Republic of Iraq
Ministry of Higher Education
and Scientific Research
AL-Nahrain University
College of Science

Preparation and Characterization of Doxorubicin Loaded Tri-Block Copolymer (PCL-PEG-PCL) and its Cytotoxic Effect on Breast Cancer Cell Line MCF-7

A dissertation
Submitted to the council of College of Science, University of Al-Nahrain as a partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Biotechnology

By
Randa Mohammed Dhahi Alwan

B.Sc. Biotechnology 2005
M.Sc. Biotechnology 2009

Supervised by

Dr. Hameed M. Jasim
Professor

Dr. Mayssaa Chassib
Assisst. Professor

August 2015 A.D **Thul-Qi'da 1436 A.H**

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

قَالُواْ سُبْحَانَكَ لاَ عِلْمَ لَنَا إِلاَّ مَا
عَلَّمْتَنَا إِنَّكَ أَنتَ الْعَلِيمُ الْحَكِيمُ

صدقَ الله العَظيم
سورة البقرة ألاية " ٣٢ "

ألإهداء

إلى من بلغ الرسالة وأدى الأمانة.. إلى نبي الرحمة ونور العالمين

سيدنا محمد (صلى الله عليه وسلم)

إلى من أحمل أسمه بكل فخر .. إلى من غرسني ولم يشهد آوان عطائي .. إلى عزيز قلبي

والدي العزيز (رحمه الله)

إلى بسمة الحياة .. إلى من كان دعائها سر نجاحي .. إلى أغلى ألاحبة

أمي الحبيبة

إلى الروح التي سكنت روحي .. إلى من آنسني في دراستي وشاركني همومي.. رفيق دربي تذكاراً وتقديرا..

...إلى قرة عيني و هبة الرحمن

عائلتي الصغيرة زوجي وابنتي

إلى الشموع المتقدة التي تنير ظلمة حياتي .. إلى من بوجودهم أكتسب قوة ومحبة لا حدود لها

أخواني وأخواتي

رندة

Acknowledgments

I begin with immeasurable gratitude to Almighty Allah who has sustained me, gave me strength, guided and spared my life to the present level of my educational attainment, Thankful I ever remain.

This thesis would not have been possible without the guidance and the help of several individuals for whom I would like to extend my gratitude:

First and foremost, I pay my heartfelt tribute goes to my supervisor and head of biotechnology department Prof. Dr. Hameed M. Jasim for offering me the great opportunity to learn about cutting-edge scientific research as well as his invaluable assistance, support and guidance throughout my thesis. Above all, his willingness to explore new areas made this research possible, also I would like to express my thanks and gratitude to my supervisor Ass. Prof. Dr. Maysaa Chassib for her support.

I would like to thank the Dean of College of Science/Al-Nahrain University and the staff of the Biotechnology Department.

The work presented here was performed at the laboratories of the institute for bioengineering, School of Engineering in University of Edinburgh, Scotland, United Kingdom. Sincere gratitude is also extended to Prof. Dr. Alistair Elfick, Dr. Andy Downes and Dr. Valistimile Sersen for their innate sense of enthusiasm and encouragement, particularly in times of difficulty, proved a source of motivation and helped in remaining focused on the task at hand made this work not only possible, but also very enjoyable.

In particular, I am also eternally grateful to my father soul and my mother the source of inspiration and a pillar of strength in every aspect of my life. I want to dedicate my work to my parents as a token of love and affection towards them. All my work would have not been possible without their blessings and support and an enormous thanks to my mother for her love and support over all these years, and the hard work and her prayers, I could achieve nothing without her.

I would also like to thank my brothers and my sister for their love, unlimited support, guidance and prayers throughout my life kept the internal desire for knowledge at all times.

Last but not least, words fail me to express my appreciation to my husband "Haitham" whose dedication, affection and persistent confidence in me, has taken the load off my shoulder. Without his infinitive support and his inspirational words none of this would be possible. He was helped me to keep my smile throughout my thesis work and I am look forward to support him all of our life and as he complete his own Ph. D. dissertation.

Finally, I offer my regards and blessings to all of those who supported me in any respect during the completion of the project.

Randa

August, 2015

Supervisors Certification

We certify that this dissertation entitled **"Preparation and Characterization of Doxorubicin Loaded Tri-Block Copolymer (PCL-PEG-PCL) and its Cytotoxic Effect on Breast Cancer Cell Line MCF-7"** was prepared under our supervision at the College of Science/Al-Nahrain University as a partial fulfillment of the requirements for the Degree of Doctorate of Philosophy in Biotechnology.

Supervisors

Signature	Signature
Name: **Dr. Hameed M. Jasim**	Name: **Dr. Maysaa C. Hatem**
Scientific Degree: Professor	Scientific Degree: Assist. Professor
Date:	Date:

In view of the available recommendations, I forward this dissertation for debate by the examining committee.

Signature:

Name: **Dr. Hameed M. Jasim**

Scientific Degree: Professor

Title: Head of Biotechnology Department

Date:

Committee Certification

We, the examining committee, certify that we have read this dissertation entitled "**Preparation and Characterization of Doxorubicin Loaded Tri-Block Copolymer (PCL-PEG-PCL) and its Cytotoxic Effect on Breast Cancer Cell Line MCF-7**" and examined the student "**Randa Mohammed Dhahi**" in its contents and that in our opinion; it is accepted for the Degree of Doctorate of Philosophy in Biotechnology.

(Chairman)

Signature:

Name: **Dr. Subhi J. Hamza**
Scientific Degree: Professor
Date

(Member)

Signature

Name: **Dr. Adawiya J. Haider**
Scientific Degree: professor
Date:

(Member)

Signature

Name: **Dr. Saad M. Salih**
Scientific Degree: Assist. Professor
Date:

(Member)

Signature

Name: **Dr. Ali S. Ahmed**
Scientific Degree: Assist. Professor
Date:

(Member)

Signature

Name: **Dr. Ahmed M. Al-Shammari**
Scientific Degree: Assist. Professor
Date:

(Member / Supervisor)

Signature

Name: **Dr.Hameed M. Jasim**
Scientific Degree: Professor
Date:

(Member / Supervisor)

Signature

Name: Dr.Maysaa G. Hatem
Scientific Degree: Assist. Professor
Date:

I hereby certify upon the decision of the examining committee.

Signature:

Name: **Dr. Hadi M. A. Abood**
Scientific Degree: Assist. Professor
Title: Dean of College of Science
Date:

Summary

The present study aimed to prepare, characterize and explore the cytotoxicity effect of doxorubicin (Dox) loaded biodegradable amphiphilic triblock copolymer poly caprolactone (PCL) poly ethylene glycol (PEG) poly caprolactone (PCL-PEG-PCL) micelles against breast cancer cell line MCF-7.

The physicochemical properties and *in vitro* test including cytotoxicity of the micelles were examined. PCL-PEG-PCL (PCEC) micelles were prepared by nanoprecipitation method using acetone as the organic solvent, and the PCL-PEG-PCL triblock copolymer was self-assembled into core/shell-like structured micelle nanoparticles due to the amphiphilic property of the PCL-PEG-PCL. The average particle size determined by dynamic light scattering (DLS) of obtained micelle was (226 ± 5) nm, and polydisperse index was (0.26 ± 0.034) with a narrow monodispersed unimodal size distribution pattern. The TEM image revealed that the micelles prepared by nanoprecipitation were spherical in shape.

In this study, Dox was encapsulated into micelles with encapsulation efficiency (EE) 99.7% and drug loading (DL) 28.69 %, then the release profile of Dox from the PCL-PEG-PCL micelles was studied using a dialysis method. At pH 5.6, approximately 33% of Dox within 3 hr was released, while after 46 hr approximately 92% of Dox was released, but the rate of Dox that released at pH 7.4 is notably slower compared to pH 5.6. Moreover, the percentage of Dox released was approximately 43% within 46 hr.

The *in vitro* safety evaluation of polymeric PCL-PEG-PCL micelles was performed and it was concluded that these nanoparticles did not induce hemolysis with the concentration of (100 µg/ml) on human erythrocyte comparing with the negative control (normal saline).

The *in vitro* cytotoxicity of PCL-PEG-PCL micelles at different concentrations (0.1, 0.2, 0.4, 0.6, 0.8 and 1 µg/ml) on the MCF-7 cell line was evaluated by the tetrazolium dye MTT method, and the analyzed results

revealed that PCL-PEG-PCL micelles possessed negligible toxicity to MCF-7 cells even at higher concentrations, the viability percentage was above 80%, indicating good biocompatibility of these polymeric triblock.

Additionally, the morphological observations of Dox loaded PCL-PEG-PCL against MCF-7 cell line by inverted microscope revealed that after 24 hrs of treatment, no morphological changes were observed in the MCF7 cells. Whereas, after 48 hrs, the cells became rounded and detached. After 72 hrs, the number of treated cells was reduced, and they were shrinkage, rounded, detached and suspended in the culture media. Therefore, this study showed an effective delivery characteristic and the inhibition of cell growth of Dox that loaded into PCL-PEG-PCL polymeric micelles.

The *in vitro* cytotoxicity was investigated by MTT assay of (0.01, 0.05, 0.1, 0.4, 0.8, 1.2 µg/ml) of free Dox and Dox loaded PCL-PEG-PCL micelles against MCF-7 cell line after 24, 48 and 72 hrs. MCF-7 was more sensitive to free DOX as compared to PCL-PEG-PCL/Dox for 24hrs and 48hrs, whereas at 72hrs, it was observed that MCF-7 cells was more sensitive to PCL-PEG-PCL/Dox than free Dox. moreover, it was found that the cell viability decreased significantly at $p \leq 0.05$ when the treatment time was increased within the experimental period.

The inhibition concentration values (IC_{50}) (inhibitory concentration to kill 50% cell death) of free Dox and Dox loaded PCL-PEG-PCL against MCF-7 cells after 72 hr were 0.56 and 0.5 respectively.

To track the Dox loaded PCL-PEG-PCL micelles and Dox molecules in MCF-7 living cells, label-free techniques such as coherent anti-stock Ramman spectroscopy (CARS) and two photon excitation (TPEF) was used.

The visualization of the three dimensional distribution of the Dox molecules by CARS and TPEF showed that free Dox entered nucleus quickly and it’s concentration in cytoplasm dropped over time after 10 hr and 35 min. At the same time, Dox was released from micelles and could be observed in

cytoplasm and nucleus. In addition, the cell number was relatively low of exposure MCF-7 to Dox-loaded micelles as compared with the Dox that was released after 5hr and 50 min.

List of contents

Chapter One: Introduction

Chapter Two: Literature review

Chapter three: Materials and method

Chapter four: Result and discussions

Conclusions and recommendations

List of tables

List of Figures

Figure Title **Page Number**

List of abbreviations

3D	Three dimensions
AACR	American Association for Cancer Research
BRCA1	Breast cancer type 1 susceptibility protein
BRCA2	Breast cancer type 2 susceptibility protein
CARS	Coherent Anti-Stokes Raman Scattering
CLSM	Confocal laser scanning microscope
CNS	Central nervous system
DDS	Drug delivery system
DL	Drug loading
DLS	Dynamic Light Scattering
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
Dox	Doxorubicin
DPBS	Dulbecco's Phosphate-Buffered Saline
EE	Encapsulation efficiency
EPR	Enhanced permeability and retention
FBS	Fetal bovine serum
FDA	Food and drug administration
IARC	International Agency for Research on Cancer
IC_{50}	The drug concentration at which 50% of the cell growth is inhibited
kDa	Kilo Dalton
LSD	Least significant differences
MCF-7	Human breast carcinoma cell line (Michigan Cancer Foundation)
MDR	Multi drug resistance

MRP	Multidrug resistance associated protein
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
MPS	Mononuclear phagocyte system
MWCO	Membrane molecular weight cut-off
NLO	Nonlinear optical imaging
nm	Nanometer
NPs	Nanoparticles
PBS	Phosphate buffer saline
PCEC	Poly caprolacton-poly ethylene glycol-poly caprolacton
PCL	Poly caprolacton
PEG	Polyethylene glycol
PGA	poly glycolic acid
P-gp	P-glycoproteins, multidrug resistance protein
PLA	Poly lactic acid
PMDDS	Polymeric Micelles Drug Delivery System
PMs	Polymeric micelles
PMT	Photomultiplier tubes
RES	Reticular Endothelial System
TEM	Transmission Electronic Microscope
TB	Trypan blue
TPEF	Two photon excitation fluorescence
UV	Ultraviolet
WHO	World Health Organization

Chapter One

Introduction

1.1. Introduction

Cancer is a group of diseases which is defined as the uncontrolled cell division and spread of abnormal cells in patients (Garcia, *et al*., 2007). If this spread is not controlled, cancer will lead to death. Cancer has been known for many centuries, but concerns and death from this disease, have not been as high as they are today. The word cancer was first introduced by Hippocrates (460-370 BC), the Greek physician and Father of Medicine. He coined the term carcinoma from the Greek word "Karcinos" meaning "crab", after discovering a shellfish-like shaped tumor (Rettig, 2005).

Breast cancer is the most frequent malignant disease in women worldwide. In Iraq, breast cancer ranks first among cancers diagnosed in women, the incidence of female breast cancer has risen in Iraq (Al-Hashimi and Wang, 2014). It is estimated that more than one million new cases of breast cancer are diagnosed all over the world annually (Curado, 2011).

Recent years have witnessed intense research on the modification of drug release and absorption. So, the development of new drug delivery systems will offer additional advantages and may facilitate the launch of poorly soluble drugs and also will facilitate more patient-friendly administration, thus resulting in patient increased compliance and satisfaction. Drugs incorporated into nanosized polymeric micelles are promising nanocarrier systems for drug delivery, because the polymeric micelles have several advantages, such as controlled drug release, enhanced tumor-penetrating ability, reduced side toxicity, increased stability, increased loading capacity and specific-tissue target ability (Nguyen and Nguyen, 2010).

Nanotechnology is the study, design, creation, synthesis, manipulation, and application of materials, devices, and systems at the nanometer scale. The prefix nano is derived from the Greek word "dwarf". One nanometer is equal to one billionth of a meter, that is, 10^{-9} m Nanotechnology provides an

important method to overcome the poor water solubility of hydrophobic drugs. Hydrophobic drugs were manipulated to be entrapped into nano-scale particles, which could be well dispersed in aqueous solution to form stable and homogeneous suspension, therefore met the requirements of clinic administration (De *et al*. 2008).

Micelles prepared from synthetic biodegradable block copolymers are widely applied in drug delivery system (DDS) owing to their intrinsic core–shell architecture which demonstrates a series of attractive properties for increasing drug solubility, enhancing drug stability, and passive target effects (Croy and Kwon 2006). Poly ethylene glycol (PEG) and poly caprolactone (PCL) are both biocompatible and have been used in several FDA-approved products (Hamaguchi *et al*. 2007). Polyethylene glycol (PEG) is a common constituent for the hydrophilic shell. Its high water-solubility and low cytotoxicity makes PEG a widely used material for medical applications including drug carriers. Furthermore, drugs that are encapsulated by small-sized polymeric micelles with a hydrophilic outer shell can potentially increase the circulation time of drugs and can prevent recognition by macrophages of the reticuloendothelial system (RES) after intravenous injection. (Shuai *et al*., 2003).

Polycaprolactone (PCL) is a polymer that suitable for controlled drug delivery due to a high permeability to many drugs excellent biocompatibility and its ability to be fully excreted from the body once bioresorbed (Sinha *et al*., 2004)

For decades, microscope has been a key tool in the investigation of biological processes, imaging of cellular structures and the localization of molecules within cells. The identification of different molecular species on the microscopic scale is still a considerable challenge in many areas of biology. The multimodal imaging approach has a great potential in diagnosis of diseases like cancer and arterial disease, and provides a fast-track method to detect and

evaluate drug response with high temporal and spatial sensitivity, which is crucial to understanding the drug action and subsequent response of the cell.

1.2 Aims of the study

This study was aimed to the following:

1- Preparation of polymeric PCL-PEG-PCL micelles.

2- Loading of anticancer drug doxorubicin (Dox) into (PCL-PEG-PCL) micelles to meet the requirements of drug delivery system and *in vitro* release study of Dox from nanoparticle at different pH.

3- *In vitro* safety evaluation of polymeric nanoparticle within *in vitro* hemolytic test and MTT assays.

4- *In vitro* study of free Dox and Dox loaded PCL-PEG-PCL micelles against breast cancer MCF-7 cell line at different concentrations using MTT assay

5- Monitoring uptake of free Dox and Dox loaded PCL-PEG-PCL micelles by MCF-7 cell line with multimodal spectroscopy by CARS and TPEF microscope.

Chapter Two

Literature Review

2. Literature review

2.1 Cancer

A cell is the smallest living unit in the body and it is known as the structural and functional unit of all living organisms, normal body cells grow, divide and die in an orderly fashion and all the functions of a cell including rate of cell growth, division, differentiation and death are regulated by a specified set of genes, which act as triggers. A loss of function of these genes, sometimes lead to a state of uncontrolled cell growth without any differentiation termed as "Neoplasia or Cancer" (Khurana, 2009). Cancer has been identified as a chronic disease (Hanahan and Weinberg, 2011), cancer is a class of disease in which abnormal cells divide without control, invade other near end tissues and finally metastasize via blood and lymph node vessels to other organs (Sahai, 2007).

Cancer is one of the major causes of mortality, and the worldwide incidence of cancer continues to increase. The discovery of cytotoxic agents was revolutionary for cancer treatment in the last century to improve the survival rates and the quality of life for patients with different types of cancers. However, the development of agents that combine efficacy, safety, and convenience remains a great challenge due to the narrow therapeutic index of some drugs, the fact that they may damage not only cancer cells but also healthy and normal tissue, and the occurrence of resistance (Ferrari, 2005).

Cancer is a generic term for a large group of diseases that can affect any part of the body, other terms used are malignant tumors and neoplasms, one of the defining feature of cancer is the rapid creation of abnormal cells that grow beyond their usual boundaries, and which can then invade adjacent parts of the body and spread to other organs, the latter process is referred to as metastasizing. Metastases are the major cause of death from cancer, figure (2.1) (World Health Organization, 2014).

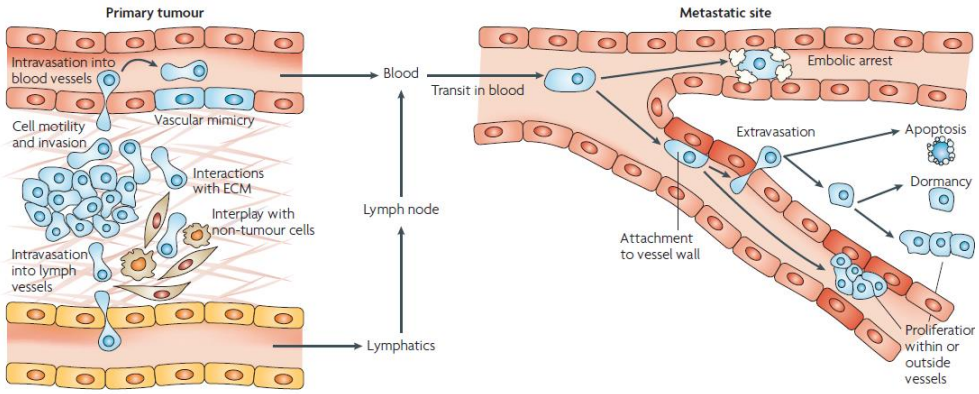


Figure (2.1) Development of cancer from the primary tumor to metastatic site (Sahai, 2007).

Cancer is a major public health problem because it is one of the leading causes of burden, although cancer disease has been exiting for many centuries, it becomes the most common disease all over the world. There were 12.4 million new cancer cases and 7.6 million cancer deaths in 2008 as reported by World Health Organization (WHO), it was also estimated by the International Agency for Research on Cancer (IARC) that the new cancer incidence was expected to rise from 12.4 million in 2008 to 26.4 million in 2030 with the growth in the world population from 6.7 million in 2008 to 8.3 million by 2030, figure (2.2)**,** (WHO, 2008).

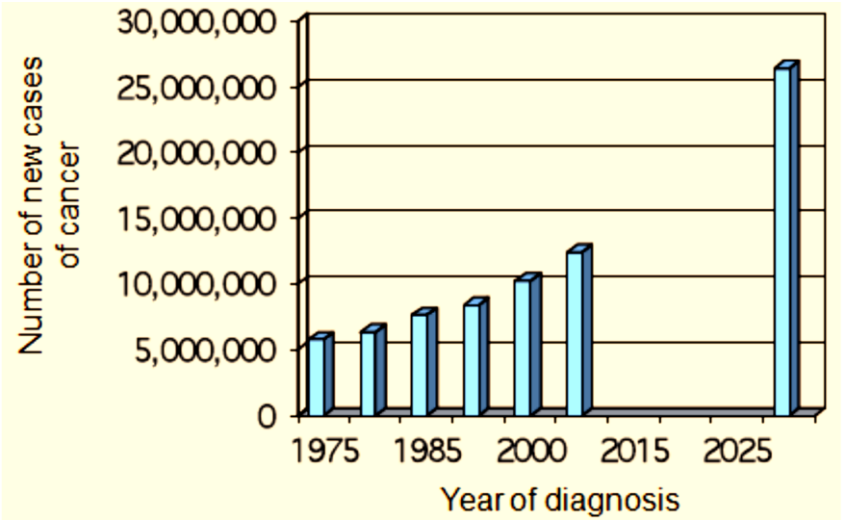


Figure (2.2) Estimated global cancer incidences (1975-2030), (WHO, 2008).

Due to the huge worldwide health burden of cancer, the ultimate efforts of scientists, researchers and societies have been put on the improvement of diagnostic devices and treatments over decades. In Iraq, cancer incidence relatively high and the trends are up going in terms of quantity and variables related like age, sex, etc., so the prevention and management of cancer are still inadequate. The number of cancer cases registered in Iraq was 5720 cancer (31.05) case per 100,000 in 1991 then raised to 14,180 case (44.46) per 100,000 population in 2008 as shown in figure (2.3) (Husain and Al-Alawachi, 2014).

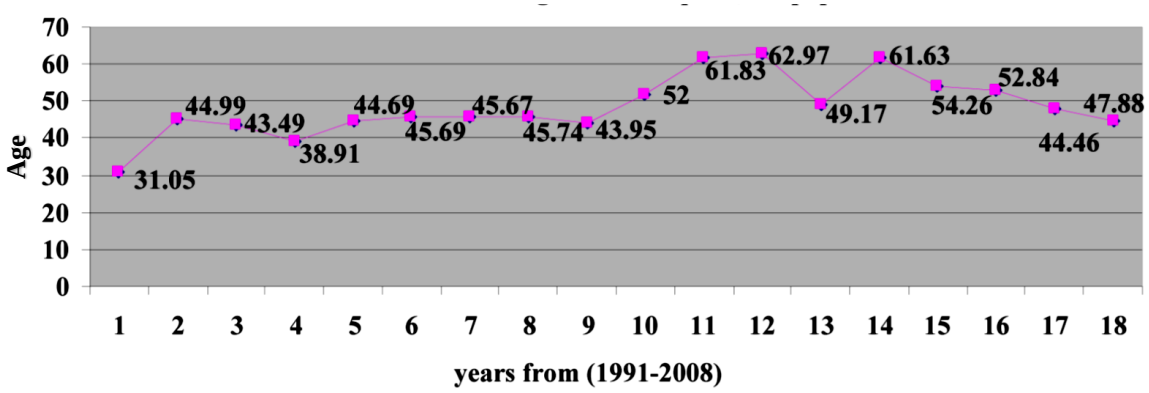


Figure (2.3) Incidence of cancer rate registered in Iraq 100,000 population between 1991 and 2008. (Husain and Al-Alawachi, 2014).

It was found that the breast cancer was recorded the highest number of cases among the other types of cancers in Iraq as shown in table (2.1) followed by lung cancer, leukemia, bladder cancer, brain and central nervous system (CNS), non-Hodgkin's lymphoma, colorectal cancer, stomach cancer, skin cancer excluding melanoma, larynx cancer which is similar to many other studies in the region (Janet, 2012).

Table (2.1) The most common cancer types in Iraq in 2008, (Husain and Al-Alawachi, 2014).

Cancer site	No. of cases	Male	Female	% of cancer type to the all sites	Registered case/100,000 population
Breast Cancer	**2729**	**92**	**2637**	**19.25**	**8.56**
Bronchus and lung	1375	975	400	9.70	4.31
Leukemia	860	555	405	6.77	3.01
Urinary Bladder	881	667	214	6.21	2.76
Brain & other CNS	780	406	374	5.50	2.24
Colorectal cancer	693	378	315	4.89	2.17
stomach	471	274	197	3.32	1.48
Skin (excluding melanoma)	367	215	152	2.59	1.15
Larynx	334	251	83	2.36	1.05
Total 10	9306	4247	5059	65.63	29.18
All sites	14,180	6589	7591	100.00	44.46

2.1.1 Cancer biology

Cancer has been recognized as a genetic disease in which malignant cells have undergone mutations and epigenetic changes caused by interaction of many external factors and individual susceptibility (Weinberg, 2007). These external factors, including chemicals, radiation, viral infections, and so on, can directly damage DNA, act as mutagens or induce the missense and frameshift of multiple genes and an epigenetic change of somatic cells (figure 2.4). When these damages overtake the DNA repair ability of normal somatic cells, or existing DNA repair is deficient, the underlying carcinogenic gene will turn a normal cell into a cancer cell; otherwise, the spontaneous mutations arising from an inherent error rate in the fidelity of DNA replication and/or repair could give rise to the initiation of cancer. A gold standard of tumorigenesis is that malignant tumors arise from a single cell transformed by the above mentioned

carcinogenic agents. Once the initiated cell starts to undergo clonal expansion, according to genetic instability, it will lead to invasive metastatic cancer (Thiery, 2002).

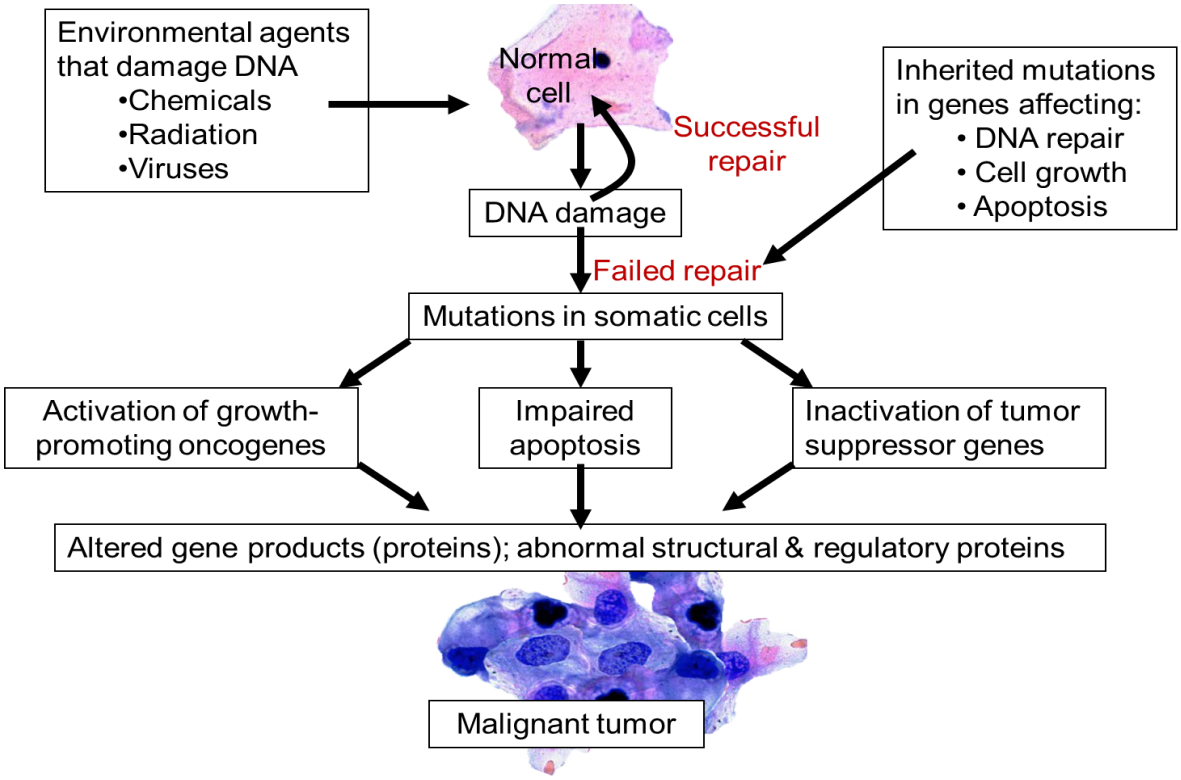


Figure (2.4) Overview of carcinogenesis (LaMorte, 2014)

Molecular and genetic analysis of some common cancers signify that at least five gene defects are frequently present in cancers of the colon (Fearon and Vogelstein, 1990), breast (Wood, *et al*., 2007), lung (Sato, *et al.*, 2007) and pancreas (Maitra, *et al*., 2006), whereas fewer gene defects may cause development of precancerous precursor lesions found in these cancers.

Non-lethal genetic damage to any one of the following types of genes may lead to carcinogenesis:

- The growth promoting proto-oncogenes
- The growth inhibiting, tumor suppressor genes
- Genes regulating programmed cell death (apoptosis)

- Genes involved in DNA damage repair.

The carcinogenic effects of environmental and occupational carcinogens date back to 16th century. However, the first ever association of such kind was documented in the 18th century. Waldron indicated that the high incidence of scrotal cancer in chimney sweeps could be related to their occupational exposure to soot and tar (Waldron, 1983).

Dipple *et al.;* later identified the carcinogens in coal and tar to be polycyclic aromatic hydrocarbons (Dipple, *et al*., 1984). The work of Case *et al*. depicted an association between tumors of the urinary bladder and azo dyes, workers employed in chemical manufacturing and textile dying, were found to be at a greater risk for bladder cancers (Case, *et al*., 1954). Studies have indicated that cigarette smoking increases the risk for the cancers of the lung, oral cavity, pharynx, larynx, esophagus, bladder, renal pelvis and pancreas (Vineis, *et al*., 2004). However, it has been reported that most and possibly all types of human cancer share common traits that are acquired during tumor development (figure 2.5) (Hanahan and Weinberg, 2000):

1. self-sufficiency in growth signals,
2. insensitivity to anti-growth signals,
3. evasion of programmed cell death or apoptosis,
4. limitless replicative potential,
5. sustained angiogenesis,
6. Tissue invasion and metastasis.

In his 2010 NCRI conference talk, Hanahan proposed four new hallmarks. These were later codified in an updated review article entitled "Hallmarks of cancer: the next generation" (Hanahan and Weinberg, 2011).

7. Deregulated metabolism,
8. Evading the immune system,
9. Unstable DNA,
10. Inflammation.

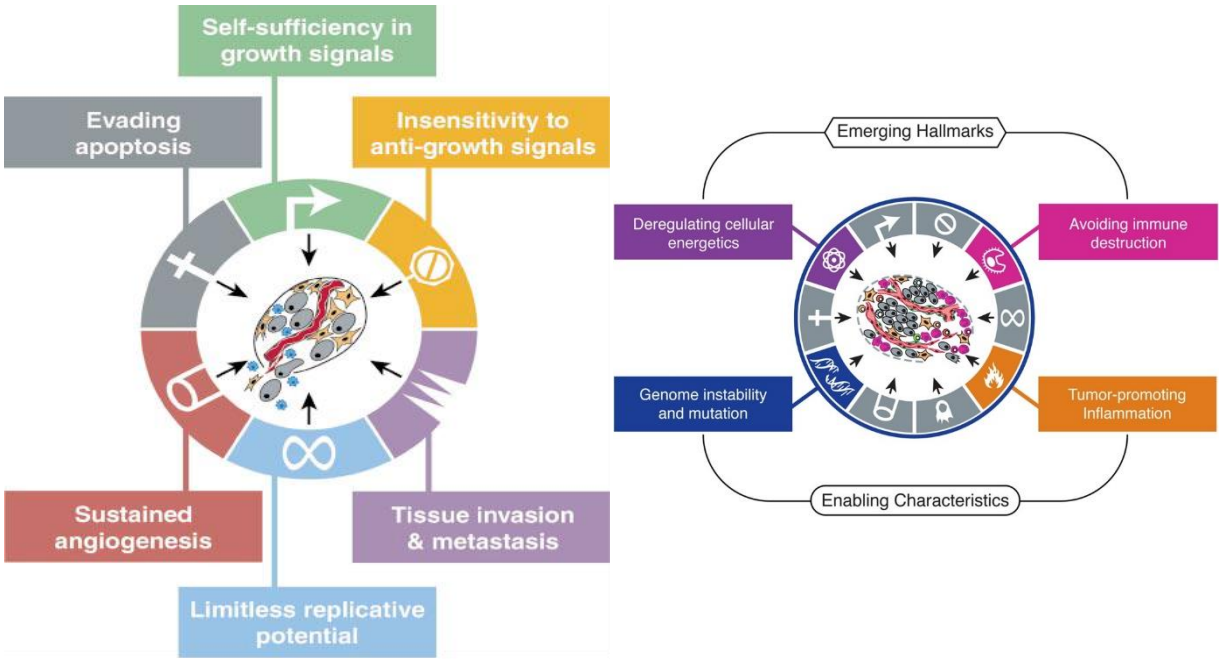


Figure (2.5) Common traits that are acquired during tumor development (Hanahan and Weinberg, 2000).

2.1.2 Cell growth

It is essential that cell division is regulated to guarantee the proper structure and function of tissues and organs. A loss in regulation can lead to uncontrolled cell growth, which is a characteristic of cancer cells (Wolkers *et al*., 2004). The process of cell growth is known as "the cell cycle", which can be divided in two brief phases: interphase and mitosis. During interphase, the cell grows, accumulating nutrients needed for mitosis and duplicating its DNA. Interphase can be divided in three sub-phases: G1, S, and G2.

The sub-phase from the end of the previous M phase to the beginning of DNA synthesis is known as G1, during which cells synthesize various enzymes for DNA replication. The S phase is the DNA synthesis phase, during this phase, the amount of DNA in the cell doubles, though the ploidy of the cell remains the same. The cell then enters the G2 phase, which lasts until mitosis commences. During the G2 phase, biosynthesis occurs, mainly involving the increase in cell size and the number of cell components. Following the progression from G2 to the M phase, the cell divides to produce two daughter

cells, each of which will immediately enter the G1 phase. Most somatic cells remain in G0, the quiescent phase, unless the cell is induced by the growth factor (figure 2.6). Each phase of the cell cycle contains checkpoints that are used to inspect the progress of the cell cycle in order to ensure the fidelity of cell division (Hartwell and Kastan, 1994).

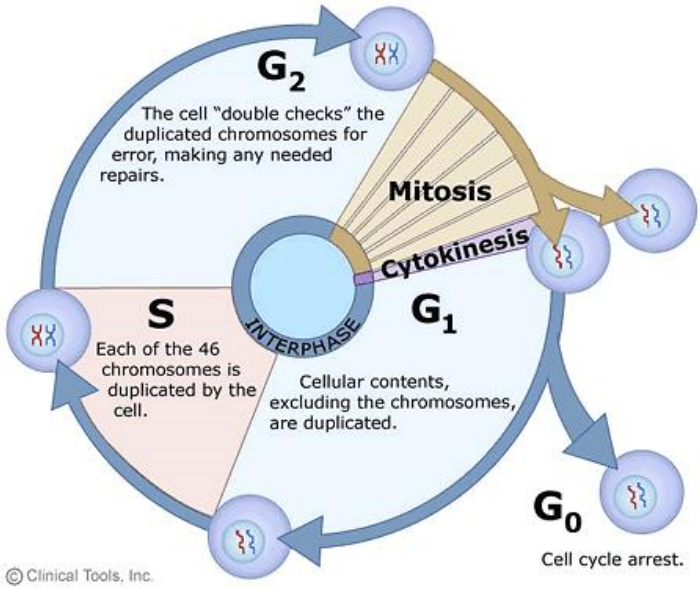


Figure (2.6) The Eukaryotic cell cycle

An important function of many checkpoints is to evaluate DNA damage, which is detected by sensor mechanisms. When damage is detected, the checkpoint uses a signal mechanism to halt the cell cycle until repairs are made. If the damage is too extensive to repair, a cell may initiate the apoptosis process, which is also known as "programmed cell death". All the checkpoints that assess DNA damage appear to use the same sensor-signal-effector mechanism. The gene encoding proteins that promote cell‘s progression through the cycle is known as proto-oncogene (Kroemer, 1997). Contrarily, the gene encoding proteins that inhibit the cell cycle are known as "tumor-suppressor genes". There are two types of tumor suppressor genes: caretaker and gatekeepers.

The functions of the caretaker gene involve maintaining the integrity of the genome (Vogelstein and Kinzler, 2002). The proteins coded by the caretaker gene repair the structural damage of chromosomes, correct mutations in the DNA sequence and sort the chromosomes into daughter cells during cell division, while the function of proteins, encoded by gatekeepers, is to prevent a cell from irregularly proceeding through the cell cycle (figure 2.7), the most representative gatekeeper gene is p53 gene (Donehower *et al*., 1992).

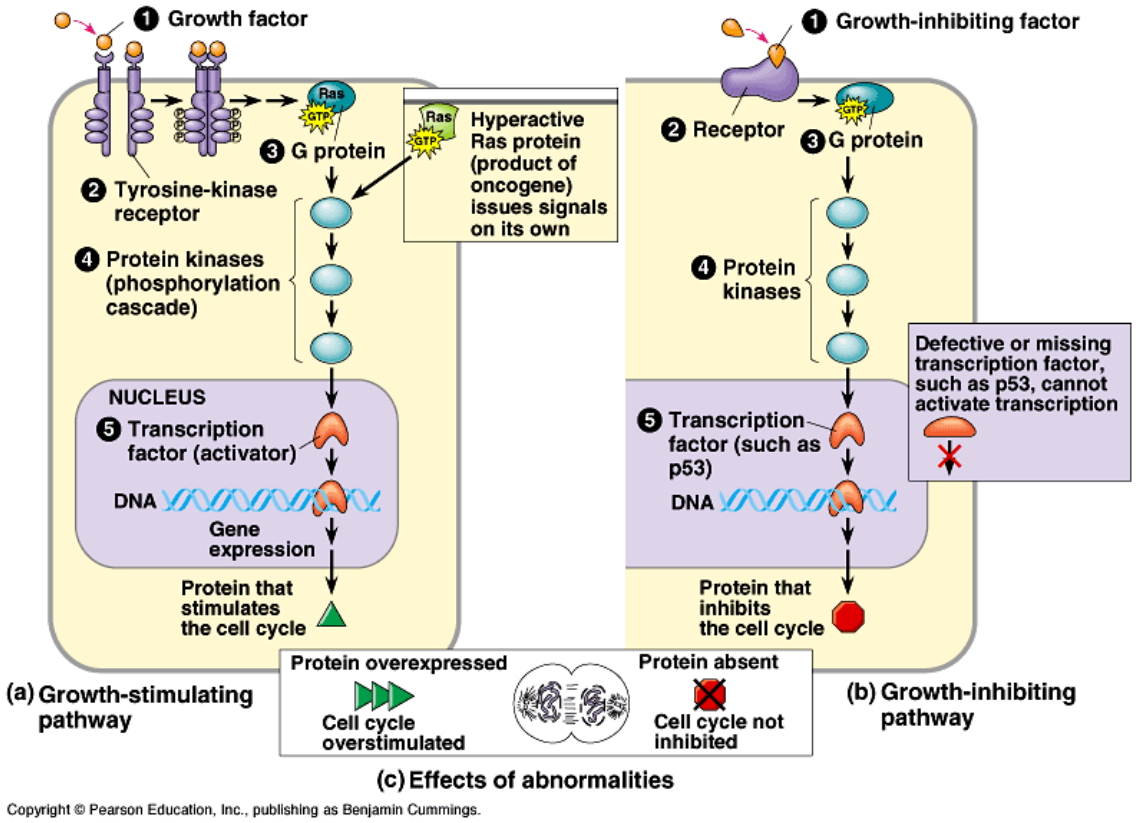


Figure (2.7) The tumor suppressor gene and proto-oncogene (www.zo.utexas.edu/faculty/sjasper/images/19.14.gif)

The protein encoded by the p53 gatekeeper gene functions at the checkpoints late in the G1 and S phases, and late in the G2 phase. This gatekeeper gene acts as a transcription factor regulating the expression of many genes that active cell cycle arrest, DNA repair and apoptosis. Almost 50% of cancers are the result of the mutation of the p53 gene that causes unchecked

division of damaged or transformed cells, ultimately resulting in tumor formation (Levine *et al*., 2004).

2.1.3 Breast cancer

Breast cancer is a malignant, uncontrolled growth of epithelial cells lining the ducts or lobules of breast tissue arising through a series of molecular mutations at the cellular level (Lippman, 2005). It is the most commonly occurring cancer and the leading cause of cancer death in females worldwide. In 2008, about 1.38 million new cases of breast cancer were diagnosed and 458,400 women died of breast cancer (Jemal *et al*., 2011). The causes of most breast cancer cases remain unknown. However, numerous advances in the identification of various risk factors including gender, environment, hormones, genetic factors, and lifestyle (Michaud *et al*., 2008).

Studies have shown that there is a genetic predisposition to a large number of cancer types (Knudson, 2002; Narod, 2006). Inheritance of a single autosomal dominant mutant gene greatly increases the risk of developing the tumor. Such cancers are termed as autosomal dominant inherited cancer syndrome. BRCA1, BRCA2, p53, PTEN are some of the genes associated with hereditary breast cancer predisposition. In addition to the inheritance of a single autosomal dominant mutant allele, recent studies have shown that familial susceptibility to cancer may also depend on multiple low-penetrance alleles (Easton, *et al*., 2007). The traditional treatment of breast cancer often comprises treatment with surgery, radiotherapy, or both for local diseases, and treatment with chemotherapy, endocrine therapy, or combinations of these for systemic diseases (Carlson *et al*., 2009).

In Iraq, breast cancer is the most frequent cancer among women and there was a significant increase in breast cancer among other types in compared to other types of cancer as shown in table (2.1)

A study by Al-Hashimi and Wang in 2014 shows that in Iraq, the proportion of breast cancer in females (33.8%), as compared with very similar proportion in Lebanon, lower than that observed in several Arab countries such as Jordan. It's higher than that observed in USA, Asia, Africa and several Arab countries such as Saudi Arabia, UAE, and in non-Arab neighboring countries such as Turkey and Iran (figure 2.8).

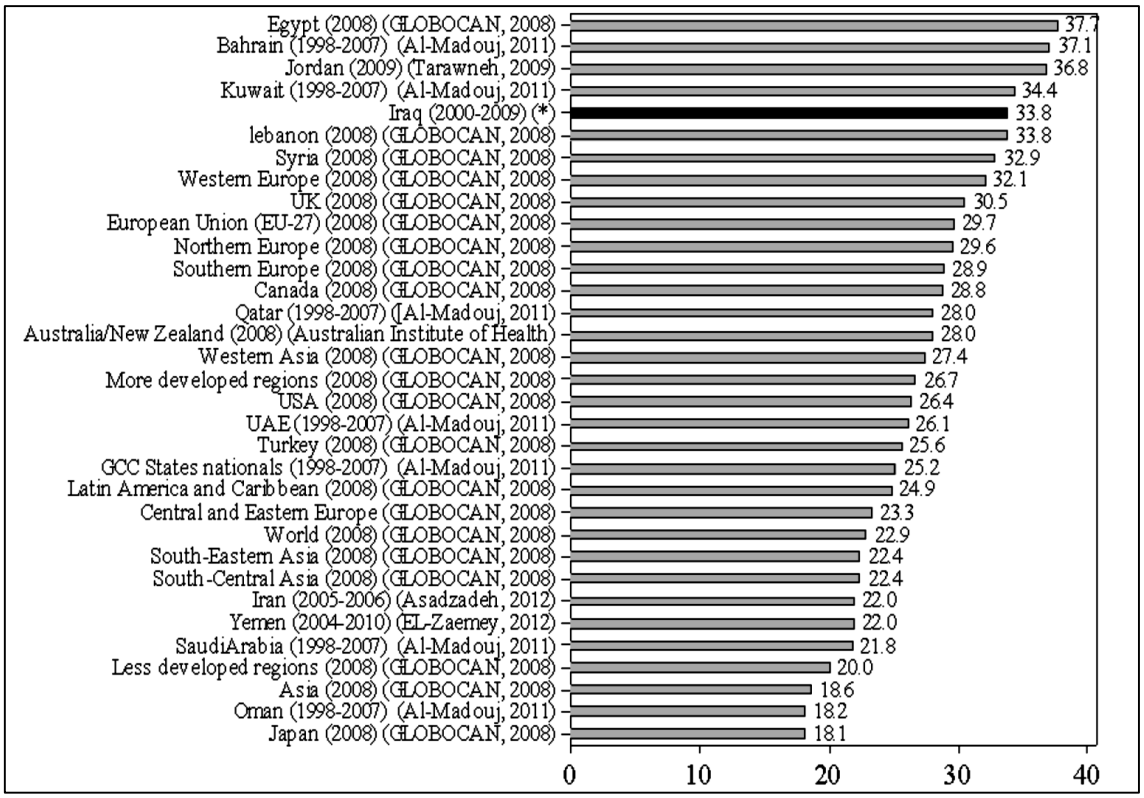


Figure (2.8) Proportion of breast cancer in females in Iraq compared to other countries (Al-Hashimi and Wang , 2014).

2.2 Conventional therapies for cancer

The traditional strategies for cancer treatment includes surgery, radiation, and chemotherapy or combined strategies of these treatments, sometimes these are supplemented by more specialized therapies such as immunotherapy or hormone therapy which can be applied only for some tumor types (Miller *et al*., 1981). The oldest form of cancer treatment is surgery; it was one of the most

important weapons against cancer. Radiotherapy is the second major weapon against cancer, it involves use of high-energy particle beams or waves (radiation), such as X-rays, gamma rays or neutrons for treating cancer (Thorell and Larson, 1978). One of the major obstacle of chemotherapy and radiotherapy is that it is cannot treat only tumor cells without affecting the surrounding healthy cells hence damage also healthy cells as well.

2.2.1 Standard chemotherapy of cancer

Cancer chemotherapy was first successfully used in the 1950s when nitrogen mustard was found to be useful in inhibiting tumor growth. However, due to its toxicity, chemotherapy with anticancer drugs took until the 1960s to be widely applied and it started to gain popularity in 1970s as a means to cure or inhibit the growth of certain types of cancers (Feng and Chien, 2003). Traditional cancer chemotherapy is the treatment by using one or more small-molecule anticancer drugs which aim to destroy the rapidly dividing cells via their specific mechanisms to the cells. Chemotherapeutic drugs are very strong to fight against a spectrum of cancers from the early stage to the metastatic stage due to their broad range of mechanism to cancer cells. Although the mechanisms of action are different among them, they all rely on the rapid and uncontrolled proliferation and division properties of cancer cells. They attack the cell division and apoptosis pathways (Prakash *et al*., 2011).

Traditional chemotherapeutic drugs attack the proliferation of normal cells that causes toxic to healthy tissues with serious side-effects including hair loss, appetite loss, nausea, vomiting, anemia, nerve damage, memory loss, and permanent organ damage to heart (De Keulenaer *et al*.,, 2010), lung, (Yao, 2007), liver (Rybak, 2007) and kidneys (Lukenbill and Kalaycio, 2013; Pavlidis and Pavlidis, 2013). As a result, off-target cancer drug delivery causes serious side effects and systemic damage to a human body going through chemotherapy. For example, doxorubicin (DOX), the most effective and widely

used anticancer drug, is reported to cause adverse effects including nausea, vomiting, anorexia and heart damage (cardiotoxicity), which considerably limit its applicability. A series of laboratory and clinical studies during the 1960s and 1970s have tested the concept that breast cancer is a metastatic disease that cannot be cured with loco-regional therapy alone (Barratt, 2000).

2.2.1.1 Doxorubicin

Doxorubicin is an anthracycline antibiotic figure (2.9) and the history of its discovery can be traced back to the 1950s, when a bright red pigment, later named daunorubicin, was isolated from *Streptomyces peucetius*. The clinical use began in 1960s for treating acute leukemia and lymphoma, although fatal cardiac toxicity was recognized in 1967 (Tan *et al.*, 1967). In 1969, a mutant strain of *Streptomyces* produced a different, red-colored antibiotic, Dox which exhibited better anticancer activity than daunorubicin but the cardiotoxicity remained (Arcamone, *et al*., 1969).

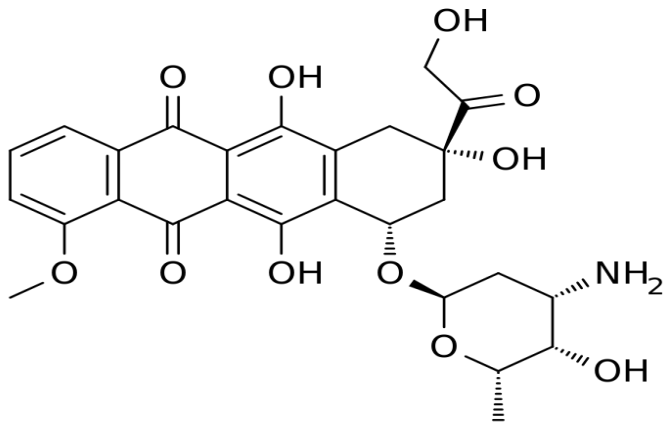


Figure (2.9) Chemical structure of doxorubicin (Cao Na, 2007)

Despite Dox has been frequently used in the treatment of numerous human malignancies, the exact mechanism of the action is still somewhat unclear. Several mechanisms have been proposed and often subject to controversy. One

of the most popular explanations is the capability of Dox to inhibit DNA synthesis which may be due to:

- DNA intercalation or inhibition of DNA polymerase activity such as Topoisomerase II (Tanaka and Yoshida 1980; Gewirtz 1999),
- Effects on signaling issues of growth arrest and p53 function (Kastan, Onyekwere *et al*. 1991),
- Induction of enzymatic or chemically activated DNA adducts (Cullinane *et al*., 1994) and DNA cross-linking (Skladanowski and Konopa 1994),
- Interference with DNA strand separation and helicase activity (Fornari *et al*. 1994).

The use of Dox in cancer treatment is limited because it was reported that its achieving a chemotherapeutic dosage through systemic delivery due to the lake of specificity and selectivity of conventional delivery systems (Fan and Alekha, 2001), this resulted in a narrow therapeutic index and significant increases in the high dose distribution to healthy normal tissues (Jia *et al*., 2012) and among the major side effects of Dox which limit the clinical use of the drug are cardiotoxicity, myelosuppression and mucositis, low water solubility and inherent multi-drug resistance effects (Jia *et al*., 2012). Although DOX is one of most effective chemotherapeutic agents with a wide anticancer spectrum, its clinical use is still limited by the serious side effects including nausea, vomiting, mild alopecia, neutropenia, and the most serious one, cardiotoxicity. DOX can not only cause acute cardiovascular changes but also lead to life-threatening chronic effects such as hypotension, tachycardia, cardiac dilation and ventricular failure after several weeks or months and even years (Edward, *et al.*, 1973; Singal, *et al*., 2000).

On the basis of mechanisms of the action, the cardiotoxicity mechanisms of Dox can be attributed to free radical induction, Ca^{2+} overload accumulation, toxic doxorubicin metabolites, production of prostaglandins and platelet

activating factor, simulation of histamine release, and direct interaction with the actin-myosin contractile system (De Beer *et al.*, 2001).

Besides these serious irreversible side effects, other drawbacks also limit the clinical use of Dox is the multi-drug resistance (MDR) that is one of the serious limitations in the treatment of cancers through overexpression of MDR transporter proteins such as P-glycoproteins (P-gp) and multidrug resistance associated protein (MRP). P-gp and MRP can be overexpressed in malignant cells, as well as liver, kidney, and colon cells, to pump anticancer drugs out of the cancer cells, significantly restricting the intracellular level of the drug for effective therapy. Dox is the substrate of P-gp and MRP, which results in its shot half-life in circulation and low therapeutic efficacy (Krishna and Mayer 2000). Dox is a tetracyclic molecule, which is due to its structure exhibiting an intrinsic fluorescence (figure 2.10), belongs to that its containing three planar and aromatic hydroxyanthraquinone rings, which function as chromophore, (Sturgeon and Schulmann, 1977).

The basic structure of Dox molecule contains four cyclic groups, which make this molecule appropriate for chromphore functioning, which interacts with the base pair of DNA (Pigram *et al*., 1972; Aubel-Sardon and Londos Gagliardi, 1984), so, it's convenient for probing and visualization with various microscopic imaging technologies.

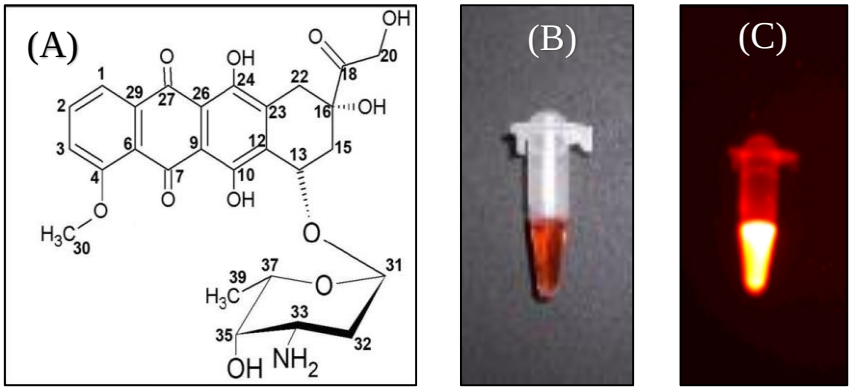


Figure (2.10) The structure and photographs of Dox:
A: Simple molecular structure of Dox, B: Photograph of solution of Dox in the ambient light. C: Fluorescence photograph of solution of Dox, λex = 480 nm, λem = 600 nm, exposition time: 6 seconds.

2.3 Enhanced permeability and retention (EPR) effect

Tumor vasculature is characterized as “leaky” due to its irregular-shaped, dilated, disorganized, and poorly-aligned endothelial cells (Modi, *et al*., 2006; Dreher *et al*., 2006; Fang *et al*., 2010). Additionally, poor lymphatic drainage results in leakage of plasma components from the circulation into the interstitial space of the tumor. This phenomenon, originally described by Matsumura and Maeda, is called "enhanced permeability and retention" (EPR) effect (Matsumura and Maeda, 1986). As seen in figure (2.11), normal, healthy vasculature displays continuous morphology where pores are 2-6 nm in size (Takakura *et al*., 1998).

Tumor vasculature has larger pores than the normal vessels that size ranges from 100 to 2000 nm (Hashizume *et al*., 2000; Hobbs, et al., 1998; Yuan *et al*., 1995). Increased cutoff pore size for tumor vasculature allows increased permeability of plasma proteins for tumor and lack of functional lymphatic vessels within tumor decreases the rate of clearance. The EPR effect now has become the “gold standard” in anticancer drug delivery that takes advantage of the unique anatomical-pathophysiological nature of the tumor blood vessels.

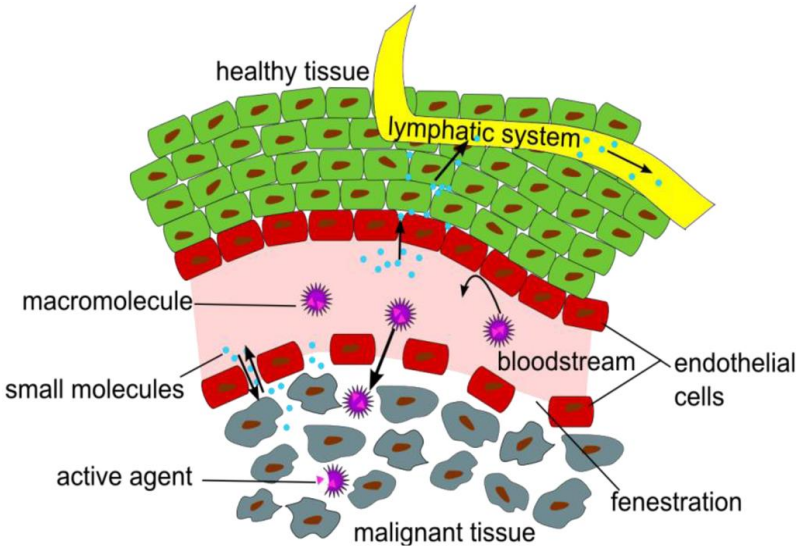


Figure (2.11) Illustration of the Enhanced Permeation and Retention (EPR) effect (Katharina *et al*., 2014)

The EPR effect is a molecular weight dependent phenomenon that only occurs in the tumor tissue. Particles larger than 40 kDa selectively leak out from the tumor vessel to accumulate in the tumor tissue. The increased accumulation of less than 40 kDa are due to prolonged circulation time and decreased clearance rate from the body. In attempt to prolong the drug residence time and selectively trap the nanoparticle in the tumor cells, poly ethylene glycol is commonly used to prevent rapid clearance of the nanoparticle by the reticuloendothelial system (Gupta *et al*., 2005).

2.4 Nanobiotechnology

Bionanotechnology and nanobiotechnology are terms that refer to the intersection of nanotechnology and biology (Ehud, 2007). Bionanotechnology and nanobiotechnology serve as blanket terms for various related technologies. These two terms are often used interchangeably. When a distinction is intended, though, it is based on whether the focus is on applying biological ideas or on studying biology with nanotechnology. Bionanotechnology generally refers to the study of how the goals of nanotechnology can be guided by studying how biological "machines" work and adapting these biological motifs into improving existing nanotechnologies or creating new ones(Nolting, 2005).

The first hint of nanotechnology can be traced back to a talk entitled “There’s Plenty of Room at the Bottom”, given by physicist Richard Feynman (Feynman, 1960). The term ‘Nanotechnology' was then coined and popularized in 1980's by K. Eric Drexler (Drexler, 1986). Nanotechnology is the exciting multidisciplinary field that involves the design and engineering of nanoobjects or nanotools 500 nanometers (nm) in size figure (2.12) (Ferrari, 2005).

The prefix “nano” is derived from the Greek word “dwarf”. One nanometer is equal to one billionth of a meter, that is, 10^{-9} m. Nanotechnology and nanoscience are widely seen as having a great potential to bring benefits to many areas of research and applications (Yan and Deng, 2005).

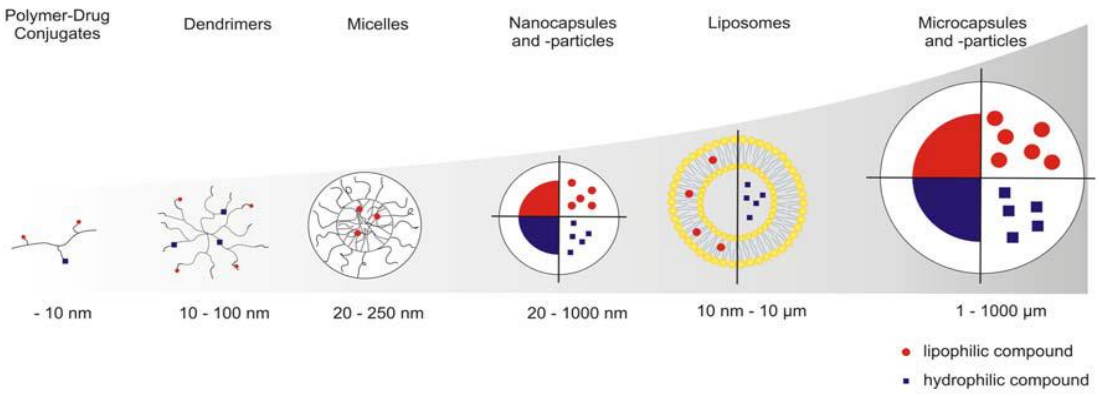


Figure (2.12) The nanoperspective (Hans *et al*., 2002)

Cancer nanotechnology seeks to characterize the interaction of nanoscale devices with cellular and molecular components specifically related to cancer diagnosis and therapy. The potential of cancer nanotechnology lies in the ability to engineer vehicles with unique therapeutic properties that, because of their small size, can penetrate tumors deeply with a high-level specificity.

The American Association for Cancer Research (AACR) website mentions that the component size for nanotechnology ranges from 5 to 500 nanometers (Ahmad, 2013). Although there is still no strict definition of the size range for nanoparticles, particles with size from a few nanometers to a few hundred nanometers have been commonly considered as nanoparticles**.**

Nanomanufacturing methods can be classified according to whether their assembly into two approaches i) the so called 'bottom-up' approach, where smaller components of atomic or molecular dimensions self-assemble together, according to a natural physical principle or an externally applied driving force, to give rise to larger and more organized systems (Grill *et al*., 2007), or ii) the 'top-down' approach, a process that starts from a large piece and subsequently uses finer and finer tools for creating correspondingly smaller structures. These two approaches are schematically presented in figure (2.13) (Pease and Chou, 2008).

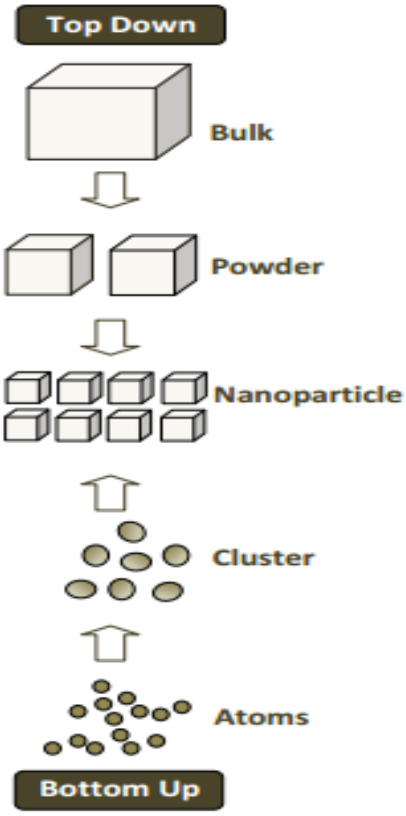


Figure (2.13) Schematic representation of the formation of nanostructures via the to down and bottom-up approaches (Pease a Chou, 2008).

Drug delivery is one of the most promising fields of utility for nanotechnology. So, in the field of drug delivery for cancer therapy, with the aid of nanotechnology, researchers are resolving the numerous challenges facing the efficient delivery of chemotherapy drugs for effective cancer therapy at an unprecedented speed (Chen, 2009).

2.5 Drug delivery system for cancer therapy

In order to overcome the limitations of the traditional chemotherapeutic, the drug delivery technology was designed and by using suitable carriers that can efficiently encapsulate anticancer drugs, overcome drug-resistance, and increase selectivity of drugs towards cancer cells while eliminating their

toxicity to normal tissues, anticancer drugs should be delivered by molecular carrier systems which are: hydrophilic (Bourzac, 2012), biocompatible and nontoxic; exhibit prolonged circulation in the blood stream that have sustained delivery property, and have higher selectivity and affinity to tumor cells than healthy cells (Seymour *et al*., 2005).

Drug delivery and related pharmaceutical development in the context of nanomedicine should be viewed as science and technology of nanometer scale complex systems (10–1000 nm), consisting of at least two components, one of which is a pharmaceutically active ingredient (Duncan 2003; Ferrari 2005).

Although the definition identifies nanoparticles as having dimensions below 1000 nm or 100 nm, especially in the area of drug delivery relatively large (size >100 nm) nanoparticles may be needed for loading a sufficient amount of drug onto the particles (Duncan 2003). Drug delivery systems have been developed to generate new therapeutic systems with better treatment efficacy and lower side effect, so, numerous drug delivery systems have been developed with different designs including liposomes, micelles, nanoparticles, polymer-drug conjugates, dendrimer, silica nanoparticle, carbon nanotubes, and metallic particles (Shapira *et al*., 2011) figure (2.14).

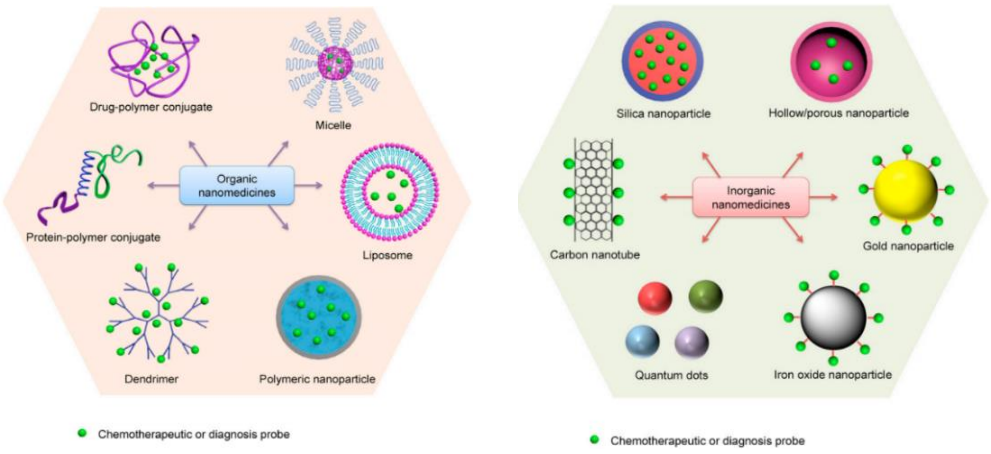


Figure (2.14) Examples of nanomedicines for cancer diagnosis and Therapy (Tang, and Cheng, 2013)

Although the designs and materials of these delivery systems are different, they are all developed based on the same aims which are able to deliver the right dose of drugs in the active condition to the targeted tissues without causing side-effects or drug resistance to tumor cells.

The carriers used for drug delivery system have four unique properties that distinguish them from other cancer therapeutics as described by Heath and Davis:

1) by itself ,have therapeutic or diagnostic properties and which can carry a complex and highly concentrated therapeutic,
2) Biodegradability, biocompatibility and low toxicity.
3) Can be attached to multivalent targeting ligands which yield high affinity and specificity for target cells; cancer therapy, either simultaneously or serial.
4) Can bypass multiple drug resistance mechanisms typical for traditional chemotherapeutics. (Heath and Davis 2008).

The therapeutic index of nearly all drugs currently being used could be improved if they were more efficiently delivered to their biological targets through appropriate application of nanotechnological tools (Sahoo and Labhasetwar 2003; Vasir *et al.*, 2005). On the other hand, those drugs that have previously failed clinical trials because of toxicity concerns may be re-examined using nanoparticulated preparations (Kipp, 2004).The first drug-loaded carrier (Doxil) was approved in 1995 using polyethylene glycol (PEG) modified-liposome to encapsulate Dox. Doxil was designed with 100 nm size, hence it is delivered selectively to tumor tissues while excluding from the healthy tissues. By encapsulating Dox into nano-carriers, the serious side-effects caused by the toxicity of Dox had been reduced. As the result, the heart damage incidence of Doxil treated patients had been reduced by 3 times as compared with that of traditional Dox treated patients (Bourzac, 2012).

At the tumor level, the accumulation mechanism of the drug delivery system relies on the diffusion or convection across the leaky tumor vasculature. As presented in figure (2.15), drug-loaded carriers with nano-size have higher accumulation into cancer tissues by the enhanced permeability and retention (EPR) effect due to the leaky blood vessels and the dysfunctional lymphatic drainage of tumors (Matsumura, and Maeda, 1986; Maeda, 2000; Maeda, 2001; Peer *et al*., 2007).

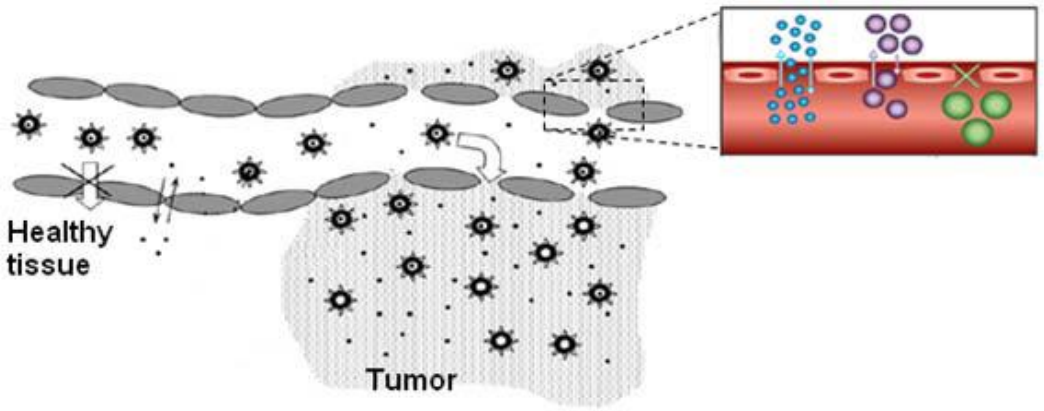


Figure (2.15) Schematic of delivery mechanism of drug-loaded carriers to tumor cells (Peer *et al*., 2007; Torchilin , 2010)

2.5.1 Types of nanoparticles that used as drug delivery systems

Nanoparticles applied as drug delivery systems are sub-micron sized Particles (about 3-200 nm), devices, or systems that can be made using a variety of materials including polymers (polymeric nanoparticles, micelles, or dendrimers), lipids (liposomes), viruses (viral nanoparticles), and even organometallic compound (nanotubes) (Cho *et al*., 2008):

2.5.1.1 Polymer-based drug carriers

Depending on the method of preparation, the drug is either physically entrapped in or covalently bound to the polymer matrix (Rawat *et al*., 2006). The resulting compounds may have the structure of capsules (polymeric

nanoparticles), amphiphilic core/shell (polymeric micelles), or hyperbranched macromolecules (dendrimers) (figure 2.16), polymers used as drug conjugates can be divided into two groups of natural and synthetic polymers:

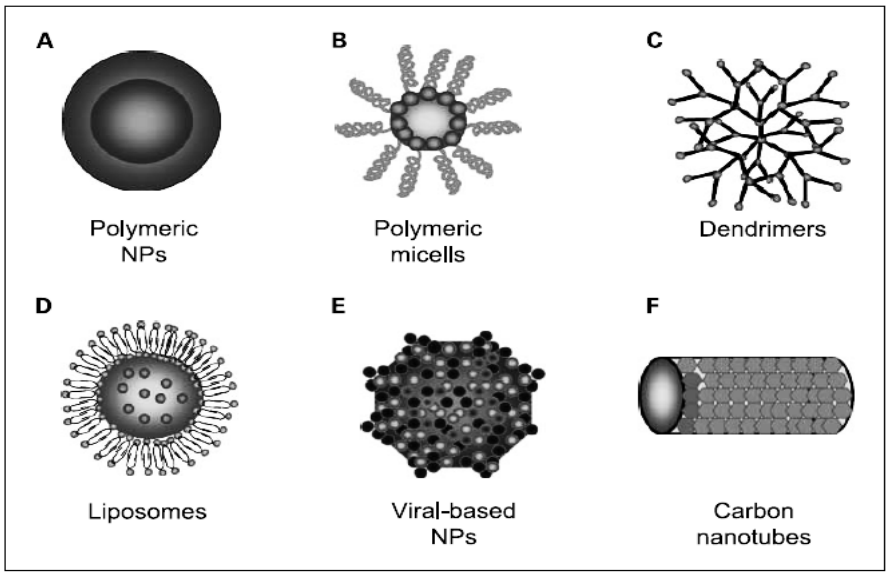


Figure (2.16) Types of nanocarriers for drug delivery (Cho *et al*., 2008).

2.5.1.2 Polymeric nanoparticles (polymer-drug conjugates)

Polymers such as albumin, chitosan, and heparin occur naturally and have a material of choice for the delivery of oligonucleotides, DNA, and protein, as well as drugs. Recently, a nanoparticle formulation of paclitaxel, in which serum albumin is included as a carrier (nanometer-sized albuminbound paclitaxel (Abraxane)); (figure 2.16 A), has been applied in the clinic for the treatment of metastatic breast cancer (Gradishar *et al*., 2005). Besides metastatic breast cancer, Abraxane had also been evaluated in clinical trials involving many other cancers including non–small-cell lung cancer (phase II trial) and advanced non-hematologic malignancies (phase I and pharmacokinetics trials(Nyman *et al*., 2005 ;Green *et al*., 2006) . Among synthetic polymers such as N-(2-hydroxypropyl)- methacrylamide copolymer

(HPMA), polystyrene-maleic anhydride copolymer, polyethylene glycol (PEG), and poly-L-glutamic acid (PGA),poly caprolacton (PCL), PGA was the first biodegradable polymer to be used for conjugate synthesis. Several representative chemotherapeutics that are used widely in the clinic had been tested as conjugates with PGA *in vitro* and *in vivo* and showed encouraging abilities to circumvent the shortcomings of their free drug counterparts (Li, 2002). Among them, Xyotax (PGA paclitaxel) (Sabbatini *et al*., 2013) and CT-2106 (PGA-camptothecin) (Bhatt *et al*., 2003) are now in clinical trials. HPMA and PEG are the most widely used non-biodegradable synthetic polymers (Duncan, 2003).

PK1, which is a conjugate of HPMA with doxorubicin, was the synthetic polymer-drug conjugate to be evaluated in clinical trials as an anticancer agent. A phase I clinical trial had been completed in patients with a variety of tumors that were refractory or resistant to prior therapy such as chemotherapy and/or radiation (Vasey *et al*., 1999).

2.5.1.3 Dendrimers

A dendrimer is a synthetic polymeric macromolecule of nanometer dimensions, which is composed of multiple highly branched monomers that emerge radially from the central core (figure 2.16 C). Properties associated with these dendrimers such as their monodisperse size, modifiable surface functionality, multivalency, water solubility, and available internal cavity make them attractive for drug delivery. The easily modifiable surface characteristic of dendrimers enables them to be simultaneously conjugated with several molecules such as imaging contrast agents, targeting ligands, or therapeutic drugs, yielding a dendrimer based multifunctional drug delivery system (Svenson and Tomalia, 2005). Polyamidoamine dendrimer, the dendrimer most widely used as a scaffold, was conjugated with cisplatin (Malik *et al.*, 1999).

2.5.1.4 Lipid-based drug carriers

Liposomes are self-assembling closed colloidal structures composed of lipid bilayers and have a spherical shape in which an outer lipid bilayer surrounds a central aqueous space (figure 2.16 D). Currently, several kinds of cancer drugs have been applied to this lipid-based system using a variety of preparation methods. Among them, liposomal formulations of the anthracyclines doxorubicin (Doxil, Myocet) and daunorubicin (DaunoXome) are approved for the treatment of metastatic breast cancer and AIDS-related Kaposi's sarcoma (Markma, 2006; Rivera, 2003; Rosenthal *et al*., 2002). Besides these approved agents, many liposomal chemotherapeutics are currently being evaluated in clinical trials (Hofheinz *et al.*, 2005). The next generation of liposomal drugs may be immunoliposomes, which selectively deliver the drug to the desired sites of action (Wu *et al*., 2006).

2.5.1.5 Viral nanoparticles

A variety of viruses including cowpea mosaic virus, cowpea chlorotic mottle virus, canine parvovirus, and bacteriophages have been developed for biomedical and nanotechnology applications that include tissue targeting and drug delivery (figure 2.16 E). A number of targeting molecules and peptides can be displayed in a biologically functional form on their capsid surface using chemical or genetic means. Therefore, several ligands or antibodies including transferrin, folic acid, and single-chain antibodies have been conjugated to viruses for specific tumor targeting in *vivo* (Manchester and Singh, 2006). Besides this artificial targeting, a subset of viruses, such as canine parvovirus, have natural affinity for receptors such as transferrin receptors that are up-regulated on a variety of tumor cells (Singh *et al*., 2006).

2.5.1.6 Carbon nanotubes

Carbon nanotubes are carbon cylinders composed of benzene rings (figure 2.16 F) that have been applied in biology as sensors for detecting DNA and protein, diagnostic devices for the discrimination of different proteins from serum samples, and carriers to deliver vaccine or protein (Bianco *et al*., 2005). Carbon nanotubes are completely insoluble in all solvents, generating some health concerns and toxicity problems. However, the introduction of chemical modification to carbon nanotubes can render them water-soluble and functionalized so that they can be linked to a wide variety of active molecules such as peptides, proteins, nucleic acids, and therapeutic agents (Bianco *et al*., 2005). Antifungal agents (amphotericin B) or anticancer drugs (methotrexate) have been covalently linked to carbon nanotubes with a fluorescent agent. In an *in vitro* study, drugs bound to carbon nanotubes were shown to be more effectively internalized into cells compared with free drug alone and to have potent antifungal activity (Pastorin *et al*., 2006).

2.5.1.7 Polymeric micelles (amphiphilic block copolymers)

In the science of nanomedicine, one of the most useful modalities for efficient drug delivery is the micelle. Micelles are block copolymers that assemble themselves (self-assembly), with sizes ranging between 10 to 500 nm. They are characterized by their hydrophobic core and hydrophilic shell structure. The shell is usually poly ethylene glycol (PEG) whereas the hydrophobic drug is loaded into the core. Micelles display an advantage over other nanocarriers due to their easy method of preparation, simplicity of drug loading into the core, stability, and the fact that drug release from micelles can be controlled with relative ease and their thermodynamic stability *in vitro* and *in vivo*. This stability does not allow for rapid dissociation, which is advantageous. Furthermore, micelles have a long circulation time in blood and their small size allows them to escape renal excretion, while passive targeting

ability to tumor tissues (Chen, 2006) and successfully extravasating in the tumor (Husseini and Pitt, 2008) and.

Generally, in comparison with other drug delivery methods, these biodegradable polymer systems can keep drug levels at an optimum range over a longer period of time, which increase the efficacy of the drug and maximize patient compliance, biodegradable polymer NPs can enhance the ability to use highly toxic, poorly soluble, or relatively unstable drugs. A combination of targeted delivery with controlled release technology would significantly benefit to targeted therapeutic approaches, thus allowing for a large amount of drug to be delivered to cancer cells (Ferrari 2005). The advantages of block copolymer micelles include their nanosize, biodegradability, biocompatibility, long-circulation times, controllable drug-release profile, and tissue penetrating ability, currently, polymeric micelles are popular pharmaceutical carriers for the delivery of anticancer drugs due to their advantages over other systems (Mu *et al*., 2005).

They are small size with a narrow distribution that is considered ideal for stable and long term circulation in the blood stream because it evades the RES uptake Moreover, while remaining stable in the blood over a long time period, the carriers are small enough to pass through small blood vessel pores of less than 400 nm (Jain, *et al*., 2002). Polymeric micelles are an effective delivery system in term of the enhanced permeability and retention (EPR) effect and overcoming the RES system (Bae and Kataoka, 2005). The second advantage is the high static and dynamic structural stability (Calderara *et al*., 1994; Wang *et al*., 1995). The functional properties of micelles are based on amphiphilic block copolymers, which assemble to form a nano-sized core/shell structure in aqueous media (figure 2.16 B). The hydrophobic core region serves as a reservoir for hydrophobic drugs, whereas the hydrophilic shell region stabilizes the hydrophobic core and renders the polymers water-soluble, making the particle an appropriate candidate for i.v. administration (Adams *et al*., 2003).

The drug can be loaded into a polymeric micelle in two ways: physical encapsulation (Batrakova *et al*., 1996) or chemical covalent attachment (Nakanishi *et al*., 2001). Micelles prepared from synthetic biodegradable block copolymers are widely applied in drug delivery system (DDS) owing to their intrinsic core–shell architecture which demonstrates a series of attractive properties for increasing drug solubility, enhancing drug stability, and passive target effects (Croy and Kwon, 2006).

The first polymeric micelle formulation of paclitaxel, Genexol-PM (PEG-poly (D,L-lactide)-paclitaxel), is a cremophor- free polymeric micelle-formulated paclitaxel. A phase I and pharmacokinetic study has been conducted in patients with advanced refractory malignancies (Kim *et al*., 2004). Multifunctional polymeric micelles containing targeting ligands and imaging and therapeutic agents are being actively developed (Nasongkla *et al*., 2006).

2.5.2 Block copolymers as drug delivery systems

Block copolymers are defined as polymers that have two or more blocks or segments arranging in the main chain and can be classified according to their architecture (figure 2.17) as AB-type diblock, ABA- or BAB-type triblock, and multiblock, where A represents the soluble block in a selected solvent and B designates the insoluble block (Kumar *et al*., 2001), Because of the intrinsic affinity interactions of those segments with the same physicochemical properties, block copolymers often show a tendency to form self-assemblies in solvents. However, block mobility is quite restricted for steric reasons, and the self-assembled domains composed of identical blocks consequently fall into nano-or microsized scale and are segregated into the most stabilized state. The detailed features of self-assembled domains are sensitive to the architecture of the block copolymer.

It is practical to tune the physicochemical properties of the polymer to give a functionalities of either the core or the surface of such a self-assembly. Block

copolymers self-assemble into micelles showing promise as long circulating vehicles for drug delivery (Bae and Kataoka, 2005).

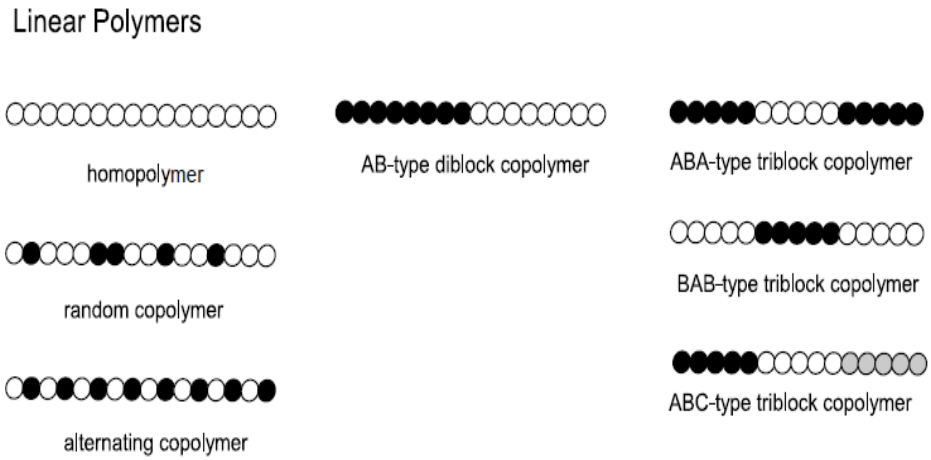


Figure (2.17) Linear copolymeric architecture

Amphiphilic block polymers specifically refer to those having both hydrophilic and hydrophobic blocks in the same polymer chain, which can then build spherical polymeric assemblies in aqueous solution, called "polymeric micelles", with nanosized and core-shell segregated domains. Studies on polymeric micelles were initiated in 1960s. But the first attempt to utilize block copolymer micelles as drug carriers was not reported until 1984 by Bader (Bader *et al.*, 1984) and Pratten *et al.* (Pratten *et al* 1985). Unlike homopolymers made of identical monomeric units, copolymers include two kinds of monomeric units of different solubility. Thus, in solution and at low concentration, these amphiphilic molecules exist as unimers, while at increasing concentrations, aggregation takes place. The final aggregates (called micelles), characterized by spherical shape when the hydrophilic segment is longer than the core block (Zang and Eisenberg, 1995).

In past decades, the application of the synthetic copolymers in drug delivery system (DDS) as drug carriers led to technological advances which bypassed the pharmacokinetic limitations of conventional, rapid release dosage forms.

The improved DDS based on synthetic polymers generally appears in three types, micro/nanoparticles, implants (containing hydrogels) and fibers (Lin *et al*., 1999; Kopecek *et al*., 2001; Takeuchi *et al*., 2004; Ideta *et al*., 2005; Lin *et al*., 2006; Cai *et al*., 2007; Nakayama *et al*., 2007).

2.5.2.1 Polycaprolactone

Polycaprolactone or Poly-ε-caprolactone (PCL) with chemical formula $(C_6H_{10}O_2)_n$ (figure 2.18) was one of the earliest polymers synthesized by the Carothers group in the early 1930s (Van Natta *et al*., 1934). It became commercially available following efforts to identify synthetic polymers that could be degraded by microorganisms (Huang, 1985). PCL is a hydrophobic, semicrystalline polymer; its crystallinity tends to decrease with increasing molecular weight. The good solubility of PCL, its low melting point (59-64 Cº) and exceptional blend-compatibility has stimulated extensive research into its potential application in the biomedical field (Chandra and Rustgi, 1998; Okada, 2002; Nair, 2007).

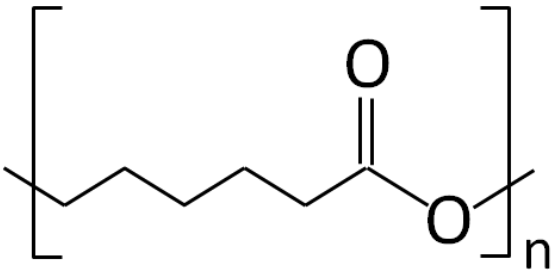


Figure (2.18) Chemical structure of Poly Caprolactone (PCL)

PCL is suitable for controlled drug delivery due to a high permeability to many drugs excellent biocompatibility and its ability to be fully excreted from the body once bioresorbed. Biodegradation of PCL is slow in comparison to other polymers, so it is most suitable for long-term delivery extending over a period of more than one year. PCL also has the ability to form compatible

blends with other polymers which can affect the degradation kinetics which fulfill desired release profiles (Merkli, *et al*., 1998; Freiberg, 2004; Sinha *et al*., 2004). PCL, a semi-crystalline linear resorbable aliphatic polyester, is subjected to biodegradation because of the susceptibility of its aliphatic ester linkage to hydrolysis (Lu *et al*., 2009). PCL is one of the most promising synthetic polymers which degrades in aqueous media or when in contact with microorganisms and thus can be used to make stable polymeric devices (Jarrett *et al*., 1984). The addition of hydrophilic polyether blocks to PCL chains has been used to enhance hydrophilicity compared to the parent homopolymer. Poly (ethylene glycol) (PEG) has been used to form various block copolymers with PCL (Li *et al*., 1998).

2.5.2.2 Poly ethylene glycol (PEG)

Poly ethylene glycol (PEG) with chemical formula $C_{2n}H_{4n+2}O_{n+1}$ (figure 2.19) is currently the most used polymer in the biomedical field of drug delivery and the only polymeric therapeutic that has market approval for different drugs. The success of PEG is based on its hydrophilicity, decreased interaction with blood components, and high biocompatibility. However, scientific results obtained in recent years show that it may also have possible drawbacks, such as interaction with the immune system, possible degradation under stress, and accumulation in the body above an uncertain excretion limit (Knop *et al*., 2010)

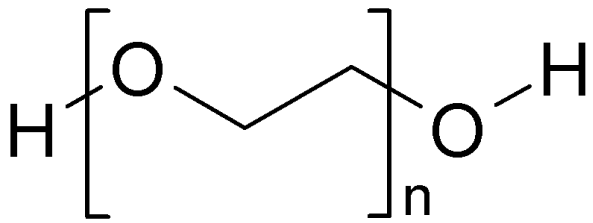


Figure (2.19) Chemical structure of Poly ethylene glycol (PEG).

For its hydrophilicity, nontoxicity and absence of antigenicity and immunogenicity, PEG can be selected to be attached to PCL, forming PCL–PEG copolymers. Thus, their hydrophilicity, biodegradability and mechanical properties can be improved and may find much wider applications (Moon *et al*., 2002; Huang *et al*., 2004). Even though the use of micelle-forming amphiphilic polymers as drug-delivery vehicles was already proposed by Ringsdorf *et al*. in the 1970s, Kabanov *et al*. were the first whose propose the use of PEG as a hydrophilic part of linear block copolymers for micellization in 1989 (Kabanov, *et al*., 1989).

There are several reasons for using PEG in polymeric micelles drug delivery system (PMDDS): it is non-toxic, it is one of the few synthetic polymers already approved by FDA for use in the drug products, in aqueous environment, PEG is highly hydrated and can move rapidly to sweep out a large exclusion volume. This particular properties has generated a lot of excitement in delivery of highly potent compounds such as anticancer agents, which would benefit in terms of efficacy and safety profiles (Lu and Park, 2013).

2.5.2.3 Polyethylene glycol-Polycaprolactone Copolymers in drug delivery system

PCL and PEG have been widely used in biomedical field, because they are materials that are biocompatible and have been used in several FDA approved products. Since Perret and Skoulios (1972) firstly prepared a series of block copolymers containing PEG and PCL, these copolymers consisting of PCL blocks and PEG blocks have been widely studied, due to the integration of respective advantages of PEG and PCL, PEG–PCL copolymers might have even wider applications in biomedical field. (Li *et al*., 2006). Generally, amphiphilic block copolymers composed of hydrophilic and hydrophobic segments can form a micelle-like structure with a hydrophobic inner core and a hydrophilic outer shell in selective solvent (Tanodekaew *et al*., 1997).

In PCL/PEG polymeric micelles, hydrophobic core formed by PCL is surrounded by water-soluble polar groups of PEG that extended into an aqueous medium. Therefore, the drugs with a hydrophobic character can be easily incorporated into the core of nanoparticles by covalent or non-covalent bonding through hydrophobic interactions in aqueous media (Singh and Muthu, 2007). The core-shell structure give the triblock copolymers with several attractive features:

- Convenient preparation and well-defined structure.
- Favorable stability under physiological neutral conditions.
- Cleavability in the acidic environment, leading to an efficient drug release inside tumor cells, and Good biocompatibility (Wang *et al*., 2013).

Nanoparticles can look like viruses to the immune system, and they may be rapidly taken up by cells of the mononuclear phagocyte system (MPS), part of the body's defense against invasion by bacteria, and viruses. Uptake by MPS cells can cause intravenously injected nanoparticles to be shuttled to the liver and spleen, preventing them from delivering their chemotherapeutic payloads to tumors, so, the researcher think about nanoparticles in terms of two fundamental components: the core, which doesn't interact with the environment, and the surface layer or "corona," which does. So, the hydrophobic core is a key component in determining the micelle's capacity to solubilize a poorly water-soluble compound (Grossman and McNeil, 2012).

Most cell membranes have a net negative charge, so, nanoparticles with cationic coronas may have an easier time to get into the cells and to deliver their payload. But they may also bind more readily to healthy cells. So instead, researchers commonly coat their nanoparticles with polyethylene glycol (PEG), a charge-neutral molecule that reduces both protein binding and MPS uptake, and thus increases the length of time that the particles circulate in the blood and the likelihood of their reaching to its target, also the length of the PEG polymer

chain and the density of PEG coating both affect nanoparticle protein binding and distribution in the body (Jokerst *et al*., 2011).

Micelles composed of MPEG-PCL of about 20nm in size were obtained that used for drug encapsulation of poorly water-soluble drugs ketoprofen and furosemide were evaluated (Dwan'isa *et al*., 2008). Also some studies on enhancing the solubility of geldanamycin with MPEG–PCL micelles were reported (Forrest *et al*., 2006) which inhibits heat shock protein 90 (Hsp90) and has shown significant *in vivo* antitumor activity. There were other studies which have concerned with PCL/PEG copolymers as carriers for hydrophobic drugs. Wei *et al*. (2009) found a significant difference between rapid releases of free honokiol and much slower and sustained release of honokiol (HK) loaded PCL–PEG–PCL micelles in the *in vitro* release tests. Besides, micelles of MPEG–PCL as vehicles for the solubilization and controlled delivery of cyclosporine A were prepared by Aliabadi *et al*. (2005).

With the same material, solubilization of fenofibrate was achieved (Jette *et al*., 2004). Self-assembled micelles encapsulating ketoprofen and furosemide were obtained using PEG-PCL modified with trimethylene carbonate (Latere *et al*., 2008). In addition to that, MPEG–PCL nanoparticles containing hydrophilic drug doxorubicin were also prepared to optimize drug releasing profiles (Hsieh *et al*., 2008; Yadav *et al*., 2008). PCL-PEG-PCL triblock copolymers have been also utilized for protein delivery, in fact, the release behaviors of two model proteins, including bovine serum albumin (BSA) and horseradish peroxidase (HRP), from a gel-forming controlled drug delivery system were studied in detail(Ma *et al*, 2010).

Furthermore, in 2009, Gong *et al.* studied a PEG-PCL-PEG hydrogel system for basic fibroblastic growth factor (bFGF) antigen delivery. Another study conclude that the biodegradable (PCL-PEG-PCL) copolymers were loaded with curcumin were produced as a novel drug-delivery method (Feng *et al*., 2012).

Synthesis and Characterization of PEG–PCL–PEG Triblock Copolymers as Carriers of Doxorubicin for the Treatment of Breast Cancer were studied by Nguyen (Nguyen *et al*., 2010).

2.5.3 Drug delivery strategies

Traditional cancer treatments often kill healthy cells and cause toxicity to the patient (figure 2.20 A). Therefore, two main mechanisms of delivery of drug-loaded nanoparticles to tumors have been reported (Figure 2.20):

(i) Passive targeting through leaky vasculature surrounding the tumors, described as the enhanced permeability and retention effect (figure 2.20 B).

(ii) Active targeting by grafting specific ligands of cancer cells or angiogenic endothelial cells to the surface of the nanocarrier (figure 2.20 C).

Passive targeting exploits the characteristic feature of tumor biology that allows carriers to accumulate in the tumor by the enhanced permeability and retention (EPR) effect. Active approaches achieve this by conjugating carriers containing chemotherapeutics with molecules that bind to overexpressed antigens or receptors on the target cells (Prakash *et al*, 2011).

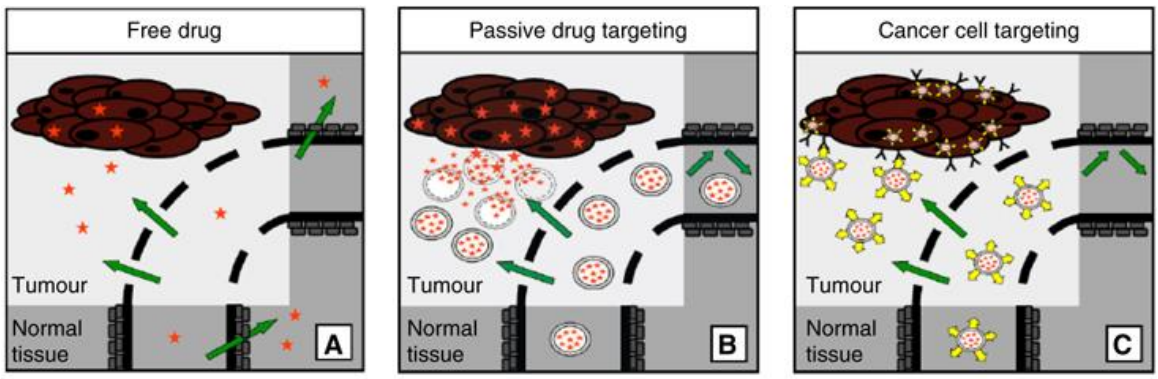


Figure (2.20) Strategy for drug delivery system (Lammers *et al.*, 2008)

Passive targeting refers to the extravasation of the nanomedicine-associated drug into the interstitial fluid at the tumour site, exploiting the locally increased vascular permeability (figure 2.20 B). In addition, solid tumours tend to lack functional lymphatics, and extravasated nanomaterials are retained within the tumour site for prolonged periods of time. The exploitation of this so-called 'enhanced permeability and retention' (EPR) effect is currently the most important strategy for improving the delivery of low molecular weight chemotherapeutic agents to tumours (Maeda *et al*, 2000; Torchilin, 2005).

2.6 Multimodal microscopy for drug delivery monitoring and cancer tissue imaging

For decades, microscope has been a key tool in the investigation of biological processes, imaging of cellular structures and the localization of molecules within cells, and the identification of different molecular species on the microscopic scale is still a considerable challenge in many areas of biology, but do not provide chemical specificity. Many of these techniques, however, require the use of exogenous labels that often disturb the system of interest (Zipfel *et al*., 2003).

With its distinguished capabilities of three-dimensional (3D) sectioning with tightly focused excitation and larger penetration depth due to the use of IR wavelength excitations, two-photon excitation fluorescence (TPEF) is the most widely employed nonlinear optical (NLO) imaging technique by utilizing intrinsic fluorescence or extrinsic fluorescent labels and has become a strong tool for various studies in biology. In addition to these imaging modalities, third-order NLO microscopy based on coherent anti-Stokes Raman scattering (CARS) allows chemically selective imaging (Mouras, 2010).

As a highly promising imaging tool, CARS microscopy provides a label-free and fast method with 3D sectioning capabilities. It has been widely applied to visualize morphological as well as chemical contrast in tissue samples

without labeling or staining. The multimodal imaging approach has a great potential in diagnosis of diseases like cancer and arterial disease, and provides a fast-track method to detect and evaluate drug response with high temporal and spatial sensitivity, which is crucial to understanding the drug action and subsequent response of the cell.

Multimodal microscopy (figure 2.21) has been used to visualize living cells with contrast of different vibrational modes, including the phosphate stretch vibration (DNA), amide I vibration (protein) (Cheng *et al*., 2002), OH stretching vibration (water) (Dufresne *et al*., 2003), and the CH group of stretching vibrations (lipids) (Nan *et al*., 2006). Among these modes, the signal from lipids is so high that single phospholipid bilayers can be visualized (Potma and Xie 2005).

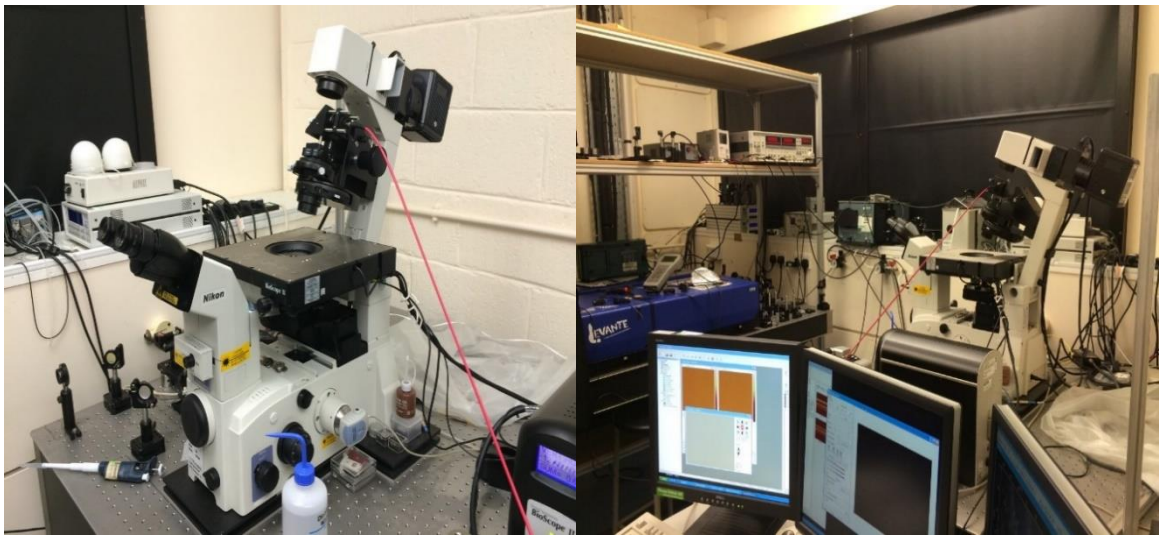


Figure (2.21) CARS system (photo was taken by author in CARS laboratory/center for bioengineering at university of Edinburgh/Scotland/UK 2014).

A study by Mouras (Mouras *et al*., 2010) showed that NLO microscopy is able to monitor Dox delivery in chemo-sensitive MCF-7 wild-type living cells from their chemo-resistant variants MCF-7 and this study demonstrates the potential of NLO for real-time imaging of drugs and cancer diagnosis.

Chapter Three

Materials and Methods

3.1 Materials

3.1.1. Equipment and apparatuses

The following equipment and apparatuses were used in this study:

Equipment and apparatus	Company (origin)
Centrifuge	Eppendorf (Germany)
Coherent Anti-stock Raman Scattering spectroscope (CARS) and Two photon excitation fluorescence(TPEF) multimodal microscopy	The system was set by lab manager (England)
Distillator	Gallenkamp (England)
Hematocytometer	Hausser Scientific (USA)
Hot Plate with Magnetic Stirrer	Gallenkamp (England)
Incubator with CO_2	Sanyo (Japan)
Inverted Microscope	Olympus (USA)
Laminar Air Flow cabinet	Air clean (USA)
Laser source	PicoTrain, High-Q laser (USA)
Micropipette	Eppendorf (Germany)
Millipore pore filter unit	Milipore (England)
Modulus Microplate Reader	Turner BioSystems (USA)
Multichannel Pipette	Eppendorf
pH Meter	Bibby Scientific (England)
photodiode	Perkin Elmer (Canada)
photomultiplier tubes	Hamamatsu R3896 (Japan)
Refrigerator	Fisher scientific (England)
Sensitive Balance	Mettler (USA)
Short-pass and band-pass filters	Chroma (USA)
Transmission electronic microscope	Hitachi (Japan)
Vortex	Grant Bio (England)
Water Bath	Gallenkamp (England)
Zetasizer 3000HSA	Malvern Instruments Ltd. (England)

3.1.2 Tissue culture vessels

Plastic ware and glass ware type	**Supplier (origin)**
5ml single-use glass sterile pipettes (2 ml, 5 ml, 10 ml, 25 ml and 50 ml	Fischer scientific company (UK)
beakers (50,100,200,500 and 800 ml)	
bottles (75,200 and 1000 ml)	
cylinders	
Disposable sterile plastic tissue culture flasks with different surface areas (T-25 cm^2 and T-75 cm^2)	
funnels	
intracel glass bottom dishes	
multi-well plates with flatted bottom and fluorescence multi-well plates (96-well,48-well plate,12-well plate)	
Pasture pipettes	
polystyrene conical tubes 15ml and polystyrene round-bottom tubes	
sterile polypropylene conical tubes (15 ml and 50 ml)	
volumetric flasks (50,250 and 5000 ml)	
Syringes. (1, 10 and 20ml)	Millipore (Ireland)
syringe driven filters (0.22 and0.45 μm	
Eppendorf tubes (100,200,500 and 1000 μl	Eppendorf (Germany)
amicon ultra	Millipore corporation (USA)

3.1.3 Chemicals

chemical	supplier
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)	Fisher scientific (England)
Acetone	Fisher scientific
Dibasic sodium phosphate anhydrous	Fisher scientific
Dipotassium phosphate (K_2PO_4)	Fisher scientific
Disodium hydrogen phosphate (NA_2HPO_4)	Fisher scientific
Doxorubicin	Sigma Aldrich (USA)
Dulbecco's Modified Eagle Medium (DMEM)	Sigma Aldrich
Dulbecco's Phosphate-Buffered Saline (DPBS)	Sigma Aldrich
Fetal Bovine Serum	Sigma Aldrich
Glutamine	Sigma Aldrich
Hydrochloride acid (HCL)	Fisher scientific
Isopropanol	Fisher scientific
Monobasic potassium phosphate KH_2PO_4	Fisher scientific
Nondet P-40 (NP40)	Fisher scientific
Phosphate Buffer Saline	Fisher scientific
Poly caprolacton(PCL) $_{5000}$- Poly ethylene glycol (PEG) $_{10000}$- Poly caprolacton (PCL) copolymer $_{5000}$	Polysciences (USA)
Trypan blue	Sigma Aldrich
Trypsin	Fisher scientific
Virkon	Fisher scientific

3.1.4 Buffer

Sorenson's buffer was prepared as a stock solutions and it was combined prior to use to achieve desired pH as the following:

a. **For NA_2HPO_4 preparation (solution A):** by adding (1.889)g of dibasic NA phosphate anhydrous into 100 ml of distilled water.
b. **For KH_2PO_4 preparation (solution B):** by adding (1.186)g monobasic K-phosphate into 100 ml of distilled water.

3.1.5 Dye

Stock solution of Trypan blue was prepared by adding (1)g of Trypan Blue stain in 100 ml of phosphate buffer saline (PBS) solution pH (7.2-7.3), then sterilized by filtration through 0.45 µm filter unit. After that, stored at 4 ˚C, and then it was diluted (1:10) in PBS as working solution.

3.1.6 Reagents and solutions

1. **MTT Solution:** MTT stock solution was prepared by adding (5)mg/ml of MTT in DMSO. This solution is sterilized by filtration with 0.45 µm filter unit after adding MTT to remove insoluble residue. MTT working solution was prepared with 1:10 dilution of stock in DMSO.
2. **MTT Solvent:** It was prepared to solubilized the MTT solution by adding (800) µl of HCl (0.04 N) and 200 µl 0.1% Non det P-40 (NP40) in (14) ml of absolute isopropanol.

3.1.7 Preparation of tissue culture media

Breast cancer cell line MCF-7 cells was cultured in complete liquid form of the high glucose version of Dulbecco's modified Eagle's medium (DMEM) supplied from (Sigma Aldrich, Germany) that was prepared as follows:

- 50 ml of DMEM.
- 30 ml of fetal bovine serum.
- 5 ml of L-glutamine.
- 1 ml of streptomycin/penicillin.

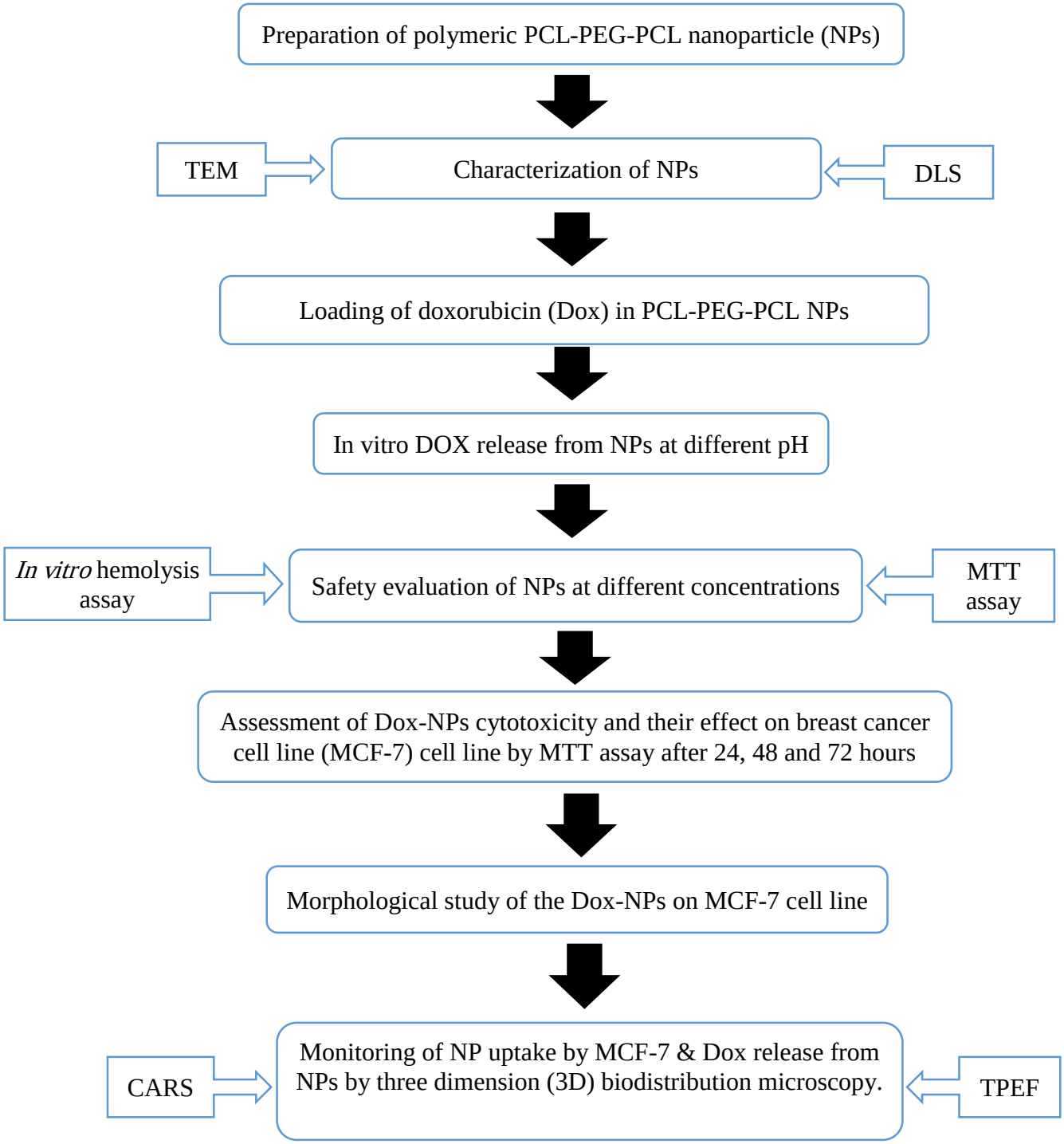
all components were mixed together, The medium was replaced twice per a week. The cells were grown in T25 flasks in complete medium and passaged by trypsinization when confluent (70%-80%), and check them with inverted microscope. The cell culture was maintained in a 37 ºC incubator with a humidified 5% CO_2-containing atmosphere.

3.1.8 MCF-7 cell line

Human breast carcinoma cell line (MCF-7) was obtained from the European Collection of Cell Cultures and cultured according to standard mammalian tissue culture protocols and sterile techniques. A breast cancer cell line MCF-7 was isolated in 1970 from a 69-year old Caucasian woman. MCF-7 is the acronym of Michigan Cancer Foundation-7 (now known as the Barbara Ann Karmanos Cancer Institute), where the cell line was established in 1973 by Herbert Soule and co-workers (Soule *et al*., 1973). Prior to MCF-7, it was not possible for cancer researchers to obtain a mammary cell line that was capable of living longer than a few months (Glodek, 1990). The MCF-7 cells were the source of much of current knowledge about breast cancer (Soule *et al*., 1973; Levenson, 1997).

3.2 Methods

The main steps of the research work plan are summarized in the scheme (3.1) :



Scheme (3.1) Main steps of the research work

3.2.1: Preparation and characterization of free and DOX loaded PCL-PEG-PCL nanoparticles

3.2.1.1 Preparation of free PCL-PEG-PCL nanoparticle

The triblock copolymer PCL_{5000}-b-PEG_{10000}-b-PCL_{5000} was purchased from Polysciences Company shown in figure (3.1). The number listed under each of polymer indicated the approximate molecular weight of the block segment. The PEG terminal groups are blocked as methyl ethers, but the caprolactone end group are hydroxyl and are suitable for functionalization.

PCL-PEG-PCL

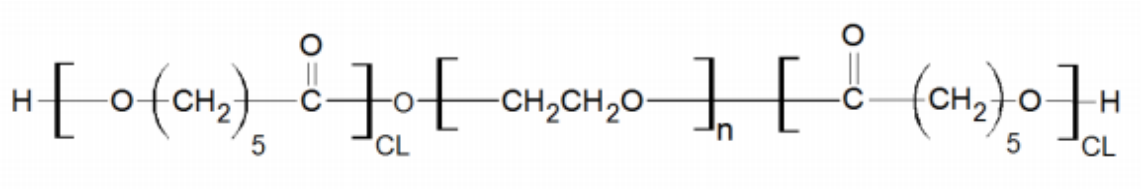
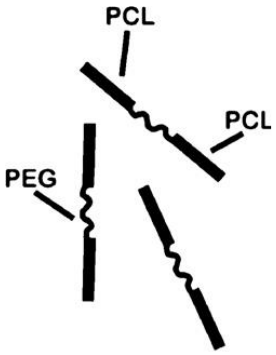


Figure (3.1) Chemical structure of PCL-PEG-PCL triblock copolymer

The PCL-PEG-PCL (PCEC) nanoparticles were prepared using nanoprecipitation method (figure 3.2) by dissolved five milligrams of PCL-PEG-PCL polymers in ten milliliter of acetone until completely dissolved by vortexing and stirring with vortex and magnetic stirrer respectively, then the solution was added dropwise into 10 ml miliQ water under moderate stirring at 25 Cº for three hours, the obtained solution was passed through dialysis membrane molecular weight cutoff (MWCO 3-8 KDa) for 24 hrs, the miliQ water was replaced hourly for the first three hours, then the solution was filtrated throughout 0.45 µm filter membrane to remove aggregates and clumps, and then it was freeze-dried for further application and characterization as described by (Gou *et al*., 2005).



Five milligram of PCL-PEG-PCL copolymer triblock

+

Ten milliliter of acetone (organic solution)

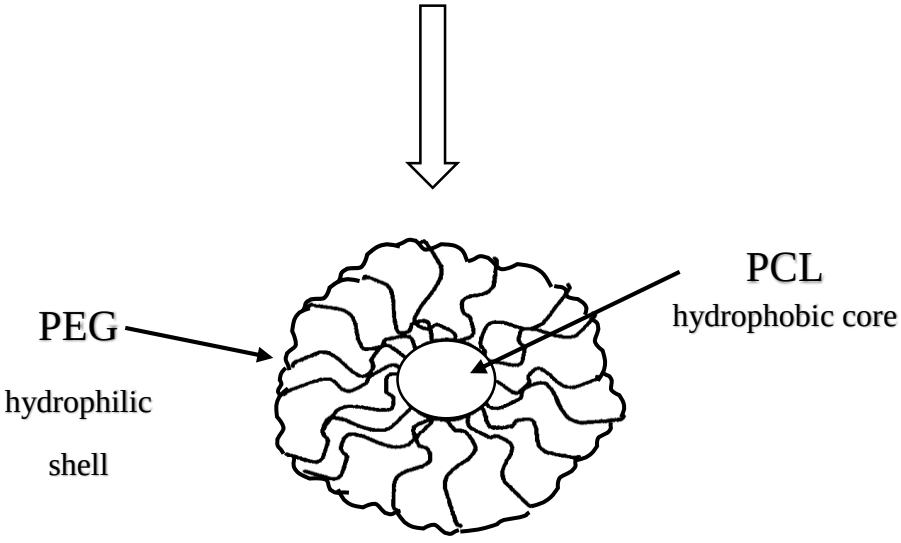
by

vortex and stirrer then adding to 10 ml of mili Q water for 3 hr then dialysis

(Nanoprecipitaion technique)

+

Characterization with DLS and TEM



PCL-PEG-PCL micelle nanoparticles

Figure (3.2) proposed scheme of PCL-PEG-PCL micelles prepared by nanoprecipitaion technique.

3.2.1.2 Loading of Dox into PCL-PEG-PCL micelles

The Preparation of doxorubicin solution was achieved according to (Xiang, *et al*., 2013) as follows:

1. A weight (5) mg of Dox was added to (1) ml of distilled water, and span at 3000 rpm for 3 minutes.
2. The mixture was incubated in water bath for 2 min at 25 Cº.
3. The mixture was vortexed gently until all particles of drug was completely dissolved.
4. The aqueous solution of Dox was used for loading into PCL-PEG-PCL micelles**.**

Doxorubicin-loaded PCL-PEG-PCL micelles were prepared by pH induced self-assembly protocol according to (Gou *et al*., 2009):

1. Aliquot of (50) µl of Dulbecco's Phosphate-Buffered Saline DPBS (10X, pH 7.4) was added to (0.35) ml of nanoparticle slurry.
2. A volume of (0.1) ml of aqueous solution of Dox was added drop by drop into nanoparticles solution under moderate stirring.
3. Thirty minutes later, the suspension were centrifuged at 12000 rpm for 30 minutes.
4. The resulting solution was placed into a dialysis bag and dialyzed against 100 ml of miliQ water for 72 hours with (MWCO 10 KDa) using amicon ultra centrifugal filter unit for purification.
5. The amount of Dox loaded in micelle was quantified by determining fluorescence of the solution at 490 nm by using Modulus™ II Microplate Multimode Reader. The Dox solutions of various concentrations were prepared, and the fluorescence of the solutions were measured to obtain a calibration curve as shown in figure (3.3):

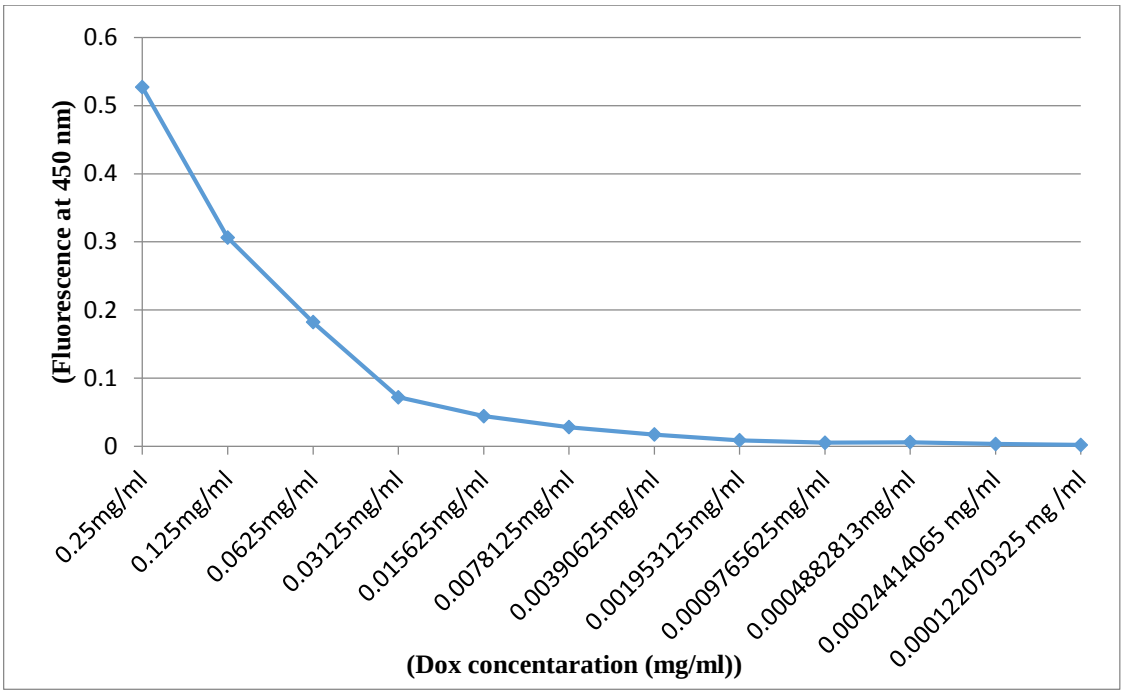


Figure (3.3) Calibration curve obtained by measuring the fluorescence of the Dox solutions at various concentrations.

6. Because of the low solubility of Dox in DPBS at a pH 7.4, Dox was self-assembled into the hydrophobic core of PCL-PEG-PCL nanoparticles. Few minutes later, DOX-loaded PCL-PEG-PCL (Dox-PCEC) nanoparticles were obtained.

3.2.1.3 Characterization of PCL-PEG-PCL nanoparticle

3.2.1.3.1 Particle size

Particle size and size distribution are the most important characteristics of nanoparticle systems. Dynamic light scattering (also known as photon correlation spectroscopy) is the fastest and the most routine method of determining particle size. The particle size of polymeric micelles was determined by dynamic light scattering (DLS) at 25 °C using a Zetasizer with an excitation of 633 nm illuminated at a fixed angle of 90°. Aqueous micelle solutions were prepared using 1:20 (vol/vol) dilution of the NPs suspension in (DI) deionized water. The concentration of polymeric micelles was kept at 1

mg/ml. The micelle solutions were sediment for 4h then it was filtered through 0.2 µm centrifuge filter before measurement. The average values were calculated from three measurements performed on each samples. The results were expressed as the size ± SD. The size of NP's was confirmed with TEM.

3.2.1.3.2 Transmission electron microscope (TEM)

The morphology of the prepared micelles was observed under a TEM. micelles were diluted with distilled water and one drop of NP solution was placed on a carbon film-coated copper grid (400 mesh) at room temperature followed by negative staining with 2% phosphotungstic acid for 20 seconds, excess solution was absorbed with filter paper, and air drying, prior to electron microscope for analysis.

3.2.1.3.3 Evaluation of Drug loading and encapsulation efficiency

The drug loading and encapsulation efficiency of Dox-PCEC were determined by a subtraction method as described by Hairong, *et al*., (2013). Amount of 0.2 ml was centrifuged using filter tube with a MWCO 10 KDa. Although the free DOX could pass through, the doxorubicin-encapsulated PCEC nanoparticles could not pass through the filter. The unbounded doxorubicin was quantified by determining the fluorescence spectroscopy with excitation at 490 nm and emission wavelength of (510 – 570) nm, with a slit width of 5 nm.

The drug loading (DL) and encapsulation efficiency (EE) were calculated according to the following formulas:

$$\text{DL (\%)} = \frac{\text{Concentration of (Total Drug} - \text{ Free Drug)}}{\text{Concetration of (polymer} + \text{total drug} - \text{Free Drug)}} * 100$$

$$\text{EE}\ (\%)\ = \frac{\text{Concentration of (Total Drug} - \text{ Free Drug)}}{\text{Concentration of Total Drug}} * 100$$

3.2.3 *In vitro* drug release

The *in vitro* release behavior of Dox from the drug loaded PCL-PEG-PCL micelles was done using modified dialysis method described by (Gong, *et al*., 2012) as follows:

1. A volume of 5 ml of the Dox-loaded micelle solutions were put into a dialysis membrane (MWCO 10kDa) and dialyzed against 30 ml of Sorenson's Buffer pH 5.6 and pH 7.4 of at 37 ºC (2.5 ml of Dox-loaded micelle solution was added to each tubes).
2. Sorenson's Buffer stock solutions was prepared to achieve desired pH as following protocol described by Ruzin (1999):
3. A volume of each solution (A) and solution (B) of Sorenson's buffer were mixed to obtain the desired pH as following:

	Volume (ml)	
pH	**Na_2HPO_4 (solution A)**	**KH_2PO_4 (solution B)**
5.6	5.0	95.0
7.4	80.4	19.6

4. A volume of 5 ml of the released solution from dialysis bag was withdrawn for fluorescence measurements and replaced by 5 ml of fresh buffer solution after different time intervals (0.5, 3, 4, 5, 22, 24, 30, 34, and 46) hr.
5. The concentration of Dox was determined by fluorescence spectroscopy with excitation at 490 nm and emission wavelength of 510 – 570 nm.

6. Each experiment was carried out in triplicate, and average values plotted. The percentage of the released drug was calculated using the equation proposed by Sanson *et al*., 2010:

$$\% \text{ Drug Releasing } = \frac{1 - \text{Fluorescence (t)}}{\text{Fluorescence } (t_o)} * 100$$

Where (t) is the time at which the fluorescence is measured, and ($t_\circ$) the initial time. All release experiments were conducted in the dark by covering tubes with aluminum foil.

3.2.4 *In vitro* hemolytic test

The *in vitro* hemolytic test was performed on PCL-PEG-PCL micelles was achieved to evaluate an *in vitro* toxicity of the micelles according to Gou *et al*., (2009) by diluting 100 µg/ml of PCL-PEG-PCL micelles to a volume of 2.5 ml with normal saline and added to 2.5 ml of erythrocyte suspension (2%) in normal saline at 37 ◦C.

Normal saline and distilled water were chosen as negative and positive controls, respectively. Three hours later, the erythrocyte suspension was centrifuged at 1500 rpm for 10 min and the color of the supernatant was compared with the negative control. If the supernatant was totally achromatic, it proved that there was no hemolysis. Oppositely, hemolysis occurred when the supernatant color was red.

3.2.5 Trypan blue assay for cell viability

One of the earliest and most common method for measuring MCF-7 cell viability is the trypan blue (TB) assay, Trypan blue staining is a simple way to evaluate cell membrane integrity (and thus assume cell proliferation or death), the following protocol described by Freshney (1987) was used to assay viability.

1. A volume of 1 ml of trypsinized cells suspension was added to 0.1 ml of a 0.4% Trypan Blue Solution in 1:9 ratio (1 part of 0.4% Tb and 9 parts of cell suspension) and mixed well and incubated for 2 minutes at room temperature, but longer because viability of cells will decrease due to Trypan blue toxicity.
2. About 10 µl of Trypan blue-cell suspension mixture was loaded on the each sides of haemocytometer by micropipette. The edge of the cover-slip was carefully touched by using the pipette tip and each chamber filled by capillary action.

   Then, MCF-7 cells were counted under microscope in two 1 mm^2 squares in each champers of haemocytometer and as a total number of cells and recorded the data as the following table.

Total cells	Stained(dead)	Unstained (Viable)	%

   Non-viable cells will stain blue, while the others were stay unstained, if the percentage was greater than 10% of the cells appear clustered, the procedure was repeated entirely to ensure that all the cells were dispersed by vigorous pipetting in the original cell suspension as well as the Trypan blue-cell suspension mixture.
3. The total number of cells overlying one of 1 mm^2 should be between (20-50 cells/square), if the cell density is higher than 200, the procedure should repeated until adjusting to an appropriate dilution factor.
4. Then, the cells concentration and the total cell number and cell viability were calculated using the following formula:

- $\text{Cells /ml} = (\text{the average count / square}) * (\text{dilution factor}) * 10^4$
- $\text{Total Cells} = (\text{cells / ml}) *$
  (the original volume of suspension from which cell sample was removed)
- $\text{Cell viability (\%)} = \frac{\text{Total viable cells (unstained)}}{\text{Total cells (stained and unstained)}} * 100$

3.2.6 *In vitro* cytotoxicity of blank PCL-PEG-PCL and DOX loaded PCL-PEG-PCL nanoparticles

The ability of PCL-PEG-PCL micells and Dox-loaded micelles to inhibit the proliferation of tumor cell line was evaluated using MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) viability assay.

In this study, MTT assay was achieved according to the protocol that was described by Alley, *et al*., (1988):

- The trypsinized MCF-7 cells were seeded at a density of 5×10^3 cells/well in 96-well plates and incubated for 24 h with CO_2 incubator.
- After 24 and 48 hours, When MCF-7 cells confluent (70%-80%), it was treated with blank PCL-PEG-PCL at different concentrations (0.1, 0.2, 0.4, 0.6, 0.8 and 1) µg/ml, and another MCF-7 cultured 96-well plate was treated with free Dox and Dox loaded with PCL-PEG-PCL nanoparticles after 24,48 and 72 hours at different concentrations (0.01, 0.05, 0.1, 0.4, 0.8, and 1.2) µg/ml, included one set of wells contain MCF-7 cells without Dox and nanoparticle as a control.
- After 24 hours, a volume of 20 µl of 5 mg/ml MTT solution was added to each well of 96-well plate and incubated for 3 hours at 37 $^{\circ}$C in CO_2 incubator.
- After three hours, the purple crystal formation was checked under microscope, and the media with MTT solution was aspirated carefully without disturbing the cells and replaced with 150 µl of MTT solvent by

multichannel micropipette, and covered with tinfoil and agitated cells with orbital shaking for 15 min by microplate reader.

- Then, the absorbance was measured at 450 nm.
- The cell viability was calculated using the following formula:

$$\text{Cell viability } (\%) = \{(\text{Abs}_t - \text{Blank})/(\text{Abs}_c - \text{Blank})\} * 100$$

Where Abs_t stands for the absorbance of the treated cells and Abs_c stand for absorbance of the untreated cells that used as a control. The experiment was achieved with 6 replica and with 24, 48, and 72 hrs of treatment with free Dox and Dox loaded nanoparticles.

In mitochondria of living cells, yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) was reduced to purple formazan. This colored solution can be quantified by measuring the absorbance at a certain wavelength. The crystals was dissolved in acidified isopropanol. The resulting purple solution is spectrophotometrically measured. An increase in cell number results in an increase in the amount of MTT formazan formed and an increase in absorbance (Slater *et al*., 1963; Alley *et al*., 1988; van de Loosdrecht, *et al*., 1994). Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, yielding purple MTT formazan crystals which are insoluble in aqueous solutions (figure 3.4).

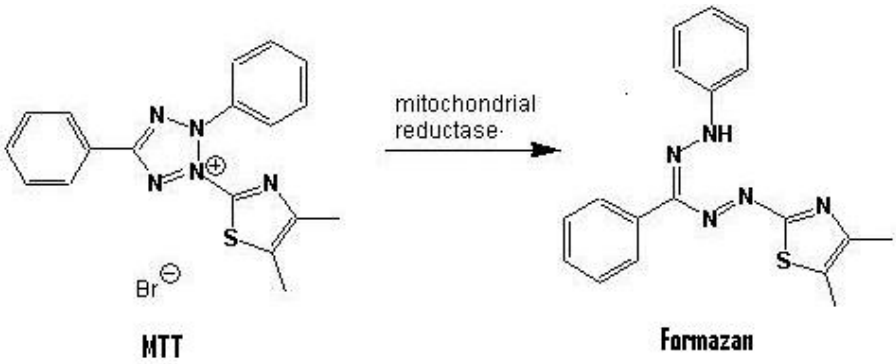


Figure (3.4) The mechanism of MTT assay.

3.2.7 Inhibition concentration (IC_{50})

The IC_{50} is the concentration of an inhibitor where the response is reduced by half. This quantitative measure indicates how much of a particular drug or other substance (inhibitor) is needed to inhibit a given biological process (or component of a process by half. According to the FDA, IC_{50} represents the concentration of a drug that is required for 50% inhibition *in vitro*. The half maximal inhibitory concentration (IC50) is a measure of the effectiveness of a compound in inhibiting biological or biochemical function. In other words, it is the half maximal (50%) inhibitory concentration (IC) of a substance (50% IC, or IC_{50}), the concepts of IC_{50} is fundamental to pharmacology (Salazar *et al*., 2012).

For IC_{50} calculation of free Dox and Dox loaded PCL-PEG-PCL, the equation: Y = mx + C was applied after calculating the % inhibition at different concentrations of free Dox and Dox loaded PCL-PEG-PCL by excel and insert Scatter Graph, after getting the equation with r^2 value, which is based on Y = mx + C.

Where:

Y= 50% Inhibition of MCF-7 cells growth

M, C = Constant

X= IC_{50} (for free DOX, DOX loaded PCL-PEG-PCL)

R^2 = correct coefficient

3.2.8 MCF-7 morphological changes study

Inverted microscope was used to study cell morphology, viability and cell death. Cell changes were studied as follows:

- MCF-7 cells was cultured in 60 mm cell culture dish, after confluence (70%-80%), cells were treated with various concentration of free Dox and Dox loaded with PCL-PEG-PCL nanoparticles from (0.01 to 1.2) µg/ml for 72 h.

- The morphological changes of cells were examined under inverted microscope at 40x magnifications and captured by digital camera after 24h, 48h, and 72 h incubation.
- Apoptotic characteristics were identified by the appearance of cell shrinkage**.**

3.2.9 Cellular uptake of Dox with 3D multimodal imaging using CARS and TPEF microscopy

3.2.9.1 Multimodal microscope set up

The experimental setup used in this study was described by Mouras, *et al.*, (2010) as shown in figure (3.5):

A mode-locked Nd: YVO4 laser source produces the Stokes pulse (6 ps, 1064 nm) used in the CARS process. The source also produces a 5-ps, frequency-doubled, 532-nm beam, which was used to pump a picosecond optical parametric oscillator (OPO). The OPO delivers a signal tunable in the range 700–1000 nm, which was used as a pump in the CARS process resulting in an observable range of ~600–4000 cm^{-1} covering all the biologically relevant molecular vibrations. The two beams were combined by a dichroic mirror (DM) and focused onto a single-mode fiber (Thorlabs SM980-5.8–125, single modefrom ~780 to>1064 nm) for ease of alignment, which is connected to the input of a laser-scanning confocal inverted optical microscope. The pulses weresynchronized in time by adjusting a micrometer-driven delay stage. These wavelengths are reflected towards a 60× oil immersion objective with 1.4 numerical aperture (NA) by a DM which removes most of the backscattered laser light.

The configuration of the system enabled both backward (epi-) and forward detection schemes. The backward signal is collected by the same objective and directed either to two different photomultiplier tubes (PMTs) or to an avalanche photodiode through a multimode fiber. The forward signal is collected by an air condenser (NA 0.55) and detected by a third PMT detector.

An appropriate set of short-pass and band-pass filters are used to selectively transmit the non-linear (NLO) signals. Although picosecond pulse widths are far less efficient than femtosecond pulses (of the same average power) for TPEF imaging, more power with picosecond sources was significantly used. Photo damage occurs at far higher powers with a picosecond source, and hence imaging rates for two photon microscopy (TPEF) are quite similar for picosecond and femtosecond sources.

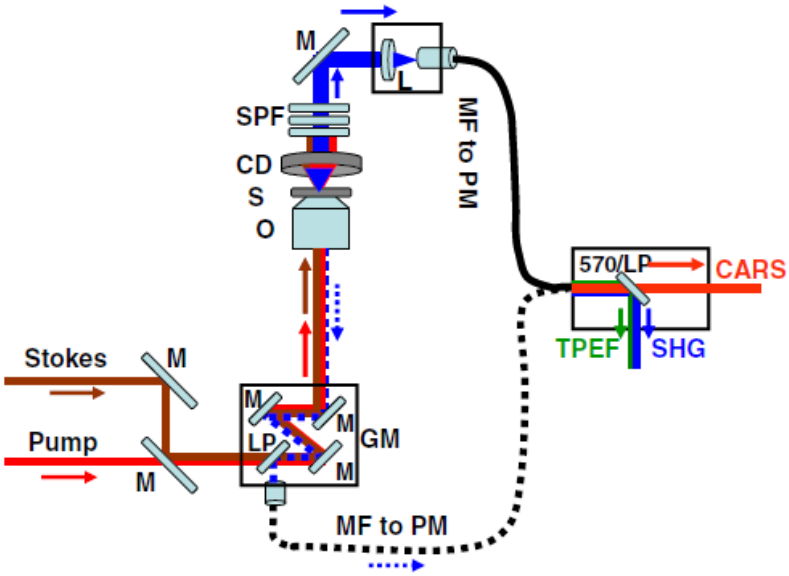


Figure (3.5) Multimodal imaging using CARS and TPEF set up.
M: mirror; LP:long pass dichroic mirror;
GM: galvano mirrors; O:objective;
S: sample; CD: condenser; SPF:short-pass filters set; L:focusing lens;
MF:multimode fibre, PM: photomultiplier. (Mouras *et al.*, 2013)

3.2.9.2 Cell culture and fixation for imaging

The MCF-7 cells with confluent (70%-80%) were cultured in intracel glass bottom dishes with DMEM supplemented with 15% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine, then the cell line was maintained at 37 C^0 in a humidified 5% CO_2-containing atmosphere. The cultured cells were fixed using formaldehyde and kept in solution. The fixed cells were used for imaging at different wave numbers

(Mouras *et al*, 2009). For drug uptake monitoring, cells were treated as following:

1- MCF-7 cells as control
2- MCF-7 cells with free Dox ,
3- MCF-7 with Dox loaded PCL-PEG-PCL
4- MCF-7 with nanoparticle alone.

Cells were kept at 37 ◦C in CO_2-containing incubator for up to 10 hours and transferred onto a CARS microscope equipped with a heating stage to maintain the cells at 37 ◦C at different time of incubation.

3.2.10 Statistical Analysis

Comparisons of quantitative data were computed by means of ANOVA tests. Statistical analysis was performed using the statistical package SPSS for windows (version 13, SPSS Inc., Chicago, IL, USA). A *P* value less than 0.05 was adopted to indicate statistical significance for each test.

Chapter Four

Results and Discussion

4.1 Preparation of blank and loaded polymeric PCL-PEG-PCL nanoparticles

To improve the hydrophilicity and to regulate the biodegradation rate of PCL, triblock copolymers of PCL with PEG was chosen and successfully was self-assembled to form nano-sized micelles by bottom-up approach starting from amphiphilic block copolymers that self-assemble in acetone to form micellar aggregates consisting of a hydrophilic outer shell and a hydrophobic inner core in aqueous media, such micelles with core-shell structure can readily incorporate anticancer drug doxorubicin (Dox) into their cores, while the hydrophilic shell can provide stabilization for the micelles without the need for additional stabilizers, thus making them a suitable carrier for Dox by nanoprecipitation techniques.

In this study, PCL:PEG:PCL triblock copolymer in molecular weight 5000:10000:5000 respectively was used. Amphiphilic block copolymer with longer hydrophilic PEG block are more hydrophilic and they can diffuse easily in aqueous medium also, the longer PEG chains will give a denser hydrophilic corona thus increasing stealth properties and increase the circulation time *in vivo* (Kedar *et al*., 2010).

During the preparation of PCL-PEG-PCL nanoparticles with long PEG chains, water cannot freely penetrate the inner part of the PCL core due to the strong hydrophobic and crystallized character of the PCL block. Thus, the hydrolytic degradation of ester bond first takes place at the interface between the PCL core and PEG shell, resulting in the partial cleavage of ester bonds of PCL-PCL and PCL-PEG on the surface of the PCL core, resulting PCL-PEG-PCL with core-shell micelles formation. The formation of the hydrophobic PCL inner core and the PEG outer core in an aqueous solution has been reported by Liu *et al*., 2007.

Due to this different arrangement and steric constraints, the B-A-B aggregates of triblock copolymer display larger core and great encapsulation

capacity that fit bulkier molecules like doxorubicin than the A-B and A-B-A counterparts (Zhang *et al*., 2012)**.**

The nanoprecipitation method was chosen to prepare the micelles from the triblock copolymers because the amphiphilic block copolymer micelles could not be formed in water alone because of the characteristic of the hydrophobic block. Nanoprecipitaion technique was first developed by Fessi *et al*. in 1986, nanoprecipitation is the simplest, the fastest, most reproducible, and industrially suitable procedure of nanoparticles preparation (Vauthier and Bouchemal, 2009).

The selection of polymer is a crucial step to obtain particles that are suitable for a well-defined application, some polymers like poly ethyleneglycol (PEG) copolymerized in order to decrease nanoparticle recognition by the reticular endothelial system (RES) (Mazzaferro *et al*., 2012). The arrangement of blocks along the polymer backbone is an additional parameter that affects the micellar structure, the self-aggregation behavior and the drug release kinetics to undergo self-assembly, the hydrophobic blocks of B-A-B amphiphilic triblock fold on themselves giving place to the so-called "flower-like" polymeric micelles (PMs) (Venkataraman *et al*., 2011).

On the other hand, depending on the nature of the hydrophobic block, these polymeric micelles often require production methods employing water-miscible organic solvents and a final drying step to increase their physical stability, and prevent secondary aggregation or micellar fusion and phase separation. This phenomenon is very common in PMs containing poly caprolactone as the hydrophobic component (Venkataraman *et al*., 2011; Moretton *et al*., 2012).

The PCL-PEG copolymer displays a desirable properties for drug delivery applications (Remant *et al*., 2007), amphiphilic block copolymers consisting of hydrophobic block and water-soluble hydrophilic block have been widely studied as carriers for controlled drug delivery (Liu *et al*., 2007). The main

difference between the di-block and tri-block micelles was referred to a PEG chain anchored to the micelle. For PEG diblock copolymer, PEG chain anchors to the micelle through one terminal group, and for PEG triblock copolymer, PEG anchored to the micelle through both terminal groups of PEG. So, when a protein approaches a micelles, the PEG chain anchored to the micelle core through both ends can more effectively prevent any interaction with blood proteins (opsonins) because of a more effective conformational PEG cloud, furthermore, these micelles are biodegradable and they will be degraded into small molecular after delivering drugs in a period of time and can be easily excreted from the body (Peracchia *et al*., 1997).

The PCL-PEG-PCL triblock copolymer architecture have several advantage over PEG-PCL-PEG triblock copolymer; First, the PCL-PEG-PCL triblock copolymer can be synthesized in one step without using any coupling agent; second, PCL-PEG-PCL have a wider gel window; third, PCL-PEG-PCL can persist for a longer period, about six weeks, compared to PEG-PCL-PEG about two weeks. Both PCL-PEG-PCL and PEG-PCL-PEG are biodegradable, and can sustain drug release in an extended period. The choice of using PCL-PEG-PCL or PEG-PCL-PEG was depending on application (Ma *et al*., 2010).

The triblock copolymer of PCL-PEG-PCL contains the hydrophobic part, enabling the encapsulation of Dox in micelle's core. Dox is limited by it's acute toxicity to healthy tissues, low water solubility and inherent multi-drug resistance (MDR) effects. In an attempt to overcome these disadvantages and increase selectivity towards cancer cells, the hydrophobic Dox was physically entrapped in the core of PCL-PEG-PCL micelles by a self-assembly method. This procedure for preparing Dox loaded PCL-PEG-PCL micelles was simple and easy to scale up, also there is no surfactants and organic solvents were applied in this procedure. The Dox was physically encapsulated into the copolymeric micelle due to several factors such as; the hydrophobic interaction of the drug and the PCL core, the structure of the hydrophobic core, and core–

drug interaction. After Dox was entrapped into the core of the amphiphilic polymeric micelles, drug-loaded micelles could be well and stably dispersed in water solution to meet the requirement of intravenous injection. The ability of the core to encapsulate Dox is largely dependent upon the compatibility between the hydrophobic core and the drug molecule. In their study, Yan *et al*., (2011) found that there is compatibility between Dox and PCL.

The amount of Dox that loaded in PCL-PEG-PCL micelles was determined by calculation the Drug Loading (DL) and the Encapsulation Efficiency (EE) with initial concentration ratio of copolymer to Dox (table 4.1).

Recently, attention have been focused on effective delivery system by use nanotherapuetic delivery systems which have some advantages and offer promising results in solving the major drawback in conventional delivery system. Such advantages are achieved through increase of drug solubility, tumor targeting, enhanced accumulation in tumor cells and tissues, decreased systemic toxicity and increased maximum tolerate dosage (Patil *et al*., 2012).

Drug delivery systems protect active pharmaceutical ingredients from degradation, enhance biopharmaceutical properties and could provide passive or active targeting delivery (Miladi *et al*., 2013).

Hydrophilic blocks form a hydrated outer shell which may conceal the hydrophobic core preventing its quick uptake by the reticuloendothelial system (RES) and more active clearing organs such as kidneys, liver, spleen and lung. Therefore, the hydrated outer shell can increase the blood circulation times of the nanoparticles. The predominant characteristics of this system have been reported to reduce toxic side effects of antitumor agents, passive targeting to specific sites, solubilization of hydrophobic drugs, and stable storage of drugs, longer blood circulation, favorable biodistribution, thermal stability, and lower interactions with the RES (Kataoka *et al*., 1993).

A biodegradable triblock copolymer PCL-PEG-PCL with good biocompatibility, was adopted as drug carrier as reported by Zhou *et al*., 2003.

Recently, biodegradable polymeric micelles are highlighted as advanced drug delivery systems for cancer therapy due to their unique core-shell architecture, which could solubilize hydrophobic Dox into the hydrophobic inner core and release the drug in a controlled manner at a later stage (Zhang *et al*., 2012).

4.2 Characterization of PCL-PEG-PCL nanoparticles

4.2.1 Morphology

Transmission electron microcope (TEM) was used to ascertain the shape and morphology of PCL-PEG-PCL micelles. TEM image revealed that the micelles that prepared by nanoprecipitation were spherical in shape as shown in figure (4.1). The micelles was shown by light spherical entities surrounded by dark staining, it apparent that the hydrophobic PCL part were assembled in the micelle core and the hydrophilic PEG backbone was exposed to the shell.

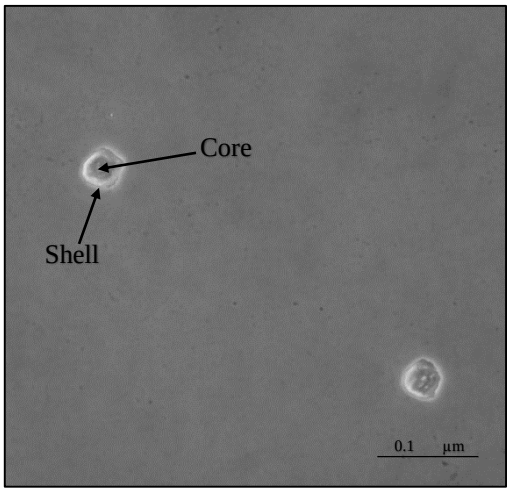


Figure (4.1) TEM image of PCL-PEG-PCL micelles

Note: magnification: 8000x; scale bars correspond to 0.1 µm.

The distinct spherical shape for polymeric micelles was formed when PEG segments are hydrated, and then water can cross PEG shell freely and contact the surface region of PCL core, resulting in the swelling of PCL-PEG-PCL micelles.

In aqueous solution, the relative difference in hydrophobicity between PEG and PCL allows the formation of self-assembled micelle with PCL core and PEG shell. PEG chain, located at the surface of the micelles, could provide limited affinity among the particles to prevent the formation of aggregates. Therefore, the stability of micelles might be mainly contributed to stereospecific block formed by hydrophilic PEG chain located at the surface of the micelles.

The shape is a critical parameter for drug delivery of nanoparticles. Recent studies have shown that the shape of nanoparticles have an important effect on particle functions, especially in biological processes, including internalization, transport through the blood vessels and targeting diseased sites, as well as targeting in cancer drug delivery (Gratton *et al*., 2008). Regardless of the mode of administration, transport of particles in the body will be affected by nanoparticle shape. Just as diameter dictates particle velocity, diffusion and adhesion to walls in blood vessels, airways and intestine, shape will also affect these properties but in more complex ways. Movement of spheres is easier to predict due to their inherent symmetry, but non-spherical nanoparticles may align or tumble in the presence of flow. Alignment or tumbling issues will be compounded when nanoparticles flow through filtering organs, such as spleen or liver, or when branching in the vessels are encountered, spherical nanoparticles also were internalized from any point of attachment, due to their symmetry (Moghimi *et al*., 2001).

4.2.2 Particle size

To investigate the size of nanoparticles of the triblock copolymer in an aqueous solution, Dynamic Light Scattering (DLS) was employed to evaluate the size and size distribution and polydispersity index (PDI) of the obtained nanoparticles. The particle size and PDI was illustrated in figure (4.2). DLS studies indicated that the average particle size of obtained micelle was (226±5)nm and polydispersity index (PDI) was (0.26± 0.034) with a narrow monodispersed unimodal size distribution pattern.

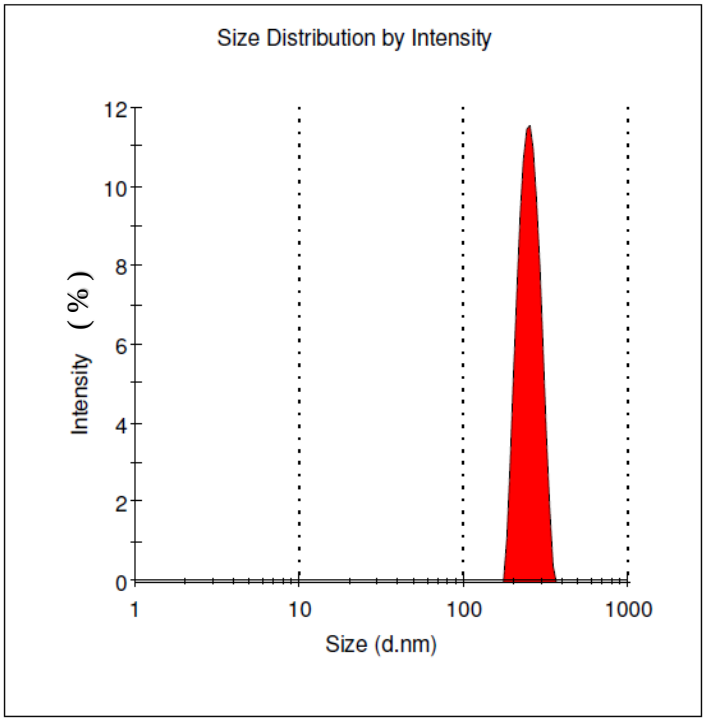


Figure (4.2) particle size distribution of PCL-PEG-PCL micelles determined by Dynamic Light Scattering (DLS). Mean ± standers deviation.

Nanoparticles can escape from the circulation through openings which are called "fenestrations of the endothelial barrier", although nanoparticles should be smaller than 150 nm to cross the endothelial barrier, many literatures reports the penetration of particles larger than the limits of fenestrations that can undergo modifications under various pathological conditions (Hirano *et al*.,

1994). For instance, tumor growth induces the development of neovasculature characterized by discontinuous endothelium with large fenestrations of (200–780) nm (Hobbs *et al*, 1998) that allowing nanoparticles passage.

The size of nanoparticles used in a drug delivery system should be large enough to prevent their rapid leakage into blood capillaries but small enough to escape capture by fixed macrophages that are lodged in the RES, such as the spleen and liver. The size of micelles is normally in the range of (10-250) nm which is small enough to avoid filtration by the lung and spleen. In addition, if the hydrophilic shell is composed of flexible polymers such as PEG and its derivatives, the outer shell can also help these micelles to escape from the RES after I.V. administration (Jones and Leroux, 1999; Zambaux *et al*., 1999; Gref, *et al*., 2000).

4.2.3 Drug loading and encapsulation efficiency

Drug loading and encapsulation efficiency is very important in a drug delivery system, it is one of the parameters used to evaluate the usability of nanocarriers. As indicated in table (4.1), drug loading content has significant effect on the encapsulation efficiency**.**

Table (4.1) The concentration, drug loading and encapsulation efficiency of DOX/PCL-PEG-PCL nanoparticles.

Drug/ Nanoparticle	Concentration of nanoparticle (mg/ml)	Concentration of Drug (mg/ml)	Drug loading (%)	Encapsulation Efficiency (%)	Ratio of PCL-PEG-PCL micelles:DOX (%)
DOX /PCL-PEG-PCL solution	12.4	5	28.69	99.7	70:30

The drug loading (DL) and encapsulation efficiency (EE) are directly affected by the copolymer/drug feed ratio and the interaction between Dox and the crystallinity of the hydrophobic core PCL. The increase of the DL and EE of Dox in PCL-PEG-PCL may be caused by the strong hydrophobic interaction between the hydrophobic PCL block chain and Dox. Most of anticancer hydrophobic drugs are encapsulated in the inner core by hydrophobic interactions (Nah *et al*., 1998; Wang *et al*., 2007). Table (4.2) showed different studies that evaluate the DL and EE for different polymeric nanoparticles:

Table (4.2) studies that evaluate the DL and EE for different polymeric nanoparticles

Type of polymeric nanoparticles	**Drug**	**DL(%)**	**EE (%)**	**Reference**
PCL_{6000}-PEG_{10000}-PCL_{6000}	nimodipine	4.80%	44.42%	Ge *et al*., 2002
PCL_{4000} PEG_{8000}-PCL_{4000}	Paclitaxel	28.98 %	94.36%,	Zhang *et al*., 2012
PCL–PEG–PCL	camptothecin	6.1%	85.7%	Dai *et al*., 2008
PCL-PEG-PCL	honokiol	6.4%,	44.0%	Wei *et al*., 2009
PCL-PEG-PCL	doxorubicin	4.2%	91.7%	Gou *et al*., 2009

There are several factors may affect drug loading content and drug encapsulation efficiency of the core-shell structured nanoparticles prepared by nano-precipitation method:

- The affinity of the loaded drug with the core-forming polymer
- The hydrophobic core
- Drug solubility in water

- Drug – core interaction, i.e. its ability to self-aggregate.
- The compatibility between the drug and core. Among them, the compatibility between the drug and the core-forming block is said to be the essential factor **(**Allen *et al*., 1999).

4.3 *In vitro* release of doxorubicin from nanoparticle

The release profile of Dox from PCL-PEG-PCL micelles was studied using a dialysis method. In this study, pH 5.6 was selected to mimic pH of cancer cells, while pH 7.4 was selected to mimic pH of the healthy cells. As shown in figure (4.3), it could be observed that Dox release from Dox-PCEC faster at pH 5.6 than at pH 7.4, this was obvious also when the color of incubation media was changed after 46 hrs. of incubation, figure (4.4).

During the releasing process, Dox was first released inside the hydrophobic core region of the polymeric micelles due to the attachment of Dox to PCL the core region, then, Dox diffused out from the micelle, eventually, into the incubation medium.

This delay of drug release indicates the nanoparticle applicability in drug carrier to minimize the exposure of healthy tissues while increasing the accumulation of therapeutic drug in the tumor site. The profile of Dox released indicates two different phases of release at pH 5.6, first is the burst or fast release of approximately 33% of the Dox within 3 hr and secondly is the sustained released of Dox for 46 hr where approximately 92% of Dox was released.

The rate of Dox released at pH 7.4 is notably lower than that at pH 5.6. On other hand, the percentage of Dox released at pH 7.4 was approximately 43% within 46 h.

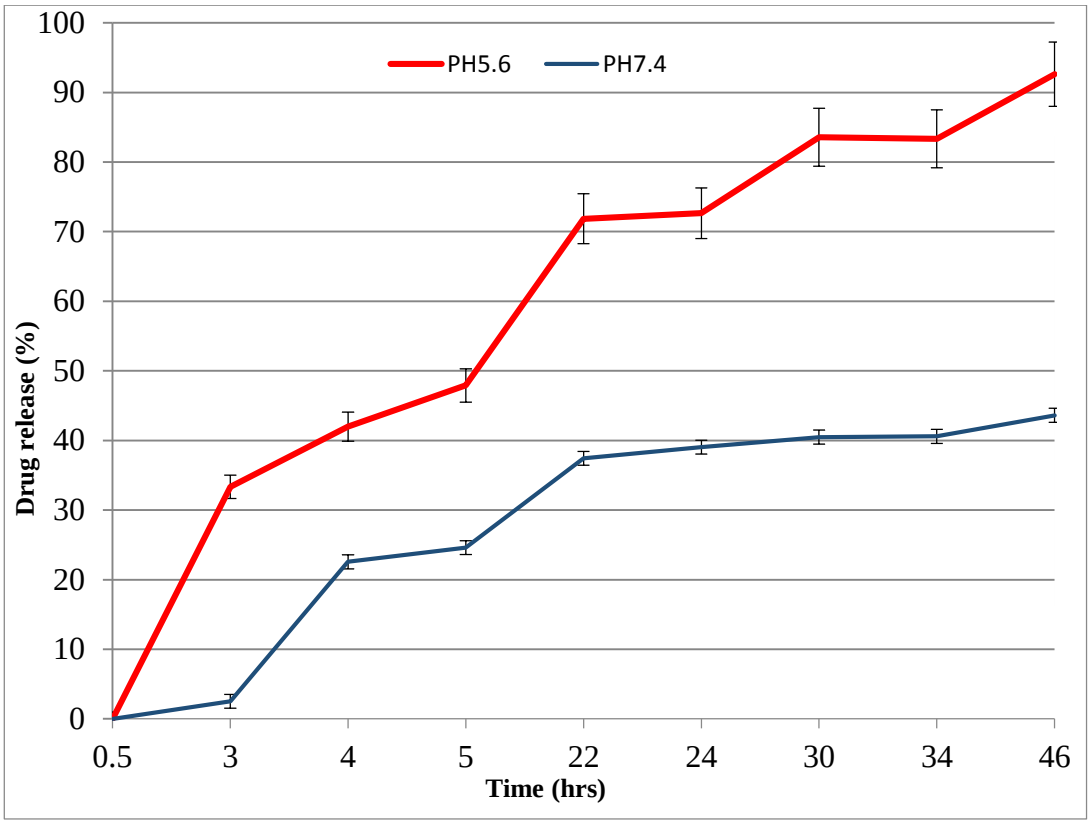


Figure (4.3) *In vitro* release profiles of Dox from PCL-PEG-PCL micelles during time of incubation with Sorenson's Buffer solutions at different pH.

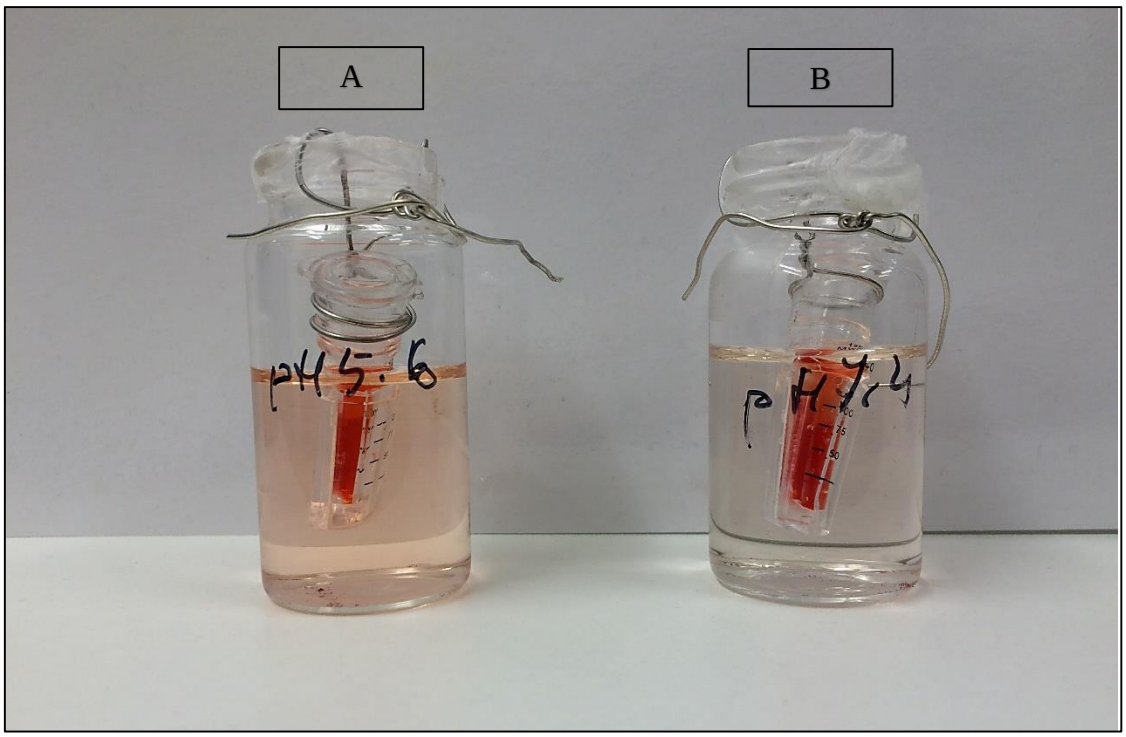


Figure (4.4) The *in vitro* Dox release in buffer solution at different pH (A: pH 5.6), at acidic buffer, the color of incubation media was changed into red, while no colored change at neutral buffer (B: pH 7.4) after 46 hr of incubation.

This pH dependent releasing behavior was interesting in achieving the tumor-targeted Dox delivery with nanoparticles. It was expected that Dox could be very slowly released in the plasma under normal physiological conditions (pH 7.4), but quickly released at the solid tumor site (pH 5.6). This indicates that envelopment of Dox in the micelles resulted in significantly sustained release of the drug, which is beneficial for drug action. $P<0.05$ indicates significant difference between release profiles of Dox from PCL-PEG-PCL micelles during 46 hours of incubation with phosphate buffer solutions at different pH .

The stability of Dox may be affect by the different pH environment (pH dependent). This is probably due to the increase in solubility of Dox at mildly acidic pH values, because the diffusion driving force of the concentration gradient is enhanced and the therapeutic efficacy will also be enhanced, since therapeutic effectiveness is closely related to the release of drug from the carrier system.

At pH 7.4, the slow release of Dox is the result of the strong hydrophobic interaction between Dox and PCL and slow degradation of the micelle, while, the relatively fast release rate of Dox at pH 5.6 from the micelle could be attributed to the weak interaction of Dox molecules and hydrophobic core of the polymeric micelle, and short PCL segment (Allen *et al*., 1999).

This pH-dependent releasing behavior might be due to the reprotonation of the amino group of Dox and faster degradation of PCL-PEG-PCL nanoparticles at lower pH (Shuai *et al*., 2004). Therefore the slow release of Dox from PCL-PEG-PCL polymeric micelles at normal blood pH or normal physiological pH is an indication of excellent anticancer drug carrier properties in view of the fact that most of the conventional methods of chemotherapeutic delivery fail to achieved therapeutic concentrations of the drugs needed at the target site or do not have specificity and selectivity for the target cells or tissues, thereby affecting also the healthy cells.

The excellent property of the understudy polymeric nanoparticle that the Dox released from PCL-PEG-PCL micelles is hardly released in normal tissues or healthy cells (pH 7.4), while at (pH 5.6) Dox may responsively release in tumor tissues, or even within cancer cells, to selectively kill the cancer cells.

Acidic extracellular pH is a major feature of tumor tissue, extracellular acidification being primarily considered to be due to lactate secretion from anaerobic glycolysis (Kato *et al*., 2013). Study by Gou *et al* 2009 shown that the release profiles of Dox-loaded PEG-PCL micelles at different pH 5.5, pH 7.0 was about 80% and 60% respectively within 30 hr. Dox release from Dox/PEG-PCL was faster at pH 5.5 than that at pH 7.0 (Gao *et al*., 2013).

In vitro release profiles of Dox from the star shaped FOL–PEG–PCL in pH 7.4 and pH 5.4, in a period of 156 h were 42% and 78% respectively (Cuong and Hsieh, 2011).

Another study was shown that at mildly acidic pH (4.5), Dox release was significantly faster compared to pH (7.4) from PS-PAA-PEG micelles (Bastakoti *et al.*, 2013). The *in vitro* release studies showed that Dox release from poly(DEAP-Lys)-b-PEG-b-PLLA flower-like micelles was accelerated by acidic tumor pH (Oh *et al*., 2009).

4.4 *In vitro* safety evaluation of PCL–PEG–PCL nanoparticles

To make sure of the safety of the PCL-PEG-PCL nanoparticle as a drug delivery system for nanomedical application in cancer therapy, the *in vitro* hemolytic test and MTT assay were applied:

4.4.1 *In vitro* hemolytic assay

The hemolytic study of PCL-PEG-PCL micelles was performed, and the result was shown in figure (4.5), that the concentration of (100 µg/ml) did not cause any hemolysis on human erythrocyte as compared with the negative

control (normal saline) by visual observation. This implies that these micelles could be safely applied by intravenous injection.

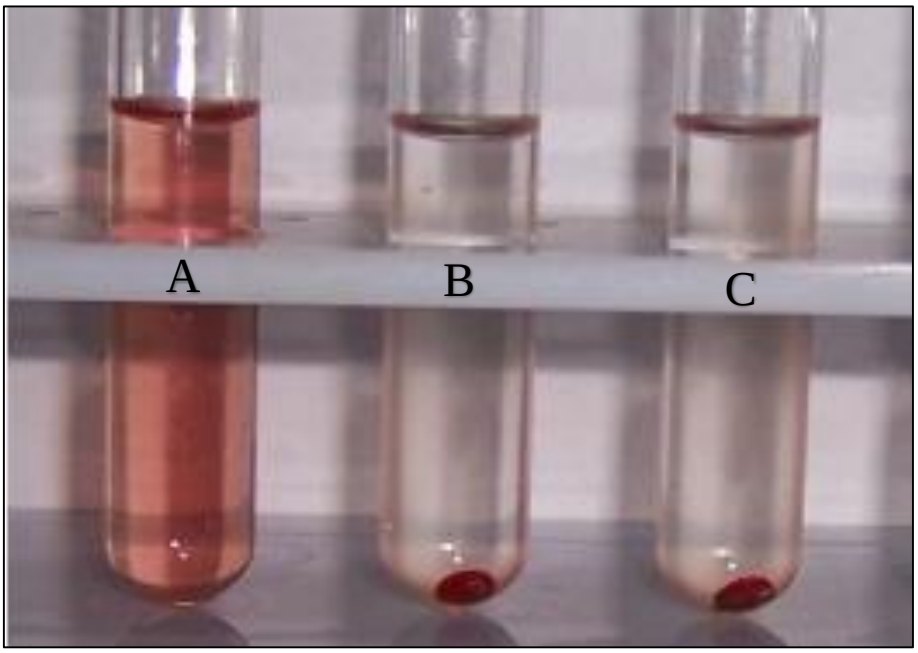


Figure (4.5) *In vitro* hemolytic assay of PCL- PEG-PCL micelles:
A) Distill water that used as positive control,
B) PCL-PEG-PCL micelles at the concentration of 100 µg/ml,
C) Normal saline used as the negative control.

4.4.2 MTT assay

Toxicity studies of any nanomaterials are the first evaluation step in cellular interaction and may lead to the cellular response, so the effect of PCL-PEG-PCL nanoparticles against MCF-7 cell line was examined by the MTT assay *in vitro* to determine the toxicity of the delivery system for the compatibility studies in biological systems.

Results illustrated in figure (4.6) showed PCL-PEG-PCL nanoparticles did not suppress the proliferation of MCF-7 cells.

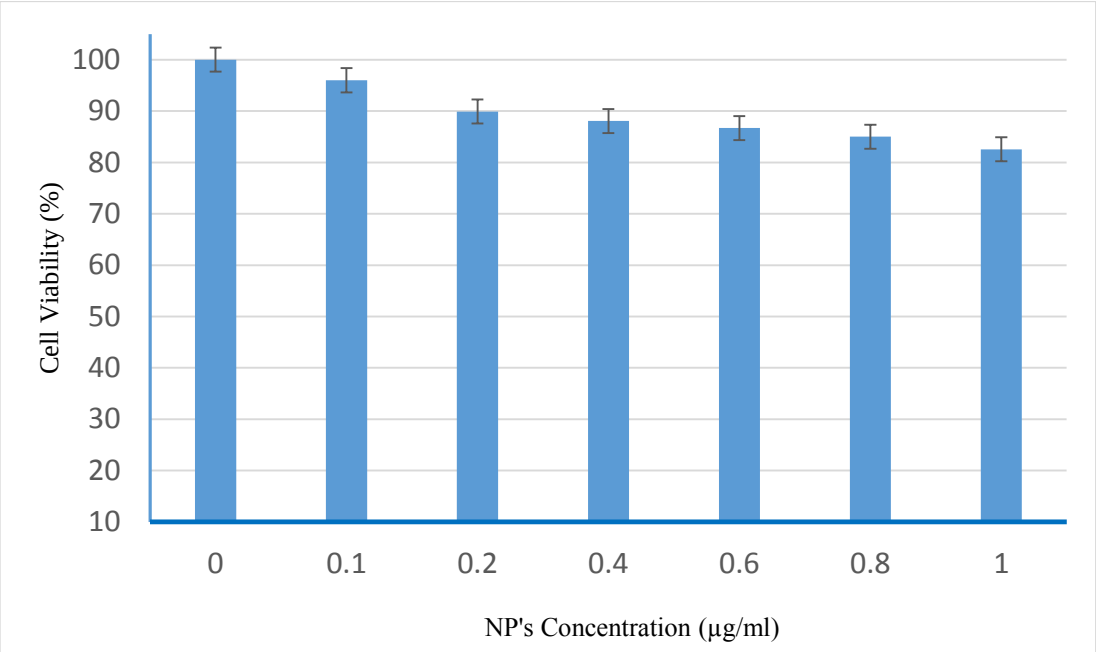


Figure (4.6) Viability of MCF-7 cell line after incubation with different concentrations of PCL-PEG-PCL nanoparticles for 48 hrs.

The results revealed a negligible toxicity of PCL-PEG-PCL against MCF-7 cells even at higher concentration of (0.1 to 1) µg/ml and the viability percentage were above 80% at this different concentration of the nanoparticles, indicating that these nanoparticles show a significant differences at ($P \leq 0.05$) when the concentration increased after 48 hrs. of incubation and did not suppress the proliferation of MCF-7 cells *in vitro*.

PEG and PCL are well-known polymers as their low cytotoxicity and good biocompatibility, making them suitable for various applications in biomedical fields such as drug carriers, tissue engineering (Cai *et al*., 2012; Cheng *et al*., 2012).

On other hand, it was found that PCL-PEG-PCL nanoparticle has cyto-compatibility which make them a potential material for biomedical applications and could be regarded as a safe drug delivery carrier.

4.5 Morphological observation of cancer cells change

Microscopic observation was applied for determining and quantifying cell viability and cell death. Breast cancer cell line MCF-7 was treated with PCL-PEG-PCL/Dox at different concentrations for a period of 24, 48 and 72 h. Results illustrated in figure (4.7) showed that there is no morphological changes observed after 24 h of treatment with drug, while after 48 h, the cells become rounded and detached from the surface of the culture dish.

After 72 h, the majority of the treated cells were rounded and many were detached and suspended in the culture media, while other cells appearance with shrinkage. The number of treated cells was reduced as compared to the control. Therefore, these results showed effective delivery of anticancer drug and inhibition of cell growth by the Dox that were loaded into PCL-PEG-PCL polymeric nanoparticles.

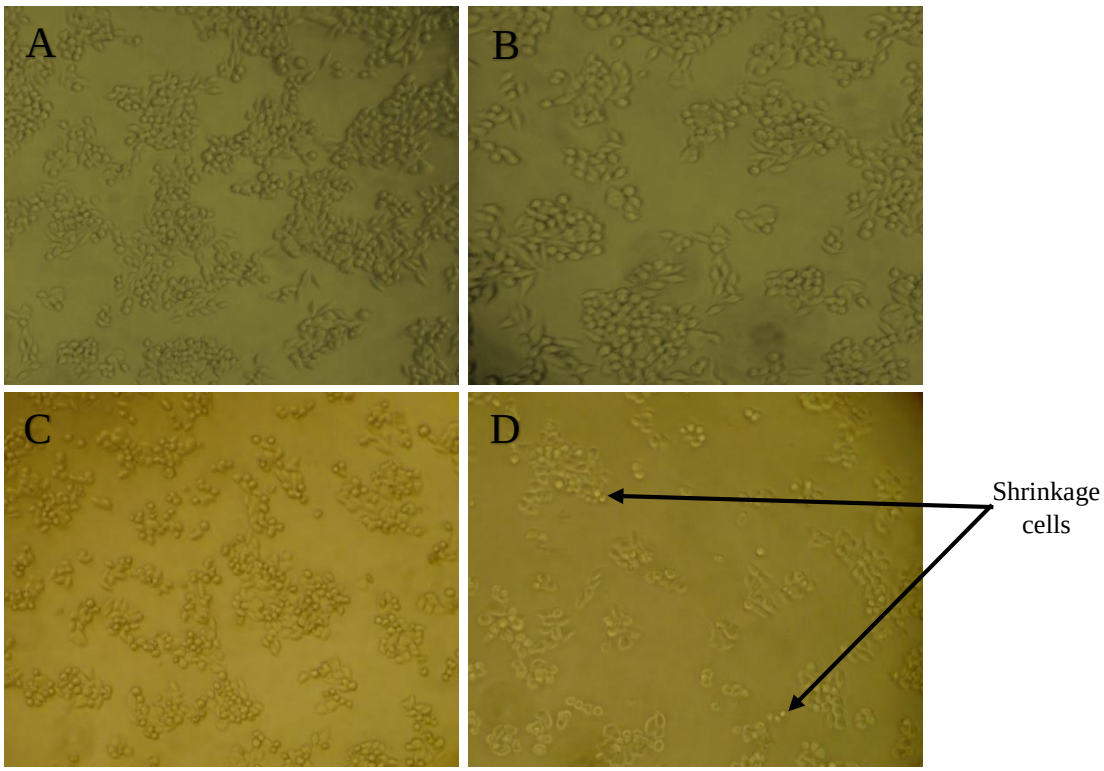


Figure (4.7) Morphological changes in MCF-7 cells by inverted microscope at (40X) after treatment with polymeric PCL-PEG-PCL nanocarrires loaded with Dox:
(A) Untreated MCF-7 (control) (B) MCF-7 cells after 24 hr.
(C) MCF-7 cells after 48 hr. (D) MCF-7 cells after 72 hr.

Nanoparticles may deliver anticancer drug to specific sites by size-dependent passive targeting, passive targeting is also dependent on both tumor and the surrounding inflamed tissues structure. Nanoparticulate delivery systems may exploit a characteristic of solid tumors such as the EPR effect, in which tumor tissues display several unique characteristics such as hyper vasculature, defective vascular architecture and a deficient lymphatic drainage which leads macromolecules and particulates to be accumulated preferentially and to be retained for a longer time in tumors (Natalie and Mandal, 2007).

4.6 *In vitro* cytotoxicity assay of DOX loaded PCL-PEG-PCL nanoparticles

The *in vitro* cytotoxicity of free Dox and Dox loaded PCL-PEG-PCL nanoparticles against the MCF-7 cell line was investigated by MTT assay to verify whether the released DOX is still pharmacologically active, and to evaluate the cytotoxic activity of the drug-loaded polymeric micelles. Results illustrated in figure (4.8) showed the viability of MCF-7 cells after 24, 48, and 72 hours of treatment with different concentrations of Dox and Dox-loaded polymeric micelles ranged between (0.01 to 1.2) µg/ml.

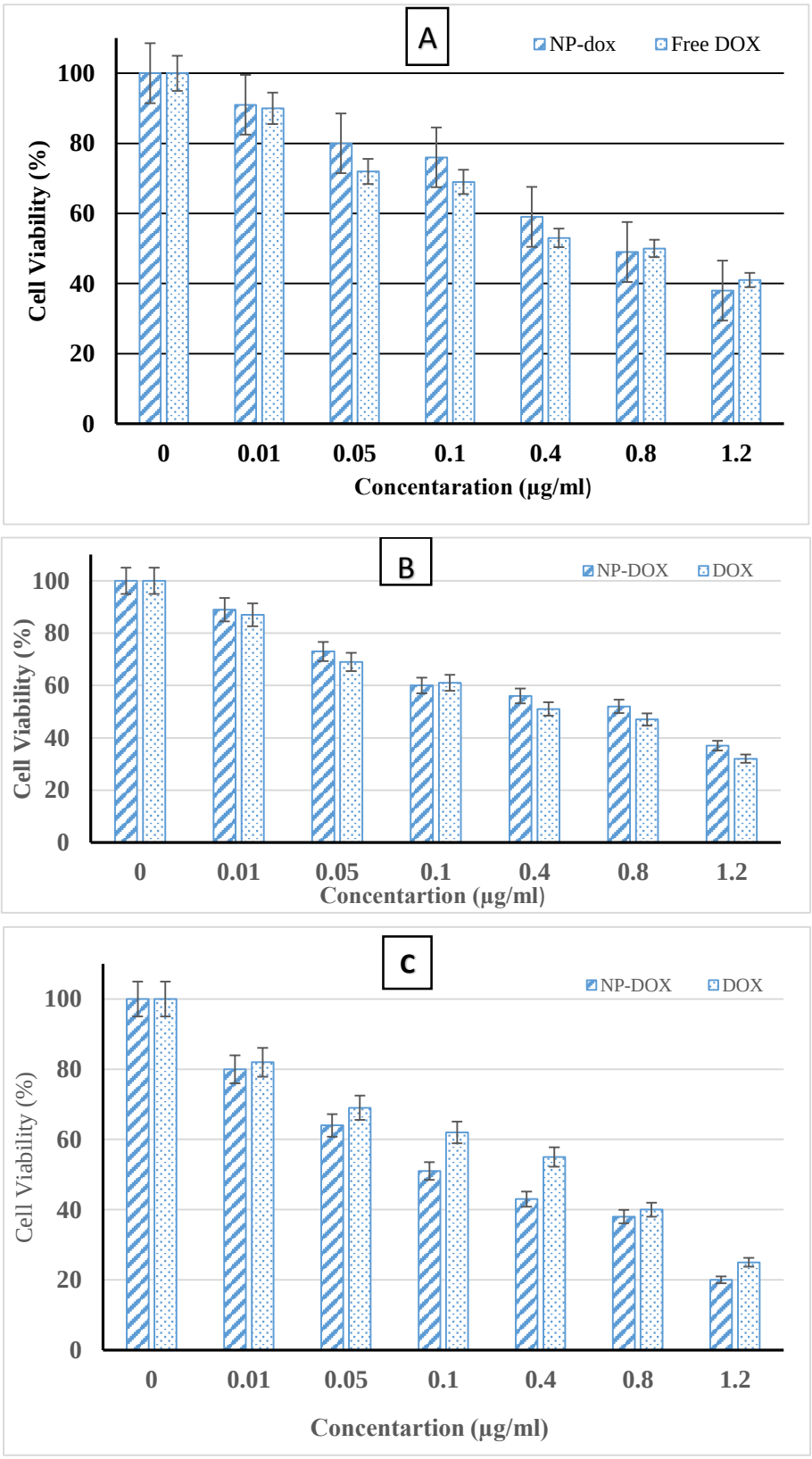


Figure (4.8) cytotoxicity of Dox and Dox loaded PCL-PEG-PCL nanoparticles on MCF-7 breast cancer cell line after: A) 24 hr treatment , B) 48 hr treatment and C) 72 hr treatment.

Corresponding figure (4.7) was shown in the previous section, the MTT assay showed that PCL-PEG-PCL/Dox has dose and time dependent cytotoxicity against the MCF-7 breast cancer cell line. It was also observed that the cell inhibition rate is dependent on incubation periods.

On other hand, results illustrated in figure (4.8) showed that MCF-7 cell line was more sensitive to free Dox for 24 and 48 hrs of incubation, while Dox loaded PCL-PEG-PCL showed more toxicity than free Dox after 72 hrs. of incubation. Therefore, as time and concentration increase, the rate of Dox release increases, it could suggest that the inhibiting of the cell growth is a time dependent and PCL-PEG-PCL/Dox was more effective than free Dox after 72 hrs., and the cell viability decreased significantly ($p \square 0.05$) with the increased time in drug concentration at various time of incubation. The reason that Dox-loaded micelle did not show cytotoxicity after a 24 h incubation could be attributed to the lag phase of Dox (Eliaz *et al*., 2004).

It can be seen from figure (4.8) that the Dox/PCL-PEG-PCL nanoparticles exhibited significantly higher cytotoxicity after 72 hrs. at low drug concentration or at least comparable cytotoxicity at high drug concentration in comparison with the free Dox. This advantage became more significant for longer time cell culture.

The cellular uptake of free Dox occurs through a passive diffusion mechanism whereby it may be trapped at the P-gap junction and thereby affect the healthy cells while in the case of Dox loaded PCL-PEG-PCL, the drug has to be released in a time-dependent manner from the PCL-PEG-PCL micelles before exerts its effects on the cells (Gillies and Frechet, 2005; Chittasupho *et al*., 2009).

The cellular uptake of Dox loaded PCL-PEG-PCL micelles was through non-specific endocytosis which may lead to reduced effect of cytosolic free Dox for the P-glycoprotein pumping action, this mechanism of Dox loaded

PCL-PEG-PCL micelles delivery to tumors may circumvent the effect of MDR proteins which are present in cancer cells (Larsen *et al*., 2000).

The IC_{50} for free Dox and Dox loaded nanoparticles was also studied. The result indicated in table (4.2) showed that IC_{50} of Dox loaded PCL-PEG-PCL was (0.83 µg/ml) after 24 hrs as compared with free Dox that had IC_{50} (0.76 µg/ml) at the same time, so the IC_{50} of Dox loaded nanoparticles is higher than that by using Dox alone.

On the other hand, the IC_{50} was decrease for both treatments free Dox and Dox loaded PCL-PEG-PCL on MCF-7 cells were 0.63 and 0.75 µg/ml respectively for 48 hrs of incubation. After 72 hrs. of treatment, the IC_{50} for Dox loaded PCL-PEG-PCL was decrease into (0.5 µg/ml) while the IC_{50} for Dox was (0.56 µg/ml), so it could be conclude that the Dox loaded PCL-PEG-PCL has more toxicity than free Dox.

These results showed that after 72 hrs that Dox loaded PCL-PEG-PCL nanoparticles have cytotoxic effect against MCF-7 cell line compared with the cytotoxicity of free Dox due to prolonged release of Dox from micelles as demonstrated by the *in vitro* Dox release profile shown in figure (4.3) and the Dox encapsulated in PCL-PEG-PCL could increase the intracellular concentration of Dox through different transport process from Dox solution.

Table (4.3) The IC_{50} of free Dox and Dox loaded polymeric micelles against MC7-7 cell line after period of time.

Incubation Time (hour)	IC_{50} for free Dox (µg/ml)	IC_{50} for Dox/PCL-PEG-PCL (µg/ml)
24	0.76	0.83
48	0.63	0.75
72	0.56	0.5

This delay of Dox release indicates the potential applicability of nanoparticles in drug carrier to minimize the exposure of healthy tissues while increasing the accumulation of therapeutic drug in the tumor site. Finally, this result indicates that encapsulation of Dox in PCL-PEG-PCL micelles enhanced the cytotoxicity of Dox

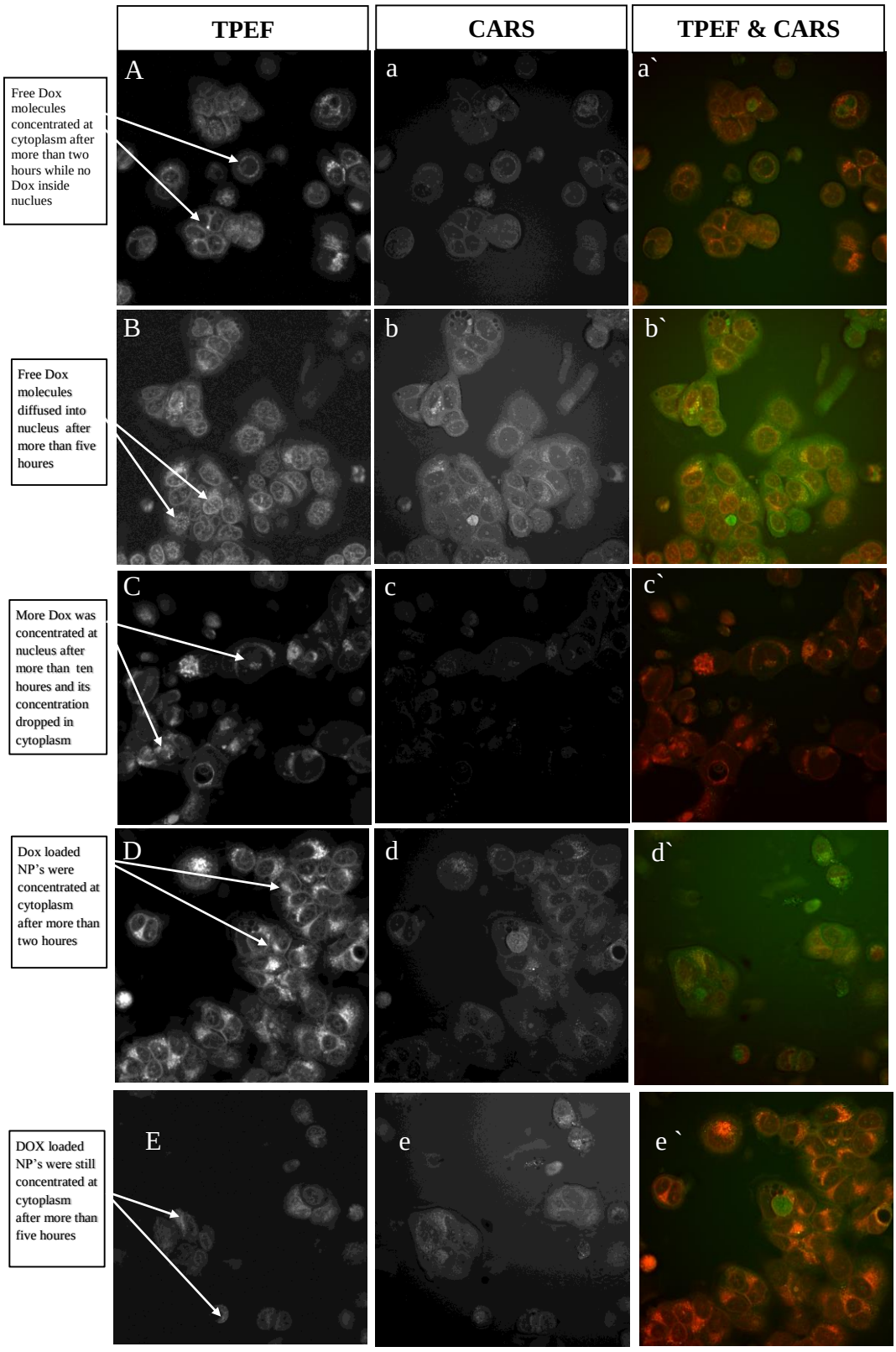
4.7 Cellular uptake of Dox loaded with PCL-PEG-PCL by MCF-7 breast cancer cell line using CARS and TPEF spectroscopy

Because Dox is fluorescent, it can be used directly to track it's uptake by cancer cell without introducing additional fluorescent probes. The intracellular uptake of free Dox and Dox loaded micelles was observed in the MCF-7 cell line by nonlinear optical microscopy with CARS and TPEF.

In order to monitor the drug uptake, the MCF-7 cells was treated with medium containing free Dox and a micellar Dox formulation. Results illustrated in figure (4.9) showed Two-photon excitation fluorescence (TPEF) images are on the left line, CARS on the middle line and combined (both TPEF and CARS) in colored (red-green) images (TPEF as red, CARS as green) on the right line that revealed the differences between the uptake of PCL-PEG-PCL/Dox and free Dox, the Dox-free medium was used as a control.

The TPEF images shown in figure (4.9) as (A,B,C,D,E,F,G) only show the presence of Dox, CARS images shown in figure (4.9) (a,b,c,d,e,f,g) and combined TPEF and CARS (a`,b`,c`,d`,e`,f `,g`) where CARS and TPEF have been acquired on the same microscope in the backscattered direction.

To evaluate the spatial resolution of nonlinear optical imaging (NLO) imaging system, a glass cover slip in air was used. The images consists of a Z-stack of 10 images taken at 0.5 µm steps, 200µm size, 21 seconds per frame.


TPEF
CARS
TPEF & CARS
A
a
a`
Free Dox molecules concentrated at cytoplasm after more than two hours while no Dox inside nuclues
B
b
b`
Free Dox molecules diffused into nucleus after more than five houres
C
c
c`
More Dox was concentrated at nucleus after more than ten houres and its concentration dropped in cytoplasm
D
d
d`
Dox loaded NP's were concentrated at cytoplasm after more than two houres
E
e
e `
DOX loaded NP's were still concentrated at cytoplasm after more than five houres

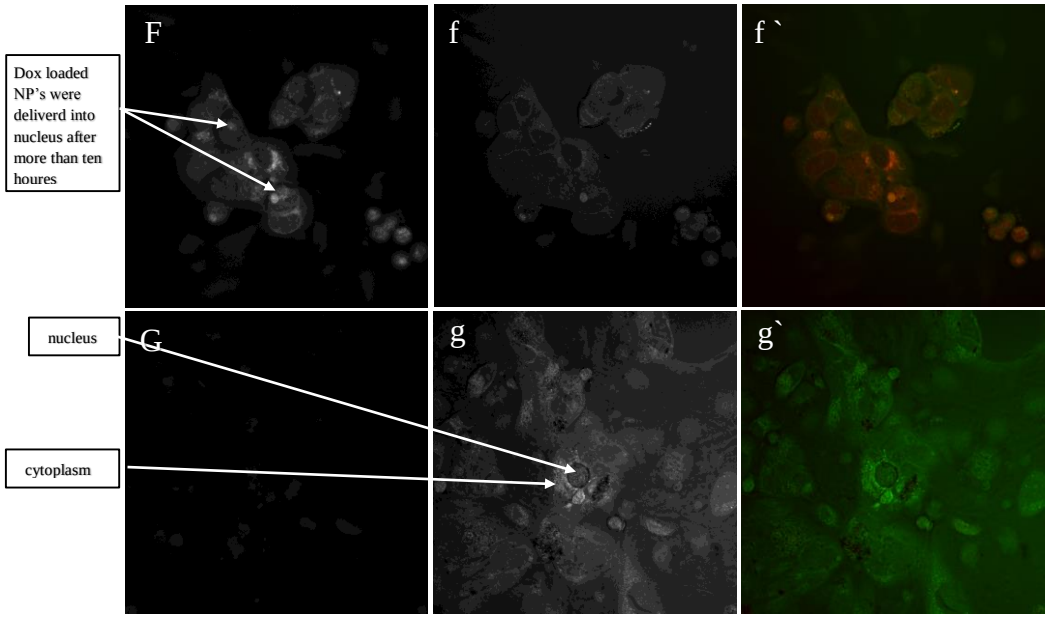


Figure (4.9) TPEF and CARS images of MCF-7 cells after incubation at the equivalent DOX 0.01 mg/ml, NP 7.31 mg/ml:
A,a,a`) Free Doxorubicin (after 2h, 20 min),
B,b,b`) Free Doxorubicin (after 5h,50min)
C,c,c`) Free Doxorubicin (after 10 h ,35min),
D,d,d`) Nanoparticles + Doxorubicin (after 2h,20m)
E,e,e`) Nanoparticles + Doxorubicin (after 5h,50m),
F,f,f `) Nanoparticles + Doxorubicin (after 10h,35m)
G,g,g`) Control (no Nanoparticles, no Doxorubicin).

The CARS data have been demonstrated to be a useful tool for understanding the distribution of the elastin–collagen network of the extracellular matrix of the tumor cell. While, the intracellular distribution of Dox was studied by TPEF due to its inherent fluorescence of Dox.

For TPEF, The MCF-7 Dox-free medium (control) is highlighted in figure 4.9 (G), and MCF7-free Dox figure 4.9 (A,B,C), MCF7-NP/Dox figure 4.9 (D,E,F). Additionally, for CARS microscopy, MCF-7 (control) was highlighted in figure 4.9(g), MCF7-Dox in figure 4.9(a,b,c) and MCF-7 NP-Dox in figure 4.9 (d,e,f) by probing the CH_2 stretching vibration.

The visualization of the 3D distribution of Dox by CARS and TPEF showed that nuclei of MCF-7 accumulate a high concentration of free DOX after 10h,35m figure 4.9 (C,c), while after 5h,50m, Dox appeared to be exclusively located within the cytoplasm and concentrated in a perinuclear area with negligible levels accumulation in nuclei, figure 4.9 (A,a, B, b).

The nuclear accumulation of free Dox can be ascribed to diffusion. These observations demonstrate that Dox was distributed in living MCF-7. Monitoring Dox over time showed that these drug molecules diffused into the nucleus after 10h,35min and most cells underwent apoptosis.

As shown in figure (4.9) the distribution of free Dox in MCF-7 cells is different from that of Dox loaded micelles. The cellular uptake of free Dox is faster than that of Dox-loaded micelles in MCF-7.

After 2 h,20 min,(figure 4.9: A,a) the MCF-7 cells that had been incubated with free Dox exhibited strong fluorescence of Dox, and the intensity of fluorescence continued to increase after 10h,35min of incubation time.

Furthermore, after 10h,35min (figure 4.9, C,c) when the MCF-7 cells were incubated with free Dox, fluorescence signals were observed only near the nuclei of cells but not in the cytoplasm. This is reasonable since Dox is a small molecule and could transport freely through both the plasma membrane and nuclear membrane via a passive pathway of diffusion which may result in trapping of the drug at the P-gap junction and counteractive effects in healthy cells (Manaspon *et al*., 2012; Liang *et al*., 2013).

However, the results suggested that free Dox was taken up by cancer cells with a greater efficiency than Dox-loaded micelles. These data, which are in agreement with the characterization of cytotoxicity discussed in the previous section, indicated once again that the free Dox has higher toxicity than Dox-loaded micelles toward MCF-7.

The increased concentration of Dox in nucleus as compared with cytoplasmic Dox can be ascribed to the known nuclear intercalation effect of Dox with DNA molecule (Ashikawa *et al*., 1985; Bodley *et al*., 1989).

In the case of relatively larger Dox-loaded micelles, strong Dox fluorescence was observed only in the cytoplasm, whereas the fluorescence in the nuclei was dim (figure 4.9, D,d,E,e).

After 2hr,20 min, strong Dox fluorescence was observed only in the cytoplasm highlighted in figure 4.9 (D,d), the observation of fluorescence in cytoplasm indicated that the Dox loaded PCL-PEG-PCL micelle was internalized by the cells through endocytosis and Dox was distributed in the cytoplasm after escaping from the endosome and/or the lysosome then diffused from the cytoplasm to the nucleus where it will intercalate in the DNA in the cells (figure 4.9:F,f), and MCF-7 show the early signs of their reaction to the drugs by shrinking suggesting that Dox may delivered into nuclei and successfully inhibited the proliferation of MCF-7 cells. However the diffusion of Dox from the cytoplasm depends on the time taken during the incubation (Liu *et al*., 2007).

Dox loaded micelles is released in a controlled manner from the micelles as shown in figure (4.9 F,f) and eventually enters the nucleus where Dox is known to exert its cytotoxicity during DNA synthesis.

The release mechanism of Dox from nanoparticles could be attributed to the acidic environment inside the tumor cells resulting in the dissociation of Dox-loaded micelles and rapid release of the Dox. As a small molecule, the uptake of free Dox is a dynamic process and they can freely escape from the cells. While for the Dox-loaded micelles with larger sizes, it is likely that the prolonged circulation and passive tumor-targeting delivery process caused by the EPR effect will enhance the delivery of Dox into the tumor cells and once the micelles were internalized, it is not easy for them to escape from the cells (Ge and Liu, 2013), figure (4.10).

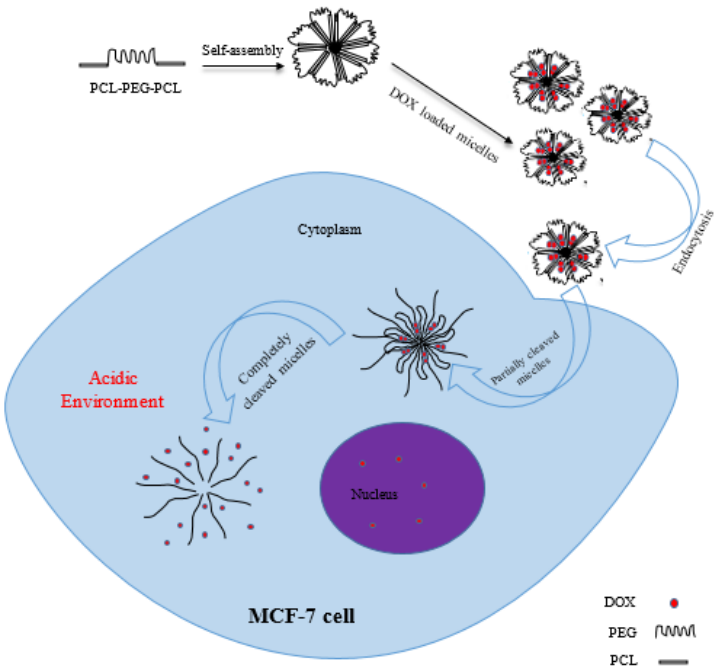


Figure (4.10) The proposed behavior of polymeric Dox carrier and Dox release in MCF-7 cell.

It could be conclude that the quick nuclear accumulation of free Dox can be ascribed to diffusion, while PCL-PEG-PCL/Dox was taken up by the cells into small vesicles, and concentrations remain mostly in the cytoplasm with negligible nuclear accumulation observed for incubation more than two hours. These findings are characteristic for uptake of PCL-PEG-PCL-Dox via endocytosis but not via diffusion.

The Dox forms π-π stacks with the aromatic groups of the DNA base pairs, locally reducing the exposure of Dox to external quenchers (like: dissolved oxygen), and this leads to the increases in fluorescence (Dai *et al*., 2008). Dox is positively charged and tends to bind to membranes just like any other positively charged molecule. However, Dox that bound to membranes was localized at the cell periphery and on vesicle membranes.

This clearly showed a direct contact of the Dox to the cells which was highly concentrated in the nucleus of the cells. This mode of Dox delivery is among the shortcomings of traditional chemotherapy where it shows no specificity and selectivity to cancerous cells. Increasing the concentration of free Dox leads to decreased lifetimes in the nuclear domains, suggesting the onset of fluorescence self-quenching. Increasing the concentration from 0.02 to 0.05 mg/ml reduced lifetimes from 3.8 nanoseconds to 2.8 nanoseconds, whereas cytoplasmic lifetimes remained unchanged (Dai *et al.*, 2008).

However, it was found that the cytotoxic actions of Dox has been proposed to be the intercalation of Dox with DNA inhibiting the progression of enzyme topoisomerase II, then unwinding DNA for transcription. Therefore, a decreased chemotherapeutic action of encapsulated Dox is usually observed in comparison with that of free Dox (Liu, *et al*., 2006; Shen, *et al*., 2011).

Many experiments conducted by different researchers reported differences in the mechanism of cellular uptake between free Dox and the Dox delivered by nanoparticles.

As for the micellar doxorubicin, although it enters into the cells at a lower rate than free Dox, migration of free Dox into the cells may mostly rely on a passive diffusion process. This may explain why free Dox entered the cells more quickly than micellar Dox. Similar results were also obtained by Wang *et al*., (2013).

Their studies showed that the patterns of cellular distribution of Dox-loaded PCL-PEG-PCL micelles and free Dox are different in HeLa cells. They demonstrated by confocal laser scanning microscope (CLSM) that micelles are internalized into HeLa cells via an endocytosis mechanism after 12 h, while free Dox entered into the cells by diffusion. Coung *et al*.,(2010) also investigated cellular internalization of PEG-PCL-PEG copolymer micelles in MCF-7 cells after 2 h, and the internalization process of the micelles was found

to fulfill the basic criteria of endocytosis were it was time, temperature, pH, and energy-dependent.

After releasing the drug what is the fate of polymeric micelles inside the body? The answer of this question is, both of PCL and PEG biodegradable and biocompatible, so they are easily removed from the body after a specific period without inducing any incompatible reaction with the body components. The ester bonding of PCL backbone makes it biodegradable *in vivo*, and the degradation product, 6-hydroxycaproic acid, can be removed from the body via the citric acid cycle.

Also, both of polymers should have the characteristics that they should not be excreted without accumulating at the tumor site and should induce no effect after releasing drug at the tumor site. Before being excreted, polymeric micelles may aggregates or disassembled inside the cell (figure 4.11).

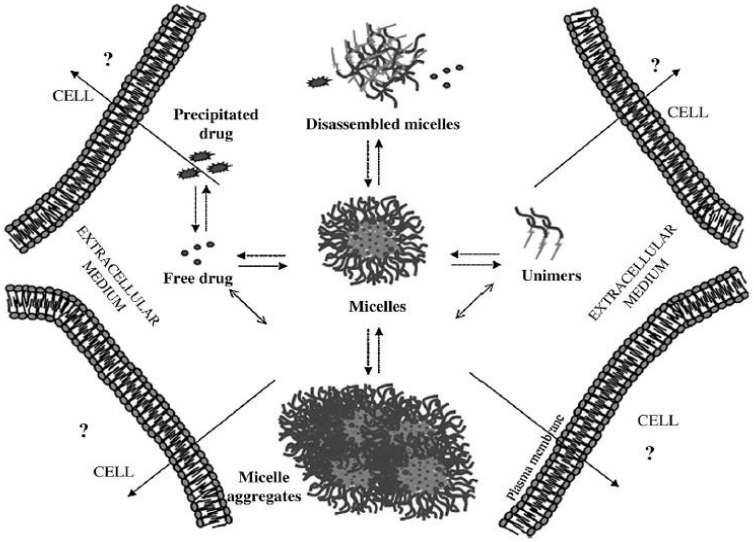


Figure (4.11) *In vivo* behavior of the polymeric micelles (Kedar *et al*., 2011).

The amphiphilic copolymers upon dissociation might be excreted, having a molecular weight less than the cutoff limit for glomerular filtration. Volume of

distribution and rate of removal of the copolymers from the body influences the concentration of the copolymer in the blood (Kedar *et al*., 2011).

To the best of our knowledge, using CARS spectroscopy has not been reported in the literature in the drug delivery system research so, it can be said that the first application of CARS in polymer-mediated drug delivery research is presented in this work.

The fluorescent nature of the Dox molecule can exploit in the imaging of organs or cells that provide accurate information of drug biodistribution (Mohan and Rapoport, 2010).

The loading of Dox into the PCL-PEG-PCL nanoparticle core for the formation of PCL-PEG-PCL/Dox is reflected in the fact that non-fluorescent nanoparticle becomes fluorescent.

The results that were obtained demonstrate the usefulness of multimodality for understanding the distribution of the elastin–collagen network of the extracellular matrix in addition to the localization of tumors, as well as their size and shape. These data show the ability of multimodal microscopy to be translated into clinical applications

The polymeric based drug delivery system may offer a successful and promising potential application for many therapeutic agent with more confidence in Dox for the clinical treatment of breast cancer and for efficient intracellular delivery of hydrophobic anti-cancer drugs.

Conclusions and Recommendations

I. Conclusions

Based on the obtained results, it is possible to reach the following conclusions:

1. The biocompatible and non-toxic triblock copolymer micelle was successfully prepared in a self-assembly with nanoprecipitation method and employed to load doxorubicin by an easy procedure.
2. The encapsulation of Dox in PCL-PEG-PCL nanoparticles improved the anticancer activity of Dox on MCF-7 cell line *in vitro* after 72 hr of treatment.
3. The *in vitro* cytotoxicity study demonstrated that the micelle was safe on red blood cells according to *in vitro* hemolysis assay and biocompatible, and the optimal time for MTT assay was 72hr.
4. The Dox was release faster from Dox-PCL-PEG-PCL at pH 5.6 than at pH 7.4.
5. The polymeric based delivery drug system may offer a successful and promising potential application for many therapeutic agent with more confidence in Dox for the clinical treatment of breast cancer.

II. Recommendations

1. Further characterization for polymeric nanoparticles with different techniques such as proton nuclear magnetic resonance (1H NMR), Fourier transform infrared spectroscopy (FT-IR), X-ray diffraction (XRD), differential scanning calorimetry (DSC) and Scanning electronic microscope (SEM).
2. Preparing polymeric PCL-PEG-PCL nanoparticle using different methods like emulsion diffusion to increase nanoparticle yield and perform the *in vivo* cytotoxicity evaluation.
3. Studying the cytotoxicity against other cancer cell lines and *in vivo* model.

4. Flow cytometry analysis of Dox loaded PCL-PEG-PCL nanoparticles for apoptosis pathway.
5. Development of multifunctional ‘‘smart’’ nanoparticles that may facilitate the realization of individualized cancer therapy.
6. Conjugate targeting agent to the PCL-PEG-PCL for the anticancer drug active targeted delivery.

References

A

Adams M.L., Lavasanifar A., Kwon G.S. (2003). Amphiphilic block copolymers for drug delivery. J. Pharm. Sci.; 92*:*1343*–55.*

Ahmad H. (2013). A special issue on polymer and hybrid particles for biomedical applications. J. Colloid. Sci. Biotechnol. 2:153–4.

Ahmed, F., Pakunlu R.I., Brannan A., *et al*., (2006). Biodegradable polymersomes loaded with both paclitaxel and doxorubicin permeate and shrink tumors, inducing apoptosis in proportion to accumulated drug. J Control Release, 116 (2):150-158.

Ahmed, F.; Discher, D. E. (2004). Self-porating polymersomes of PEG-PLA and PEG-PCL: hydrolysis-triggered controlled release vesicles. J. Controlled Release, 96 (1), 37–53.

Al-Hashimi M.M.Y. and Wang X.J: (2014). Breast cancer in Iraq: Incidence trends from 2000-2009. Asian Pac J Cancer Prev, 15 (1), 281-286.

Aliabadi H.M., Mahmud A., Sharifabadi A.D., Afsaneh L. (2005). Micelles of methoxy poly (ethylene oxide)-b-poly(q-caprolactone) as vehicles for the solubilization and controlled delivery of cyclosporine A. J. Control. Rel. 104:301–311.

Allen C., Han J., Yu Y., Maysinger D. Eisenberg A. (2000). J. Control. Release 63 275.

Allen C., Maysinger D., Eisenberg A. (1999). Nano-engineering block copolymer aggregates for drug delivery, Colloids Surf., B. Biointerfaces 16: 3 -27.

Alley M.C., *et al*. (1988). Cancer Res. 48: 589-601.

An J.H., Kim H.S., Chung D.J., Lee D.S. (2001). Thermal behavior of poly (caprolactone) –poly (ethylene glycol)–poly(caprolactone) tri-block copolymer. J. Mater. Sci. 36:715–722.

Arcamone, F., G. and Cassinelli, *et al*., (1969). Adriamycin, 14-hydroxydaunomycin, a new antitumor antibiotic from *S. peucetius* var. *caesius*. Biotechnology and Bioengineering 11(6): 1101-1110.

Asadi H., Rostamizadeh K., Salari D., Hamidi M. (2011). Preparation of biodegradable nanoparticles of triblock PLA-PEG-PLA copolymer and determination of factors controlling the particle size using artificial neural network. J. Microencapsul. 28:406–16.

Ashikawa I., Kinosita K., Ikegami A. (1985). Increased stability of the higher order structure of chicken erythrocyte chromatin: nanosecond anisotropy studies of intercalated ethidium. Biochemistry. 24:1291-7.

Aubel-Sadron G., Londos-Gagliardi D. (1984). Daunorubicin and doxorubicin, anthracycline antibiotics, a physicochemical and biological review. Biochimie. 66:333-352.

B

Bader H., Ringsdorf H., and Schmidt B. (1984). Water soluble polymers in medicine. Angew. Chem. 123Y124:457Y485.

Bae S.J., Suh J.M., Sohn Y.S., Bae Y.H., Kim S.W., Jeong B., (2005). Thermogelling poly (caprolactone-b-ethylene glycol-caprolactone) aqueous solutions. Macromolecules 38:5260–5265.

Bae Y. and Kataoka K. (2005). Polymer assemblies: intelligent block copolymer micelles for the programmed delivery of drugs and genes. In G. S. Kwon (ed.), Drug and the Pharmaceutical Sciences. Volume 148. Polymeric Drug Delivery Systems, Taylor & Francis Group, Boca Raton; 491Y532.

Bae, Y. and Kataoka K. (2005). Polymer assemblies: intelligent block copolymer micelles for the programmed delivery of drugs and genes. Polymeric Drug Delivery Systems, G.S. Kwon, Editor, Taylor & Francis. 652.

Barratt M. (2000). Therapeutic applications of colloidal drug carriers. Pharmaceutical Science & Technology Today, 3, (5), 163-171.

Bastakoti B.P., Wu K., Inoue C., Yusa M., Nakashima S., Yamauchi Y.(2013). Multifunctional core-shell-corona-type polymeric micelles for anti-cancer drug-delivery and imaging. Chemistry 19:4812-4817.

Batrakova E.V., Dorodnych T.Y., Klinskii E.Y., et al. (1996). Anthracycline antibiotics non-covalently incorporated into the block copolymer micelles: *in vivo* evaluation of anti-cancer activity. Br J Cancer; 74:1545–52.

Bianco A., Kostarelos K., Partidos C.D., Prato M. (2005). Biomedical applications of functionalised carbon nanotubes. Chem. Commun.; 5:571-7.

Bianco A., Kostarelos K., Prato M. (2005). Applications of carbon nanotubes in drug delivery. Curr. Opin. Chem. Biol.; 9:674-9.

Bodley A, Liu L.F., Israel M., Seshadri R., Koseki Y., Giuliani F.C., *et al*. (1989). DNA topoisomerase II-mediated interaction of doxorubicin and daunorubicin congeners with DNA. Cancer Res. 49:5969-78.

Bogdanov B., Vidts A., Van Den Bulcke A., Verbeeck R., Schacht E. (1998). Synthesis and thermal properties of poly (ethylene glycol)–poly (caprolactone) copolymers. Polymer 39:1631–1636.

Bourzac, K. (2012). Nanotechnology carrying drugs. Nature, 491: S58-S60.

C

Cai S., Vijayan K., Cheng D., Lima E.M., Discher D.E.(2007). Micelles of different morphologies advantages of worm-like filomicelles of PEO–PCL in paclitaxel delivery. Pharm. Res. 24:2099–2109.

Cai T., Li M., Neoh K., Kang G E. T., Mater J. (2012). Chem. 22: 16248 -16258.

Calderara F., H.Z., Hurtrez G., Lerch J.P, Nugay T., Riess G.(1994). Investigation of olystryrene-poly (ethylene oxide) block copolymer micelles formation in organic and

aqueous solutions by nonradiadiative energy transfer experiments. Marcromolecules. 27:1210-1215.

Campagnola P. J., Loew L. M. (2003). Nat. Biotech. 21:1356.

Cao Na (2007). Doxorubicin conjugated to D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS): in vitro cytotoxicity, in vivo pharmacokinetics and biodistribution. Ph.D. Thesis, Singaphore.

Carlson, R. W., Allred, D. C., Anderson, B. O., Burstein, H. J., Carter, W. B., *et al*.,. (2009). Breast cancer. Clinical practice guidelines in oncology. J Natl Compr Canc Netw, 7(2), 122-192.

Case, R.A.,. Hosker, Mc D.D. and Pearson J.T. (1954). Tumours of the urinary bladder in workmen engaged in the manufacture and use of certain dyestuff intermediates in the British chemical industry. I. The role of aniline, benzidine, alpha-naphthylamine, and beta-naphthylamine. Br. J. Ind. Med. 1954; 11: 75-104.

Cerdan S., *et al*. (1999). In vivo imaging of extracellular pH using ^{1}H MRSI. Magn. Reson. Med. 41:743-50.

Cerrai P., Guerra G.D., Lelli L., Tricoli M., Sbarbati Del Guerra R., Cascone M.G.,Giusti P. (1994). Poly (ester-ether-ester) block copolymers as biomaterials. J.Mater. Sci.: Mater. Med. 5, 33–39.

Champion J, Mitragotri S. (2006). Role of target geometry in phagocytosis. Proc. Natl Acad. Sci. USA *:* 103(13):4930–4934.

Chandra R., Rustgi R. (1998). Biodegradable polymers. Progress in Polymer Science. 23:1273-335.

Chanv J., Samantha F., Sebastian W, Thomas H. (2008). Raman spectroscopy and microscopy of individual cells and cellular components. Laser & Photon. Rev. 2(5):325–349.

Charbonneau C., Marli M. D. S. L., Christophe C., Olivier C. Taco N. (2013). Structure of pH sensitive self-assembled amphiphilic di- and triblock copolyelectrolytes: micelles, aggregates and transient networks. Phys.Chem. Chem.Phys.15, 3955.

Chen D.R., Bei J.Z., Wang S.G. (2000). Polycaprolactone microparticles and their biodegradation. Polym. Degrad. Stab. 67:455–459.

Chen M. (2009). Nanotechnology for efficient delivery of short therapeutic oligonucleotides (antisense ODN and siRNA) and codelivery with chemical anticancer drugs for effective cancer therapy. Ph.D. Dissertation. USA.

Chen, C., Yu C. H., Cheng Y. C., Yu P. H. F. Cheung M. K.(2006) . Biodegradable nanoparticles of amphiphilic triblock copolymers based on poly (3-hydroxybutyrate) and poly (ethylene glycol) as drug carriers.Biomaterials, 27 (27), 4804–4814.

Cheng J., Ji R., Gao S. J., Du F. S., Li Z. C. (2012). Biomacromolecules, 13:173–179.

Cheng JX, Jia YK, Zheng G, Xie XS. (2002). Laser-scanning coherent anti-Stokes Raman scattering microscopy and applications to cell biology. Biophys. J. 83:502–9.

Cheng R., Wang X. Y., Chen W., Meng F. H., Deng C., Liu H. Y., Zhong Z. Y., Mater J. (2012). Chem. 22: 11730–11738.

Chittasupho C.; Xie S.X., Baoum A., Yakovleva T., Siahaan T.J., Berkland C.J. (2009). ICAM-1 targeting of doxorubicin-loaded PLGA nanoparticles to lung epithelial cells. Eur. J. Pharm. Sci. 37, 141–150.

Cho K., Wang X, Nie S., Chen Z., Dong M. (2008). Therapeutic Nanoparticles for Drug Delivery in Cancer. REVIW. Clin Cancer Res.; 14(5) .

Cho K., Xu W., Shuming N., et al. (2008). Therapeutic Nanoparticles for Drug Delivery in Cancer. Clin Cancer Res, 14:1310-1316.

Chorny M., Fishbein I, Danenberg H.D., Golomb G. (2002). Lipophilic drug loaded nanospheres prepared by nanoprecipitation: effect of formulation variables on size, drug recovery and release kinetics. J Control Release. 83:389–400.

Couvreur P., *et al* (1995). Controlled drug delivery with nanoparticles: current possibilities and future trends, European Journal of Pharmaceutics and Biopharmaceutics, 41(1); 2-13.

Croy S.R., Kwon G.S. (2006). Polymeric micelles for drug delivery. Curr. Pharm. Des. 12:4669–4684.

Cullinane, C., S. M. Cutts, *et al*. (1994). Formation of adriamycin-DNA adducts in vitro. Nucl. Acids Res. 22(12): 2296-2303.

Cuong N. , Jian Lin J., Yu Lun L., Jim Ray C., Shyh Chuan J., Ming Fa H. (2011). Doxorubicin-Loaded PEG-PCL-PEG Micelle Using Xenograft Model of Nude Mice: Effect of Multiple Administration of Micelle on the Suppression of Human Breast Cancer. Cancers. 3:61-78.

Curado M.P. (2011). Breast cancer in the world: Incidence and mortality. Salud pública de México. (53):372–384.

D

Dai X., Zhilian Y., Mark E. E. Johannes S., Nigel K.H. Clemens F. K. (2008). Fluorescence intensity and lifetime imaging of free and micellar-encapsulated doxorubicin in living cells. Nanomedicine: Nanotechnology, Biology, and Medicine. 4: 49 - 56.

Dalhaimer, P.; Engler, A. J.; Parthasarathy, R.; Discher, D. E. (2004). Targeted worm micelles. Biomacromolecules, 5 (5), 1714–1719.

Danhier, F., E. Ansorena, J.M. Silva, *et al*. (2012). PLGA-based nanoparticles: An overview of biomedical applications. J Control Release. 161(2):505-522.

De Beer, E. L., A. E., Bottone *et al*. (2001). Doxorubicin and mechanical performance of cardiac trabeculae after acute and chronic treatment: a review. European Journal of Pharmacology 415(1): 1-11.

De Keulenaer, G.W., Doggen K., and Lemmens K., (2010). The vulnerability of the heart as a pluricellular paracrine organ: Lessons from unexpected triggers of heart failure in targeted ErbB2 anticancer therapy. Circ Res,. 106(1):35-46.

De M., Ghosh P., Rotello V. (2008). Applications of nanoparticles in biology. Adv Mater 20:4225–4241.

Devissaguet J.P, Fessi H., Puisieux F. (1991). Process for the preparation of dispersible colloidal systems of a substance in the form of nanocapsules. US Patent 5049322.

Dipple A., Moschel R.C, Bigger C.A.H. (1984). Polynuclear aromatic hydrocarbons. Chemical Carcinogens. American Chemical Society;1(2):41–163.

Donehower L., Harvey M., Slagle B., McArthur M., Montgomery Jr. C., Butel J. (1992). Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature; 356:215-21.

Dong Y., Feng S.S. (2004). Methoxy poly (ethylene glycol)- poly(lactide) (MPEG-PLA) nanoparticles for controlled delivery of anticancer drugs. Biomaterials.25: 2843–9.

Downes A., Mouras R., Bagnaninchi P., Elfick A. (2011). J. Raman Spectrosc. 42, 1864.

Downes A., Mouras R., Elfick A. (2009). J. Raman. Spectrosc. 40:757.

Dreher MR, Liu W, Michelich CR, Dewhirst MW, Yuan F, Chilkoti A. (2006). Tumor vascular permeability, accumulation, and penetration of macromolecular drug carriers. J Natl Cancer Inst., 1;98(5):335-344.

Drexler, K. E. (1986). Engines of Creation: The Coming Era of Nanotechnology, Doubleday.

Dufresne E.R., Corwin E.I., Greenblatt N.S., Ashmore J., Wang D.Y., *et al*. (2003). Flow and fracture in drying nanoparticle suspensions. Phys. Rev. Lett. 91:224501

Duncan M.D., Reintjes J., Manuccia T.J. (1982). Scanning coherent anti-Stokes Raman microscope. Opt. Lett. 7:350–52.

Duncan R. (2003). The dawning era of polymer therapeutics. Nat. Rev. Drug Discov; 2:347-60.

Duncan, R., Vicent M.J., Greco F., and Nicholson R.I. (2005). Polymer-drug conjugates: Towards a novel approach for the treatment of endrocine-related cancer. Endocr-Relat Cancer, 12(1): S189-S199.

Dwan'isa J., Rouxhet L., Brewster M., Preat V., Arien A. (2008). Spontaneously self-assembled micelles from poly (ethylene glycol)-b-poly(epsilon-caprolactoneco-trimethylene carbonate) for drug solubilization. Pharmazie 63:235–240.

E

Easton, D.F., K.A. Pooley, A.M. Dunning, P.D. Pharoah, D. Thompson, D.G. Ballinger, J.P. Struewing, J. Morrison, H. Field, R. Luben, N. Wareham, S. Ahmed, C.S. Healey, R. Bowman, K.B. Meyer, *et al*., (2007). Genome-wide association study identifies novel breast cancer susceptibility loci Nature; 447: 1087-93.

Edward A. Lefrak, J. P., tcaron, *et al*. (1973). A clinic-pathologic analysis of adriamycin cardiotoxicity. Cancer 32(2): 302-314.

Ehud G. (2005). Plenty of room for biology at the bottom: An introduction to bionanotechnology. Imperial College Press, 2007, ISBN 9781860946776.

Eliaz R.E., Nir S., Marty C., Szoka F.C. (2004). Determination and modeling of kinetics of cancer cell killing by doxorubicin and doxorubicin encapsulated in targeted liposomes. Cancer Res. 64:711-718.

Ericson M. B., Simonsson C., Guldbrand S., Ljungblad C., Paoli J. Smedh M. (2008). Two-photon laser-scanning fluorescence microscopy applied for studies of human skin. J. Biophoton. 1(4): 320-330.

F

Fan H., Alekha K.D. (2001). Effect of cross-linking on the in vitro release kinetics of doxorubicin from gelatin implants. Int. J. Pharm. 213:103–116.

Fang J, Nakamura H, Maeda H. (2010). The EPR effect: Unique features of tumor blood vessels for drug delivery, factors involved, and limitations and augmentation of the effect. Advanced Drug Delivery Reviews.

Fearon, E.R. and B. Vogelstein. (1990). A genetic model for colorectal tumorigenesis Cell; 61: 759-67.

Feng R., Song Z., Zhai G. (2012). Preparation and in vivo pharmacokinetics of curcumin-loaded PCL-PEG-PCL triblock copolymeric nanoparticles. International Journal of Nanomedicine.7: 4089–4098.

Feng S. S. and Chien, S.(2003). Chemotherapeutic engineering: Application and further development of chemical engineering principles for chemotherapy of cancer and other diseases. Chemical Engineering Science, 58 (18); 4087-4114.

Ferrari M. (2005). Cancer nanotechnology: Opportunities and challenges. Nature Reviews Cancer; 5(3):161-171.

Feynman, R. P. (1960). There's Plenty of Room at the Bottom, Engineering and Science magazine XXIII.

Fornari, F. A., J. K. Randolph, *et al*. (1994). Interference by doxorubicin with DNA unwinding in MCF-7 breast tumor cells. Mol Pharmacol 45(4): 649-656.

Forrest M., ZhaoA., Won C., Malick A., Kwon G. (2006). Lipophilic prodrugs of Hsp90 inhibitor geldanamycin for nanoencapsulation in poly (ethylene glycol)- b-poly(epsilon-caprolactone) micelles. J. Control. Rel. 116:139–149.

Freiberg S., Zhu X. (2004). Polymer microspheres for controlled drug release. International Journal of Pharmaceutics. 282:1-18.

Freshney, R. (1987). Culture of Animal Cells: A Manual of Basic Technique, 117.Alan R. Liss, Inc., New York.

G

Gabizon A. and Martin F., (1997). Polyethylene glycol-coated (pegylated) liposomal doxorubicin: rationale for use in solid tumors. Drugs,54 (4); 15-21.

Garcia M., Jemal A., Ward E., Center M., Hao Y., Siegel R., *et al*. (2007). Global cancer facts & figures. Atlanta, GA: Ame.

Ge H., Yong H. Xiqun J., Daming Cheng,1 Yuyan Y., Hong B. Changzheng Y. (2002). Preparation, Characterization, and Drug Release Behaviors of Drug Nimodipine-Loaded Poly(e-caprolactone)-Poly(ethylene oxide)-Poly(e-caprolactone) Amphiphilic Triblock Copolymer Micelles Journal of Pharmaceutical Sciences. 91:1463–1473.

Ge Z. S., Liu S. Y. (2013). Chem. Soc. Rev. 42: 7289-7325.

Gelder J. D., Gussem K. D., Vandenabeele P., Moens L. (2007). J. Raman Spectrosc. 38, 1133.

Geng Y, Dalhaimer P, Cai S *et al.* (2007). Shape effects of filaments versus spherical particles in flow and drug delivery.*Nat. Nanotech.*2 (4): 249–255.

Gewirtz, D. A. (1999). A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin. Biochemical Pharmacology 57(7): 727-741.

Ghoroghchian P. P., Li G. Z.M, Levine D. H., Davis K. P., Bates F. S., Hammer D. A., Therien, M. J.(2006). Macromolecules, 39 (5):1673–1675.

Gillies E. R., Goodwin A. P., Fr´echet J. M. J. (2004). Bioconjugate Chem. 15:1254-1263.

Gillies E.R., Fréchet J.M.J. (2005). pH-responsive copolymer assemblies for controlled release of doxorubicin. Bioconjug. Chem. 16, 361–368.

Gong C., Shi, S., Dong P., Kan B., Gou M., Wang X., Li X., Luoa F., Zhaob X.,Wei Y., Qian, Z. (2009). Synthesis and characterization of PEG-PCL-PEG thermosensitive hydrogel. Int. J. Pharm. 365, 89-99.

Gong C., Wang C., Wang Y., *et al*. (2012). Efficient inhibition of colorectal peritoneal carcinomatosis by drug loaded micelles in thermosensitive hydrogel composites. Nanoscale; 4(10):3095–3104.

Gong C.Y., Wang Y, Wang Y, Wei X, QinJie W. BiLan W., PengWei D., LiJuan C. , Feng L., ZhiYong Q. (2011). Biodegradable self-assembled PEG-PCL-PEG micelles for hydrophobic drug delivery, part 2: in vitro and in vivo toxicity evaluation. J. Nanopart. Res. 13:721–731.

Gou M., XiuLing Z., Ke M, Juan Z., Lan Z., XiuHong W., Feng L., YinLan Z., Xia Z., YuQuan W, and ZhiYong Q. (2009). Poly (ε-caprolactone)-Poly (ethylene glycol)-Poly (ε-caprolactone) Nanoparticles: Preparation, Characterization, and Application in Doxorubicin Delivery J. Phys. Chem. 113:12928–12933.

Gou M., Zheng L., XinYun P., Ke M., XiuLing Z., Shi Z., Gang G., Feng L., Xia Z., LiJuan C., YuQuan W., ZhiYong Q. (2009). Poly (caprolactone)–poly (ethylene glycol) – poly(caprolactone) (PCL–PEG–PCL) nanoparticles for honokiol delivery in vitro. International Journal of Pharmaceutics 375:170–176.

Gradishar W.J., Tjulandin S., Davidson N., *et al*. (2005). Phase III trial of nanoparticle albumin-bound paclitaxel compared with polyethylated castor oil-based paclitaxel in women with breast cancer. J.Clin.Oncol.; 23 :7794-803.

Gratton S, Ropp P, Pohlhaus P *et al.* (2008). The effect of particle design on cellular internalization pathways. Proc. Natl Acad. Sci. USA105,11613–11618.

Green M.R., Manikhas G.M., Orlov S., *et al*.(2006). Abraxane, anovel Cremophor-free, albumin-bound particle form of paclitaxel for the treatment of advanced nonsmall- cell lung cancer. Ann. Oncol. ; 17:1263-8.

Gref R., Minamitake Y., Peracchia M.T., Trebetskoy V.S, Torchilin V.P., Langer R. (1994). Biodegradable long-circulating polymeric nanospheres, Science 263:1600– 1603.

Gref, R., Lueck, M., Quellec, P., Marchand, M., Dellacherie, E., Harnisch, S., Blunk, T., Mu¨ller, R. H. (2000). Colloids Surf., B: Biointerfaces, 18, 301-313.

Grill L., Dyer M., Lafferentz L., Persson M., Peters M. V. and Hecht S. (2007). Nano-architectures by covalent assembly of molecular building blocks. Nature Nanotechnology (2): 687 – 691.

Grossman H. J. and Scott E. M. (2012). Nanotechnology in Cancer Medicine Physics Today. 65(8):38.

Gupta B., Tatiana S. L., Vladimir P. T. (2005). Intracellular delivery of large molecules and small particles by cell-penetrating proteins and peptides. Advanced Drug Delivery Reviews, 57; 637 – 651.

H

Hairong W., Jinlin H., Mingzu Z., Yunfeng T., Fei L., Kam Chiu T. Peihong N. (2013). Biocompatible and acid-cleavable poly (caprolactone)-acetal-poly (ethylene glycol)-acetal-poly (caprolactone) triblock copolymers: synthesis, characterization and pH-triggered doxorubicin delivery. J. Mater. Chem. B; (1):6596.

Haka A., Motz J., Gandhi S., Scepanovic O., Kramer J., Dasari R., Feld M., J. Biomed. (2005). Opt., 10, 031113.

Hamaguchi T., Kato K., Yasui H., Morizane C., Ikeda M., Ueno H., Muro K., Yamada Y., Okusaka T., Shirao K., Shimada Y., Nakahama H., Matsumura Y. (2007). A phase I and pharmacokinetic study of NK105, a paclitaxel-incorporating micellar nanoparticle formulation. Br. J. Cancer 97:170–176.

Hanahan D. and Weinberg R. A. (2000). The six hallmarks of cancer. Cell, 100: 57–70.

Hanahan, D. & Weinberg, R. A (2011). Hallmarks of cancer: the next generation. Cell, 144 (5), 646–674.

Hans, M.L. *et al*., (2002). Biodegradable nanoparticles for drug delivery and targeting. Current Opinion in Solid State and Materials Science (6): 319-327.

Hartwell L., Kastan M. (1994). Cell cycle control and cancer. Science; 266:1821.

Hashizume H, Baluk P, Morikawa S, McLean JW, Thurston G, Roberge S, *et al.* (2000). Openings between Defective Endothelial Cells Explain Tumor Vessel Leakiness. Am J Pathol, 156(4):1363-1380.

He G., Ma L.L., Jie P., Subbu V. (2007). ABA and BAB type triblock copolymers of PEG and PLA: A comparative study of drug release properties and "stealth" particle characteristics. International Journal of Pharmaceutics 334: 48–55.

Heath J.R. Davis M.E.(2008). Nanotechnology and cancer. Annu. Rev. Med., 59:251-265.

Heller J., Barr J., Ng S. Y., Abdellauoi K. S., Gurny R. (2002). Adv. Drug Delivery Rev. 54:1015- 1039.

Hirano A., Kawanami T., Llena J.F. (1994). Electron microscopy of the blood– brain barrier in disease, Microsc. Res. Tech. 27: 543–556.

Hobbs S.K., Monsky W.L., Yuan F., Roberts W.G., Griffith L., Torchilin V.P., Jain R.K. (1998). Regulation of transport pathways in tumor vessels: role of tumor type and microenvironment, Proc. Natl. Acad. Sci. USA 95 : 4607–4612.

Hobbs SK, Monsky WL, Yuan F, Roberts WG, Griffith L, Torchilin VP, *et al*. (1998). Regulation of transport pathways in tumor vessels: Role of tumor type and microenvironment. Proceedings of the National Academy of Sciences of the United States of America;95(8):4607-4612.

Hofheinz R.D., Gnad-Vogt S.U., Beyer U., Hochhaus A. (2005). Liposomal encapsulated anti-cancer drugs. Anticancer Drugs;16:691-707.

Hsieh M., Cuong N., Chen C., Chen Y., Yeh J. (2008). Nano-sized micelles of block copolymers of methoxy poly (ethylene glycol)–poly (epsilon-caprolactone)-graft-2-hydroxyethyl cellulose for doxorubicin delivery. J. Nanosci. Nanotechnol. 8:2362-2368.

http://sphweb.bumc.bu.edu/otlt/MPH Modules/PH/PH709_Cancer/PH709_Cancer_print.html

Hu Y., Leyang Z., Yi C., Haixiong G., Xiqun J., Changzheng Y. (2004). Degradation Behavior of Poly (caprolactone)-b-poly (ethylene glycol)-b-poly (caprolactone) Micelles in Aqueous Solution. Biomacromolecules, 5, 1756-1762.

Hu, C.M.J. and Zhang L. (2012). Nanoparticle-based combination therapy toward overcoming drug resistance in cancer. Biochem. Pharmacol. 83(8): 1104-1111.

Huang S. (1985). Biodegradable Polymers. In: Mark F, Bikales N, Overberger C, Menges G, Kroshwitz J, editors. Encyclopedia of Polymer Science and Engineering. New Yord: John Wiley and Sons; 220-43.

Huang Y., Tang Z. H, Zhang X. F., Yu H. Y., Sun H., Pang X., Chen X. S. (2013). Biomacromolecules. 14:2023-2032.

Huang Z, McWilliams A, Lui H, McLean DI, Lam S, Zeng H. (2003). Nearinfrared Raman spectroscopy for optical diagnosis of lung cancer. Int. J. Cancer 107:1047–52.

Husain, h. Y., and Al-Alawachi, S. F. A. (2014). Incidence Rates, Pattern and Time Trends of Registered Cancer in Iraq (1991-2008) Population and Hospital Based Registry. Open Access Library (OALib); 1, 1-6.

Husseini, G.A. and Pitt, W.G.(2008). The use of ultrasound and micelles in cancer treatment. J. Nanosci. Nanotechnol. 8(5) : 1-11.

I

Ideta R., Tasaka F., Jang W.D., Nishiyama N., Zhang, G.D., Harada, A., Yanagi Y., Tamaki Y., Aida T., Kataoka K. (2005). Nanotechnology-based photodynamic therapy for neovascular disease using a supramolecular nanocarrier loaded with a dendritic photosensitizer. Nano Lett. 5:2426–2431.

J

Jain R., Standley S. M., Fr´echet, J. M. J. (2007). Macromolecules. 40:452-457.

Jain, R.K., Munn L.L., and Fukumura D. (2002). Dissecting tumour pathophysiology using intravital microscopy. Nat. Rev. Cancer. 2:266-276.

Janet, F. (2012). Spotlight on Top 5 Cancers in the Middle East Region (Talking Cancers in the Middle East. Cancer World Organization Cited)

Jarrett P.; Benedict C., Bell J.P., Cameron J.A., Huang S.J. (1984). Mechanism of the Biodegradation of Polycaprolactone; Polymers as Biomaterials; Plenum Press: New York, NY, USA.

Jemal, A., Bray, F., Center, M. M., Ferlay, J., Ward, E., Forman, D. (2011). Global cancer statistics. C.A.Cancer J. Clin, 61(2), 69-90.

Jette K., Law D., Schmitt E., Kwon G. (2004). Preparation and drug loading of poly (ethylene glycol)-block–poly(epsilon-caprolactone) micelles through the evaporation of a cosolvent azeotrope. Pharm. Res. 21:1184-1191.

Jia Y.H., Yuan M., Yuan H.D., Huang X.L., Sui X., Cui X.M., Tang F.Q. Peng J., Chen J.Y., Lu S.B., et al. (2012). Co-encapsulation of magnetic Fe3O4 nanoparticles and doxorubicin into biodegradable PLGA nanocarriers for intratumoral drug delivery. Int. J. Nanomed.7:1697–1708.

Jokerst J.V., Miao Z., Zavaleta C., Cheng Z., Gambhir S.S. (2011). Affibody functionalized gold-silica nanoparticles for Raman molecular imaging of the epidermal growth factor receptor. Small. 7(5):625–633.

Jones, M. C., Leroux, J. C. Eur. (1999). J. Pharmacol. Biopharmacol. 48, 101-111.

Juliene M. C., Alonso M. J., Gomez Amoza, J. L., Benoit J. P. (1992). Drug Dev. Ind. Pharm.18, 1063.

K

Kim K.T., D., Youn Y.S., Bae Y.H. (2007). Tumor pH-responsive flower-like micelles of poly (L-lactic acid)-b-poly (ethylene glycol)-b-poly (L-histidine). J. Control Release .123:19–26.

Kabanov A. V., Chekhonin V. P., Alakhov V. Y., Batrakova E. V., Lebedev A.S. , Melik-Nubarov N. S., Arzhakov , Levashov A. V., Morozov G. V.(1989). FEBS Lett., 258:343 – 345.

Kastan, M. B., O. Onyekwere, *et al*. (1991). Participation of p53 Protein in the Cellular Response to DNA Damage. Cancer Res 51, 23 (1): 6304-6311.

Kataoka K., Kwon G. S., Yokoyama M., Okano T., Sakurai Y. (1993). J. Control. Release.24, 119.

Kataoka, K., Harada A., and Nagasaki Y. (2012). Block copolymer micelles for drug delivery: design, characterization and biological significance. Adv. Drug. Deliv. Rev., 2012. 64; 37-48.

Kataoka, K., Harada, A., Nagasaki, Y. (2001). Block copolymer micelles for drug delivery: design, characterization and biological significance. Adv. Drug Delivery Rev., 47 (1):113–131.

Kate Y. J. (2011). Colloidal gold nanoparticles for cancer therapy: effects of particle size on treatment efficacy, toxicology, and biodistribution. Ph.D. dissertation. USA.

Katharina S., Johannes M. P., Hanno S., and Tobias L. R. (2014). Radiolabeling of Nanoparticles and Polymers for PET Imaging. Pharmaceuticals, 7; 392-418.

Kato Y., Shigeyuki O., Chihiro M., Yojiro M., Atsuko S., Toyonobu M. Yuh B. (2013). Acidic extracellular microenvironment and cancer. Cancer Cell International, 13:89.

Kedar U., Prasanna P., Supriya S., Tech V. K. (2011). Advances in polymeric micelles for drug delivery and tumor targeting. Nanomedicine: Nanotechnology, Biology, and Medicine. 6:714–729.

Khurana, J (2009). Development and Characterization of Polymeric Nanoparticulate Delivery System for Hydrophillic Drug:Gemcitabine. Ph.D. thesis, Creighton University.

Kim T.Y., Kim D.W., Chung J.Y., *et al*. (2004). Phase I and pharmacokinetic study of Genexol PM, a cremophor-free, polymeric micelle-formulated paclitaxel, in patients with advanced malignancies. Clin. Cancer Res.; 10:3708 -16.

Kipp J.E. (2004). The role of solid nanoparticle technology in the parenteral delivery of poorly water-soluble drugs. Int. J. Pharm.284:109-12.

Knop K., Hoogenboom R., Fischer D, Ulrich S. S. (2010). Poly (ethylene glycol) in Drug Delivery: Pros and Cons as Well as Potential Alternatives. Angew. Chem. Int. Ed., 49:6288 – 6308.

Knudson, A.G. (2002). Cancer genetics. Am. J. Med. Genet; 111: 96-102.

Koenig M.F., Huang S.J. (1995). Biodegradable blends and composites of poly-caprolactone and starch derivatives. Polymer 36:1877–1882.

Kopecek J., Kopeckova, P. Minko, T. Lu Z.R. Peterson, C.M. (2001). Water soluble polymers in tumor targeted delivery. J. Control. Rel. 74:147–158.

Krishna, R. and Mayer L. D. (2000). Multidrug resistance (MDR) in cancer: Mechanisms, reversal using modulators of MDR and the role of MDR modulators in influencing the pharmacokinetics of anticancer drugs. European Journal of Pharmaceutical Sciences 11(4): 265-283.

Kroemer G. (1997). The proto-oncogene Bcl-2 and its role in regulating apoptosis. Nature Medicine; 3: 614-20.

Kumar N., Ravikumar M. N. V, and Domb A. J. (2001). Biodegradable block copolymers. Adv. Drug Deliv. Rev. 53:23Y44.

Kwon G.S., Okano T. (1996). Polymeric micelles as new drug carriers, Adv. Drug Deliv. Rev. 21:107–116.

L

La Morte W.W. (2014). The Biology of Cancer. Boston University.

La S.B., Okano T., Kataoka K. (1996). Preparation and characterization of the micelle-forming polymeric drug indomethacin-incorporated poly(ethylene oxide)-poly(b-benzyl L-aspartate) block copolymer micelles. J.Pharm. Sci. 85:85–90.

Lammers, T., Kiessling F., Hennink W.E, Storm G. (2012). Drug targeting to tumors: principles, pitfalls and (pre-) clinical progress. J. Control Release, 161(2):175-187.

Larsen A.K, Escargueil A.E., Skladanowski A. (2000). Resistance mechanisms associated with altered intracellular distribution of anticancer agents. Pharmacol. Ther. 85, 217–229.

Lasic, D. D. (1992). Mixed Micelles in drug delivery. Nature, *355* (6357), 279–280.

Latere D.J., Rouxhet L., Brewster M.E., Préat V., Arien A. (2008). Spontaneously self-assembled micelles from poly (ethylene glycol)-b-poly(epsilon-caprolactoneco-trimethylene carbonate) for drug solubilization. Pharmazie 63: 235–240.

Levenson, AS; Jordan VC. (1997). MCF-7: the first hormone-responsive breast cancer cell line. Cancer Research 57 (15): 3071–3078.

Levine A., Finlay C., Hinds P. (2004). P53 is a tumor suppressor gene. Cell.116:S67-9.

Li C. (2002). Poly (L-glutamic acid)-anticancer drug conjugates. Adv. Drug Deliv. Rev.; 54:695-713.

Li S., Garreau H., Vert M., Petrova T.S., Manolova N., Rashkov I., (1998). Hydrolytic degradation of poly (oxyethylene)-poly (caprolactone) multiblock copolymers. J. Appl. Polym. Sci. 68, 989–998.

Li. S., Garreau H., Vert M., Petrova T.S., Manolova N., Rashkov I. (1998). Hydrolytic degradation of poly (oxyethylene)-poly (caprolactone) multiblock copolymers. J. Appl. Polym. Sci. 68, 989–998.

Liang P., Zhao D., Wang C. Q., Zong, J.Y., Zhuo R.X., Cheng S.X. (2013). Facile preparation of heparin/CaCO3/CaP hybrid nano-carriers with controllable size for anticancer drug delivery. Colloids Surf. B. Biointerfaces **.**102:783-788.

Lin J., Zhang S., Chen T., Lin S., Jin H. (2006). Micelle formation and drug release behavior of polypeptide graft copolymer and its mixture with polypeptide block copolymer. Int. J. Pharm. 336:49–57.

Lin J., Zhang S., Chen T., Lin S., Jin H. (2006). Micelle formation and drug release behavior of polypeptide graft copolymer and its mixture with polypeptide block copolymer. Int. J. Pharm. 336:49–57.

Lin W.J., Flanagan D.R., Linhardt R.J. (1999). A novel fabrication of poly (caprolactone) microspheres from blends of poly(caprolactone) and poly(ethylene glycol)s. Polymer 40:1731–1735.

Lince F., Marchisio D.L., Barresi A.A. (2008). Strategies to control the particle size distribution of poly-ε- caprolactone nanoparticles for pharmaceutical applications. J. Colloid. Interface Sci.322:505–15.

Lippman, M. E. (2005). Breast Cancer. In B. E. Fauci A.S., Kasper D.L., Hauser S.L., Longo D.L., Jameson L.I., Loscalzo J. (Ed.), Harrisons Principles Of Internal Medicine.1,(516-522). New York. McGraw-Hill.

Liu Y., Wenping W., Jianhong Y., Chengming Z., Jin S. (2013). pH-sensitive polymeric micelles triggered drug release for extracellular and intracellular drug targeting delivery. Asian Journal of Pharmaceutical Sciences 8:159-167.

Liu Y., Zhenxian Z., Jia W., Abdelslam E.G. Suming L. (2007). J. Colloid Interface Sci. 314 470.

Liu, S. Q., Wiradharma N., Gao S. J., Tong Y. W., Yang, Y. Y. (2007). Biomaterials, 28 (7):1423–1433.

Logothetidis S. (2012). Nanomedicine and Nanobiotechnology. Springer. Germany.

Lu Y., Park P. (2013). Polymeric micelles and alternative nanonized delivery vehicles for poorly soluble drugs. International Journal of Pharmaceutics 453:198– 214.

Lukenbill, J. and Kalaycio M.. (2013). Fludarabine: a review of the clear benefits and potential harms. Leukemia Res., 37(9):986-994.

M

Ma G., Miao B., Song C. (2010). Thermosensitive PCL-PEG-PCL hydrogels: Synthesis, characterization and delivery of proteins. J. Appl. Polym. Sci.116, 1985-1993.

Maeda, H. (2001). The enhanced permeability and retention (EPR) effect in tumor vasculature: the key role of tumor-selective macromolecular drug targeting. Adv. Enzyme Regul., 41(1):189-207.

Maeda, H., Wu J., Sawa T., Matsumura Y., and Hori K. (2000). Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. J Control Release, 65 (1–2):271-284.

Maitra, A., Kern S.E. and Hruban R.H. (2006). Molecular pathogenesis of pancreatic cancer Best Pract Res Clin Gastroenterol.; 20: 211-26.

Malik N., Evagorou E.G., Duncan R. (1999). Dendrimer platinate: a novel approach to cancer chemotherapy. Anticancer Drugs; 10: 767-76.

Manaspon C., Viravaidya-Pasuwat K., Pimpha N. (2012). Preparation of folate-conjugated pluronic F127/chitosan core-shell nanoparticles encapsulating doxorubicin for breast cancer treatment. J. Nanomater. 593878.

Mansfield J., Yu J., Attenburrow D., Moger J., Tirlapur U., Urban J., Cui Z., P. Winlove, J. (2009). Anat. 215:682.

Markman M. (2006). Pegylated liposomal doxorubicin in the treatment of cancers of the breast and ovary. Expert Opin. Pharmacother. ; 7:1469-74.

Martini L., Attwood D., Collett J.H., Nicholas C.V., Tanodekaew S., Deng N.J., Heatley F., Booth C. (1994) . Micellisation and gelation of triblock copolymer of ethylene oxide and poly-caprolactone, ClnEmCLn, in aqueous solution. J. Chem. Soc. Faraday Trans. 90:1961–1966.

Matsumura Y, Maeda H. (1986). A New Concept for Macromolecular Therapeutics in Cancer Chemotherapy: Mechanism of Tumoritropic Accumulation of Proteins and the Antitumor Agent Smancs. Cancer Research . 1;46, (1):6387-6392.

Matthews T. E., Wilson J. W., Degan S.,. Simpson M. J, Jin J. Y., Zhang J. Y., Warren W. S. (2011). Biomed. Opt. Expr. 2:1576.

Mazzaferro S., Bouchemal K., Maksimenko A., Skanji R., Opolon P., Ponchel G.(2012). Reduced intestinal toxicity of docetaxel loaded into mucoadhesive nanoparticles, in mouse xenograft model. J. Colloid. Sci. Biotechnol. 1:210–7.

Merkli A., Tabatabay C., Gurny R., Heller J.(1998). Biodegradable polymers for the controlled release of ocular drugs. Progress in Polymer Science. 23:563-80.

Michaud, L. B., Espirito, J., Esteva, F. J. (2008). Breast Cancer. In M. A. Chisolm-Burns (Ed.), Pharmacotherapy principles & practice, (2121 - 2258). New York: McGraw-Hill Medical.

Miladi K., Sfar S., Fessi H., Elaissari A. (2013). Drug carriers in osteoporosis: preparation, drug encapsulation and applications. Int. J. Pharm. 445:181–95.

Miller A.B., Hoogstraten B., Staquet M., Winkler A (1981). Reporting results of cancer treatment. Cancer; 47 (1):207-214.

Modi S, Jain JP, Domb AJ, Kumar N. (2006). Exploiting EPR in Polymer Drug Conjugate Delivery for Tumor Targeting. Current Pharmaceutical Design,12(36):4785-4796.

Moghimi S.M., Hunter A.C., Murray J.C. (2001). Long-circulating and targetspecific nanoparticles: theory to practice, Pharm. Rev. 53 (2):283–318.

Mohan P., Rapoport N. (2010). Doxorubicin as a molecular nanotheranostic agent: Effect of doxorubicin encapsulation in micelles or nanoemulsions on the ultrasound-mediated intracellular delivery and nuclear trafficking. Mol. Pharm. 7:1959-1973.

Moon H.T., Lee Y.K., Han J.K., Byun Y.J. (2002). Improved blood compatibility by sustained release of heparin-deoxycholic acid conjugates in a PCL–PEG multiblock copolymer matrix. J. Biomater. Sci. Polym. 13:817–828.

Moretton M.A., Chiappetta D.A., Sosnik A. (2012). Cryoprotection–lyophilisation and physical stabilization of rifampicin-loaded flower-like polymericmicelles. J. R. Soc. Interface. 9: 487–502.

Moretton M.A., Glisoni R.J., Chiappetta D.A., Sosnik A. (2010). Molecular implications in the nanoencapsulation of the antituberculosis drug rifampicin within flower-like polymeric micelles. Colloids Surf B Biointerfaces.79:467–79.

Mouras R, Rischitor G., Downes A., Salter D. Elfick A. (2010). Nonlinear opticalmicroscopy for drug delivery monitoring and cancer tissue imaging. J. Raman Spectrosc., 41: 848–852.

Mouras R. *et al*. (2010). A multi-modal, multi-photon microscope for biological imaging, Proc. SPIE 7569, 756933.

Mouras R., Bagnaninchi P, Downes A., Elfick A. (2013). Multimodal, label-free nonlinear optical imaging for applications in biology and biomedical science. J. Raman Spectrosc., 44:1373–1378.

Mu, L., Elbayoumi T.A, and Torchilin V.P. (2005). Mixed micelles made of poly (ethylene glycol)-phosphatidylethanolamine conjugate and D-a-tocopheryl polyethylene glycol 1000 succinate Int. J. Pharm. 306: 142-149.

N

Nagarajan R., Barry M. (1986). Unusual selectivity in solubilization by block copolymer micelles. Abstr. Papers Amer. Chem. Soc. 191:287–290.

Nah J.W., Jeong Y.I., Cho C.S. (1998). Clonazepam release from core-shell type nanoparticles composed of poly (γ-benzyl-L-glutamate) as the hydrophobic part and poly (ethylene oxide) as the hydrophilic part. J. Polym. Sci. B. Polym. Phys. 36:415-23.

Nair L.S., Laurencin C.T. (2007). Biodegradable polymers as biomaterials. Progress in Polymer Science. 32:762-98.

Nakanishi T., Fukushima S., Okamoto K., *et al*. (2001). Development of the polymer micelle carrier system for doxorubicin. J. Control Release; 74:295–302.

Nakayama M., Chung J.E., Miyazaki T., Yokoyama M., Sakai K., Okano T. (2007). Thermal modulation of intracellular drug distribution using thermoresponsive polymeric micelles. React. Funct. Polym. 67: 1398–1407.

Nan X., Yang W.Y., Xie X.S. (2004). CARS microscopy: lights up lipids in living cells. Biophotonics Int. 11:44.

Narod, S.A. (2006). Modifiers of risk of hereditary breast cancer Oncogene; 25: 5832-6.

Nasongkla N., Bey E., Ren J., Ai H., Khemtong C., Guthi J.S. (2006). Multifunctional polymeric micelles as cancer-targeted, MRI-ultrasensitive drug delivery systems. Nano. Lett.; 6:2427-30.

Natalie P. P. and Mandal T.K. (2007). Engineered Nanoparticles in Cancer Therapy. Recent Patents on Drug Delivery & Formulation.1:37-51 37.

Nguyen T.H.A, Nguyen V.C. (2010). Formation of nanoparticles in aqueous solution from poly (caprolactone) – poly(ethylene glycol)–poly(caprolactone). Adv. Nat. Sci.: Nanosci. Nanotechnol.1, 025012:5.

Nguyen-Van C., Jian-Lin J., Yu-Lun L., Jim-Ray C., Shyh-Chuan J., Ming-Fa H. (2011). Doxorubicin-Loaded PEG-PCL-PEG Micelle Using Xenograft Model of Nude Mice: Effect of Multiple Administration of Micelle on the Suppression of Human Breast Cancer. Cancers, 3, 61-78.

Nolting B. (2005). Biophysical Nanotechnology. In: "Methods in Modern Biophysics", Springer, ISBN 3-540-27703-X.

Nyman D.W., Campbell K.J., Hersh E., *et al*. (2005). Phase I and pharmacokinetics trial of ABI-007, a novel nanoparticle formulation of paclitaxel in patients with advanced nonhematologic malignancies. J. Clin. Oncol.;23:7785-93.

O

Oh K.T., Oh Y.T., Oh N.M., Kim K., Lee D.H., Lee E.S. (2009). A smart flower-like polymeric micelle for pH-triggered anticancer drug release. Int. J.Pharm. 375:163–9.

Ojugo A.S.E., Mcsheehy P.M.J, Mcintyre D.J.O, Mccoy C., Stubbs M., Leach M.O., *et al*. (1999). Measurement of the extracellular pH of solid tumors in mice by magnetic resonance spectroscopy: a comparison of exogenous 19F and 31P probes NMR. Biomedicine. 12:495-504.

Okada M. (2002). Chemical syntheses of biodegradable polymers. Progress in Polymer Science. 27:87-133.

P

Pastorin G., Wu W., Wieckowski S., *et al*. (2006). Double functionalisation of carbon nanotubes for multimodal drug delivery. Chem. Commun.; 11:1182-4.

Patil R., Portilla-Arias J., Ding, H., Konda B., Rekechenetskiy A., Inoue S., Black K.L., Holler E., Ljubimova, J.Y. (2012). Cellular Delivery of Doxorubicin via pH-Controlled Hydrazone Linkage Using Multifunctional Nano Vehicle Based on Poly (ßL-Malic Acid). Int. J. Mol. Sci. 13: 11681-11693.

Pavlidis, E.T. and Pavlidis T.E. (2013). Role of bevacizumab in colorectal cancer growth and its adverse effects: A review. World J Gastroentero,. 19(31): 5051-5060.

Pease R. F., and Chou S. Y. (2002). Lithography and Other Patterning Techniques for Future Electronics. Proceedings of the IEEE 96, 248.

Peer, D., Karp J.M., Hong S., *et al*. (2007). Nanocarriers as an emerging platform for cancer therapy. Nat Nanotechnol, 2(12): 751-760.

Peracchia M.T., Vauthier C., Passirani C., Couvreur P., Labarre D. (1997). Complement consumption by polyethylene glycol in different conformations chemically coupled to poly (isobutyl 2-cyanoacrylate) nanoparticles. Life Sci 61:749–761.

Perry W. Ryan M. B., Edward B. B. (2012). Two-Photon and Second Harmonic Microscopy in Clinical and Translational Cancer Research. Ann Biomed Eng. 40(2): 277–291.

Pigram W.J., Fuller W., Hamilton L.D. (1972). Stereochemistry of intercalation: Interaction of daunomycin with DNA. Nature New Bio 235:17–19.

Potma E. O., Evans C.L., Xie X.S. (2006). Heterodyne coherent anti-Stokes Raman scattering (CARS) imaging. Opt. Lett. 31:241–43.

Potma E.O., Xie X.S. (2005). Direct visualization of lipid phase segregation in single lipid bilayers with coherent anti-Stokes Raman scattering microscopy. Chem. Phys. Chem. 6:77–79.

Prakash, S., M., Malhotra, W. Shao, C., Tomaro-Duchesneau, and S. Abbasi,S.(2011). Polymeric nanohybrids and functionalized carbon nanotubes as drug delivery carriers for cancer therapy. Adv. Drug. Deliv. Rev.; 63(14-15):1340-1351.

Pratten M. K, Lloyd J. B., Horpel G., and Ringsdorf H.(1985). Micelle-forming block copolymers: pinocytosis by macrophages and interaction with model membranes. Makromol. Chem. 186:725Y733.

R

Rao J.P., Geckeler K.E. (2011). Polymer nanoparticles: preparation techniques and size-control parameters. Prog. Polym. Sci.; 36:887–913.

Rawat M, Singh D, Saraf S, Saraf S. (2006). Nanocarriers:promising vehicle for bioactive drugs. Biol. Pharm. Bull.;29 :1790-8.

Remant B.K.C, Bhattarai S.R. , Aryal S., Khil M.S. , Dharmaraj N. Kim H. Y. (2007). Colloids Surf. A 292 69.

Rettig R. (2005). Cancer crusade: The story of the National Cancer Act of 1971: Authors Choice Press.

Reyes J. M. G. *et al*. (2006). Metabolic changes in mesenchymal stem cells in osteogenic medium measured by autofluorescence spectroscopy, Stem Cells 24(5):1213–1217.

Rice W. L., Kaplan D. L, Georgakoudi I. (2010). Two-photon microscopy for non-invasive, quantitative monitoring of stem cell differentiation. PLoS ONE 5(4), e10075.

Rivera E. (2003). Current status of liposomal anthracycline therapy in metastatic breast cancer. Clin Breast Cancer; 4:S76-83.

Rosenthal E., Poizot-Martin I., Saint-Marc T., Spano J.P., Cacoub P. (2000). Group DNXS. Phase IV study of liposomal daunorubicin (DaunoXome) in AIDS-related Kaposi sarcoma. Am. J. Clin. Oncol. 25:57- 9.

Ruzin. (1999). Plant Microtechnique and Microscopy.

Rybak, L.P., (2007). Mechanisms of cisplatin ototoxicity and progress in otoprotection. Curr Opin Otolaryngo,. 15(5):364-369.

Ryu J.G., Jeong Y.I., Kim I.S., Lee J.H., Nah J.W., Kim S.H.(2000). Clonazepam release from core–shell type nanoparticles of poly (caprolactone)/ poly (ethylene glycol)/poly(caprolactone) triblock copolymers. Int. J. Pharm. 200:231–242.

S

Sabbatini P., Aghajanian C., Dizon D., *et al*. (2004). Phase II study of CT-2103 in patients with recurrent epithelial ovarian, fallopian tube, or primary peritoneal carcinoma. J.Clin. Oncol.; 22:4523-31.

Sahai, E. (2007). Illuminating the metastatic process. Nat Rev Cancer, 7(10) , 737-749.

Sahoo S.K., Labhasetwar V. (2003). Nanotech approaches to drug delivery and imaging. Drug Discov Today; 8: 1112-1120.

Salamanca-Buentello F., Persad D.L., Court E.B., Martin D.K., Daar A.S., *et al*. (2005). Nanotechnology and the Developing World. PLoS Med 2: 97.

Salazar C., El-Arabi A.M., Scmidt J.J. (2012). EC50 and IC50 measurements of TRPM8 in lipid bilayers, Biophysical journal,102 (3): 344a-345a.

Sanson C., Christophe S., Jean-François L.M., Alain S., Julie T., Elisabeth G., Sébastien L. (2010). A simple method to achieve high doxorubicin loading in biodegradable polymersomes. Journal of Controlled Release 147: 428–435.

Sato, M., Shames D.S., Gazdar A.F. and Minna J.D.(2007).A translational view of the molecular pathogenesis of lung cancer J. Thorac. Oncol. 2: 327- 43.

Seymour, L., R. Duncan, Strohalm J., and Kopecek J. (2005). Effect of molecular weight (Mw) of N-(2-hydroxypropyl) methacrylamide copolymers on body distribution and rate of excretion after subcutaneous, intraperitoneal, and intravenous adminustration to rats. Drugs Pharmaceut Sci,.148:1341-1358.

Shapira, A., Livney Y.D., Broxterman H.J., and Assaraf Y.G.(2011). Nanomedicine for targeted cancer therapy: Towards the overcoming of drug resistance. Drug Resist Updat. 14(3):150-163.

Shawn C. O., Dianna P.Y. Molly. S. S. (2012). Polymeric micelle stability. Nano. Today. 7, 53—65.

Shen Y.Y., Gao Z.G., Rapoport N. (2009). Recent advances in the applications of ultrasonic microbubbles as gene or drug vectors. 44(9):961–6.

Shuai X., Aia H., Nasongkla N., Kim S., Gao J. (2004). J. Controlled Release, 98, 415.

Shuai, X., Merdan, T., Unger, F., Wittmar M., Kissel T. (2003). Macromolecules, 36, 5751.

Simşek S., Eroğlu H., Kurum B., Ulubayram K. (2010). Brain targeting of atorvastatin loaded amphiphilic plga-bpeg nanoparticles. J. Microencapsul. 30:10–20.

Singal, P., T. Li, *et al*. (2000). Adriamycin-induced heart failure: mechanisms and modulation. Molecular and Cellular Biochemistry 207(1): 77-86.

Singh P., Destito G., Schneemann A., Manchester M. (2006). Canine parvovirus-like particles, a novel nanomaterial for tumor targeting. J. Nanobiotechnology; 4:2.

Singh S., Muthu M. (2007). Preparation and characterization of nanoparticles containing an atypical antipsychotic agent. Nano Med. 2: 233–240.

Sinha V.R., Bansal K., Kaushik R., Kumria R., Trehan A. (2004). Poly-epsilon-caprolactone microspheres and nanospheres: an overview. Int. J. Pharm.278:1-23.

Skladanowski, A. and Konopa J.(1994). Interstrand DNA crosslinking induced by anthracyclines in tumour cells. Biochemical Pharmacology 47(12): 2269-2278.

Slater T. *et al*. (1963). Biochem. Biophys. Acta 77:383.

Soares A.S.P. (2013). A special issue on applications of microencapsulation. J. Colloid. Sci. Biotechnol.2:77.

Soule, HD; Vazquez J; Long A; Albert S; Brennan M. (1973). A human cell line from a pleural effusion derived from a breast carcinoma. Journal of the National Cancer Institute 51 (5): 1409–1416.

Sturgeon R.J., Schulman S.G. (1977). Electronic absorption spectra and protolytic equilibria of doxorubicin: direct spectrophotometric determination of microconstants. J Pharm Sci. 66 (7):958-61.

Svenson S., Tomalia D.A. (2005). Dendrimers in biomedical applications reflections on the field. Adv. Drug Deliv. Rev.; 57:2106- 29.

T

Takakura Y, Mahato RI, Hashida M. (1998). Extravasation of macromolecules. Advanced Drug Delivery Reviews ;34(1):93-108.

Takeuchi Y., Ichikawa K., Yonezawa S., Kurohane K., Koishi, T., Nango M., Namba Y., Oku N. (2004). Intracellular target for photosensitization in cancer antiangiogenic photodynamic therapy mediated by polycation liposome. J. Control. Rel. 97:231–240.

Tan, C., H. Tasaka, *et al*. (1967). Daunomycin, an antitumor antibiotic, in the treatment of neoplastic disease. Clinical evaluation with special reference to childhood leukemia. Cancer, 20(3): 333-353.

Tanaka, M. and S. Yoshida. (1980). Mechanism of the Inhibition of Calf Thymus DNA Polymerases {alpha} and {beta} by Daunomycin and Adriamycin.J Biochem. 87(3): 911-918.

Tang, L. and Cheng J. (2013). Nonporous silica nanoparticles for nanomedicine application. Nano Today. 8(3): 290-312.

Tanodekaew S., Pannu R., Heatley F., Attwood D., Booth C. (1997). Association and surface properties of diblock copolymers of ethylene oxide and d,l-lactide in aqueous solution. Macromol. Chem. Phys. 198: 927–944.

Thiery J. (2002). Epithelial mesenchymal transitions in tumor progression. Nature Reviews Cancer; 2:442-54.

Thorell JI, Larson SM (1978). Radioimmunoassay and Related Techniques. Methodology and Clinical Applications. Saint Louis, The C. V. Mosby Company.

Tonhauser C., Sch¨ull C., Dingels C., Frey H. (2012). ACS Macro Lett. 1:1094-1097.

Torchilin, V. P. (2007). Micellar nanocarriers: pharmaceutical perspectives. Pharm. Res., 24 (1), 1–16.

Torchilin, V.P. (2010). Passive and active drug targeting: Drug delivery to tumors as an example. Drug Deliv. 3-53.

V

V**an de Loosdrecht A.A., *et al*. (1994).** J. Immunol. Methods 174: 311-320.

Van Natta F.J., Hill J.W., Carruthers W.H. (1934). Polymerization and ring formation, ε-caprolactone and its polymers. J. Am. Chem. Soc.; 56:455-9.

Vasey P.A., Kaye S.B., Morrison R., *et al*. (1999). Phase I clinical and pharmacokinetic study of PK1 (N-(2-hydroxypropyl) methacrylamide copolymer doxorubicin): first member of a new class of chemotherapeutic agents drug polymer conjugates. Clin. Cancer Res.; 5:83-94.

Vasir J.K., Reddy M.K., Labhasetwar V. (2005). Nanosystems in drug targeting: opportunities and challenges. Curr Nanosci ; 1:47-64.

Vauthier C., Bouchemal K. (2009). Methods for the preparation and manufacture of polymeric nanoparticles. Pharm. Res.; 26:1025–58.

Venkataraman S., Hedrick J.L., Ong Z.Y., Yang C., Ee P.L.R., Hammond P.T., *et al*. (2011). The effects of polymeric nanostructure shape on drug delivery. Adv. Drug Deliv. Rev. 63: 1228–46.

Vilara G., Judit Tulla P., Fernando A. (2012). Polymers and Drug Delivery Systems. Current Drug Delivery. 9 (4):1- 27.

Vineis, P., M. Alavanja, Buffler P., Fontham E., Franceschi S., Gao Y.T., Gupta P.C., Hackshaw A., Matos E., Samet J., Sitas F., Smith J., Stayner L., Straif K., Thun M.J., Wichmann H.E., Wu A.H., Zaridze D., Peto R. and Doll R.(2004). Tobacco and cancer: recent epidemiological evidence. J. Natl.Cancer. Inst; 96: 99-106.

Vogelstein B. and Kinzler K. (2002). The genetic basis of human cancer: McGraw-Hill Professional; 2002.

W

Waldron, H.A. (1983). A brief history of scrotal cancer Br. J. Ind. Med.; 40: 390-401.

Wang H., Jinlin H., Mingzu Z., Yunfeng T., Fei L., Kam C. T. and Peihong N. (2013). Biocompatible and acid-cleavable poly (3 caprolactone) - acetal-poly (ethylene glycol)-acetal-poly (3 caprolactone) triblock copolymers: synthesis, characterization and pH-triggered doxorubicin delivery. J. Mater. Chem. B, 1:6596.

Wang Y., K.C.-M., Chun M., Quirk R-P, Mattice W.L. (1995). Exchange of chains between micelles of labeled polystyrene-block-poly (oxyethylene) as monitored by nonradiatice singlet energy transfer. Marcromolecules. 28: 904-911.

Wang Y., Yu L., Han L., Sha X., Fang X. (2007). Difunctional Pluronic copolymer micelles for paclitaxel delivery: synergistic effect of folatemediated targeting and Pluronic-mediated overcoming multidrug resistance in tumor cell lines. Int. J. Pharm. 337:63-73.

Wei X.Y., Gonga C, Goua M., Fua S, Guoa Q, Shia S., Luoa F., Guoa G., Qiub L., Qiana Z.(2009). Biodegradable poly (caprolactone)-poly(ethylene glycol) copolymers as drug delivery system. International Journal of Pharmaceutics 381:1–18.

Weinberg R. (2007). The biology of cancer. Garland Science New York.

Wolkers M.C., Brouwenstijn N., Bakker A.H., Toebes M., Schumacher T.N. (2004). Antigen bias in T cell cross-priming. Science. 304:1314-7.

Wood, L.D., Parsons D.W, Jones S., Lin J., *et al*.,(2007). The genomic landscapes of human breast and colorectal cancers, Science; 318: 1108-13.

World Health Organization (WHO) (2014). "Cancer Fact sheet N°297".

World Health Organization (WHO)(2008). World Cancer Report. International Agency for Research on Cancer.

Wu J., Liu Q., Lee R.J. (2006). A folate receptor-targeted liposomal formulation for paclitaxel. Int. J. Pharm.; 316:148-53.

www.zo.utexas.edu/faculty/sjasper/images/19.14.gif.

Y

Yadav A.K., Mishra P., Jain S., Mishra P., Mishra A.K., Agrawal G.P.(2008). Preparation and characterization of HA–PEG–PCL intelligent core-corona nanoparticles for delivery of doxorubicin. J. Drug Target 16: 464–478.

Yan Z., Bin Y. and Deng Y. H. (2005). Take the initiative to drug-loaded liposomes prepared by vincristine sulfate and the determination of encapsulation efficiency Chinese Pharm. J. 10 1559.

Yao, X., Panichpisal, K. Kurtzman, N. and Nugent K. (2007). Cisplatin nephrotoxicity: a review. Am J Med Sci., 334(2): 115-124.

Yokoyama, M., Inoue S., Kataoka, K. *et al*., (1989). Molecular design for missile drug: Synthesis of adriamycin conjugated with immunoglobulin G using poly (ethylene glycol)-block-poly (aspartic acid) as intermediate carrier. Makromol. Chem., 190: 2041-2054.

Yong H., Leyang Z., Yi C., Haixiong G., Xiqun J., Changzheng Y. (2004). Degradation Behavior of Poly (caprolactone)-b-poly (ethylene glycol)-b-poly (caprolactone) Micelles in Aqueous Solution. Biomacromolecules, 5, 1756-1762.

Youlden D.R., Cramb S.M., Dunn N.A., Muller J.M., Pyke C.M., Baade P.D. (2012). The descriptive epidemiology of female breast cancer: an international comparison of screening, incidence, survival and mortality. Cancer Epidemiol. 36(3):237–248.

Yuan F, Dellian M, Fukumura D, Leunig M, Berk DA, Torchilin VP, *et al*.(1995). Vascular Permeability in a Human Tumor Xenograft: Molecular Size Dependence and Cutoff Size. Cancer Research.;55(17):3752-3756.

Z

Zambaux M.F., Bonneaux F., Gref R., Dellacherie E., Vigneron C. (1999). MPEO–PLA nanoparticles: effect of MPEO con tent on some of their surface properties, J. Biomed. Mater. Res. 44:109–115.

Zang, L., Eisenberg, A. (1995). Multiple morphologies of crew-cut aggregates of polystyrene-b-poly (acrylic acid) block copolymers. Science, 268: 1728-1731.

Zhang L., He Y., Ma G., Song C., Sun H. (2012). Paclitaxel-loaded polymeric micelles based on poly (ε -caprolactone)-poly (ethylene glycol)- poly(ε -caprolactone) triblock copolymers: in vitro and in vivo evaluation. Nanomed: Nanotechnol Biol. Med.8: 925–34.

Zhao H., Yung L.Y.L. (2008). Selectivity of folate conjugated polymer micelles against different tumor cells. Int. J. Pharm. 349:256-268.

Zhou S., Deng X., Yang H. (2003). Biodegradable poly (caprolactone)-poly (ethylene glycol) block copolymers: characterization and their use as drug carriers for a controlled delivery system. Biomaterials 24: 3563–3570.

Zhou S., Deng X., Yang H. (2003). Biodegradable poly (caprolactone)-poly (ethylene glycol) block copolymers: characterization and their use as drug carriers for a controlled delivery system. Biomaterials 24:3563–3570.

Zipfel W.R., Williams R.M., Webb W.W. (2003). Nonlinear magic: multiphoton microscopy in the biosciences. Nat. Biotechnol. 21:1369–7775.

الخلاصة

استهدفت هذه الدراسة إعداد وتوصيف واستكشاف التأثير السمي الخلوي لعقار الدوكسوروبسين (doxorubicin) المحمل في الدقائق النانوية ثلاثية البوليمرات القابلة للتحلل (PCL-PEG-PCL) ضد الخط الخلوي لسرطان الثدي MCF-7 .

تمت دراسة الخواص الفيزيائية والكيميائية للدقائق النانوية واختبارها خارج الجسم الحي وتضمن الاختبار دراسة التأثير السمي الخلوي للدقائق. تم تحضير الدقائق النانوية (PCL-PEG-PCL) باستخدام طريقة الترسيب النانوي (nanoprecipitation) باستخدام الأسيتون كمذيب عضوي، وتم تجميع البوليمرات ذاتيا الى دقائق نانوية بشكل شبيه بتركيب shell/core اعتمادا على خاصية ازدواج الالفة لبوليمرات (PCL-PEG-PCL). بلغ متوسط حجم الدقائق النانوية (226 ± 5 نانومتر) الذي تم تحدديه بواسطة تقنية (DLS)، وكانت قيمة PDI (0.034 ± 0.26) مع نمط توزيع أحادي. كشفت صورة المجهر الالكتروني النافذ TEM أن الدقائق النانوية المعّدة بطريقة الترسيب النانوي كانت كروية الشكل.

في هذه الدراسة، تم تغليف عقار الدوكسوربوسين بواسطة الدقائق النانوية بكفاءة تغليف تساوي (99.7 %) وتحميل عقار (28.69%). بعد ذلك تمت دراسة تحرير عقار الدوكسوروبوسين من الدقائق النانوية باستخدام طريقة الديلزة. حيث تحرر من العقار مايقارب (33%) في الوسط الحامضي (pH= 5.6) بعد مرور180 دقيقة، بينما تحرر مايقارب (92%) من العقار في نفس الوسط بعد مرور 2760 دقيقة لكن معدل تحرر العقار في الوسط المتعادل ابطء مقارنة مع تحرره في الوسط الحامضي اذ تحرر ما يقارب (43%) من العقار بعد مرور 2760 دقيقة في (pH =7.4).

تم إجراء تقييم السلامة خارج الجسم الحي للبوليمرات النانوية واثبتت الدراسة أن هذه البوليمرات لم تحرض انحلال كرات الدم الحمراء البشرية في التركيز (100 ميكروغرام / مل) مقارنة مع محلول السيطرة (المحلول الملحي).

تم تقييم سمية الخلايا خارج الجسم الحي للدقائق النانوية بتراكيز مختلفة (0.1، 0.2، 0.4، 0.6، 0.8 و1 ميكروغرام / مل) على الخط الخلوي MCF-7 بطريقة MTT ، وكشفت النتائج أن الدقائق النانوية كانت تمتلك سمية ضئيلة ضد خلايا MCF-7 حتى بأعلى تركيز وكانت النسبة المئوية لحيوية الخلايا أكثر من 80%، مشيرة إلى التوافق الحيوي الطبي الآمن لهذه البوليمرات.

بالإضافة إلى ذلك، اظهرت الدراسات الشكلية بواسطة المجهر المقلوب لعقار الدوكوروبيسين المحمل ضد خلايا MCF-7 أنه بعد 24 ساعة من العلاج لم يلاحظ أي تغيرات شكلية للخلايا، في حين بعد 48 ساعة اصبحت الخلايا مدورة ومنفصلة. بعد 72 ساعة، انخفض عدد الخلايا المعالجة وأنكمشت

وأصبحت معلقة في الوسط الزرعي. لذلك، أظهرت هذه الدراسة خاصية التوصيل لعقار الدوكسوروبيسين المضاد للسرطان المحمّل في الدقائق النانوية وايضاً تثبيطه لنمو الخلايا السرطانية.

لقد تمت دراسة السمية الخلوية خارج الجسم الحي بواسطة فحص MTT لعقارالدوكسوروبيسين الحر وعقار الدوكسوروبيسين المحمل بالدقائق النانوية بتراكيز مختلفة (0.01، 0.1، 0.4، 0.8 و 1.2 ميكروغرام / مل) ضد الخط الخلوي MCF-7 بعد 24 و 48 و 72 ساعة. اظهرت النتائج ان خلايا MCF-7 أكثر حساسية للدوكسوروبيسين الحر مقارنة مع الدوكسوروبسين المحمل بعد مرور 48 ساعة، بينما لوحظ أن خلايا MCF-7 كانت أكثر حساسية للعقار المحّمل من العقار الحربعد مرور 72 ساعة، و انخفضت حيوية الخلايا بشكل كبير وملحوظ في ($P\leq0.05$) مع ازدياد وقت العلاج خلال فترة الدراسة.

كانت قيم IC_{50} (التركيز المثبط المسبب لـ 50% من موت الخلايا) للعقار الحر والعقار المحمّل بالدقائق النانوية ضد خلايا MCF-7 0.56 و 0.5 على التوالي بعد 72 ساعة.

استخدمت التقنيات الخالية من استخدام واسم لمتابعة العقار المحمّل بالدقائق النانوية PCL-PEG-PCL والعقار الحر في خلايا MCF-7 الحية، مثل تقنيات التصوير بواسطة CARS وTPEF.

أظهرالتوزيع ثلاثي الابعاد لجزيئات عقار الدوكسوروبيسين بواسطة CARS وTPEF أن العقار الحر وصل الى داخل النواة بسرعة وانخفض تركيزه في السايتوبلازم بعد 10 ساعة و 35 دقيقة. في الوقت نفسه تحرر العقار من الدقائق النانوية بحيث يمكن ملاحظته في السايتوبلازم والنواة، وكان عدد خلايا MCF-7 المعالجة بالعقار المحّمل بالدقائق النانوية منخفض نسبياً بالمقارنة مع التي تم معالجتها بالعقار الحر بعد 5 ساعات و 50 دقيقة.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم
قسم التقانة الاحيائية

تحضير وتوصيف عقار الدوكسوروبيسين المحمول في الدقائق النانوية البوليمرية (PCL-PEG-PCL) وتأثيره السمي الخلوي على خط خلايا سرطان الثدي MCF-7

أطروحة مقدمة إلى مجلس كلية العلوم/ جامعة النهرين
وهي جزء من متطلبات نيل درجة دكتوراه فلسفة في
علوم التقانة الاحيائية

من قبل
رندة محمد ضاحي علوان

بكالوريوس تقانة أحيائية 2005
ماجستير تقانة أحيائية 2009

بإشراف

أ.د. حميد مجيد جاسم

أ.م.د. ميساء جاسب حاتم

ذوالقعدة 1436 هـ

آب 2015 م