THE EFFECTS OF DOXORUBICIN CONTAINING POLY (SEBACIC ANHYDRIDE) NANOCAPSULES ON GLUTATHIONE S-TRANSFERASE ACTIVITY

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ABSTRACT

THE EFFECTS OF DOXORUBICIN CONTAINING POLY (SEBACIC ANHYDRIDE) NANOCAPSULES ON GLUTATHIONE S-TRANSFERASE ACTIVITY

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Nanocapsules are used as drug delivery system commonly. Poly (sebacic anhydride) is a good candidate for the nanocapsule preparation since they are ideal for controlled release application with respect to biocompatibility, low cost, and the approval by US Food and Drug Administration (FDA). The overactivity of glutathione S-transferase is generally related with the resistance of chemotherapy and this problem can be overcome through drug delivery systems. In this study, poly (sebacic anhydride) - poly (ethylene glycol) copolymer with the molecular weight of 5202 g/mol was synthesized successfully. In addition, doxorubicin loaded nanocapsules form this copolymer were prepared properly and the size of these nanocapsules were 200 nm approximately. The loading efficiency of these nanocapsules is 71.9%. The realese study indicated that these nanocapsules follows sustain drug release profile. Moreover, the effect of these nanocapsules on glutathione S-tranferase activity was examined and almost 60% inhibiton on the ezyme activity was observed.

Keywords: Polysebasic anhydride, Nanocapsule, Controlled Release, Glutathione S-transferase

ÖZ

DOKSORUBİSİN İÇEREN POLİSEBASİK ANHİDRİT BAZLI NANOKAPSÜLLERİN GLUTATYON S-TRANSFERAZ AKTİVİTESİ ÜZERİNE ETKİSİ

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Nanokapsüller yaygın bir şekilde ilaç taşıyıcı sistemi olarak kullanılır. Nanokapsül üretimi için polisebasik anhidrit kullanımı idealdir çünkü biyolojik olarak uyumludur, üretimi düşük maliyetlidir ve FDA tarafından onaylanmıştır. Glutatyon S-tranferaz (GST) enziminin yüksek aktivitesi genel olarak kanser ilaçlarına karşı dirençliliğe sebep olur. GST enzim kaynaklı dirence karşı ilaç taşıyıcı sistemler umut verici bir yöntemdir. Bu çalışmada moleküler ağırlığı 5202 g/mol olan polisebasik anhidrit- polietilen glikol kopolimeri başarı ile sentezlenmiştir. Bu kopolimerden boyutları yaklaşık 200 nm olan doksorubisin yüklü nanokapsüller sentezlenmiştir ve bu nanokapsüllerin ilaç yükleme kapasitesi %71,9 olarak tespit edilmiştir. Üretilen nanokapsüller üzerinde yürütülen salım çalışması, bu kapsüllerde kontrollü ve sürekli ilaç salımı olduğunu göstermiştir. Ayrıca, üretilen nanokapsüllerin glutatyon S-transferaz aktivitesi üzerine etkisi incelenmiş ve enzim aktivitesinde %60 azalma gözlemlenmiştir.

Anahtar Kelimeler: Polisebasik anhidrit, Nanokapsül, Kontrollü Salınım, Glutatyon S-transferaz

To My Sister

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TABLE OF CONTENTS

ABSTRACT v

ÖZ vi

ACKNOWLEDGEMENTS viii

TABLE OF CONTENTS x

LIST OF FIGURES: xiii

LIST OF TABLES: xv

LIST OF SYMBOLS: xvi

CHAPTERS 1

1. INTRODUCTION 1

1.1 Cancer 1

1.1.1 The Reasons for Cancer 2

1.1.2 Cancer Treatment Ways 3

1.1.3 Chemotherapy 4

1.2 Drug Delivery Systems 5

1.2.1 Passive Targeting 8

1.2.2 Active Targeting 10

1.2.3 Stimuli Responsive Targeting 13

1.2.4 Nanoparticles 16

1.3 Glutathione S-Transferase 34

1.3.1 Drug Metabolism and GST Function 37

1.3.2 The Interaction between Doxorubicin and GST 40

1.4 The Aim of This Study 50
2. THE EXPERIMENTAL PART 53
2.1 Materials 53
2.2 Polymer Synthesis 54
2.2.1 PSA Prepolymer Synthesis 55
2.2.2 PSA Polymer Synthesis 56
2.2.3 PSA-PEG Prepolymer Synthesis 57
2.2.4 PSA-PEG Copolymer Synthesis 57
2.2.5 Polymer Characterization 57
2.3 Nanocapsule Preparation 58
2.3.1 Characterization of Doxorubicin Loaded Nanocapsules 59
2.3.2 Drug Loading Capacity and Encapsulation Efficiency Calculation 59
2.3.3 DOX Release Profile of the Nanocapsules 60
2.4 GST Enzyme Assay of Nanoparticles 61
2.4.1 Isolation of GST Enzyme from Bovine Liver 61
2.4.2 GST Enzyme Activity Assay 62
3. RESULTS AND DISCUSSION 65
3.1 Polymer and Copolymer Characterization 65
3.2 Characterization of the Nanocapsules 74
3.3 Release Profiles of Nanocapsules 81
3.4 GST Enzyme Activity Assay 84
4. CONCLUSION 95
REFERENCES 97
APPENDICES 121

CALIBRATION CURVE OF DOX..121

LIST OF FIGURES:

FIGURES

Figure 1.1: Drug Release Profile (MTC- Minimum Toxic Concentration, MEC- Minimum Effective Concentration) a) Conventional Drug Application b) Controlled Drug Delivery System 7
Figure 1.2: Passive Targeting and EPR Effect 9
Figure 1.3: Endothelial Cell Targeting and Tumor Cell Targeting 13
Figure 1.4: Stimuli Responsive Targeting 15
Figure 1.5: The Structure of a) Nanosphere b) Nanocapsule and c) Nanoconjugates 17
Figure 1.6: a) Bulk Erosion b) Surface Erosion 20
Figure 1.7: General Formula of Poly (anhydrides) 24
Figure 1.8: Synthesis of Poly (Sebacic Anhydride) 25
Figure 1.9: Degradation of PSA a) Hydrolysis into Carboxylic Acid b) Intermolecular Interaction 26
Figure 1.10: The Structure of Poly (ethylene glycol) 30
Figure 1.11: The Effect of PEGylation on Macrophage Detection 31
Figure 1.12: The Conjugation of GST with Substrate 34
Figure 1.13: The Reaction between CDNB and GSH 36
Figure 1.14: Drug Metabolism 37
Figure 1.15: Mercapturic Acid Formation 39
Figure 1.16: Doxorubicin Structure 41
Figure 1.17: DOX Redox Reaction 42
Figure 1.18: GST-JNK Interaction 43
Figure 2.1: The experimental Setup for Prepolymer Synthesis 56
Figure 2.2: Nanocapsule Preparation by Double Emulsion Method 58
Figure 2.3: The Microplate Map for GST Enzyme Activity Assay 63
Figure 3.1: ^{1}H-NMR Result of PSA Polymer 66

Figure 3.2: ^{1}H-NMR Result of PSA-PEG1000 Copolymer 67
Figure 3.3: ^{1}H-NMR Result of PSA-PEG6000 Copolymer 68
Figure 3.4: ^{1}H-NMR Result of PSA-PEG14000 Copolymer 69
Figure 3.5: FTIR Analysis of PSA and PSA-PEG1000 70
Figure 3.6: FTIR Analysis of PSA and PSA-PEG6000 71
Figure 3.7: FTIR Analysis of PSA and PSA-PEG14000 72
Figure 3.8: Z- Average of PSA-DOX Nanocapsules 76
Figure 3.9: Z- Average of PSA-PEG1000-DOX Nanocapsules 76
Figure 3.10: Z- Average of PSA-PEG6000-DOX Nanocapsules 77
Figure 3.11: Z- Average of PSA-PEG14000-DOX Nanocapsules 77
Figure 3.12: SEM Image of PSA-DOX Nanocapsules 79
Figure 3.13: SEM Image of PSA-PEG1000-DOX Nanocapsules 79
Figure 3.14: SEM Image of PSA-PEG6000-DOX Nanocapsules 80
Figure 3.15: SEM Image of PSA-PEG14000-DOX Nanocapsules 80
Figure 3.16: The DOX Release Profile at pH 4 82
Figure 3.17: The DOX Release Profile at pH 7.4 82
Figure 3.18: GST Inhibition Profile for PSA-PEG6000-DOX Nanocapsules 84
Figure 3.19 GST Inhibition Profile for Free DOX 85
Figure 3.20: GST Inhibition Profile for PEG6000 86
Figure 3.21: GST Inhibition Profile for PSA-PEG6000-DOX Nanocapsules at 1st and 6th Days of Evaluation 88
Figure 3.22: GST Inhibition Profile for PSA-PEG6000-DOX Nanocapsules at 8th and 13th Days of Evaluation 88
Figure 3.23: GST Inhibition Profile for PSA-PEG6000-DOX Nanocapsules at 15th Day of Evaluation 89
Figure 3.24: GST Inhibition Profile for PSA-PEG6000-DOX Nanocapsules from the Stocks of 2013 and 2015 90
Figure 3.25: GST Inhibition Profile of PSA-PEG6000 Nanocapsules at 6th Week (After 2 Years Shelf Life) 91
Figure 3.26: PSA-DOX Stability Results 92

LIST OF TABLES:

TABLES

Table 1.1: Advantages and Disadvantages of Nanoparticles 18
Table 1.2: Synthetic Biodegradable Polymers used in Controlled Release 21
Table 2.1: The Materials used for the Experiments 53
Table 2.2: The Composition of Polymers and Copolymers 55
Table 2.3: The Volume of KP Buffer used According to the Volume of the Nanocapsule Solution 63
Table 3.1: Yields, Molecular Weights, and PDI of the Synthesized Polymers and Copolymers 74
Table 3.2: The Nanocapsule Characterization Results 75

LIST OF SYMBOLS:

1H-NMR	proton nuclear magnetic resonance
AFB1	aflatoxin B1
CDNB	1-chloro-2, 4-dinitrobenzene
DCM	dichloromethane
DDS	drug Delivery System
DLS	dynamic light scattering
DMSO	dimethyl sulfoxide
DOX	doxorubicin
DTT	ditiyotreitol
EDTA	ethylenediaminetetraacetic acid
EE	encapsulation efficiency
FDA	Food and Drug Administration
FTIR	Fourier transform infrared spectroscopy
GPC,	gel permeation chromatography
GST	glutathione s-transferase
HBDL	hyperbranched dendritic-linear
HE	histidine-glutamic acid
HPMA	N-(2-Hydroxypropyl) methacrylamide
IC50	half maximal inhibitory concentration

JNK	c-Jun N- terminal kinases
KP buffer	potassium phosphate Buffer
LC	loading capacity
MAP	model amphipathic peptide
MAP kinase	mitogen-activated protein kinase
MAPEG	membrane associated proteins
MDR	multiple drug resistance
MT	melatonin
NCs	nanocapsules
P85	pluronic 85
PBS	phosphate buffered saline
PEG	poly (ethylene glycol)
PL	piperlongumine
PSA	poly (sebacic anhydride)
PTX	paclitaxel
PVA	polyvinyl alcohol
QC	quercetin
QDs	quantum dots
RES	reticuloendothelial systems
ROS	reactive oxygen species
SEM	scanning electron microscopy
SLN	solid lipid nanoparticles

UV	ultraviolet

CHAPTERS

1. INTRODUCTION

1.1 Cancer

Cancer is the fact of changing the normal cells into rapidly dividing cells in an uncontrolled way. Hippocrates (460-370 BC) firstly introduced the term ‘carcinoma’ coming from the Greek word ‘carcinos’, which means crab, to depict tumors. Then, the word ‘carcinos’ was converted into the Latin word ‘cancer’ by Celsus (25 BC- 50 AD). With the improvement in microscopes in the late nineteenth century, the cells were observed in more detail. It was understood that the cancer cells are different from the normal cells in the same tissue with respect to appearance and behavior [1]. As time passed, almost the whole story about cancer cells was revealed. Normally, healthy cells in the body grow and divide in a controlled way. When there is a mutation in a cell, it loses its ability to respond appropriately to the chemical signals. As a result, it goes to apoptosis due to repair mechanisms found in the cell. However, if the repair mechanisms of the cell do not work properly, the apoptosis process cannot be achieved. The abnormal cell continues to grow and divide, eventually it becomes cancerous cell. With the increase in the number of the unconsciously dividing cells, there may be formation of a mass of tissue called tumor.

Tumors can be benign or malignant. Benign tumors are not cancerous. Although they do not respond to the repair mechanisms, they can interact with the other cells. This type of tumor does not spread to the other parts of the body and if they are removed, they do not reappear. On the other hand, malignant tumors are cancerous. They can invade nearby tissues and other parts of the body by traveling through bloodstream or lymphatic system.

This process called as metastasis. By removing of the malignant tumor, there is still the risk of survival of the cancerous cells which can continue to divide uncontrollably causing cancer to recur [2]. The occurrence of the cancer can depend on several factors.

1.1.1 The Reasons for Cancer

There are two main reasons for cancer formation; the environmental and genetic factors. While 5-10 % of cancer risk depends on genetic factors, 90-95 % of cancer is based on environmental factors such as smoking, alcohol, diet, infections, environmental pollution, radiation etc. The effect of smoking on lung cancer was revealed in 1964. Then, it was understood that more than 14 types of cancer is the result of tobacco use since tobacco contains several types of carcinogens, the chemical agents causing cancer [3]. Another factor increasing cancer risk is alcohol. The strong link between alcohol use and esophageal cancer was demonstrated in 1910 [4].

Additionally, alcohol use has also influence on other types of cancer e.g. mouth, liver, pancreas, breast, etc. The study conducted by Longnecker et al. in 1995 demonstrated that 4% of breast cancer diagnosed in USA is mainly results of alcohol use [5]. One other factor causing cancer is diet. For instance, the people whose diet contains large amount of red meat are more prone to gastrointestinal tract, prostate, bladder, pancreatic, oral, breast, and colorectal cancer. According to the research of Chan et al. in 2013, there is 14% increase in the risk of colorectal cancer with the increase in red meat and processed meat consumption in everyday diet [6]. Furthermore, infections are other environmental factor for cancer risk. Generally, viruses (human papilloma virus, HIV, HBV, and HCV) can be the cause of cancer. Moreover, it has been found out that some parasites and some bacteria like *Helicobacter pylory* can also cause cancer [3]. For example, 15 to 80% of the world is infected with *Helicobacter pylory* and 1% of them suffers from gastric cancer [7].

There is also strong relationship between environmental pollution and various types of cancer e.g. leukemia, lung cancer, testicular cancer, gastric cancer, etc. Tobacco smoke, formaldehyde, volatile organic compounds, food additives, pesticides, and polycyclic aromatic hydrocarbons (PAHs) are the types of pollution that can develop cancer [3]. In

China, for instance, PAHs emission is approximately 20% of the total emission around the world. Therefore, it can be concluded that the lung cancer is one type of cancers resulting in most deaths in China due to air pollution [8].

The last but not least, radiation has an important effect on cancer development. It can emerge from radioactive substances, ultraviolet, and, electromagnetic fields and it can cause thyroid cancer, leukemia, lymphoma, skin cancer, lung cancer, and breast cancer. An obvious example for the effect of radiation on cancer induction is Chernobyl nuclear power plant explosion. After Chernobyl accident, there was 100 times rise in childhood thyroid cancer occurrence [3, 9]. Nowadays many people are suffering from cancer and future projections estimate that unless a solution is found, 12 million people may die because of cancer until 2020 [3]. That is why many efforts put on development of new and effective treatment ways for this fatal disease.

1.1.2 Cancer Treatment Ways

There are several cancer treatment methods depending on the type and place of the cancer, age, health status, and additional personal characteristics. Generally, combination of these therapies is applied. The main cancer treatment methods are surgery, immunotherapy, hormone therapy, gene therapy, radiation therapy, and chemotherapy.

In surgery, patient can be cured completely by removing the tumor or organ from the body if there is no metastasis. This method is generally applied to prostate, breast, or testicle cancers. On the other hand, radiation therapy removes cancer cells by applying high-energy rays on cancer cells. It uses high-energy gamma-rays or high-energy x-rays. It can be combined with other treatment methods in addition to cure for leukemia and lymphoma. Another therapy method is immunotherapy in which immune system tries to kill the cancer cells via the introduction of the antibodies in to the body. Because of the relationship among some cancer diseases (generally breast and prostate cancer) and some types of hormones, hormone therapy is used to alter hormone production in the body; so that, cancer cells stop growing or are killed completely [10, 11]. For instance, the hormone therapies for breast cancer often focus on reducing estrogen levels by using tamoxifen.

Jaiyesimi et al. stated in 1995 that women treated with tamoxifen, a blocking chemical for estrogen receptor, displayed 39% reduction in contralateral primary breast carcinoma [12]. Different from the other methods, in gene therapy, genes that got damage are replaced with healthy ones. For example, researchers are trying to replace the damaged gene that signals cells to stop dividing (the p53 gene) with a copy of a working gene [10]. Chemotherapy, which is the focus of this work, is another way for cancer treatment and it is used most commonly among other treatment methods.

1.1.3 Chemotherapy

Chemotherapy uses chemicals like alkylating agents, anti-metabolites, anthracyclines, plant alkaloids, and topoisomerase inhibitors etc. that interfere with the cell division process - damaging proteins or DNA (deoxyribonucleic acid). As a result, cancer cells commit suicide. Since chemotherapeutic drugs can travel whole body, chemotherapy is commonly used to treat cancer that has spread or metastasized [10, 11].

The birth of chemotherapy term was in the early 1900s by Paul Ehrlich, who defined chemotherapy as the cure of diseases by chemicals. An important discovery for the effect of chemicals on cancer treatment was done by Gustaf Lindskog in 1943. Based on the observations for the diminution effect of nitrogen mustard used during WWI (World War I) on bone marrow and lymph nodes, he decided to apply this chemical to his patients with non-Hodgkin's lymphoma. He observed a notable suppression in cancer development. This discovery resulted in the application of nitrogen mustard on the cure of lymphomas in US (United States) commonly. Another important invention about chemotherapeutic agents in history was done by Heidelberger et al. in 1950. They developed a drug called as 5-fluorouracil (5-FU) for solid tumors which is still used for the treatment of colorectal cancer today. In addition, this drug is the first one for targeted therapy.

In 1960s, the problem with the treatment of metastatic cancers was solved with the administration of chemicals in addition to surgery and radiotherapy. Combination chemotherapy was started to use in order to cure advanced metastatic cancers in 1970s. For instance, Einhorn et al. combined cis-platinum, vinblastine, and bleomycin for the

treatment of metastatic testicular cancer and they observed 50% increase in the cancer suppression. After that, the studies were improved continuously to find more effective chemotherapeutic agents [13]. In spite of all admirable discoveries about chemotherapy, this treatment way has an important disadvantage as both cancerous cells and healthy cells are affected from the application of chemotherapeutic agents. This situation creates uncomfortable side effects such as abdominal pain, dizziness, weight gain or loss, vomiting, anemia, depression of the immune system, hair loss, etc. Moreover, these drugs can develop heart, liver, kidney damage, and damage to the inner ear on the patient body. Furthermore, low molecular weight of these agents resulted in rapid clearance from the body, high hydrophobicity of them that ends up with a high immunological response, and the multidrug resistance due to the presence of some protection enzymes in the cells decrease the success of the chemotherapeutic agents drastically [10, 11]. These disadvantages forced the researchers to develop drug delivery systems to allow alternative dosing and routed ways of chemotherapeutic agents, new therapeutic targets, and targeted therapeutics. Moreover, clinical trials indicate that patients are open for the application of these carriers [14].

1.2 Drug Delivery Systems

The clinical definition of drug is given as “a therapeutic agent; any substance other than food, used in the prevention, diagnosis, alleviation, treatment, or cure of diseases” [15]. Since ancient times, various types of drugs have been discovered and used in order to cure diseases or to improve health quality of the patients. For example, ancient Chinese and Indian people have used vaccination for the treatment of smallpox. The milestone for the drug mechanisms has occurred with the improvement in pharmaceutical technology and biotechnology [16].

Drugs have many applications like anti-inflammatories, analgesics, antibiotics, muscle relaxants, and chemotherapeutic agents etc. The typical drug administration ways are oral, topical, inhalation, and injection. However, the typical drug administration requires a duration of time which is greater than half-life of some drugs. Not only this but also low permeability of the drugs in the membrane, and high toxicity of them with the systematically delivery in high doses make the typical drug administration ways inconvenient for many therapies. For instance, chemotherapeutic agents are very toxic for body especially at high doses; thus, their concentration in blood should be maintained constant and below the toxicity level over prolonged period of time in order to reduce severe side effects. Furthermore, for all drugs three parameters are very important: the dose to be administrated, the time between doses, and the total period of the use. In conventional drug administration methods, to obtain optimum conditions for these parameters is very hard since the concentration of drugs in blood change throughout time. In order to prevent this change, drug delivery systems can be the solution. The aim of the drug delivery systems is to reach maximum therapeutic effect and minimum side effects of the drugs by creating controlled release of them. Moreover, with the targeting ability of these systems side-specific delivery can be accomplished [15]. The difference between controlled delivery system and conventional drug application methods can be observed in figure 1.1 below:

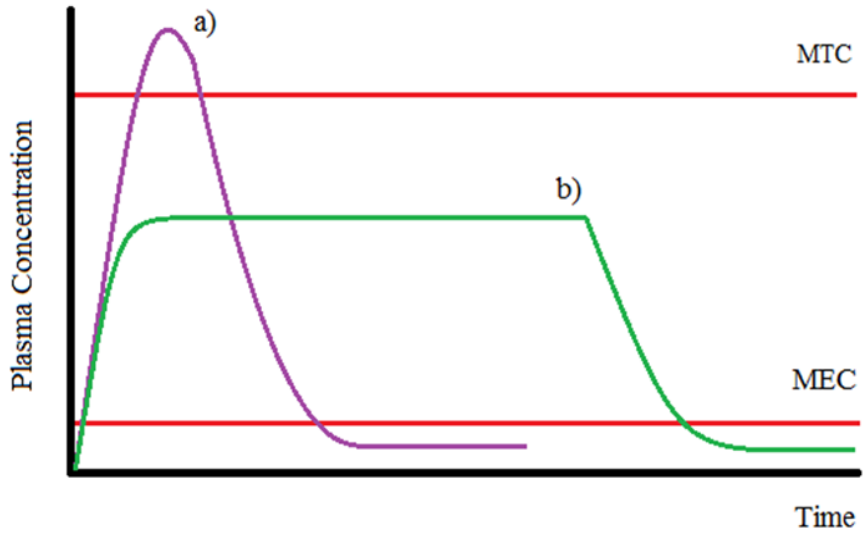


Figure 1.1: Drug Release Profile (MTC- Minimum Toxic Concentration, MEC- Minimum Effective Concentration) a) Conventional Drug Application b) Controlled Drug Delivery System

There are several important criteria for ideal drug delivery systems:

- Should have no toxic and immunological effect
- Should keep their physical and chemical stability for in vivo and in vitro conditions
- Should have uniform capillary distribution
- Should have controllable rate of drug release and the release rate should not affect the drug action
- Should have minimum drug leakage before reaching the target site
- Should be biodegradable (eliminated from the body without side effects)
- Should be easily prepared, stored, and should have low cost

Drug delivery can be achieved in three ways; passive targeting, active targeting, and triggered targeting.

1.2.1 Passive Targeting

In passive targeting, enhanced permeability and retention effect (EPR effect) in tumor sites is used. The EPR effect is observed first by Matsumura and Maeda in 1986. After this discovery, they used poly (Styrene-co-Maleic Acid)-NeoCarzinoStatin (SMANCS) (drug-polymer conjugate) that can bind to albumin in the blood. By means of labeled albumin, they observed that larger protein conjugates with respect to molecular weight shows longer retention time in tumor region [17]. One reason is the irregular and leaky vasculature structure in the tumor site that results in easy entrance of the carriers into tumor tissues. The other reason is the lack of functional lymphatics in tumor structure since normally lymphatic system eliminates the carriers from the tumor site (Figure 1.2). Moreover, high degree of angiogenesis, insufficiency of pericytes, and imbalanced blood flow can be considered as the other reasons [18]. As a result, drug carrier systems can enter easily and circulate longer in the tumor region.

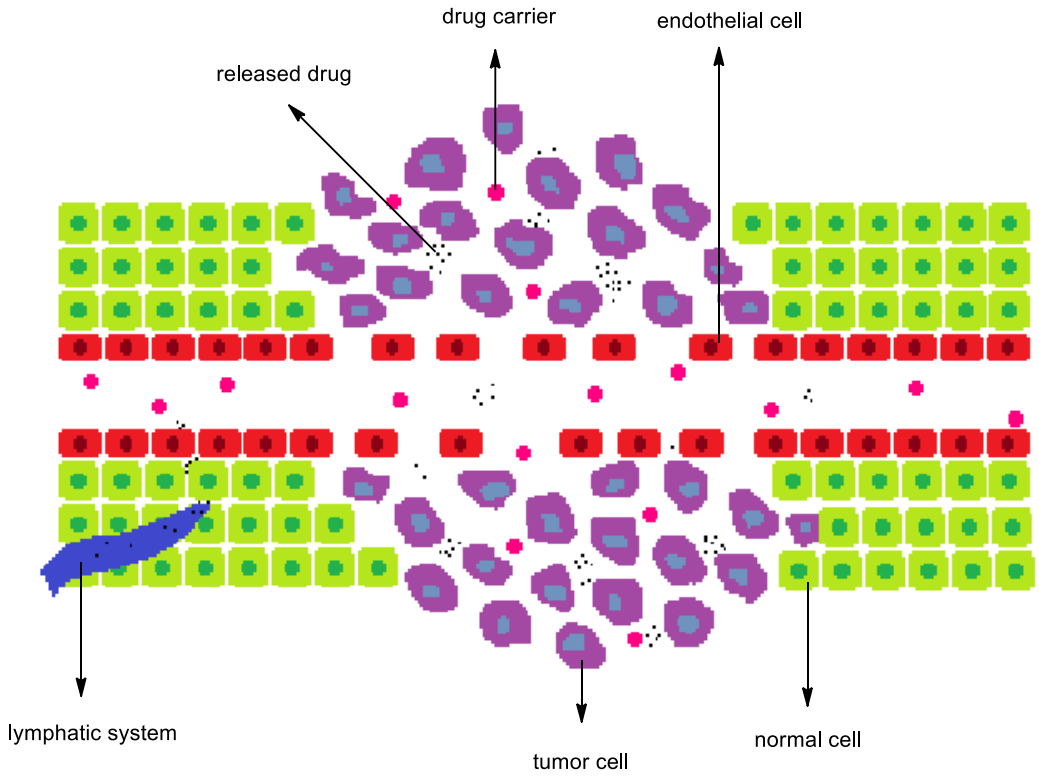


Figure 1.2: Passive Targeting and EPR Effect

Myocet® (doxorubicin containing liposome for breast cancer treatment), Doxil® (doxorubicin containing PEGylated liposome for breast cancer, ovarian cancer, multiple myeloma, and Kaposi Sarcoma treatment), Daunoxome® (daunorubicin containing liposome for Kaposi Sarcoma treatment), Abraxane® (albumin-paclitaxel conjugate for breast cancer treatment) are examples for clinically approved drug carrier systems which use passive targeting [19]. However, there are important obstacles of passive targeting in terms of EPR effect. One drawback is the change of EPR effect from subject to subject. This means one application of carrier system can be successful on a patient and it can be unsuccessful on one another. This can be the consequent of difference in vascular permeability or density of perivascular lining. One solution can be the characterization of

vascularization and monitoring the drug accumulation in tumor site through labeled drug carriers and imaging methods [19]. Thus, the patients who have a good response to the treatment of passive targeting can be selected in advance. Moreover, the applied dose can be adjusted during the treatment. For example, Theek et al. in 2014 tried to use contrast-enhanced functional ultrasound (ce-US) imaging to be able to monitor EPR effect in passive targeting. They used near-infrared-fluorophore-labeled polymeric nanoparticles (pHPMA-Dy750) in mice. They observed that with the increase in degree of tumor vascularization, higher amount of drug accumulation was detected [20]. Another drawback is that the insufficient retention time of drug carriers in tumor site because of the strong barriers, the high interstitial fluid pressure, and the high density of tumor cells. Due to these obstacles the carries cannot reach to the target site and these carriers can be removed from the target site before releasing all the agent. For this reason, researchers put effort on developing new targeting ways to increase retention time of drug delivery systems.

1.2.2 Active Targeting

Active targeting, which can be considered as a solution for obstacles in passive targeting, is another way to carry drug delivery system to target site. The logic in this type of targeting is based on the increase in retention time of the carriers at the tumor site by using the interaction between ligand attached to the carrier and receptors on the tumor cells. The ligand can be monoclonal antibodies (mAbs), antibody fragments, proteins (epidermal growth factor, transferrin, etc.), peptides (such as RGD peptides), nucleic acids (like aptamer), sugars (such as galactose), vitamins (like folic acid), and extracellular matrix receptors (heparin, sulphate, etc.). The receptors used for targeting are expressed higher in the tumor cells than the healthy cells. Therefore, the maximum target interaction and the minimum interaction in the healthy cell are achieved. The size, density, charge, and orientation of the ligand are important parameters for the success of active targeting since too big size, extreme density, improper for the attraction on the particle surface, and the improper orientation can end up with a failure of the delivery system by improper ligand

attachment, steric hindrance or macrophage activity [17, 18]. For instance, the quantitative analysis of ligand density on tumor cells by using imaging methods can provide the information of optimal ligand density on the carrier system since the receptor density changes target to target. According to the study by Tang et al. in 2014, they observed optimal amount of the folic acid (FA) ligand for the targeting of nanoparticles to HeLa cells with the aid of red fluorescence. In addition, they observed the increase in cellular uptake of nanoparticles with the increase in the density of FA up to a point [21].

The ligand can be attached on the carrier system in two ways which are pre-conjugation and post-conjugation to the carrier system. For post-conjugation, functional groups such as carboxylic acid-amine reaction as covalent approach or avidin-biotin interaction as non-covalent method are used for the ligand attachment and all types of ligands can be used in this method. In pre-conjugation, the ligand is combined with the carrier material firstly; then, the carrier formation occurs. At the end of the process, the infused ligand can stuck inside the particle and unsuccessful ligand-receptor interaction can be observed although this method is less complicated and can allow the use of the different types of ligands. Generally, peptides and aptamers are used for this method [17, 22].

In active targeting, there are two important tumor targets; targets for endothelial cells constructing blood vessels or tumor and targets found in tumor cells (Figure 1.3). In epithelial cell targeting, the ligands commonly used are L19 (antibody fragment), RGD and NGR peptides (oligopeptides), etc. Endothelial cell targeting can be a good way to overcome some problems in active targeting like thick cell layers among endothelial and tumor cells, high density of tumor cells, and high interstitial fluid pressure inside the tumor. The reason is the independence on extravasation and thick tumor cell layer diffusion. For cancer cell targeting; folate, transferrin, galactosamine, etc. are employed as ligands. Even though the specificity of cancer cell targeting is an important advantage, the carriers should overcome many different barriers such as high interstitial fluid pressure and thick cell layers formed by pericytes, muscle, and fibroblasts before reaching the target cells. As a consequence, the ligand can be lost without constructing ligand-receptor interaction and this situation creates failure in this type of targeting [19].

Herceptin® (anti-ERBB2 for adenocarcinoma and breast cancer), Avastin® (anti-VEGF for cervical cancer), Zevalin (anti-CD20 for lymphoma), and Ontak® (anti-CD25 for lymphoma) are types of actively targeted carriers that are approved clinically [18, 19]. It is believed that active targeting increases the rate of tumor site accumulation. However, this is the common misconception for active targeting. Although actively carried particles improves retention time on tumor site, the accumulation of them still occurs through EPR-effect. Hence, it cannot be assumed that one type of targeting is better than the other but active targeting can still be a solution to the retention time problem observed in passive targeting. For example, folic acid attached and ligand free PEG-PLA dendrimers were used to compare active and passive targeting on nude mice in the research of Sykes (2014). They detected longer plasma circulation time for folic acid attached dendrimers [23]. The probable disadvantage of active targeting is the increase in immunological response of the body due to the presence of ligands [19].

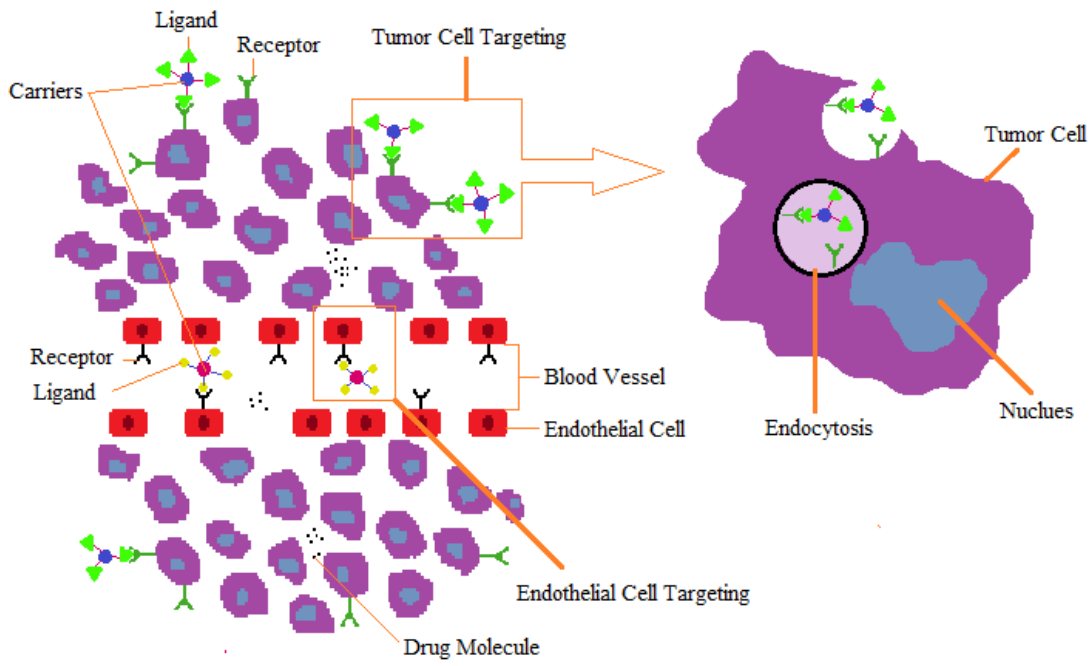


Figure 1.3: Endothelial Cell Targeting and Tumor Cell Targeting

1.2.3 Stimuli Responsive Targeting

In order to increase the selectivity and bioavailability of targeting carriers, stimuli responsive targeting has been introduced (Figure 1.4). The aim of stimuli responsive targeting is the release of cargo at the target site by the effect of internal or external stimuli [18]. The internal stimuli can be pH difference, temperature, redox potential, and enzyme. pH shows difference between tumor site (~6.5) and blood (7.4). When a carrier system which is sensitive to low pH is injected into blood, the payload is not released until the carrier reaches the target site with low pH. As a result, site-specific release is obtained. The same logic is considered for temperature. For some tumor types, the temperature is higher than 37ºC (e.g. 42ºC for human ovarian carcinoma). The carriers which are

sensitive to high temperature release the drug at the target tissue [24]. Redox potential is another way for stimuli responsive drug delivery. The purpose is use of the fact that there is much more reductive environment inside the cell than extracellular matrix. For example glutathione-glutathione disulfide (GSH-GSSG) is a type of cellular redox pair and 100-1000 fold higher concentration of GSH is observed in the cells. The carriers composed of disulfide bonds are used for this type of targeting [25]. Another internal stimulus for stimuli responsive targeting delivery is enzyme. There is high expression rate of specific enzymes in cancer cells. Thus, carrier system, which is modified with enzyme cleavable linker by covalent bond, releases the drug inside the target cells. The linker can be protein, polysaccharide, or phospholipid and the enzymes for systems are proteases, glycosidases, and lipases, [26]. There are also studies on biomaterials that are responsive to more than one stimulus for targeting purpose. For instance, in the research paper of Gao et al. (2014), they developed nanoparticles made of amphiphilic thiolated carboxymethyl chitosan which shows response to low pH and high GSH concentration by swelling. They observed 19 wt% release rate at pH 7.4 and 10 μM GSH concentration while 93 wt% release rate at pH 5.0 and 20 μM GSH concentration [27].

The external stimuli are magnetic field, ultrasound, heat, and light. With the aid of magnetic field, the drug is accumulated at the tumor site. Generally, superparamagnetic nanoparticles such as ferrite oxide-magnetite (Fe_3O_4) are used. These particles gain magnetic property with the effect of magnetic field but they become nonmagnetic in the absence of magnetic field. Therefore, specific targeting of carriers is enabled [28]. Other external stimulus is the ultrasound which allows local drug delivery in harmless way and monitoring carrier system at the same time. For drug delivery, ultrasound creates three important effect; heating for the release of drugs at target site, cavitation to increase the permeability of tumor tissue, and radiation to increase diffusion rate of the drugs. The carriers which are sensitive to mechanical force resulted in ultrasound pressure and temperature are used for this type of targeting [29]. Heat and light are the other external stimuli for targeting drug delivery. Thermo-responsive materials such as lipids, proteins, and polymers are used for targeting with heat stimulus. These materials generally have phase transition temperature above 40ºC. Thus, with the application of heat these materials

change their phase and allow drug release. For light sensitive carriers, photo responsive groups called as choromophores (like azobenzene) are incorporated on the surface or inside of carriers. With the application of UV with specific wavelength or NIR light (near-infra red light), photoreaction occurs and the structural change results in the drug release [30].

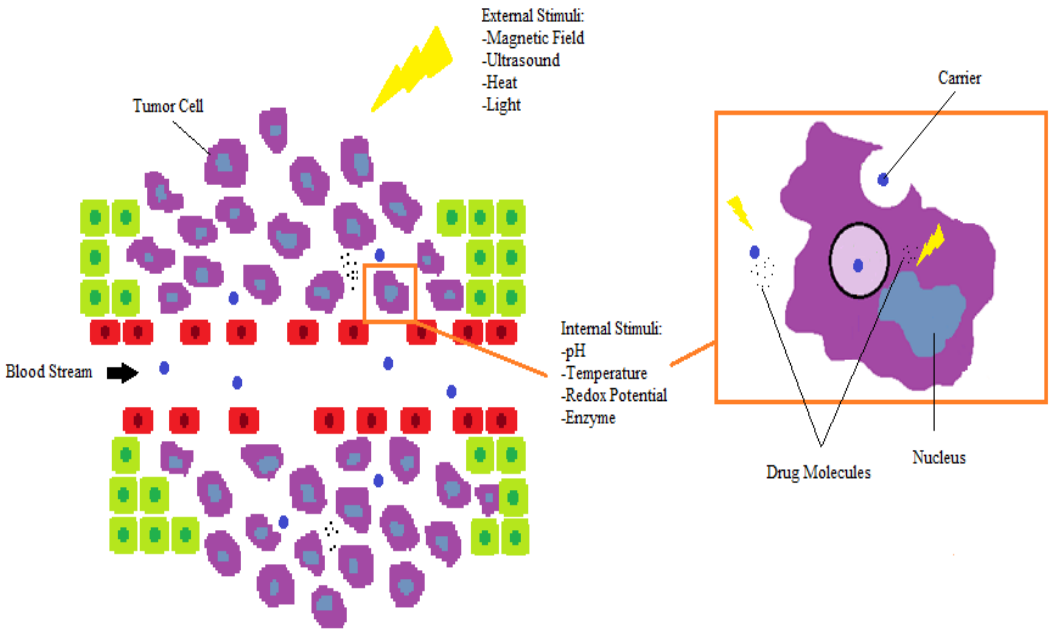


Figure 1.4: Stimuli Responsive Targeting

In addition to these stimuli responsive targeting systems, a new approach called as theragnosis has been introduced for cancer therapy. It is the combination of diagnosis and targeted therapy by the application of diagnostic agent, therapeutic agent, and targeting ability at the same time [31]. Diagnostic agent can be light sensitive dyes, quantum dots,

gold and iron oxide nanoparticles. The research conducted by Seo et al. in 2015 is based on the theragnostic drug delivery. They used nanoblend that is composed of a low-bandgap π-conjugated polymer (LB-CP) and polystyrene as the photonic core by incorporation of photosensitive dye. With the application of NIR, they obtained tissue imaging without giving damage and successful targeting [32]. This study is a good example for the combination of stimuli responsive targeting and imaging. The disadvantages for stimuli responsive carriers are the long and multistep production process, too resistant particles to stimuli at the target site, and systemic toxicity [26]. Due to these drawbacks, stimuli responsive targeting is needed to be developed more.

Although the delivery systems have some disadvantages like rapid clearance, immune reactions, and insufficient localization of targeted systems into tumors, the advantages like the simplified drug administration protocols, improved patient adaptation, reduction in the drug dose, decrease in the cost of the therapy, and the site-specific and maximized drug concentration make the drug delivery system favorite for the cure of many diseases including cancer [33]. In addition to different targeting ways, there are several types of carriers. Drug carriers can be liposomes, micelles, nanoparticles, etc. Among them, nanoparticles are commonly investigated for cancer therapy since it is believed that the problems like low rate of cell uptake observed in micro-size delivery agents, solubility problem for hydrophobic agents, multidrug resistance, and macrophage uptake before reaching the disease site can probably be overcome through the nanoparticles [34].

1.2.4 Nanoparticles

Nanoparticles are submicron (<1μm) colloidal systems composed of biocompatible polymers (PLGA-polylactic-co-glycolic acid, PCL- polycaprolactone, PLA- polylactic acid, chitosan, gelatin, HA- hyaluronan, etc.) generally. Depending on the production process, they can be nanospheres, nanocapsules, or nanoconjugates. Nanospheres (Figure 1.5-a) are called as matrix-type nanoparticles in which the drug is dispersed throughout the particles while nanocapsules (Figure 1.5-b) are reservoir type nanodevices where the drug is in the aqueous or oily cavity coated by a membrane. Nanoconjugates generally

consist of polymeric chains attached with drug molecules (Figure 1.5-c) [35]. Nanoparticles can generally be used for the targeting of antineoplastic agents, anti-inflammatory drugs, ocular drugs, cutanously applied drugs, diagnostic agents, proteins, peptides, genes, hormones, agents for vaccines, agents for blood brain barrier, and poorly soluble drugs [36].

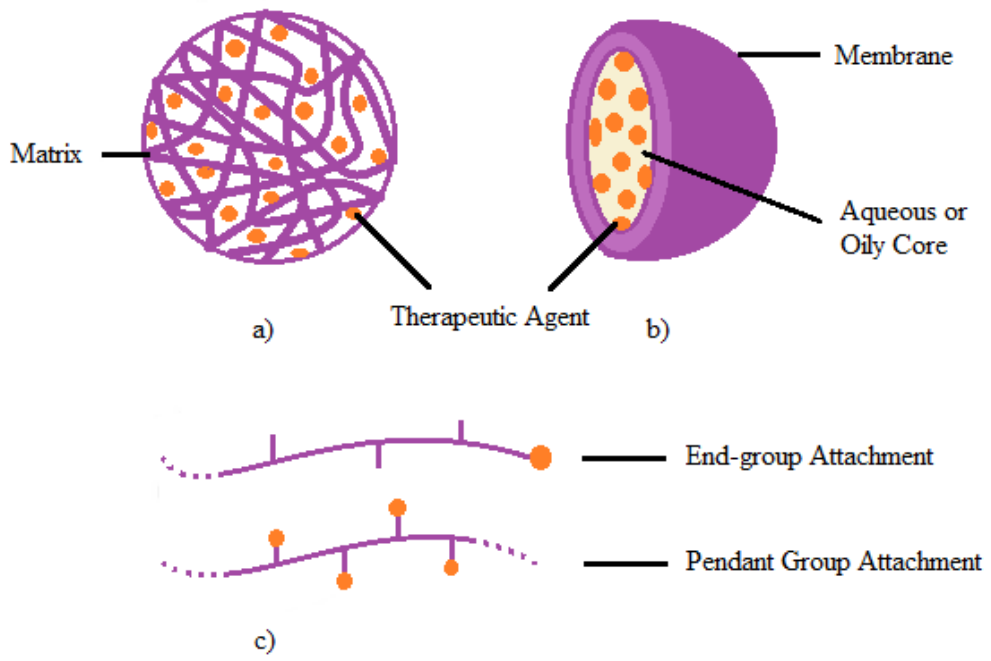


Figure 1.5: The Structure of a) Nanosphere b) Nanocapsule and c) Nanoconjugates

There are many advantages of nanoparticles for drug delivery systems besides their disadvantages (depicted in table 1.1) [35].

Table 1.1: Advantages and Disadvantages of Nanoparticles

Advantages	Disadvantages
Their particle size and surface characteristics can easily be manipulated for both active and passive targeting.	They have higher manufacturing cost.
The release of drugs can be controlled and the side effects can be reduced.	They have low encapsulation efficiency.
They have long shelf stability.	Water-soluble drugs can be rapidly leaked out in the presence of blood components.
They have ability to carry both hydrophilic and hydrophobic molecules.	They are more prone to aggregation because of small size and large surface area.
They can be administrated in different ways (oral, nasal, parental, etc.).	They can trigger immune responses and allergic reactions.
The circulation time in the body can be longer.	

From the historical view, administration of drugs intravenously in a suspension form could not be imagined before the invention of nanotechnology. After discovery of nanotechnology in 1960s, lipid vesicles were used as drug delivery system [37]. Many advantages in pharmaceutical field were provided by using organic and inorganic biomaterials as nanocarrier systems. The first study on nanoparticles for controlled release of drug delivery system was done by Speiser and Birrenbach in 1976 for vaccination purpose. They produced polymeric nanoparticles for the slow release rate of the antigen, which resulted in a better immune response. After this invention, in 1977, first nanocapsules that can carry drugs into cells was developed by Couvreur et al. However,

these studies and others was mainly done by using non-biodegradable polymers like polyacrylamide, polymethylmethacrylate etc. An important advance for nanocarriers has done with the invention of biodegradable and biocompatible polymers such as albumin, polyalkylcyanoacrylate, polylactate-co-glycolate. Still, many studies are conducted on material science for the production of the ideal nanocarrier system [38]. Many nanocarriers have been accepted for clinical application. For example, paclitaxel is a type of chemotherapeutic agent with very low solubility. In order make this drug soluble, a toxic solvent called cremophor was used but this solvent causes severe side effects. For this reason, researchers developed a nanocarrier system called as Nab-paclitaxel (130 nm) (Abraxane) in order to carry paclitaxel without the toxic solvent. They attached the drug molecules to albumin. As a result, they observed larger volume distribution with respect to Taxol with lower side effects. In the light of these advantages, Nab-paclitaxel was approved by FDA for the treatment of breast cancer. Another example for clinically approved polymeric nanoparticle is Taxane. It is composed of PEG and poly-(D, L- lactic acid) block copolymeric micelles and paclitaxel (Genexol-PM) (20 to 50nm). Today, it is widely applied for the treatment of breast and lung cancer in Korea [39]. Beside these good examples, nanoparticles for drug delivery should still be developed in terms of high immune response, rapid clearance from the body, aggregation problem for the nanoparticles etc. These problems may be fixed in the near future with the aid of material science.

1.2.4.1 Polymers for Nanoparticles

Polymers for the production of nanoparticles can be non-biodegradable and biodegradable as stated before. However, non-biodegradable polymers for the drug carrier systems lead to problems such as need of elimination from the body after application. As a result, biodegradable polymers are preferred for the application of the drug delivery systems. Generally, biodegradation can form by hydrolysis and enzyme cleavage in the polymeric backbone or by cleavage of a side-chain resulted in water soluble polymeric products. From the aspect of biodegradability, polymers degrade (bond cleavage) or erode

(depletion of material). The erosion process can take place in two different ways; bulk erosion and surface erosion. In bulk erosion, water molecules can pass through the bulk in a faster rate than erosion of the polymeric matrix such as polyesters. The release kinetic in bulk erosion is more complex than surface erosion. On the other hand, polymers with surface erosion consist of highly labile groups for rapid hydrolysis of polymer chains. Thus, the surface erosion rate in polymeric matrix is much faster than penetration of water molecules into the bulk. In surface erosion, the zero-order drug release is observed. For the zero-order drug release, there is no fluctuation of the drug concentration in blood [40].

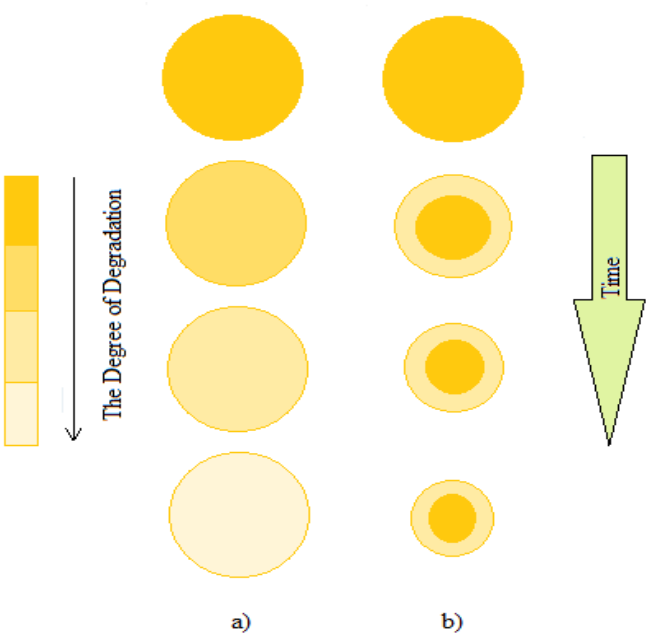


Figure 1.6: a) Bulk Erosion b) Surface Erosion

Biodegradable polymers used in drug delivery system can be natural polymers (e.g. cellulose, starch, chitosan) or synthetic polymers (e.g. polyesters, polyamino acids, polyorthoesters, polyphosphazenes, and polyanhydrides indicated in table 1.2). Due to limited manipulation of bulk material in natural polymers and easy modification of special properties in synthetic polymers, synthetic ones are preferred for controlled release systems [40].

For drug delivery applications, polymeric matrix should possess some special physical and chemical properties like being hydrophobic, stable, strong, flexible, and soluble in organic solutions. In addition, low melting point, and linear degradation over time in an aqueous environment are important factors for the polymeric matrix.

Table 1.2: Synthetic Biodegradable Polymers used in Controlled Release

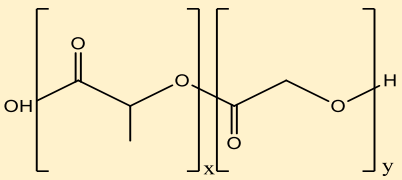
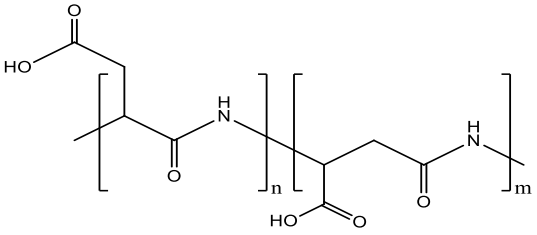
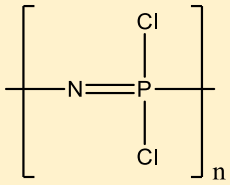
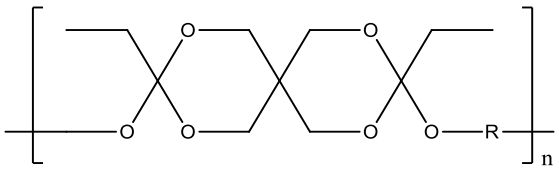
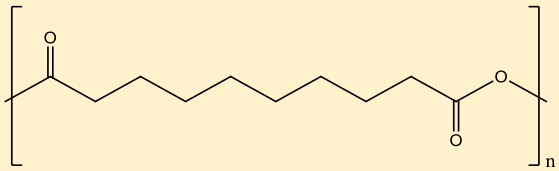
Polymer	Example	Application
Polyesters	Poly (lactic acid-co-glycolic acid) 	Nanocapsules for the controlled release of growth factor in bone tissue engineering [41]
Poly (amino acids)	Poly (aspartic acid) 	Adriamycine bounded poly (ethylene glycol)-poly (aspartic acid) nanoparticles for the treatment of leukemia [42]

Table 1.2 (Continued)

Polymer	Example	Application
Poly (phosphazenes)	Poly (dichlorophosphazene) 	PEGylated pH sensitive poly (phosphazene) nanoparticles for the delivery of adriamycine into drug resistant tumor cells [43]
Poly (ortho esters)	3,9-diethylidene- 2, 4, 8, 10-tetraoxaspiro[5.5]undecane-based polymers (DETOSU) 	Poly (ortho ester) nanoparticles loaded with epinephrine for the ocular drug delivery [44]
Poly (anhydrides)	Poly (sebacic anhydride) 	PEGylated poly (sebacic anhydride) nanoparticles loaded with paclitaxel for cancer therapy [45]

Poly (anhydrides) (Figure 1.7) demonstrates many of these important properties mentioned before. That is why they are perfect candidates for drug delivery applications. Moreover, they degrade into nontoxic [46, 47], non-mutagenic [46], and non-

inflammatory [46] monomers inside the body. Poly (anhydrides) are consisted of water-soluble diacid monomers linked to each other by anhydride bonds. These anhydride bonds are relatively unstable against water; hence, surface erosion for zero-order release is observed. By means of this water reactivity, controlled degradation rates can be obtained. Another reason for surface erosion in poly (anhydrides) is hydrophobicity that limit water diffusion into the bulk. The degradation of poly (anhydrides) is based on simple hydrolysis and the degradation rate can be arranged by modifying hydrophilic and hydrophobic ratio of monomeric units. This hydrolysis rate depends on pH of the surrounding medium and the solubility of polymeric compound in the medium. Solubility of the degradation products is higher at basic pH because poly (anhydrides) have carboxylic acid groups in the structure [40, 48]. The first prove of being good candidate of poly (anhydrides) in controlled release application was done by Rosen et al. in 1983. They produced poly (anhydride) matrix loaded with steroid and they observed the release profile close to zero-order release kinetic [49]. After this finding, many efforts have been put on the invention of drug delivery systems with different poly (anhydrides). For example, Gliadel wafer composed of 1,3-bis(p-carboxyphenoxy)propane (CPP) and sebacic acid (SA) copolymer in the 20:80 ratio loaded with BCNU (chemotherapeutic agent) was approved by FDA in 1997 for the treatment of glioma and very successful results have been obtained clinically. One type of poly (anhydrides) that is applied for the drug delivery systems is poly (sebacic anhydride).

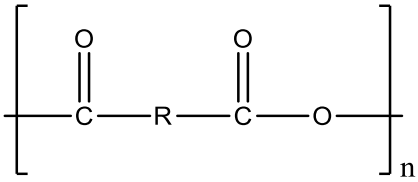


Figure 1.7: General Formula of Poly (anhydrides)

1.2.4.2 Poly (Sebacic Anhydride)

Poly (sebacic anhydride) (PSA) is a type of aliphatic poly anhydrides that is used commonly for the drug delivery systems. Since it contains all advantages of poly anhydrides like zero-order release, biocompatibility, etc., it is a perfect choice as drug delivery system. In addition, low cost and high availability are the other factors that make PSA a good candidate for drug delivery application. The synthesis of this polymer is generally conducted through melt condensation polymerization [50, 51]. First synthesis of PSA was conducted by Hill and Carothers in 1932 (Figure 1.8). They mixed sebacic acid (diacid monomer) and acetic anhydride and refluxed the mixture under nitrogen gas. With the effect of heat, monomeric units may combine with each other by discarding terminal groups in the adjacent molecules. Then, they used melt condensation polymerization reaction to produce PSA polymer from prepolymer in vacuum oven [52].

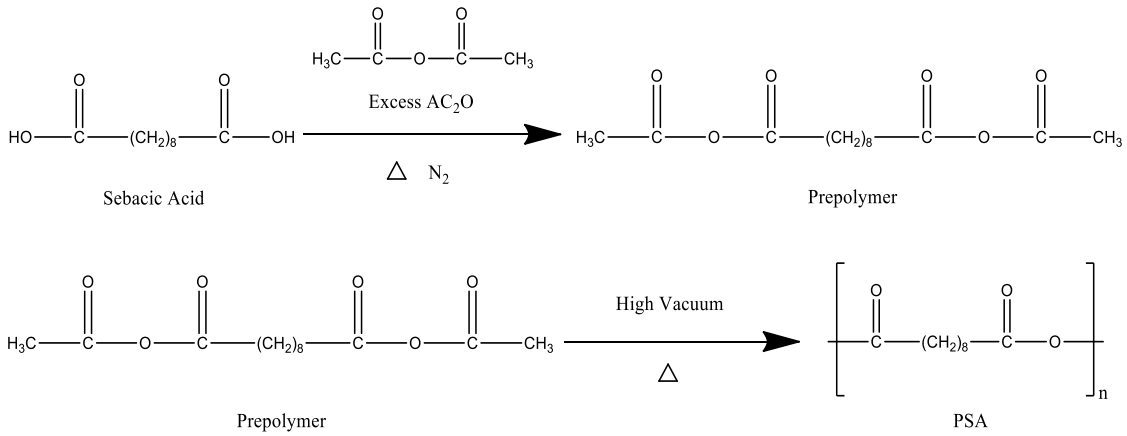
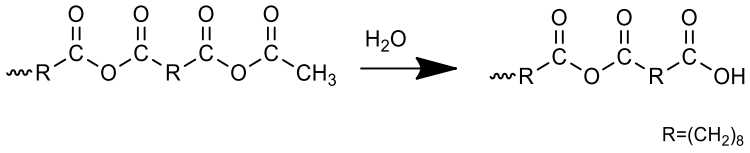


Figure 1.8: Synthesis of Poly (Sebacic Anhydride)

The degradation of PSA depends on many factors such as temperature, humidity, molecular weight of polymer etc. The study of Domb and Langer in 1989 indicates depolmerization rate is affected by temperature. The minimum depolymerization rate was observed at -20°C and the depolymerization rate increases as temperature increases. The storage condition of PSA should be below 0°C and under nitrogen gas [53]. The molecular weight is also very important. With higher molecular weights, the lower degradation rate is observed. According to the studies, it is believed that degradation of PSA can take place through two different mechanism which are hydrolysis and intermolecular interaction (Figure 1.9) [53, 54].

a)



b)

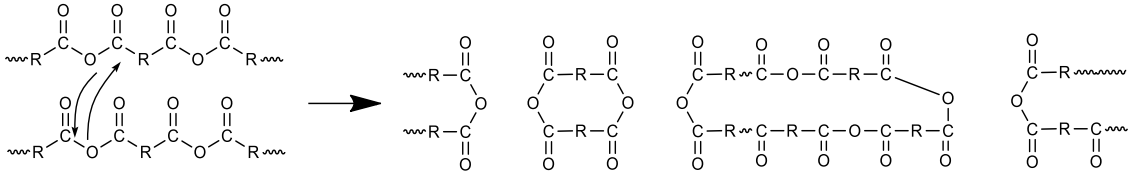


Figure 1.9: Degradation of PSA a) Hydrolysis into Carboxylic Acid b) Intermolecular Interaction

The degradation products of PSA are metabolized through β-oxidation pathway producing acetyl co-A in the body. This product can further be used in different important metabolic pathways such as energy production in citric acid cycle and synthesis of neurotransmitter [48]. The biocompatibility of PSA was investigated by Leong et al. in 1986. They used PCCP-SA copolymer matrix for cytotoxicity and mutagenicity tests on bovine aortic endothelial cells. There was no cytotoxic and mutagenic effect of PSA so that cells continued to grow normally. Moreover, they implanted this polymeric matrix into rabbit corneas to study the tissue response. There was no sign of the inflammation or the abnormal cell attachment [46]. Furthermore, in the research of Laurenci et al. (1990) the biocompatibility of PCCP-SA copolymer was proved in rat by exploring hematological results, systemic toxicological results, and the histological result of implant site. There was no evidence for toxicology of this biomaterial [47]. For this reason, PSA can be considered as biocompatible polymer.

There are many types of drug delivery systems made up of PSA in the form of disc, film, injectable gel, microspheres, micelle, and nanocapsule. In the literature, PSA was generally combined with other polymers in order adjust degradation rate of polymer matrix according to the need. Liang et al. (2013) produced poly (sebacic anhydride) and poly (sebacic anhydride) containing glycol (PSAG) in the disc form. They obtained slower degradation and drug release rate through PSAG disc compared with PSA film [55]. The degradation study on film of PSA and PLA blends was conducted by Davies et al. in 1996. As the PLA content increased in the film structure, degradation rate became slower which is closer to degradation rate of PLA [56]. Another example for PSA as drug delivery system is thermo responsive injectable gel composed of PEG-(SA-LA)-PEG triblock copolymer containing 5-FU as chemotherapeutic agent. This system was developed by Zhai et al. in 2009 and applied in situ rat model. After this application, the longer and better sustained release was obtained [57]. PSA can also be used in the form of microsphere for drug delivery. Microspheres composed of poly (sebacic anhydride-co-Pluronic F68/F127) loaded with nifedipine showed a good sustain release profile and the release profile became better with increase in the molecular weight raito of pluronic in the copolymer structure [58]. PSA-PEG-PSA block copolymer was synthesized by Zhang and Guo (2006) and they produced micelles from this block copolymer. They optimized several factors such as organic phase, water phase, and molecular weight of PSA that affect micelle size [59]. Fu et al. (2002) designed a photo-responsive nanocapsules composed of PSA and phthalocyanine. The average particle size was 166 nm and zero order release kinetic was followed [60]. In addition to the modifications of PSA with respect to the drug release rate, other modifications can be applied such as introduction of poly (ethylene glycol) (PEG) into the polymer structure to decrease the immunological response of the body.

1.2.4.3 Opsonization

As mentioned in the section 1.2.4.2, PSA is generally combined with other polymers in order to get a desired drug release rate. Another reason for the introduction of a new polymer into the PSA structure is to solve the problem of fast removal of the nanoparticles from blood circulation. This problem is called as opsonization. Normally, nanoparticles should travel throughout the blood circulation without loss of any properties before arriving to the site of action. As a result, a successful drug delivery system is obtained. However, opsonization process in which a foreign structure is marked for the destruction by phagocytes causes very short circulation time of nanoparticles. This process is conducted by reticuloendothelial systems (RES) containing different types of macrophages such as Kupffer cells or macrophages found in liver. Phagocytes detect the nanocarriers through opsonin proteins found in blood circulation. Opsonin proteins use in random Brownian motion in the blood plasma and when they become close enough to nanoparticles, van der Walls, electrostatic, ionic, and hydrophobic forces cause the attachment of opsonin proteins on the nanoparticle surfaces. This attachment results in conformational change of these proteins and they become active. Then, phagocytic cells identify these active proteins, bind to them, and phagocytosis of these particles takes place [61].

Opsonization can be affected by two main factors which are hydrophobicity and surface charge of the particles. Hydrophobic particles can go into interaction with opsonin proteins easily which was proved by Carrstensen et al. in 1992. They compared naked hydrophobic latex particles and latex particles coated with Poloxamine 908 which is more hydrophilic. They observed less blood clearance rate for the coated nanoparticles [62]. The particles with positive charge can be subjected to opsonization easier than neutral or negatively charged particles. In the study of Roser et al. (1998), the albumin particles with nearly zero zeta potential has lower opsonization binding comparing with the positive and negative charged particles. The highest opsonization rate was observed in the positively charged particles [63]. The reason of this fact is that opsonin proteins cannot interact easily with neutral charges to create weak bonds with the particles. Higher opsonization is

expected for positively charged nanoparticles than negatively charged since opsonin proteins are negatively charged molecules. As a result, repulsive forces is created for the negatively charged molecules making interaction harder and attractive forces for positively charged molecules making the interaction easier.

In order to overcome opsonization problem, surface adsorption or grafted groups are used for blocking electrostatic and hydrophobic interaction between nanoparticles and opsonin proteins. For this purpose, long hydrophilic polymer chains such as PEG and nonionic surfactants are used generally [61]. First try to overcome opsonization problem was done in 1970 by Abuchowski et al. They used methoxypolyethylene glycols with two different molecular weigths. They linked bovine serum albumin with this PEG covalently and injected into rabbit. Following this, they observed similar blood circulation time for PEG attached molecule like native bovine serum albumin [64]. After this finding, many efforts was put on modification of nanoparticles with PEG to reduce the binding probability of opsonin proteins onto the particle surfaces.

1.2.4.4 Poly (Ethylene Glycol)

Poly (ethylene glycol) (PEG) (Figure 1.10) is a type of polyether with linear or branched form which has many application in biotechnology and biomedical engineering. It is available with different molecular weights. Since it is soluble in water and most of the organic solvents and it can easily go into chemical alteration and attachment, the application of PEG on drug delivery systems is an easy process. In addition, PEG is nontoxic, nonimmunogenic, and FDA approved which make this polymer a good candidate for the application on human body [65, 66].

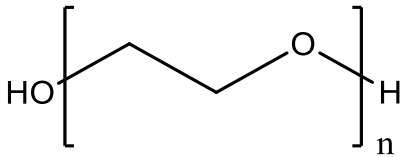


Figure 1.10: The Structure of Poly (ethylene glycol)

Although PEG is generally used as polymer-protein pair, the application on drug delivery system is also popular as due to its ability to prevent opsonization process. One popular application of PEG as drug delivery system clinically approved by FDA in 1995 is Doxil (OrthoBiotech) for the treatment of ovarian cancer and multiple myeloma. Through this liposome-PEG conjugate, increase in the half-life of particles, enhancement in the tumor deposition, and remarkable decrease in the cardiotoxicity were observed [67].

The incorporation of PEG into drug delivery system is called as PEGylation and PEG can be attached to the drug carrier in two ways. First one is the attachment by means of physical interaction such as electrostatic interaction. The second way is the attachment by covalent bond. Physical attachment is used for surface adsorption of PEG but this is not as strong as attachment by covalent bond since with covalent bond is my more stable. This fact was proved by Harper et al. in 1991. They produces PEG incorporated poly (styrene) microspheres by means of charge interaction and covalently binding. They observed more stable particles in blood circulation with covalent attachment [68].

The feature of PEG that prevents the nanoparticles from opsonization process can be explained based on the chemical and physical features of PEG. Because of its hydrophilic and flexible properties, PEG chains on the surface can move more freely than the other conjugated structure. For this reason, opsonin proteins contact with the PEG chains easier and they bind to these chains through van der Wall forces that results in compression in PEG chains. This compression further causes conformational change which forms repulsive forces. This repulsive force masks attractive force coming from the other

conjugate in the structure. Thus, the probability of binding of opsonin proteins decreases effectively. As a result, macrophage recognition rate decreases sharply [61]. This fact can be demonstrated roughly in figure 1.11:

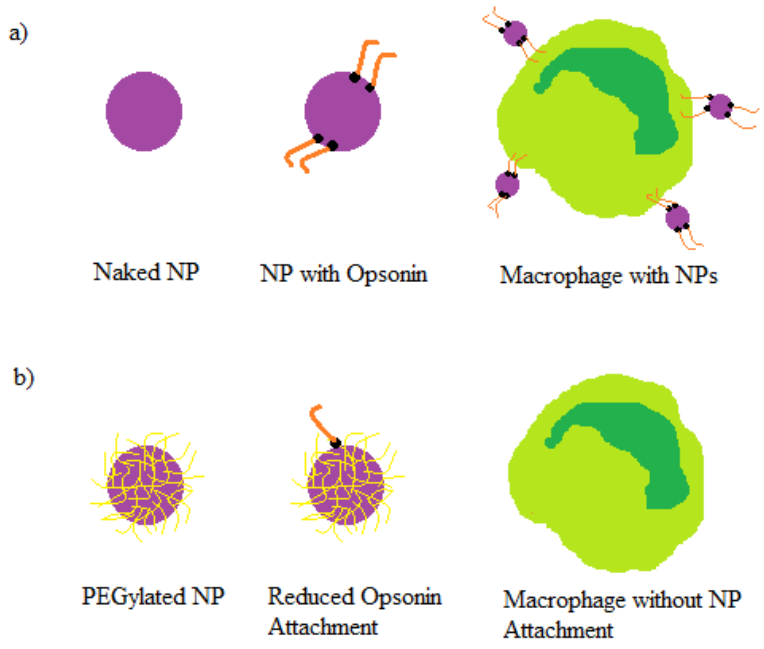


Figure 1.11: The Effect of PEGylation on Macrophage Detection

Another evidence for the effect of PEGylation on opsonization process was suggested by Tan et al. in 1993. The blood circulation time of PEG-5000 coated and uncoated polystyrene latex nanoparticles were analyzed. When these particles were injected into rats, half-life of uncoated particles was 20 min while half-life of coated particles was 13 h

[69]. Molecular weight of PEG is a very important parameter for the success of stealth capability. For smaller particles PEG with larger molecular weight is used. As particle size increases, molecular weight of PEG decreases. For example, nanoparticles with 50-100 nm size, PEG with 3400-10000 Da is used. The effect of molecular weight of PEG on blood circulation time of nanoparticles was shown by the study of Mori et al. in 1991. The liposomes composed of N-(monomethoxy polyethyleneglycol succinyl) phosphatidylethanoamine (PEG-PE) was attached with different molecular weights of PEG (5000, 2000, and 750). The largest blood circulation time was detected with PEG 5000 [70]. Generally, PEG with molecular weight between 2000 and 20000 Da is preferred for drug delivery system since molecular weight lower than 2000 Da is not flexible enough and molecular weight higher than 20 kDa is more prone to liver accumulation. However, PEG with up to 30 kDa can be excreted easily with urinary system [71].

Because of the fact that PEG is one of the best choice to prevent rapid clearance of drug delivery systems, there are several studies in literature performed on PSA-PEG copolymer. The first try for the production of PSA-PEG copolymer was done by Peracchia et al in 1997. They produced diblock copolymer of PSA and PEG and the nanoparticles from this copolymer. In addition, they examined the drug release profile of these nanoparticles. The result showed that the presence of PEG or molecular weight of PEG does not affect the total amount of drug release. However, higher molecular weight of PEG reduced the burst effect and slowed down the drug release process [72]. Another study for PSA-PEG diblock copolymer suggested that the degradation of nanoparticles fits to first-order kinetic and the rate of degradation depends on pH and temperature [73]. Another type of drug carrier system with PSA-PEG copolymer was produced by Fu et al. (2002). In that research, the microparticles were applied on the lung for which the controlled release and the carrier localization is very difficult. The carrier system loaded with a model drug were deposited suitable in the regions of the lung through inhalation [74]. The difference between PSA-PEG copolymer and blend with respect to mechanical properties was investigated by Chan and Chu in 2003. The copolymer structure showed higher viscous behavior and crystallinity than the blend structure did. This shows that the

copolymer structure is more appropriate for the application of controlled release [75]. In the copolymer structure PSA/PEG ration is crucial for the particle size. Zhang and Guo (2006) proved this fact by synthesizing PSA-PEG block copolymer with different PSA/PEG ratios. They observed increase in the micelle size with the increase in molecular weight of PSA block. Moreover, the size of micelle increased as the concentration of the copolymer dissolved in the organic phase increased [59]. In the same year, Zhang et al. obtained the same result by trying linear, three-, and four-armed block copolymers [76]. As mentioned before, surface erosion characteristic of PSA is very special for the controlled release application. In some studies, researchers tried to adjust erosion rate of the carrier system while preserving this special surface erosion characteristics. For example, Hou et al. (2007) synthesized disk shape carrier system composed of SA-CPP-PEG copolymer. With the increase in PEG content, the erosion rate of the copolymer increases while the mechanical strength decreases [77]. Same copolymer content was tried by Zhao et al. (2010) in order to produce thermosensitive micelles loaded with doxorubicin. The successful burst release followed by sustained release was obtained [78]. The mucus barrier can easily remove foreign particles; thus, penetration through this barrier is very difficult. Tang et al. (2009) produced PSA-PEG nanoparticles for delivery by inhalation. They compared PLGA nanoparticles with these particles and they observed 3300 fold increase in the diffusion rate of PSA-PEG nanoparticles [79].

In conclusion, PEG can be used for different drug delivery systems for different purposes since it can be modified by chemical methods easily. By means of PEG, the degradation rate of polymeric drug delivery system can be adjusted. In addition, the stealth ability of PEG is the most important feature since it solves rapid clearance problem which is mostly observed for drug delivery systems. All these advantages and its biocompatibility, non-toxicity, high solubility in water make PEG the best choice for the drug delivery application.

1.3 Glutathione S-Transferase

Glutathione S-transferase (GST) is a complex multigene family of phase II metabolic isozymes that can be found in both eukaryotic and prokaryotic systems [80]. Many endogenous and exogenous electrophilic compounds e.g. carcinogens, drugs, medicinal plant extracts, pesticides, herbicides, and oxidative stress products are conjugated to reduced glutathione (GSH) through GST by nucleophilic attack on carbon, sulphur, or nitrogen atoms of substrates (Figure 1.12) [81, 82, 83]. As a result, cellular macromolecules like proteins can be prevented from attack of reactive electrophiles, which causes stress responses, toxicity, and cell death [81, 84].

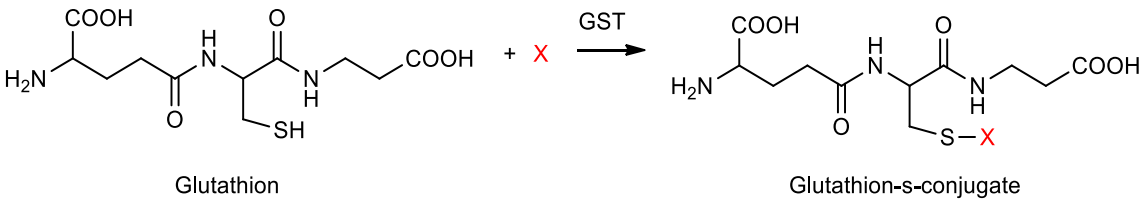


Figure 1.12: The Conjugation of GST with Substrate

GST family is divided into three main groups: cytosolic, mitochondrial, and microsomal MAPEG (membrane associated proteins). Among them, cytosolic GSTs are dimeric and the most abundant type. They are classified according to their structural similarities, substrate specificity, and immunological properties as alpha, zeta, theta, mu, pi, sigma,

and omega [82, 83]. All subgroups of cytosolic GSTs have different functions that are displayed in table 1.3 [85, 86].

Table 1.3: Human Cytosolic GSTs

Type of Cytosolic GST	Location in Body	Function
GST α	Testis, liver, kidney, adrenal, pancreas	▪ conjugation of fatty acids ▪ isomerism of steroids ▪ important in stress signaling
GST μ	Skeletal and cardiac muscles	▪ Ca^{2+} channel regulation ▪ important for cardiac and muscle pathology
GST π	Brain, heart, lung, testis, kidney, and pancreas	▪ drug resistance against several anticancer drugs because of its over activity ▪ aging ▪ neurodegeneration
GST π and μ	Brain region called black substance	▪ formation human brain tumors
GST σ	Fetal liver and bone marrow	▪ involved in prostaglandin synthesis ▪ xenobiotic conjugation with GSH
GST θ	Kidney, liver, small intestine, brain, and prostate	▪ xenobiotic metabolism ▪ signaling mechanism in tissue
GST ζ	Fetal liver and skeletal muscle	▪ catabolism of phenylalanine and tyrosine amino acid residues ▪ glyoxalate formation

In human, there are only one type of mitochondrial GST called as Kappa. It is found in liver, kidney, stomach, and heart. It is believed that Kappa has an important role in β-oxidation of fatty acids. The last type GST in human is microsomal MAPEG and mostly found in liver, pancreas, small intestine, skeletal muscle, and spleen. It has participation in the production of leukotrienes, prostanoids, and endogenous lipid signaling molecules [83, 85]. These three types of GSTs are different in structure but most of their subtypes can catalyze the reaction of GSH and 1-chloro-2, 4-dinitrobenzene (CDNB) conjugation (Figure 1.13) [83].

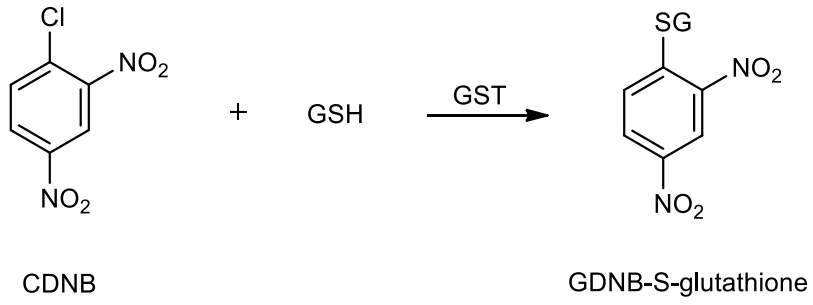


Figure 1.13: The Reaction between CDNB and GSH

1.3.1 Drug Metabolism and GST Function

Although GST has different kinds of biological functions, the drug metabolism function of GSTs is the scope of this context. The drug metabolism is the transformation of hydrophobic agents into more polar compounds which can be excreted from the body easily. It takes place in smooth endoplasmic reticulum of hepatocytes and it is made up of three phases called as phase I, phase II, and phase III [87].

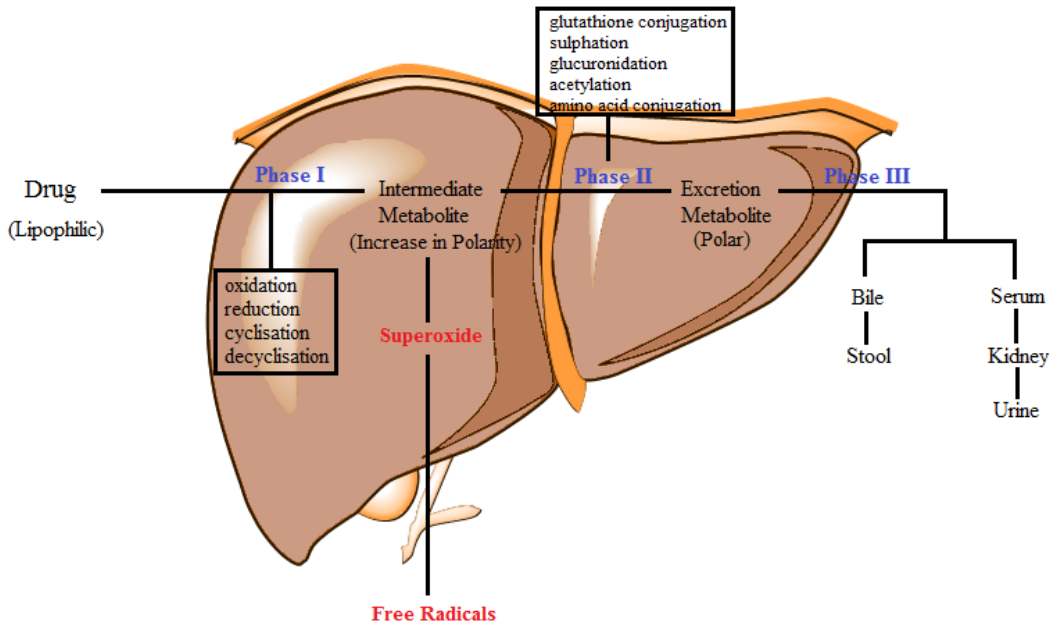


Figure 1.14: Drug Metabolism

Phase I reactions are nonsynthetic and can be oxidation, reduction, hydrolysis, cyclisation, and decyclisation. The most common enzyme in phase I is cytochrome P450, which is for oxidation-reduction reaction. These reactions are conducted to make the drug molecules more polar by means of alternating the reactive groups of drugs. The product can be excreted from the body if it is polar enough. If it is not, the product go into phase II reactions. Phase II reactions are called as conjugation reactions because polar groups of the products coming from phase I reactions are combined with charged compounds like glutathione (GSH), sulphonates, glucuronic acid, and amino acids (glycine, taurine, glutamine, ornithine, and arginine). These reactions occur with the help of transferases that are glutathione S-transferase (GST), N –acetyltransferases, epoxide hydrolases, and glucuronyl transferases [88]. Phase III reactions are mainly composed of transport and excretion process. This process is accomplished by the multidrug resistance protein (MRP) family which remove the final metabolite by means of the secretion by using adenosine triphosphate. As a result, the detoxification process is finished. In some cases, the conversion of metabolites into more polar compounds can continue in phase III e.g. the conversion of glutathione conjugate into mercapturic acid conjugates [89, 90].

The first discovery about GST-GSH interaction has revealed by Boyland and Chasseaud in 1969. They discovered that GST protects the cell from electrophilic agents. After this discovery, researchers have tried to understand the exact detoxification function of GST. It is found that detoxification reaction occurs through mercapturic acid pathway (Figure 1.14) [91]. The reaction starts with the GSH conjugation to electrophilic compound through the thiol group of GSH. Then, catabolism occurs by means of γ-glutamyl-transpeptidase to eliminate γ-glutamyl part. After that, cysteinyl-glycinase enzyme hydrolyze cysteinyl-glycine molecule to remove glycine groups from the conjugate. Thus, cystein conjugate is formed. This conjugate is then converted into mercapturic acid with the aid of N-acetylase. The mercapturic acid is removed from the cells by MRP (multidrug resistance associated protein) [83]. These reactions generally occur in liver and condensed cysteine-aromatic compound is removed from the body by means of urine. As a result, detoxification process is completed.

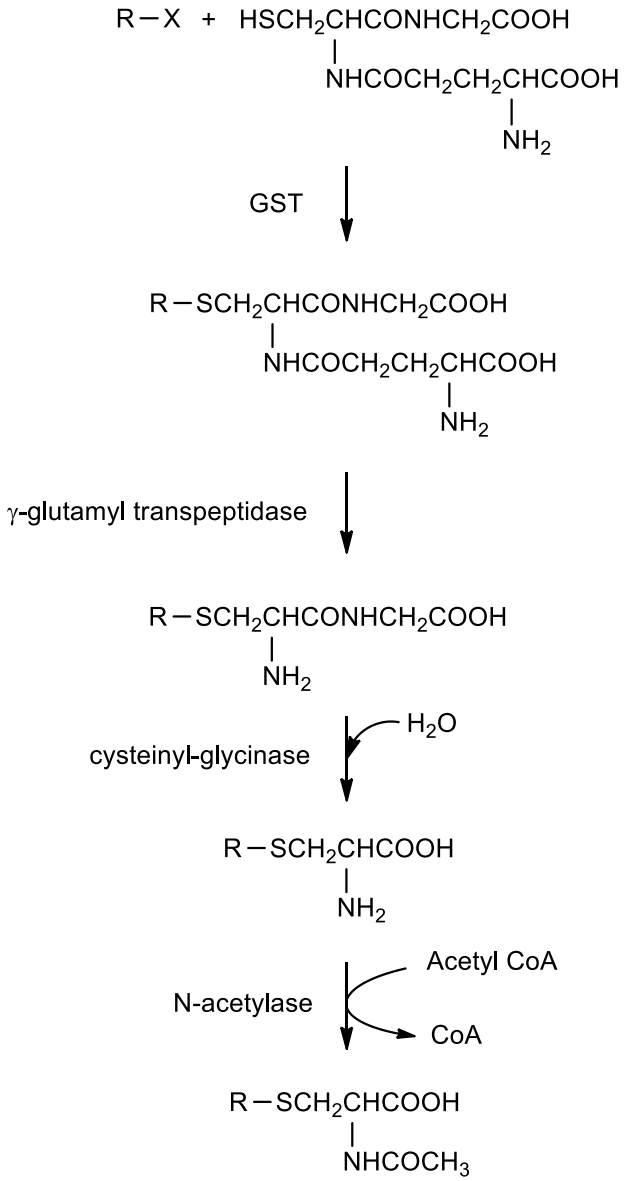


Figure 1.15: Mercapturic Acid Formation

However, this direct detoxification process is valid only for the chemotherapeutics which are substrates of GST like chlorambucil, nitrogen mustard, and melphalan, etc. For the chemotherapeutics that are not substrate for GST like mitomycin C, cisplatin, and doxorubicin, etc, the mechanism is different [81]. First, they should be converted into more electrophilic compound by redox reduction. Then, they interact with GST and be removed from the cells [92].

With the overexpression of GST and GSH gene, resistant cells to chemotherapeutic drugs can be produced. The resistance can arise in two ways: direct detoxification and inhibition of MAP kinase pathway [81]. In direct detoxification, as concentration of GST and GSH increases, the capacity of removing drugs from cells also increases. For the inhibition of MAP kinase pathway which results in cell apoptosis, high concentration of GST and GSH causes high rate of inhibition cell apoptosis process. This fact has been proved with the observation of GST and GSH in high concentration in many types of resistant cancer cells [93].

1.3.2 The Interaction between Doxorubicin and GST

Doxorubicin (DOX), which is also known as Adriamycin® or hydroxydaunorubicin, is a cancer drug commonly used in the treatment of hematological malignancies and several types of solid tumors (Figure 1.16). It is a kind of anthracyclines that was first isolated from *Straptomyces peucetius* [94]. Even though the exact mechanism of DOX is still not clear for cancer treatment, two mechanisms have been proposed. In first mechanism, doxorubicin behave as topoisomerase II inhibitor by intercalating into DNA base pair. Normally, topoisomerase II cuts DNA strands to remove supercoils and reseals the DNA strand after duplication. In the presence of DOX, topoisomerase II enzyme cannot work properly and it cannot reseal it because of stabilization. As a result, the cell goes apoptosis. The second action mechanism makes the cell go apoptosis by producing reactive oxygen species (ROS). The produced ROS give damage to mitochondrial membrane and cytochrome c is released. Therefore, caspases start for apoptosis [95, 96].

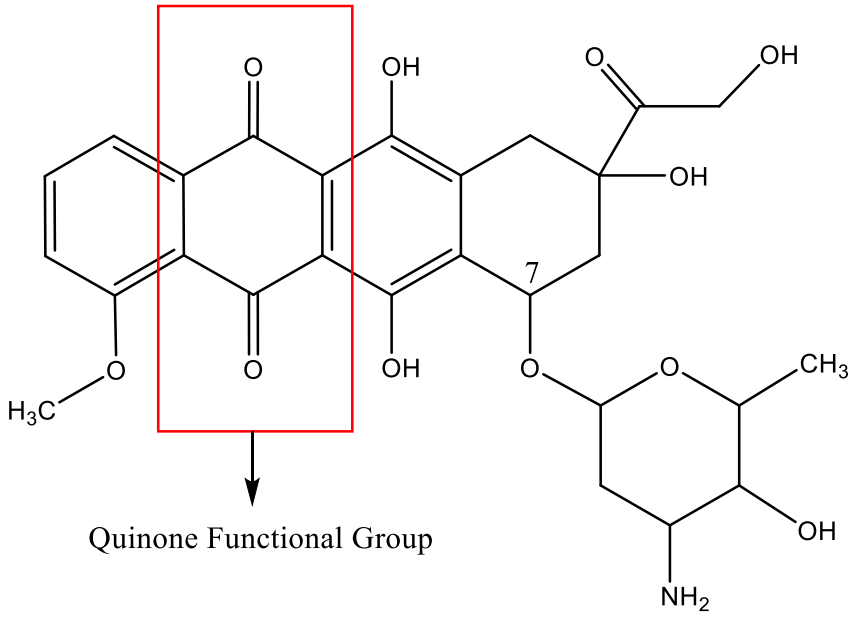


Figure 1.16: Doxorubicin Structure

DOX is normally in quinone form which behave as electron acceptor. By means of phase I enzyme P450, it can easily be reduced into its semiquinone form. ROS can be produced through non-enzymatic oxidation of this semiquinone form [97, 98]. This mechanism is shown in figure 1.17:

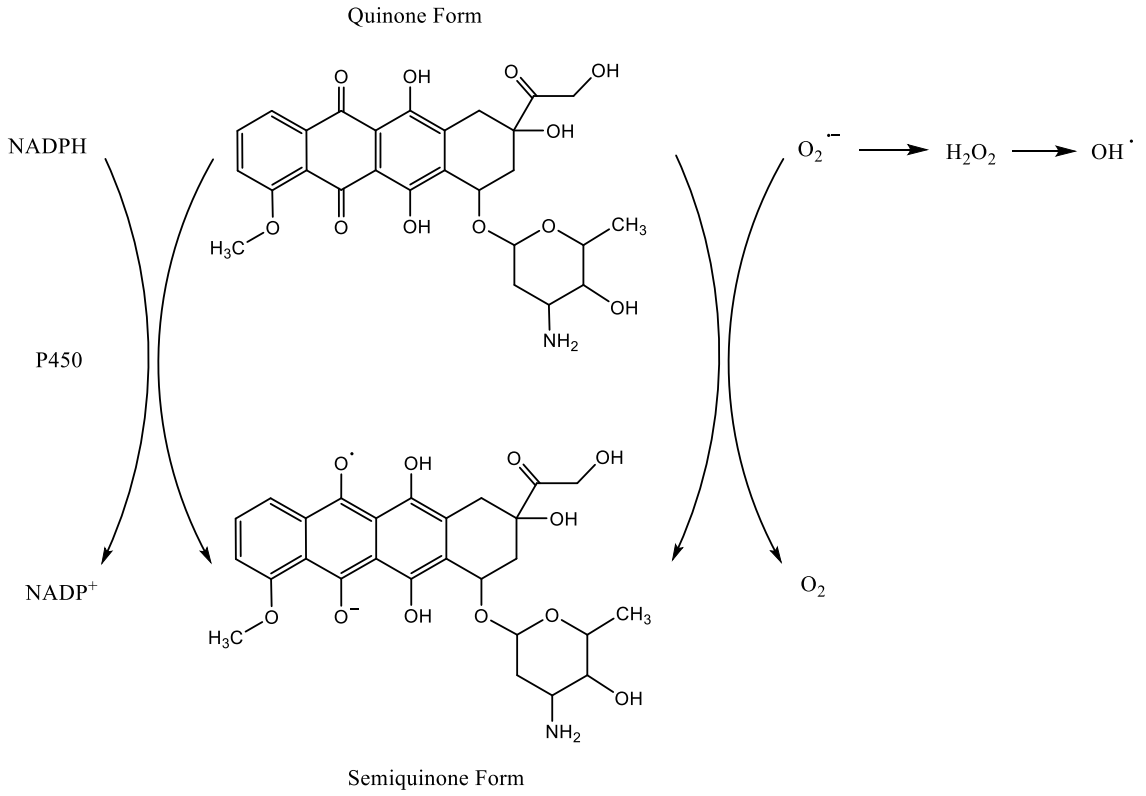


Figure 1.17: DOX Redox Reaction

After the production of ROS, the exact mechanism that leads to cell apoptosis is unclear. However, several studies proved that cell apoptosis depends on GST-JNK (c-Jun N-terminal kinases) interaction [99, 100, 101, 102, 103]. JNK is a protein that belongs to mitogen-activated protein kinase (MAPK) and its activation is crucial for cell proliferation, differentiation, and apoptosis. Normally, JNK is found as inactive form in the cell by the protein: protein interaction with GSTP1-1. Reduced DOX conjugates with GSH which inhibits the GST activity so GSTP1-1-JNK interaction is lost. As a result, JNK becomes active and it phosphorylates c-Jun which starts apoptosis. For dissociated GSTs,

it is believed that they form dimers or multimeric products. The process is depicted in figure 1.18.

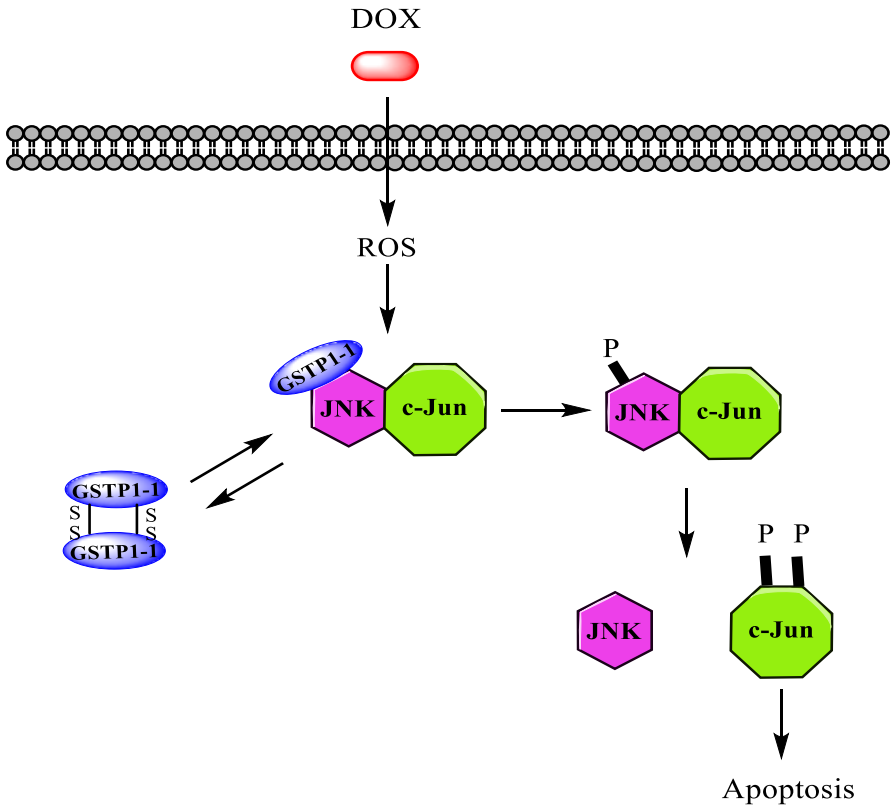


Figure 1.18: GST-JNK Interaction

The overexpression of GST gene and other proteins special for MDR system results in drug resistance as mentioned before. Drug delivery system can overcome this problem through increasing retention time at the target site and controlled release that forms high drug concentration at the target site. Subsequently, the components cause drug resistance reach the saturation and removing drug molecules from the cell becomes harder. Moreover, it has been proved that some polymers have inhibition effect on resistant cells of cancer. For example, Alakhov et al. (1996) studied Pluronic 85 [poly (oxyethylene-b-oxypropylene-b-oxyethylene)] block copolymer loaded with DOX on MDR resistant human ovarian carcinoma cells [104]. The ATP depletion effect of P85 inhibits P-glycoprotein 1 (P-gp1), which is a MDR protein working in the presence of ATP. Thus, efflux pump cannot work to remove drug from the cell. For the examination of GST activity with the application of Pluronic 85, other studies were also conducted. In 2003, Batrakava et al. used poly (ethylene oxide)-block-poly (propylene oxide)-block-poly (ethylene oxide) and DOX in MRP overexpressing cells [105]. Two hours after the application of the polymer, significant reduction in GST activity was observed in MDCKII cell lines. Moreover, the decrease in the GST activity was found with the increase in the concentration of P85. Another example for P85 is the study of Shen et al. in 2012 [106]. PTX loaded P85 nanoparticles reduced GST activity and increased PTX accumulation in resistant A549 human lung cancer cells. In addition to these examples, many studies for GST-DDS relationship is observed in literature not only on chemotherapeutic agents but also on other types of drugs. The researches can be classified according to the material of DDSs like polymeric, metallic, carbon, and others. For polymeric DDSs, generally PLA, PLGA, chitosan, Pluronic P85, etc. were used. Baras et al. in 2000 studied on a type of vaccine to treat a chronic parasite disease [107]. GST is very important for defense to defeat host immune system. The nanoparticles composed of PLA polymer was loaded with recombinant GST and their application resulted in good immune response. For the effect of nanoparticles composed of PLA-PEG copolymer, Zhang et al. (2007) observed the decrease in the expression of GSTP-1 and resistance gain of mouse hepatic cells by the application of large of dose of the nanoparticles [108]. This shows the degradation product of PLA-PEG copolymer has effect on GST activity. Quercetin (QC) is a type of

antioxidant drug used to remove ROS from cells. PLA nanoparticles loaded with QC showed higher GST activity than free QC in the research paper of Das et al. (2008) [109]. This result proves the importance of DDS to control GST activity. For the cytotoxicity assay of DDS, the microparticles composed of PLA was loaded with PhSeZnCl to analyze the effect of this delivery system on different cell line in the study conducted by Bartolini et al. in 2015 [110]. 10- 20 fold lower GST activity was seen for encapsulated PhSeZnCl than free PhSeZnCl. This shows the cytotoxic effect of PhSeZnCl can be eliminated by drug delivery system. The QC was also loaded with a different type of polymer (PLGA) by Ghosh et al. in 2009 [111]. Higher GST activity was observed for QC loaded nanoparticles than free QC again. Nowadays curcumin is very popular substance to enhance cancer treatment since it has supportive effect on GST activity. Shahani and Panyam showed higher GST activity in the liver samples of mice treated with PLGA-curcumin microparticles than that in mice with no treatment [112]. Chitosan based DDS was also examined for the effect on GST activity in literature. Gibaly et al. (2003) investigated chitosan based microcapsules loaded melatonin (MT) to reduce the carcinogenic effect of aflatoxin B1 (AFB1) by increasing the activity of GST [113]. Two times higher GST concentration was observed in mouse liver cells treated with microcapsule compared to the untreated liver cells. Chitosan is used as drug conjugate for delivery purpose. Chakraborty et al. (2012) used chitosan-vancomycin nanoconjugate for the treatment of *Staphylocossus aureus* infection which creates ROS in high amount and decreases GST activity [114]. They observed a 48% increase in GST activity in swiss mouseliver cells with the application of these particles. The research of Tripathy et al. (2013) on malaria infected swiss mice indicated 19.71% and 24.83 % increase in GST activity in free chloroquine and chitosan conjugate nanochloroquine, respectively [115]. Decrease in GST activity was also detected in aramide nanoparticles loaded chiral N-phthaloyl valine moieties in the research of Hassan et al. (2012) [116]. Researchers discovered other types of polymers that inhibits GST activity directly like P85 does. Xia et al. in 2006 proved the reduction effect of polystyrene on GST and Minko et al. in 2010 discovered the inhibition effect of HPMA on GST by applying DOX-HPMA conjugate on resistant human ovarian carcinoma cells [117, 118]. Since peptide and gene delivery

through DDS are very difficult for their sensitive nature, this type of DDS is prone to failure. A solution for this problem can be seen in the study of Buhrman et al. in 2013 [119]. In this study, GST was used in a different aspect. Bacterial peptide (melittin) was stabilized by means of GST-GSH interaction on the surface of hydrogel microspheres composed of poly (ethylene glycol) diacrylate (PEGDA). A successful delivery was obtained. Another research on gene delivery was conducted by Yin et al. in 2013 [120]. PBD-PCL nanoparticles loaded shRNA for apoptosis inhibiting genes and DOX were applied on MDR resistant MCF7 cells. The particle concentration with 10 μM, 20 μM, and 50 μM resulted in 40%, 52%, and 84% inhibition in GST activity. JNK pathway and MGST1 for mitochondrial mediated apoptosis are very important for activation of apoptosis. For this reason, DDS that activates JNK pathway and MGST1 can be a good solution to overcome drug resistance problem. A good proof for this hypothesis is the study of Zeng et al. (2014) on nanoparticles made of hyperbranched dendritic-linear (HBDL) polymer and loaded with DOX [121]. The researchers observed that these nanoparticles can change subcellular drug distribution by a special endocytosis. Therefore, MGST1 levels and JNK pathway can be modified. As dendrimeric carrier system, polyuria dendrimers were used to carry small interfering si(DNA) in the research of Restani et al. (2014) [122]. By means of GST activity assay, GSH depletion, which indicates high oxidative damage, was observed in HepG2 hepatocellular liver carcinoma cell line with the application of this carrier system. In addition, as dose increases, more reduction in GST activity was obtained. Piperlongumine (PL) and chalcones are two cytotoxic drugs used in treatment of cancer. However, their free form is highly toxic. The solution for this problem can be the use of DDSs like in the studies for poly (ethylene glycol) methyl ether nanoparticles loaded with PL (Liu et al. 2014) and polysorbate 80 nanoparticles loaded with chalcones (Winter et al. 2014), respectively [123, 124]. In both studies, the problem of GST activity reduction and GSH depletion was not seen with the use of DDS. Therefore, cytotoxic effect was reduced properly. GST can be applied for different purposes in addition to testing cytotoxicity or getting rid of resistance. For example, Zaro et al. in 2012 managed the conjugation of pH sensitive histidine-glutamic acid (HE) copolymer with model amphipathic peptide (MAP) by means of GST [125]. GST can also be used as

delivery agent. Fei et al. in 2014 synthesized pH sensitive HE-MAP and GST was attached for the delivery to mouse model of human breast cancer [126]. GST reached its target without losing its activity and property.

For metallic DDS, biocompatibility is the main problem for their application because they increase ROS and decrease in GSH and GST concentration causing cellular damage. For this reason, GST activity was used as the indication of particle toxicity in the literature. The decrease in GST activity indicates the high production of ROS and toxicity. For example, Fonseca et al. in 2011 observed decrease in GST activity in lung tissue of rats by the application of TiO_2 nanoparticles that causes high amount of ROS formation [127]. The size effect of metallic nanoparticles on toxicity was also studied through GST activity. In the study of Wang et al. (2008), 80 and 150 nm TiO_2 nanoparticles were used and nanoparticles with 150 nm showed more reduction effect on GST activity [128]. It was proved that smaller size of the nanoparticles display less toxicity. Exposure time of metallic carrier systems is also important for toxicity detection. The research on Fe_2O_3 nanoparticles for the effect on MRC-5 lung fibroblast cells was conducted by Radu et al. (2010) [129]. With the exposure time of 48 h and 72 h, the GST activity increased 21% and 32%, respectively since ROS concentration increased. Dose effect of metallic carrier systems is also very important for the formation of toxicological reactions. For instance, Al_2O_3 nanoparticles with 500, 1000, and 2000 mg/kg doses were applied on the liver cells of Wistar rats in the research of Prabhakar et al. (2011) [130]. As dose of the nanoparticles increased, GST activity became higher which is the indication of the increase in ROS. Another study which investigates the dose effect of copper nanoparticles on kidney cells of mice belongs to Sarkar et al. (2011) [131]. With the increase in the concentration of copper nanoparticles, the decrease in the GST activity was seen. Cisplatin made of platinum (IV) is a popular drug for several types of cancer treatment. However, the resistance is a very important obstacle for this drug application. To overcome the resistance of the cells, GST inhibitors like ethacrynic acid (EA) can be applied. Ang et al. in 2005 produced platinum (IV)-EA conjugate and applied the conjugates on A549 cisplatin resistant lung carcinoma cell line [132]. The least GST activity was observed for platinum (IV)-EA conjugate (up to 22.6%) whereas for cisplatin up to 63.6% and for EA

up to 78.5% activity were observed. The interaction between GST and platinum (IV)-EA conjugate was understood through the study of Parker et al. (2011) [133]. It is revealed that platinum (IV)-EA conjugate binds to GSP1-1 from H-site. The solution of the biocompatibility problem of metallic nanoparticles can be surface modification. This can be performed by means of GSH. For instance, Ag nanoparticles are very effective to monitor and photodynamic therapies of tumors. On the other hand, they are not biocompatible and show high toxicity for the cells. Wu et al. (2008) solved this problem by surface coating of nanoparticles with GSH [134]. K562 human leukemic cells shows high inhibition of cell proliferation and high biocompatibility. To examine cytotoxicity of water dispersible CuO nanoparticles (14 nm) on MCF7 cells, Kumaran et al. (2014) measured the expression level of GST gene [135]. They concluded that the particle cytotoxicity was not significant since there were no significant change in GST gene expression. The particles can be used for diagnosis and therapeutic purposes.

GST activity was also investigated for carbon based nanoparticles. Iwata et al. in 1998 found 16 % inhibition in GST activity for C_{60} nanoparticles in rodent and human liver cells [136]. In 2006, Wang et al. studied the effect of gadolinium endohedral metallofullerend nanoparticles on GST activity in hepatoma cell (H22) in mice [137]. High GST activity is the indication of the fight for the removal of ROS from the cell. In this study, the decrease in the GST activity with the application of these nanoparticles was examined. It shows the helping effect of the nanoparticles to get rid of ROS. $C_{60}(OH)_{20}$ which suppresses carcinoma metastasis was also analyzed with respect to GST activity in the research paper of Jiao et al. (2010) [138]. They used breast cancer metastasis model and they observed decrease in the GST activity in $C_{60}(OH)_{20}$ treated mice with respect to untreated mice. Carbon nanotubes such as carboxylic acid functionalized single wall carbon nanotubes (COOH-SWCNT) are also very popular for the reduction of cancerous cell viability yet Pichardo et al. in 2012 could not see GST activity difference on the human intestinal cell line Caco-2 after 24 h [139]. However, GSH disappeared after 24 h application of the carbon nanotubes that is the indication of high production of ROS.

For biomedical applications like imaging, diagnosis, and targeting, quantum dots (QDs) have become very popular. They are made up of a cap and a metalloid crystalline core that triggers ROS formation. For this reason, the investigation of their effect on GST activity is very crucial. In a study carried out by Zhao et al. in 2010, the effect of QDs on L02 cells toxicity developed by Cu^{2+} [140]. It was proved that QDs helped the induction of GST activity by 22%. Another study by Nguyen et al. in 2013 is related with the toxicity effect of cadmium telluride quantum dots (CdTe-QDs) on Hep62 cells [141]. The inhibition action of CdTe-QDs on GST activity was proved through monitoring 1.95 fold decrease in the GST activity. There are studies about GST activity on silica based DDSs also. Munteanu et al. (2010) examined the antioxidative response of silica nanoparticles in MRC-5 cells [142]. It was found out that, GST activity increased as time passes in order to eliminate oxidative stress caused by these particles. The biocompatibility of highly pure biogenic silica nanoparticles was tested on human lung fibroblast cells (hLFCs) by Alshatwi et al. in 2015 [143]. Even though these particles increased GST activity and ROS production, they still showed more biocompatibility than synthetic silica nanoparticles. Selenium nanoparticles can be used for cancer prevention since they increase GST activity that resulted in high detoxification of carcinogens. However, the particle size is very important factor for this mechanism which is analyzed by Peng et al. in 2007 [144]. For the particles with 36 nm sizes, better GST activity was obtained than for the particles with 90 nm sizes. For metastasis, selenium nanoparticles can be a good solution. Wang et al. in 2014 applied elemental Se nanoparticles on H22 hepatocarcinoma cell [145]. They concluded that Se nanoparticles can be more effective to increase GST activity by comparing sodium selenite nanoparticles. For poor water-soluble drugs and gene delivery, solid lipid nanoparticles (SLN) can be used as DDS. Unfortunately, SLN can cause high cellular damage. This fact was studied by Doktorovova et al. in 2014 on HepG2 cells [146]. There was no difference in GST activity between control groups and SLN applied cells. Therefore, there is no any drawback to apply these particles on the body. GST can also be used to detect the presence of biological molecules such as thrombin, to carry drugs, bioactive conjugates, or proteins, and to trigger immune response of the body in the form of fusion protein [147, 148, 149]. Moreover, GST can be used as prodrug for the

GST overexpressing cancer cells like in the study of Maciag et al. in 2015 [150]. The prodrug releases its drug with the presence of GST. As a result, targeting delivery can be accomplished.

1.4 The Aim of This Study

Chemotherapy is a very risky method for cancer treatment since it involves highly toxic chemicals that can lead to patient death. Nevertheless, it is the most common method. In order to solve toxicity problem, DDSs are very efficient way because the cure can be achieved with smaller doses of chemotherapeutic agents by diminishing uncomfortable side effects. In addition to its side effects, the resistance is another problem for the application of chemotherapeutical agents. Recently, several efforts have been put on the development of DDSs to overcome this problem as stated in literature research.

The aim of this study is to develop a polymeric drug delivery system loaded with doxorubicin for cancer treatment. As a result, the systemic side effects of doxorubicin can be eliminated and more efficient chemotherapeutic effect can be obtained. First, polysebacic anhydride and poly (sebacic anhydride)-poly (ethylene glycol) copolymer with three different M_w of PEG (1000, 6000, and 14000 g/mol) were synthesized. The synthesized polymers were characterized by different polymer characterization techniques like GPC, FTIR, and ^{1}H-NMR. Then, the nanocapsules loaded with doxorubicin were produced from these polymers. The size and size distribution of the nanocapsules were characterized by DLS and SEM. Moreover, the drug release behavior and loading efficiency of these nanocapsules were studied.

GST activity is very important for the success of drug delivery system on cancer cells as explained before. In literature, there are many studies about the effect of drug delivery systems on GST activity. The inhibition of GST activity was observed for some kind of polymeric systems by the effect of the degradation product. However, there is no study on the effect of poly (sebacic anhydride) for GST activity both in the cytotoxic and resistance manners. In this study different from the literature, the effect of nanocapsules made of

PSA and PSA-PEG copolymer on GST activity was studied. For this purpose, GST was isolated from beef liver. Then, the enzyme was mixed with nanocapsules at pH 6.5. The change in total GST activity was measured against the substrate, 1-chloro-2, 4-dinitro-benzene (CDNB), by monitoring the thioether (GSH-CDNB conjugate) formation at 340 nm. As a result, the effect of PSA on GST activity was determined.

2. THE EXPERIMENTAL PART

2.1 Materials

The materials used for the experiments were listed below:

Table 2.1: The Materials used for the Experiments

Chemicals	**Company**
Poly (sebacic acid)	Fluka (Gillinham-UK)
Poly (ethylene glycol) (Mw= 1000 Da)	Merck (Schuchard, München)
Poly (ethylene glycol) (Mw= 6000 Da)	Fluka (Switzerland)
Poly (ethylene glycol) (Mw= 14000 Da)	Aldrich (USA)
Acetic anhydride	Sigma Aldrich (Gillinham-UK)
Ethyl ether	Sigma Aldrich (Gillinham-UK)
Petroleum ether	Sigma Aldrich (Gillinham-UK)
Poly vinyl alcohol (13000-23000)	Sigma Aldrich (Gillinham-UK)
Adrimisin	(Istanbul- Turkey)
Hydrochloric acid	R&D Systems (CA-USA)

Table 2.1 (continued)

Chemicals	**Company**
Sodium hydroxide	J.T. Baker (Holland, Netherland)
Dichloromethane	Sigma Aldrich (Gillinham-UK)
Chloroform	Sigma Aldrich (Gillinham-UK).
1-chloro-2, 4-dinitro-benzene (CDNB)	Fluka (India)
Ethyl alcohol	Merck (USA)
Potassium chloride	Merck (USA)
Sodium chloride	Merck (USA)
Dimethyl sulfoxide (DMSO)	Riedel-de Haen (Germany)
Potassium mono phosphate	Riedel-de Haen (Germany)
Potassium di phosphate	Riedel-de Haen (Germany)
Glutathione	Fluka (Switzerland)

2.2 Polymer Synthesis

In the literature, there are several examples for the synthesis of PSA and PSA-PEG copolymers [75, 151]. For this study, the modified methods based on the published ones were used. The monomers used in the synthesis of PSA polymer and PSA-PEG copolymers (which were prepared by using different molecular weight PEGs) are given in Table 2.2 and the methods for the synthesis are explained in the following sections.

Table 2.2: The Composition of Polymers and Copolymers

Groups	**Monomers**
PSA	Sebacic acid
PSA-PEG1000	50:50 Sebacic acid + PEG (Mw=1000 Da)
PSA-PEG6000	50:50 Sebacic acid + PEG (Mw=6000 Da)
PSA-PEG14000	50:50 Sebacic acid + PEG (Mw=14000 Da)

2.2.1 PSA Prepolymer Synthesis

10 g sebacic acid and 100 mL acetic anhydride were added into three-neck flask. After the temperature increased to 140°C in oil bath, this mixture was refluxed under dry nitrogen for 30 min. The produced acetic acid and unreacted acetic anhydride were removed by means of rotary evaporation at 50°C. Then, the remaining part was dissolved in 40 mL chloroform. The crude prepolymer was separated from unreacted sebacic acid by adding the chloroform solution into 400 mL and 1:1 mixture of dry petroleum ether and diethyl ether. The prepolymer precipitation was gathered by filtration and dried in vacuum desiccator.

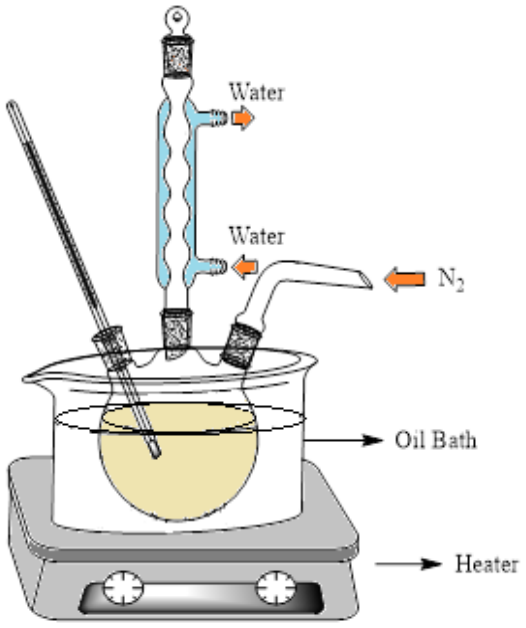


Figure 2.1: The experimental Setup for Prepolymer Synthesis

2.2.2 PSA Polymer Synthesis

For the production of PSA, condensation polymerization method was conducted by curing 6 g prepolymer at 180°C for 90 min. The cured polymer was purified by mixing with 80 mL dichloromethane. After that, the mixture was precipitated again in 150 mL dry ethyl ether and dried under vacuum. The dried polymer was desiccated under dry N_2 and stored in -20°C to prevent the polymer degradation.

2.2.3 PSA-PEG Prepolymer Synthesis

6 g sebacic acid, 6 g PEG-1000, and 60 mL acetic anhydride were added into three-neck flask. After the temperature increased to 140°C, this mixture was refluxed under dry nitrogen for 30 min by rigorous mixing. The produced acetic acid and unreacted acetic anhydride were removed by means of rotary evaporation at 50°C. Then, the remaining part was dissolved in 50 mL chloroform. The crude prepolymer was separated from unreacted sebacic acid and PEG by adding the chloroform solution into 400 mL and 1:1 mixture of dry petroleum ether and diethyl ether. The precipitation was gathered by filtration method and dried in vacuum desiccator. For the synthesis of PSA-PEG-6000 and PSA-PEG-14000 prepolymers, the same procedure was applied.

2.2.4 PSA-PEG Copolymer Synthesis

For the production of PSA-PEG-1000 copolymer, condensation polymerization method was conducted by curing 6 g prepolymer at 180°C for 90 min like the production of PSA polymer. The cured copolymer was purified by mixing with 80 mL dichloromethane. After that, the mixture was precipitated in 150 mL dry ethyl ether again and dried under vacuum. The dried copolymer was desiccated under dry N_2 and stored in -20°C to prevent the polymer degradation. For the synthesis of PSA-PEG-6000 and PSA-PEG-14000 copolymers, the same procedure was applied.

2.2.5 Polymer Characterization

The chemical structures of synthesized polymer and copolymers were characterized by ^{1}H-NMR (Bruker, Bremen, Germany) by dissolving them in the deuterium chloroform. The analysis of polymer and copolymer structure formation was detected through FTIR

(PerkinElmer, MO, USA). Molecular weights of synthesized polymer and copolymers were measured by gel permeation chromatography (GPC) (PL-GPC 220, CA, USA) with the experimental conditions of 30°C in tetrahydrofuran with flow rate of 1 mL/min and polystyrene was used as standard.

2.3 Nanocapsule Preparation

The nanocapsule preparation method was adopted from Ashjari et al. in 2012 [152]. The double emulsion method and solvent evaporation method were applied for the preparation which is showed in figure 2.2.

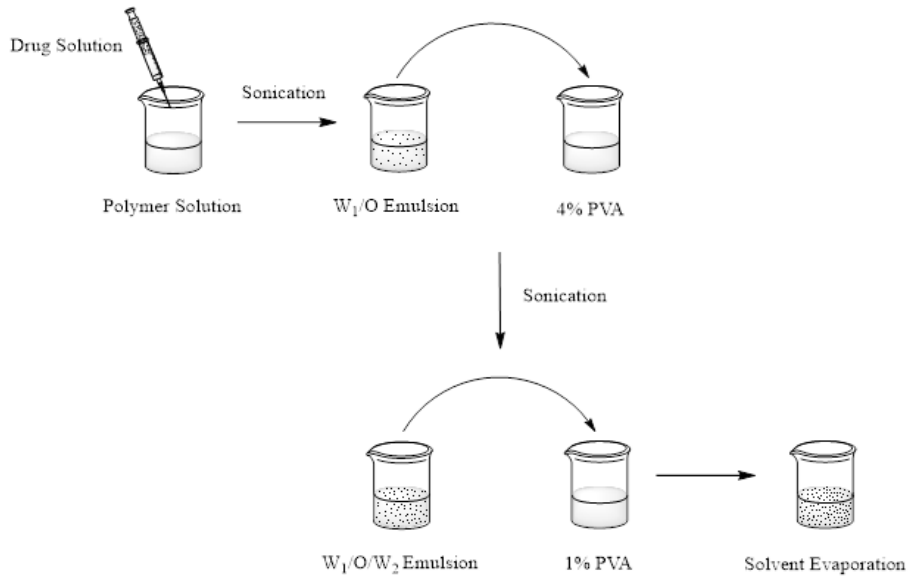


Figure 2.2: Nanocapsule Preparation by Double Emulsion Method

50 mg polymer was dissolved in 4.5 mL DCM in order to prepare the organic phase. Then, 2.5 mL of DOX (2 mg/mL) or distilled water (to synthesize nanocapsules without drug) was added to the solution as water phase. The organic phase was emulsified in the water phase by means of sonicator probe at an output 60 W for 15 s. As a result, W1/O phase was obtained. After that, the emulsion was poured into an aqueous 4 wt/vol% PVA solution (W2) immediately and the mixture was ultrasonicated again for 30 s. The resulted emulsion (W1/O/W2) was diluted in an aqueous 1wt/vol% PVA solution with mechanical stirring. The DCM was removed by solvent evaporation through 4 h at room temperature. The resulting nanocapsules was cleaned by repeating centrifugation at 14000 rpm and resuspending in distilled water for three times. After the third centrifugation, the supernatants were removed and the nanocapsules were dried by freeze-drying and stored at 4℃.

2.3.1 Characterization of Doxorubicin Loaded Nanocapsules

The yields of NCs preparation were calculated by dividing the mass of NCs produced into the mass of polymer used. The morphology of NCs was monitored through scanning electron microscopy (SEM, QUANTA 400, Oregon, USA). The size and size distributions of NCs were analyzed by a particle size analyzer (Malvern Mastersizer,Worcestershire, UK).

2.3.2 Drug Loading Capacity and Encapsulation Efficiency Calculation

Drug loading capacity of NCs was calculated by:

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

in which;

LC: Loading Capacity, $W_{(DOX)}$: Amount of DOX in NCs, and $W_{(NC)}$: Mass of NCs

Encapsulation efficiency was obtained by:

$EE\% = [W_{(DOX)}/W_{(I)}] \times 100$

in which;

EE%: Encapsulation efficiency, $W_{(DOX)}$: The Amount of DOX in NCs, and $W_{(I)}$: The Initial Amount of DOX

The absorbances coming from the DOX content inside the nanocapsules were measured at 486 nm by means of UV spectrophotometer (Wallac Victor, Turku, Finland) and the DOX concentrations were calculated by means of calibration curve (Figure A.1) with R^2= 0.992 which was prepared from different concentration of DOX at pH 7.4.

2.3.3 DOX Release Profile of the Nanocapsules

The DOX release profile of the synthesized PSA and PSA-PEG (1000, 6000, and 14000) nanocapsules was examined in PBS (0.01 M) at pH 4 and 7.4 by dialysis technique and all the release experiments were conducted as duplicate. First of all, 4 mg nanocapsules of each group was resuspended in 0.5 mL double distilled water. Then, they were placed into seamless cellulose dialysis tubes (12400 MWCO) and dialyzed immediately against the 4.5 mL of PBS medium in 100 ± 2 rpm at 37 ± 0.5℃. The release study was conducted as triplicate). At predetermined time intervals (including time zero), 0.5 mL aliquots were removed from the medium and 0.5 mL fresh PBS was added. The absorbance of removed aliquots was measured at 486 nm through UV spectrophotometer. The amount of released DOX was calculated by using the DOX calibration curve that was prepared before. The graphics of the cumulative percentages of the released drug versus time were drawn. The

experiments were conducted as duplicate and for the drawing of the graph the average values of absorbance were taken.

2.4 GST Enzyme Assay of Nanoparticles

In the literature, many different types of nanoparticles were investigated with respect to GST enzyme activity. For this study, the effect of poly (sebacic anhydride) based nanocapsules on GST activity was analyzed.

2.4.1 Isolation of GST Enzyme from Bovine Liver

The livers from well bled bovine about 6- 12 months were obtained from Kazan slaughter house, Ankara. Connective and fatty tissues from sample weighing 25- 30 gr were removed. Then, whole liver was cut into small pieces with scissors and the pieces were washed with cold distilled water to remove excess blood. After that, the liver pieces were ground by using meat grinder. All the subsequent steps were carried out at 4°C in the presence of 10 mM (pH 7.0) potassium phosphate buffer which contains 1 mM EDTA (ethylenediaminetetraacetic acid), 1 mM DTT (ditiyotreitol), and 0.15 M KCl. The tissue sample was homogenized in this potassium phosphate buffer by means of Potter-Elvehjem homogenizer coupled with a motor (Black and Decker, V850, multi-speed drill)-driven Teflon pestle at 2400 rpm (5 to 10 passes). The obtained homogenate was centrifuged in 12000 g for 25 min at 4°C. After that, the collected supernatant was centrifuged in 134000 g for 50 min at 4°C. The supernatant containing GST enzyme was passed through cheesecloth to remove the floating lipid part and the remaining part was stored at -80°C.

2.4.2 GST Enzyme Activity Assay

GST enzyme activity assay was adopted from the study of Habig et al. in 1974 [153]. It was miniaturized for microplate application which was developed by Isgor et al. (2013) [82]. For this purpose, the stock solutions of PSA-DOX nanocapsules, PSA-PEG6000-DOX nanocapsules, and PEG6000 polymer were prepared. The 1 mg /mL stock solutions of these particles and the polymer were prepared through dissolving 2 mg nanocapsules in 2 mL DMSO and 2 mg PEG6000 in 2 mL dH_2O. Predetermined volumes from the stocks and 200 µL assay mixture, which is composed of 50 mM CDNB, 200 mM GSH, and 200 mM KP (potassium phosphate) Buffer (pH 6.5), were added into the wells (Figure 2.3). Moreover, 5 µL cytosol and KP buffer, which was adjusted in different volumes according to the volume of the drug solution (Table 2.3), were added, respectively. Then, the absorbance was read at 340 nm by Spectramax M2e, Multi-Mode Microplate Reader (Molecular Devices Corporation, Sunnyvale, CA, USA) for 288 sec. The GST enzyme assay was repeated many times as duplicate in predetermined time periods.

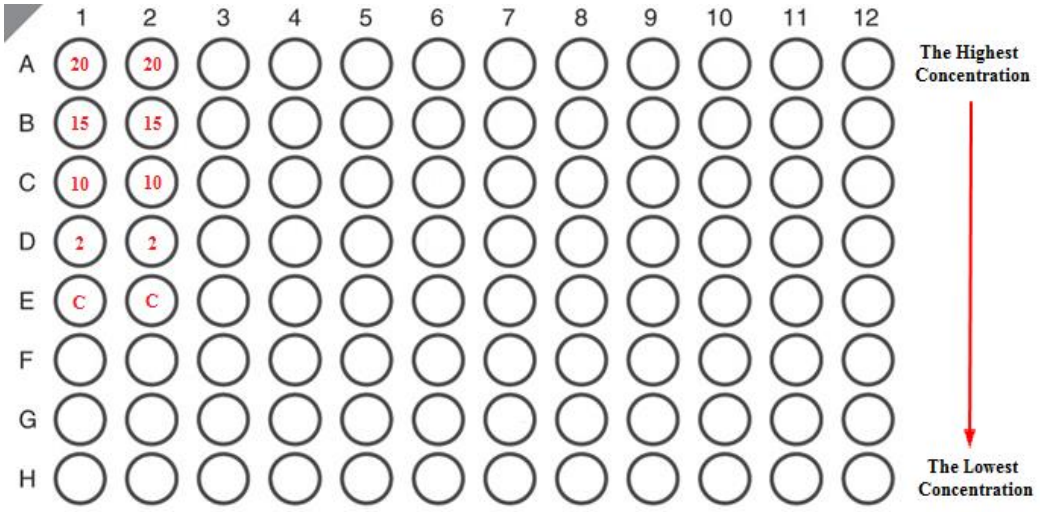


Figure 2.3: The Microplate Map for GST Enzyme Activity Assay

Table 2.3: The Volume of KP Buffer used According to the Volume of the Nanocapsule Solution

Drug (µL)	**KP Buffer 200 mM, pH 6.5 (µL)**
20	25
15	30
10	35
2	43
Control (5 µL DMSO)	40

The enzyme activity was calculated according to the formula depicted below:

EA (IU/mL)=(OD340/min)×(1/ Ɛ340)×(Dilution Factor)

Ɛ= Extinction Coefficient (for CDNB 5.3 $mM^{-1}.cm^{-1}$ for 96-well plate)

After that, the percent inhibition versus drug concentration graphs were drawn by means of GraphPad Prism (GraphPad Sofware Inc.). In addition, the stabilities of PSA-DOX and PSA-PEG6000-DOX nanoparticles were investigated by repeated experiments of their effect on the GST ezyme activity for predetermined time periods. Moreover, the effect of free DOX on the GST enzyme activity was analyzed.

3. RESULTS AND DISCUSSION

3.1 Polymer and Copolymer Characterization

PSA polymer and PSA-PEG copolymers were produced through melt condensation polymerization. First of all, prepolymers were synthesized; and then, the polymerization process was conducted. The success of this synthesis processes was proved by several polymer characterization methods like ^{1}H-NMR, FTIR, and GPC. For PSA polymer, ^{1}H-NMR result showed chemical shifts at δ= 1.3 (a), 1.6 (b), 2.2 (c), and 2.4 (d) ppm which are belong to the protons of the functional groups in PSA structure. Each shift was represented as a, b, c, and d indicated in figure 3.1 [75].

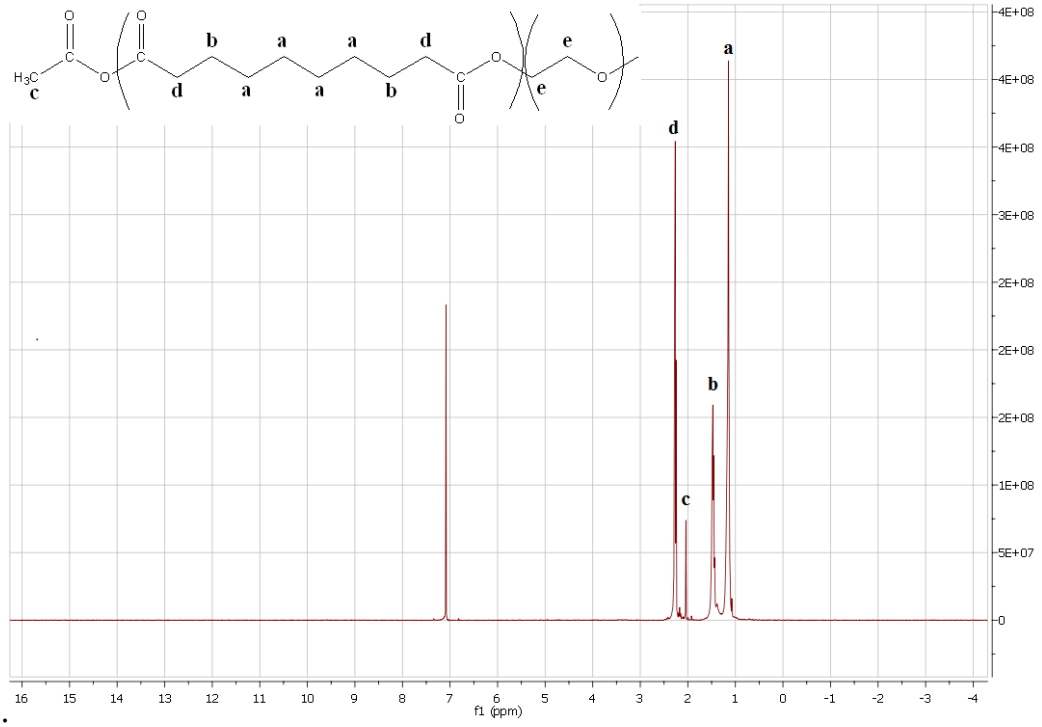


Figure 3.1: ^{1}H-NMR Result of PSA Polymer

With the introduction of PEG into the PSA polymer structure results in an additional chemical shift at δ= 3.6 ppm (e) which is the indication of the presence of PEG in the structure [75]. For three copolymer structures, the ^{1}H-NMR results are depicted in figure 3.2, 3.3, and 3.4.

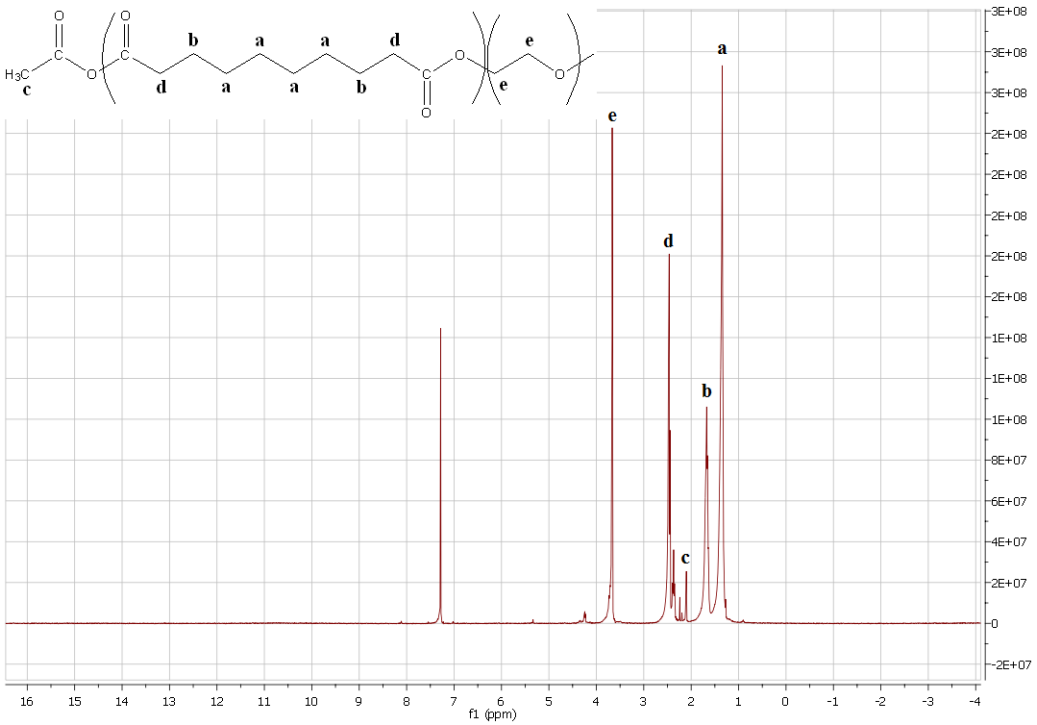


Figure 3.2: ^{1}H-NMR Result of PSA-PEG1000 Copolymer

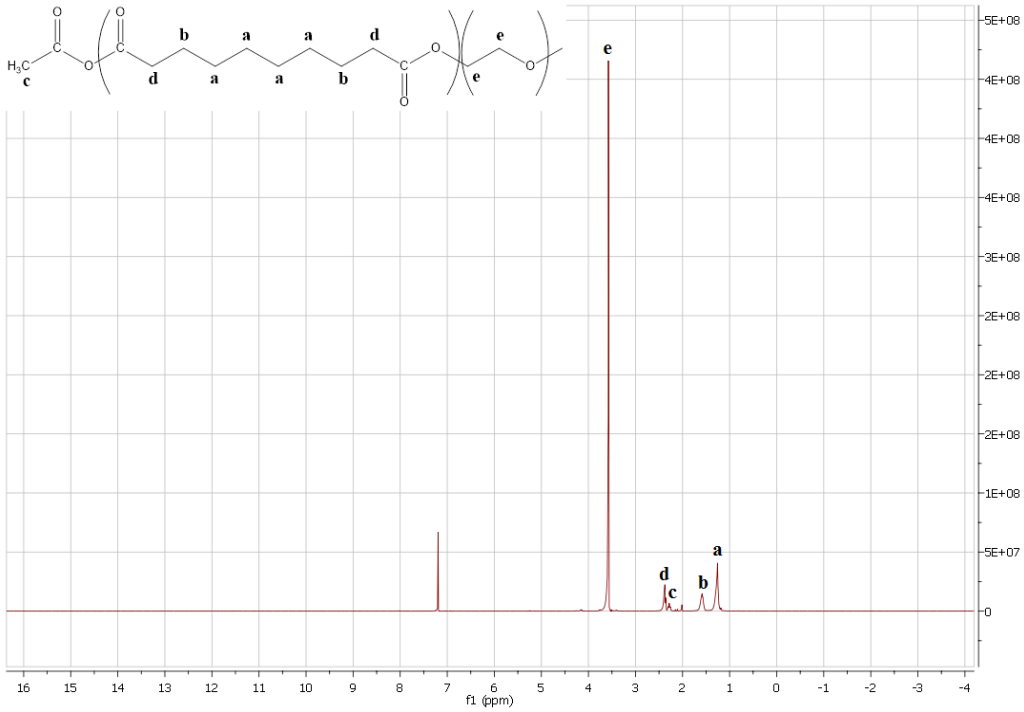


Figure 3.3: ^{1}H-NMR Result of PSA-PEG6000 Copolymer

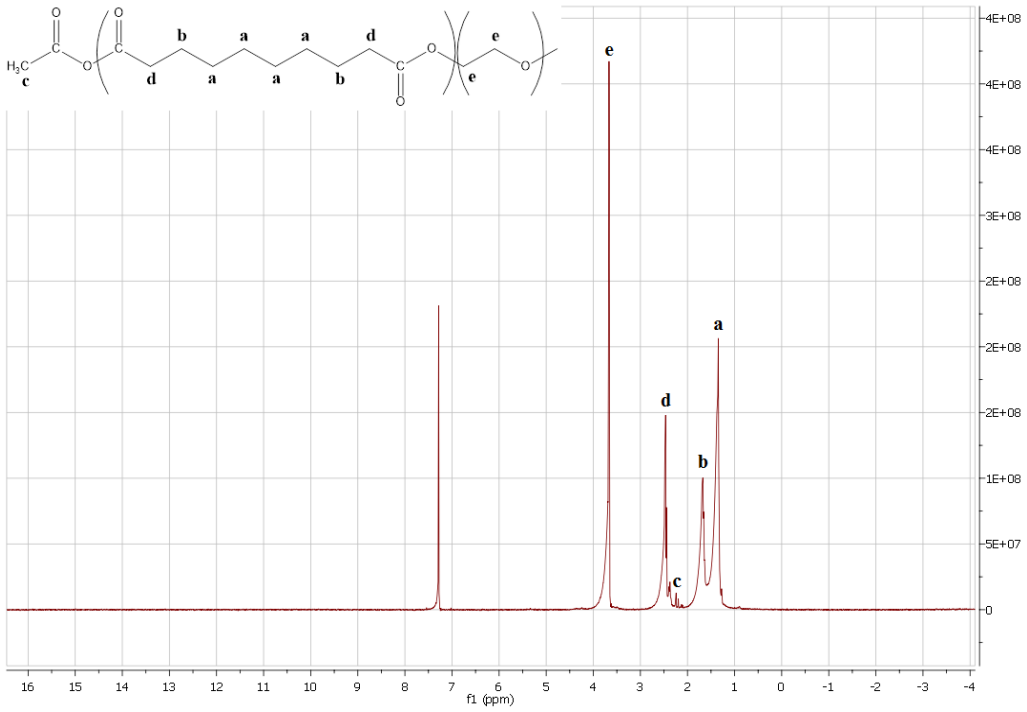


Figure 3.4: ^{1}H-NMR Result of PSA-PEG14000 Copolymer

All the peaks were seen properly for all polymeric substances. Therefore, the structures of polymer and copolymers are fitted with the expectation.

In addition to ^{1}H-NMR, FTIR analysis was conducted for the detection of polymer and copolymer chemical structures. The peaks at 1810 cm^{-1} and 1740 cm^{-1} refers to the asymmetric and symmetric stretching modes of carbonyl group (C=O) of PSA. In addition, the peaks at 1080 cm^{-1} and 1040 cm^{-1} depicts the asymmetric and symmetric stretching modes of C-O for anhydride groups. For the presence of ethylene glycol segments (C-O-C), peaks at 950 cm^{-1} and 1120 cm^{-1} are the indicators. All described peaks were observed properly in the structures and they are shown in figure 3.5, 3.6, and 3.7 below.

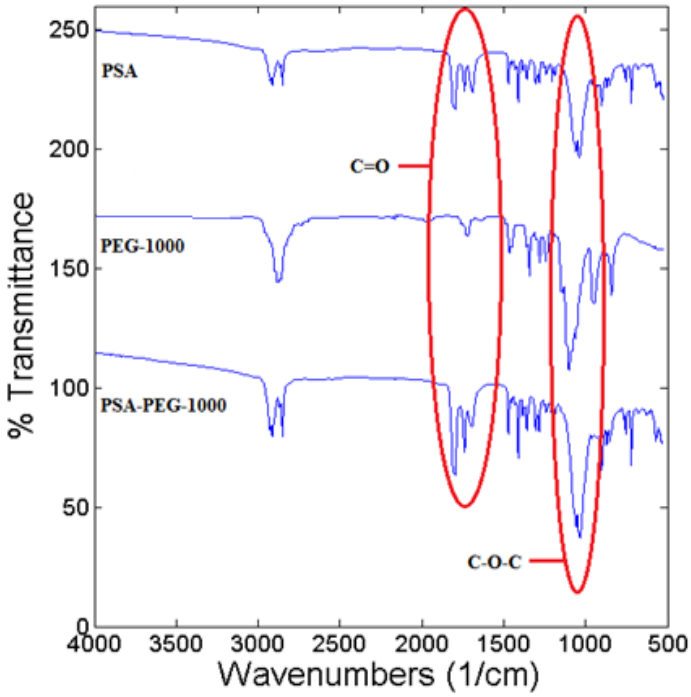


Figure 3.5: FTIR Analysis of PSA and PSA-PEG1000

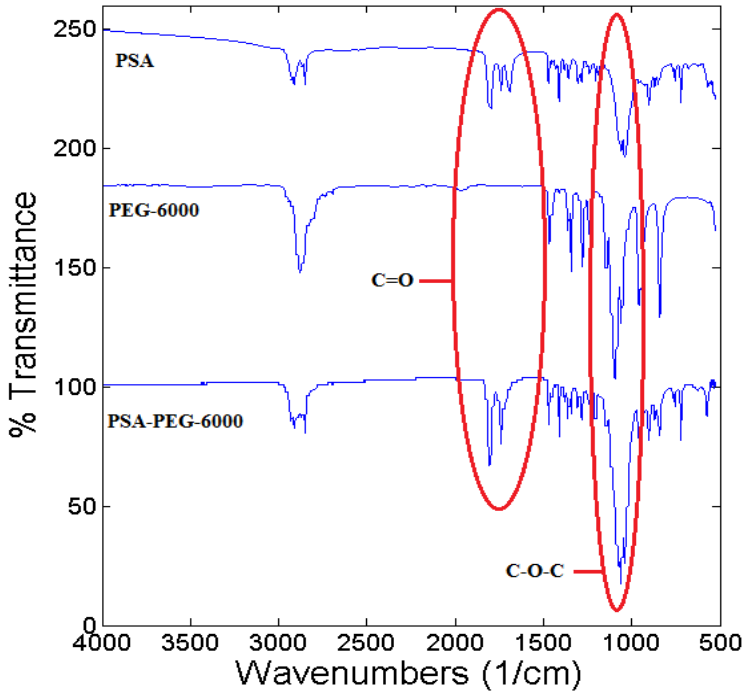


Figure 3.6: FTIR Analysis of PSA and PSA-PEG6000

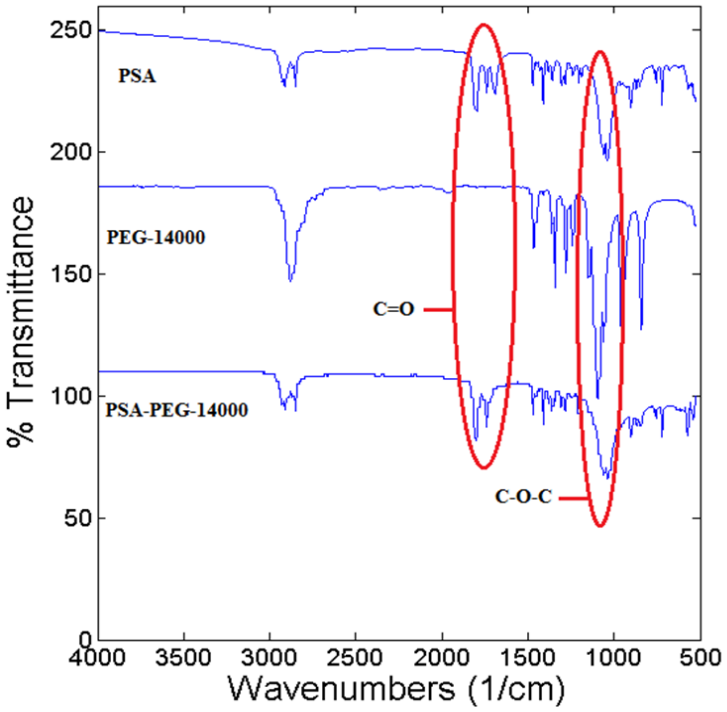


Figure 3.7: FTIR Analysis of PSA and PSA-PEG14000

In the light of ^{1}H-NMR and FTIR analysis, it can be concluded that the polymers and copolymers were synthesized successfully.

The average molecular weight and polydispersity index of the polymer and copolymers were measured by GPC analysis. The average molecular weights of PSA, PSA-PEG1000, PSA-PEG6000, and PSA-PEG14000 are 2600, 1356, 5202, and 12976 g/mol respectively. Although the same procedure was applied on the synthesis of the polymer and copolymers, different average molecular weights were observed. The reason is the different average molecular weights of PEG that introduced into the copolymer structure. The average

molecular weight of the copolymers was expected between the molecular weight of PSA and the molecular weight of PEG used during the synthesis. For PSA-PEG1000 and PSA-PEG6000 copolymers, the average molecular weights fit with the expectation. However, for PSA-PEG14000 the average molecular weight is less than the expectation which should be higher than 14000 g/mol. The reason may be that during drying process of the PSA-PEG14000 copolymer in the desiccator, the vacuum did not work properly. Thus, the copolymer interacted with the air resulted in a small amount of the degradation and decrease in the average molecular weight. The polydispersity index (PDI) of PSA, PSA-PEG1000, PSA-PEG6000, and PSA-PEG14000 are 1.27, 1.34, 1.70, and 1.78, respectively. PDI value shows the uniformity of the chain lengths in the polymer structure. The more uniform chain lengths give the value closer to 1. The PDI results for this study are close to 1 which are quite good. As molecular weight increases, the rise in the PDI was observed. The reason is the increase in the difference between chain lengths in the polymer and copolymer structure when the molecular weight becomes higher. Moreover, the yields of the groups are consisted with the literature. For PSA the yield is 75-88% [51], for PSA-PEG copolymer the average yield is 70% [60, 76] in the literature. For this study, the yield is approximately 70% that is close to the values found before. The GPC, PDI and yield results can be summarized in table 3.1.

Table 3.1: Yields, Molecular Weights, and PDI of the Synthesized Polymers and Copolymers

Groups	**Yield %**	$\overline{Mn}$ **(g/mol)**	**PDI**
PSA	79.1	2600	1.27
PSA-PEG1000	69.3	1356	1.34
PSA-PEG6000	76.6	5202	1.70
PSA-PEG14000	65.3	12976	1.78

3.2 Characterization of the Nanocapsules

The nanocapsules loaded with DOX were prepared by double emulsion and solvent evaporation method. Since these particles designed for passive targeting, the particle size is very important for the EPR effect. The nanoparticles with the size of between 100 and 300 nm is proper for the medicinal application [154]. To understand the suitability of the synthesized nanocapsules for the drug delivery application, the size distribution, loading efficiency, yield, and zeta potentials were examined (Table 3.2).

Table 3.2: The Nanocapsule Characterization Results

Groups	Average Size of Nanocapsules (nm)	Loading Capacity (%)	Encapsulation Efficiency (%)	Yield (%)	Zeta Potential (mV)
PSA-DOX	256.6	31.4	78.5	65	-15.6
PSA-PEG1000-DOX	120.8	25.28	63.2	57	-13.1
PSA-PEG6000-DOX	136.6	28.76	71.9	63	-14.6
PSA-PEG14000-DOX	160.8	29.28	73.2	51	-13.6

The average particle size for all groups is between 100nm and 200 nm which is proper for the passive targeting application. The size distributions are consistent with this expectation. The particle size analysis was conducted as duplicate and for the description of the data nonlinear regression analysis were conducted. As the molecular weight of copolymers increases, the nanoparticles size also increases. For PSA, the biggest nanoparticles size observed although the average molecular weight of PSA is not the biggest. The reason can be the seasonal difference (temperature difference) between nanoparticle productions since PSA nanocapsules were produced in winter while the nanocapsules made of the copolymers were prepared in summer. The graphical depictions of the size distributions are shown in figure 3.8, 3.9, 3.10, and 3.11.

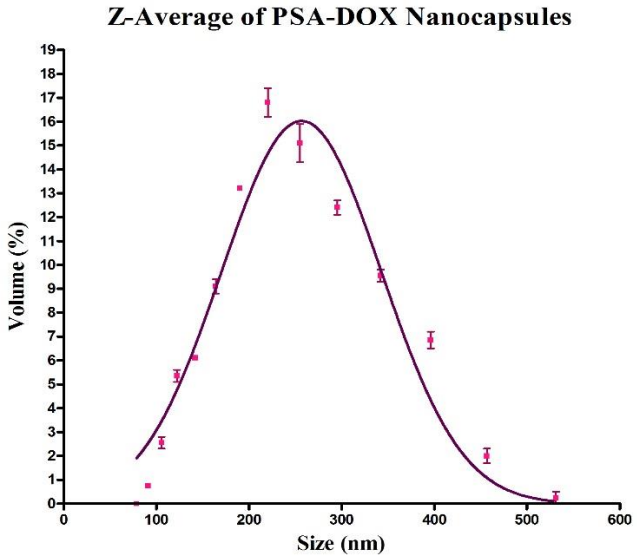


Figure 3.8: Z- Average of PSA-DOX Nanocapsules

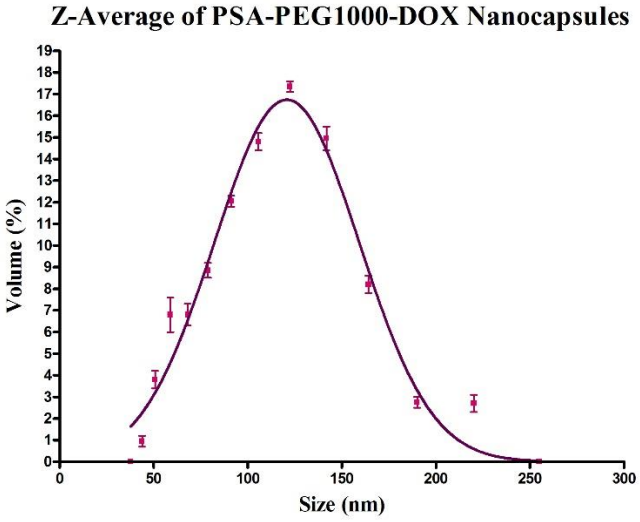


Figure 3.9: Z- Average of PSA-PEG1000-DOX Nanocapsules

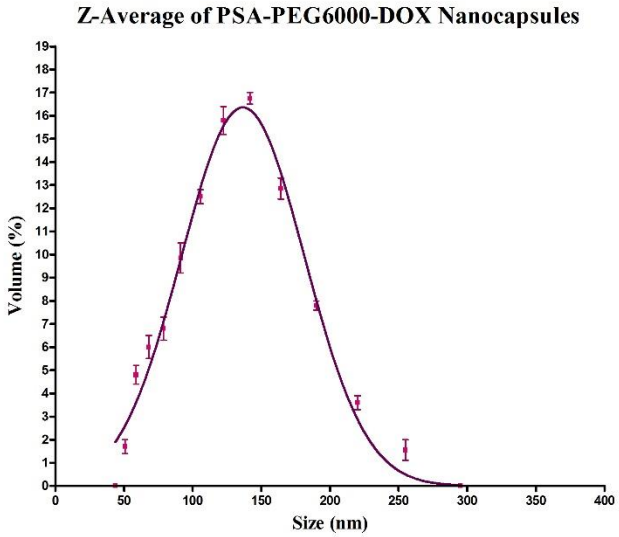


Figure 3.10: Z- Average of PSA-PEG6000-DOX Nanocapsules

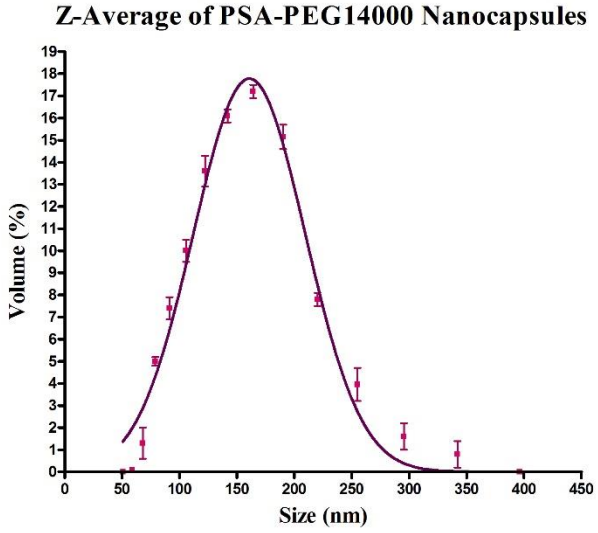


Figure 3.11: Z- Average of PSA-PEG14000-DOX Nanocapsules

Loading capacity and encapsulation efficiency are important parameters for drug delivery applications. Only with the high LC and EE, the advantage can be created over conventional drug application [155]. In the literature, loading capacity and encapsulation efficiency for PSA and PSA-PEG based nanocapsules are around 70% and 80%, respectively [72, 156]. The LC and EE results for the nanocapsules in this study indicates around 30% LC and 70% EE. LC is a little low by comparing the literature. The capacity can be increased by changing the surfactant type or some experimental conditions. On the other hand, EE seems consistent with the literature. It can be concluded that the nanocapsules are proper for the drug delivery application. Moreover, with respect to the yield, all types of nanoparticles showed close yield values around 60%. It can be concluded that the differences can be the result of experimental errors.

Zeta potential analysis was conducted to determine surface charge of the nanocapsules. The magnitude of the zeta potential shows the colloidal stability. The value should be greater than +25 mV or less than -25 mV [157]. As a result, the nanoparticles can show high stability. The zeta potential results of nanocapsules showed that they are quite stable which means less prone to agglomeration. In addition, the negative zeta potentials are an important advantage for the prevention of opsonization process.

Furthermore, nanocapsule morphologies were examined through SEM and the results are depicted in figure 3.12, 3.13, 3.14, and 3.15.

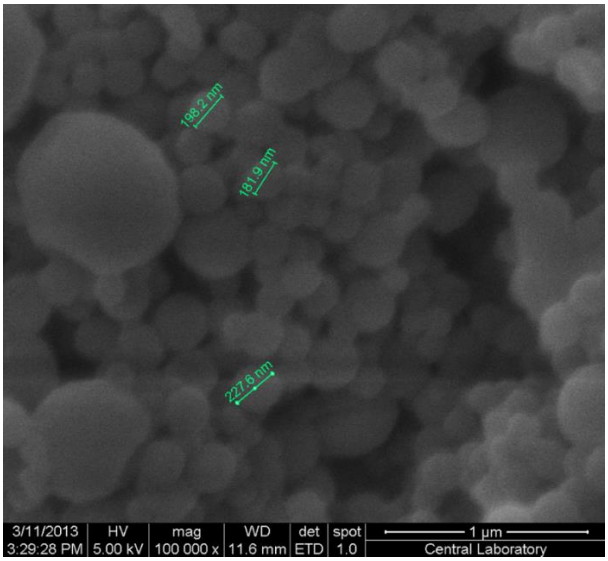


Figure 3.12: SEM Image of PSA-DOX Nanocapsules

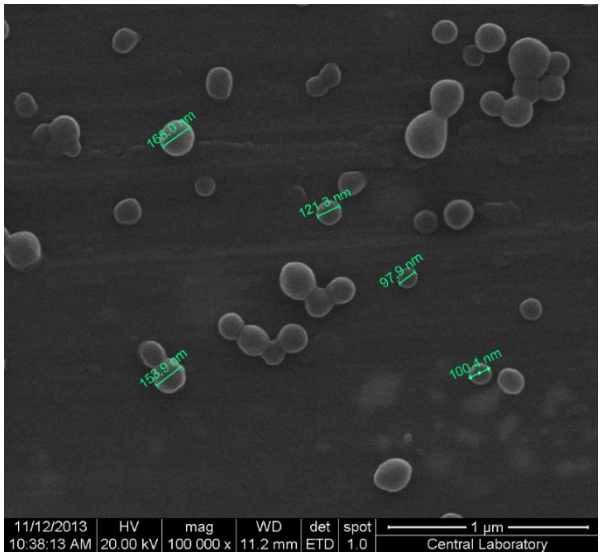


Figure 3.13: SEM Image of PSA-PEG1000-DOX Nanocapsules

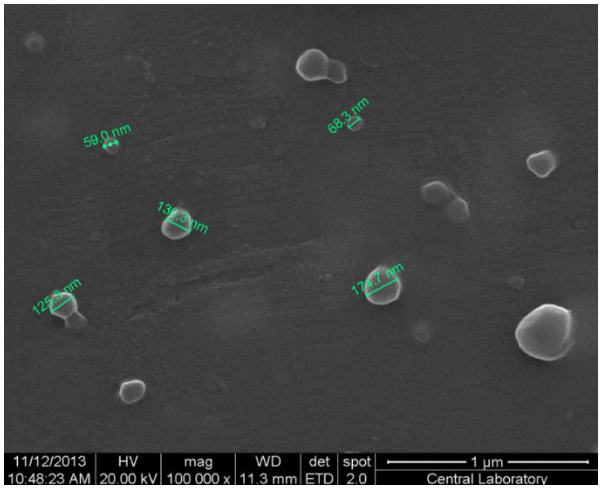


Figure 3.14: SEM Image of PSA-PEG6000-DOX Nanocapsules

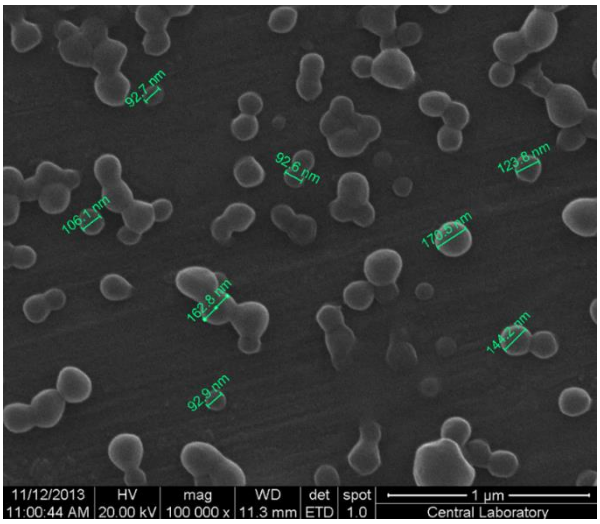


Figure 3.15: SEM Image of PSA-PEG14000-DOX Nanocapsules

The synthesized nanocapsules have porous surface since PSA is a crystalline polymer and solvent evaporation method also causes this porosity [158]. The nanocapsules represents smooth spherical shapes which is the indication of a successful nanocapsule preparation process. However, some capsule structures lost its spherical shape during SEM analysis. The reason is the degradation of PSA because of the focusing process of SEM since PSA is a type of heat sensitive polymer.

3.3 Release Profiles of Nanocapsules

Controlled release is the release of the drug in desired period of time with or without the effect of stimuli. Therefore, dose frequency can be adjusted through controlled release systems. For polymeric nanoparticles the release rate can be affected by particle size, morphology, and shape of the nanoparticles in addition to characteristic of the polymer [40]. For this study, the release experiments were highly reproducible; thus, the averages of the absorbance data were calculated to draw the graphs. In figure 3.16 and 3.17, the DOX release data that belong to different nanocapsule groups at different pH showed biphasic drug release profile; burst and sustained release. In the burst release phase, there is high increasing rate of drug concentration with respect to time. After 6 hours, the release profile turns into the sustain release profile where there is no fluctuation in the drug concentration throughout the time.

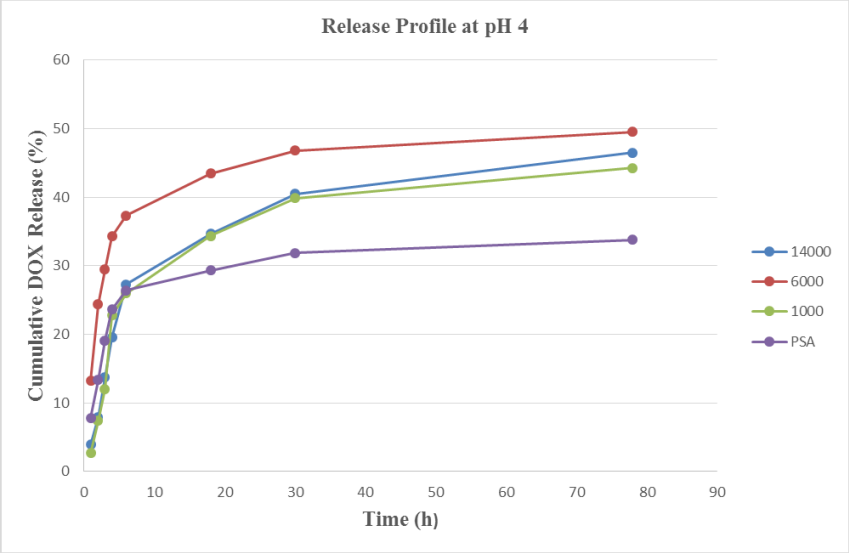


Figure 3.16: The DOX Release Profile at pH 4

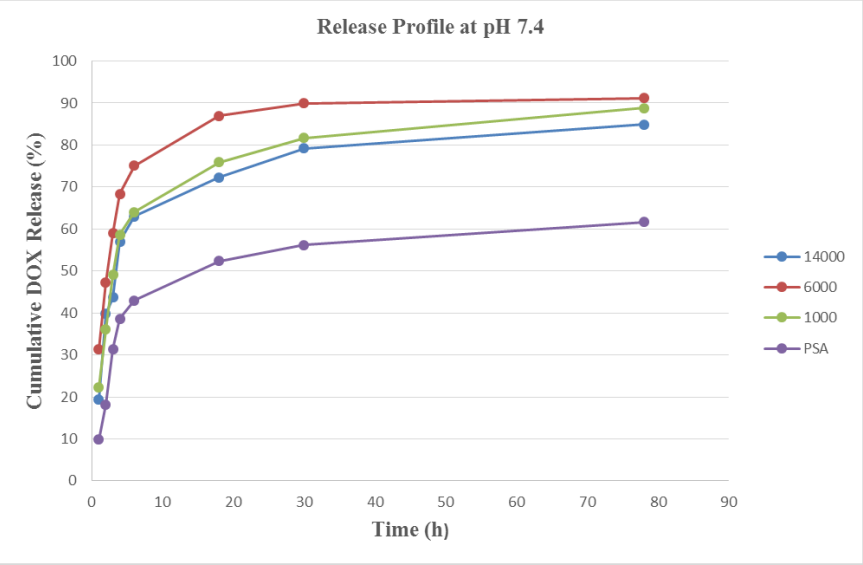


Figure 3.17: The DOX Release Profile at pH 7.4

For pH 7.4, faster burst release at the beginning for PSA-PEG based nanocapsules can result from the phase separation of PEG segments in the nanocapsule structure [159,160]. PEG is a hydrophilic polymer and PSA is a hydrophopic polymer. Therefore, PEG degrades faster than PSA in the hydrophilic environment like PBS. In addition, the burst release effects can be the result of the localization of PEG at the outer part of the capsule structure. As a result, PEG was degraded first which caused the release of some drug content. After that, the PSA core part started to degrade and the release profile turned into sustained release. Moreover, higher release rate can be seen for PSA-PEG nanoparticles than PSA nanoparticles. This is also caused by the hydrophilicty of PEG in the copolymer structure. By introducing PEG into copolymer structure, the hydrophilicity of the structure increases. As a result, the degradation rate and drug release rate rises. The molecular weight of the copolymers also affects the release rate. The highest release rate was observed for PSA-PEG6000 nanocapsules. For PSA-PEG1000 nanocapsules, less release rate was observed by comparing the release rate of PSA-PEG6000 nanocapsules. This can be due to less PEG content; so that, less water solubility. For PSA-PEG14000 nanocapsules, again less release rate was observed than for PSA-PEG6000 but higher release rate was seen than PSA-PEG1000. The difference can come from higher PEG content in PSA-PEG14000 than in PSA-PEG1000. For the comparison of PSA-PEG14000 nanocapsules with PSA-PEG6000 nanocapsules, the molecular weight is much higher for PSA-PEG14000 copolymer. Thus, degradation process of PSA-PEG14000 takes longer time that resulted in slower release rate. Furthermore, slower drug release rate was observed at pH 4 than at pH 7.4 for all the nanocapsule groups. For example, for PSA after 80 h, 30% DOX release was observed at pH 4 while 60% release was obtained at pH 7.4. The reason is the slower degradation rate of PSA at lower pH [161]. In conclusion, DOX release analysis for the nanocapsules showed a successful sustain release profile which is very important for the effective treatment and disappearance of the side effects of DOX.

3.4 GST Enzyme Activity Assay

High GST activity can result in an unsuccessful chemotherapy application for cancer. For this reason, drug delivery systems can be used to overcome this problem such as inhibition of GST activity by means of P85 DDS [104]. To understand the interaction between PSA-PEG6000-DOX and GST activity, GST enzyme activity assay was conducted as triplicate. Predetermined nanocapsule volumes were added into the assay mixture and the absorbance was read at 340 nm. Then, enzyme activity was calculated and percent inhibition was drawn against log concentration (figure 3.18). For PSA-PEG6000-DOX nanocapsules, approximately 60% inhibition in GST activity was observed.

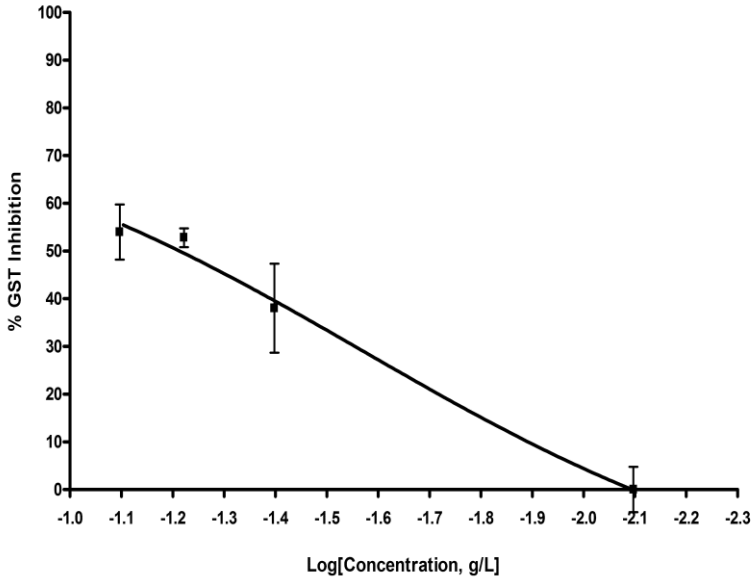


Figure 3.18: GST Inhibition Profile for PSA-PEG6000-DOX Nanocapsules

Since nanoparticles are loaded with DOX and DOX can have inhibitory effect on GST enzyme activity, the effect of free DOX on the enzyme was analyzed in order to understand whether the inhibition effect comes from polymer structure or DOX. The result for free DOX is depicted in the figure 3.19.

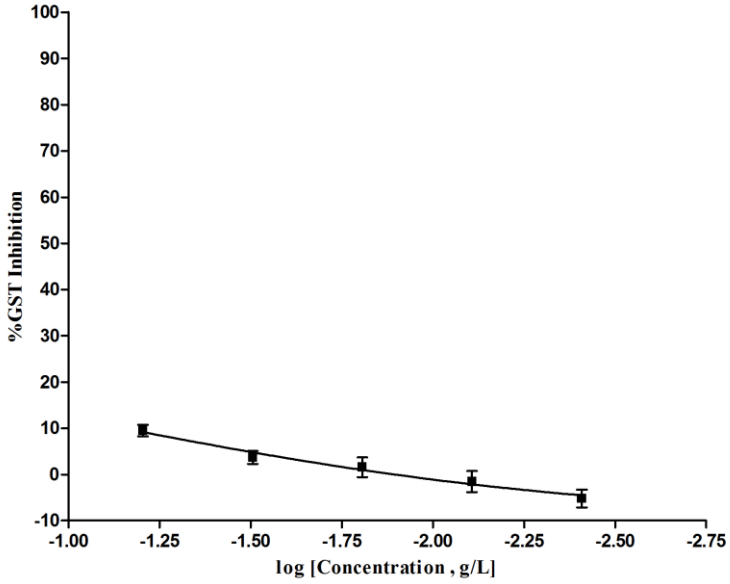


Figure 3.19 GST Inhibition Profile for Free DOX

There is a significant difference between the inhibitory effect of PSA-PEG6000 –DOX nanocapsules and free DOX on GST activity. For example, at the same DOX concentration (0.060 g/L), PSA-PEG6000-DOX nanocapsules shows approximately 60% inhibition whereas free DOX represents only 10 % inhibition. This result is meaningful

since DOX can show inhibitory effect on GST after its conversion into semiquinone form. Due to the absence of the special enzymes in the assay mixture that converts DOX into its semiquinone form, there was no ROS formation; so that, no GST inhibition. Finally, it was concluded that for free DOX only nonenzymatic inhibition was observed since less than 30% of the inhibition is not considered as a significant inhibition for enzyme assays [162]. At this point, the main concern becomes that which polymer in the nanocapsule structure shows this 60% inhibitory effect. To investigate this phenomena, the effect of PEG6000 on the GST enzyme activity was tested and the result is shown in figure 3.20.

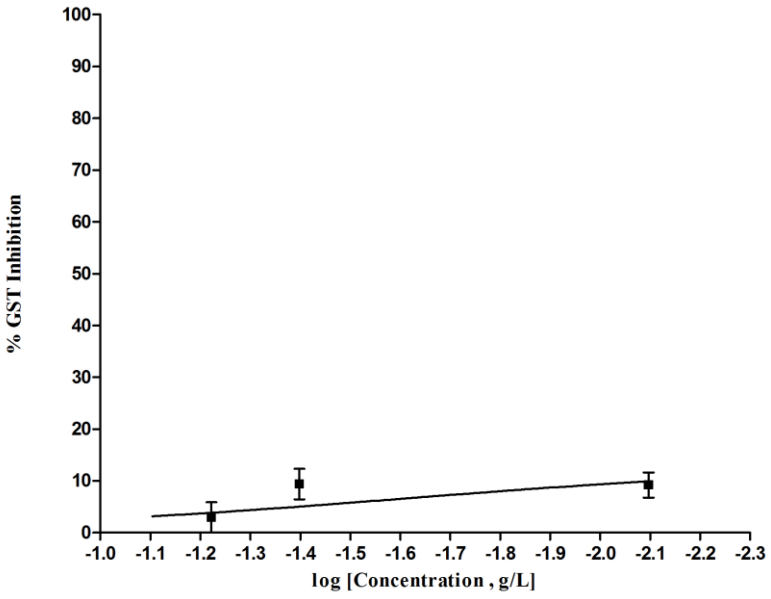


Figure 3.20: GST Inhibition Profile for PEG6000

The result for PEG6000 shows that there is no significant inhibition for GST enzyme. In figure 3.20, the maximum inhibition is 10% and it can be concluded that PEG6000 has no inhibitory effect on GST enzyme. Moreover, the result proves that PSA is the cause of 60% GST inhibition. The reason for the inhibition effect may be the steric effect like steric hindrance and steric shielding of the large polymer chains on the enzyme active site.

For the GST enzyme activity assay, stock solutions from PSA-DOX and PSA-PEG6000-DOX nanocapsules were prepared as stated in methods part. The nanocapsules were totally dissolved in DMSO and the polymer structure started to degrade. As time passes, the polymer degradation should continue in the stock solution. Therefore, PSA should lose its inhibitory activity since the big polymeric chains that hide the enzyme active site becomes smaller. This was proved by using the same stock solution for GST activity assay at different time periods for the detection of inhibition effect. The results coming from 15 days long evaluation of GST inhibition for PSA-PEG6000-DOX nanocapsules are given in figure 3.21 to 3.23.

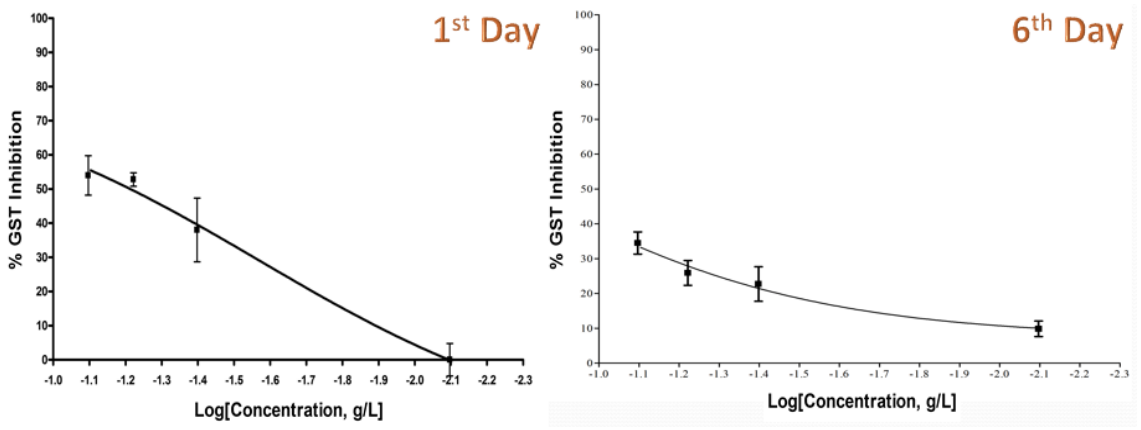


Figure 3.21: GST Inhibition Profile for PSA-PEG6000-DOX Nanocapsules at 1st and 6th Days of Evaluation

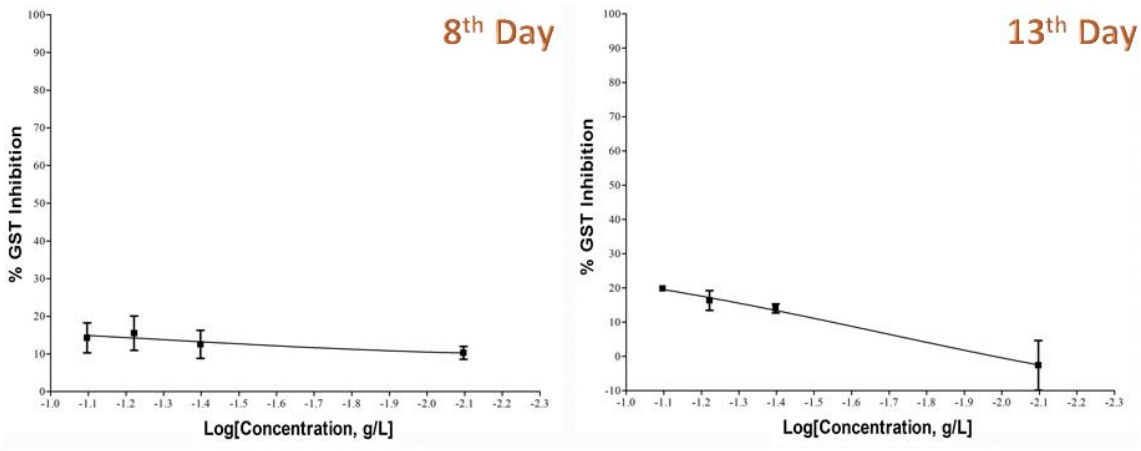


Figure 3.22: GST Inhibition Profile for PSA-PEG6000-DOX Nanocapsules at 8th and 13th Days of Evaluation

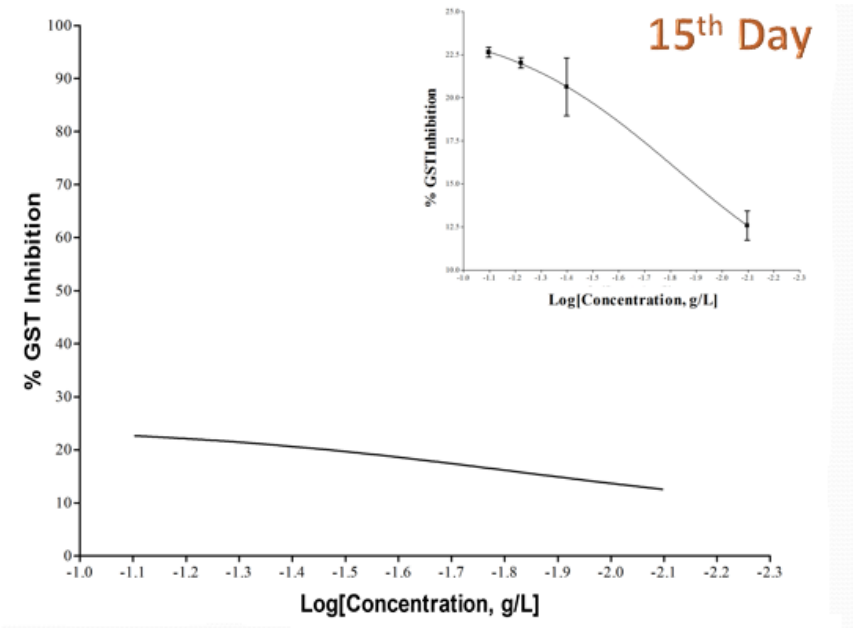


Figure 3.23: GST Inhibition Profile for PSA-PEG6000-DOX Nanocapsules at 15th Day of Evaluation

Although there is a little decrease in the inhibition effect of the PSA-PEG6000-DOX nanocapsules after 6 days, the inhibition on the GST activity decreases approximately 40% after 8 days. It can be concluded that this decrease results from PSA degradation. After 8 days, there is no significant change in the inhibition effect of the nanocapsules. Thus, it can be concluded that most of the polymer content was degraded completely in DMSO in 8 days.

Moreover, PSA-PEG6000-DOX nanocapsules were stored at -20ºC for 2 years (the storage from 2013 to 2015). After 2 years, GST enzyme activity assay was repeated for the nanocapsules that dissolved in DMSO again at the same laboratory conditions. The result is shown in figure 3.24.

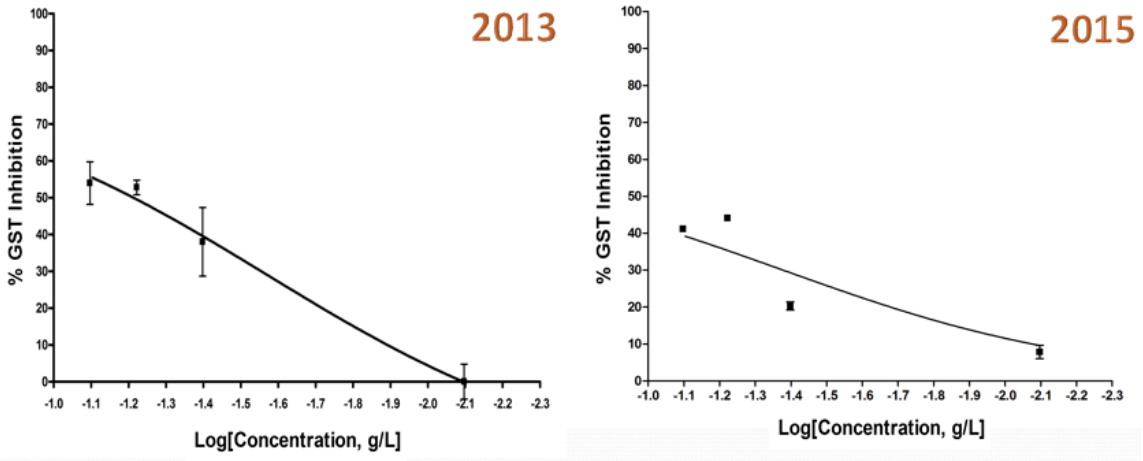


Figure 3.24: GST Inhibition Profile for PSA-PEG6000-DOX Nanocapsules from the Stocks of 2013 and 2015

Almost 20% decrease in the inhibition effect of PSA-PEG6000-DOX nanocapsules was observed. This indicates that the nanocapsules lose their shelf stability as time passes even though they are stored at the optimum storage conditions. The stock solution of the nanocapsules coming from 2015 was kept for 6 weeks in DMSO. Another GST enzyme activity assay was conducted again to observe the degradation effect of the copolymer structure on the percent inhibition (Figure 3.25). Only 10% enzyme inhibition was observed after 6 weeks storage. It is obvious that PSA-PEG6000-DOX nanoparticles lose their inhibitory effect on GST after 6 weeks because of the polymer degradation.

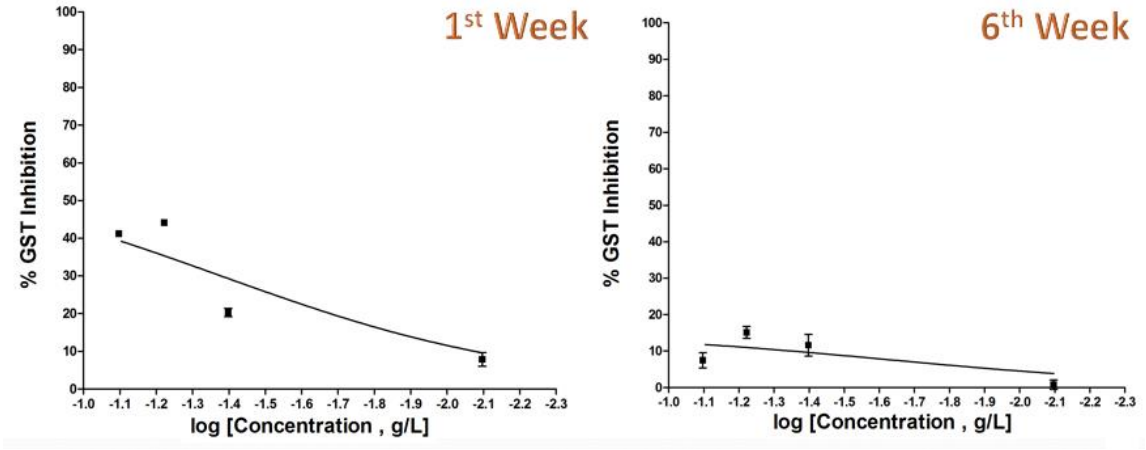


Figure 3.25: GST Inhibition Profile of PSA-PEG6000 Nanocapsules at 6th Week (After 2 Years Shelf Life)

For PSA-DOX nanocapsules, the stability experiment was also conducted by comparing the nanocapsule samples containing 0.04 g/mL DOX content with control group which contains only DMSO. The GST enzyme assay was repeated at 1st week, 4th week and 6th week. The results are shown in figure 3.26.

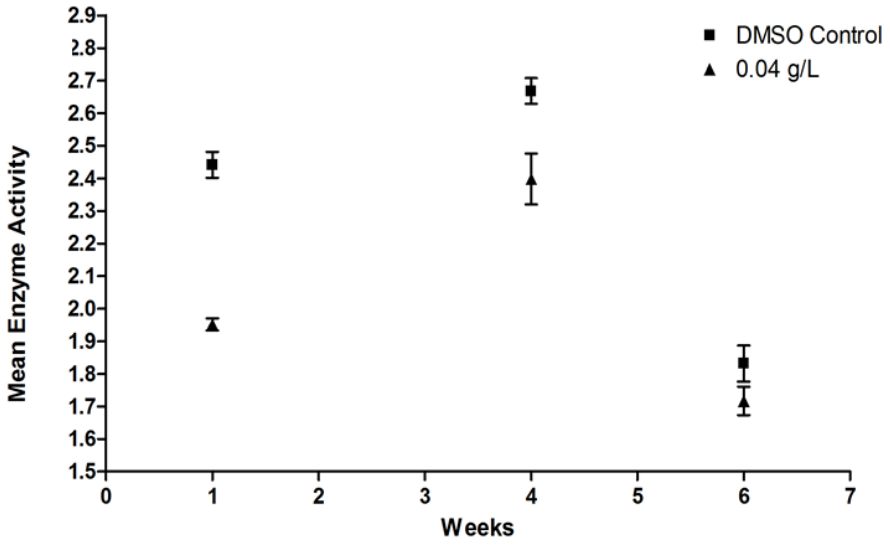


Figure 3.26: PSA-DOX Stability Results

The enzyme activities for week 1, 4 and 6 were calculated as 79.96%, 89.87%, and 93.72%, respectively. The decrease in the enzyme activities was observed for PSA-DOX nanocapsules with respect to the control group for each weeks since PSA-DOX nanocapsules showed inhibitory effect on GST enzyme activity. On the other hand, control had no inhibitory effect on the GST activity because it did not contain the nanocapsule solution. As time passes, the enzyme activities of PSA-DOX nanocapsules increased. This result shows that the nanocapsules lose its stability inside DMSO throughout the time. The reason again depends on the degradation of the polymer structure. This result is consistent with the stability result of PSAPEG6000-DOX nanoparticles for 6 week (Figure 2.35).

In brief, PSA-PEG6000-DOX nanocapsules have a powerful effect on the inhibition of GST in which the inhibitory effect comes from PSA. The same inhibition effect could not be obtained by means of free DOX. In addition, the inhibition effect of PSA decreased as

the polymer structure degraded. Moreover, the nanocapsules still represented the inhibitory effect on GST after 2 years although they lose some of their inhibitory effect.

4. CONCLUSION

Drug delivery systems are very effective to apply controlled release of the drugs to treat different types of illness such as cancer. For the cancer treatment, controlled release system has some advantages like decrease in side effects, increase in therapeutic efficiency etc. Biodegradable polymers can be used as controlled release system to carry both hydrophilic and hydrophobic agents by adjusting the release rate. PSA is one of the most suitable type of the polymer that is used to get sustained release.

In this study, nanocapsules made of PSA and PSA-PEG copolymers were synthesized with average particles size around 200 nm and loaded with DOX which is a popular chemotherapeutic agent. PEG is used to increase biocompatibility of the nanocapsules by the stealth ability. The nanocapsules were produced based on the passive targeting and their sizes are suitable for the application of cell and animal studies. However, the loading capacity of the nanocapsules can be improved by changing the nanocapsule preparation parameters. The encapsulation efficiencies of the nanocapsules are quite good to obtain the effective dose of DOX during the drug release process. The release rate of these nanocapsules were analyzed for pH 4 and 7.4 and the nanocapsules show DOX release with biphasic profile which contains burst release and sustained release. The burst release at the beginning can be a problem for the application on the body. This problem can be solved by some surface modification of the nanocapsules.

The success of the cancer treatment through chemotherapy can be affected by many factors. One is the defense mechanisms of the cells by means of special enzymes such as GST. GST is an important detoxification enzyme which works to remove toxic substances from the cells. However, its overactivity can create a disadvantage for the application of chemotherapy. It removes the chemotherapeutic agents from the cells before reaching their target site. Drug delivery systems can be a solution and some special polymers can be used for the production of the drug delivery system. In the literature, some polymeric materials were proposed to inhibit GST activity. However, there is no study which investigates the

relationship between PSA and GST enzyme activity up to now. In the light of this study, the significant inhibitory effect of PSA on GST activity was proven. Almost 60% inhibition effect of PSA on GST enzyme activity was observed. This is a promising result to use PSA polymer as DDS for cancer treatment. Through the inhibition effect of PSA on GST, chemotherapeutic agents can successfully reach to the target site in the cells Therefore, the resistance problem which is very challenging for the treatment of cancer can also be overcome. On the other hand, the stability results indicated that the nanocapsules lose its stability but not totally after 2 years. The stability of nanocapsules can be enhanced with some surface modifications.

In conclusion, drug delivery systems are very promising for the treatment of cancer. The type of targeting, the agent that is carried, size and shape of the carriers are important factors for the success of the DDS. Moreover, there is another powerful factor that can affect the success of DDS. The materials for the production of DDS can have a synergetic effect with the agent for the success of the DDS. The research conducted here demonstrated that the obstacles not only the chemotherapeutic agent and targeting way are important but also the biomaterial selection is very crucial for the effective treatment through DDS. This study can be developed further with the examination of these nanocapsules for cell culture and animal studies. In addition, the nanocapsules developed in this study can be modified and adopted into active or triggered targeting to be able to increase the bioavailability and selectivity of the carrier system.

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APPENDICES

CALIBRATION CURVE OF DOX

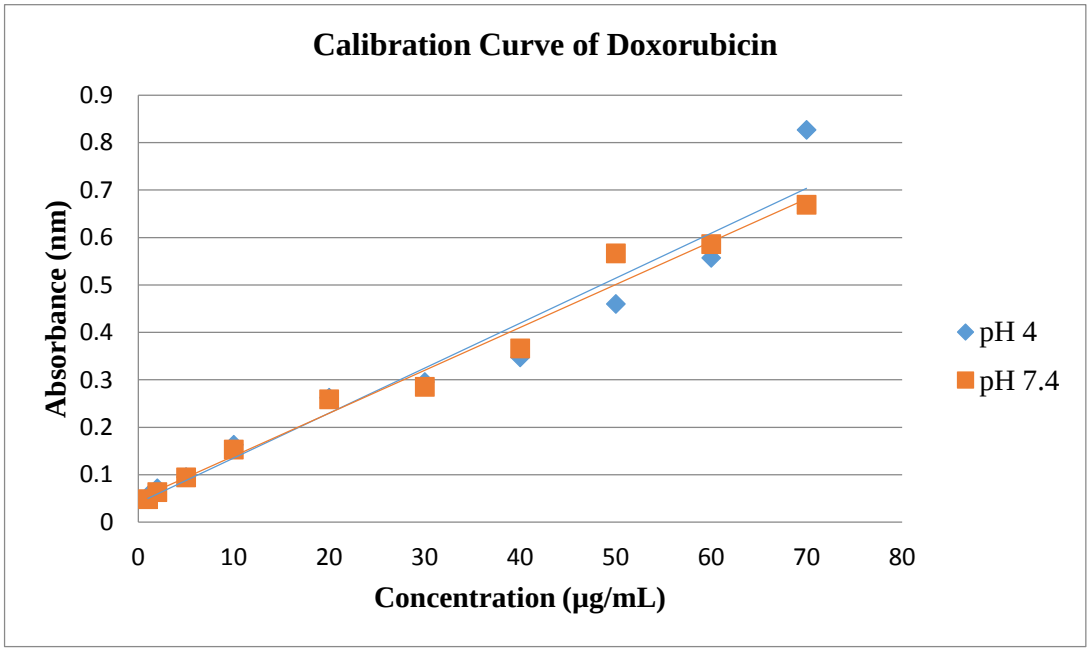

Figure A.1: Calibration Curve of Doxorubicin for pH4 and pH 7.4