School of Sciences and Engineering

**PEGylated Chitosan / Doxorubicin Nanoparticles and conjugated with Monoclonal Antibodies for Breast Cancer Therapy**

A Thesis Submitted to
The Nanotechnology Master's Program
In partial fulfilment of the requirements for
The degree of Master of Science
By:

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18th December, 2019
The American University in Cairo

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Affiliation

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Dept. Chair/Director Date Dean Date
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Abstract

The potential of nanobiomedical field for developing a promising therapeutic nano-sized drug delivery system is seen to be a great pharmaceutical trend for encapsulation and release of various antineoplastic drugs. In this context, the current work is targeting preparation of biodegradable chitosan nanoparticles (CSNP) that have been intended for selective and sustained release of doxorubicin (DOX) within breast tumor microenvironment. Surface modification of these CSNP with Polyethylene glycol (PEG) was performed in order for enhancing its blood circulation time without being opsonized or captured by immunogenic reticuloendothelial system (RES). PEG has maintained high particles stability profile and enhanced its surface positive charge for the sake of intracellular attachment via electrostatic attachment with negatively charged tumor cell membrane. Advanced tumor selectivity has been achieved through functionalization of two different types of breast cancer specific monoclonal antibodies (mAb); anti-human mammaglobin (Anti-hMAM) and anti-human epidermal growth factor (Anti-HER2) in two different separate nano formulations. This functionalization has the potential of evading systemic side effects of parenteral free DOX and promoting cancerous endocytosis through receptor mediated interaction. In-vitro cytotoxicity effects of PEGylated DOX loaded CSNP, free DOX, Anti-HER2 PEGylated DOX loaded CSNP and Anti-hMAM PEGylated DOX loaded CSNP were tested against breast cancer cell line (MCF7) and normal fibroblast cell line (L929). Notably, Anti-hMAM PEGylated DOX loaded CSNP and Anti-HER2 PEGylated DOX loaded CSNP formulations were the most cytotoxic against MCF7 cancer cells than L929 normal cells compared to free DOX. Confirmatory bright filed images of the two cell lines have exhibited cancerous cellular damage after 24 hours exposure time to these nano formulations. Finally, we believe that dose dependent system toxicity of freely ingested DOX can be hindered with such targeted nano formulated drug delivery system.

Scheme 1: Graphical diagram of DOX encapsulation and release within tumor cell
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List of Abbreviations

Anti-HER2: Anti-Human Epidermal Growth Factor Receptor antibody
Anti-hMAM: Anti-Mammaglobin antibody
Cs: Chitosan
CSNP: Chitosan Nanoparticles
DLS: Dynamic Light Scattering
DOX: Doxorubicin
EDC: 1-Ethyl-3- (3-Dimethylaminopropyl) Carbodiimide
EE: Encapsulation Efficiency
EPR: Enhanced Permeability Retention
FTIR: Fourier Transmission Infra-Red Spectroscopy
H NMR: Proton Nuclear Magnetic resonance
IPN: Inter Penetrating Network
LC: Loading Capacity
mAb: Monoclonal Antibody
PBS: Phosphate Buffer Solution
PDI: Poly Dispersity Index
PEG: Polyethylene Glycol
RES: Reticuloendothelial System
SEM: Scanning electron microscope
TEM: Transmission Electron Microscope
TPP: Tri-Poly Phosphate
UV-VIS: Ultraviolet-Visible Spectroscopy
XRD: X-ray Diffraction
Z average: Zeta potential average
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Thesis Scope and Objectives
Thesis Scope and Objectives:

The current study is an indicative attempt to apply readily available and naturally existent polymers; chitosan (CS), in order to enhance the pharmacotherapeutic efficacy of an anti-cancer drug; Doxorubicin (DOX) and its smart delivery system against breast cancer cell line. The main objective here is to design a nano-formulation platform that will be able to travel inside the human physiological blood circulatory system. This circulatory journey is oriented to be from a certain site of administration till reaching the selected site of action by the aid of cell specific ligand(s) known as smart antibodies. This partially invasive drug delivery system aims to deliver, localize, and prolong the release of the loaded DOX. Besides, it is an efficient system to minimize the undesirable immunogenic uptake of this entire therapeutic platform by the effect of mono-nuclear phagocyte system (MPS) that is known, also, as reticuloendothelial system (RES). Thereby, facilitating the accumulation of effective doses of DOX within the tumor environment without harming the other non-cancerous organs. Thus, minimizing its severe side effects like heart failure, bone marrow suppression, mucositis and alopecia (1).

The referred nanoparticles conjugated system will be based on a biodegradable and biocompatible polymeric chitosan nanoparticles (CSNP) which will be functionalized with Polyethylene Glycol (PEG), DOX and biological marker(s) over the different chapters and activities of this study. In activity 1, the CSNP will be prepared within a bottom-up approach using ionotropic gelation method (2). The resultant nanoparticles will be characterized using different characterization analysis in order to confirm the consistency of their crosslinking, size, shape, distribution, morphology and surface charge. Various characterization techniques have been exploited for this purpose such as Dynamic Light Scattering (DLS), Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM), X-Ray Diffraction (XRD), Ultraviolet-Visible spectrophotometry (UV-VIS) and Infrared spectroscopy using Fourier Transform Infrared Spectroscopy (FTIR). In Activity 2, the prepared CSNPs have been loaded with a polymeric Polyethylene Glycol (PEG). The main purpose behind adding this polymer has been to ensure a prolonged and extended circulation of the nanoparticles till reaching the breast tumor area without being uptake by RES. These PEGylated CSNP have been characterized, also, using the same characterization analysis as activity 1. (3)
Encapsulation of DOX inside the PEGylated CSNP has been done as the third activity. This step has been done with very minute concentrations of DOX. These minute concentrations have been tested in terms of encapsulation efficiency (EE%), loading capacity (LC%) and cell cytotoxicity. The dye compound 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide for colorimetric assay (MTT assay) has been an indicative test of the most effective DOX concentration that can kill the highest percentage of MCF-7 breast tumor cells (3). The concentration of choice has been tested for the drug release behavior in order to monitor and expect its behavior inside the tumor microenvironment. Besides cell cytotoxicity test, this activity has been characterized also with FTIR, TEM, Zeta-sizer, Zeta potential and UV-Vis spectroscopy. These characterization analyses have confirmed the successful encapsulation and stability of DOX inside the PEGylated CSNP. Achieving such dose reduction with maintaining good stability of this nano-formula in normal saline pH is an outstanding step for obtaining efficient delivery process of highly effective doses of DOX. Therefore, the integration of such multidisciplinary nanosystem into pharmaceutical DOX drug delivery process has empowered its activity against breast tumor cells.

Scheme 2: Full schematic diagram of different work activities in this study
Chapter 1

General Introduction

&

Literature Review
1.1. Cancer

For many decades, cancer has been recognized as one of the top unresolved threatening health problems that represents the second leading cause of death worldwide according to some contemporary reports by World Health Organization (WHO). Typically, cancer can start within any organ in the body at any time for many identified and unidentified etiologies as shown in Figure (1.1) (4). The main problem with cancer is the fast cellular growth of a group of mutated cells that are known as tumor cells. Basically, Cancer starts with one normal healthy cell that develops a damage or unexpected change in its Deoxyribonucleic Acid (DNA) molecule. This change, which may be inherited or developed by many factors such as radiation, oxidative stress, infection, smoking and hormonal disturbances can cause gene mutation and rather interfere with the normal cell division instructions. Therefore, it turns from a normal cell to a cancerous cell that grows out of control. Tumor is developed when this cancer cell continues to grow and divide.

Figure 1.1: Initiation of cancerous tumor by the action of genetic alteration that results from various environmental factors. (5)
Tumor cells don’t have any specific physiological function in the human body, and they can easily avoid the immune system. Besides, they ignore the physiological signals that aim to control or stop cell division. The tumor grows and gets bigger by the flood of oxygen and different types of nutrients using blood supply through cancerous angiogenesis formation as illustrated in Figure (1.2) (6).

![Blood Vessel Overgrowth on Cell](image)

**Figure 1.2:** The angiogenesis pathway of a cancerous tumor with overexpression of VEGF glycoprotein results in initiation of new microblood vessels that feed and carry nutrients and oxygen to tumor area so that, the tumor cells can divide and grow easily (7)

The increased numbers of cancer diseased cases worldwide are tremendously growing every day, the mortality rate at 2018 has been 9.6 million deaths (5). In Asia, the highest mortality rate, the estimated number of death cases has been more than 5 million. While the remaining amount of death cases has been localized in different areas worldwide including Europe, Africa, Oceania and North America as shown in Figure (1.3) (B). On the other hand, the estimated economic expenses that has been accompanied with cancer treatment in US is about $1,16 Trillion per year. Besides, more than 300,000 new cancer cases among the age of 0-19 years are being annually diagnosed. Therefore, it has been proposed by some WHO guidelines that patient awareness and early screening of a tumor can improve the treatment access (8).
Figure 1.3: (A) Total cancer prevalence rate in 2018 including various types of cancer with various physiological sites in human body, with total estimated number of 18,078,957 cancer patients. (B) estimated rate of death cases because of cancer all ages and all types in by the end of 2018 that reached around 9.6 million worldwide (8)

1.2. Breast cancer

Breast cancer is the highest comon cancer type within female population, and it has been ranked worldwide as one of the top leading causes of high death rates among the same demographic population section. Being one of the top international public health issues over the century, breast cancer has a direct influence on different dimensions of human lives including physiological, psychological, mental and social aspects. Meanwhile, it has an incidence rate of more than 2.1 million new cases every year as shown in Figure (1.3 A). In 2018, the mortality rates of breast cancer have been 627,000 deaths as shown in Figure (1.4 A).

Figure 1.4: (A) Different types of cancer with variable death rates including breast cancer (6.6%) death rate in 2018, (B) the incidence rate of Breast cancer in different world zones for 2018 (8)
Anatomical picture of a female’s breast tissue is composed of a group of lobes that are estimated as 10-20 lobes (9). Each of these lobes is made up of many smaller lobules which function as glands that produce and carry the breast milk. These lobules are connected together by ducts that act as a passing canal for milk drainage from the lobules to the nipples. The second part of breast tissue is called stroma which is a supporting tissue that integrates the surrounding connective tissue and fatty tissue. Typically, breast cancer originates at the cells of either the lobules (lobular carcinoma) or ducts (ductal carcinoma), or any type of these supporting tissues as shown in Figure (1.5).

**Figure 1.5:** Schematic representation of breast tissue anatomy (10)

### 1.3. Types of breast cancer:

#### 1.3.1. Preinvasive breast cancer:

It is a less frequently dominated cancer type which originates only and locally inside either the milk ducts or milk lobules. It doesn’t spread outside these specific two sites. They can be considered as the early stage or precancerous states of breast cancer. Typically, this type of cancer can easily respond to early treatment. It represents around 25% of all known types and subtypes of breast cancer. For instance, in United Kingdom, around 7400 women get such preinvasive breast cancer every year which may originate in breast duct known as ductal
carcinoma in situ (DCIS), or in breast lobule known as lobular carcinoma in situ (LCIS) (11). A Full detailed description of the different types of breast cancer is illustrated in Figure (1.16).

1.3.1.1. Ductal Carcinoma in Situ (DCIS):

Ductal carcinoma can be diagnosed as cancerous cells that grow locally inside the walls of ducts and don’t invade the external surrounding breast tissues. Approximately, all women with such type of tumor can be cured easily and the early detection of such type is, also, another helpful factor for efficient treatment. Because such tumor type is confined locally within the ductal tissue only, DCIS is also known as intraductal or non-invasive cancer. It is not a life-threatening subtype, but in rare cases it can develop a more severe invasive type. Usually, women develop such breast cancer subtype after the age of 48 yrs (12). DCIS can be subcategorized into three types; low grade, intermediate grade and high grade. The last two types can be screened as a thick mass accompanied with some nipple discharge.

![Schematic illustration of ductal carcinoma in situ](image)

**Figure 1.6:** Schematic illustration of ductal carcinoma in situ (13)

1.3.1.2. Lobular Carcinoma in Situ (LCIS):

This type of cancer tumor is another confined and localized tumor. The cells grow exclusively inside the lining lobules only without harming the adjacent walls and tissues or cells. Although it is physiologically confined, it can however be diagnosed hardly using such advanced screening as mammograms. Some studies have proposed that such type may not be considered as a real type of breast cancer, but they consider it as a marker for future tumor. It is detected as a change in the cellular structure in a biopsied tissue. It differs from DCIS as if it was left without treatment, it won’t proceed to such aggressive invasive subtype (13, 14).
1.3.2. Invasive breast cancer:

This is a non-confined breast cancer tumor in which the cells are growing out of control and spread outside the ductal and lobular tissues. The tumor grows outside the confined areas as shown in figure (1.8) (A). It represents around 75% of breast cancer tumor types and has been estimated that the US is developing 180,000 invasive breast cancer cases every year (14). The majority of this number is diagnosed with invasive ductal carcinoma. It can affect women population randomly at any stage of their ages. Besides, invasive breast cancer can affect men population as well.
1.3.2.1. **Invasive ductal carcinoma:**

This type of aggressive breast cancer represents around > 80% of the invasive carcinoma. It initiates mainly in the ductal lining and spread out within the nearby breast tissues as shown in figure (1.9) (A). Such tumors use physiological blood stream and lymphatic pathways in order to move outside the breast tissue which is known as metastasis. Its main diagnosis starts with screening of a lump or mass that the physician can identify. The use of X-ray supported Mammogram is highly considered (16).

1.3.2.2. **Invasive Lobular carcinoma:**

It is the second most common type of aggressive breast tumors that accounts for around 15% of the breast tumor cases. It spreads out of the lobular tissue through the fatty and connective tissues as shown in figure (1.9) (B). Usually, the lobular carcinoma in situ (LCIS) is the main precursor of this type. Its metastasis is mainly located within the abdominal area such as colon, uterus, and ovary.

![Image](image_url)

**Figure 1.9:** Illustrative figure shows the difference between invasive ductal carcinoma and invasive lobular carcinoma (17)

1.3.3. **Molecular subtypes (Receptor mediated breast cancer):**

Clinically, breast cancer tumors have been categorized according to the presence of certain hormonal known receptors and the status of these receptors. Histologically, there are three types of cellular receptors that are attached to breast tumor cells; estrogen receptors (ER), progesterone receptors (PR) and human epidermal growth factor receptor 2(HER2) as shown in Figure (1.10) (18).
1.3.3.1. **Estrogen receptor mediated breast cancer:**

Around 65 % of breast cancer tumors are estrogen receptors positive (ER+) meaning that these tumor cells have estrogen receptors in the outer surface of their cellular membranes. Physiologically, estrogen receptors are functioning as stabilizers for the effect of the endogenous estrogen and, in some cases, responding to other therapeutic agents (20). Only 15-20 % are considered to be estrogen receptors negative (ER-). This type of breast cancer is riskier than ER+. Patients with ER+ tumors are typically treated with hormonal therapy, immunotherapy and/or chemotherapy. They respond well to such types of therapies as shown in Figure (1.11).

![Figure 1.10: Three basic types of receptors within breast tumor cell (19)](image1)

![Figure 1.11: The effect of anti-estrogenic drug Tamoxifen up on placebo and tamoxifen treated breast cancer patients. (21)](image2)
1.3.3.2. Progesterone receptor mediated breast cancer:

Progesterone (PR) is one of the endogenous steroidal hormones that is involved in different physiological processes such as embryogenesis, pregnancy and menstruation cycles. It has been stated that progesterone is involved, also, in breast cancer tumor progress. Physiologically, PR is functioning as an activator for rapid activation of protein kinase that is involved in some modification processes of proteins. Additionally, it acts as a mediator for signals transduction pathways within the breast tissues. Therefore, it has been found that the presence of such PR receptors can play a role with the development, growth and even the treatment of the breast cancer. Likewise, in ER mediated tumors, some patients have been found to efficiently respond to hormonal treatment. Those are patients with PR+, respond to anti-progesterone drugs as shown in Figure (1.12) While others with PR- don’t respond well to hormonal therapy and they respond, majorly, to chemotherapy and immunotherapy (22).

![Diagram](image.jpg)

**Figure 1.12:** The anti-progesterone action with a breast cell mediated progesterone (23)

1.3.3.3. human epidermal growth factor receptor 2(HER2) breast cancer:

HER2 is an over expressed oncogene that is naturally existent on the external surface of normal breast and tumor breast cells. Normally, in a healthy woman, HER2 is responsible for cell growth, division and self-repair. Basically, HER2 has been discovered during early 1980s, and it has been stated to be one of the key factors that participate in the development of breast tumor within 15 – 20 % of the total patient population. This oncogene is responsible for encoding various types of fundamental proteins that are responsible for maintaining persistent tumors
growth. The overexpression of HER2 gene in breast cancer patients is originated by its cellular mutation related over amplification, and this results in initiation of too many HER2 receptors as shown in figure 1.13. Theses receptors are responsible for uncontrolled growth and division of tumor cells (24).

**Figure 1.13:** Difference between HER2 positive and HER2 negative breast tumor cell (25)

HER2 (+ve) tumor cells provide their own tumor growth through a proliferative, angiogenic and cell progressive pathway as shown in figure (1.14). This pathway is enzymatic based network that is supported with many different signals that develop a cascade manner which ends up with uncontrolled growth of the tumor (26).

**Figure 1.14:** HER2 signaling pathway with a cascade manner that ultimately target the final breast tumorigenesis (26)
1.3.4. Triple Negative Breast Cancer (TNBC):

This is a major heterogeneous challenging aggressive type of breast carcinoma that occurs in young women. TNBC is mainly characterized by the absence of the most important three types of receptors that feed the tumor cells; ER, PR and HER2 receptors. This represents a distinct challenge in front of so many trials for controlling the growth of such type. The distinct aggressive nature associated with this type is related to the lack of targeted therapy besides its critical pathological nature. It has been estimated that TNBC represent around 20% of all worldwide invasive breast cancer patients as shown in figure (1.15) (A) (27).

Figure 1.15: The Incidence rates of molecular types of breast cancer (A) and subtypes of TNBC (B) (28)

TNBC has many different molecular subtypes as shown in Figure (1.15) (B) and Table (1.1); Luminal A, Luminal B, Immunomodulatory (IM), mesenchymal stem-like (MSL), mesenchymal (M), basal like 1 and 2 (BL1, BL2), luminal androgen receptors (LAR) and normal tumors (29). The molecular profile for TNBC entails that the majority of infected patients are basal like category. This category is characterized by overexpression of CK5 and CK14 genes, which are used immunohistochemically for diagnosis of solid breast carcinomas like intraductal papilloma with usual ductal hyperplasia (IPUDH).

Table1.1: Various subcategories of TNBC with their relative genetic characteristic behaviors (30)

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Gene expression profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal-like 1 (BL-1)</td>
<td>High in the expression of genes involved in cell cycle progression, cell division, and DNA damage response pathways.</td>
</tr>
<tr>
<td>Basal-like 2 (BL-2)</td>
<td>High in the expression of genes involved in cell cycle progression, cell division, and growth factor signaling.</td>
</tr>
<tr>
<td>Immunomodulatory (IM)</td>
<td>High in the expression of genes involved in immune processes and cell signaling.</td>
</tr>
<tr>
<td>Mesenchymal (M)</td>
<td>High in the expression of genes involved in motility and extracellular matrix.</td>
</tr>
<tr>
<td>Mesenchymal stem-like (MSL)</td>
<td>High in the expression of genes involved in motility, extracellular matrix, and growth factor signaling; consistent with claudin-low Intrinsic subtype.</td>
</tr>
<tr>
<td>Luminal androgen receptor (LAR)</td>
<td>High in the expression of genes involved in hormonally regulated pathways.</td>
</tr>
</tbody>
</table>
Breast cancer pathogenesis and histologic vs. molecular subtypes

Histological subtypes

<table>
<thead>
<tr>
<th>Preinvasive cancer</th>
<th>Ductal carcinoma in situ (DCIS)</th>
<th>Lobular carcinoma in situ (LCIS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25%</td>
<td>80%</td>
<td>100%</td>
</tr>
<tr>
<td>Cells limited to basement membrane</td>
<td>May spread through ducts and distort duct architecture</td>
<td>Usually unilateral</td>
</tr>
<tr>
<td>1% progress to invasive cancer per year</td>
<td>1% progress to invasive cancer per year</td>
<td></td>
</tr>
</tbody>
</table>

Invasive cancer

<table>
<thead>
<tr>
<th>Extension beyond the basement membrane</th>
<th>Invasive ductal carcinoma (IDC)</th>
<th>Invasive lobular carcinoma (ILC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>75%</td>
<td>70%</td>
<td>10%</td>
</tr>
<tr>
<td>Usually from DCIS precursor</td>
<td>Usually from IDC precursor</td>
<td>Usually from LCIS precursor</td>
</tr>
<tr>
<td>Causes fibrous response, producing a palpable mass on examination</td>
<td>Minimal fibrous response, presents less often with palpable mass</td>
<td>Metastasis through abdominal venules to GI, ovaries, uterus</td>
</tr>
<tr>
<td>Metastasis through lymphatics and blood</td>
<td>Metastasis through abdominal venules to GI, ovaries, uterus</td>
<td></td>
</tr>
</tbody>
</table>

Molecular subtypes

<table>
<thead>
<tr>
<th>% of breast cancers</th>
<th>Receptor expression</th>
<th>Histologic grade</th>
<th>Prognosis</th>
<th>Response to medical therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triple negative (ER-, PR-, HER2-)</td>
<td>15-20%</td>
<td>HER2</td>
<td>High (grade III)</td>
<td>Chemotherapy, Trastuzumab</td>
</tr>
<tr>
<td>HER2+</td>
<td>10-15%</td>
<td>ER+/PR+</td>
<td>Low (grade I)</td>
<td>Trastuzumab, Endocrine therapy</td>
</tr>
<tr>
<td>Luminal B</td>
<td>20%</td>
<td>ER+/PR+</td>
<td>Good</td>
<td>Endocrine therapy</td>
</tr>
<tr>
<td>Luminal A</td>
<td>40%</td>
<td>ER+/PR+</td>
<td>Good</td>
<td>Endocrine therapy</td>
</tr>
</tbody>
</table>

Normal breast stem cells or progenitor cells transform into breast cancer cells. The cancer cells are similar in phenotype to the normal basal and luminal cells of the ductal structure.

Basal or myoepithelial cells
- Contractile cells for milk ejection
- Estrogen receptor –
- Progesterone receptor –

Luminal or epithelial cells
- Respond to hormonal stimulation for milk production
- Estrogen receptor +
- Progesterone receptor +/-

Cancer cell phenotype
- Basal
- Basoluminal
- Luminal
1.4. Stages of breast cancer:

Many worldwide health and treatment organizations that are concerned with the global public health have proposed and classified different stages of breast cancer according to many factors. For instance, size of the tumor, hormonal receptors overexpression, type of the tumor, how deep the tumor is within the healthy unaffected breast tissues, metastatic tendency to nearby organs and spreading to the nearby lymph nodes. However, the most often applied system for staging has been the American Joint Committee on Cancer (AJCC) (32). This system is called TNM system that stands for (Tumor Node Metastasis), and it classifies breast cancer according to two main sub-classifications; clinical and pathological (Table 1.2)

Table 1.2: TNM grading system for different stages of breast cancer (33)

<table>
<thead>
<tr>
<th>TNM</th>
<th>Description</th>
<th>Stage</th>
<th>patients (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tumor (T)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T0</td>
<td>Tumor is not formed</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>T1</td>
<td>Tumor &lt; 2 cm</td>
<td>1</td>
<td>6.8%</td>
</tr>
<tr>
<td>T2</td>
<td>Tumor (2 cm – 5 cm)</td>
<td>2</td>
<td>19.7%</td>
</tr>
<tr>
<td>T3</td>
<td>Tumor ≥ 5 cm</td>
<td>3</td>
<td>28.4%</td>
</tr>
<tr>
<td>T4</td>
<td>Tumor of any size</td>
<td>4</td>
<td>45.4%</td>
</tr>
<tr>
<td><strong>Lymph node (N)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>No tumor in lymph nodes</td>
<td>0</td>
<td>20.9%</td>
</tr>
<tr>
<td>N1</td>
<td>Non movable tumor</td>
<td>1</td>
<td>24.9%</td>
</tr>
<tr>
<td>N2</td>
<td>Movable Tumor</td>
<td>2</td>
<td>31.7%</td>
</tr>
<tr>
<td>N3</td>
<td>Tumor in 4 – 10 lymph nodes</td>
<td>3</td>
<td>22.5%</td>
</tr>
<tr>
<td>N4</td>
<td>NA</td>
<td>4</td>
<td>NA</td>
</tr>
<tr>
<td><strong>Metastasis (M)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M0</td>
<td>No metastasis</td>
<td>0,1,2,3</td>
<td>83%</td>
</tr>
<tr>
<td>M1</td>
<td>Distant metastasis</td>
<td>4</td>
<td>17%</td>
</tr>
</tbody>
</table>
Stage 0:
This is the earliest stage of breast cancer that is characterized by a noninvasive nature of the tumor, and sometimes called in-situ carcinoma. The cancerous and non-cancerous cells are separated from each other and there is no evidence of any cancerous invasion. The prognosis and treatment possibilities in this stage are high, since there are separate boundaries between the growing cancerous cells and healthy cells. DCIS can be considered as example of such stage.

Stage 1:
This is the secondary stage that provides a descriptive picture of invasive carcinoma. Sometimes microscopic examination of such invasion is a rigid evidence of this clinical grade. Through this examination, physicians can define the general size of the tumor and the involvement of the nearby lymph nodes. Some studies differentiate between stage 1A and 1B (33). In stage 1A, the tumor’s size can reach 2 cm while keeping the adjacent lymph nodes unaffected. While in stage 1B, the tumor’s size may reach 0.2 mm while the adjacent lymph nodes are affected and involved in the tumor growth.

Stage 2:
The size of the tumor in this stage is < 5 cm and having the adjacent lymph nodes affected with the tumor. Likewise, stage 1, this stage is classified into two sub classes; 2A which has a tumor growing in the axillary or sentinel lymph nodes, while the breast tissue has no tumor. On the other hand, in 2B subclass, the tumor is large and reaches to 5 cm, though it doesn’t reach the adjacent lymph nodes.

Stage 3:
This is one of the advanced stages of breast tumor in which the axillary lymph nodes are highly involved with having a large tumor size of 5 cm. It can be sub-classified into three stages; A, B and C. Breast tumor of stage 3A, tumor is found to be between 5 – 9 axillary lymph nodes. In stage 3B, Tumor is found in 9 axillary or sentinel lymph nodes. This type is called inflammatory breast cancer since it has a swelling of the affected skin area. In stage 3C, tumor is found in more than 10 lymph nodes.

Stage 4:
This stage of breast cancer is known as metastasis, and characterized by the spread of tumor cells to the nearby organs including distant organs such as liver, bone, colon and brain and
distant lymph nodes. Physicians may call this tumor type ‘De Novo’ and it has some possibility at this stage that the tumor is a recurrence of a previous breast cancer.

Table 1.3: Five years survival rates of different stages of breast cancer (34)

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
<th>5 years survival rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stages 0 and 1</td>
<td>Primary diagnosed cancer (high curability rate)</td>
<td>90 %</td>
</tr>
<tr>
<td>Stage 2</td>
<td>Early stage breast tumor (intermediate curability rate)</td>
<td>70%</td>
</tr>
<tr>
<td>Stage 3</td>
<td>Advanced carcinoma (augmented therapy is required)</td>
<td>48%</td>
</tr>
<tr>
<td>Stage 4</td>
<td>Late stage metastatic breast carcinoma (very low curability rate)</td>
<td>20%</td>
</tr>
</tbody>
</table>

1.5. Breast cancer current therapy:

1.5.1. Surgery

Surgery is the main traditional management strategy that has a prominent role in evading breast tumor. This strategy is prioritized over many other treatment techniques for those patients whose tumors are localized and did not metastasize to further areas of the nearby body organs (35). Additionally, surgery is highly considered, also, for such patients who are at late complex stages of breast cancer. In these stages, the removal of axillary or sentinel lymph nodes and other breast lesions are the main targets for performing a surgery as showed in Figure (1.17).

Figure 1.17: (A) Removal of sentinel lymph nodes with blue colored drainage, (B) removal of axillary lymph nodes (35)
Mastectomy:

This is one of the well-known breast surgeries that entails the removal of the whole breast tissue including the outer skin tissue and axillary lymph nodes. According to many studies, this is one of the options that are offered for early stages patients such as primary stages; 1,2 and 3 and non-invasive DCIS and LCIS patients. Some preventive procedures, also, may recommend such surgery for patients who are believed to be at high risk of having later breast carcinoma (36).

Lumpectomy:

It is a partial surgical procedure that is less inclusive than mastectomy. It entails the removal of a benign part of the breast. In other words, lumpectomy is a partial mastectomy that is highly recommended for patents whose malignant tumors are surrounded by healthy non-cancerous tissues including lymph nodes. Notably, most surgeons and patients as well recommend lumpectomy than mastectomy owing to their high concern with losing breast-based asexuality (37).

1.5.2. Radiotherapy:

It is one of the highly considered treatment methodologies that proved its effectiveness in evading and control many breasts malignant tumors’ growth. Radiotherapy could be a completely curative treatment for some breast cancer cases. A combination therapy that combines radiotherapy with many other therapeutic techniques may enhance the survival benefits in many breast carcinomas. Wang et al has recently conducted a rigid conclusion of minimizing the breast tumor recurrence rates of 50% within 10 years survivals when radiation therapy has been combined with breast tumor surgery (38). Moreover, the death rate, also, has been decreased by 20% for 15 years.
Clinically, many randomized and retrospective studies have reviewed the acute and chronic toxicity profiles associated with adjuvant radiotherapy on breast cancer patients. The size of the breast is a one indicative factor that may enhance or diminish the radiotherapy toxicity as shown in figure (1.19).

Harsolia et al. has demonstrated a strong relationship between the breast edema, dermatitis and chronic hyperpigmentation relative to the intensity of the breast size. Besides, Shah et al. has concluded the same sever rates of these aforementioned adverse effects. Moist desquamation, subcutaneous fibrosis and breast shrinkage have been reported by Pignol et al. for 5 years simple radiation therapy as shown in Figure (1.20) (41).
1.5.3. **Hormonal therapy:**

In premenopausal patients, the prevention of estrogen action is achieved through two major techniques; ovarian ablation or the use of therapeutic estrogen analogues such as Luteinizing hormone releasing hormone (LHRH) like goserelin (Zoladex®). In the same context, selective estrogen receptor modulators (SERM), like tamoxifen and raloxifene, is frequently used as adjuvant treatment for ovarian cancer and breast cancer. It has been reported by a collaborative study that tamoxifen has shown a significant reduction of the tumor recurrence rate by 74%, it could also minimize the death rate by 26% (43). Another adjuvant hormonal treatment that has attracted some oncological interest is selective estrogen receptor degrader (SERD) like fulvestrant. This type of therapy is used to damage or degrade the estrogen receptors. It is often applied for post-menopausal women and sometimes it can be combined with goserelin in order for augmenting their antagonizing effect against breast tumor.

Postmenopausal breast cancer patients, who represents quiet higher rates than premenopausal patients, have 80% of their breast tumors ER+. Physiologically, estrogen in postmenopausal women is produced in minute quantities through some organs such as breast tissues, liver and adrenal glands. That is because their ovaries are no longer producing sufficient estrogen. It has been shown according to Czick et al. that some hormonal treatment, such as tamoxifen and aromatase inhibitors (AIs), can effectively stop the conversion of androgens hormones to estrogens through inhibition of the aromatase enzyme as shown in Figure (1.21) (44).
1.5.4. Chemotherapy:

Anthracyclines (a subcategory of chemotherapy) has been reported by many studies as the most potent class of chemotherapeutics that can interfere with such enzymatic associated cell growth (46). The initiation of redox reactions by anthracyclines has a powerful role on generating reactive oxygen species (ROS). These ROS causes oxidative stress and cancerous cell death. In the same context, anthracyclines interfere with topoisomerase II enzyme whom inhibition arrest the cancerous cell growth. Therefore, the use of chemotherapy is highly recommended in sever cancer types, such as TNBC and HER2+, including stages 3 and 4. These late stages have characteristic inclusion of metastasis and lymphatic carcinomas with independence of size of the tumor, patient age and nodal status (47).

On the other hand, having various therapeutic alternatives is always recommended owing the wide adverse effects that can be clinically decisive in shifting between different treatment options. Chemotherapy has been listed by WHO as one of the top recommended treatment plans of breast carcinoma. Chemotherapy has been reported as a source of generating ROS, this caused a programed cardiotoxicity for patients who receive various chemotherapeutic agents. It’s been for many years with a great conflict of the imbalance between the risks and
befits of using chemotherapy and the evoked cardiotoxicity. Besides, the significant adverse effect of hair loss associated with chemotherapy has been discussed with many retrospective studies (48). The other mild to severe adverse reactions of chemotherapy have been reported, also, by many clinical studies are listed in Table (1.4). They include nausea, vomiting, fever, abdominal pain, photosensitivity, constipation, mucositis, azoospermia, co-enzyme Q10 deficiency, alopecia, injection site reaction, rash, pruritis and infection (49).

Table 1.4: Various adverse effects of using chemotherapy after one-month treatment (50)

<table>
<thead>
<tr>
<th>Side effect</th>
<th>Frequency (%)</th>
<th>Incidence rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Any side effect</td>
<td>204 (84)</td>
<td>0.03</td>
</tr>
<tr>
<td>Chest pain</td>
<td>26 (11)</td>
<td>0.60</td>
</tr>
<tr>
<td>Constipation</td>
<td>184 (76)</td>
<td>0.55</td>
</tr>
<tr>
<td>Diarrhoea</td>
<td>180 (74)</td>
<td>0.52</td>
</tr>
<tr>
<td>Dyspnœa</td>
<td>176 (72)</td>
<td>0.78</td>
</tr>
<tr>
<td>Fatigue</td>
<td>200 (82)</td>
<td>0.53</td>
</tr>
<tr>
<td>Mucositis</td>
<td>176 (72)</td>
<td>0.54</td>
</tr>
<tr>
<td>Pain</td>
<td>179 (74)</td>
<td>0.54</td>
</tr>
<tr>
<td>Rash</td>
<td>181 (74)</td>
<td>0.54</td>
</tr>
<tr>
<td>Vomiting</td>
<td>162 (67)</td>
<td>0.44</td>
</tr>
</tbody>
</table>

1.5.4.1. **Doxorubicin:**

It is one of the chemotherapeutic anthracycline type of antibiotics that is obtained from a specific bacterium species called *Streptomyces Peucetius* as shown in figure (1.22). DOX has been used for the curing of solid based cancerous tumors in all ages; children and adults. It has been used, also, for effective treatment of soft tissues tumors such as ovarian cancer, bladder cancer, neuroblastoma, thyroid cancer, soft tissue sarcoma and breast cancer. It has been approved by FDA at 1974 as one of the safe medicines for cancer treatment. Additionally, it has been managed to be one of the tops WHO lists of the most effective drugs against cancer by many health practitioners (52).
1.5.4.2. Mechanism of action of DOX:

DOX have been elucidated with its intense ability to confine and limit the cancerous tumor’s growth with various modes of actions.

- First, generating stressful ROSs. DOX and anthracyclines, in general, contain hydroxyquinone which is chelating structure for elements like iron. By the aid of some mitochondrial mediators such as NADH dehydrogenase and cytochrome P450 reductase, iron-DOX complex enhances the conversion of electrons from glutathione to oxygen and its derivatives. This results in accumulation of ROS and production of free radicals as shown in Figure (1.23) (53).

- Hydroxyl radicals, single oxygen, superoxide ions and hydrogen peroxide are accumulated inside the cell and can’t be detoxified. This causes DNA damage and gradually trigger cancerous cell apoptosis. The cell membrane lipids are exposed to peroxidation, and this causes rabid destruction of cell membrane. Loss of energy and activated lipid metabolism are other findings for such the oxidative stresses (54).
Second, it interferes with the actions of Topoisomerase-II (TOP II) enzyme which is fundamental enzyme for the completion of DNA structural stabilization. Therefore, DOX arrests the relegation of the small DNA breaks and, thereby, block the cellular growth. Then, finally, it enhances gradual apoptosis.

Third, DOX has an intercalation activity between the double helical base pairs of the cancerous DNA. Therefore, it interferes with the synthesis of DNA and RNA, and this in turn inhibit cancerous cell division (55)

Finally, it has been reported by Chapner et al. that DOX interferes with the function of vascular nitric oxide synthase, thus disrupt the vascular tone of the angiogenic blood vessels that transfer blood and nutrients to the tumor area (56). It is effective, also, to kill the disseminated cancerous cells that are resistant to hormonal therapy or are metastatic. Various generic pharmaceutical products are listed in table (1.5).

Table 1.5: Examples of generic pharmaceutical market products (57, 58)

<table>
<thead>
<tr>
<th>Generic drug</th>
<th>Conc.</th>
<th>Administration</th>
<th>company</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adriamycin®</td>
<td>2mg/mL</td>
<td>Intravenous</td>
<td>West-Ward Pharmaceutical Corps</td>
<td>1996</td>
</tr>
<tr>
<td>DOX HCL®</td>
<td>2mg/mL</td>
<td>Intravenous</td>
<td>Fresenius Kabi</td>
<td>2000</td>
</tr>
</tbody>
</table>
1.5.4.2.1. Limitations of non-targeted DOX:

The main hindrance to DOX clinical anticancerous application has been its non-selective pharmacological activity against healthy and tumor cells. This poor selectivity generates dose dependent toxicity and severe side effects within normal cells. One of the widely conducted severe toxicity is its known intense cardiotoxicity. The second considerable hindrance is the elevated levels of DOX cellular resistance. These two main reasons besides many other adverse drug reactions are the top restricting clinical effects that cause treatment failure (59).

It has been reported that 11% of patients receiving DOX are experiencing these adverse reactions within the first few days of treatment. Furthermore, Oikonomou et al. has reported that cardiac arrhythmias have been manifested in 26% of DOX receiving breast cancer patients during the first 5 years of treatment. Another clinical study, Luu et al., has revealed a 50% mortality within the first year of DOX administration being manifested with acute cardiotoxicity and congestive heart failure (CHF) (60). Some other drug-drug interactions are summarized in table (1.6).

Another drawback for the conventional use of DOX is the elevated drug resistance levels owing to the multiple frequent dosing system. The severity and clinical grade of the tumor are the two main reasons for such multiple doses. This elevated resistance is associated with many reasons; first, the overexpression of multiple drug resistance -1 gene (MDR1) and multi drug resistance protein (MRP). Second, impaired DNA repairing, which improve the tumor cell apoptotic resistance. Third, mutation and enzymatic degradations of Topoisomerase II, which change the expression level of topoisomerase II. Finally, alteration of the cellular membrane fatty acid composition that deteriorate some cellular repair mechanisms (61).
Table 1.6: Drug-drug interaction with resulted adverse reactions of doxorubicin vs some frequently administered types of drugs for a cancer patient

<table>
<thead>
<tr>
<th>Co-administered drug</th>
<th>Drug-drug interaction</th>
<th>Mechanism</th>
<th>Solution</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-nitrosouria (streptozocine)</td>
<td>Increased DOX toxicity and bone marrow suppression</td>
<td>Prolong the half-life elimination of DOX</td>
<td>Decreased doses of DOX</td>
<td>(62)</td>
</tr>
<tr>
<td>Digoxin</td>
<td>Decreased blood serum level of digoxin</td>
<td>Reduced intestinal absorption of digoxin</td>
<td>Changing the does regimen of digoxin</td>
<td>(63)</td>
</tr>
<tr>
<td>Quinolone antibiotics</td>
<td>I. Increased DOX toxicity</td>
<td>I. Inhibited cytochrome P450 3A4 and cytochrome P450 1A2; responsible for dox metabolism</td>
<td>Caution and dose monitoring</td>
<td>(64, 65)</td>
</tr>
<tr>
<td></td>
<td>II. Decreased antibacterial activity of quinolones</td>
<td>II. Decreased systemic absorption of quinolones</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>Added cardiotoxicity</td>
<td>Reduced metabolism of DOX</td>
<td>Avoided</td>
<td>(66)</td>
</tr>
<tr>
<td>Barbiturates (phenobarbital)</td>
<td>Decreased DOX efficacy</td>
<td>Increased plasma clearance of DOX</td>
<td>One of them should be stopped based on a risk-benefit ratio evaluation.</td>
<td>(67)</td>
</tr>
<tr>
<td>Cyclosporine</td>
<td>Increased DOX related cardiotoxicity with a high serum level of more than 50%</td>
<td>Cyclosporines interfere with P-glycoprotein and reduce DOX metabolism by reducing CYT P450 enzyme.</td>
<td>Caution with regular checking</td>
<td>(68)</td>
</tr>
<tr>
<td>Antiviral (stavudine and zidovudine)</td>
<td>Minimize the antiviral activity</td>
<td>Decreased intracellular activation</td>
<td>Avoided</td>
<td>(69)</td>
</tr>
</tbody>
</table>
1.6. Targeted drug delivery:

In the same sense and over the past decade, researchers have combined the use of pharmaceutical sciences to nanoscience as a trial for achieving highly effective targeting treatment. This combination has brought different optimized formulas for the targeted delivery of wide variety of components such as proteins, antibodies, narrow therapeutic indices drugs, poorly water-soluble drugs, organic based drugs and polymeric drugs. Besides, the implication of nanoscience has provided as new controlled release drug delivery systems which is therapeutically outstanding and cost effective as well. Nanoparticles could effectively prove its durability and efficacy as a successful nano-based platform for the synthesis of promising anticancer treatment. The scientific concept behind this system is shown in Figure (1.24). The nanoparticles act as a protective carrier that carries an entrapped drug. This drug, whether water soluble or insoluble, is carried either inside it or upon the particle’s surface. The loaded amount of drug is released in a controllable manner over certain time. The nanoparticle is marked with certain biological marker in order for targeted and specific delivery of the entrapped drug. This biological marker can be antibody, protein or DNA molecule (70).

![Figure 1.24: Simplified illustration of a nano-based drug delivery system (70)](image)

1.7. Nanotechnology:

Nanotechnology has been shown to the light of medicinal and bio-medicinal applications for many years. It could open the door in front of fabrication of various nanosized material formulations that have been used within many various applications. Various Nano formulated shaped material have been reported in many studies including dendrimers, polymeric nanoparticles, quantum dots, core-shell nanoparticles and carbon nanotubes (figure 1.25). All these nano- formulations have been fabricated with high control over their chemical, electrical, physical and biological safety applications (71). Generally, a material within the nanoscale
behave as if it is completely new material in terms of optical, magnetic, chemical and physical performance, especially when its intended application is size dependent.

![Nanometer scale with relative sizes of various biological objects](image)

**Figure 1.25:** Nanometer scale with relative sizes of various biological objects (72)

Synthesis of nanoparticles can be engineered for performing various surface and core modifications within the nanoparticles. Two main approaches for manufacturing nanoparticles; bottom-up and top-down approaches. During both methodologies, nanoparticles can be conjugated with different types of ligands including functional chemical groups, biological molecules, permeation enhancers, targeting moieties, magnetic dyes or surface cross-linkers. Furthermore, the beauty of nanotechnology, in general, is that there is a good control over the resulted final geometrical shape of the nanostructure. For instance, nano-rod, nano-spheres, nano-cubes, nanofibers and nanowires as shown in Figure (1.26).

![TEM images of various shapes of nanostructures](image)

**Figure 1.26:** TEM images of various shapes of nanostructures; (A) nanoparticles, (B) nanocubes and (C) nanorods (73)
Biologically, nanotechnology develop another spectacular manipulation of some serious specifications such as stability in different pH, surface charges, and drug release. These properties are extremely critical whenever the intended application has been biological or therapeutic. Therefore, there are five available routes of administration for any nano-based structures as illustrated in Figure (1.27). That is because there are many variables included to affect the intended nanoparticles such as immune response, enhanced permeability retention (EPR), lymphatic drainage and the pH of the diseased or affected organ area. All these factors can strengthen or weaken the pharmacological action of the loaded drug. Breast cancer has some characteristic pathological features for which nanotechnology can be a considerable solution for such problematic issues.

![Figure 1.27: Five routes of administration for nano based drug delivery systems](image)

1.8. **Nanotechnology enhanced pH manipulation:**

First, breast tumor environment is generally acidic in nature. Some reports have reported that the microenvironment of the breast tumor is ranging between pH (6.5 – 6.9). While the normal blood stream pH value is neutral to slightly alkaline; ranging between pH=7.2 – 7.5 (75). The reason for this pH difference is known as Warburg effect (76). This phenomenon differentiates between healthy and cancerous cells. Normally, mitochondrial phosphorylation is the main source of energy for the cell to obtain ATP. While cancerous cell uses metabolic glycolysis to obtain its energy. This glycolysis ends up with accumulated levels of pyruvate and lactate. This
occurs within anaerobic conditions that are attained by the action of tumor acidosis which minimize the amount of oxygen that is required for keeping the organ area healthy as shown in Figure (1.28).

![Diagram of mitochondrial phosphorylation and glycolysis](image1)

**Figure 1.28:** Schematic representation of mitochondrial phosphorylation and glycolysis (78)

Furthermore, DOX has been reported by Fukamachi et al. as one of the chemotherapeutic drugs whose therapeutic efficacy is not changed between the acidic and alkaline environments. Indeed, it rather showed more cytotoxic efficacy within highly acidic and larger tumor sizes than smaller less acidic. These results are agreed, also, with Hiroshi et al. (79). In other words, the larger the tumor size, the higher therapeutic efficacy that DOX has Figure (1.29).

![Diagram of pH dependency with tumor size](image2)

**Figure 1.29:** pH dependency scale relative to cancerous tumor size (79)
1.9. **Nanotechnology enhanced surface charge manipulation:**

Second, and on the same context, cancerous cell membrane of the tumor cell is negatively charged. That is because the accumulated lactate anions tend to remove the positive ions and leaving the cancerous cell membrane with negative charges. The poor blood perfusion is a supporting factor for this cellular reaction. Therefore, the fabricated nanoparticles that are intended to work in such environment should have certain specifications. They are preferred to have a net positive charge upon their surfaces, so that an electrostatic interaction can maximize the internalization rate of the positively charged nanoparticles. Besides, it has been developed by Osaka et al. that the accumulation rates of nanoparticles would be greater when electrostatic interaction between the nanoparticles and cancer cell membrane takes place. This would provide better drug diffusion with a controlled release behavior as shown in Figure (1.30) (80).

![Figure 1.30: Electrostatic interaction between nanoparticles (+ve) and cancerous cell wall (-ve) has developed instant drug release (80)](image)

1.10. **Nanotechnology enhanced biomarker attachment:**

One of the main effective advantages that entails the great role of nanotechnology in therapeutics is the ability of attaching targeting moieties to the surfaces of the drug carriers. These moieties are acting as a guiding monitor that direct the nanoparticle or nanocarrier from the site of drug introduction to the site of action which is breast area. Attaching such targeting moiety has been a great next step for a new era in designing effective drug delivery systems. Nanotechnology enables many researchers to conduct various types of biomarker attached nano-formulations that are directed for the treatment of various diseases. Some examples for various nano-based drug delivery systems are shown in Table (1.7). The guidance of such nano formulations is going to be routed in one of two targeting pathways; active and passive targeting Figure (1.31) (81).
1.10.1. Passive targeting:

Passive targeting entails the accumulation of the injected nano formulations within the tumor tissue with enhanced extravasation out of the tumor. Theses targeted nano formulations could efficiently penetrate the tumor through some factors such as their small nano-size, leaky vasculature, disorganized permeable blood vessels. The main reasons for such leaky nature of these blood vessels are hypoxia, inflammation and lack of nutrients due to high metabolic rate. This leakage tendency is referred as enhance permeability retention (EPR). Within these leaky permeable vessels, larger pores are formulated and allow transfer of particles with size up to 400 nm (82). Some studies have assumed a much broader range of these pores’ sizes of (380 – 780) nm (53).

1.10.2. Active targeting:

Active targeting is highly efficient ligand-receptor interaction that promote a targeted and precise drug release within the tumor environment. This interaction is taken place between the overexpressed surface epitopes of the tumor cell and the attached ligand biomarkers. It is more efficient than passive targeting (83). The entrapped drug is guaranteed to be released and diffuse inside the cell with active targeting. Once the ligand receptor conjugation takes place, plasma membrane close so that an internalization endosome is formulated.

**Figure 1.31:** Schematic illustration that differentiate between active (A) and passive targeting (B) (84)
Table 1.7: Example of different nanostructured drug delivery formulas that are assisted with various biological targeting moieties.

<table>
<thead>
<tr>
<th>Nanostructure</th>
<th>Monitored disease</th>
<th>Targeting moiety</th>
<th>Target</th>
<th>Entrapped drug</th>
<th>Mode of action</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multiwalled carbon nanotube</td>
<td>Colon cancer</td>
<td>Hyaloronic acid</td>
<td>CD44 receptors that are over expressed on colon tumor cells</td>
<td>Gemcitabine (chemotherapeutic anticancer) (Gemzar ®)</td>
<td>Intracellular</td>
<td>(85)</td>
</tr>
<tr>
<td>Liposomes</td>
<td>Non-small cell lung tumor</td>
<td>GE11 peptide</td>
<td>Epidermal growth factor receptors (EGFRs)</td>
<td>DOX (chemotherapeutic anticancer)</td>
<td>Intracellular</td>
<td>(86)</td>
</tr>
<tr>
<td>Dendrimers</td>
<td>Ovarian cancer</td>
<td>Primary antibody (mAbK1)</td>
<td>Mesothelin, a glycosyl-phosphatidyl inositol (GPI) protein</td>
<td>Paclitaxel (chemotherapeutic anticancer)</td>
<td>Intracellular</td>
<td>(87)</td>
</tr>
<tr>
<td>Polymeric micelles</td>
<td>Pancreatic cancer</td>
<td>anti-tissue factor (TF) antibody (clone 1894)</td>
<td>Pancreatic overexpressed tissue factors antigen receptors.</td>
<td>Oxaliplatin (platinum derived chemotherapy) (Eloxatin ®)</td>
<td>Intracellular</td>
<td>(88)</td>
</tr>
</tbody>
</table>
1.11. Nanotechnology enhanced prolonged biological circulation:

One of the critical factors that affect the therapeutic efficacy of the intended Nano formulated drug is its rapid biological clearance. During this biological journey, this nano-formulation encounter many biological defending mechanisms that slow down their migration and, in turns, speed up their elimination through lymphatic drainage. Immunologic reticuloendothelial system (RES) and mononuclear phagocyte system (MPS) are phagocytic systems that is majorly existent in the liver (89). This system is capable of capturing the nanocarrier and deteriorate its action through immunological phagocytosis.

Generally, in order for a drug carrier to be therapeutically effective, it must have some criteria available upon its biological ingestion. It must be (1) non captured and escape from the RES and MPS, (2) of high stability profile along over its biological journey till reaching the tumor area, (3) circulate freely within the systemic blood circulation without being encountered by various immunological responses and (4) accumulate within the tumor area so that a therapeutic concentration of the drug is released (90). For this purpose, some polymers are being suggested to be used so that we ensure high versatility and solubility of the nanocarrier. Hao et al. have suggested the use of polyethylene glycol (PEG) and poly (lactic co-glycolic acid) (PLGA) as hydrophobic co polymers in order for solubilization of water insoluble drugs. Owing to the biological performance, this study has reported the preference of coated nanoparticles than uncoated nanoparticles. Another study, Nadeem et al., has developed the use of poly-vinyl alcohol (PVA) as a coating material for iron oxide nanoparticles-based drug delivery system (91).

1.12. Chitosan as a nanoparticle platform:

CS has been therapeutically used as a polymer based nano drug platform (s) for the delivery of various drug types that are intended for the treatment of many diseases like breast cancer. Different nano-formulations of CS have been suggested to be used for such biomedical application such as CS nanoparticles, nano fibers, nanoliposomes and nanogels (92). CS possesses a prolonged circulation behavior and low immune clearance rates. Besies, it has what is called shielding effect against RES system that retard its detection (93). Furthermore, CS has the ability to efficiently penetrate the tight junction of the epithelial membranes, therefore, it has an improved permeation through this junction.
Chitosan nanoparticles (CSNP) have been used as a successful drug carrier of many anticancer agents for breast cancer therapy. Many studies have conducted a better antitumor activity for CSNP loaded with various anticancer drugs compared to these free unloaded drugs. Table (1.8) is showing the potential of some CSNP to be used as efficient drug delivery systems in breast cancer therapy. CSNP can be synthesized with many applicable preparation techniques such as ionotropic gelation, emulsification-crosslinking, co-precipitation, reverse micellization, microemulsion, desolation, solvent evaporation and coacervation (94).

![Diagram of Chitosan synthesis](image)

**Figure 1.32:** preparation of CS by deacetylation of chitin (95)

Ionotropic gelation is a simple and direct preparation technique that allow synthesis of crosslinked CSNP within aqueous conditions. It represents an electrostatic bridging between positively charged CS (through amine NH$_2$ group) and negatively charged polyanionic polymer such as tripolyphosphate (TPP), genipen and glutaraldehyde. The result of such bridging is formation of a complex gel like structure that represent crosslinked CSNP. Therefore, ionotropic gelation is more preferred than other techniques. Besides, it doesn’t require the use of extensive preparation conditions such as high-tech procedures and the burdens of using organic solvents (96).
Table 1.8: Some CSNP formulations used for breast cancer drug delivery

<table>
<thead>
<tr>
<th>Nano-formulations</th>
<th>Coating agent</th>
<th>Particles size</th>
<th>Synthesis</th>
<th>Drug</th>
<th>Target cancer</th>
<th>Route of administration</th>
<th>Mechanism of action</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSNP + PLGA</td>
<td>PLGA</td>
<td>(90 – 105) nm</td>
<td>Ionotropic gelation</td>
<td>DOX</td>
<td>Breast cancer</td>
<td>Topical (implant)</td>
<td>Passive targeting (EPR, extracellular)</td>
<td>(97)</td>
</tr>
<tr>
<td>CSNP</td>
<td>Uncoated</td>
<td>(100 – 150) nm</td>
<td>solvent evaporation</td>
<td>Tamoxifen</td>
<td>Breast cancer</td>
<td>Systemic (intravenous)</td>
<td>pH responsive (extracellular)</td>
<td>(98)</td>
</tr>
<tr>
<td>CSNP (micellar form)</td>
<td>α-tocopherol succinate</td>
<td>81.4 ± 10.5 nm</td>
<td>coupling reaction</td>
<td>Paclitaxel and Docetaxel</td>
<td>Breast cancer</td>
<td>Systemic (intravenous)</td>
<td>Charge affinity (intracellular)</td>
<td>(99)</td>
</tr>
<tr>
<td>CSNP (core-shell)</td>
<td>PEG</td>
<td>(34 – 48) nm</td>
<td>Copolymer grafting</td>
<td>Anti-HER2 drug (trastuzumab) + DOX</td>
<td>Breast cancer</td>
<td>Systemic (intravenous)</td>
<td>Targeting ligand</td>
<td>(100)</td>
</tr>
<tr>
<td>CSNP</td>
<td>Thiolation</td>
<td>(200 – 700) nm</td>
<td>Self-assembly</td>
<td>DOX</td>
<td>Breast cancer</td>
<td>Systemic (intravenous)</td>
<td>Targeting ligand enhanced antibody-antigen interaction</td>
<td>(101)</td>
</tr>
<tr>
<td>CSNP</td>
<td>Uncoated</td>
<td>473 ± 41 nm</td>
<td>Ionotropic gelation</td>
<td>DOX</td>
<td>General cancer therapy</td>
<td>Systemic (intravenous)</td>
<td>EPR</td>
<td>(102)</td>
</tr>
</tbody>
</table>
TPP has been shown to be more biologically safe than other polyanionic gelation polymers as a cross-linker for synthesizing CSNP. It is one of the considerable factors that influence many specifications of the resulted nanoparticulate system such as size average, surface charge, and particles distribution (see appendix SI). Moreover, the mixing technique of TPP into acidic CS aqueous solution affect these parameters as well. For instance, dropwise addition, one-shot addition and dilution techniques are three different mixing procedures that lead to variation of size, distribution and potential of the resulted CSNP (103).

![Figure 1.33: chemical formula of Sodium tripolyphosphate](image)

1.13. Polyethylene glycol (PEG):

PEG is a synthetic hydrophilic based polymer that has been used in numerous pharmacotherapeutic applications such as wound dressing and drug delivery. It has unique chemical properties such as biocompatibility, solubility, neutrality, non-irritating and odorless. It is composed of a linear chain of polyether with hydrophilic terminal groups of OHs as shown in figure (1.34). This hydroxyl group is responsible for its hydrophilicity, and it acts as electron donor. This electronic donation is responsible for many chemical characteristics of PEG including its good compatibility with organic solvents through weak hydrogen bonding interaction. This allow rapid release of the entrapped pharmaceutical drug. PEG has low melting temperature and fast solidification rate; therefore, it can be used preparation of chemical and organic dispersions (105). It has been approved as biodegradable polymer with non-immunogenicity and nonantigenicity effect for safe biological applications by FDA (106). For these reasons, it is widely used as encapsulating or coating agents with various nanoparticles-based drug delivery systems.

MAM has been demonstrated as a protein-based biomarker antigen that is overexpressed on the cellular surfaces of all breast tumor cancer types. Many studies have encountered the analysis of MAM as breast cancer biomarker. The results recommend the independent use of MAM as a diagnostic and therapeutic biomarker. These promising results have put MAM as one of the targeting ligands that entails an antigen-antibody interaction upon the membrane surface of breast tumor cells (108).

In this context, in breast tumor cells, the membrane associated MAM interaction can play a very important role in designing an actively targeting drug delivery system for minimizing the growth of such tumor tissue. By conjugating MAM to a nanoparticulate carrier that entrap antineoplastic drug will promote its therapeutic activity within the acidic based microenvironment of breast tumor area (109). This open the door in front of nanoscience and biopharmaceutical science for improving new methodologies of breast cancer curing. However, the number of reports that have been conducted on the use of this biomarker for active targeted breast cancer drug delivery systems are yet low. The potential of anticipated frequent application of such biomarker is still growing. Lian et al. has conducted a nano-based drug delivery of doxorubicin to the breast tumor area. Anti-hMAM has been used as a specific targeting ligand for specific targeting of breast tumor tissue (110). The results of this study came promising with efficient specific membrane targeting and functional drug carrying signaling system.

MAM has been discovered first in 1994 by Watson-Fleming within breast tissue cells. They could identify MAM-A and MAM-B. The overexpression of MAM-A receptors is restricted to breast
cancer patients only, while MAM-B is related to various tumor organs such as ovary, thyroid, uterus and breast (110, 111). Leygue et al. has experimentally concluded the diagnostic capability of MAM-A for diagnosis of breast cancer in a clinical study. The study has applied reverse transcriptase polymerase chain reaction (RT-PCR) to test the over-expression of the MAM-A coding RNA in 20 breast tumor affected females with advanced lymph nodes tumors. The results have confirmed its over expression with a recommendation of using MAM-A as a diagnostic target in breast cancer.
Chapter 2

Materials & Methods
2.1 Materials:

The materials used for the current experimental work were purchased from different local and international sources. CS polymer of low molecular weight (LMW) with 89.9% deacetylation degree and 65 cp viscosity was purchased from Priemex ehf, Chitoclear®, Iceland. Polyethylene glycol (PEG), acetic acid 100%, potassium bromide (KBr) (FTIR grade, ≥99% trace metals basis), and Dulbecco’s modified Eagle’s medium (DMEM), were purchased from Sigma Aldrich, Germany. DMEM reagent was supplemented with 4500 mg/L glucose, L-glutamine, sodium pyruvate, sodium bicarbonate, 10% FBS, and 5% penicillin/streptomycin. Anhydrous sodium tri-poly phosphate (TPP) (technical grade 85%) was purchased from Sigma Aldrich®, Missouri, USA. Doxorubicin hydrochloride (molecular weight of 579.96 g/mol) was purchased from Sigma Aldrich®, Switzerland (Suisse). Anti HER2 and anti Mammoglobin antibodies (originated from rabbit source) were purchased from Sigma Aldrich®, Steinheim, Germany. Phosphate buffered saline (PBS) was purchased from BioWhittaker® Reagents, Lonza, Walkersville, USA. For cleaning and sterilization purposes, acetone and Ethanol 99.9% were purchased from Medical Pure Life®, Europe and Alamia®, Egypt, respectively. 1-Ethyle-3-(3-dimethylaminopropyl) carbodiimide (EDC) was purchased from Sigma Aldrich®, Switzerland (Suisse). MTT reagent, 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide and dimethyl sulfoxide (DMSO), were purchased from Serva Electrophoresis, Heidelberg, Germany. Fetal bovine serum (FBS), Trypsin EDTA, Penicillin/Streptomycin, were obtained from Lonza, Switzerland. CO₂ incubator (Heracell incubator, Thermo Scientific, USA). Most of the other aforementioned chemicals and materials were stored in a well-controlled storage conditions including ventilation, humidity and temperature.

2.2. Preparation of Chitosan nanoparticles (CSNPs):

Chitosan nanoparticles (CSNPs) have been fabricated using Ionotropic gelation technique as referred by many different studies with some modifications including various processing factors as described in the supporting information appendix; part 1 (SI.1) (112). Ionotropic gelation method is a soft and straightforward technique in which CS NPs can simply be formulated via electrostatic interaction between acidic phase and alkaline phase. In other words, whitish clouds
of nanoparticles were spontaneously prepared upon addition of TPP (alkaline phase) as a negatively charged cross-linking agent, to the positively charged poly-cationic chitosan solutions (acidic phase) as shown in Figure (2.1). Various concentrations of acidic chitosan solutions (0.05%, 0.25%, 0.5% and 1%) were used against various concentrations of TPP aqueous solution (0.1%, 0.4%, 0.5% and 0.7%) as illustrated in the appendix (SI.2.1). The chitosan acidic solution and TPP solution were filtered first with syringe filters of 0.4 and 0.2 µm pores sized filters respectively before the crosslinking addition (113).

Figure 2.1: Cross-linked CS with TPP (113)

TPP was dropped to chitosan acidic solution in a dropwise manner within a moderate stirring speed. The mixture was left for proper mixing while keeping the applied mixing technique, time and rate within acceptable ranges Figure (2.2). The stirring time was adjusted to 30 min with low stirring speed of 250 rpm at room temperature. By comparing various mixing techniques such as hand stirring, magnetic stirring, homogenization or sonication, the magnetic stirring was
efficiently applied over other stated methods. It is simple and possesses a strong effect on reducing the sizes of CS NPs (114).

Figure 2.2: General scheme for preparation of CS NPs

In order for ensuring high stability of the formulated CS NPs, some factors such as polymers’ concentrations, pH, molecular weights of chitosan, stirring time and stirring speed were studied in a more detailed manner as shown in the appendix (SI.2). All these factors have a direct influence on size, particle charge and stability of the resulted nano-system. Each factor was experimented and studied independently, and the resulted recommended parameters that were applied for our current study are listed in Table (2.1).

Table 2.1: Optimum conditions for the applied factors in preparation of CS NPs

<table>
<thead>
<tr>
<th>Preparation Factors</th>
<th>Applied parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight of chitosan</td>
<td>Low molecular weight chitosan, DA 89.9%</td>
</tr>
<tr>
<td>Concentration of chitosan</td>
<td>1% Acetic acid aqueous solution (1%)</td>
</tr>
<tr>
<td>Concentration of TPP</td>
<td>0.4% Aqueous solution</td>
</tr>
<tr>
<td>pH</td>
<td>5</td>
</tr>
<tr>
<td>Stirring time</td>
<td>30 minutes</td>
</tr>
<tr>
<td>Stirring speed</td>
<td>250 RPM</td>
</tr>
<tr>
<td>Temperature</td>
<td>Ambient room temperature</td>
</tr>
</tbody>
</table>
The resulted samples of the synthesized CS NPs were representing a mélange of various concentrations of chitosan and TPP (see the appendix, SI.2 and Table S2). After successful formation of whitish clouds of nanoparticles, the mixtures were centrifuged for 30 min at 10,000 rpm. These specific centrifugation parameters were enough to separate the formulated nanoparticles (115). The resulted biphasic system, after centrifugation, was divided into two parts as shown in the appendix (SI.1.3 and table 1). The first part was the supernatant, which was discarded since it contained the unreacted polymers and acetic acid residues. The second part separated nanoparticles pellet was re-suspended in fresh distilled water to be washed from any excess un-reacted chitosan and TPP. The washing process was done twice at 10 min centrifuging time at 5000 rpm, and this part was divided according to the sampling process for further characterization experiments (116).

2.3. Stability of CS NPs:

Stability of the formulated CS NPs as a candidate for the following anticancer tests was crucial step. For investigating the stability of the surface of the resulted nanoparticles, their mean particles diameter size, surface zeta potential and poly distribution index (PDI) were analyzed by using Malvern Zeta sizer (NANO ZS, Malvern, UK) (see appendix SI.3, and Table 6) (117). An indicative stability profile was developed, and the samples were incubated in fresh distilled water with pH of 7.2 for three weeks at room temperature, and the required data of the size, distribution and surface zeta potential were collected over this stated period.

2.4. Coating of CS NPs with Polyethylene Glycol (PEG):

CS NPs showed different stability profiles with different conditions, resulted in some variations for successful delivery of the entrapped material inside these nanoparticles. Some specifications within the physical or chemical structure of the resulted CS NPs could be modified or tailored so that they could fit within the desired intended application. For instance, the poor water solubility of sole chitosan in water revealed some drawbacks with some chitosan-based drug delivery systems of some hydrophilic drugs (118). Increasing hydrophilicity of CS NPs and enhancing the
stability of an entrapped hydrophilic drug are two examples of specific properties that could be, fortunately, modified with a hydrophilic polymer like polyethylene glycol (PEG) (119). The modification adjusted by grafting this copolymer to the basic structure of CS NPs was reported in the literature as successful platform of maintaining high nanoparticles’ stability. This process is known as “PEGylation” and the resulted PEGylated CS NPs are called “stealth” (120).

Table 2.2: Sample variations relative to the polymeric concentrations of PEG while keeping TPP and Cs concentrations fixed

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cs (%)</th>
<th>TPP (%)</th>
<th>PEG (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.4</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>√</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>3</td>
<td>√</td>
<td>√</td>
<td>√</td>
</tr>
</tbody>
</table>

PEGylated CS NPs were prepared following Calvo et al. with some modifications (121, 122). This aims to provide high particles stability profile and enhance application durability on the final selected PEG concentration. Three different aqueous solutions of PEG (1%, 5% and 10%) were prepared as shown in table 2.2 by using a simple stirring speed around 500 rpm for 30 min at room temperature. All other preparation parameters were kept constant, so that the optimal functioning conditions can provide a sole explanation of the physical and chemical properties of the resulted nanoparticles. After dissolving PEG in distilled water, we could identify three different PEG concentrations whose viscosities varied according to the weighted amount of PEG powder. The dissolution of PEG didn’t require addition of any excipients like acetic acid or any additives. That’s because it is readily water-soluble polymer and has an FDA (Food and Drug Administration) approval for safe pharmaceutical and food applications (123).
The three aforementioned prepared PEG concentrations were added drop-wisely to the chitosan acidic solution (CS 1%). Then, the three mixtures were left for mixing for 30 min with stirring speed of 250 rpm. This provided three homogenous mixtures of both polymers; chitosan and PEG. The PEGylated CS NPs were formulated upon drop wise addition of TPP solution to chitosan acidic solutions containing the previously stated PEG concentrations as shown in Figure (2.3) and (2.4). Ionotropic gelation process started upon cross-linking of PEGylated chitosan solutions with the polyanion TPP. This process took place immediately, and the three mixtures were left on the magnetic stirrers for 30 min at low stirring speed of 250 rpm at the ambient temperature. Then, the PEGylated CS NPs were centrifuged and separated from the excess unreacted chitosan, TPP, PEG and acetic acid residues. The separation was performed by centrifuging at 10,000 rpm for 30 min at 24°C using HERMILE Labotechnik 36 HK centrifuge (Germany) (see Appendix SI.4.4). The resulted pellet of PEGylated CS NPs was later divided into three parts like those non-PEGylated CS NPs that were discussed in the appendix (SI. 1.3 and table 1) (124).
Some vital parameters were identified such as size, external morphology, surface zeta potential and PDI of the resulted PEGylated CS NPs. The effect of various concentrations of PEG on these parameters was analyzed. It was confirmed that change in PEG concentration could affect such parameters of these nanoparticles. This was confirmed before by some previously published reports (125). The effects of PEGylation process on the external positive amino groups and hence the stability of PEGylated CSNPs were studied also in accordance with another previously published study (112).

2.5. Encapsulation of Doxorubicin (DOX) within CS NPs:

The capability of PEGylated CS NPs to effectively entrap anticancer DOX drug was evaluated in this phase. After successful preparation of stable, biologically durable and biocompatible PEGylated CS NPs, we have studied different protocols that were intended for prosperous encapsulation of a hydrophilic drug inside the core of PEGylated CS NPs’ structure (127). These
protocols have identified the use of different drugs including DOX. PEGylated CS NPs were the same nanostructured matrix that was used as the platform to deliver these drugs. This study is mainly dependent on the efficient use and modification of DOX’s pharmacological action. Many reasons that were related to the physical and chemical properties of the intended drugs were analyzed for the specific use of DOX over other anticancer drugs as will be discussed in more details in the next chapter (128).

Integrating DOX within such nan-formulation was expected to provide many effective pharmacological and non-pharmacological indications over sole non encapsulated DOX. Furthermore, PEGylated CS NPs were expected to entrap such hydrophilic DOX and travel inside the bloodstream without leaking the loaded drug or being captured by the immunologic responding reticuloendothelial system (129). All preparation parameters were fixed in order to investigate the effect of different concentrations of DOX (table 2.3) on the encapsulation efficiency. Additionally, the preparation procedure was performed in light protected environment due to the photodegradation nature of DOX (130).

Table 2.3: Sample variation relative to DOX concentrations while keeping the polymeric concentrations for Cs, TPP and PEG fixed.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cs (%)</th>
<th>TPP (%)</th>
<th>PEG (%)</th>
<th>DOX (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>0.4</td>
<td>5</td>
<td>1.25</td>
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<td>1</td>
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</table>

After dissolving PEG in acidic chitosan solution, it was left to be mixed for 30 min as shown in Figure (2.5) with stirring speed of 250 rpm. DOX was added to the CS-PEG mixture while maintaining light protective performing conditions. Four different concentrations of DOX were used; 1.25 µg/mL, 2.5 µg/mL, 5 µg/mL and 10 µg/ml. Four different drug concentrations were obtained by the end of this step. The color of the four solutions were varied with red color intensity according to their corresponding concentration. The drug-copolymers were left for homogenous
mixing in order to achieve complete chemical interaction between the positive amino NH$_2$ group of DOX and negative polyanion of TPP, likewise the noncovalent encapsulation between DOX and of CS / PEG polymers. They were left for 1 hour within a mild stirring speed of 250 rpm. This period of time was quiet enough to allow efficient interaction capability between DOX, TPP and the polymers.

**Figure 2.5:** illustrative scheme for encapsulation of DOX inside PEGylated CS NPs

Finally, the polyanion TPP was added to the four solutions in order to allow sufficient cross-linking between DOX and the other polymers. The solutions were kept mixing under mild stirring conditions within a speed 250 rpm for 30 min. The formation of white turbid clouds inside the three solutions appeared instantaneously upon drop-wise addition of TPP under these mild stirring conditions. These whitish clouds, simply, represent aggregates of DOX loaded PEGylated CS NPs. These clouds were kept in light protected conditions to avoid photo-degradation of any remaining DOX.

The formulated CS NPs were directly separated by using centrifuging parameters at 10,000 rpm for 30 min using HERMLE Labotechnic 36 HK (Germany) in order to be ready for further analysis and testing. The sedimented NPs required considerable multiple washing in order to ensure collecting pure DOX loaded CS NPs without any residue or unreacted chemicals. The unused supernatant was analyzed each time for measuring the amount of non-encapsulated DOX. CARY 500 SCAN varian (Hi-tech, NJ, USA) UV-VIS spectrophotometer was used for characterizing the
presence of DOX in the produced supernatants. The instrumental response for the supernatant at each round was plotted against the previously and basically issued regression equation of calibration curve of DOX.

The fresh isolated pellets of DOX loaded CS NPs were divided into two main parts. The first part had vigorous mixing or redispersion of these formulated nanoparticles in a fresh media, like distilled water, using ultrasonic probe sonicator; Ploytron (Thermo Fisher, USA). The applied sonication parameters were 50% Amplitude for 3-5 min. The sonicated part of NPs was, then, diluted with distilled water so as to provide a liquid nano-system with a concentration of 1mg/ml. This DOX loaded CS NPs suspension was characterized later for particles’ surface morphology, shape, size, distribution and zeta potential using TEM and DLS. The second part was characterized for the chemical interaction between DOX with the applied polymers using FTIR (Thermo Fisher Scientific Nicolet 380 Spectrophotometer, USA). For this purpose, the second part of the NPs pellet was freeze-dried using BIOBASE freeze drier (Jinan, China) (see appendix SI 4.2).

### 2.6. Conjugation of Monoclonal Antibodies (mAbs):

Attaching biological ligands to the surfaces of the nanoparticles was expected in enhance the specificity and sensitivity of the entire nanocarrier. Anti-Human Epidermal Growth Factor Receptors (Anti-HER2) and Anti Human Mammaglobin antibody (Anti-hMAM) have been suggested to be used as the targeting moieties. Such conjugation may promote the cancerous endocytosis and cancerous sell uptake. In turn, it may minimize the normal cells attachment. This has been expected to decrease the systemic adverse drug reactions that are of the entrapped DOX drug and minimize its oxidative cardiotoxicity. The conjugation of the mAbs has been done according to collected procedure using some previous reports (130, 131).

In this sense, after selecting the nano-formula with 5 Ug/ml Loaded DOX, the nanoparticles have been freeze dried. EDC has been used to activate the COOH gp on the surfaces of DOX loaded PEGylated CSNP. After around 15 minutes slight mixing, centrifugation of the solution has resulted in a pellet of nanoparticles that have activated COOH group on the surface. BPS has been used for washing the nanoparticles to remove the excess EDC. On the other side, mAbs has been
shacked with micropipette and then was introduced to the nanoparticles mixed with EDC. EDC is extremely hygroscopic; therefore, its storage should be highly considered (132).

Then mAbs have been mixed to the COOH activated nanoparticles. They have been left on the orbital shaker with slight speed of 150 RPM for 2 hours. Then the two samples with two separates different mAbs have been centrifuged at 20,000 RPM for 30 minutes. Then PBS has been used for washing for 2 times at least with 5 minutes centrifuging at 8000 RPM. The resulted pellet has been resuspended in fresh PBS with slight shaking in order to resuspend the nanoparticles with surface conjugated mAbs. The ratios between the chemicals were different. For instance, the ratio between EDC to the nanoparticles was 5:1 and the ratio between nanoparticles and mAbs was 1:50. H\textsuperscript{1}NMR spectrum and FTIR spectrum have been conducted to ensure the surface conjugation of these mAbs. Besides, TEM imaging have been conducted as well in order for confirming the surface decoration and spherical shape of the resulted nano-systems.

\[
\text{Degree of substitution (DS)} = \frac{\text{Mod}(\text{COOH})}{\text{Tot}(\text{COOH})} \times 100 \quad \ldots \ldots \text{Eq. (2.1) (246)}
\]

2.7. Characterization of CSNPs, PEGylated CSNPs and DOX loaded

PEGylated CSNPs:

2.7.1. Dynamic light scattering (DLS):

The main application of criteria of the NPs were the smallest size, least aggregation tendency and smallest PDI value as measured by Dynamic light scattering (DLS) enhanced Zeta sizer instrument. The concentration of all different samples was kept constant at 1mg/ml.

2.7.2. Electrophoretic light scattering:

In the current study, we mainly used zeta potential analysis during different phases of the study in order for investigating the surface charges of the resulted CS NPs, PEGylated CS NPs and DOX loaded CSNPs. Any variation in the surface zeta potential indicated a change in the surface structure, which was a good indicator of surface attachment and hence the charge density would
be affected with that attachment and the stability rate of the prepared NPs. The higher the value of zeta potential, whether positive or negative, the higher the tendency of the colloidal dispersion to resist particles attraction or aggregation. The information obtained from zeta potential analysis have had a good directionality of which sample will be more stable and more efficient to be a platform for DOX drug delivery.

2.7.3. Fourier transform Infrared (FTIR) spectroscopy:

FTIR spectroscopy was applied using FTIR (Thermo Fisher Scientific, Nicolet 380) spectrophotometer to develop a graphical fingerprint of the chemical structures of the control nanoparticulate samples (CS, TPP and PEG), to be compared with the prepared nano-formulations including CS NPs, PEGylated CS NPs and DOX loaded CS NPs. The samples were lyophilized for at least 72 hours under -60 °C temperature. Then KBr was used with all samples to prepare the sample pellet. KBr was lyophilized first in order not to affect the sample absorbance since KBr is optically transparent, and acts as inactive sample carrier within the IR region (133).

2.7.4. X-Ray Diffraction Spectroscopy:

XRD spectroscopy analysis were carried out using Bruker D8 (MS, USA) in order to investigate the internal crystalline structures of all samples including the cross-linked CS NPs, and PEGylated CS NPs. The linkage of some polymers like TPP and PEG would provide some effect on the entire crystalline structure of CS. Therefore, the entrapment efficiency of these polymers would be shown with XRD analysis.

2.7.5. Scanning Electron Microscopy (SEM):

The particle’s morphology and size of the produced NPs with attachment of PEG were characterized by using SEM. The primary characterization of CSNP and PEGylated CSNP was done with an oven dried sample. These samples were diluted as 1mg/mL, and then moved to a spread aluminum sheet, over which it was dried at 60°C temperature for 10 min. After drying, the sample was gold sputtered for 15 min so that the surface of the sample become conductive and, hence, interact with the bombarded electrons and also to enhance the resolution and quality of the images.
2.7.6. Transmission Electron Microscopy (TEM):

TEM analysis were performed using Ultra-High Resolution schotty Transmission Electron Microscope SU 7000. The preparation of TEM sample was done with diluting the prepared solutions to 0.02 mg/mL. Three different types of samples; CS NPs, PEGylated CS NPs and DOX loaded PEGylated CS NPs were prepared. The provided information by TEM images was related to the morphology, topography, size and particle distribution was helpful to show the successful attachment of PEG and DOX. One drop of the diluted sample was dried and dyed with phosphotungestin dye to keep the surface of the nanoparticles conductive; and therefore, the electrons can be captured easily resulting in better image resolution.

2.7.7. UV-Vis Spectroscopy:

UV-Vis spectroscopy analysis were performed using CARY500 SCAN Varian UV-VIS spectrometer (Hi-tech, NJ, USA) for different characterizations analysis that were related to DOX concentrations. First, a calibration curve of 6 different concentration of DOX aqueous solution using deionized water was plotted. Then, the absorbance of the unloaded free DOX that was measured in the supernatant of DOX loaded CSNP preparation was determined at wavelength of 480 nm using clear quartz processing cell. This allowed the determination of the loading capacity of such NPs relative to very minute concentrations of DOX that were reaching few micro liters. Besides, the release profile of DOX loaded CS NPs has been detected with the same UV spectrophotometer. PEGylated CS NPs was used as a black. The scattering of UV-Vis light beam that was done by such PEGylated CSNP solution was excluded. Moreover, the UV-Vis absorption of many different samples with various concentrations that were related to various time slots were analyzed in order to reveal the releasing behavior of the nano formulations.

2.8. Drug Loading, Release Profile and Stability of NPs:

2.8.1. Drug loading:

Loading of DOX to PEGylated CS NPs was performed using different weights of DOX; 0.125 mg, 0.25 mg, 0.5 mg and 1 mg in order to define the most suitable drug concentration that could achieve the best loading capacity. DOX loaded PEGylated CS NPs were dissolved in PBS medium and kept on stirrer for 24 hours at 100 rpm. This was done within light protected environment under
ambient temperature. Thereafter, the DOX loaded NPs were centrifuged at 20,000 RPM for 30 min under 4 °C temperature. The concentration of free DOX drug in the supernatant layer was detected using UV-Vis spectroscopic analysis at wavelength of 482 nm. Drug loading capacity has been detected using the given equation (Eq. 2.2).

\[
\text{Loading capacity (LC\%)} = \frac{(\text{Total drug added} - \text{drug in supernatant})}{\text{Weight of nanoparticles}} \times 100 \quad \ldots\ldots\text{Eq. (2.2)} (134)
\]

\[
\text{Encapsulation efficiency} = \frac{(\text{Total drug added} - \text{drug in supernatant})}{\text{Total drug added}} \times 100 \quad \ldots\ldots\text{Eq. (2.3)} (134)
\]

2.8.2. Swelling test:

CSNP has been shown by many studies to have a swelling tendency which may differ according to the pH of the dispersive media (135). Swelling test in the current study has been performed in order to assess the influence of various pH levels on the swelling of the DOX loaded PEGylated CSNP and predict their effect on the entrapped drug. Therefore, swelling indices have been calculated for the nanoparticles in two different pH media; 6.6 and 7.4. These two specific pH media are two model values that are related to the breast tumor pH and normal blood pH levels respectively (136). Swelling index has been obtained using the given equation Eq. (2.4), where Sw is the swelling of the nanoparticles after time t, Wo is the weight of nanoparticles before incubation or in a dry form and Wt is the weight of nanoparticles after time t or after incubation time.

\[
\% Sw = \frac{Wt - W_0}{W_0} \times 100 \quad \ldots\ldots\text{Eq. (2.4)} (137)
\]

2.8.3. Stability of DOX PEGylated CS NPs:

The highest LC\% of DOX was determined from the previous experiments. Thereafter, the stability profile of the highest DOX loaded sample was investigated. The stability of the samples was confirmed after incubation in BPS of pH 7.4 for two weeks at 37 °C. UV-Vis spectroscopy was applied to detect the release profile of DOX by measuring its concentration at wavelength of 480 nm.
2.8.4. Drug Release Profile:

Release profile of the highest DOX loaded PEGylated CS NPs was studied. The sample was tested in two different pH media. The first one mimicking the normal blood circulatory pH 7.4, while the second one was mimicking the tumor microenvironment pH 6.6. The samples were kept inside the incubator for around 72 hours at 37 °C temperature. Within various time intervals, the concentration of the released DOX was determined by using UV-Vis spectroscopy as well at wavelength of 480 nm. The applied time slots were (1, 3, 6, 9, 12, 18, 24, 36, 48 and 72) hours. Re-compensation for the withdrawn amount with another fresh media was carried out.

2.9. Cell culture analysis:

2.9.1. Cell line maintenance:
The anticancer activity of the fabricated CS NPs, CS NPs-PEG, and CS NPs/DOX was analysed in-vitro against human isolated breast cancer cell line (MCF-7) and a normal mouse fibroblast cell line (L929). The cell lines were propagated as a monolayer separately in DMEM. They were passaged three times per week in 75 cm² cell culture flasks and safely incubated at 37°C in 5% CO₂ incubator. Regular cell detachment was proceeded before passaging times and the specified MTT assays using trypsin (0.25%) containing 0.1% EDTA. Trypan blue has been applied in cell counting using hemocytometer.

2.9.2. Direct Cytotoxicity (MTT assay):
The anticancer property of DOX before and after encapsulation inside CS NPs against MCF-7 cells and the cyto-compatibility of fabricated samples against L-929 cells were investigated based on ISO10993-5 standard procedure (138). Colorimetric 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye analysis (MTT cell viability analysis) has been used to detect the viability profile of both types of cells with 24 hrs incubation in a 96 well plate. In order to attain a semi-confluent cell growth after 24 hrs; MCF-7 cells seeded at density of 25 × 10³ cells/well, while L-929 cells seeded at density of 3 × 10³ cells/well in 96 well plate. Following that, a direct addition of 50 ul/well from previously prepared serial dilutions of CS NPs, CS NPs-PEG, and CS NPs/DOX (1.25, 2.5, 5 and 10) µg/mL. After 24 hrs, MTT reagent has been used to replace the media with 1 mg/ml conc. The replacing volume was 100 µl that has been incubated for 4 hours. PBS was used for washing and removal of MTT medium. This washing process was done tow times. 100
µl of 100% DMSO was applied in order to dissolve insoluble Formazan crystalline products, that are expected to be cytotoxic and damaging to the cells. A microplate reader (SPECTROstar Nano, BMG LABTECH, Germany) has been used at Abs of 570 nm to detect the cell viability. Control sample has showed 100% viability, while the viability of the remaining wells has been identified using Eq. (2.5)

\[
\text{Cell Viability Rate} = \frac{\text{Sample ABS}}{\text{Control ABS}} \times 100 \quad \ldots \quad \text{Eq. (2.5)} (139)
\]

2.10. Theoretical background:

2.10.1. Zeta-Sizer and Zeta-Potential (MALVERN®) :

This is a main dual systematic instrument that includes both dynamic light scattering (DLS) that is used to assess the nanoparticle’s sizes and distribution, and electrophoretic light scattering (ELS) that is used to determine the surface charge of a particles within a solution medium.

![Figure 2.6: Systematic setup of the basic principle for dynamic light scattering with detecting different scattering angles of the laser beam (140)](image)

The basic principle of DLS is simple as a monochromatic laser beam is directed towards the sample cuvette. When the incident laser beam illuminates the particles in the cuvette, it gets scattered in all directions. A photon detector is used to receive the scattered light beam at a certain scattering angle \( \theta \). In another words, when the particles scatter the laser beam, they provide information about their size, motion and distribution. Physically, the scattering is related to the hydrodynamic radius
(Rh) of the particles (141). The correlation of the laser scattering intensity is done through software analysis or the digital correlator as shown in the DLS scheme in Figure (2.7).

![Dynamic Light Scattering Instrument](image)

**Figure 2.7:** Illustrative description of different components of dynamic light scattering instrument (141)

By defining the diffusion parameters from Eq. (2.6), we can calculate the hydrodynamic radius Rh that corresponds to the particles size. \( D \) represents diffusion coefficient, \( k_B \) represents Boltzmann constant, while \( T \) is the temperature and \( n \) is the sample viscosity of the sample. By defining such parameters, we can calculate the. The concentration of the sample should be very low, because this equation is valid for single scattered light. When sample’s concentration is too high, multiple scattering occur and this equation become invalid.

\[
D = \frac{k_B T}{6\pi \eta R} \quad \text{Eq. (2.6) (143)}
\]

The surface charge of any nanoparticle is a key indicator of the aggregation or stability tendency. Zeta potential represent the electro-kinetic potential of nanoparticles or any other particles in general within a colloidal system. The higher the charge value on the particles surface, the more stability suspension or colloidal system.

The electrophoretic light scattering instrument is processing with an electric field is applied the sample cell. This electric filed cause the particles to move because of the interaction between them. Through this interaction between the particles and the applied field, they acquire some charge. This charge affects the speed and direction of particles movement. Besides, the strength of the
applied electrical field and the nature of the colloidal medium are two important factors also that affect them (144).

![Figure 2.8](image)

**Figure 2.8:** Schematic diagram showing the principle of electrophoretic light scattering with a multiple layer of illustrative charged layers (A), and the shape of the sample cell kit (B) (144)

### 2.10.2. Fourier Transform Infrared (FTIR) Spectroscopy:

It is one of the well-known analytical techniques that is mainly used to characterize various materials types including organic, in-organic and polymeric chemical materials. The change in the surface structure or surface functional group(s) can be monitored and analyzed by FTIR. This allow us to understand the significant change of the physico-chemical properties of the material after addition of certain polymer or chemical substance to the surface or the core of the main material. Besides, the durability and structural stability of a material can be another feature that can be easily monitored through FTIR (145).

Some characteristic structural moieties of a chemical material can be represented in a specific pattern within a certain range of infra-red absorbance value. Analytically, the ability of these moieties to absorb the incident infra-red spectrum in the range of 400 to 4000 provide a graphical analysis of the chemical information of the applied sample. through FTIR, we can also release a chemical comparison between the polymerized material and the original or the standard ones through the intensity, position and behavior of the absorption peaks. It can be used, also, to identify material’s decomposition, oxidation, deterioration or any undesired contaminants. Therefore,
FTIR can provide a rigid justification that supports a successful change in the chemical and physical properties of the applied material (146).

![Figure 2.9: schematic representation of the FTIR working principle (147)](image)

The working principle of FTIR is mainly focusing on the interaction between the sample material and a light source within the infrared region in a surrounding electromagnetic field. Once the sample disc is subjected to the IR light, it absorbs the light and get excited. The inner structural molecules are excited also and moving to a higher vibrational level at a certain frequency. The energy difference between the ground energy level and excitation level is basically equal to the light energy that the molecules have absorbed.

Light is sent to the sample through an instrumental device called interferometer. The IR radiation is sent to the sample material through a beam splitter. As a result, the radiation light is divided into two halves. The first one is going to a fixed mirror, while the second half is going to a continuously moving mirror. Then the two split beams recombine again at the detector and pass through the sample again. In this case, the sample absorb all characteristic wavelengths, in an interference pattern, from the interferogram. The energy variation is detected through the detector which read it versus time. Through Fourier Transform mathematical technique, a software generated procedure provides a relationship between time and frequency. This relationship is identified later.
with against referenced libraries that develop the spectrum information of the sample that is considered as a fingerprint for such specific material (148).

2.10.3. X-ray diffraction:

X-ray diffraction is advanced new characterization techniques that is used to analyze a crystalline material and studying its atomic spacing as well. Besides three-dimension structural analysis, XRD is efficient also to study a unit cell dimensions. XRD is mainly based on the interaction between X-rays and a crystalline sample. the main source of X-rays emission is a cathode ray tube that is directed to the sample with a monochromatic radiation pattern. When the X-rays hit the powdered sample, it diffracts with a diffraction angle $\theta$ (149). This constructive interference and the relationship between the angle of diffraction $\theta$ and the electromagnetic radiation wavelength is expressed as Bragg’s law that is shown in (figure 2.10) and Eq.2.

\[
n\lambda=2d \sin \theta. \quad \ldots \quad \text{Eq. (2.7) (150)}
\]

Where $\theta$ is the scattering angle or the angle between the incident X-ray beam and the lattice plane, $\lambda$ is the wavelength of the incident electromagnetic waves, $d$ is the interplanar distance (Figure 2.10). $n$ represents the positive integer factor or the order of reflection.

![Figure 2.10: illustrative scheme of X-ray diffraction with diffraction angle $\theta$ (Bragg’s law) (151)](image)

The X-ray cathode tube produces an X-ray beam by heating the filament of the tube that produces a series of electrons. These electrons are directed towards the sample material and accelerated by
applying voltage. These electrons are going to bombard the sample and expel its inner shell electrons as shown in (figure 2.11). In order to do that, the incident electrons must have sufficient high energy. By dislodging the inner shell electrons, X-ray is emitted. A crystal monochromator is used to produce monochromatic rays. As the sample and X-ray detector are rotated, the diffracted emissions are recorded by the processing detector. When the dimensional arrangement of the incident rays satisfies Bragg’s law, the detector record the signal and convert it to mathematical counts that is illustrated through a computer monitor (152).

![Figure 2.11: X-ray beam bombarding the Inner shell electrons (152)](image)

**2.10.4. Ultra-Violet / Visible Spectroscopy:**

UV-Vis spectroscopy is a frequently used characterization analyses that mainly target the optical properties of different samples. Through this analysis technique, we can develop qualitative and quantitative analysis, detect the presence of undesired impurities and detect the presence or absence of certain compositional functional groups. Generally, the electromagnetic spectrum of sunlight consists of a range of radiation regions including the UV-Vis region which is located at the range of 210-900 nm. The interaction of the sample to light in this region is the principle source of UV-Vis spectroscopic analysis. When light hit the sample’s molecules, the atomic electronic transition takes place immediately between different energy levels or, typically, from the higher occupied molecular orbital (HUMO) or ground state to lower unoccupied molecular orbital (LUMO) or excited state as shown in (figure 2.12). This intermolecular transition is accompanied by absorption of electromagnetic radiation in this region.
HUMO and LUMO orbitals are called also bonding shells and antibonding shells, and the difference between them is known as band gap. In order for absorption to take place, the UV light photons must have energy amount that matches to this band gap. In this concern, different molecules will definitely have different band gap energies and, therefore, will have different absorption spectra. Once the sample was hit with a beam of UV light, it absorbs part of it and the intensity of the released beam will exponentially decreased as shown in (figure 2.13) and absorbance equation Eq. (6). However, the transmittance has the same relationship between the incident light ($I^o$) and the transmitted light ($I$), but absorbance is the counteractive principle of transmittance.

$$T = \frac{I}{I^o} \quad \ldots \quad \text{Eq. (2.8)} \quad (154)$$

$$A = \log_{10} \frac{I^o}{I} \quad \ldots \quad \text{Eq. (2.9)} \quad (154)$$
UV-VIS spectroscopy is mainly based on the ability of measuring the spectrum of a certain sample containing chromophore or light absorbing molecules. This allow us to obtain a graphical relationship between the wavelength or frequency at which light is absorbed, and the absorption rate that is detected by the amount of chromophore exist in the sample. therefore, the higher the amount of this chromophore in the applied sample, the more dense the amount of the absorbed light. This relationship reveals another direct relationship between the sample concentration and wavelength or frequency of the emitted UV light. Beer’s Lambart law has exploited this optical relationship in order to develop a linear relationship between concentration and absorbance as shown in Eq (7).

\[ A = \varepsilon c l \]  

…….. Eq. (2.10) (155)

A: the absorbance

\( \varepsilon \): absorption coefficient

\( c \): molar concentration of the solute

\( l \): path length

This equation (Beer’s Lambart law) can be used analytically to relates the concentration of a substance in a sample to the color intensity of the same sample. when we have large number of samples and different concentration in each sample, we can use the developed linear relationship that this law provides. However, we need first to issue a calibration curve. This calibration or
regression curve is based on plotting 3 to 4 concentrations of the sample and getting their corresponding absorbance values. This plot will work as a reference from which we can obtain the concentration of any unknown concentration of the same sample (155).

Figure 2.14: schematic representation of UV-VIS spectrometer principle (154)

2.10.5. Scanning Electron Microscopy (SEM):

SEM is a powerful and reliable analysis techniques that is used for imaging samples. This technique is much advanced than simple light microscope since it uses electrons to image the sample unlike light microscope that use light. The wavelength of electrons is much smaller than that of light, therefore the magnification and resolution of SEM is more preferred than traditional light microscope. SEM provides such electron-oriented magnification through the use of a set of coils that scan the electrons bombarded from the sample (156).
As shown in (figure 2.16), the raster pattern of the coils is used to scan the sample through the generated electrons for the electron source at the top of the instrument. Basically, these electrons are produced when their thermal energy exceed that of the main electron sources. The role of the positive anode that is fixed below the electron source is to attract and accelerate the speed of the emitted electrons under protective vacuum. This vacuum has a critical indirect effect on the overall quality of the released images by protecting the electrons from interacting with any external atoms, molecules, noise or contamination (158). On the other hand, the use of such condenser lens and objective lens is mainly applied for adjusting the electron path, and this ends up with controlling the resolution. The integration of apertures may be considered in some SEM systems in order to control the size of the beam and control the current as well. This may give a good opportunity for controlling the resolution of the resulted images (159). Typically, the main use of SEM is to analyze different types of samples such as insulating, thin organic, biological and vacuum sensitive samples with satisfying resolution and magnification power.
2.10.6. Transmission Electron Microscopy (TEM):

TEM is a powerful and versatile tool for imaging of many different types of samples. It is widely used in biological fields, forensic analysis, material sciences, medical and nanotechnology applications. The main use of TEM is to provide some information about the crystalline structure, size, particles distribution, morphological, chemical structure, structural defects and topographical properties. Similar to SEM, the source of these information is basically dependent on the interaction of the sample to a very high energy electron beam that is much stronger than SEM. The electron beam is accelerated with applying voltage approximately more than 200Kv. The electrons are accelerated to a very high speed near the speed of light. While the wavelength of the electrons in the electron beam is million times shorter than normal electromagnetic wavelength of light, the spatial resolution and magnification quality of TEM images is much higher than normal light microscopy (161).

TEM is operated in a similar process to the simple light microscope. However, TEM uses a stream of electrons produced by an electron gun (figure 2.17) instead of using light source. The electron beam is directed towards the sample in a very high speed that is enough to pass through the sample. A primary condenser lens is used to focus the emitted electrons into a thin and coherent electron beam. Once the electrons could pass through the sample, they are scattered. These scattered electrons are then focused by an electromagnetic lens called objective lens. This lens focuses the received electrons into an image that is transmitted through a projector lens. This projector lens is
used mainly to expand the electron beam into a monitor or camera supported projector so that the image is seen by the user (162).

![Schematic representation of the different components of TEM](image)

**Figure 2.17**: Schematic representation of the different components of TEM (163)

**Table 2.4**: comparison between TEM and SEM (162-164)

<table>
<thead>
<tr>
<th>Item</th>
<th>TEM</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>Very thin</td>
<td>Thick and conductive</td>
</tr>
<tr>
<td>Resolution</td>
<td>Very high, ~ 0.5 Å</td>
<td>High, ~ 0.5 nm</td>
</tr>
<tr>
<td>Electron beam</td>
<td>Pass through the sample</td>
<td>Surface analysis</td>
</tr>
<tr>
<td>Sample preparation</td>
<td>Extensive</td>
<td>Simple</td>
</tr>
<tr>
<td>Surface modification</td>
<td>Dyeing</td>
<td>Gold sputtering</td>
</tr>
<tr>
<td>Voltage</td>
<td>Very high</td>
<td>Medium</td>
</tr>
</tbody>
</table>

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Chapter 3

Results and Discussion

Synthesis and Characterization of PEGylated Chitosan / Doxorubicin Nanoparticles and Conjugated with Monoclonal Antibodies for Breast Cancer Therapy
3.1. Preparation of CSNP:

In order to successfully prepare a conjugated drug delivery system that is effective against breast cancer, CSNP have been used to encapsulate anticancer drug like DOX, and the nano-system was further conjugated with monoclonal antibodies. Several parameters have been considered and found to have considerable effects on the preparation of the final formulations such as: particles size, size distribution, surface charge, stability in different pH media, surface functionalization, physiological circulation time, biocompatibility, biodegradability, swelling matrix, and drug diffusion rate. Ionotropic gelation method was used to prepare CSNP due to its efficiency and the resulted high particles’ stability for longer periods (165).

3.1.1. Ionotropic Gelation:

As shown in Table (3.1), the integration of different concentrations of CS polymer and TPP, as a cross-linker, for preparation of CSNP. TPP has been shown to be biologically safe, non-toxic and non-carcinogenic owing to its hydrolyzed phosphate ions (166). The resulted CSNP in Table (3.2) had various sizes and PDI values. Moreover, the final appearance of the formulated CSNP was slightly different from each other. Some samples have shown whitish uniform colloidal systems of suspended CSNP. While others have shown high tendencies of particles aggregation with whitish turbid solution (112).

<table>
<thead>
<tr>
<th>TPP conc (%)</th>
<th>Chitosan concentration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.05</td>
</tr>
<tr>
<td>0.1</td>
<td>S1</td>
</tr>
<tr>
<td>0.3</td>
<td>S5</td>
</tr>
<tr>
<td>0.5</td>
<td>S9</td>
</tr>
<tr>
<td>0.7</td>
<td>S13</td>
</tr>
</tbody>
</table>

The polymeric matrix of CSNP, by ionic gelation technique, is controlled and tunable through an electrostatic interaction between (+vely) charged NH$_2^+$ from CS and (-vely) charged polyanions from TPP (166). This gelling formation interaction has been found to be affected by the
concentrations of the two polymers, and the resulted particles sizes are, also, affected as shown in Table (3.2).

**Table 3.2:** Particle size and distribution of different samples with various concentrations of TPP and chitosan

<table>
<thead>
<tr>
<th>Sample</th>
<th>Particle size (nm)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>3668.6 ± 967.6</td>
<td>0.31 ± 0.17</td>
</tr>
<tr>
<td>S2</td>
<td>4986 ± 1300</td>
<td>0.64 ± 0.07</td>
</tr>
<tr>
<td>S3</td>
<td>1607 ± 63.66</td>
<td>0.43 ± 0.01</td>
</tr>
<tr>
<td>S4</td>
<td>518.76 ± 28.28</td>
<td>0.86 ± 0.05</td>
</tr>
<tr>
<td>S5</td>
<td>1594.5 ± 195.8</td>
<td>0.86 ± 0.02</td>
</tr>
<tr>
<td>S6</td>
<td>3894 ± 376.7</td>
<td>0.69 ± 0.26</td>
</tr>
<tr>
<td>S7</td>
<td>1582.5 ± 228.3</td>
<td>1.37 ± 0.36</td>
</tr>
<tr>
<td>S8</td>
<td>190 ± 5.6</td>
<td>0.15 ± 0.02</td>
</tr>
<tr>
<td>S9</td>
<td>1979.5 ± 64.3</td>
<td>0.22 ± 0.05</td>
</tr>
<tr>
<td>S10</td>
<td>1895 ± 161.3</td>
<td>0.41 ± 0.07</td>
</tr>
<tr>
<td>S11</td>
<td>678.66 ± 15.02</td>
<td>0.85 ± 0.06</td>
</tr>
<tr>
<td>S12</td>
<td>690.9 ± 28.42</td>
<td>0.24 ± 0.02</td>
</tr>
<tr>
<td>S13</td>
<td>5645.3 ± 685.5</td>
<td>0.45 ± 0.11</td>
</tr>
<tr>
<td>S14</td>
<td>2362 ± 678.34</td>
<td>0.24 ± 0.06</td>
</tr>
<tr>
<td>S15</td>
<td>2265.6 ± 141.78</td>
<td>1.33 ± 0.31</td>
</tr>
<tr>
<td>S16</td>
<td>1300.6 ± 664.8</td>
<td>0.66 ± 0.32</td>
</tr>
</tbody>
</table>

By demonstrating all of these previous results, sample 8 has been the sample of choice. It has demonstrated the smallest size with acceptable poly dispersity index. The applied CS:TPP ratio has been close to 3:1. The size of this sample has been 190 ± 5.6 with a good particle’s distribution of PDI 0.51 ± 0.002. Biologically, this size can develop many pharmacokinetic and therapeutic benefits compared to larger sized drug carriers (112). Many factors have been shown to affect the size and PDI of the resulted CSNP such as processing pH and deacetylation degree of CS (167). Therefore, the effect of these parameters has been explored on this sample.

**3.1.2. Factors affecting size of CSNP:**

![Figure 3.1: Effect of processing pH (A) and deacetylation degree (B) of CS on the size of CSNP](image-url)
o **Processing pH:**

Size has been shown to be decreased with decreasing the processing pH media. That’s may be because of the high protonation rate at highly acidic media. It increases the reactivity of the terminal NH2 groups of chitosan and improve the ionic strength of the dissolution media (168). Therefore, the lower the pH value, the lower the size we have as shown in Figure (3.1A). However, the distribution of the resulted particles has been non uniform with high PDI value of the resulted CSNP. Consequently, lowest pH media are not always the best solution, the current study recommends adjusting pH of the processing medium to 5 (169).

o **Deacetylation degree:**

On the other hand, degree of deacetylation is another critical factor that has been discussed with many published reports (170). The results of the current work have revealed that sizes of CSNP decrease as the degree of deacetylation of CS increases as shown in Figure (3.1B). This may be related to the amount of deacetylated amine groups. In another words, high deacetylation degree provides high density of positive NH\(_2^+\) charged groups. This high positive charge density enhances higher crosslinking interaction with TPP. Although, this may contradict with Vivek et al. that proposed that the surface charge of CSNP is -35 (171), however, Phaechamud et al. and Ling et al agreed on this (172). Therefore, CS with high deacetylation degree has been used in the current study.

o **CS: TPP ratio:**

The relationship between CSNP’s size increment and TPP concentration has been demonstrated in Figure (3.2). Whenever the concentration of TPP is increased or decreased than the selected concentration (0.3%), the sizes and PDI of the resulted CSNP are going to massively increase with having some of them reaching micro sized ranges.

When TPP concentration is over increased, the cross-linking rate of CS is increased. That’s may be due to the accumulation of the excess TPP molecules upon the outer surfaces of the already crosslinked CSNP. This may enhance more intramolecular interaction, and higher occupation rate of free NH\(_2^+\) groups of chitosan by TPP. This can provide what is called oversaturation of CSNP’s
surface and become more reactive to proceed another cross-linking process. Therefore, the size becomes greater

![Diagram](image)

**Figure 3.2:** Effect of TPP concentration on the final size of CSNP

On the other side, at very lower TPP concentrations, the size of the crosslinked CSNP tends to increase, and whitish turbid clouds are seen in the preparation media. At very high conc of CS relative to lower TPP, neutralization of the protonated cationic NH$_2^+$ groups of CS by TPP is decreased, and this led to higher sedimentation rate of chitosan polymer, especially, when chitosan acidic solution is rich with acetic acid (113, 173). Therefore, the current work may conclude that there is an optimum ration of CS: TPP which is expected to be near 3:1 (168).

### 3.1.3. Stability of the selected CSNP sample:

Surface charge of CSNP is a remarkable factor that is considered as an indicative factor of the particle’s stability and their tendency to form aggregates. The colloidal suspending appearance of CSNP is highly affected by the charge on their surfaces. This charge is influenced by the same previous parameters (174, 175). In order to make sure of the surface stability of the selected CSNP sample; S8, a three weeks stability profile has been developed as shown in Figure (3.3).
Figure 3.3: Stability profile of the selected CSNP sample; S8 for three weeks at pH 7.4

Furthermore, size stability profile has been confirmed with calculating size increment percentage, which is an indication of size variation along the stability period of time. It has been calculated according to the given equation (Eq.3.1). Owing to the high charge surface positive zeta potential value of the selected CSNP sample with Zeta value of 42.8 mEv, the particles have been shown to have a small size increment percentage as 8.9%. That because of a direct relationship between having high zeta potential value and showing a good stability profile for longer time (166).

$$\text{Size increment } \% = \frac{\text{Size at the beginning} - \text{Size after 22 days}}{\text{Size at the beginning}} \times 100 \quad \ldots \ldots \quad \text{Eq. (166)}$$

Finally, the size of the developed CSNP as drug carrier is a substantial factor that needs to be studied very well before drug encapsulation or PEGylation process. This has a strong influence on the efficiency of the developed drug delivery system. As the size of the nanoparticles decreases, the drug cellular uptake increases. Besides, a direct relationship between nanoparticle size and pharmacokinetic drug distribution is considered. Nanoparticles’ sizes below 100 nm have 6-fold more cellular uptake rate than micro sized particles (177). Furthermore, CSNP within a size range less than 200 nm provided more potent dosage forms than larger micro range particles. The advantages of nanoparticles in this size ranges are enhanced muco-adhesion, biocompatibility,
increased therapeutic index, enhanced stability and less carcinogenicity. This has been consistent with Pawar et al, that could achieve > 95% drug entrapment efficiency within a size range (100 – 200) nm (178). Therefore, the recommended size of the prepared CSNP in the current study has been less than 200 nm.

3.2. PEGylation of CSNP:

Coating of the prepared CSNP’s surface with PEG has been reported in many studies showing high NP surface stability and solubilization. The increased blood circulation time and immunological RES resistance of CSNP that are coated with PEG is a guiding factor for seeking PEGylation process. PEG aims to protect CSNP by blocking their surface positive charges that can be included in the opsonization process by RES (179, 180).

Table 3.3: Size, PDI and zeta potential of PEGylated CSNPs

<table>
<thead>
<tr>
<th>Samples</th>
<th>Size</th>
<th>PDI</th>
<th>Zeta Pot.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (CSNP)</td>
<td>192 ± 4.1</td>
<td>0.19 ± 0.01</td>
<td>+43 ev ± 0.4</td>
</tr>
<tr>
<td>CSNP-PEG 1%</td>
<td>175 ± 4.8</td>
<td>0.25 ± 0.03</td>
<td>+ 41.8 ev ± 0.6</td>
</tr>
<tr>
<td>CSNP-PEG 5%</td>
<td>169 ± 5.8</td>
<td>0.15 ± 0.01</td>
<td>+ 35.6 ev ± 1.1</td>
</tr>
<tr>
<td>CSNP-PEG 10%</td>
<td>173 ± 2.9</td>
<td>0.21 ± 0.02</td>
<td>+ 39 ev± 1.2</td>
</tr>
</tbody>
</table>

Table (3.3) provides the results of PEGylation process using various concentrations of PEG; 1%, 5% and 10%. The resulted PEGylated CSNP have had various sizes, PDI and zeta potential values. The surprising observation about the PEGylated CSNP is that their sizes were found to be smaller than those free non-PEGylated CSNP by 8.7%, 12% and 9.8% respectively. This can be demonstrated by the effect of PEG surface brushes acting as colloidal stabilizer on CSNP surfaces. Moreover, the polymeric layer of PEG has provided “stearic stability” upon conjugation to the surface of CSNP. This has affected their size, PDI and surface zeta potential values (181).

PEG forms an internal structural network with chitosan which is known as semi interpenetrating network (IPN) (112). This network has been proposed to be formulated during crosslinking of chitosan. It can increase the stabilization and compactness of the surfaces of CSNP. Therefore,
their sizes and surfaces charges decrease after PEGylation. Besides, zeta potential value of CSNP has decreased because of the attribution of blocking surface terminal \( \text{NH}_2^+ \) groups of chitosan and, thereby, decreasing their zeta potential values (182).

The recommended PEG surface coverage with PEG conjugation has been attributed to 5% PEG concentration. The resulted nanoparticles have had lower size than 1% and 10% PEG concentrations. Besides, the particles distribution and surface charge values of the selected PEG concentration have pointed out its tendency to be stable. Assuming that further increase in PEG concentration can destabilize the nanoparticles due to the repulsive behaviors of the neighboring PEG chains (183). Further stability profile of the three PEG concentrations has been conducted in the current study as illustrated in Figure (3.4). PEGylated CSNP that have 5% PEG has been the most stable as referred by the blue arrow. While those with 10% PEG and 1% PEG were less stable.

![Figure 3.4: stability profile of PEGylated and non-Pegylated CSNP](image)

Basically, it is very rational to give high concern to surface stability issue of the PEGylated CSNP. Verreechia et al. has indicated its pharmacokinetic preference towards PEGylated CSNP with
stable surface conjugated PEG. The study has reported that PEGylated CSNP have higher blood plasma level with lower hepatic excretion rates compared to non-PEGylated ones (184).

The developed system in the current work tend to be positively charged, that aims to enhance its later electrostatic interaction to further tumor cells in the coming stages of the study. Generally, positive nano-carrier systems are not biologically favored, because they are easy to be captured by phagocytes and RES. Besides, they initiate electrostatic interaction with negative blood proteins (185). Furthermore, thrombogenic embolization and blood hemolysis are two adverse reactions that are expected to be initiated by positive systems.

Therefore, grafting PEG can promote the biological safety and circulatory retention time of CSNP without casing such toxic adverse blood reactions. Furthermore, PEGylation improves the uniqueness of CSNP as an ideal nano carrier for later chemotherapeutic DOX encapsulation process. For instance, Clin et al. has reported higher liposomal accumulation of PEGylated DOX liposomes within a tumor area than non-PEGylated ones (186). This may affect the cancerous cellular drug uptake.
The conjugation reaction or PEGylation of CSNP could be confirmed through XRD patterns of CS, CSNP, PEG, PEGylated CSNP as shown in Figure (3.5). Basically, this conjugation has been formulated between the amino group NH$_2^+$ group from chitosan and C-O-C of PEG (187). This is in agreement with Calvo et al. that assumes, also, inter and intra molecular H-bonding between CS and PEG (112). The patterns show some representative peaks which indicates the crystalline behaviors of the tested structures. Chitosan powder, for instance, has two characteristic peaks at the angles of 21° and 10° as shown in the diffractogram, this demonstrated a high degree of crystallinity (188).

On the other side, the diffractogram of CSNP shows no peaks, which is an indication of the destroyed crystalline structure of chitosan by TPP entry. This is related to the amorphous structure of CSNP which is composed of dense of cross linked counterions (188). In the diffractogram of
PEGylated CSNP, the peak at $2\theta$ of 10° has been diminished. This is related to the decreased crystallinity of CSNP by the entry of PEG. The pattern of PEG has surpassed that of chitosan with its characteristic peaks at 19.12° and 23.1°, and this resulted in peaks shifting (189). Therefore, there is a good evidence for the efficient PEGylation of CSNP.

CSNP could maintain their spherical morphological shape after grafting of PEG on their surfaces. The PEGylation layer have demonstrated a brush like structure that surrounded CSNP. This has been confirmed with TEM pictures of both PEGylated; Figure (3.2 B and D) and non-PEGylated CSNP; Figure (3.6 A and C).
3.3. Encapsulation of Doxorubicin:

Doxorubicin has been selected as effective chemotherapeutic agent against all different types of breast cancer including both solid and soft tumors (190). DOX has been encapsulated successfully within the PEGylated CSNP via a pre-crosslinking entrapment. In other words, DOX has been introduced into CS-PEG solution before drop wise addition of TPP solution. Achieving good encapsulation with lower drug leakage has been fairly expected by the presence of PEG stabilizer. This enhances initiation of intramolecular hydrogen bonding between DOX and the reacting polymers (191).

Slight mixing has been required besides pH adjustment from 5 to 6.5. This is assumed to enhance the entrapment of DOX inside the polymeric matrix. Both factors; the deprotonation $pKa$ of chitosan at 6.6, besides the hydrophilic nature of DOX promote more intracellular hydrogen interactions (192). Coinciding with that, PEG brushes are being maintained in order for enhancing the system biocompatibility (193). Non-covalent interaction is therapeutically more preferred than covalent entrapment (194). Covalent interaction in a biological drug delivery system is more difficult to manage or predict.

3.3.1. DOX Standard Curve:

Experimentally, variable concentrations of DOX have been fed in order to evaluate the highest loading and entrapment content of DOX; 1.25 µg/mL, 2.5 µg/mL, 5 µg/mL and 10 µg/mL. This has required a prior quantification of DOX, that has been issued by plotting its calibration curve.
UV-VIS spectrophotometer has been used to detect the absorbance values of the prepared known concentrations of DOX. The plotted concentrations have been as following; 1 µg/mL, 10 µg/mL, 30 µg/mL, 50 µg/mL, 70 µg/mL and 90 µg/mL. The linear pattern of the graph has been translated into a fitting regression equation as shown in Figure (3.3) and Eq 3.2.

\[ Y = 8.05 X - 0.0245 \] \hspace{1cm} \text{Eq. (3.2)}

![Graph showing standard calibration curve of DOX in PBS](image)

**Figure 3.7**: standard calibration curve of DOX in PBS

### 3.3.2. DOX Loading Capacity (LC%) and Encapsulation Efficiency (EE%):

On the other hand, the resulted DOX loaded PEGylated CSNP have been analyzed with DLS in order for detecting the modification effect of DOX entrapment on the size, PDI and zeta potential values of the nanoparticles. Table (3.4) shows the results of all prepared formulations. The sizes of the resulted formulations have been slightly smaller than the PEGylated CSNP. Besides, the surface net charge values have been slightly lower also. Based on this two information, this slight change in size and surface charge may be attributed to the entrapped amount of DOX (97).
Table 3.4: DLS analysis of DOX loaded and unloaded CSNP

<table>
<thead>
<tr>
<th>Nano-formulation</th>
<th>Size</th>
<th>PDI</th>
<th>Zeta Pot.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSNP</td>
<td>195.4 ± 3.8</td>
<td>0.22 ± 0.005</td>
<td>+42.5 mV ± 1.3</td>
</tr>
<tr>
<td>CSNP - PEG</td>
<td>188.6 ± 0.5</td>
<td>0.19 ± 0.002</td>
<td>+38.4 mV ± 3.8</td>
</tr>
<tr>
<td>CSNP - PEG - DOX 1.25 µg/ml</td>
<td>177.8 ± 2.6</td>
<td>0.18 ± 0.002</td>
<td>+35 mV ± 1.6</td>
</tr>
<tr>
<td>CSNP-PEG - DOX 2.45 µg/ml</td>
<td>180.6 ± 4.3</td>
<td>0.20 ± 0.008</td>
<td>+33.4 mV ± 0.9</td>
</tr>
<tr>
<td>CSNP-PEG – DOX 5 µg/ml</td>
<td>178.5 ± 4.7</td>
<td>0.21 ± 0.006</td>
<td>+34.6 mV ± 2.6</td>
</tr>
<tr>
<td>CSNP-PEG – DOX 10 µg/ml</td>
<td>176 ± 5.7</td>
<td>0.22 ± 0.005</td>
<td>+33.6 mV ± 3.59</td>
</tr>
</tbody>
</table>

The LC% and EE% are two main important parameters that define the efficacy of the entire drug delivery nano-carrier system. The results are given in averages of triplicate experimented values. As showed in Table (3.5), the LC% and EE% are independent from each other. They continued to increase gradually with increasing the fed DOX concentration, till certain concentration (10 µg/ml) then decreased. At this point the internal chitosan network has been saturated with DOX, besides, the drug has reached its saturation solubility (102). Therefore, higher drug concentrations are expected to be eluted in the loading supernatant media (196).

<table>
<thead>
<tr>
<th>Drug formula</th>
<th>LC%</th>
<th>EE%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.25 µg/ml</td>
<td>0.033 %</td>
<td>71.44 %</td>
</tr>
<tr>
<td>2.45 µg/ml</td>
<td>0.08 %</td>
<td>77.5 %</td>
</tr>
<tr>
<td>5 µg/ml</td>
<td>0.114 %</td>
<td>80.4 %</td>
</tr>
<tr>
<td>10 µg/ml</td>
<td>0.09 %</td>
<td>78.5 %</td>
</tr>
</tbody>
</table>

The potentials of PEGylated CSNP to load such DOX concentrations are proposed to be dependent on its internal spacing structure besides capillary forces (197). When the initial fed DOX, concentrations have been compared to the calculated loaded amounts, 5 Ug/ml concentration has been selected as the highest LC% and EE%, for the following experiments (101, 198). Furthermore, the cell viability tests have confirmed the lowest viable rate of breast tumor cells (MCF-7) with 24 hours incubation after ingestion of 5 µg/ml nano-drug formulation using 96 well plate. This will be discussed in more details in cell cytotoxicity part. The selected sample of DOX conjugated to CSNP has been confirmed with spherical morphology through TEM images as shown in Figure (3.8).
3.3.3. UV-VIS spectrum of DOX loaded PEGylated CSNP:
In order to confirm the efficiency of the prepared nano-system to effectively encapsulate DOX, the UV-Vis spectra of free DOX, unloaded CSNP and DOX loaded PEGylated CSNP are shown in Figure (3.9). The nano particulate samples have been incubated in slightly acidic pH media for 24 hours in the ambient conditions before centrifuging. Then, the resultant supernatant has been matched to the UV spectrum of free DOX which has a characteristic absorption peak at wavelength of (481 nm) (199). The unloaded CSNP have revealed a broad absorption band at the area of 200-300 nm. This band is thought to be related to C=O group.

Figure 3.8: TEM images of DOX loaded PEGylated CSNP (A, B), and schematic diagram of DOX loaded PEGylated CSNP
Figure 3.9: UV-Vis spectra of DOX loaded CSNP, non-loaded CSNP and free DOX

While the supernatant of DOX loaded PEGylated CSNP showed stronger peak on the spectrum with a characteristic peak at 480 nm wavelength, which is thought to be related to DOX. In fact, CSNP has a characteristic swelling behavior that can be generally noted in acidic pH media. That’s because the amino group related pKa value of chitosan is around 6.5. Therefore, the crosslinked framework of CSNP start degrading in this acidic pH. The protonated NH$_2$ groups of CSNP in acidic pH enhances its solubility. This can improve the release of the entrapped DOX (200).

3.3.4. FTIR Spectroscopy analysis:

FTIR spectroscopy tests of CS powder, CSNP, DOX, PEGylated CSNP and DOX loaded PEGylated CSNP are shown in Figure (3.10). In chitosan powder, broad and strong peak at 3446 cm$^{-1}$ might be related to O-H stretching mode. This peak has been shifted to lower wavenumber at 3422.3 cm$^{-1}$ and 3411 cm$^{-1}$ in CSNP and PEGylated CSNP respectively. This probably means that the more polymeric cross linkage with TPP and PEG, the lower peak shifting wavenumber. This refer to the enhancement of H-bonding in the two nano formulations (201). Therefore, the bonds in PEGylated CSNP is stronger than those in CSNP (96).
The peak at 1600 cm\(^{-1}\) in chitosan represents bending vibration amide I bond N-H. In CSNP, it has been shifted to 1540 cm\(^{-1}\). Likewise, the peak at 1650 cm\(^{-1}\) amide II stretching in chitosan powder has been shifted to 1612 cm\(^{-1}\) in nanoparticulate systems. This supports the assumption of successful TPP crosslinking of CSNP (202). Amide III bond peak of N-O has appeared in PEGylated CSNP at 1380 cm\(^{-1}\) region. (131). Furthermore, a characteristic P=O peak has been observed at 1155 cm\(^{-1}\) region of the formulated nanoparticulate systems, this peak can be related to the linkage between NH\(_2\) of chitosan and phosphoric of TPP.

A peak at 2888.3 cm\(^{-1}\) wavenumber has appeared in PEGylated CSNP can be attributed to -CH\(_2\) stretching vibration. This may enhance the assumption of successful PEGylation of CSNP (204). Another PEGylation specific peak is vibrational C-O-C peak that appeared with high intensity at 1101 cm\(^{-1}\) (205). After introduction of DOX to the system, two characteristic peaks at 1590 cm\(^{-1}\) and 1651 cm\(^{-1}\) have appeared in the IR spectrum compared to the spectrum of free DOX. These two peaks are assumed to be related to stretching C=C of DOX inside the PEGylated CSNP matrix. Moreover, OH stretching band has been shifted to 3430 cm\(^{-1}\) with more intensity and broader
appearance, which may represent increased strength of its hydrogen bonding $. Another peak at 1021.3 in PEGylated DOX loaded CSNP may refer to C—O (25).

3.3.5. Swelling Studies of DOX loaded PEGylated CSNP

After 24 hours of incubation, the samples have been weighed and their swelling indices have been determined in two different pH media; 6.6 and 7.4. DOX loaded PEGylated CSNP have had higher tendency for swelling at acidic 6.5 pH media than slightly neutral 7.4 pH. Swelling indices at 6.6 pH and 7.4 pH were 62.5 % and 7.89 % respectively. The difference in swelling behavior has been investigated, also, with a TEM image as shown in Figure (3.11). The nanoparticles in 7.4 pH could maintain their polymeric spherical shapes as referred by the blue arrows. While in 6.6 pH, the polymeric matrix of the nanoparticles has been destroyed (206).

![Figure 3.11: TEM images of DOX loaded PEGylated CSNP at pH 7.4 (a), pH 6.5 (b) after 24 hrs incubation and schematic illustration of the difference between non-swelled (pH7.4) and swelled nanoparticles (pH 6.6) (c)
There is a relationship between having a high swelling rate and the release of the entrapped drug from CSNP, which may be due to the pores formed in the matrix network of the nanosystems. Swelling has been shown to be affected by some parameters such as temperature, swelling time, crosslinking degree, and pH. By maintaining all these parameters fixed and changing the pH of the media, the effect of pH has been demonstrated. This variation between pH 6.6 and pH 7.4 may be related to the hydrophilicity of CSNP and its polymeric chains. The presence of some active groups such as —COOH, —OH and NH$_2$— may have another contribution (206).

Besides, the protonation of chitosan (pKa 6.6) at lower pH levels is increasing, and its positive amino groups induce its swelling. While at higher pH levels, chitosan is less protonated and uncharged, thus its swelling and solubility decrease (207). Another rationale is that, in higher pH levels, water tends to form hydrogen bonds with functional active groups such as —COOH, —OH and NH$_2$— groups. These H-bonds tend to fill the spaces inside the nano matrix and, hence, minimize swelling. Furthermore, the rheological behavior of DOX loaded CSNP and sizes of counter anions at these pH levels are participating in this swelling behavior (208). In conclusion, DOX loaded CSNP demonstrates a pH dependent swelling, and this has a direct effect on the release of the entrapped drug.

3.3.6. *In-vitro* Release Profile:

![Figure 3.12: DOX release profile of PEGylated DOX loaded CSNP at pH 6.6 and pH 7.4](image)

Figure 3.12: DOX release profile of PEGylated DOX loaded CSNP at pH 6.6 and pH 7.4
The *in-vitro* release behavior in our study has exhibited a selective pH dependent DOX release rate. This has been investigated with two aforementioned pH levels; 7.4 and 6.6, that mimic the physiological and cancerous conditions at 37°C as shown in Figure (3.12). The retention of DOX inside PEGylated CSNP should be equilibrated between its stability in normal noncancerous environment versus its targeted release at cancerous acidic conditions (209). In nanosystems, the release profile can be demonstrated in three main phases; desorption, swelling then erosion as shown in Figure (3.13). The *In-vitro* release behavior of DOX loaded nano-system in acidic 6.6 pH has showed basic burst release that has been followed by controlled release of DOX. The burst release has been related to the diffusion of DOX out of the nano matrices. This is known as burst effect that entails a combination of polymeric CSNP degradation and drug desorption (210).

![Illustrative diagram of DOX release mechanism](image)

**Figure 3.13**: Illustrative diagram of DOX release mechanism

The burst release of DOX has been identified during the first 3 hours with around 45% at pH 6.6 as shown in Figure (3.12). While less than 14% of the drug has been released at the pH 7.4. This can be a good predicting indication of the polymeric stability in normal blood pH compared to acidic tumor environment. This can be related to the pH media in a way that the protonation of the amino NH$_2$ groups of both CS and DOX is increasing in acidic media compared to neutral one. Such protonation can be a main causative factor for the polymeric diffusion and erosion as we
mentioned before. After 3 hours of burst drug release, the drug has exhibited a slow sustained release over 72 hours incubation time. This controlled release may be related to simultaneous occurrence of minute molecules diffusion and the degradation of the remaining polymeric structures (97). Our results suggest that DOX release is affected with pH level of the ingestion media. The drug release rate has decreased by increasing the processing pH of the media. The breast tumor environment is acidic because of the high anerobic glycolysis that enhance the acidity levels. Besides, the endocytosis uptake pathway of DOX loaded PEGylated CSNP is expected at such slightly acidic pH levels; however, it has been reported that its release decreases with extremely acidic conditions (211). The massive oxidative stress at the tumor area enhances such extreme acidity. Therefore, it is assumed that the tumor pH is a preferable media for the nanoparticles to release the entrapped DOX compared to normal physiological conditions.

3.3.7. *In-vitro* release kinetics:

The releasing kinetics of DOX loaded PEGylated CSNP have been demonstrated in order to predict physiological performance of the released drug. The relationship between the drug concentration and release rate has been described with different mathematical models; Zero-order, First order, Hixson crowell, Higuchi and Korsemeyer Peppas (212). According to the results, DOX release may fit with Higuch model at the acidic 6.6 pH. This system hypothesized that DOX particles are smaller than the nano-system thickness. Additionally, this model entails that the release rate is related to the pH and the nano carrier matrix has a good solubility rate. On the other side, at pH 7.4, the release rate follows Korsemeyer Peppas model that assume one dimensional drug release relative to the drug dosage form (213).

<table>
<thead>
<tr>
<th>pH</th>
<th>Zero order</th>
<th>First order</th>
<th>Hixson Crowell</th>
<th>Higuchi</th>
<th>Korsemeyer Peppas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K</td>
<td>r2</td>
<td>K</td>
<td>r2</td>
<td>K</td>
</tr>
<tr>
<td>6.6</td>
<td>0.253</td>
<td>0.859</td>
<td>0.004</td>
<td>0.843</td>
<td>0.005</td>
</tr>
<tr>
<td>7.4</td>
<td>0.173</td>
<td>0.767</td>
<td>0.007</td>
<td>0.694</td>
<td>0.007</td>
</tr>
</tbody>
</table>

Table 3.6: Release kinetics of the released DOX according to different systems equations
3.4. Functionalization of Monoclonal Antibodies:

The attachment of mAb to the surface of DOX loaded PEGylated CSNP has been suggested for enhancing the intracellular uptake and endocytosis. This may be achieved through specific surface receptors attachment of mAb to cancer cells. The functionalization of Anti-HER2 (Anti Human Epidermal Growth Factor II) mAb and Anti-hMAM (Anti Human Mammaglobin) mAb to the surfaces of the nanoparticles has slightly increased their sizes as shown by the TEM images in Figure (3.14) and (3.15). Each mAb has been immobilized to the surface of the nanoparticles separately by EDC crosslinking. Supported activation of COOH terminal groups on the PEGylated surfaces of DOX loaded CSNP has prepared the surface of these nanoparticles for further attachments of the mAbs. In other words, this may facilitate the entry of mAb for successful surface conjugation. Therefore, ratio between EDC to the mAbs was 5:1. This may increase the opportunities of surface satisfaction of the nanoparticles with the antibodies separately. This could be confirmed with FTIR spectrum. Furthermore, the size of the nanoparticles is greater than the mAbs, therefore the ratio of nanoparticles to the mAbs was high (214).

The presence of PEG layer besides a stronger EDC supported linkage, may together help the nanocarriers to proceed their way without detachment of the surface mAbs (146). This is from a
biological point of view. Furthermore, high consideration has been given to the use of PBS for frequent washing in order to eliminate the presence of the unfavorable attached EDC molecules. This may grasp further circulating molecules (216).

In order to explore the surface chemistry of the particles surfaces that are functionalized with mAbs, $^1$H NMR spectrum has been conducted as shown in Figure (3.16). Peaks at 2.4, 2.6 and 2.7 ppm are responding of H protons of CH$_2$ groups of mAb functionalized nanoparticles. Besides, the appearance of triplicate peaks at 3.2 and 3.3 ppm are representing amino NH$_2$ groups, which may

![Figure 3.15: TEM images of Anti-hMAM DOX loaded PEGylated CSNP (A, B) and Anti-HER2 DOX loaded PEGylated CSNP (C, D)](image)
confirm the conjugation of the antibodies to the PEGylated surface. The increased amount of nitrogen within the surface of nanoparticles can be illustrated with the increased immobilization of the mAbs. Moreover, FTIR spectrum has been conducted as well, and it confirm this assumption of surface coverage with mAbs related NH$_2$ groups (217).

**Figure 3.16:** HNMR spectrum of Anti-hMAM / DOX loaded PEGylated CSNP (A) and Anti-HER2 / DOX loaded PEGylated CSNP (B)
The FTIR spectrum of Anti-HER2 DOX loaded PEGylated CSNP and Anti-hMAM DOX loaded PEGylated CSNP has been explored to define the change in the absorption peaks relative the non-functionalized DOX loaded PEGylated CSNP. This is shown in Figure (3.17). The shifting of OH peak from 3430 cm\(^{-1}\) to 3300 is an indication of the pronounced COOH group to the surface of the CSNP. Moreover, the increased intensity of the peak at 1600 cm\(^{-1}\) can be attributed to the increased surface coverage with NH\(_2\) groups. These groups may be related to the immobilized mAbs (216). The disappearance of the characteristic peak of PEG at 1094 cm\(^{-1}\) indicates, also, the conjugation of the mAbs to the surfaces.

![FTIR spectrum of mAbs/DOX loaded PEGylated CSNP and non-functionalized DOX loaded PEGylated CSNPs](image)

**Figure 3.17:** FTIR spectrum of mAbs/DOX loaded PEGylated CSNP and non-functionalized DOX loaded PEGylated CSNPs

### 3.5. Cytotoxicity Analysis:

The cytotoxic effect of different nano-formulated systems on MCF7 cells have been more prominent than various free DOX drug concentrations. L929 cells were less responsive to the different nano-formulations as shown in Figure (3.18) (102).
Figure 3.18: Colorimetric MTT assay of DOX loaded PEGylated CSNP (1.25, 2.5, 5 and 10 Ug/ml) (A), Free DOX concentrations (1.25, 2.5, 5 and 1 Ug/ml) (B) and Anti-HER2 and Anti-hMAM functionalized DOX loaded PEGylated CSNP.
Various nano-formulations have been screened including unloaded CSNP, PEGylated CSNP, DOX loaded PEGylated CSNP (variable concentrations), free DOX, Anti-HER2 functionalized PEGylated DOX loaded CSNP and Anti hMAM functionalized PEGylated DOX loaded CSNP. The cytotoxicity results suggest that nanoformulated DOX systems have shown greater toxicity on cancerous MCF7 cells. As shown in Figure (3.14A), PEGylated DOX loaded CSNP with drug concentrations of 1.25, 2.5, 5 and 10 µg/ml could achieve MCF7 cell viability rates of 24.7± 4.2 %, 18.66 ±2.3%, 14.98± 1.5% and 39.44± 3.1% respectively. Normal L929 cells have been less affected by these nano-formulated drug concentrations and less responsive to the released drug, although both types of cells had identical same incubation conditions of time, type of media and drug interval concentrations. The released drug could achieve successful endocytosis in case of MCF7 and killed more than 50% of the seeded cells. (130).

The nanoparticles encapsulating 5 µg/ml DOX has shown the highest cytotoxicity effect with 14.89% viable MCF7 cells. Le et al. has reported the minimal cytotoxic effect of the released DOX when the drug concentration is increasing than proposed concentrations in this study (218). On the other hand, the effect of the same 5Ug/ml nano-DOX formula on normal L929 has been the least compared with other concentrations. This may put some confirmatory assets on its therapeutic durability and biological safety (102).

Free DOX concentrations have demonstrated higher viability rates of MCF7 cells with 30 ± 1.98%, 34 ± 1.45%, 38 ± 4.31% and 45 ± 2.21% viability rates for similar free drug concentrations of the nano formulated DOX. Therefore, their cytotoxic effect on cancerous cells is lower than nano DOX formulations. We have demonstrated their partial dose dependent cytotoxicity on MCF7 and L929. The most efficient free DOX concentration could revel around 30 ± 1.98 % viability rate. While in nano formulated DOX, the lowest viability rate has been 14.89 ± 1.5%. This suggests a good indication that our PEGylated CSNP system has improved the cellular uptake and intracellular delivery of DOX with one-time higher rate, under the same exposure time relative to free DOX (53). Confirmatory bright filed images of MCF7 and L929 after exposure to different drug formulations are shown in Figures (3.19) -(3.22).
Figure 3.19: Bright field images of L929 cells with white arrows referring to living cells (A), and MCF7 cells with white arrows referring to dead cells (B) after exposure to DOX–PEGylated CSNP (0.5 µg/ml)
Figure 3.20: Bright field images of L929 cells with white arrows referring to dead cells (A), and MCF7 cells with white arrows referring to living cells (B), after exposure to Free DOX
Figure 3.21: Bright field images of L929 cell line with white arrows referring to living cells (A), and MCF7 cell line with white arrows referring to dead cells (B) cells after exposure to Anti-HER2 functionalized DOX loaded PEGylated CSNP
Figure 3.22: Bright field images of L929 cells with white arrows referring to living cells (A) and MCF7 with white arrows referring to dead cells (B) cells after exposure to Anti-hMAM functionalized DOX loaded PEGylated CSNP
Functionalization of monoclonal antibodies to the surfaces of DOX loaded PEGylated CSNP as a targeting moiety was assumed to be a productive therapeutic conjugate in terms of cancer cell cytotoxicity. This has empowered the cellular uptake and cell viability rates of these nano formulations. In this sense, Anti HER2 and Anti hMAM antibodies have been used for functionalization of DOX loaded PEGylated CSNP separately. This could enhance the tumor cell susceptibility and higher intracellular accumulation of the released DOX. Our results have showed the potential of the nano formulated DOX functionalized with these monoclonal antibodies to increase its cytotoxic effect in MCF7 three times as free DOX, while maintaining higher viability rates of normal L929 cells (131).

Anti-HER2 and Anti-hMAM functionalized nano formulations have shown MCF7 viability rates of 1.79 ± 0.06% and 2.99 ± 0.023% respectively. While Normal L929 have been less responsive and less affected by the same formulations. This may be a good marking point that MCF7 are more affected by these antibodies functionalized nano formulations, while normal L929 cells are less susceptible and less affected. Specific antigen-antibody mediated drug accumulation my developed more efficient cellular endocytosis and, hence, higher drug efficacy on MCF7. On the other hand, L929 normal cells lack such tumor cell specific receptors like HER2, for instance. Therefore, such intracellular drug uptake is not expected, and receptor mediated coupling mechanism hiders drug release or accumulation with normal cells (219). The bright field images of the cells after identical exposure to antibodies functionalized DOX loaded PEGylated CSNP have demonstrated the viability profile. The difference in cellular shape and uniformity responses of both types of cells may exhibit their different susceptibilities.

The MTT test has been performed in CO₂ incubator under processing temperature of 37 °C. The temperature has been proposed to affect the intracellular action of DOX and affect its diffusion. In this sense, Miller’s law of diffusion entails a strong relationship between the temperature and drug diffusion rates (220). Therefore, the diffusion and cytotoxicity profiles of all our experimented formulations, including loaded and unloaded DOX, are less likely to be affected by this relationship. That’s because the experimental processing temperature was 37°C, which is higher than the intermolecular self-aggregation temperature of DOX. In this temperature, intermolecular
π–interaction may be formulated specially less than 4°C, which minimize the diffusion of DOX. However, our study has been performed at physiological 37°C temperature (221).

3.6. Size increment profile:

Over the different activities of our study, variable sizes of CSNP have been obtained. The size increment over all the activities didn’t exceed 10%. This is a good indication for the surface stability. The size increment after PEGylation of CSNP, encapsulation of DOX to PEGylated CSNP, functionalization of Anti-HER2 to DOX loaded PEGylated CSNP and Functionalization of Anti-hMAM to DOX loaded PEGylated CSNP have been 10%, 7.23%, 4.3% and 4.72% (222).

Figure 3.23: TEM images and the relevant DLS histograms of non-PEGylated CSNP (A, B) and PEGylated CSNP (C, D)
Figure 3.24: TEM images and their relevant DLS histograms of DOX loaded PEGylated CSNP (E, F), Anti-HER2 DOX loaded PEGylated CSNP (G, H), and Anti-hMAM DOX loaded PEGylated CSNP (I, J)
Appendix

(supporting Information)
Supporting Information:


SI. 1.1. Dissolving Chitosan:

Chitosan acidic solution has been prepared simply through dissolving LMW chitosan powder with different weights including 1 gm, 0.5 gm, 0.25 gm and 0.05 gm in 1% acetic acid. The pH has been adjusted to 5.0 using 0.1N NaOH. These prepared solutions have been left to be mixed and dissolve for 24 hours under magnetic stirring at 750 RPM within the ambient temperature. The resulted four solutions have showed different viscosities and some dissolved traces have been removed out using 0.4 Um syringe filter mesh. All preparation parameters and conditions have been controlled within this step-in order to obtain homogenous chitosan acidic solutions. The stirring beakers have been covered with protective par-film sheets in order for avoiding the entrance of any contaminants, and suitable sizes of magnetic bars have been used for the mixing process.

SI. 1.2. Dissolving Sodium tri-polyphosphate:

Sodium tri-polyphosphate is a chemical cross linker that has been used in this work in order to induce the ionic gelation process through which, chitosan nanoparticles would be formulated. TPP has been dissolved in purified distilled water within four different concentrations; 0.1%, 0.4%, 0.5% and 0.7%. Suitable sizes of magnetic bars have been used for stirring process. The solutions have been rapidly mixed within 35 minutes at 500 RPM within the ambient conditions.

SI. 1.3. Preparation of Crosslinked chitosan nanoparticles:

After formation of homogenous chitosan and TPP solutions, they have been mixed together. Whitish clouds of chitosan nanoparticles have been spontaneously formed upon addition of the two polymers together. In order for separating these nanoparticles, we have applied efficient centrifuging forces. Therefore, the reacted polymers part, that formulated crosslinked chitosan nanoparticles, could be separated from the unreacted eluted polymers. The resulted pellet of
chitosan nanoparticles has been used for further investigative characterization techniques. For this purpose, the pellet has been divided into three sections as shown in figure SI. 1 and table SI. 1.

Table SI. 1: variable conditions for characterizing chitosan nanoparticles

<table>
<thead>
<tr>
<th>Nanoparticles pellet</th>
<th>Action</th>
<th>Characterization Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>First part (minor)</td>
<td>Oven drying</td>
<td>Scanning electron microscopy (SEM),</td>
</tr>
<tr>
<td>Second part (moderate)</td>
<td>Sonication + dilution (distilled water)</td>
<td>Dynamic light scattering (DLS), Ultraviolet-Visible spectrophotometry (UV-VIS) and Transmission electron microscopy (TEM)</td>
</tr>
<tr>
<td>Third part (major)</td>
<td>Freeze drying</td>
<td>Fourier transmission infra-red spectroscopy (FTIR), X-ray Diffraction (XRD)</td>
</tr>
</tbody>
</table>

Figure SI. 1: Divided pellet undergoes further investigation techniques with a prior sample preparation technique such as oven drying, freeze drying and resuspending in distilled water.

SI. 2. Factors affecting chitosan nanoparticles:

The morphological chemical properties of chitosan nanoparticles are significantly affected with various parameters and factors. This is a part of the reason of why chitosan is a remarkable biopolymer and possess attractive advantages for various biological and non-biological applications. Changing some parameters such as polymers concentration, mixing rate and mixing time can obviously change the size and shape of the formulated nanoparticles. It has a simple
analogy like tailoring a specific nano-carrier or nanosystem with providing certain specific resulted features and properties, by playing with these stated parameters.

**SI. 2.1. Effect of polymers concentrations:**

The particle size, shape, distribution and surface charge of the prepared chitosan nanoparticles can be influenced by the concentrations of chitosan and TPP. In order to investigate this influence, various concentrations of both polymers have been applied with keeping all other preparation parameters constant. Identical volumes of both polymer solutions have been applied, while their concentrations have been varied. Four different concentrations of chitosan acidic solutions have been used; 0.05%, 0.25%, 0.5% and 1% (w/v).

On the other hand, sodium tri-poly phosphate concentrations were 0.1%, 0.4%, 0.5% and 0.7% (w/v). Sixteen samples of whitish clouds of chitosan nanoparticles have been formulated up on drop wise addition of sodium tri-polyphosphate to chitosan acidic solutions with a dropping syringe. Based on the number of concentrations of both polymers, the optimization of the nanoparticles has taken place in a quadruple manner as shown in table 2.

**Table SI. 2:** General scheme for the applied polymers concentrations; Cs and TPP

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cs (w/v) %</th>
<th></th>
<th></th>
<th></th>
<th>TPP (w/v) %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.05</td>
<td>0.1</td>
<td>0.5</td>
<td>1</td>
<td>0.1</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The mixture of chitosan solution and TPP has been left for mixing on the magnetic stirring under the previously stated preparation parameters in terms of mild speed and time within protected ambient conditions. The resulted sixteen samples of chitosan nanoparticles have been visually analyzed as turbid suspensions of undissolved white clouds of nanoparticles. After using particles size analyzer which has been based on dynamic light scattering physical principle (Malvern Zetasizer®), sample 8 has been the sample of choice in terms of size and distribution.

### SI. 2.2. The effect of Stirring speed:

Another significant factor that has a direct influence on the resulted chitosan nanoparticles is related to the identified mixing technique. Stirring speed has been reported to directly affect the NP’s size and distribution (223). Although it has reported by Oliviera et. al., that stirring time and speed have low influence on the size and distribution of the formulated nanoparticles (224). We have investigated that this contradict with our study. Four main different stirring speeds have been investigated and analyzed to produce various sizes and distributions; (250, 500, 750 and 1000) RPM. All other parameters have been constant including polymers concentrations. The sample of choice, sample 8, has been investigated in these four different stirring speeds as shown in table 2.

<table>
<thead>
<tr>
<th>Stirring speed</th>
<th>Particle size</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>250 RPM</td>
<td>182 ± 5.3</td>
<td>0.37 ± 0.004</td>
</tr>
<tr>
<td>500 RPM</td>
<td>345.9 ± 1.3</td>
<td>0.517 ± 0.003</td>
</tr>
<tr>
<td>750 RPM</td>
<td>357.2 ± 4.45</td>
<td>0.512 ± 0.041</td>
</tr>
<tr>
<td>1000 RPM</td>
<td>391.4 ± 2.2</td>
<td>0.491 ± 0.007</td>
</tr>
</tbody>
</table>

### SI. 2.3. The effect of stirring time:
Stirring time is one of the main influential factors that affect the size and distribution uniformity of the formulated chitosan nanoparticles. In order to investigate that notion, we have performed this preparation procedure with keeping all other preparation factors constant. The polymers concentrations, stirring speeds and temperature have been kept constant with changing the performing stirring time. The influence of changing stirring time on particle size and distribution has been studied with three different time slots; (15, 30 and 60) minutes as shown in figure 3. According to the resulted smallest particles sizes and smallest PDI, the optimized procedure has been selected. (225, 226)

Table SI. 4: Variation of the stirring time provides variation of particles size and distribution.

<table>
<thead>
<tr>
<th>Stirring time</th>
<th>Particle size</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 min</td>
<td>245 ± 7.6</td>
<td>0.67 ± 0.004</td>
</tr>
<tr>
<td>30 min</td>
<td>182.2 ± 5.45</td>
<td>0.304 ± 0.009</td>
</tr>
<tr>
<td>60 min</td>
<td>209.3 ± 25.45</td>
<td>0.609 ± 0.082</td>
</tr>
</tbody>
</table>

SI. 2.4. The effect of chitosan molecular weight:

Molecular weight of raw chitosan powder is another effective factor that influence the size of the resulted chitosan nanoparticles (227). It has been reported by many studies that there is a direct relationship between the molecular weight of chitosan relative to particles size and distribution of the resulted chitosan nanoparticles (228). Considerably, the effect of varying the molecular weights of chitosan on the resulted nanoparticles has been studied with holding the initial chitosan and TPP constant. The molecular weights of chitosan have been changed within three different preparations; Low molecular weight chitosan (LMWC), medium molecular weight chitosan (MMWC) and high molecular weight chitosan (HMWC). However, all other preparation parameters and factors have been kept constant, and the optimized molecular weight has been selected based on the resulted particle size and distribution.

Table SI. 5: Variation of chitosan molecular weight provides variation of particles size and distribution
The external morphology mean sizes and uniform distribution of chitosan nanoparticles can be affected by different pH of chitosan solution. The relation between the crosslinking process of chitosan/TPP and changing the pH of chitosan solution has been reported by some studies (229). For investigating this effect, we have adjusted the pH of chitosan solution from 2.6 to 5.5 (2.6, 3.1, 3.5, 4.5, 5 and 5.5). The protonation degree of chitosan polymer can greatly control the crosslinking process of CSNP. On the other hand, this protonation degree can be affected with chitosan solution pH, therefore we need to investigate this factor and whether it needs to be controlled within specific range that doesn’t harm the intended physiological environment in which the nanoparticle would deliver certain drug or not.

### SI. 3. Stability of chitosan nanoparticles:
Throughout all the optimization procedure and manipulation of the different preparation factors of the resulted chitosan nanoparticles, we could identify the optimum procedure through which we can obtain chitosan nanoparticles with satisfying physical and chemical properties. The main characteristic features of the nano-system that we have investigated are particle size, poly distribution and zeta potential value as shown in table 6.

<table>
<thead>
<tr>
<th><strong>Chitosan Molecular weight</strong></th>
<th><strong>Degree of Deacetylation</strong></th>
<th><strong>particles size</strong></th>
<th><strong>PDI</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>LMWC (110)</td>
<td>89.9 %</td>
<td>187 ± 2.7</td>
<td>0.282 ± 0.015</td>
</tr>
<tr>
<td>MMWC (400)</td>
<td>84.8 %</td>
<td>357 ± 80.5</td>
<td>0.81 ± 0.14</td>
</tr>
<tr>
<td>HMWC (800)</td>
<td>76 %</td>
<td>444.2 ± 106.9</td>
<td>0.52 ± 0.07</td>
</tr>
</tbody>
</table>

### SI. 2.5. The effect of pH:

**Table SI. 6:** various processing pH provide variation of particles size and distribution

<table>
<thead>
<tr>
<th><strong>pH</strong></th>
<th><strong>Size</strong></th>
<th><strong>PDI</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>5.5</td>
<td>196 ± 4.1</td>
<td>0.42 ± 0.06</td>
</tr>
<tr>
<td>5</td>
<td>182 ± 3.7</td>
<td>0.22 ± 0.005</td>
</tr>
<tr>
<td>4.5</td>
<td>180.8 ± 4.4</td>
<td>0.34 ± 0.003</td>
</tr>
<tr>
<td>3.5</td>
<td>162 ± 4.6</td>
<td>0.35 ± 0.008</td>
</tr>
<tr>
<td>3.1</td>
<td>147 ± 1.9</td>
<td>0.41 ± 0.05</td>
</tr>
<tr>
<td>2.6</td>
<td>136 ± 6.1</td>
<td>0.47 ± 0.005</td>
</tr>
</tbody>
</table>
Table SI. 7: Stability profile for the prepared chitosan nanoparticles along over three weeks

<table>
<thead>
<tr>
<th>Time</th>
<th>Particle size</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 day</td>
<td>183.7 ± 5.8</td>
<td>0.20</td>
</tr>
<tr>
<td>2 days</td>
<td>187 ± 3.9</td>
<td>0.192</td>
</tr>
<tr>
<td>4 days</td>
<td>187.6 ± 4.6</td>
<td>0.223</td>
</tr>
<tr>
<td>6 days</td>
<td>195 ± 2.12</td>
<td>0.253</td>
</tr>
<tr>
<td>8 days</td>
<td>198.5 ± 3.5</td>
<td>0.262</td>
</tr>
<tr>
<td>10 days</td>
<td>193.4 ± 6.1</td>
<td>0.292</td>
</tr>
<tr>
<td>12 days</td>
<td>184.5 ± 5.7</td>
<td>0.239</td>
</tr>
<tr>
<td>14 days</td>
<td>192.2 ± 8.4</td>
<td>0.209</td>
</tr>
<tr>
<td>16 days</td>
<td>200.1 ± 2.1</td>
<td>0.190</td>
</tr>
<tr>
<td>18 days</td>
<td>196 ± 3.4</td>
<td>0.242</td>
</tr>
<tr>
<td>20 days</td>
<td>204.4 ± 4.6</td>
<td>0.240</td>
</tr>
<tr>
<td>22 days</td>
<td>201.1 ± 5.2</td>
<td>0.189</td>
</tr>
</tbody>
</table>

Zeta potential is an indicative parameter to the surface charge and hence the aggregation tendency or size stability in the intended suspending medium as shown in table 7 (230). This is a very important parameter whenever the formulated nano-system would be intended to be injected in a biological tissue within biomedical applications. In other words, the size stability of the in-vivo colloidal nanoparticles system can be assessed with zeta potential value.

Table SI. 8: Various ranges of Zeta potential values with their corresponding aggregation tendencies (231)

<table>
<thead>
<tr>
<th>Zeta potential value</th>
<th>Stability profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 - ±5</td>
<td>Rapid aggregation</td>
</tr>
<tr>
<td>±10 - ±30</td>
<td>Low stability</td>
</tr>
<tr>
<td>±30 - ±40</td>
<td>Moderate stability</td>
</tr>
<tr>
<td>±40 - ±60</td>
<td>High stability</td>
</tr>
<tr>
<td>≥ ±60</td>
<td>Excellent stability</td>
</tr>
</tbody>
</table>
SI. 4. Theoretical and Instrumental background:

SI. 4.1. Ultrasonic Probe Sonicator (Polytron, Thermo-fisher):

Ultrasonic probe sonicator is an instrument that is used for mixing and extraction of various chemical or polymeric compounds. This device is working by generating powerful energy in the form of sound waves that are enough to agitate or suspend micro and nano sized particles in a solution (232). These sound waves are ultrasonic waves (frequency > 20 kHz). They provide rapid and violent vibration of the probe tip, and this creates a kind of cavitation that collapse within the solution or medium as shown in (Figure 2).

![Compression Waves](image)

**Figure SI. 2:** controlled compression and expansion energy that bring particles to larger sizes which later breakdown into much smaller ones (232)

This cavitation intensity is controlled by the setting agitation time and amplitude settings. It creates what is called cavitation bubbles that grow in size until it reaches a critical size that can aggressively disrupt the intermolecular covalent bonding of the applied medium as shown in (figure 2). Therefore, the greater the frequency, the stronger the waves, the higher cavitation intensity and hence the stronger particles agitation. It has been recommended by many studies that ultrasonic probe sonicator is a recommended high-speed mixing technique and put it as the first technique of choice for mixing (233)
Ultrasonic probe sonicator is mainly composed of three main parts; generator, converter and probe tip (figure 3). Generator is the processing unite through which, we can control the sonication parameters. It provides high voltage pulses of electrical energy that is produced basically form normal alternating current and convert into such powerful pulses through that generator unite (234).

Converter is a piezoelectric unite of the ultrasonic instrument. It is typically controlled through the high voltage pulses that are created by the generator. These pulses provide such enough powerful energy at a certain un-fluctuated frequency of 20 kHz. The main function of the converter is to convert the produced electrical energy into mechanical energy. Therefor there is a high voltage cable that connect between the converter and the generator. The outer shape of converter, as shown in figure X, is cylindrical. The third part of the ultrasonic sonicator is the probe or horn. This the instrumental part of the device the is put in direct contact to the applied sample. It has threaded ends whose longitudinal tip expands and contract during processing (235).

SI. 4.2. Freeze drying (BIOBASE, Jinan, China):

Freeze drying is a dehydration process or removal of solvent and it is called also lyophilization. It is a frequently used technique in many pharmaceutical industries, different scientific laboratories and food industries. Basically, freeze drying is applied for drying heat labile samples so that it becomes more stable in a powdered form without presence of moisture any solvent after being frozen (236). Samples like proteins, tissues, plasma, dead cells and pharmaceuticals are extremely
heat labile and can’t be dried by oven drying technique, therefore we can use freeze drying for keeping them in more a stable state. The basic scientific concept behind freeze drying is related to sublimation phenomenon. This phenomenon states that solid iced material can undergo phase transition to become gaseous vapor without being exposed to the liquid state. This occurs by applying low pressure and low temperature that allow the frozen water or solvent to sublimate as shown in (figure 4). This diagram, also, represents water phase diagram. In other words, it represents the physical state of water under different conditions including temperature and pressure. Through the diagrams, sublimation occurs when pressure is lowered, and heat is applied to the frozen sample. Sublimation rate increases more with vacuum. This is a simple explanation of the freeze-drying process and its scientific concept.

![Figure SI. 4: Theoretical illustration of freeze-drying](237)

Sublimation is the main target for a freeze drier unite in order to completely dry the sample and evaporate the remaining excess water by breaking the bonds between the sample and water. Three main phases for the freeze drier to pass through as shown in (figure 5); freezing, primary drying (vacuum sublimation) and secondary drying (adsorption) (238).
SI. 4.2.1. Freezing:
In this phase, the sample is completely frozen in a vial that is done by freezer or even a chilled bath. The sample is cooled down to reach below the triple point in order to ensure that the sample will not undergo melting but rather with exposure to sublimation.

SI. 4.2.2. Primary drying (vacuum sublimation):
This is the stage in which around 95% of the entire drying process takes place. The application of vacuum in this stage is a reason of rapid sublimation. It is a slow phase in which pressure is lowered and moderate heat is applied because excessive heat may cause breakdown or change in the structure of the sample. The released water vapor is condensed and solidified within the condenser.

SI. 4.2.3. Secondary drying:
It is called, also, adsorption phase in which the ionic bonds between water and the applied material is broken and removed. This breakage is owing to the increased temperature in the freeze drier to a degree higher than the primary drying phase so that the remaining 1-5% of the residual moisture is removed. The dried samples can be preserved within dry un-moisturized environment such as silica jar or refrigerator so that they can be prepared for later phases of the study.

The main purpose of using freeze drier during the current study has been to provide a stable form of free unloaded CSNP, PEGylated CSNP and DOX loaded CSNP. The dried forms of these nanoparticles have been used for further characterization analytical techniques such as FTIR and

Figure SI. 5: Graphical illustration for different stages of freeze-drying process (238)
XRD. Additionally, these dried forms have had more stability profile and longer shelf life as a powered form. They can be used anytime with a definite amount of micro and milligrams of the nanoparticles. During drug loading and release studies, we have used to weigh certain amppunts of nanoparticles in order to investigate some critical analytical tests such as release, loading and cell viability percentages.

**SI. 4.3. Magnetic stirrer (ISG®, China)**

It is a laboratory device that is frequently used in most chemistry, physic and biology laboratories for mixing of different fluids. It is a simple device in which a magnetic bar is allowed to stir at the bottom of certain sample as shown in figure 6. This rotating bar is stirring within a rotating magnetic field. The sited bar is immersed inside the vessel of liquid sample, and it must be of suitable size relative to the applied vessel.

![Figure 6: Magnetic stirrers (239)](image)

In case of glass vessel, it can be broken if the size of the stirring bar is quite large and strong. Another important factor that affect the stirring process is the viscosity of the liquid. When the applied liquid has high volum or high viscosity, additional mechanical stirring is required. The main controllers with magnetic stirrer device are rotating speed, that is defined as round per minute (RPM), time that is defined in minutes and temperature, that is defined by Celsius °C (240). The size of the stirring bar is another considerable factor that variably affect mixing process. As shown in figure 7, there is a variety of sizes and shapes of the used magnetic bars. The selection of a specific size or shape is basically related to the purpose of mixing process and the size of the container in which we perform mixing of the sample.
Centrifugation is one of the fast separation techniques that involves sedimentation of suspended particles to the base of a liquid medium. This sedimentation can take place naturally without any external interference, but this may take very long time. Many factors affect the particles sedimentation or separation including particles shape, density and size, in addition to the viscosity and temperature of the suspending medium. It has wide application in most biological and chemical experiments such as DNA separation and proteins purification.

It is a simple instrument in which the sample is placed in a vessel called centrifuging tube. This tube is allowed to rotate in a rotor whose speed is adjusted before the sedimentation process. As the rotor speed gets higher, a centrifugal force is moved and each particle in the medium will sense that force as shown in (figure 8). This result in immediate sedimentation of these particles. The sedimentation rate is directly proportional to the difference between the density of the medium and the density of its suspended particles. Additionally, the viscosity and some other physical and chemical properties influence the sedimentation rate. If we fixed the viscosity of the medium and centrifugal forces, the molecular weight of the dissolved particles will be directly proportional to the sedimentation rate.
SI. 4.5. Oven drying (TITANOX, Italy):

Drying by oven is the process of removing any solvent that is mixed with the applied sample, and this sample must be non-heat labile in order to avoid its destruction. It is applicable in small scales like laboratories and larger industrial scale as well. The main purpose of oven drying remains the same at both scales is to remove the mixed solvent and avoiding moisture. It is completely made of stainless steel and have a key and lock door as shown in (figure 9). During our experiment, drying oven has been used to dry PEGylated and non-PEGylated chitosan nanoparticles so as to be ready for SEM analysis. Each sample has been spreaded over an aluminum foil that has been fixed on a simple watch glass, then entered to the oven that has been adjusted at 60°C for 10 minutes in order to evaporate the solvent. In few times, the drying oven has been used to sterilize and dry the applicable used glassware. (245)
Chapter 4

Conclusion and Future Perspectives
Conclusion:

The current study has developed a therapeutic assumption of developing a selective targeted drug delivery system for the treatment of breast cancer. The proposed system has intended chitosan nanoparticles in order to entrap DOX inside, with maintaining the external surface of the nanoparticles decorated with PEG polymer. In order to enhance its specificity and sensitivity, the nano-system has been functionalized with two different types of breast cancer specific monoclonal antibodies; Anti-HER2 and Anti-hMAM. Along over the study, some characterization techniques have been applied as confirmatory procedure to ensure the durability of the final therapeutic cargo. Examples of which, DLS, TEM, XRD, FTIR, UV-VIS and 1H NMR. In this sense, primary size adjustment of blank unloaded CSNP has been performed to put some control over the size, surface charges and stability in different pH media. Then PEG has been grafted on the external surface of CSNP in order to enhance its stability against immunogenic responses and prevent its lymphatic uptake or RES uptake. Three PEG concentrations have been demonstrated; 5% PEG had higher stability compared with other proposed concentrations with the lowest particle size. This is because the final size of the nano-system has been supposed to be less than 200 nm. That is because the RES capturing size limit is known to be hindered for size range less than 200nm. After confirming stability of the PEGylated CSNP and its size stability below that RES opsonization range, DOX has been encapsulated in different concentrations with various nano-formulations. 5Ug/ml concentration formula has been the most effective one in terms of loading capacity and surface stability. Therefore, it has been selected for the functionalization of mAbs. Each one of the mAbs has been functionalized separately; having two final types of nano-functionalized formulations. The cytotoxicity analysis has been conducted against MCF7 breast cancer cells and L929 fibroblasts has been conducted. Various nano-formulations have been tested against the effect of free DOX on the same two types of cells with maintaining fixed temperature, drug concentration and incubation period. The Anti-HER2 and Anti-hMAM functionalization could promote the MCF7 cytotoxicity, with achieving less than 3% cell viability. While, L929 cells were less reactive and less responsive than MCF7. Therefore, the assumption of encapsulating DOX into a selective targeted nano-system has enhanced its in-vitro therapeutic effect with promoted cancer cell endocytosis. Bright filed images have exhibited cancerous and normal cell responses to the developed formulations. Therefore, the current study recommend the application of polymeric PEGylated chitosan nanocarrier with mAbs functionalization as a novel therapeutic system for
selective release of the entrapped DOX. This may provide one of the nanoscience based biomedical solutions for breast cancer treatment.

**Future Perspectives:**

During this study, some tests and experiments were required to be implemented in order to give a clear understanding of the structural and therapeutic efficiency of the developed nanoformulations. The structural stability of the developed nano-formulations can be empowered with the following:

- **Body fluid analysis**: This gives a better prediction of the biological behavior of the developed formulation. It will detect the efficacy of some parameters that have been already tested in this study. Particles stability along over the its site of introduction till reaching its site of action can be demonstrated with this test. Besides, the convenience of the nanoparticles’ sizes developed in this study can be confirmed with this test.

- **Animal testing**: May improve the pharmacokinetic and pharmacodynamic profiles of the developed nano-formulations. Detecting some parameters such as plasma level and elimination rate of the injected drug may promote a better understanding about the assigned dose of the final drug. Besides, monitoring *In-vivo* blood release of the drug can be another confirmatory procedure that is achieved with such animal testing. It illustrates a clear relationship between the release rate and different pH levels.
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