nature 1998; 30: 5-10

Laurencin C, Domb A, Morris C, Brown V, Chasin M, et al.. Poly(anhydride) administration in high doses in vivo: Studies of biocompatibility and toxicology. J Biomed Mater Res. 1990; 24(11): 1463-81

Lee E S (a), Shin H J, Na K, Bae Y H. Poly(L-histidine)–PEG block copolymer micelles and pH-induced destabilization. J. Control. Release 2003; 90: 363–74

Lee E S (b), Na K, Bae Y H. Polymeric micelle for tumor pH and folate-mediated targeting. J. Control. Release 2003; 91: 103–13

Lee E S, Na K, Bae Y H. Doxorubicin loaded pH-sensitive polymeric micelles for reversal of resistant MCF-7 tumor. J. Control. Release 2005; 103: 405–18

Lee W C, Chu I M. Preparation and Degradation Behavior of Polyanhydrides Nanoparticles. J Biomed Mater Res B Appl Biomater 2008; 84: 138-46

Leong K W, Brott B C, Langer R. Bioerodible polyanhydrides as drug-carrier matrices. I: Characterization, degradation, and release characteristics. J Biomed Mater Res. 1985; 19(8): 941-55

Leong K W, D'Amore P, Marlettart M, Langer R. Bioerodible polyanhydrides as drug-carrier matrices. II. Biocompatibility and chemical reactivity. J Biomed Mater Res. 1986; 20(1): 51-64

Li N, Wang J, Yang X, Li L. Novel nanogels as drug delivery systems for poorly soluble anticancer drugs. Colloids Surf B Biointerfaces 2011; 83(2): 237-44

Li X, Hirsh D J, Cabral-Lilly D, Zirkel A, Gruner S M, et al. Doxorubicin physical state in solution and inside liposomes loaded via a pH gradient. Biochim Biophys Acta. 1998; 1415(1): 23-40

Liang Y, Xiao L, Zhai Y, Xie C, Deng L,, et al.. Preparation and characterization of biodegradable poly(sebacic anhydride) chain extended by glycol as drug carrier. J Appl Polym Sci. 2013; 127(5): 3948-53

Liu J, Qiu Z, Wang S, Zhou L, Zhang S. A modified double-emulsion method for the preparation of daunorubicin-loaded polymeric nanoparticle with enhanced in vitro anti-tumor activity. Biomed. Mater. 2010; 5: 065002

Liu R, Li D, He B, Xu X, Sheng M. Anti-tumor drug delivery of pH-sensitive poly(ethylene glycol)-poly(L-histidine-)-poly(L-lactide) nanoparticles. J Control Release. 2011 May 30;152(1):49-56

Lodish H, Berk A, Zipursky S L, Matsudaira P, Baltimore D, et al.. Molecular cell biology, 4th edition, section 12.4 DNA Damage and Repair and Their Role in Carcinogenesis New York: W. H. Freeman; 2000. ISBN-10: 0-7167-3136-3

Lukyanov A N, Elbayoumi T A, Chakilam A R, Torchilin V P. Tumor-targeted liposomes: doxorubicin-loaded long-circulating liposomes modified with anti-cancer antibody. J Control Release 2004; 100(1): 135-44

Luo Y, Chen D, Ren L, Zhao X, Qin J. Solid lipid nanoparticles for enhancing vinpocetine's oral bioavailability. J Control Release 2006; 114(1): 53-9

Ma Z, Yeoh H H, Lim L Y. Formulation pH modulates the interaction of insulin with chitosan nanoparticles. J Pharm Sci. 2002; 91: 1396-404

Ma Z, Lim T M, Lim L Y. Pharmacological activity of peroral chitosan-insulin nanoparticles in diabetic rats. Int J Pharm. 2005; 293: 271-80

Madden T D, Harrigan P R, Tai L C, Bally M B, Mayer L D. The accumulation of drugs within large unilamellar vesicles exhibiting a proton gradient: a survey. Chem Phys Lipids. 1990; 53(1): 37-46

Maeda H, Wu J, Sawa T, Matsumura Y, Hori K. Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. J Control Release 2000; 65(1-2): 271-84

Majeti N V, Kumar R. Nano and microparticles as controlled drug delivery devices. J Pharm Pharmaceut Sci. 2000; 3(2): 234-58

Masamune A, Shimosegawa T, Masamune O, Mukaida N, Koizumi M, et al.. Helicobacter pylori-dependent ceramide production may mediate increased interleukin 8 expression in human gastric cancer cell lines. Gastroenterology. 1999; 116: 1330–41

Mathiowitz E, Saltzman W M, Domb A, Dor P, Langer R. Polyanhydride microspheres as drug carriers. II. Microencapsulation by solvent removal. J App Poly Sci. 1988; 35(3):755 - 74

Mathiowitz E, Amato C, Dor P, Langer R. Polyanhydride microspheres: morphology and characterization of systems made by solvent removal. J. Polymer 1990; 31: 547–55

Meyer D E, ShinB C, Kong G A, Dewhirst M W, Chilkoti A. Drug targeting using thermally responsive polymers and local hyperthermia. J Control Release 2001; 74(1-3): 213-24

Minotti G, Menna P, Salvatorelli E, Cairo G, Gianni L, Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. Pharmacol Rev. 2004; 56(2): 185-229

Mo Y, Lim L Y. Paclitaxel-loaded PLGA nanoparticles: potentiation of anticancer activity by surface conjugation with wheat germ agglutinin. J Control Release. 2005; 108: 244-62

Najafi F, Sarbolouki M N. Biodegradable micelles/polymersomes from fumaric/sebacic acids and poly(ethylene glycol). Biomaterials 2003: 24; 1175–82

Noel S P, Courtney H, Bumgardner J D, Haggard W O. Chitosan films: A potential local drug delivery system for antibiotics. Clin Orthop Relat Res. 2008; 466(6): 1377–82

Owens D E 3rd, Peppas N A. Opsonization, biodistribution and pharmacokinetics of polymeric nanoparticles. Int J Pharm. 2006; 307(1): 93-102

Papahadjopoulos D, Allen T M, Gabizon A, Mayhew E, Matthay K, et al.. Sterically stabilized liposomes: improvements in pharmacokinetics and antitumor therapeutic efficacy. Proc. Natl Acad. Sci. USA 1991; 88: 11460–64

Park B.J., L. S. (1992). “Introduction to biomaterials” in “Biomaterials: An introduction" (2nd edition ed.). London: Plenum Press.

Patchornik A, Berger A, Katchalski E. Poly (L-histidine). J. Am. Chem. Soc. 1957; 79: 5227-30

Pfeifer B A, Burdick J A, Langer R. Formulation and surface modification of poly(ester-anhydride) micro- and nanospheres. Biomaterials 2007; 26: 117-24

Pirollo K F, Chang E H. Does a targeting ligand influence nanoparticle tumor localization or uptake? Trends Biotechnol. 2008; 26(10): 552-8

Preis I, Langer R S. A single-step immunization by sustained antigen release. J Immunol Methods 1979; 28(1-2): 193-7

Qiu L Y, Zhu K J, Design of a core-shelled polymer cylinder for potential programmable drug delivery. Int. J. Pharm. 2001; 219: 151–60

Qiu Y, Park K. Environment-sensitive hydrogels for drug delivery. Adv Drug Delivery Rev. 2001; 53: 321–39

Ramji K, Cairns D R, Rajeswari S. Synergistic inhibition effect of 2-mercaptobenzothiazole and tween-80 on the corrosion of brass in NaCl solution. Appl Surf Sci. 2008; 254: 4483-93

Reddy C V S, Walker E H, Wicker S A, Williams Q L, Kalluru R R. Characterization of MoO3 nanorods for lithium battery using PVP as a surfactant. J Solid State Electrochem 2009; 13: 1945–49

Rofstad E K, Mathiesen B, Kindem K, Galappathi K. Acidic extracellular pH promotes experimental metastasis of human melanoma cells in athymic nude mice. Cancer Res. 2006; 66: 6699–707

Ron E, Mathiowitz E, Mathiowitz G, Domb A, Langer R. NMR characterization of erodible copolymers. Macromolecules. 1991; 24: 2278-82
Ron E, Turek T, Mathiowitz E, Chasin M, Hageman M, et al. Controlled release of polypeptides from polyanhydrides. Proc Natl Acad Sci U S A. 1993; 90(9): 4176-80

Sahana D K, Mittal G, Bhardwaj V, M. N. V. Kumar M N. PLGA nanoparticles for oral delivery of hydrophobic drugs: influence of organic solvent on nanoparticle formation and release behavior in vitro and in vivo using estradiol as a model drug. J Pharm Sci. 2008; 97: 1530-42

Sairam M, Babu V R, Krishna Rao K S V, Aminabhavi T M. Poly(methylmethacrylate)-poly(vinyl pyrrolidone) microspheres as drug delivery systems: Indomethacin/Cefadroxil loading and in vitro release study. J Appl Polym Sci 2007; 104: 1860–65

Santos C A, Freedman B D, Leach K J, Press D L, Scarpulla M, et al.. Poly(fumaric–co-sebacic anhydride) A degradation study as evaluated by FTIR, DSC, GPC and X-ray diffraction. J Control Release 1999; 60: 11–22

Schiffman M, Castle P E, Jeronimo J, Rodriguez A C, Wacholder S. Human apillomavirus and cervical cancer. Lancet 2007; 370(9590): 890-907

Shao H, Huang Y, Lee H S, Suh Y J, Kim C O. Effect of PVP on the Morphology of Cobalt Nanoparticles Prepared by Thermal Decomposition of Cobalt Acetate. Curr Appl Phys. 2006; 6S1: 195–7

Shelke N B, Aminabhavi T M. Synthesis and characterization of novel poly(sebacic anhydride-co-Pluronic F68/F127) biopolymeric microspheres for the controlled release of nifedipine. Int J Pharm. 2007; 345(1-2): 51-8

Shen E, Pizsczek R, Dziadul B, Narasimhan B. Microphase separation in bioerodible copolymers for drug delivery. Biomaterials 2001; 22: 201-10

Shen E, Kipper M J, Dziadul B, Lim M K, Narasimhan B. Mechanistic relationships between polymer microstructure and drug release kinetics in bioerodible polyanhydrides. J Control Release 2002; 82(1): 115-25

Shikanov A, Vaisman B, Krasko M Y, Nyska A, Domb A J. Poly(sebacic acid-co-ricinoleic acid) biodegradable carrier for paclitaxel: in vitro release and in vivo toxicity. J Biomed Mater Res A. 2004; 69(1): 47-54

Shikanov A, Vaisman B, Shikanov S, Domb A J. Efficacy of poly(sebacic acid-co-ricinoleic acid) biodegradable delivery system for intratumoral delivery of paclitaxel. J Biomed Mater Res A. 2010; 92(4): 1283-91

Shoaib M H, Tazeen J, Merchant H A, Yousuf R I. Evaluation of drug release kinetics from ibuprofen matrix tablets using HPMC. Pak. J. Pharm. Sci. 2006; 19: 119-24

Singh M N, Hemant K SY, Ram M, Shivakumar H G. Microencapsulation: A promising technique for controlled drug delivery. Res Pharm Sci. 2010; 5(2): 65–7

Skwarczynski M, Zaman M, Urbani C N, Lin I, Jia Z, et al.. Polyacrylate dendrimer nanoparticles: a self-adjuvanting vaccine delivery system. Angew. Chem. Int. Ed. 2010; 49: 5742 –45

Soma C E, Dubernet C, Barratt G, Benita S, Couvreur P. Investigation of the role of macrophages on the cytotoxicity of doxorubicin and doxorubicin-loaded nanoparticles on M5076 cells in vitro. J Control Release. 2000; 68(2): 283-9

Smet M, Heijman E, Langereis S, Hijnen N M, Grüll H. Magnetic resonance imaging of high intensity focused ultrasound mediated drug delivery from temperature-sensitive liposomes: an in vivo proofof-concept study, J. Controlled Release 2011; 150: 102–10

Srivastava V, Babu E S, Han S K, Lee H S, Lim D S, et al.. Hydrothermal synthesis of ZnO nanorods in the presence of a surfactant. J Nanosci Nanotechnol 2012; 12(2): 1328-31

Stephens D, Lia L, Robinson D, Chen S, Chang H C, et al.. Investigation of the in vitro release of gentamicin from a polyanhydride matrix. J Control Release 2000; 63(3): 305-17

Suo A, Qian J, Yao Y, Zhang W. Galactosylated poly(ethylene glycol)-b-poly (l-lactide-co-β-malic acid) block copolymer micelles for targeted drug delivery: preparation and in vitro characterization. Int J Nanomedicine. 2010; 5: 1029–38

Tang B C, Dawson M, Laia S K, Wang Y, Suk J S, et al.. Biodegradable polymer nanoparticles that rapidly penetrate the human mucus barrier, PNAS 2009; 106: 19268–73

Tong R, Tang L, Yin Q, Cheng J. Drug-Polyester Conjugated Nanoparticles for Cancer Drug Delivery, Conf Proc IEEE Eng Med Biol Soc. 2011; 2011: 8337-9

Torchilin V P: Nanoparticulates as Drug Carriers. Imperial college press 2006; pages 2-4

Torchilin V P. Passive and Active Drug Targeting: Drug Delivery to Tumors as an Example. Handb Exp Pharmacol. 2010; 197: 3-53

Totiger S B, Hiremath J G. Paclitaxel loaded poly(sebacic acid-co-ricinoleic ester anhydride)-based nanoparticles. J Pharm 2011; 5: 225-30

Uhrich K E, Cannizzaro S M, Langer R S, Shakesheff K M. Polymeric systems for controlled drug release. Chem. Rev. 1999; 99: 3181-98

Ulery B D, Phanse Y, Sinha A, Wannemuehler M J, Narasimhan B, Bellaire B H. Polymer chemistry influences monocytic uptake of polyanhydride nanospheres. Pharm Res. 2009; 26(3): 683-90

Uster P S, Deamer D W. pH-dependent fusion of liposomes using titratable polycations. Biochemistry 1985; 24(1): 1-8

van Vlerken LE, Vyas TK, Amiji MM. Poly(ethylene glycol)-modified nanocarriers for tumor-targeted and intracellular delivery, Pharm Res. 2007 Aug;24(8):1405-14. Epub 2007 Mar 29.

Vauthier C, Couvreur P. Development of nanoparticles made of polysaccharides as novel drug carrier systems. In: Wise DL, editor. Handbook of pharmaceutical controlled release technology. New York: Marcel Dekker; 2000. p. 13–429

Veronese F M, Pasut G. PEGylation, successful approach to drug delivery. Drug Discov Today 2005; 10(21): 1451-8

Wang C Y, Huang L. Polyhistidine mediates an acid-dependent fusion of negatively charged liposomes. Biochemistry1984; 23(19): 4409-16

Wang H, Qia X, Chen J, Wang X, Ding S. Mechanisms of PVP in the preparation of silver nanoparticles. Mater Chem Phys. 2005; 94: 449–53

Wang S B, Xu F H, He H S, Weng L J. Novel alginate-poly(L-histidine) microcapsules as drug carriers: in vitro protein release and short term stability. Macromol Biosci. 2005; 5(5): 408-14

Wong H, Bendayan R, Rauth A M, Wu X Y. Development of solid lipid nanoparticles containing ionically complexed chemotherapeutic drugs and chemosensitizers. J Pharm Sci. 2004; 93: 1993-2008

Wu C, Fu J, Zhao Y. Novel nanoparticles formed via self-assembly of poly(ethylene glycol-b-sebacic anhydride) and their degradation in water. Macromolecules 2000; 33: 9040-43

Xu H B, Zhou Z B, Huang K X, Lei T, Zhang T, et al.. Preparation and properties of poly(dimmer acid-sebacic acid) copolymer and poly(dimer acid-tetradecaneidoic acid) copolymer. Polym Bull 2001; 46: 435-42

Xue H Y, Wong H L. Solid lipid-PEI hybrid nanocarrier: an integrated approach to provide extended, targeted, and safer siRNA therapy of prostate cancer in an all-in-one manner. ACS Nano 2011; 5: 7034–47

Yang S R, Kim S B, Joe C O, Kim J D. Intracellular delivery enhancement of poly(amino acid) drug carriers by oligoarginine conjugation. J Biomed Mater Res A. 2008; 86: 137-48

Yilgor P, Hasirci N, Hasirci V. Sequential BMP-2/BMP-7 delivery from polyester nanocapsules. J Biomed Mater Res A. 2010; 93: 528-36

Zamboni WC. Liposomal, nanoparticle, and conjugated formulations of anticancer agents. Clin Cancer Res. 2005; 11:8230-8234

Zhang C, Wang W, Liu T, Wu Y, Guo H. Doxorubicin-loaded glycyrrhetinic acid-modified alginate nanoparticles for liver tumor chemotherapy. Biomaterials 2012; 33(7): 2187-96

Zhang J, Liang Y, Li N, Zhao X, Hu R. Poly(ether-ester anhydride)-based amphiphilic block copolymer nanoparticle as delivery devices for paclitaxel. Micro & Nano Letters 2012; 7(2): 183-7

Zhao X, Li H, Lee R J. Targeted drug delivery via folate receptors. Expert Opin Drug Deliv. 2008; 5(3): 309-19

Zhao A, Zhou Q, Chen T, Weng J, Zhou S. Amphiphilic PEG-based ether-anhydride terpolymers: synthesis, characterization, and micellization. J. Appl. Polym. Sci. 2010; 118: 3576–85

Zheng C, Qiu L, Yao X, Zhu K. Novel micelles from graft polyphosphazenes as potential anti-cancer drug delivery systems: drug encapsulation and in vitro evaluation. Int J Pharm 2009; 373: 133–40

Zhu L, Huo Z, Wang L, Tong X, Xiao Y, Ni K. Targeted delivery of methotrexate to skeletal muscular tissue by thermosensitive magnetoliposomes. Int J Pharm 2009; 370: 136–43.
Zhu L, Mahato R I. Lipid and polymeric carrier-mediated nucleic acid delivery. Expert Opin. Drug Deliv 2010; 7(10): 1209–26

Zhu L, Torchilin V P. Stimulus-responsive nanopreparations for tumor targeting, Integr. Biol. 2013; 5: 96-107

APPENDICES

APPENDIX A: CALIBRATION CURVE

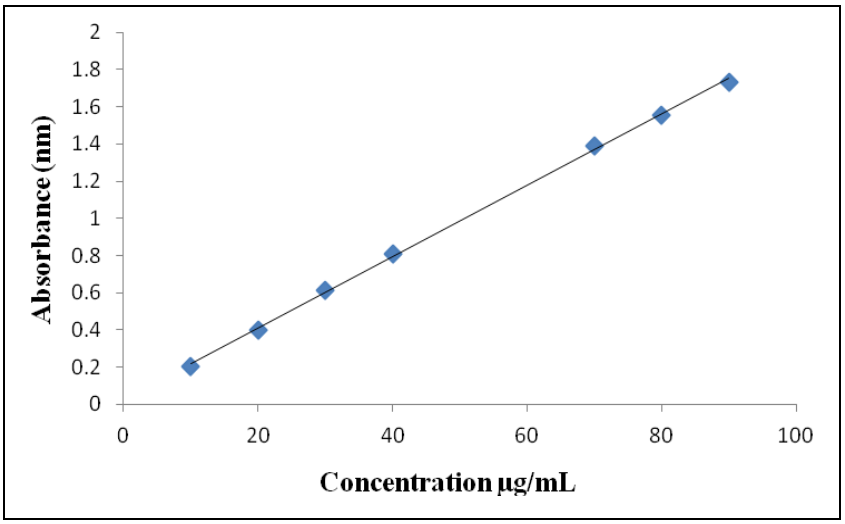


Figure A.1 Calibration curve of DOX in PBS 0.01 M, pH 7.4

APPENDIX B: DOSE CURVE

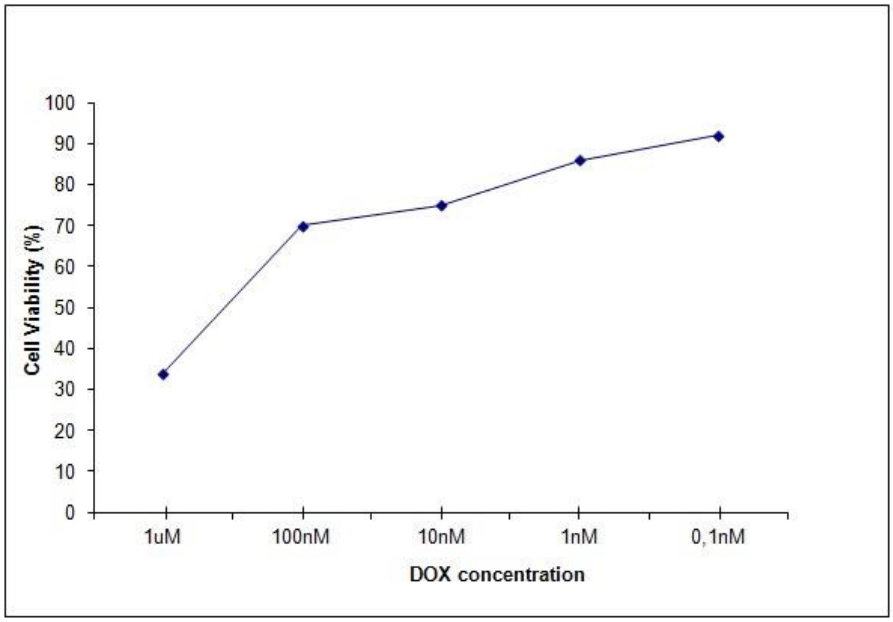


Figure B.1 Cell viability of MDA-MB-231 cells versus different concentrations of DOX.