SYNTHESIS AND CHARACTERIZATION OF PEG CAPPED TAMOXIFEN LOADED ZINCOXIDE NANOPARTICLES AND THEIRTHERAPUTIC APPLICATIONS

Submitted By

MAHNOOR MALIK

00000170546

School of Mechanical and Manufacturing Engineering

National University of Sciences and Technology

H-12 Islamabad, Pakistan

November 2018

SYNTHESIS AND CHARACTERIZATION OF PEG CAPPED TAMOXIFEN LOADED ZINCOXIDE NANOPARTICLES AND THEIR THERAPUTIC APPLICATIONS

A thesis submitted in partial fulfillment of the requirement for the degree of Master of Science

In

Biomedical Sciences

By

MAHNOOR MALIK

170546

Supervised By: Dr. Adeeb Shehzad

School of Mechanical and Manufacturing Engineering

National University of Sciences and Technology

H-12 Islamabad, Pakistan

November 2018

Declaration

It is hereby declared that this research study has been done for partial fulfillment of requirement for the degree of Master of Sciences in Biomedical Sciences. This work has not been taken from any publication. I hereby also declare that no portion of work referred to in this thesis has been submitted in support of an application for another degree or qualification in this university or other institute of learning.

FORM TH-4

MASTER THESIS WORK

We hereby recommend that the dissertation prepared under our supervision by: **Ms. MahnoorMalik**170546Titled: **"Synthesis and characterization of PEG capped Tamoxifen loaded zincoxide nanoparticles and their therapeutic applications"**be accepted in partial fulfillment of the requirements for the award of **MS Biomedical Sciences** degree. (Grade_____)

Examination Committee Members

1. Name: Dr. Umer Gillani Signature: _____

2. Name: Dr.Umer Ansari Signature: _____

3. Name: Dr. Murtaza Najabat Signature: _____

Supervisor : Dr. Adeeb Shehzad Signature: _____

Date: _____

_____ _____

Date Head of Department

COUNTER SIGNED

_____ _____

Date Principal

I dedicate my thesis to my parents and my brother for their immense support, motivation & love.

ACKNOWLEDGEMENTS

Foremost, thanks to Almighty Allah for enabling me to complete my Masters in time. I would like to express my sincere gratitude to my supervisor, Dr. Adeeb Shehzad for his guidance, continuous support; motivation and providing me with the resources that came in handy throughout my project. I would like to acknowledge Director IBGE, KRL, Dr. Muhammad Ismail for his assistance in my project. I am obliged to Maam Nousheen Fatima (HoD BMES) and Dr. Nabeel Anwar for their valuable advices and administrative support.

I would like to express my sincerest thanks to Rector NUST, Lt. General Naweed Zaman and Principal SMME, Dr. Abdul Ghafoor for providing us with an excellent research environment in SMME, NUST.

I am highly gratified to my siblings and friends for being with me in every thick and thin throughout my Masters. I would like to say thanks to Sundus Shah and Muswaria Iftikhar for their assistance, motivation and scientific discussions.

I owe my gratitude to my friends Hinna Nayyab, Palwasha Kiffyat and Sadia Bhatti for their valuable advices and bearing with me during hard times. Finally, an honorable mention goes to my family for their support.

Table of Contents

List of Abbreviations

UV-Vis	Ultra violet visible absorption spectroscopy
FTIR	Fourier transform infrared spectroscopy
SEM	Scanning electron microscopy
TAM	Tamoxifen
XRD	X-ray Diffraction
EDX	Energy Dispersive X-rays
ZnONPs	Zincoxide nanoparticles
PEG	Polyethylglycol
DPPH	1-Diphenyl-2-picryl-hydrazyl (DPPH)
NaOH	Sodium Hydroxide
Rpm	Rotation per minute
nm	Nanometer
µL	Microliter

List of figures

List of Tables

ABSTRACT

Background

Metallic nanocarriers belong to a class of nanocarriers having strong anticancerous activity through the generation of ROS and by the induction of oxidative stress in the targeted cells. They are widely used in diagnostics, therapeutic and pharmaceutical industries. Studies showed that ZnONPs have potential anticancerous, antimicrobial, anti-inflammatory and anti-angiogenesis activities.

Methods

ZnO-NPs were chemically synthesized by wet chemical precipitation method using zinc nitrate tetra hydrate and NaOH as precursor. Tamoxifen (Tam), a widely used chemotherapeutic drug for breast cancer, is conjugated with zincoxide nanoparticles and further coated with the polymer poly ethyl glycol (PEG), to enhance their therapeutic efficacy, better solubility, biocompatibility and increases its retention time. The final product (PEG-Tam-ZnONPs) is delivered to MCF-7 breast cancer cells. The antioxidant, hemolytic activity and invitro stability was tested through DPPH, hemolytic assay and salt, temperature and pH testing. The morphology and characteristics of the ZnONPs, Tam-ZnONP's and PEG-Tam-ZnO were investigated by UV, XRD, SEM, EDX and FTIR.

Results

The characterization techniques confirm the synthesis of ZnONPs and drug conjugation. Cytotoxicity evaluation of PEG-Tam-ZnONPs Tam-ZnONPs and ZnONPs showed that PEG-Tam-ZnONPs have improved and better anticancerous activity than its counterparts. The hemolytic assay confirmed that the PEG-Tam-ZnONPs has less hemolytic and clotting activity than tamoxifen when used alone. PEG-Tam-ZnONPs exposed cells generate more reactive oxygen species and free hydroxyl radical when compared to ZnONPs, Tam- ZnONPs. The current study suggests that nanoparticle coated drug formulation can contribute to the development of a suitable anticancer drug delivery system in the nearby future.

Keywords: Zincoxide nanoparticles **(**ZnONPs), Tamoxifen (Tam), Polyethyl glycol (PEG)

CHAPTER 1

INTRODUCTION

1.1 Breast Cancer

Breast cancer (BC) is most commonly diagnosed cancer types with the leading morbidity and mortality rate among the women worldwide. It's the second most common cause of death among the women, with nearly 1.7 million newly diagnosed cases and annually causing approximately 40,000 deaths worldwide (Siegel et al., 2013). The risk of developing breast cancer increases with age, approximately 1 in every 9 women (>50 years). Breast cancer cells mainly have three types of receptors: estrogen (ER) and progesterone receptors, HER-2+ receptor and triple negative breast cancer. Estrogen and progesterone can be treated with hormonal therapy or the drugs targeting the mutated hormones while HER2+ cancer cells type respond well to drugs such as the monoclonal antibody trastuzumab. Another breast cancer type lacking all three types of receptor (estrogen receptors, progesterone receptors, or HER2) are called triple-negative, although they frequently express androgen and prolactin receptors (Plevova et al., 2009). Although over the past few decades, there have been significant advances in breast cancer treatment; still current therapeutic approaches are limited by multi drug resistance, non-specific systemic distribution and inadequate drug concentrations reaching the targeted site. Due to non-specific distribution system in the body, large quantity of chemotherapeutic drug administration is required. Patients undergoing breast cancer chemotherapy experience some serious treatment related adverse toxicities such as nausea, vomiting, fatigue, anorexia, weight gain, head ache, menopausal problems, ovarian infertility and nonspecific targeting, effecting both healthy cells and cancerous and at the same time. Therefore to minimizes or eliminates the side effects by these chemotherapeutic drugs there is a necessity of an effective approach for specific targeted drug delivery system (Banu et al., 2015). The limitations relating to chemotherapy can be overcome by the application of nanotechnology. The enhanced surface properties of nanoparticles not only helps in easy diffusion inside the tumor cells but also delivers an increased concentration of drug selectively inside the tumor cells with significantly reduced toxicity (Varadharajaperumal, Subramanian, & Santhanam, 2017).

1.2 Metallic Nanoparticles as nanomedicines

Metallic nanoparticle, with their greater stability, small size, high surface properties, non-cytotoxicity and biocompatibility has been extensively used in biomedical applications. They are being prominently used in medicine, biosensors, prophylaxis, and targeted drug delivery system. Among these metallic nanocarriers, ZnONPs with their unique properties have acquired tremendous interest in cancer drug delivery. Zinc as an essential trace element and the main component of various enzyme systems, takes part in body's metabolism and have an important role in proteins and nucleic acid synthesis, hematopoiesis, and neurogenesis (Khalil, Al-Qunaibit, Al-zahem, & Labis, 2014). Nano-ZnO, with their small particle size, makes zinc more easily absorbed by the body and provides some exciting opportunities in targeted drug delivery system for much more safety and effective cancer treatment. Nanoparticle-based drug delivery system effectively targets the specific sites of cancer cells which could reduce the overall drug dosage used and thus minimizes the undesirable side effects (VickyV Mody, Siwale, Singh, & Mody, 2010). Compared with other nanomaterials, ZnONPs have got more attraction due to their high degree cancer cell selectivity, low toxicity and biodegradable characteristics. They could preferentially serve as a foundation for developing novel cancer therapeutics as they actively targets the rapidly proliferating cancerous cells.

Tamoxifen (TMX) is widely used hormonal treatment for all the stages of breast cancer and has been approved for the prevention and treatment of breast cancer in high-risk patients (Thakkar & Mehta, 2011). It is selective estrogen receptor modulator (SERM) and depending upon its target, it has both estrogenic (bones, liver, lungs) and antiestrogenic (mammary epithelium and brain cells) properties at the same time. However, tamoxifen normally induces dose-dependent side effects such as, retinopathy, severe depression, corneal opacities and increased blood clotting (Teft, Mansell, & Kim, 2011).In this study, TMX has been formulated in nanoparticulate carrier systems and further coated with synthetic polymer PEG, to overcome the disadvantages of chemotherapeutic drug. In addition, cytotoxicity, cell viability, hemolytic and antioxidant activity of the nanoformulate were studied to evaluate their effectiveness in drug delivery systems.

1.3 Phase 1- Synthesis of ZnONPs, Drug Conjugation &Coating with PEG and Characterization of the prepared samples:

This research has been divided into two phases; in the first phase, our research focus on the chemical synthesis of nanoparticles and drug conjugation. For this approach, zincoxide nanoparticles were chemically synthesized by the conventional, wet chemical precipitation method and tamoxifen was loaded on the surface of zincoxide particles. To make the prepared suspension biologically more compatible and to prevent it from opsonization and for better solubility, Tam-ZnO was coated with PEG. The prepared samples were then characterized for size, shape, structure determination and to find the purity of the prepared sample.

1.4 Phase II- Cell Cytotoxicity Assays:

In the II Phase of the study, the effect of nanoformulation PEG-Tam-ZnO and commercially available chemotherapeutic drug Tamoxifen alone were compared for efficient anticancerous activity on MCF-7 cell lines. The invitro stability of nanoconjugates was investigated through pH, salt and temperature testing to evaluate the potential of chemically synthesized nanoparticle. The antioxidant activity and the presence of free hydroxyl radicals produced by PEG-Tam-ZnO were confirmed through DPPH assay. Nanoconjugates showed more hemocompatibility (caused less than 7 % Hemolysis) and enhanced biological activity as compared to parent drug and the amount of drug required for its activity were reduced.

1.5 Aim

To investigate the anticancerous and therapeutic efficacy of chemically synthesized ZnONPs, PEG-Tam-ZnO on MCF-7 breast cancer cell lines.

Objectives

- Chemical synthesis of ZnONPs , drug loading and coating with PEG.
- Characterization of the synthesized nanoparticles.
- To analyses the therapeutic efficacy through MTT Assay, antioxidant, hemocompatibility assays and invitro and invivo stability testing.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Breast cancer is the most commonly diagnosed cancers in women with approximately worldwide 1.7 million new cases. It is still the most significant cancer-related cause of female mortality but despite of high incidence rate 5-year survival rate is nearly 90% in Western and developed Asian countries(Banu et al., 2015). Although, earlier cancer diagnosis and improvements in treatment have reduced breast cancer mortality but young age is still a risk factor for poorer survival (McGuire, 2016).Tumor markers identification, expression profile of cytokeratin positive cells from bone marrow, gene amplifications and mutations confirms their malignant nature. Breast cancer cells mainly have three types of receptors: HER-2 receptors, estrogen (ER) and progesterone receptors, and triple negative breast cancer type. HER2+ type breast cancers are generally considered more aggressive than HER2- breast cancers (Stephens et al., 2012), but HER2+ cancer cells respond well to drugs such as the monoclonal antibody trastuzumab (in combination with conventional chemotherapy), and resulted in improved and significant the prognosis significantly (Romond et al., 2015). ER+ cancer cells can be treated with drugs to block estrogen effects as they require estrogen for their growth. (e.g tamoxifen), and generally shows improved prognosis. Cells that do not have any of these three receptor types (estrogen receptors, progesterone receptors, or HER2) are called triple-negative, although they frequently express hormonal receptors, such as androgen and prolactin receptor(Stuckey & Onstad, 2015). Amongst the current fighting strategies for breast cancer treatment and management, chemotherapy plays a vital role. Chemotherapeutic agents like cyclophosphamide, tamoxifen, methotrexate, docetaxel, doxorubicin and fluorouracil can be used alone or in combination with other treatments as in adjuvant therapy or neo-adjuvant therapy (Plevová et al., 2009).

Infiltrating ductal breast tumors, the most frequent histological type of breast cancer with estrogen receptors (ERs) representing nearly 65% of cases among women diagnosed before

menopause and 80% among postmenopausal patients (Anderson, Chatterjee, Ershler, & Brawley, 2012). Almost 75% of women diagnosed with ER-positive breast cancer are in postmenopausal stage, representing approximately three-quarters of cases in this subtype of breast cancer (Economopoulou, Dimitriadis, & Psyrri, 2015).

According to the American Cancer Society's 2014 report: an estimated 235,030 new cases of breast cancer are being diagnosed annually, worldwide and among them approximately 15-20% of these cases are likely to be hereditary (Stuckey & Onstad, 2015). It may be due to the amplification or alteration in oncogenes or due to the mutations in tumor suppressor genes (TSGs). Among these inherited cases, most of these attributed cases are due to the defects in the BRCA1 and 2 genes. The altered oncogenes genes involve in uncontrolled cell growth and proliferation are: HER2, RAS, and the ER genes, c-MYC while tumor suppressor genes include RB, TP53, and PTEN. Several cellular pathways are also involved in uncontrolled cell growth and proliferation of breast cells, such as the MAPK, RB/E2F, P13K/AKT/mTOR, and TP53 regulated by various genes (Peng et al., 2011). The studies on HER2 amplification and overexpression shows the importance of molecular biology in therapeutics, as today specific antibodies against Her2 are designed to treat breast cancer (Slamon et al., 2001). P53 is most also frequently mutated gene accounting for ¼ of breast tumors and its germ-line mutations are linked to the Li-Fraumeni syndrome (LFS) (Economopoulou et al., 2015).

2.2 Tamoxifen

Tamoxifen is nonsteroidal triphenylethylene derivative prodrug, efficiently targeting the estrogen receptor. It is used both for the treatment and prevention of breast cancer. It is also used as adjuvant or additional therapy following primary treatment for early stage breast cancer and for advanced metastatic breast cancer for many years. Being a prodrug, tamoxifen extensively undergoes the metabolism by an efficient enzymes family cytochrome P450, namely CYP2D6 and CYP3A4 and form an active metabolite afimoxifene (4-hydroxytamoxifen; 4-OHT) and endoxifene (*N*-desmethyl-4-hydroxytamoxifen),inside the body(Hoskins, Carey, & McLeod, 2010).

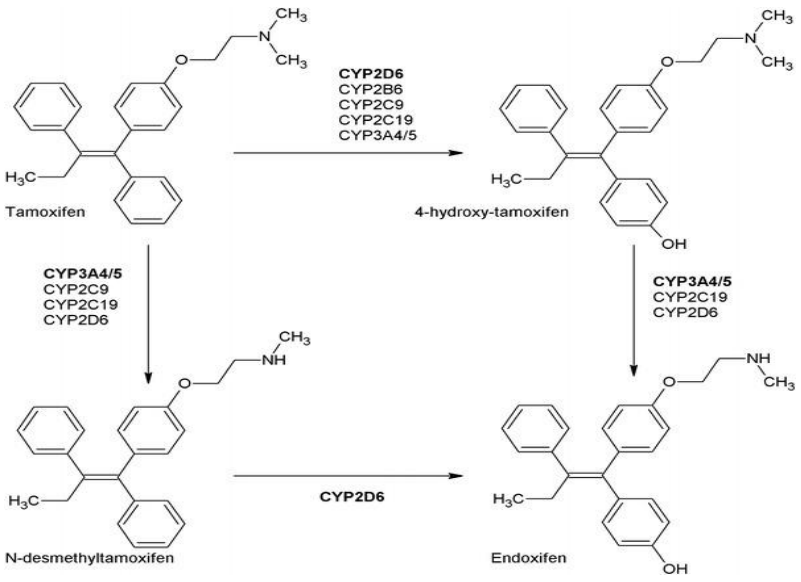


Figure 2.1: Structure of Tamoxifen and its active metabolites (Endoxifen, 4-hydroxy-tamoxifen, N-desmethyltamoxifen)

Depending on the targeted tissues, tamoxifen has both estrogenic and anti-estrogenic actions. Tamoxifen has strong antiestrogenic activity on mammary epithelium, where it blocks the growth factor proteins such as ErbB2/ HER2. After the drug administration,4-OHT (tamoxifen analog) binds to ER particularly in the tumor cells, producing a nuclear complex that inhibits estrogen effects by preventing the DNA synthesis (Martínez, Benito-Miguel, Iglesias, Teijón, & Blanco, 2012). Tamoxifen arrests the cell cycle and causes cells to remain in the G_0 and G_1phases. Proestrogenic action of tamoxifen in bone matter provides the signals required for bone maintenance by mimicking the effects of estrogen on bone, preventing the osteoclasts and reduces the risk of osteoporosis in menopausal women (Higgins & Stearns, 2009).

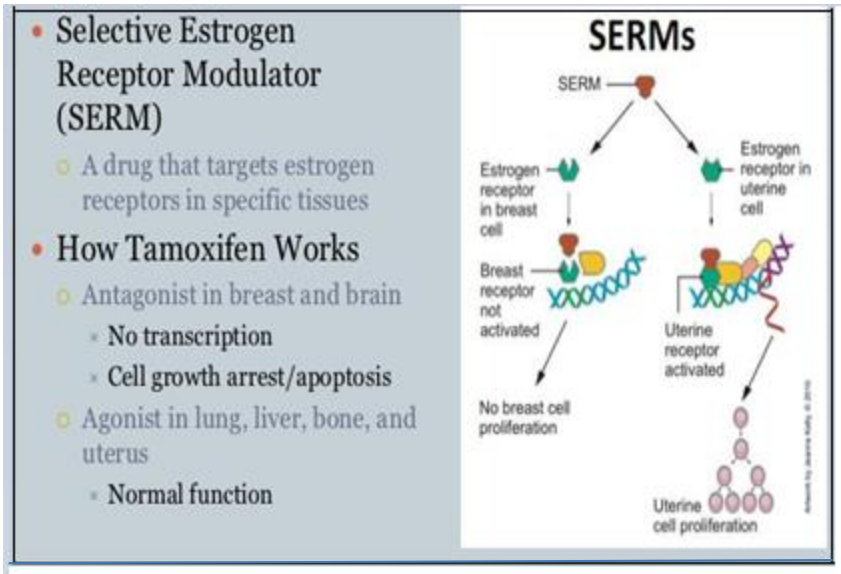


Figure 2.2: Mechanism of action of Tamoxifen

TMX as an estrogen-agonist, likely to increases the chance of developing endometrial cancer among other side effects (Hurtado et al., 2008).Moreover, retinopathy, blood clotting and atherosclerosis and low water solubility, limits the use of this drug. To overcome these issues, the development of nanocarrier holding the require drug is the need of time. The nanocarriers with their increased water solubility, permeability and extremely small size, have an easy access to the leaky vasculatures of tissues. ER-positive tumors that has initially responded to antiestrogen therapy develops resistance after long term therapy with tamoxifen (Facades, 2014). This type of tamoxifen resistance may result in tamoxifen-dependent/stimulated growth or tamoxifen-nonresponsive while still expressing ER. However, all these side effects are time and dose-dependent(Cui et al., 2010). Therefore, for efficient delivery of TMX the use of biologically compatible targeted nanocarrier that provides maximal pharmacological effects with minimal inadvertent side effect is the need of time.

2.3 Nanotechnology

Nanotechnology a combination of science and engineering refers to materials, with the size in the range of 10-250nm.Nanoparticles because of their extremely small size and unique bulk properties have got a great importance from the last few decades. Their extremely small size

helps in forming a bridge between the large bulk molecules and the small entities at an atomic and molecular level (Jokerst, Lobovkina, Zare, & Gambhir, 2011). These nanosized particles have strong communications with the cell surface biomolecules that can be decoded to various biochemical and physiochemical properties of the cell (VickyV Mody et al., 2010). The literature showed that these nanostructures have significant toxicity that not only kill the cancerous cells but also provide protection against cancer (Wahab et al., 2013.) Nanomedicine offers various advantages over conventional pharmaceutical agents, due to potential application in drug delivery system and noninvasive imaging. Nanomedicine also have strong ability to cross the biological barriers, increased biocompatibility, effective hydrophobic drugs delivery mechanism, and preferentially site specificity.(Vicky V Mody, Nounou, & Bikram, 2009). Moreover, nanoparticles due to their large surface-to-volume ratio, provides the surface to attach multiple copies of therapeutic substance on their surface and in this way increase the concentration of therapeutic substances at the pathological site. For specific targeting, conjugating nanoparticle with an appropriate ligand system to recognize the targeted cells such as cancer cells, results in specific binding with the target cells. During whole process of nanoparticles conjugation with ligand, the control in particle size (i-e in the range of 10- 80nm) , increases the chance of easy entry into the targeted cells, provide veil against reticuloendothelial system and also increases the retention time (Bao, Shi, & Xiang, 2012).

2.4 Metallic Nanocarriers

Metallic nanoparticles such as iron, copper, zinc, gold and silver, due to their great physical and chemical properties have been continuously used in diagnostic and therapeutic agent for many years (Rasmussen, Martinez, Louka, & Wingett, 2010). Metallic nanoparticle particularly ZnO nanoparticle (ZNP), has grown to be the most promising nanomaterials due to their multifunctional application. Currently zinc is one of the five compounds which are recognized as safe for humanly use by US Food and Drug Administration (21CFR182.8991) (Chivukula, Ciplys, Shur, & Dutta, 2010). ZnO nanoparticles with their unique therapeutic properties exhibit strong ultraviolet emission with the band gap of 3·37 and binding energy of 60 meV. It has wide applications in cosmetics, medicine, drug delivery, biosensors, electrodes, solar cells and in catalysis (Jiang, Pi, & Cai, 2018). ZnO is potentially a biocompatible metal oxide and various

monitoring process, its properties could be tailored such as by controlling their synthesis parameters, size, shape, pH, temperature, solvent type and conjugating different drugs with nanoparticles to increase their efficiency (Mishra et al., 2012). Morphologically different nanostructures of ZnO such as nanorods, nanosphere, nanotubes, nanowires and nanoneedles have been successfully synthesized and their functions and actions varied accordingly (Yahya et al., 2010).

Cancer is an uncontrolled division and differentiation of normal cells and during the past several decades several treatment modalities has been designed including chemotherapy, radiation, and surgery against it(Sam et al., 2014). Although all these therapies have an effective role in cancer cell destruction and damage, still they lack site specificity and have poor water retention property along with other side effects. Now a day's nanomedicine-mediated therapies due to their active targeting mechanism, high solubility/bioavailability, biocompatibility, and multifunctionality are extensively used in drug delivery. Studies have shown that ZnO NPs have strong anticancerous activity among several metallic nanoparticles and induces strong cytotoxic effect by increased oxidative stress through the generation of reactive oxygen species (ROS),decreased mitochondrial membrane potential (MPT) and increased intracellular [Ca2+] level (Moghimipour et al., 2018). Mitochondrial electron transport chain is strongly associated with the increased generation of ROS thus disrupting the mitochondrial integrity and damaged the membrane structures (Stowe & Camara, 2009). As a result of mitochondrial dysfunction, redox balance system of the cell eventually compromised and eventually causes severe damage to intracellular macromolecules, especially DNA, disrupting the cellular balance by the loss of protein integrity and ultimately resulted in apoptosis (Bai, Zhang, Zhang, Huang, & Gurunathan, 2017).The loss of mitochondrial membrane potential (MPT) causes the exposure of cytochrome *c* protein into the cytosol causing the activation of caspases.

ZnONPs have an ability to induce various pro inflammatory protein markers including IL-12, tumor necrosis factor-α, interferon-c, in blood mononuclear cells. It also causes the activation of pro apoptotic proteins, causing the down regulation of Bcl-2 and up regulation of PARP, caspases cascades, the DNA fragmentation in human breast, fibroblasts and neural cells through mitochondrial apoptotic pathway activation (Palit, Kar, Sharma, & Das, 2015). ZnONPs triggers the induction of mitochondrial apoptotic pathway which has powerful cytotoxicity against MCF-7 breast cancer cells, probably, more than cell cycle arrest. They induce apoptosis through both

extrinsic and intrinsic pathways causing the up regulation of pro apoptotic genes p21, p53, JNK, and Bax and down regulation of anti-apoptotic genes Bcl-2, AKT1, and JERK/2. The up regulated pro apoptotic genes and down regulated anti apoptotic genes Bcl-2, AKT1, and ERK1/2 regulated in dose-dependent manner in MCF-7 cells and arrests the cell cycle in the G2/M phase and(Gupta, Bhargava, & Bahadur, 2014). To show better solubility, higher toxicity compared with individual agents and effective delivery into cancer cells, different chemotherapeutic drugs like doxorubicin, tamoxifen, paclitaxel, curcumin, could be loaded onto the ZnO NPs (Jiang et al., 2018).

Autophagy is a catabolic process activated as a response to different cellular stresses like damaged organelles, presence of any anticancer agents, ROS generation or protein aggregation. The extension of autophagy and cellular self-consumption may resulted in excessive cellular damage leading to cell death through apoptosis mechanism (Hackenberg et al., 2014). Hence, autophagy is considered as an important event in nanoparticle-induced cytotoxicity that not only promotes cell survival but also activates lethal mechanisms in cancer cells.

Although currently several anticancer chemotherapies are available, still they fail to produce a considerable anticancer response due to drug resistance or their failure of effective differentiation between cancerous and normal cells, and large quantity of drug administration (Heidari Majd et al., 2013). ZnONPs having unique anticancerous activity exhibit a high degree of cancer cell selectivity and preferentially targets the rapidly dividing cancerous cells, which could serve as a foundation for developing novel cancer therapeutics. Up to now, TMX has been formulated in nanoparticulate carrier systems using synthetic polymer PEG. The main objective is the optimization of the PEG coated TMX-loaded ZnONPs for the validation of cytotoxicity effect on MCF-7 cell lines. In addition, cell viability the hemolytic and antioxidant effect of the system was studied to evaluate their effectiveness in drug delivery systems.

CHAPTER 3
MATERIALS AND METHODS

3.1 CHEMICALS AND REAGENTS

Zinc nitrate tetra hydrate [Zn(NO3) 2.4H20], sodium hydroxide (NaOH), poly ethyl glycol (PEG- 6000) and ethanol were purchased from Sigma Aldrich. Tamoxifen (Tam) was used as drug. Deionized water and ethanol was used throughout the experiment. Shimatzu UV-Vis 1800 spectrophotometer was used for recording UV-Vis spectra.

3.2 SYNTHESIS OF ZINCOXIDE NANOPARTICLES:

Zinc oxide nanoparticles were chemically synthesized by wet chemical precipitation method. For this purpose, an ethanolic solution of 0.5 M zinc nitrate tetra hydrate [Zn(NO3)2.4H20] and 0.9M sodium hydroxide (NaOH) were made separately. Both the solutions were kept under constant magnetic stirring for complete dissolution, for 1 hour. After complete mixing, 0.5M zinc nitrate solution was added into the NaOH solution drop wise and the solution was stirred vigorously for 2 hours. The solution was then covered and left overnight, so that the precipitates settled at the bottom. The supernatant was removed next morning, and the suspension was centrifugated at 6000 rpms for 10 mins. Again, the supernatant was removed, and particles were collected, which were then washed with ethanol and distilled water thrice to remove the impurities bound with the particles. The particles were dried for 4 hours in an oven at 60 °C. After complete drying, zinc hydroxide Zn(OH)2 was totally converted into whitish precipitates of zinc oxide (ZnO) (Zhang, Ram, Stefanakos, & Goswami, 2012).

3.3 IN VITRO STABILITY STUDIES

3.3.1 Salt effect

To check the effect of higher concentration of NaCl on ZnO nanoparticles, 3 different salt concentrations (2M, 3M, 5M) were prepared. For this purpose, 2ml of the zincoxide nanoparticles solution was added into the 2ml salt solution with different molar concentrations. The sample was kept at room temperature and its absorbance was recorded after different intervals i-e after 24 hours, 48 hours, 72 hours, 1 week and 2 weeks respectively. The same protocol was usef for other salt concentrations.

3.3.2 Heat effect

Heat effect on ZnO nanoparticles was studied by taking 10ml solution of ZnO nanoparticles in a flask and heated it upto 100°C for 30 mins. The UV-vis spectrum of nanoparticle keeping at room temperature and at 100°C was recorded and compared.

3.4 DRUG CONJUGATION WITH NANOPARTICLEAND PEG-6000 COATING

For drug loading into zinc oxide nanoparticles, 10% Tamoxifen was dissolved in 100ml of distilled water .1g of sonicated ZnO NP's were added to the drug solution. The solution was then kept under constant stirring for 1 hour and was left undisturbed overnight. The next morning, suspension was centrifugated at 5000rpm for 5 mins separating the supernatant and precipitates. The amount of loaded drug was calculated by difference in original drug concentration and amount of drug in supernatant/ pallet (Palanikumar, Ramasamy, Hariharan, & Balachandran, 2013).

For poly ethyl glycol (PEG) coating, 5% PEG stock solution was prepared in distilled water. 0.125% i-e 2.5ml was taken out from original stock solution and 10mg of Tam-ZnONPs was dissolved in final 100ml volume solution. After complete mixing, the solution was sonicated for

1 hour to get homogenous solution of PEG-Tam-ZnONPs. The pH of the solution was adjusted at 7.2 (Girigoswami, Viswanathan, Murugesan, & Girigoswami, 2015).

3.5 CHARACTERIZATION

To determine different properties of ZnO, Tam-ZnO and PEG-Tam-ZnO, the samples must undergo various characterization techniques. The results of these techniques give information about the optical and structural properties of the samples.

3.5.1 UV-VIS Spectrophotometry

UV-Vis absorption spectroscopy is the most widely used technique for sample's analysis. It gives the absorption spectra in sample, when a beam of light passes through it. From the reflected beam, the absorption spectra of the sample are measured.

$$A = -\log(T)$$

UV spectroscopy obeys the Beer-Lambert law, according to that: The absorbance of the sample is proportional to the molar concentration of the sample in the cuvette. The molar absorptivity also known as the absorption value is used when comparing the spectra of different compounds. The expression of Beer-Lambert law is:

$$A = \log (I_0/I) = Ecl$$

UV- Vis spectrophotometry is the first confirmatory test that confirms the synthesis of ZnO nanoparticles. For this purpose, an ethanolic suspension of the ZnONP, Tam-ZnO and PEG-Tam-ZnO were prepared. The instrument used was Jasco V-650 spectrophotometer in the range of200-600 nm range and samples were measured relative to pure ethanol.

3.5.2 Scanning Electron Microscopy (SEM)

In scanning electron microscopy, an electron beam is used for image production and magnification of the object or sample unlike light or optical microscopes. The signals generated

from the interactions of electron-sample reveal the information about the sample morphology (texture), size and crystalline structure of the materials making up the sample.

For SEM analysis of the ZnONPs, S-4800 and EDX-350 (Horiba) FE-SEM (Hitachi, Tokyo, Japan) were used. The samples were prepared in pure ethanol and were sonicated for complete dispersion. For sample scanning, prior to FE-SEM analysis, the samples were dropped onto a glass slide, fixed and were coated with osmium tetra oxide (OsO4) using a VD HPC-ISW osmium coater (Tokyo, Japan). The average particle size was calculated using FE-SEM analysis results and using Image J software and the size of approximately 50 nanoparticles were measured. The particle size distribution graph was plotted to calculate the average particle size.

3.5.3 Energy- Dispersive X-ray (EDX)

Energy-dispersive X-ray (EDX) is a microanalysis technique complementary to SEM used to determine the elemental composition of the sample in the SEM image. It works on the principle that within the specimen, X-rays are generated by the electron beam emitting the characteristic elemental energies, so, from this energy, not only the elements but their concentrations are identified in the specimen.

3.5.4 X-ray Diffraction (XRD)

X-ray Diffraction (XRD) contain an X-ray generator (3 kW) and anode (LFF Cu) that measures the patterns of the sample with an X-ray diffractometer (X'Pert-APD Philips, The Netherlands) The Cu Kα radiation was administered at a wavelength of 1.54 Å and the scanning angle varied from 10º to 80° with the X-ray generator tension and current of 40 kV and 30 mA, respectively.

3.5.5 Fourier Transform Infrared Spectroscopy (FTIR)

Fourier transform infrared spectroscopy (FTIR) is commonly used tool for functional group detection in pure compounds, mixtures or their comparison. FTIR spectroscopy works on the

principle that sample molecules absorb energy and vibrates with frequency known as resonating frequency that exactly matches the frequencies of bonds or groups present. This type of frequency absorption is detected, based on atomic masses, shape of energy surfaces and its associated vibrionic coupling.

The FTIR analysis of the samples was done in powdered form.

3.6 CELL CYTOTOXITY ASSAYS:

Cell toxicity assays were performed to found out the cytotoxic effect of the prepared samples on the cell. The cytotoxic effect of ZnO, Tam-ZnONP's and PEG-Tam-ZnO were found out through different assays:

3.6.1 MTT Assay

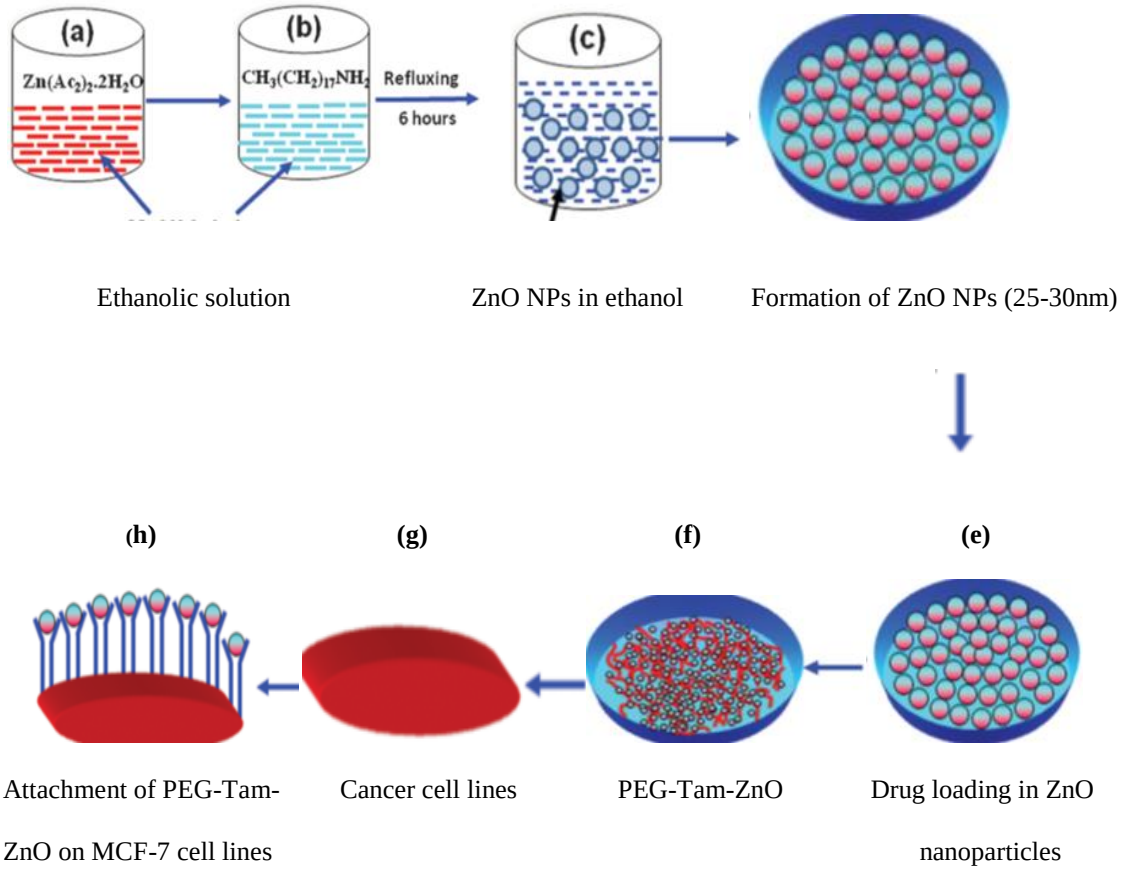
MTT assay is done to find out the effect of drug or chemicals on cells by the determination of living and dead cells count. In MTT assay, sample groups were treated with different concentrations (10, 15, 20, 25, 50, 100µg/ml) of PEG-Tam-ZnONPs and Tamoxifen alone while the seventh group served as control respectively. The cytotoxic effect of both the groups was compared. The samples were prepared by the serial dilutions of the stock solution 1mg/ml (all the dilutions were done in deionized water). Cells were cultivated in 96 well plates and were administered with drug in the following manner.

Serial no.	Drug conc. (µg/ml)	Time
1	Control (untreated)	24 hours
2	10	24 hours
3	15	24 hours
4	20	24 hours

5	25	24 hours
6	50	24 hours
7	100	24 hours

Table 3.1 Sample groups of study for MTT assay

All the sample groups were cultured and treated in triplicate, for the accuracy of results. 10µL MTT reagent was administered to the sample groups except the control group and was incubated at 37ºC for 24 hrs. The color change in MTT reagent corresponding to living or dead cells and was detected at 570nm wavelength by spectrophotometer.



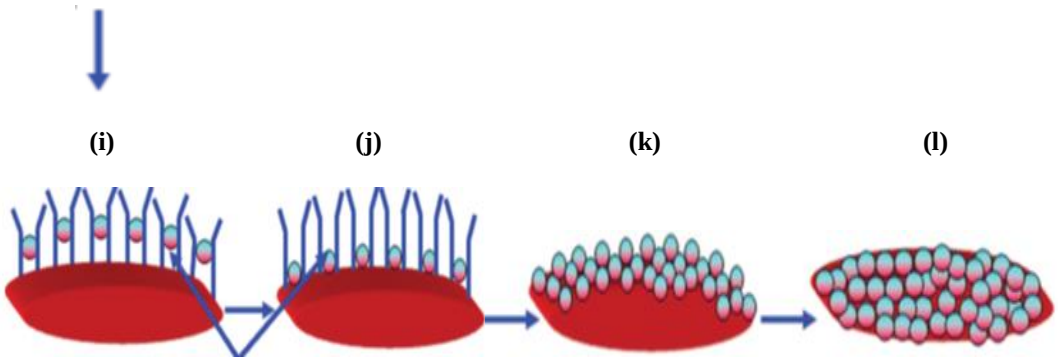


Figure 3.1: Schematic representation of synthesis, attachment and interaction of PEG-TAM-ZnO with MCF-7 Breast cancer cells.

3.6.2 DPPH

1-Diphenyl-2-picryl-hydrazyl (DPPH) possesses strong scavenging activity due to free radicals, used to calculate the antioxidant activity of any compound. For this assay 0.5 mM DPPH solution was prepared in ethanol and different concentrations (25, 50, 100, 150, 200,300 µg) of PEG-Tam-ZnONPs were prepared. Ascorbic acid was used as a standard. DPPH solution due to its high sensitivity towards light was kept in dark. The final volume of each of the sample was kept at 1.5 ml by adding distilled water. The concentration composition of the different reaction mixtures is given below:

Reaction	**DPPH solution**	**PEG-Tam-ZnO con.**	**Final volume**
1	500 µl	25 µg/ml (500 µl)	1.50ml
2	500 µl	50 µg/ml (500 µl)	1.50ml
3	500 µl	100 µg/ml (500 µl)	1.50ml
4	500 µl	150 µg/ml (500 µl)	1.50ml

5	500 µl	200 µg/ml (500 µl)	1.50ml
6	500 µl	300µg/ml (500 µl)	1.50ml

Table 3.2: Sample concentration for DPPH

The samples were kept in dark for 30-35 minutes for the reaction after mixing in required proportions. The color of the sample changes from dark purple to light yellow, showing the end of reaction. The samples were then observed through UV-VIS spectrophotometer at 517nm. The percentage of the antioxidant activity was calculated as:

% A. A= 100- [(Abs of sample) – (Abs of blank)/ Abs of control)]

3.6.3 Hemolytic Assay

To check the cytotoxic effect of Tamoxifen & PEG-Tam-ZnO on blood cells, the hemolytic assay was carried out. For this purpose, 8ml blood was taken out from a healthy donor and mixed well with 16ml PBS. The prepared sample was then centrifugated at 10,000 rpm for 10 min. The supernatant was removed, and RBC's were collected in the pallet, the collected RBC's were washed thrice with PBS at 10,00rpm and were diluted with PBS. The samples were prepared with different concentrations (20, 40, 60, 80,100 µg/ml) using PBS as a solvent and 10µl RBC's solution was added to all the prepared samples. The samples were then incubated at 37 C for 1, 3, 5 hours. 0.5% Triton X-100 served as a positive control while PBS suspension of RBC's was taken as negative control. After complete incubation, the samples were vortexed and centrifugated at 10,000 rpm for 5 mins. Supernatant collected from the samples were removed and their absorbance was analyzed at 562nm. Positive and negative control shows 100% and 0% absorbance respectively. The % hemolysis was calculated as:

Hemolysis (%) = Absorbance of sample – Absorbance of negative control /Absorbance of positive control−Absorbance of negative control* 100

CHAPTER 4

RESULTS

4.1 PHASE 1

4.1.1 UV-VIS ABSORPTION SPECTROSCOPY

The initial confirmation to check the synthesis of zincoxide nanoparticles, is through UV-VIS spectroscopy. The spectrum range for ZnONPs was set at UV-VIS 250 to 600 nm, where it gave a central absorption peak at 340nm, the characteristic peak for ZnONPs. Thus, the presence of ZnONPs was confirmed in the sample. Following is the peak obtained.

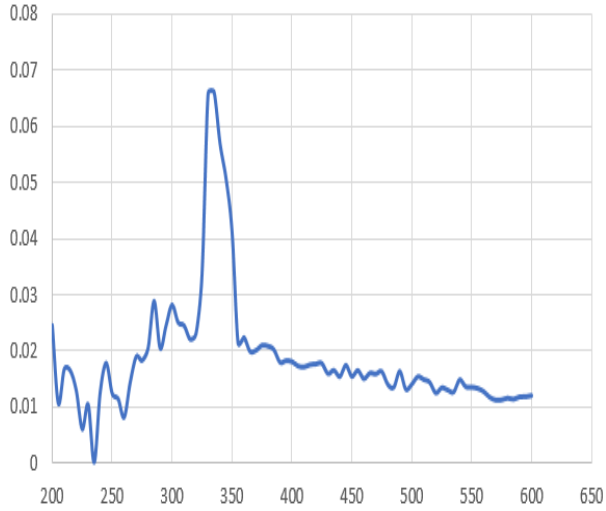


Figure 4.1: UV-VIS Absorption spectra of ZnONPs

The absorption spectra for Tamoxifen are 259 nm while ZnO showed surface Plasmon resonance (SPR) peak at 340 nm. The SPR peak of zincoxide nanoparticles shifted from 340 nm to 315 nm when conjugated with tamoxifen while PEG-Tam-ZnO show strong absorption peak at 300 nm.

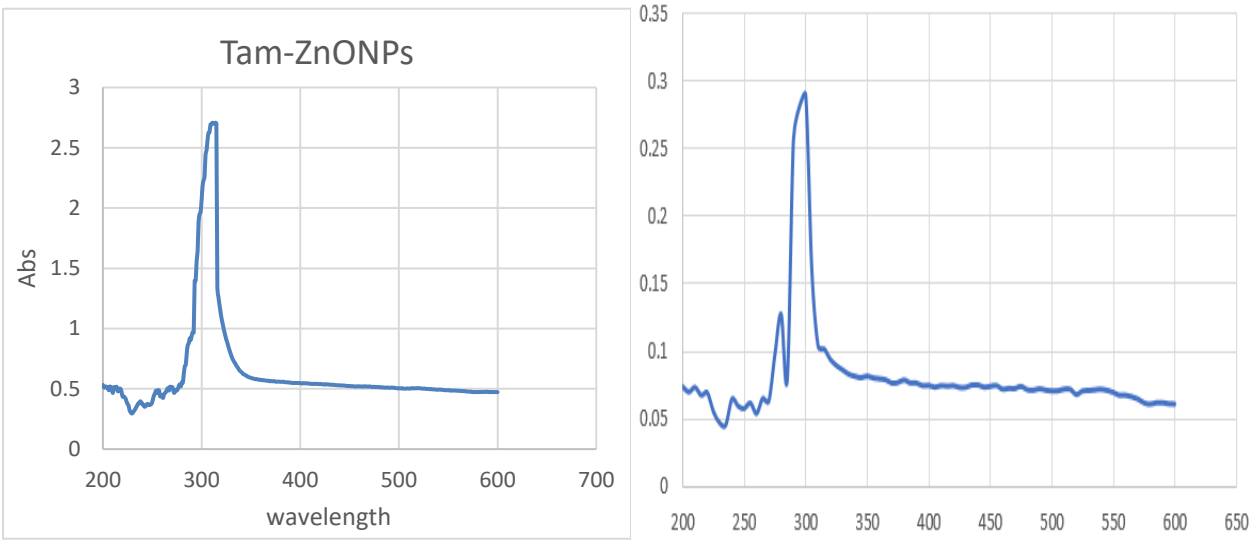


Figure 4.2: Absorption spectra of Tam- ZnONPs and PEG-Tam-ZnO

4.1.2 SCANNING ELECTRON MICROSCOPY (SEM)

Scanning Electron Microscopy was done to determine the particle size and morphology. JSM-6490 SEM model was used. Ideally the particle size should range from 10-100nm. The average particle size of these synthesized ZnONPs was 42nm which was calculated by ImageJ software. SEM also determines the morphology of the sample which was found to be hexagonal.

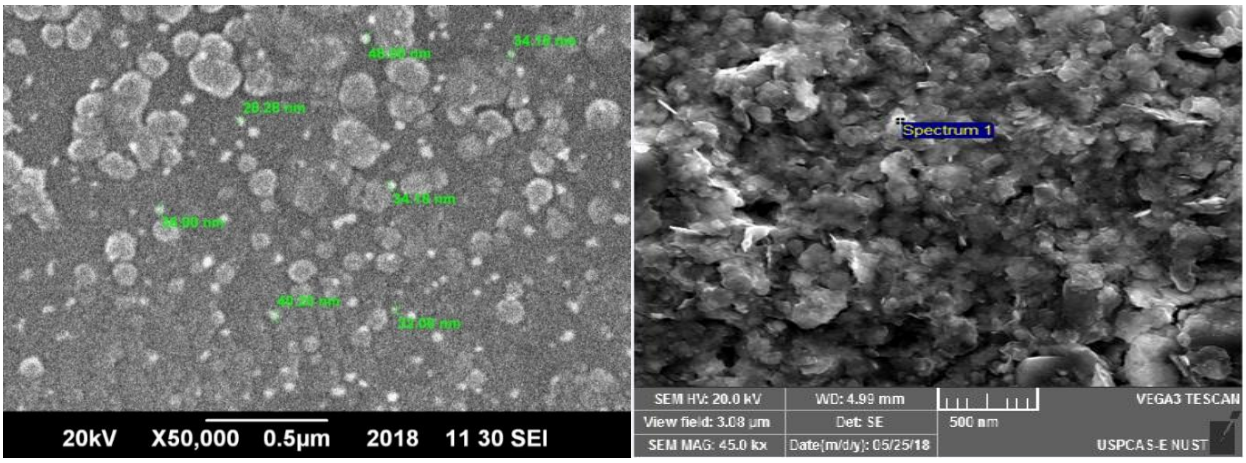


Figure4.3: SEM images ZnONP& Tam-ZnONPs

4.1.3Energy Dispersive X-ray (EDX)

Energy dispersive X-rays (EDX) is an x-rays technique used in combination with SEM, determines the elemental composition of the sample by showing the % of the elements present in the sample. Figure 1 shows the presence of Zn & O while table 1 shows percentages of its components while figure 2 shows the elemental composition of drug loaded nanoparticle while table 2 shows the percentage of tamoxifen loaded ZnO particles. EDX and XRD spectrum confirmed the presence of highly purified ZnO and Tam-ZnO nanoparticles without any impurity.

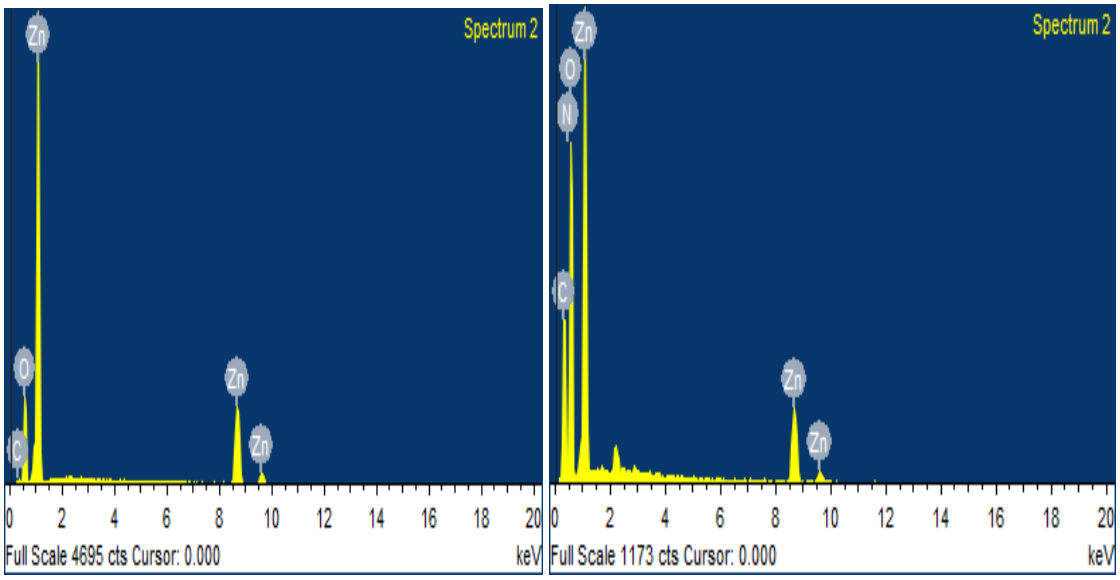


Figure 4.4: Elemental composition of ZnO, Tam-ZnO Nanoparticles

Element	Weight%	Atomic%
C K	4.83	17.34
O K	35.93	59.76
Zn K	59.24	22.90
Totals	100.00	

Table 4.1: % of Elemental composition of ZnO Nanoparticles

Element	Weight%	Atomic%
C K	21.16	40.47
N K	9.60	10.69
O K	47.12	45.94
Zn K	22.12	2.89
Totals	100.00	

Table 4.2: Elemental composition of Tam loaded ZnO nanoparticles

4.1.4 X-ray Diffraction (XRD)

The crystalline nature and hexagonal zincite phase of synthesized ZnO nanoparticles are very well demonstrated by XRD pattern (Fig. 4.5). The reflection lines of ZnO particles in hexagonal zincite phase show peaks at (100), (002), (101), (102), (110), (103), (200), and (201). No characteristic peaks of any impurities were found suggesting good-quality ZnO nanopowders. The intensity of peaks reflects high degree crystallinity of the ZnONPs . The average crystallite size of the ZnONPs was calculated from the XRD line broadening using the Scherrer equation(Patra, Mitra, Debnath, Pramanik, & Goswami, 2014) .

$$B=0.93\lambda/\beta cos\theta$$

So, the average particle size was found to be 42 nm.

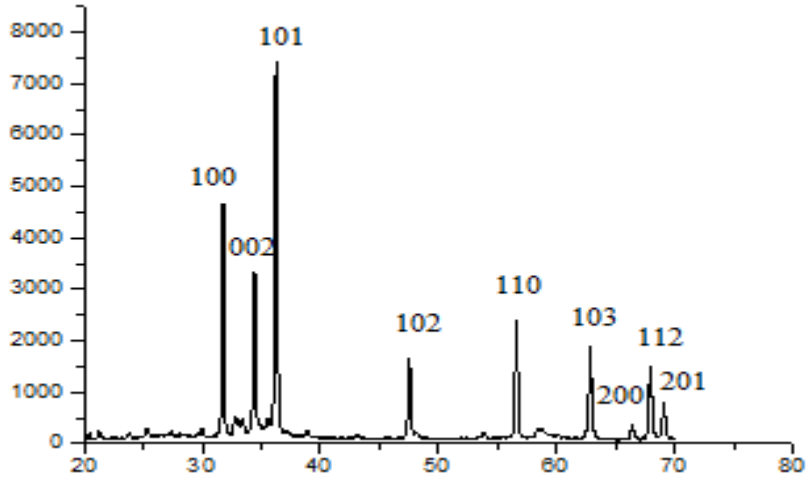


Figure 4.5: X-ray diffraction pattern (XRD) demonstrates the crystalline nature of ZnONPs

4.1.5 Fourier Transform Infrared Spectroscopy

Fourier Transform Infrared Spectroscopy (FTIR Analysis) is an analytical technique used to identify the functional groups and chemical properties of any material through infrared radiations. The FTIR analysis of the sample was done in powdered form. Synthesis of ZnONPs

and successful tamoxifen conjugation with ZnONPs was confirmed by FTIR spectra (figure 4.6 a). ZnONPs shows characteristic peaks at 3400 cm−1, 1632 cm−1 and lower bands justified O–H, C=O (carbonyl) and Zn–O stretching, respectively. The peaks obtained in the region of 1400 cm−1 and 1462 cm−1 were due to O–H and N–H bending, although the amount of N–H group was less (Patra et al., 2014).

The characteristics peaks of tamoxifen are present at 3441.29 cm−1(−OH), 1428.62 cm−1 (C–O–C) and 1010.57 cm−1 (N–H) shows the (Figure 4.6 b). In comparison, the peak at 3441.29 cm−1 of tamoxifen loaded ZnONPs was much wider and broad than ZnONPs, indicating the stretching of hydrogen bonds. The peak at 1632.78 cm−1 in ZnONPs was found to be shifted in tamoxifen loaded ZnONPs with a peak at 1428.64 cm^{-1}(Heidari Majd et al., 2013)

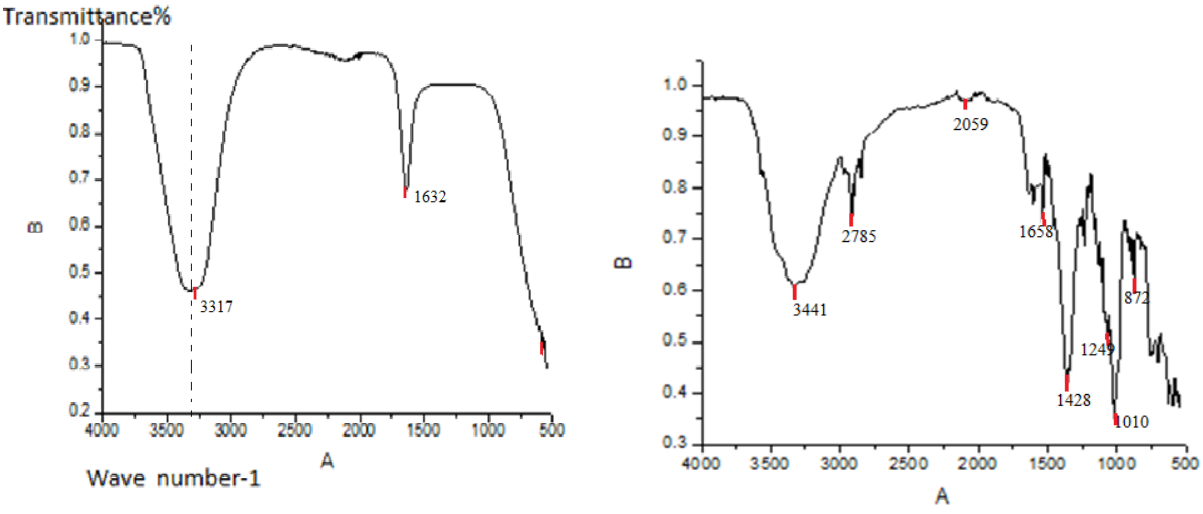


Figure 4.6: (a) FTIR Spectra of ZnO & (b) Tam-ZnONPs

4.2 PHASE II

4.2.1 INVITRO STABILITY STUDIES

The invitro stability of ZnO nanoparticles was tested through salt & temperature test. These tests check the potential of nanoparticles for in vivo studies. For salt test, three different concentrations (2M, 3M &5M) of the salt were prepared. For temperature testing, the samples were viewed at room temperature and at 100 degrees after 24 hours, 48 hours, 72 hours, 1 week and 2 weeks respectively.

4.2.2 Salt Test

Figure 4.11 showed the effect of various concentration of NaCl (2 - 4M) on SPR peak of ZnONPs. For 24-48 hours, no effect on SPR peak of ZnONPs was noticed by increasing the concentration of salt from 2M to 4M, but considerable decrease in λmax value was recorded due to higher concentration of salt for longer time resulting in decreasing the stability of nanoparticles. This absorbance value of the nanoparticles decrease is due to the aggregation of chloride (Cl-1) ions. The results showed that ZnONPs are more stable in water or ethanolic solution than in salt solution, as far as long-term stability is concerned.

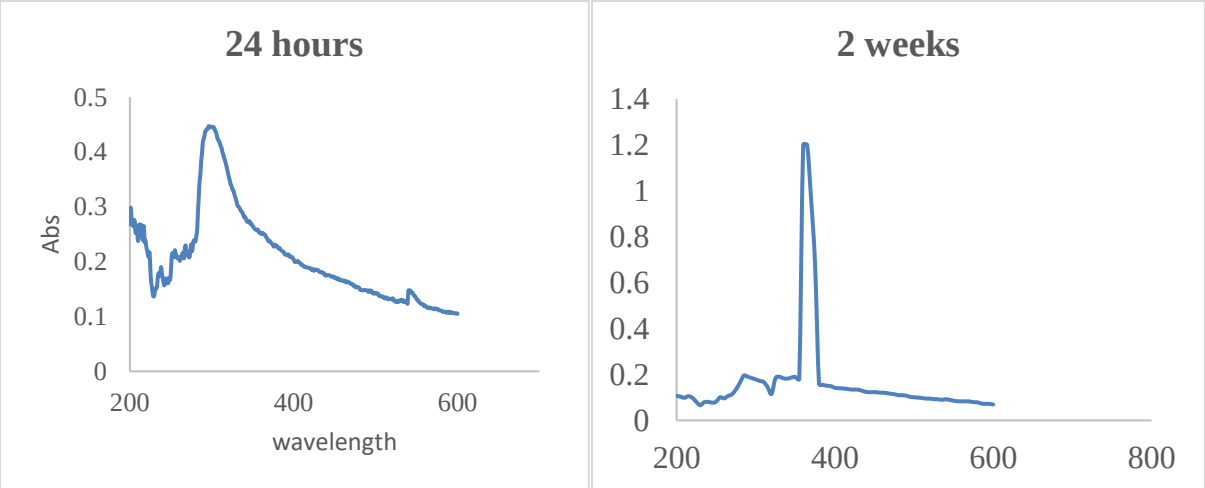


Figure 4.7: Salt effect on the stability of ZnONPs at different intervals

4.2.3 Temperature Test

Figure 4.12 showed the effect of temperature on SPR peak of ZnONPs. The results showed that the effect of temperature on ZnO nanoparticles is negligible, with no shift in plasmon peak while minute reduction in absorbance value. No signs of precipitation were observed.

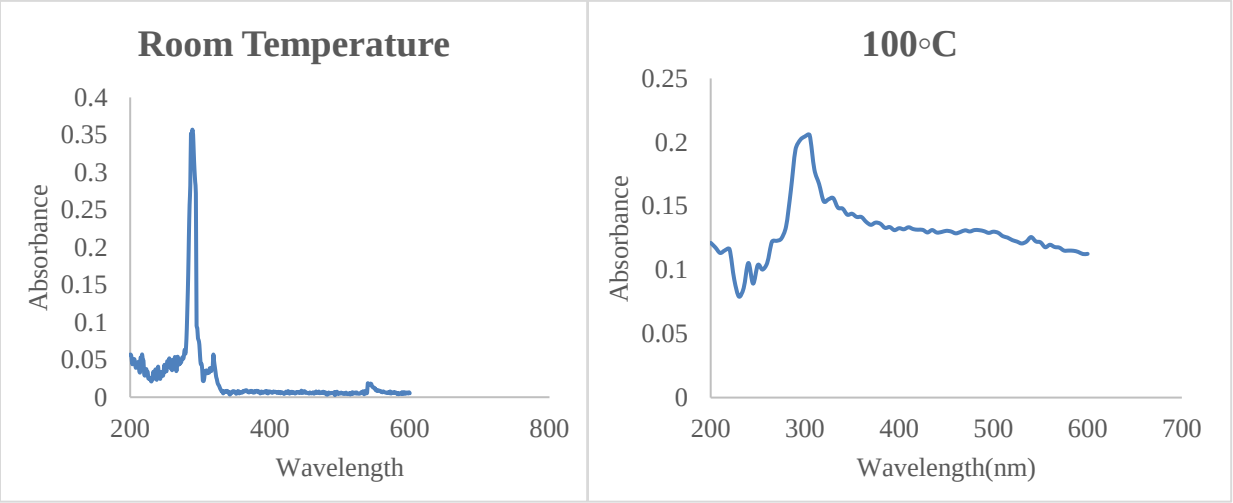


Figure 4.8: Effect of difference in temperature on the ZnONPs stability

4.3 DRUG LOADING EFFICIENCY

UV-Vis spectra of supernatant after the centrifugation of resuspended Tam-ZnO were found to be 315 nm respectively (Figure 4.6). The SPR peak of supernatant from Tam-ZnO resembles the SPR peak of Tam. Different molar concentration of Tamoxifen was prepared to determine the amount of unbound drug removed in supernatant after centrifugation, and their UV-Vis spectra were recorded. The SPR peak obtained was at 254 nm. The molar concentrations were plotted against their absorbance values and the slope obtained was 6.735 (Figure 4.5B).

From the value of slope, we can determine the concentration of unbound drug present in the supernatant.

According to Lambert-Beer Law equation: A = EcL

So, according to this equation the amount of unbound drug removed from 13mg Tam-ZnO was 6.179mg. For calculating the % drug (Tam) loading in purified Tam-ZnONPs the equation used was:

Drug loading (%)= Total amount of Drug - amount of Drug in supernatant /Total amount of Drug * 100 (Muthukumar, Prabhavathi

From this equation the amount of tamoxifen bounded with ZnO was calculated to be 48.06 %.

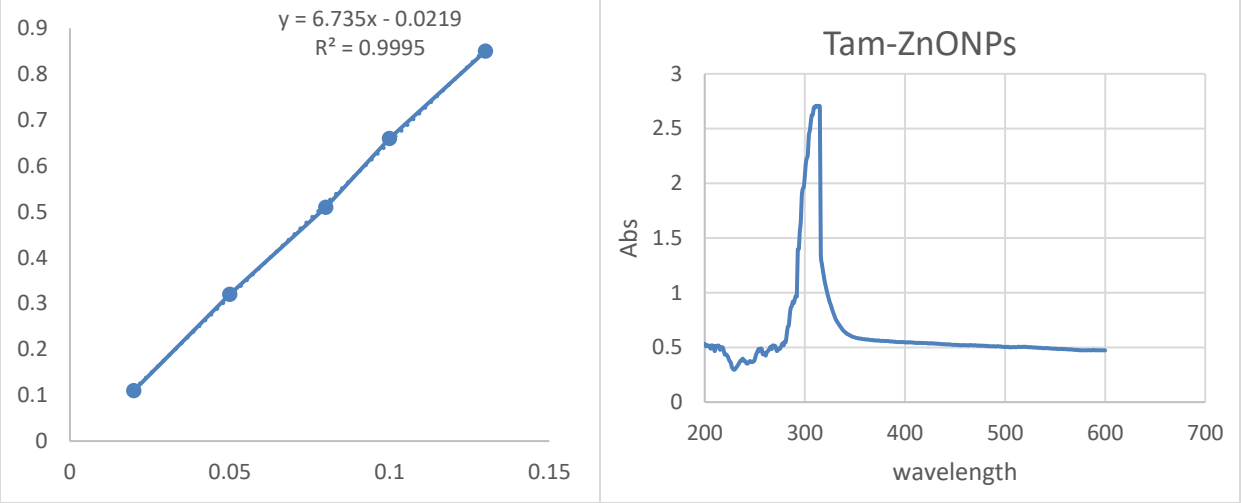


Figure 4.9: (a) Increase in absorbance value due to increase in concentration

(b) Absorption spectra of Tam loaded ZnONPs

4.4 CYTOTOXICITY ASSAYS

4.4.1 MTT Assay

MTT assay or cell viability assay was done to find out the count of living or dead cells in a sample. The activity of nano formulation i-e PEG-Tam-ZnO was checked through MTT assay

that determine the cell viability effect. Different drug concentrations were prepared for this purpose and were studied on MCF-7 cell lines. The results showed that with increasing PEG coated tamoxifen loaded ZnONPs concentration, the number of viable cells decreased. So, for 100µg concentration of the PEG-Tam-ZnO, the number of viable cells considerably decreased. The results show that, by regulating the dose concentration, this drug formulation can be used for cancer treatment in nearby future. Following results were obtained at the end of the assay.

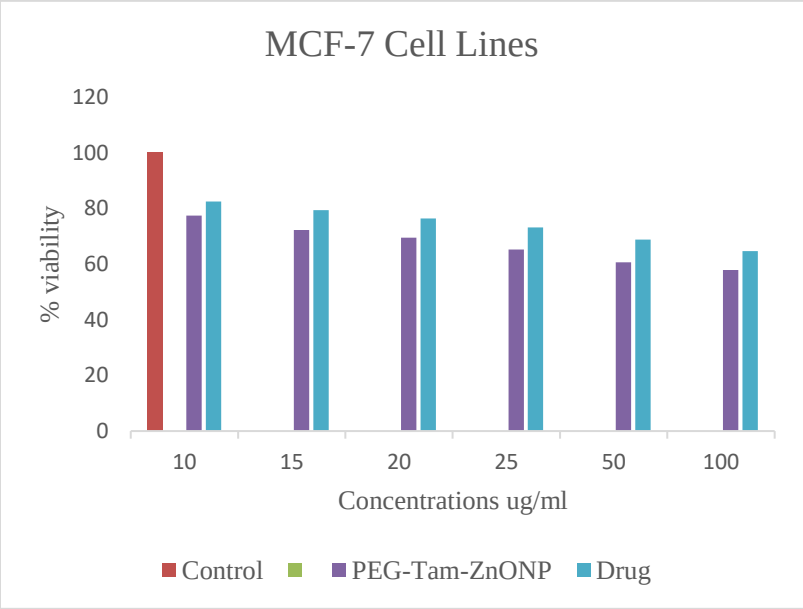


Figure 4.10: MTT assay for determination of cell viability. With increasing PEG-Tam-ZnONPs concentration the number of viable cells considerably decreases

4.4.2 HEMOLYTIC ASSAY

Hemolytic assay gave the clear indication of the extent of toxicity caused by the synthesized formulation on blood cells. For this purpose, various concentrations (1, 5, 10, 20, 50, 100, and 150 ug/mL) of tamoxifen and PEG-Tam-ZnONPs were tested on red blood cells quantitatively. The results of hemolytic assay clearly indicate that PEG-Tam-ZnONPs are more hemocompatible as compared to tamoxifen when used alone and hemolytic behavior of PEG-Tam-ZnONPs increased gradually with increasing concentration So, the % hemolysis was found

to be less than 7 % and the results indicate that these particles are more hemocompatible and according to ISO/TR 7406 this ratio is considered as the safest value.

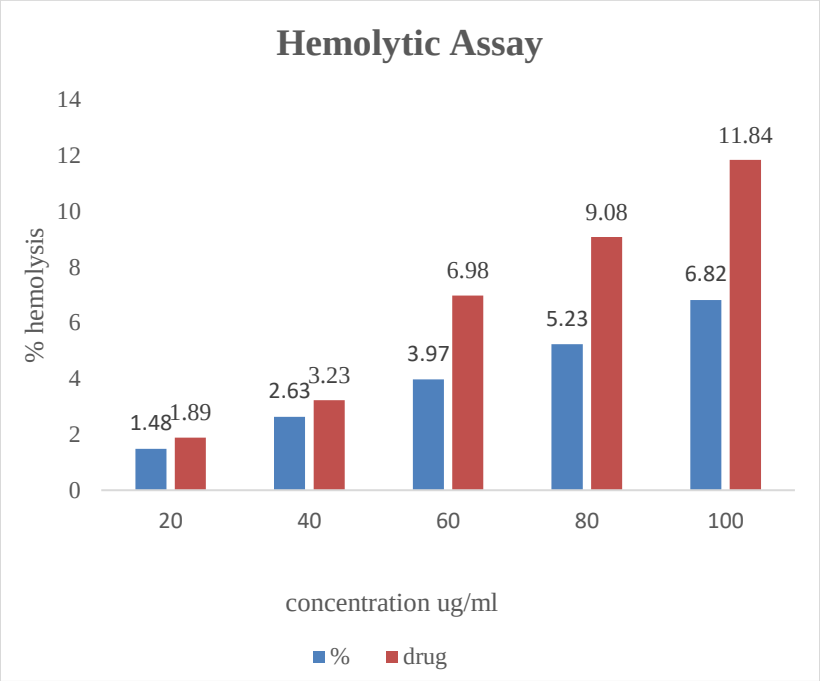


Figure 4.11: Indicate the extent of hemolysis caused by Tam and PEG-Tam-ZnO. It shows a significant difference in hemolysis % when ZnONPs were coated with Tamoxifen and PEG.

4.4.3 DPPH

The antioxidant activity of the PEG-Tam-ZnO was checked through DPPH assay by preparing its different concentrations. The reaction mixture was dark purple initially which then changes to light yellow marking the end of the reaction. Ascorbic acid was used as a standard. As DPPH is highly sensitive to light so, the experiment was carried out in dark room. At highest concentration i-e 200ug/ml DPPH assay shows the maximum antioxidant activity.

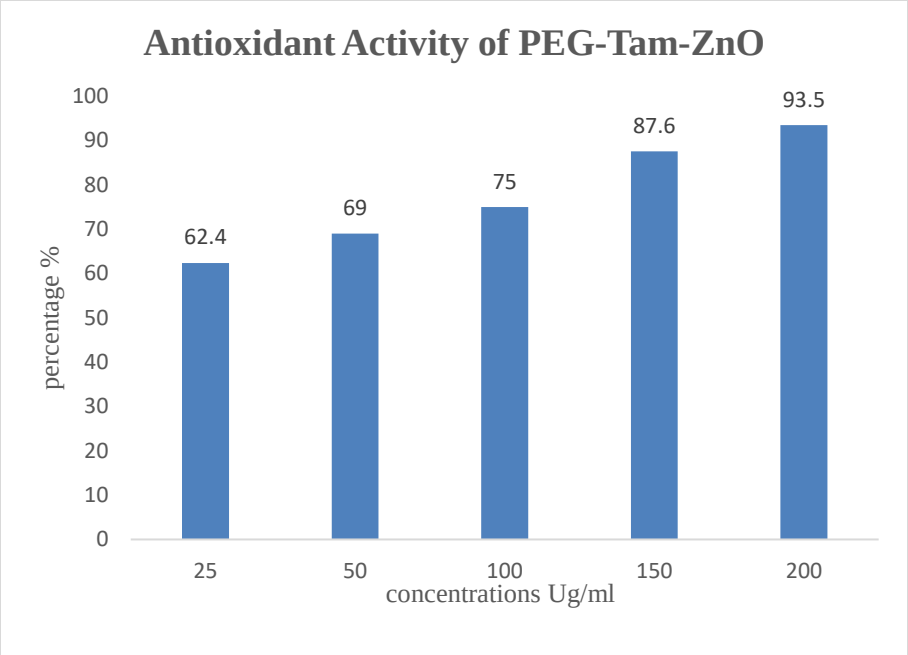


Figure 4.12: Antioxidant activity and its variation with different concentrations of samples

DISCUSSION

Zinc being an essential trace element and the main component of various enzyme systems, have an important role in proteins and nucleic acid synthesis, metabolism, hematopoiesis, and neurogenesis. The nano sized ZnO particles, helps in easy absorption of zinc by the body. ZnONPs were chemically synthesized by wet chemical precipitation method under controlled environmental condition using zinc nitrate tetra hydrate and sodium hydroxide as a precursor. The low production cost, high homogeneity, easy synthesis and controlled delivery mechanism are the main advantages for using chemical precipitation method. However, the surface effect of nanoparticles prepared by chemical precipitation method, causes the particles to agglomerates easily (Palanikumar et al., 2013). The whitish colored nano sized particles obtained were characterized for their optical and nanostructural properties. UV-vis absorption spectroscopy, a widely used technique to examine the optical properties of nanosized particles, shows an absorption spectrum of ZnONP's in the range of 300-380nm. The surface plasmon peak (SPK) of UV-VIS spectroscopy confirms the synthesis of ZnO nanoparticles, showing absorption spectra at 340nm. The average particle size was found to be 42 nm and was hexagonal in shape. Current XRD results of synthesized ZnONPs confirmed their hexagonal zincite phase with crystalline nature (Zhang et al., 2012). The XRD and EDX spectrum confirmed the presence of highly purified, crystalline and face-centered cubic ZnO and Tam-ZnO structure. All these characterization techniques confirmed the presence of pure zincoxide nanoparticles with the optimized size and properties.

After conjugating tamoxifen with zincoxide nanoparticles and coating it with PEG, the cytotoxic potential and cell viability (%) of the prepared samples were checked against MCF-7 breast cancer cell line. The pegylated nanoparticles showed higher anticancer activity and the IC50 for PEG-Tam-ZnO was less than that Tamoxifen and Zincoxide (Suk, Xu, Kim, Hanes, & Ensign, 2016).The rate of cell viability significantly dependent upon both sample concentration and culture time. The results of MTT assay showed that with increasing PEG-Tam-ZnO concentration, the number of viable cells considerably decreases and significantly inhibit cell

growth and induces cell death. The anticancer activity reveals the effect of efficient drug delivery system mediated by pegylation and additional toxicity offered by incorporated zincoxide.

Several chemotherapeutic agents, radiations and cytokines induces apoptotic response by triggering ROS generation through the increased oxidative stress in a cell. Present study also shows that PEG-Tam-ZnO produces strong antioxidant response through the generation of ROS, causing the activation of ASK-JNK pathway, disrupting the membrane potential and ultimately resulted in apoptosis. Literature reported that PEG-TAM-ZnONPs have certain cytotoxicity in cancer cells mainly due to higher intracellular release of dissolved zinc ions, followed by increased ROS induction and induced cancer cell death via apoptosis signaling pathway (Palit et al., 2015). Studies showed that Drug loaded ZnO NPs have been observed to have strong cytotoxic effect against MCF-7 cells, mainly due to apoptosis more than cell cycle arrest. PEG-TAM-ZnO NPs-induces apoptosis through extrinsic and intrinsic apoptotic pathways, downregulation of antiapoptotic genes like Bcl-2, AKT1, and JERK/2 and up regulation of proapoptotic genes like p21, p53, JNK, and Bax.

For this study we compare the cytotoxic effect of Tamoxifen and PEG-Tam-ZnO on blood cells. Literature reported that biologically compatible polymer (PEG) coated zincoxide nanoparticles, are assumed to be nontoxic due to their inherent nature. The results of hemolytic assay clearly indicate that PEG-Tam-ZnONPs are more hemocompatible as compared to tamoxifen when used alone. Hemolytic behavior of PEG-Tam-ZnONPs increased gradually with increasing the sample concentration and their % hemolysis was found to be less than 7 %. According to ISO/TR 7406 this ratio is considered as the safest value and the particles are considered as hemocompatible.

CONCLUSION

From the above experimental study carried out, it was concluded that zinc oxide nanoparticles could be easily synthesized by chemical precipitation method, have strong cytotoxic and potential effect on MCF-7 breast cell lines. For targeted drug delivery system, these nano formulations act as a vector and are biologically more compatible, strong antioxidant and low hemolytic activity as compared to drug when used alone. These nanoparticles increased the expression of caspase-3 and proteins, and tailoring of biologically compatible polymer, PEG and Tamoxifen loading would make it even more target specific for cancerous cells with minimal exposure to normal cells and enhanced activity. Further studies are needed to uncover different onco- proteins and the effect of this nano formulation on the expression mechanism of these proteins.

REFERENCES

Anderson, W. F., Chatterjee, N., Ershler, W. B., & Brawley, O. W. (2002). Estrogen receptor breast cancer phenotypes in the Surveillance, Epidemiology, and End Results database. *Breast Cancer Research and Treatment*, *76*(1), 27–36. https://doi.org/10.1023/A:1020299707510

Bai, D. P., Zhang, X. F., Zhang, G. L., Huang, Y. F., & Gurunathan, S. (2017). Zinc oxide nanoparticles induce apoptosis and autophagy in human ovarian cancer cells. *International Journal of Nanomedicine*, *12*, 6521–6535. https://doi.org/10.2147/IJN.S140071

Banu, H., Sethi, D. K., Edgar, A., Sheriff, A., Rayees, N., Renuka, N., … Vasanthakumar, G. (2015). Doxorubicin loaded polymeric gold nanoparticles targeted to human folate receptor upon laser photothermal therapy potentiates chemotherapy in breast cancer cell lines. *Journal of Photochemistry and Photobiology B: Biology*, *149*, 116–128. https://doi.org/10.1016/j.jphotobiol.2015.05.008

Bao, J., Shi, Z., & Xiang, H. (2012). Dynamic Responses of a Structure with Periodic Foundations. *Journal of Engineering Mechanics*, *138*(7), 761–769. https://doi.org/10.1061/(ASCE)EM.1943-7889.0000383

Chivukula, V., Ciplys, D., Shur, M., & Dutta, P. (2010). ZnO nanoparticle surface acoustic wave UV sensor. *Applied Physics Letters*, *96*(23), 2008–2011. https://doi.org/10.1063/1.3447932

Cui, Y., Parra, I., Zhang, M., Hilsenbeck, S. G., Tsimelzon, A., Furukawa, T., … Fuqua, S. A. W. (2006). Elevated expression of mitogen-activated protein kinase phosphatase 3 in breast tumors: A mechanism of tamoxifen resistance. *Cancer Research*, *66*(11), 5950–5959. https://doi.org/10.1158/0008-5472.CAN-05-3243

Economopoulou, P., Dimitriadis, G., & Psyrri, A. (2015). Beyond BRCA: New hereditary breast

cancer susceptibility genes. *Cancer Treatment Reviews*, *41*(1), 1–8. https://doi.org/10.1016/j.ctrv.2014.10.008

Façades, F. O. R. H. (2004). Meeting the demand for high-efficiency façades, *4*(April), 61–66. https://doi.org/10.1038/nr1317

Girigoswami, K., Viswanathan, M., Murugesan, R., & Girigoswami, A. (2015). Studies on polymer-coated zinc oxide nanoparticles: UV-blocking efficacy and in vivo toxicity. *Materials Science and Engineering C*, *56*, 501–510. https://doi.org/10.1016/j.msec.2015.07.017

Gupta, J., Bhargava, P., & Bahadur, D. (2014). Methotrexate conjugated magnetic nanoparticle for targeted drug delivery and thermal therapy. *Journal of Applied Physics*, *115*(17). https://doi.org/10.1063/1.4866080

Hackenberg, S., Scherzed, A., Gohla, A., Technau, A., Froelich, K., Ginzkey, C., … Kleinsasser, N. (2014). Nanoparticle-induced photocatalytic head and neck squamous cell carcinoma cell death is associated with autophagy. *Nanomedicine*, *9*(1), 21–33. https://doi.org/10.2217/nnm.13.41

Heidari Majd, M., Asgari, D., Barar, J., Valizadeh, H., Kafil, V., Abadpour, A., … Omidi, Y. (2013). Tamoxifen loaded folic acid armed PEGylated magnetic nanoparticles for targeted imaging and therapy of cancer. *Colloids and Surfaces B: Biointerfaces*, *106*, 117–125. https://doi.org/10.1016/j.colsurfb.2013.01.051

Higgins, M. J., & Stearns, V. (2009). Understanding resistance to tamoxifen in hormone receptor-positive breast cancer. *Clinical Chemistry*, *55*(8), 1453–1455. https://doi.org/10.1373/clinchem.2009.125377

Hoskins, J. M., Carey, L. A., & McLeod, H. L. (2009). CYP2D6 and tamoxifen: DNA matters in breast cancer. *Nature Reviews Cancer*, *9*(8), 576–586. https://doi.org/10.1038/nrc2683

Hurtado, A., Holmes, K. A., Geistlinger, T. R., Hutcheson, I. R., Nicholson, R. I., Brown, M., … Carroll, J. S. (2008). Regulation of ERBB2 by oestrogen receptor-PAX2 determines response to tamoxifen. *Nature*, *456*(7222), 663–666. https://doi.org/10.1038/nature07483

Jiang, J., Pi, J., & Cai, J. (2018). The Advancing of Zinc Oxide Nanoparticles for Biomedical Applications. *Bioinorganic Chemistry and Applications*, *2018*. https://doi.org/10.1155/2018/1062562

Jokerst, J. V, Lobovkina, T., Zare, R. N., & Gambhir, S. S. (2011). NIH Public Access. *Nanomedicine*, *6*(4), 715–728. https://doi.org/10.2217/nnm.11.19.Nanoparticle

Khalil, M. I., Al-Qunaibit, M. M., Al-zahem, A. M., & Labis, J. P. (2014). Synthesis and characterization of ZnO nanoparticles by thermal decomposition of a curcumin zinc complex. *Arabian Journal of Chemistry*, *7*(6), 1178–1184. https://doi.org/10.1016/j.arabjc.2013.10.025

Martínez, A., Benito-Miguel, M., Iglesias, I., Teijón, J. M., & Blanco, M. D. (2012). Tamoxifen-loaded thiolated alginate-albumin nanoparticles as antitumoral drug delivery systems. *Journal of Biomedical Materials Research - Part A*, *100 A*(6), 1467–1476. https://doi.org/10.1002/jbm.a.34051

McGuire, S. (2016). International Agency for Research on cancer. World Health Organizarion. World cancer report 2014. World cancer Report 2014. *Adv. Nutr.*, *7*, 418–419. https://doi.org/10.3945/an.116.012211.Genesis

Mishra, Y. K., Chakravadhanula, V. S. K., Hrkac, V., Jebril, S., Agarwal, D. C., Mohapatra, S., … Adelung, R. (2012). Crystal growth behaviour in Au-ZnO nanocomposite under different annealing environments and photoswitchability. *Journal of Applied Physics*, *112*(6). https://doi.org/10.1063/1.4752469

Mody, V. V, Nounou, M. I., & Bikram, M. (2009). Novel nanomedicine-based MRI contrast agents for gynecological malignancies ☆. *Advanced Drug Delivery Reviews*, *61*(10), 795–807. https://doi.org/10.1016/j.addr.2009.04.020

Mody, V., Siwale, R., Singh, A., & Mody, H. (2010). Introduction to metallic nanoparticles. *Journal of Pharmacy and Bioallied Sciences*, *2*(4), 282. https://doi.org/10.4103/0975-7406.72127

Moghimipour, E., Rezaei, M., Ramezani, Z., Kouchak, M., Amini, M., Angali, K. A., …

Handali, S. (2018). Transferrin targeted liposomal 5-fluorouracil induced apoptosis via mitochondria signaling pathway in cancer cells. *Life Sciences*, *194*(October 2017), 104–110. https://doi.org/10.1016/j.lfs.2017.12.026

Palanikumar, L., Ramasamy, S., Hariharan, G., & Balachandran, C. (2013). Influence of particle size of nano zinc oxide on the controlled delivery of Amoxicillin. *Applied Nanoscience*, *3*(5), 441–451. https://doi.org/10.1007/s13204-012-0141-5

Palit, S., Kar, S., Sharma, G., & Das, P. K. (2015). Hesperetin induces apoptosis in breast carcinoma by triggering accumulation of ROS and activation of ASK1/JNK pathway. *Journal of Cellular Physiology*, *230*(8), 1729–1739. https://doi.org/10.1002/jcp.24818

Patra, P., Mitra, S., Debnath, N., Pramanik, P., & Goswami, A. (2014). Ciprofloxacin conjugated zinc oxide nanoparticle: A camouflage towards multidrug resistant bacteria. *Bulletin of Materials Science*, *37*(2), 199–206. https://doi.org/10.1007/s12034-014-0637-6

Peng, S., Lü, B., Ruan, W., Zhu, Y., Sheng, H., & Lai, M. (2011). Genetic polymorphisms and breast cancer risk: Evidence from meta-analyses, pooled analyses, and genome-wide association studies. *Breast Cancer Research and Treatment*, *127*(2), 309–324. https://doi.org/10.1007/s10549-011-1459-5

Plevová, P., Novotný, J., Petráková, K., Palácová, M., Kalábová, R., Schneiderová, M., & Foretová, L. (2009). [Hereditary breast and ovarian cancer syndrome]. *Klinická Onkologie : Casopis Ceské a Slovenské Onkologické Spolecnosti*, *22 Suppl*(1), S8–S11. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/19309638\nhttp://www.ncbi.nlm.nih.gov/pubmed/19764385

Rasmussen, J. W., Martinez, E., Louka, P., & Wingett, D. G. (2010). Zinc oxide nanoparticles for selective destruction of tumor cells and potential for drug delivery applications. *Expert Opinion on Drug Delivery*, *7*(9), 1063–1077. https://doi.org/10.1517/17425247.2010.502560

Romond, E. H., Perez, E. A., Bryant, J., Suman, V. J., Geyer, C. E., Davidson, N. E., … Wolmark, N. (2005). Trastuzumab plus Adjuvant Chemotherapy for Operable HER2-Positive Breast Cancer. *New England Journal of Medicine*, *353*(16), 1673–1684.

https://doi.org/10.1056/NEJMoa052122

Sam, K. (2014). The “Other” Breast Cancer Genes. *Science*, *343*(6178), 1457–1459. https://doi.org/10.1126/science.343.6178.1457

Slamon, D. J., Leyand-Jones, B., Shak, S., Fuchs, H., Paton, V., Bajamonde, A., … Norton, L. (2001). Numb Er 11 Use of Chemotherapy Plus a Monoclonal Antibody Against Her2. *English Journal*, *344*(11), 783–792. https://doi.org/10.1056/NEJM200103153441101

Stephens, P. J., Tarpey, P. S., Davies, H., Van Loo, P., Greenman, C., Wedge, D. C., … Stratton, M. R. (2012). The landscape of cancer genes and mutational processes in breast cancer. *Nature*, *486*(7403), 400–404. https://doi.org/10.1038/nature11017

Stowe, D. F., & Camara, A. K. S. (2009). Mitochondrial Reactive Oxygen Species Production in Excitable Cells: Modulators of Mitochondrial and Cell Function. *Antioxidants & Redox Signaling*, *11*(6), 1373–1414. https://doi.org/10.1089/ars.2008.2331

Stuckey, A. R., & Onstad, M. A. (2015). Hereditary breast cancer: An update on risk assessment and genetic testing in 2015. *American Journal of Obstetrics and Gynecology*, *213*(2), 161–165. https://doi.org/10.1016/j.ajog.2015.03.003

Suk, J. S., Xu, Q., Kim, N., Hanes, J., & Ensign, L. M. (2016). PEGylation as a strategy for improving nanoparticle-based drug and gene delivery. *Advanced Drug Delivery Reviews*, *99*, 28–51. https://doi.org/10.1016/j.addr.2015.09.012

Teft, W. A., Mansell, S. E., & Kim, R. B. (2011). Endoxifen, the active metabolite of tamoxifen, is a substrate of the efflux transporter P-glycoprotein (multidrug resistance 1). *Drug Metabolism and Disposition*, *39*(3), 558–562. https://doi.org/10.1124/dmd.110.036160

Thakkar, J. P., & Mehta, D. G. (2011). A Review of an Unfavorable Subset of Breast Cancer: Estrogen Receptor Positive Progesterone Receptor Negative. *The Oncologist*, *16*(3), 276–285. https://doi.org/10.1634/theoncologist.2010-0302

Varadharajaperumal, P., Subramanian, B., & Santhanam, A. (2017). Biopolymer mediated nanoparticles synthesized from Adenia hondala for enhanced tamoxifen drug delivery in breast cancer cell line. *Advances in Natural Sciences: Nanoscience and Nanotechnology*,

8(3), aa7253. https://doi.org/10.1088/2043-6254/aa7253

Wahab, R., Dwivedi, S., Umar, A., Singh, S., Hwang, I. H., Shin, H. S., … Kim, Y. S. (2013). ZnO nanoparticles induce oxidative stress in cloudman S91 melanoma cancer cells. *Journal of Biomedical Nanotechnology*, *9*(3), 441–449. https://doi.org/10.1166/jbn.2013.1593

Yahya, N. B., Daud, H., Tajuddin, N. A., Daud, H. M., Shafie, A., & Puspitasari, P. (2010). Application of ZnO Nanoparticles EM Wave Detector Prepared by Sol-Gel and Self-Combustion Techniques. *Journal of Nano Research*, *11*, 25–34. https://doi.org/10.4028/www.scientific.net/JNanoR.11.25

Zhang, Y., Ram, M. K., Stefanakos, E. K., & Goswami, D. Y. (2012). Synthesis, characterization, and applications of ZnO nanowires. *Journal of Nanomaterials*, *2012*. https://doi.org/10.1155/2012/624520