In-vitro and In-vivo Anticancer Activity of Pegylated Methotrexate Loaded Silver Nanoparticles

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I certify that this research work titled "*In-vitro and In-vivo Anticancer Activity of Pegylated Methotrexate Loaded Silver Nanoparticles"* is my own work. The work has not been presented elsewhere for assessment. The material that has been used from other sources it has been properly acknowledged / referred.

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This Thesis Is Dedicated To My Exceptional Parents For Making Me Who I Am and My Husband, For Supporting Me All The Way Through Each and Every Step of my Life.

Abstract

Introduction: Chemotherapeutics is the first line of treatment being used worldwide by millions of people to combat cancer. Due to their specificity, insolubility, large molecular size and most importantly their adverse side effects the chemotherapeutic drugs are conjugated with silver nanoparticles. Methotrexate is one of the most widely used drugs for the treatment of many forms of cancer, but methotrexate (MTX) has poor tumor retention ability due to its high water solubility, which likely contributes to its slow or poor therapeutic response in patients. In this study, MTX was bound to silver nanoparticles by chemical reduction method and then capped with PEG to make it biocompatible. The invitro anti-tumor activity and biocompatability of the conjugated nanoparticles (AgMTX) was checked after pegylation . The *in-vivo* anticancer activity of the nanoparticles was determined by developing a two-stage tumor model in Balb/c mice by chemical carcinogenesis.

Results: The characterization of Ag-MTX nanoparticles was done through XRD, zeta potential and particle size evaluation, and drug release efficiency. PEG-AgMTX showed significantly low hemolytic behavior in comparison to AgMTX and MTX. The IC50 value for anticancer activity of PEG-Ag-MTX nanoparticles was less than that of the methotrexate alone showing them more effficient than the original drug. The results of *in-vivo* studies indicate that the mice treated with PEG-AgMTX showed relatively highest reduction in their tumor volumes, and survival rates.

Conclusion: Tailoring of PEG-AgMTX would make them more targets specific with minimal exposure to normal cells with enhanced activity so as small amount of drug causes more toxicity in cancerous cells. It is therefore worthy to investigate *in-vivo* antitumor activity as well the biodistribution of these nanoparticles.

Key Words: *Methotrexate, Silver nanoparticles, Anticancerous drug, Chemotherapeutics, Pegylatio, Tumor Model, Chemical Carcinigenesis, Histological Examination*

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CHAPTER 1: INTRODUCTION

1.1 Objective

The research work in this dissertation has been presented in two parts. The first part of the research focuses on the formulation and characterization of a novel anti-cancer drug delivery vehicle, which is targeted to the specific cancer sites. The drug selected for the study is a widely used anticancer drug methotrexate, and its nanoparticles are synthesized according to chemical reduction method, which has already been reported in our established work. Then different aspects of the nanoparticle formulation are characterized using standard tests, ultimately making them equipped for the *in-vitro* and *in-vivo* analysis.

The second part of this research is more elaborate, which focuses on the *in-vitro* and *in-vivo* analysis of anticancer activity of our nanoparticles. The *in-vitro* treatment response of the methotrexate silver nanoparticles was determined through cytotoxicity assay and biocompatibility assay, so that their efficacy and biocompatibility in cultured cells could be observed. The results of these *in-vitro* assays lead us to calculate the precise dosage and amount of nanoparticle formulation employed for *in-vivo* administration. Meanwhile, for in-vivo testing, we developed an efficient tumor model by chemical carcinogenesis in mice, in order to evaluate the role of our anti-cancer drug delivery vehicle in tumor regression and its pharmacokinetics in an *in vivo* system. The *in-vivo* analysis in animal models is very important in regard to the study of various pharmacokinetic parameters and interactions of the drug in a living system, which may vary widely from the results of characterization tests and *in-vitro* assays performed earlier. In this way, the investigation of the anticancer activity of nanoparticles in a well-established tumor model is the prime focus of our research and is the most important step in taking these nanoparticles further on the way to preclinical trials.

1.2 Nanotechnology: Introduction to a New Era

Nanotechnology refers to the manipulation of matter on an atomic, molecular, and supramolecular scale, with at least one dimension sized from 1 to 100 nanometers (Drexler and Eric 1986). The concepts that seeded nanotechnology were first discussed in 1959 by renowned Noble laureate Richard Feynman in his lecture ‘There's Plenty of Room at the Bottom’, in which

he described the possibility of synthesis via direct manipulation of atoms (Drexler and Eric 1992). Japanese researcher Norio Taniguchi, in 1970 (Corbett, McKeown et al. 2000), defined nanotechnology for the first time as "Nanotechnology majorly consist of processing of, seperation of, deformation and consolidation of materials by atom or by molecules". In 1980, K. Eric Drexler worked on promotion of technological significance at nano level.

1.2.1 Advancement of Nanotechnology

The application of nanotechnology for obtaining novel products has been going on since the ancient civilization. The ancient Romans used to colour glass with shades of mauve and yellow by using different concentrations of gold and silver (Daniel and Astruc 2004). Gold and silver nanoparticles were also used for aesthetic purpose in the famous Lycurgus cup from the 4th century, which is now placed in the British museum (Barber and Freestone 1990). Similarly, in Middle Ages, colloidal silver and gold nanoparticles were used to produce bright colored stained windows, mostly red and purple in european cathedrals. For example, in Notre Dame, the red and purple color of the rose window of cathedrals is due to presence of gold nanoparticles (Dreaden, Alkilany et al. 2012). The technique of nanoparticle synthesis was studied in the 9th century by Muslim scientists, who carried out the reduction of metal oxides upon heating at high temperature which were already deposited on ceramic surfaces (Padeletti and Fermo 2003; Daniel and Astruc 2004). This technique of glass coloring was further refined in 15th and 17th century by using precipitates of different colloids added to the glass (Daniel and Astruc 2004). The first ever documented chemical synthesis of metal nanoparticles was performed in 1857 by Michael Faraday (Faraday 1857), who reduced solution of chloroauric acid with carbon disulfide to obtain deep red colored gold nanoparticle solution, and then by Zsigmondy in 1906 (Zsigmondy 1909), who reduced choloauric acid in the presence of formaldehyde to obtain monodisperse gold solutions (Overbeek 1984). Zsigamody's method was then modified in 1951 through Turkuvish method (Turkevich, Stevenson et al. 1951) that involves chloroauric acid reduction in the presence of sodium citrate to synthesize gold nanoparticles. This method has also been employed for the synthesis of silver nanoparticles.

1.2.2 Role of Nanotechnology in Clinical Therapeutics

The emergence of nanotechnology has made a significant impact on clinical therapeutics

in the last two decades (Hu, Aryal et al. 2010) and enormous advancements have been done in developing the field of nanomedicine in cancer studies to detect, diagnose and effectively treat cancerous tissues (Babu, Templeton et al. 2013).

Nanomedicine as per national institute of health is a formulation of drug whose end product's size is less than a micron (Babu, Templeton et al. 2013). Nanomedicine has gained much advantage due to its ability to overcome biological barriers, enhances the bioavailabilty of drug (Lavan, McGuire et al. 2003), effectively deliver hydrophobic therapies, and preferentially target disease sites (Babu, Templeton et al. 2013).

1.3 Cancer and Chemotherapeutics

Cancer is one of the most common causes of death worldwide. It is a group of diseases that affects millions of people all over the world irrespective of age group and sex. The chance of getting cancer in one's lifetime is one out of every two men and one out of every three women.

Treatment strategies are strongly dependent on the type of malignancy and stage at the time of diagnosis but often involve a combination of surgery, chemotherapy, and/or radiation therapy. Chemotherapy, a first-line treatment for cancer, is often administered intravenously where it circulates throughout the body ultimately locating and destroying cancerous and normal tissues (Pronk, Stoter et al. 1995).

1.3.1 Adverse Effects of Cancer Chemotherapeutics

The hydrophobic nature of the majority of the cancer chemotherapeutics makes them poorly water soluble and therefore limits their administration at high doses(Kwon 2003; Lu, Liong et al. 2007; Kumar, Sahoo et al. 2011). Most of the chemotherapeutics have low molecular weight and so are easily excreted from the body soon after administration and so a high concentration dose is required, thus a high toxicity it causes. Furthermore, chemotherapeutics are non targeted and cause damage to healthy tissues as well. The side effects it causes includes suppression of bone marrow, sloughing of the epithelial cells of alimentary canal and the most common and most unwanted side effect in all patient subjected to chemotherapy is increased hair loss also known as alopecia (Luo and Prestwich 2002).

1.4 Applications of Nanoparticles in Medicine

The nanoparticles are the small unit whose dimensions almost resembles to the building blocks of biological macromolecules such as proteins and DNA, this feature give a benefit to nanoparticles of being used for therapeutic purpose. Surface functionalization of nanoparticles can be done by various functional groups, signaling molecules, targeted molecules to make it target specific. I can also be made biocompatible by binding with various functional groups and also it is conjugated with drug to be used as drug delivery vehicle. The surfaces of nanoparticles can be modified in such a manner so as it can bind to various functional groups that defines the fate of the nanoparticles that where should it be targeted.

One of the biggest application of using nanomaterials as biomedicine is the it has an internal core or void where the drug or the material to be targeted is encapsulated. So not only the toxicity caused by the drug is minimized but also sustained realease of the drug is achieved. Nanoparticles encapsulate radiolabelled molecules and other small molecules in its internal core or void to be used in imaging techniques. Such molecule encapsulated by nanoparticles donot cause harmful effects in the rest of the body due to its target specificity and is also biocompatible.

The most important property of using nanoparticles in medicine and diagnostics is its biocompatible nature. The outer surface of nanoparticles are modified by binding small functional group molecule or encapsulating it with polyethylene glycol (PEG) to make it biocompatible and so it do not proke immune reactions and so other inflammatory processes as well and is considered as self molecule.

Surface functionalization of nanoparticle with small functional group molecules or with other ligands make nanoparticle highly targeted, also the contolled and sustained release of drug is because the surface group attached. Furthermore, the functionalization has a lot to do with the biodistribution of drug and plays an important role in its pharmacokinetic behavior. Nanoparticle surface modified by small functional group plays an important role in the mode of excretion of nanoparticle from the body and also its biodistribution gives an idea of the type of clearance the nanoparticle follows (Bharali, Khalil et al. 2009).

1.4.1 Nanoparticle Based Drug Delivery Vehicle For Cancer Treatment

Nanoparticle drug delivery enhances therapeutic effectiveness and reduces side effects of the drug payloads by improving their pharmacokinetics (Peer, Karp et al. 2007;Davis and Shin 2008; Zhang, Gu et al. 2008). It also enhances permeability and retention effect caused by leaky tumor vasculatures for better drug accumulation at the tumor sites (Matsumura and Maeda 1986). These benefits have made therapeutic nanoparticles a promising candidate to replace traditional chemotherapy, where intravenous injection of toxic agents poses a serious threat to healthy tissues and results in dose-limiting side effects.

Nanoparticles are known to positively alter biodistribution increasing therapeutic efficiency, its pharmacological properties and reducing nonspecific toxicity of potent anticancer drugs due to its superior biocompatibility, ability to protect nucleic acids from degradation, and ability to deliver therapeutic genes to cancer cells in vivo make nanoparticles the ideal delivery vehicle (Ahmad 2002; Lu, Liong et al. 2007; Ramesh 2008; Kumar, Sahoo et al. 2011).

Nanoscale drug delivery systems hold great promise in successfully formulating and enhancing the therapeutic efficacy of a large number of anticancer agents (Wang, Langer et al. 2012).

1.4.2 Anti-cancer Nanomedicines In Practice

Two well known nanoformulations that are approved by the US food and drug administration for the treatment of cancer are Doxil and Abraxane (Bharali, Khalil et al. 2009). Doxil® has been derived from doxorubicin and has much higher therapeutic value than doxorubicin (Martin 1998; Judson, Radford et al. 2001; Park 2002; Nishiyama and Kataoka 2006). Abraxane® is a nanoformulation of paclitaxel (Moreno-Aspitia and Perez 2005; Sparreboom, Scripture et al. 2005) and is used for the treatment of metastatic breast cancer (Gradishar, Tjulandin et al. 2005). Other nanoformulations used for cancer treatment are DaunoXome® (Guaglianone, Chan et al. 1994; Forssen, Male-Brune et al. 1996) a liposomal formulation daunorubicin, DepoCyt® (Glantz, Jaeckle et al. 1999; Jaeckle, Batchelor et al. 2002),a nanoformulation for cytarabine and ONCO-TCS (2004; Immordino, Dosio et al. 2006; Zhang, Gu et al. 2008), a nanoformulation of vincristine (Hu, Aryal et al. 2010).

CHAPTER 2: LITERATURE REVIEW

2.1 Methotrexate: A Widely Used Anticancer Drug

Methotrexate (MTX) is a chemotherapy agent and an immune system suppressant. Methotrexate was originally developed and continues to be used for chemotherapy, either alone or in combination with other agents. It is effective for the treatment of a number of cancers, including breast cancer, leukemia, lung cancer, lymphoma, osteosarcoma, bladder cancer and trophoblastic neoplasms (Ohata and Marmarou 1992; Bobo et al. 1994).

Methotrexate is an antimetabolite drug, of the class antifolate. It is a folic acid analogue, used as a chemotherapeutic agent. Folate receptors are over expressed on the cell membranes of many types of cancer cells including ovarian, endometrial, colorectal, breast, lung, renal cell carcinomas, brain metastases derived from epithelial cancers (Duthie 2001), and neuroendocrine carcinomas (Ohata and Marmarou 1992; Bobo et al. 1994).

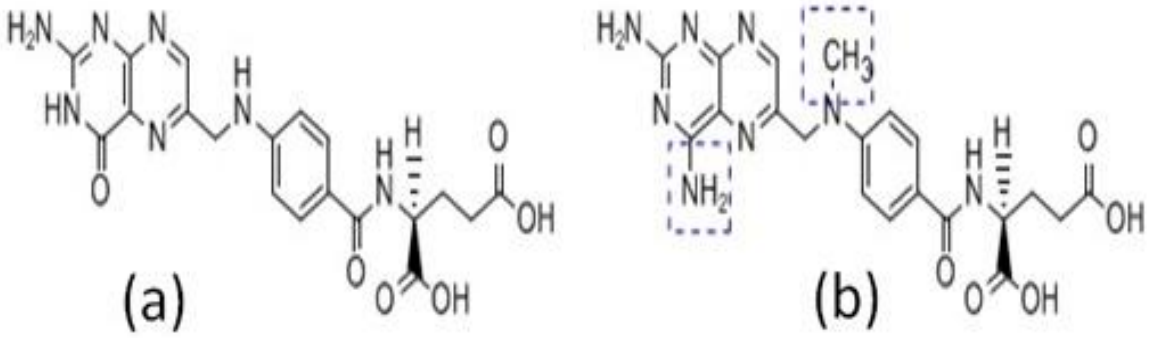

Figure 2.1(a): Strucutre of Folic Acid (b): Strucutre of Methotrexate

2.1.1 Mechanism of Action

MTX competitively inhibits dihydrofolate reductase (DHFR), an enzyme that participates in the tetrahydrofolate synthesis. The affinity of methotrexate for DHFR is about 1000-fold that of folate. DHFR catalyses the conversion of dihydrofolate to the active tetrahydrofolate. Folic acid is needed for the de novo synthesis of the nucleoside thymidine, required for DNA

synthesis. Also, folate is essential for purine and pyrimidine base biosynthesis, so synthesis will be inhibited. Methotrexate, therefore, inhibits the synthesis of DNA, RNA, thymidylates, and proteins (Sirotnak and Tolner 1999; Stella et al. 2000; Sudimack and Lee 2000).

2.1.2 Limitations of Methotrexate Chemotherapy

MTX is one of the most widely used drugs for the treatment of many forms of cancer, including tumors of the brain, breast, ovaries, and several leukemias (Messmann and Allegra 2001) , but methotrexate (MTX) has poor tumor retention ability due to its high water solubility, which likely contributes to its slow or poor therapeutic response in patients (Ohata and Marmarou 1992; Lieberman et al. 1995; Rihova et al. 2000; Di Stefano et al. 2003; Chen et al. 2007), also target cells develop resistance to MTX due to efflux of the drug by the cancerous cell membrane proteins into the extracellular environment (Banerjee et al. 2002).

The most common adverse effects of MTX include hepatotoxicity, ulcerative stomatitis, leukopenia and thus predisposition to infection, nausea, abdominal pain, fatigue, fever, dizziness, acute pneumonitis, rarely pulmonary fibrosis, and kidney failure. Central nervous system reactions to methotrexate have been reported, especially when given via the intrathecal route (directly into the cerebrospinal fluid), which include myelopathies and leucoencephalopathies. It has a variety of cutaneous side effects, particularly when administered in high doses. Another adverse effect of methotrexate is neurological damage and memory loss. Neurotoxicity may result from the drug crossing the blood–brain barrier and damaging neurons in the cerebral cortex.

2.2 Need for An Efficient Methotrexate Drug Carrier

Carriers of drugs in the form of nanoparticles are more effective as they have high stability, permeability into leaky vasculatures of tissues and their extremely small size. Drugs can be bound to nanoparticles by many different ways including physical adsorption (Reszka et al. 1997), through covalent bonding (Torchilin et al. 2001; Zhang et al. 2002) or through ionic bonding (Alexiou et al. 2002; Zhang et al. 2002). To overcome the limitations of drug, conjugates of MTX with polymers such as PEG (poly ethylene glycol) and PGA (Poly glutamic acid) were produced. These conjugates had higher retention value in the cell, thus maintaining a higher concentration of MTX within the cancerous cell (Piper et al. 1983; Riebeseel et al. 2002)

but these large sized conjugates limit the drug administration only to target specific sites, and intravenous drug delivery became a challenge. The drug carrier should be extremely small enough so as it perfuse out of the vascular system to reach its target (Howard 2003) and also to make the treatment more efficient the MTX release should be sustained.

Recent studies indicate that binding of MTX to nanoparticles alters drug's pharmacokinetic behavior, enhances its tumor targeting, reduces toxicity, and also overcomes drug-resistance mechanisms (Ohata and Marmarou 1992; Lieberman et al. 1995; Sirotnak and Tolner 1999; Stella, et al. 2000; Sudimack and Lee 2000). In this study we developed MTX-nanoparticle conjugate by binding MTX to silver nanoparticles through physical adsorption. As MTX is analogue to folic acid so due to increase expression of folate receptors on cancerous cell as compared to normal cells, MTX conjugated silver nanoparticles will be more target specific and that will highly reduce its toxicity in normal cells and its increased uptake by cancerous cells thus overcoming the problems caused in conventional chemotherapy procedures.

2.3 Significance of Metal Nanoparticles

Nanoparticles have been of great importance from the last few decades. Because of their extremely small size, they form a bridge between the large bulk molecules and the small entities at molecular and atomic level and it differs in physical properties from its bulk form (Munzuroglu and Geckil 2002; Oberdörster, Oberdörster et al. 2005; Thakkar, Mhatre et al. 2010). Among all nanoparticles used for pharmaceutical and for biomedical purposes, metal nanoparticles show very promising results because of its antibacterial property (Chaloupka, Malam et al. 2010; Lansdown 2010) and this unique property is because of large surface area to volume ratio. This is the reason that its different chemical, physical and electrical properties changes due to change in surface area, charge distribution and composition of nanoparticle(Gurunathan, Kalishwaralal et al. 2009; Kouvaris, Delimitis et al. 2012; Shameli, Bin Ahmad et al. 2012) and so due to change in their shape and size its meting point, redox potential, color, stability and their electric and magnetic behavior changes(Gurunathan, Kalishwaralal et al. 2009).

Metal nanoparticles are also very much applicable due to not only being used in therapeutics, pharmaceuticals and biomedical procedures but also they are widely used in various industries(Miura and Shinohara 2009; Park, Yi et al. 2010; Parveen, Misra et al. 2012), that

includes its usage in various food industries, agriculture, electronics(Bosi, Da Ros et al. 2003; Moghimi, Hunter et al. 2005; Surendiran, Sandhiya et al. 2009), in biomedical sciences to dealt with several different types of viruses and bacteria and most importantly in food packaging(Ahmad, Mukherjee et al. 2003). It also has several other unique properties such as its optical and electric properties (Lue 2001; Rai, Yadav et al. 2009). One of the biggest reasons of why silver nanoparticles are of great importance is their good conductive behavior, its superb stability and that it can be used as catalyst (Hussain and Pal 2008).

2.4 Potential Applications of Silver Nanoparticles

Silver nanoparticles are widely studied because of its unlimited applications in industries, biomedical sciences such as in targeted drug delivery systems(Dreaden and El-Sayed 2012), biological sciences with many physical and chemical applications and also used in many other consumer products(Scholars 2007; Higashisaka, Yoshioka et al. 2015) such as in textile industries(Benn and Westerhoff 2008), food storage(Chaudhry, Scotter et al. 2008), cosmetics, perfumes, deodorants(Chen and Schluesener 2008; Tripathi, Chandrasekaran et al. 2009), biosensors, bandages(Chen and Schluesener 2008; Rai, Yadav et al. 2009), as antimicrobial agents(Morones, Elechiguerra et al. 2005; Lok, Ho et al. 2007), in cleaning solutions, various house hold products, as therapeutic agents (Awwad, Salem et al. 2013; Nirmala, Shiny et al. 2013), as cardiovascular and orthopaedic implants, catheters, burns and wound dressings, surgical catheters, bone biomaterials(Ahamed, AlSalhi et al. 2010; Chaloupka, Malam et al. 2010; Greulich, Diendorf et al. 2011; You, Zhang et al. 2011) and due to its anti-inflammatory property silver nanoparticles are also used in wound and burn healing processes(Nadworny, Wang et al. 2008; Kwan, Yeung et al. 2014).

2.4.1 Synthesis of Silver Nanoparticles

Silver nanoparticles can be synthesized of various shapes depending upon the type of method followed and the type of reducing agent and stabilizer used. they can be spherical, rods(Xu, Wang et al. 2006), in the form of nanowires (Murphy and Jana 2002), prisms (Darmanin, Nativo et al. 2012), nanopyramids (Wiley, Im et al. 2006), cubic (Wiley, Im et al. 2006), nanobars (Wiley, Chen et al. 2007) etc. spherical silver nanoparticles are more stable thermodynamically if silver ions are reduced under controlled reaction conditions(Krutyakov,

Kudrinskiy et al. 2008). Various chemical(Liang, Wang et al. 2010), physical(Ghosh, Kundu et al. 2003; Ashkarran 2010) and biological(Pugazhenthiran, Anandan et al. 2009; Sintubin, De Windt et al. 2009; Suresh, Pelletier et al. 2010) methods are used for the synthesis of spherical silver nanoparticles. In all these methods the mostly used methodology is the chemical reduction method because it results in high yield with minimum preparation cost and also don't cause aggregation of particles(Do Kim, Han et al. 2004). This method involves the reduction of silver nitrate favoured by a reducing agent and also a stabilizing agent is there. For this method various protocols are followed depending on the type of reducing agent and stabilizers used. Different reducing agents such as trisodium citrate, NaBH4, ethylene glycol(Kim, Jeong et al. 2006), paraffin(Sato-Berrú, Redón et al. 2009) and hydrazine hydrate(Zhang, Qiao et al. 2007) are used to synthesize silver nanoparticles by chemical reduction method.

CHAPTER 3: MATERIALS AND METHODS

3.1 Experiment Design

3.1.1 Materials

All chemicals were purchased from Sigma-Aldrich (USA), unless stated otherwise. Deionized water was used throughout the study. All solutions were prepared in deionized water, unless mentioned otherwise.

3.1.2 Synthesis of Methotrexate Silver Nanoparticles

For the synthesis of methotrexate silver nanoparticles, the chemical reduction method was employed according to the same protocol as described in our previous study (Muhammad et al, 2016). 1mM MTX solution (prepared in 1mM K_2CO_3) and 1mM $AgNO_3$ solution were mixed together in a volume ratio of 1:20. The mixture was kept on shaking at 200 rpm for 20 min, followed by drop wise addition of 40mM chilled $NaBH_4$ solution as the reducing agent. These solutions were added in an optimized ratio of 20:01:02 ($AgNO_3$: MTX: $NaBH_4$). The resulting mixture was placed on shaking at 200 rpm for 4 h. For the large scale preparation of methotrexate silver nanoparticles, the mixture was transferred on to petri plates and stored at -80ºC for 10 h. These petri plates were kept in freeze dryer (Eyela FDU-1000 freeze dryer) for 40-48 h at 15 pa (Muhammad et al, 2016).

3.1.3 Synthesis of PEGylated Methotrexate Silver Nanoparticles

PEGylation of Ag MTX nanoparticles was carried out by dissolving 5 mg sample in 50% ethanol and stirring for 2 h, followed by centrifugation at 11000g for 1 h. The supernatant was separated and UV-Vis spectra of both supernatant and the pellet (dissolved in deionized water) were recorded. Purified Ag-MTX nanoparticles were dissolved in 50 % ethanol and kept on stirring followed by drop wise addition of PEG-6000 solution (2.5%). After 2 h stirring the resulting solution was centrifuged at 11000 g for 1 h and PEG-Ag-MTX nanoparticles were obtained in the form of pellet (Muhammad et al, 2016).

3.2 Characterization of PEGylated Methotrexate Silver Nanoparticles

The characterization of PEG Ag MTX nanoparticles was done to evaluate and analyze their particle size and surface charge, drug encapsulation and release efficiency, conduct further characterization tests for nanoparticles, which are described as follows.

3.2.1 UV-Vis absorption spectroscopy (UV-Vis)

UV-Vis absorption spectroscopy is one of the most widely used techniques in both clinical and chemical laboratories. It actually is the measurement of extent of absorption that occurs in sample when a beam of light passes through it and from the reflected beam the absorption is measured. In UV-Vis spectrophotometer, a beam of light is split where one half of the beam is directed through the cuvette containing the sample being analyzed and the other half is directed to a cuvette containing the solvent only (reference). Absorption can be measured both at specific wavelength and at a desired range and a spectrum is obtained that plots entire range of wavelength versus its absorption at specific wavelength. The maximum absorption at specific wavelength is called as lambda max. It measures the electronic transition of molecules and obeys the principle of Beer Lambert Law. The absorbance of the sample is proportional to the molar concentration in the sample cuvette, the absorption value known as the molar absorptivity is used when comparing the spectra of different compounds. Beer-Lambert Law says

$$A=EcL$$

Molar absorptivity E= A/ cl (where A= absorbance, c= sample concentration in moles/ liter and L= length of light path through the cuvette in cm). This law makes UV-Vs absorption spectroscopy useful for quantitative analysis.

3.2.2 X-Ray Powder Diffraction (XRD)

X-ray diffraction (XRD) is a popular analytical technique which has been used for the analysis of both molecular and crystal structures, qualitative identification of various compounds, quantitative resolution of chemical species, measuring the degree of crystallinity, isomorphous substitutions, particle sizes, etc. When X-ray light reflects on any crystal, it leads to the formation of many diffraction patterns, and the patterns reflect the physico-chemical characteristics of the crystal structures. In a powder specimen, diffracted beams typically come

from the sample and reflect its structural physico-chemical features. Thus, XRD can analyze the structural features of a wide range of materials, such as inorganic catalysts, superconductors, biomolecules, glasses, polymers, and so on. Analysis of these materials largely depends on the formation of diffraction patterns. The diffracted patterns also explain whether the sample materials are pure or contain impurities. Therefore, XRD has long been used to define and identify both bulk and nanomaterials, forensic specimens, industrial, and geochemical sample materials.

For X-ray diffraction (XRD) analysis, 0.5g of freeze dried solid Ag MTX nanoparticles were finely ground, homogenized and placed in oven at a temperature of 37ºC to completely remove any moisture. The diffraction pattern was obtained at scan angle range of 20º to 80º and scan time of 30 minutes. The resulting peaks were interpreted by comparison with standard reference patterns and measurements, thus allowing identification of the crystalline form.

3.2.3 Particle Size and Area Distribution

The particle size as determined by the transmission electron microscopy was further analyzed and area distribution of the nanoparticles was calculated by using image j software. Analysis was performed on a selected area. The command 'Analyze Particles' counts and measures objects in binary or thresholded images. It works by scanning the image or selection until it finds the edge of an object. It then outlines the object using the Wand Tool, measures it using the Measure. . . [m] command, and then resumes scanning until it reaches the end of the image or selection. Features of thresholded images can be extracted by specifying suitable Size and Circularity ranges and by choosing if particles should be traced by their outer edge or by flood filling. For Size of particles, values were given between the range of 0 and 'Infinity'. Particles with size (area) outside the range specified in this field are ignored. Circularity ranges were given from 0 (infinitely elongated polygon) to 1 (perfect circle). Particles with size circularity values outside the range specified in this field are also ignored. 8–bit binary image containing the best fit ellipse (cf. Edit . Selection . Fit Ellipse) of each measured particle (gray levels: Ellipses: 0; Background: 255) was analyzd and a histogram of particle size distribution was obtained.

3.2.4 Surface Charge and Zeta Potential

Zeta potential (surface charge) of PEG Ag MTX nanoparticles was evaluated by Dynamic Light Scattering (DLS) using Nanotrac Wave II (Microtrac® Systems). Nanotrac Wave II is an accurate particle size zeta potential analyzer ideal for characterizing materials across the widest concentration range. The sample was used in the form of dry powder, weighted and then dissolved in a solvent known to suspend the molecules- i.e., 50% ethanol. The SetZero measurement was performed with the chosen fluid in the cell. A 1:100 dilution of each formulation was made using double-deionized water followed by the determination of particle size at 25 °C. For each sample, the corresponding mean diameter ± standard deviation values were obtained from six determinations. At least three batches were analysed for mean particle size and zeta potential. The stability of the prepared PEG Ag MTX nanoparticles was determined by measuring the particle size and the surface charge using zeta potential analyzer as described (Pinto et al, 2014).

3.2.5 Drug Release Efficiency

2 ml solution containing PEG Ag MTX nanoparticles was placed into 15 ml centrifuge tubes containing 8 ml phosphate buffer. Suspensions were then placed on an electronic shaker set at 100 rpm. At various time points, 2 ml of release medium was removed and replaced with the same volume of fresh medium. Isolated samples were centrifuged at 4,400 rpm for 5 minutes and filtered through a 0.2 micron syringe filter. Analysis was carried out using a UV spectrophotometer with empty nanoparticle solution used as control (Cooper and Harirforoosh, 2014).

3.3 *In-vitro* Testing of Ag-MTX Nanoparticles

3.3.1 Cytotoxicity Assay

MCF-7 cell lines were maintained in RPMI medium. 80% confluent cells were trypsinized and seeded in 96 well plate ($1x10^3$cells/well). After 24 h incubation at 37ºC in CO_2 incubator maintained at 5% CO_2, cells were exposed to different concentrations of MTX and PEG-Ag-MTX nanoparticles for 24 h. Medium was then removed and 200 µl of light protected MTT solution (final conc 0.5mg/ml) was added. MTT solution was removed and 200 µl

solubilization solution (DMSO) was added. After 10 minutes of incubation, absorbance was recorded at 570nm. IC50 values are calculated using Graph Pad Prism 7 software.

3.3.2 Biocompatibility Assay

To access the toxicity of MTX and its nanoparticles to normal cells, Human Corneal Epithelial Cell line (HCEC) was used. Cells were maintained in DMEM supplemented with 10% FBS, 2 mM L-glutamine and 1 mM Na-pyruvate. Penicillin/streptomycin concentration was 100 U/ml. Cell viability assay was carried as described above.

3.4 Development of Chemically Induced Tumor Model in Mice

3.4.1 Acquisition of Mice

For the development of tumor model, a total of 40 female Balb/c mice (7-8 weeks) were obtained from National Institute of Health, Islamabad. These mice were maintained in the animal house at temperatures of 24 ± 3°C and 10:14 hours of light and dark. These animals were housed in polypropylene cages and fed standard cat food. Purified filtered water was provided to the animals.

3.4.2 Distribution into Groups

Mice were divided into 4 groups of 10 each. Group 1, consisting of 10 mice was assigned as the normal control group. The mice in this group were housed under normal environmental conditions, without the application of any carcinogen. The mice divided into groups 2, 3 and 4 were used for the induction of tumors.

3.4.3 Induction of Tumors

The hair on the dorsal region (back) of the mice in groups 2. 3, and 4 were shaved 3 days before the commencement of the experiment. For the induction of tumor, a two stage protocol consisting of initiation with the topical application of carcinogen 7,12-Dimethyl benz(a) anthracene (DMBA) followed by a promoter, croton oil, was employed. Mice were applied topically with a dose of DMBA over the shaved area of the skin. Two weeks later, croton oil was applied as a promoter till 5 weeks, until the appearance of visible tumors (Arya and Kumar, 2011).

3.4.4 Confirmation of Tumors

Histological examination of skin biopsies from tumor sites was carried out to confirm the formation of tumors and analyze the type and grade of tumors.

3.4.5 Evaluation And Analysis of Tumors

Tumour incidence, tumour yield, and tumour diameters were calculated after the termination of application of promoter in week 5. In order to determine tumor volumes, the tumors were considered spherical and the length (the longest dimension), width (the distance perpendicular to and in the same plane as the length), and height (the distance between the exterior tumor edge and the mouse's body) of each tumor lobe was measured with digital vernier calipers. Average latent period was calculated as time lag between the application of the promoting agents and the appearance of tumour in 50% of the animals.

3.5 Treatment Design

In order to analyze the anti-tumor activity of PEG-Ag-MTX nanoparticles, mice divided in groups 2, 3 and 4 were further taken through the experiment.

3.5.1 Group 2

Group 2 was assigned as the negative control group. The mice in this group were left untreated through the rest of the experiment, and the effect on their tumor volume was noted for comparison.

3.5.2 Group 3

Group 3 was used as the positive control group. The mice in this group were given an intravenous injection of Methotrexate for the duration of 5 weeks at the dose of 1mg/kg of the body weight. The effect on weight, tumor size and volume was noted throughout the experiment.

3.5.3 Group 4

Group 4 was allocated as the PEG-Ag MTX Nanoparticles treatment group. These mice were given an intravenous injection of PEG-Ag MTX Nanoparticles in their tail veins for a duration of 5 weeks at the dose of 1mg/kg of the body weight. The reduction in the size and volume of the tumor was noted after regular intervals for these mice.

3.5.4 Effect on Tumors

After the required treatment was carried out, the skin biopsy from the tumor tissue was obtained to perform histological examination for each group.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Visual Confirmation of Methotrexate Silver Nanoparticles

When MTX was added to the aqueous solution of $AgNO_3$, a change in color from transparent to light brown and then to greyish brown, upon drop wise addition of NaBH4 was observed that determines the formation of AgMTX (Figure 4.1 (a,b)). After freeze drying the nanoparticles solution in a freeze dryer for 40-48 h at 15 pa, the nanoparticles were obtained in the form of solid greyish black powder (Figure 4.1 (c)).

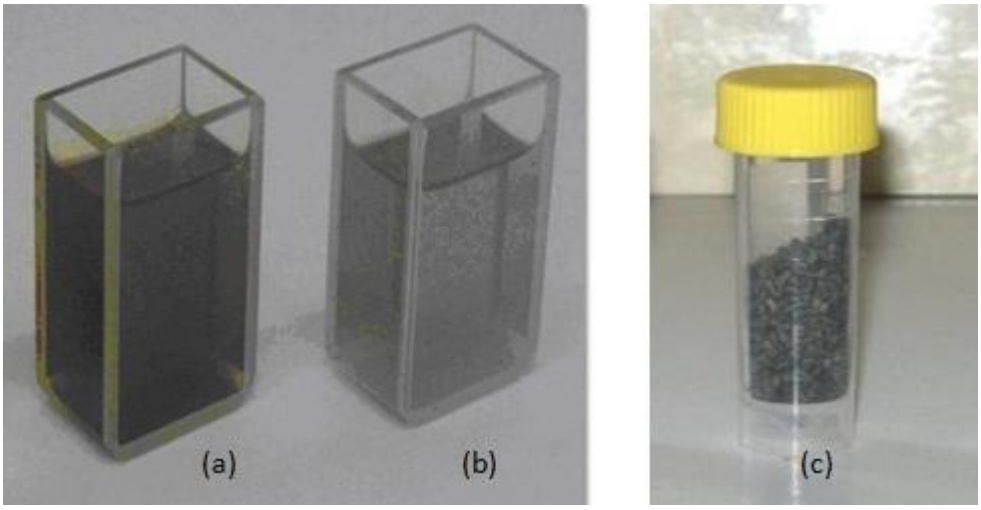

Figure 4.1 Visual Detection of (a) Ag-MTX (b) $AgNO_3$ (c) Freeze Dried Ag-MTX Nanoparticles

4.2 Characterization of PEGylated Methotrexate Silver Nanoparticles

The characterization of PEG Ag MTX nanoparticles was done to evaluate and analyze their particle size and surface charge, drug release efficiency, and many other characterization tests, which are described as follows.

4.2.1 UV-Vis absorption spectroscopy

UV-Vis absorption spectroscopy of AgMTX showed surface Plasmon resonance (SPR) peak at 397.56 nm, while that of MTX is at 370.62 nm and AgNO3 has no peak in this range (300-650 nm) that clearly determines that silver nanoparticles conjugated with drug has formed (Figure 4.2).

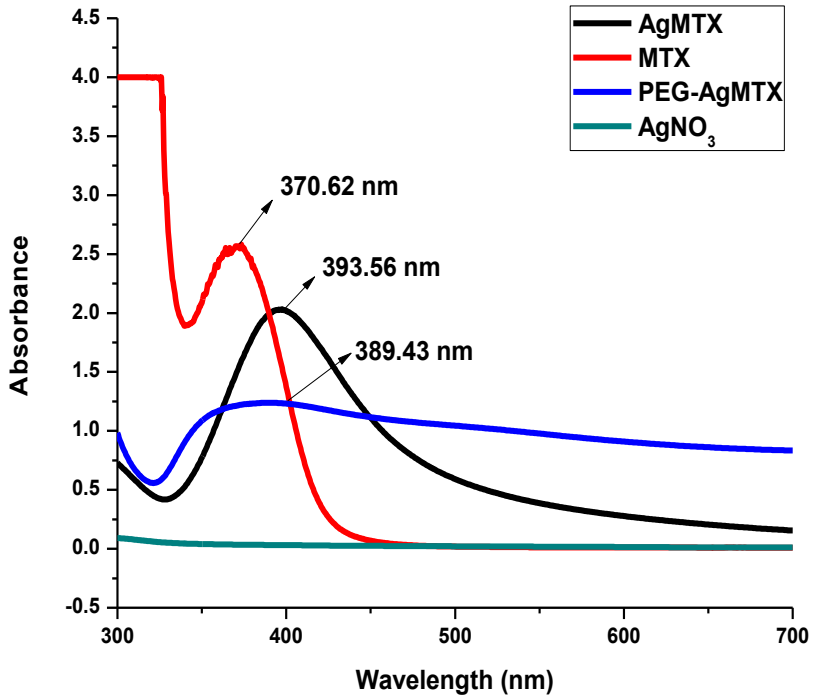

Figure 4.2 Comparative UV-Vis spectra of MTX, AgMTX, PEG-AgMTX and $AgNO_3$

4.2.2 X-Ray Powder Diffraction (XRD)

XRD is a primary technique for the identification of the crystalline nature at the atomic scale. X-ray powder diffraction is a nondestructive technique with great potential for the characterization of both organic and inorganic crystalline materials. This method has been used to measure phase identification, conduct quantitative analysis, and to determine structure imperfections in samples from various disciplines, such as geological, polymer, environmental, pharmaceutical, and forensic sciences. Recently, the applications have extended to the characterization of various nano-materials and their properties. The working principle of X-ray diffraction is Bragg's law. Typically, XRD is based on the wide-angle elastic scattering of X-rays. Although XRD has several merits, it has limited disadvantages, including difficulty in growing the crystals and the ability to get results pertaining only to single conformation/binding state.

The structure of prepared silver nanoparticles has been investigated by X-ray diffraction (XRD) analysis. Typical XRD patterns of the sample, prepared by the chemical reduction method are shown in the Fig.4.3, which illustrates the formation of silver nanoparticles. The Ag nanoparticles are characterized by the four distinct X-ray diffraction angles 2[θ] of the specimen, which show that our silver nanoparticles have a face centered cubic crystal structure. Structural information on the nanoparticles shows the XRD patterns of the nanoparticles are lying flat with their basal planes parallel to the substrate. The remarkably intensive diffraction peak at a 2θ value of 38.04 from the {111} lattice plane of face-centered cubic silver unequivocally indicates that the particles are made of pure silver and that their basal plane, i.e., the top crystal plane, should be the {111} plane. It has been suggested that this plane may possess the lowest surface tension.

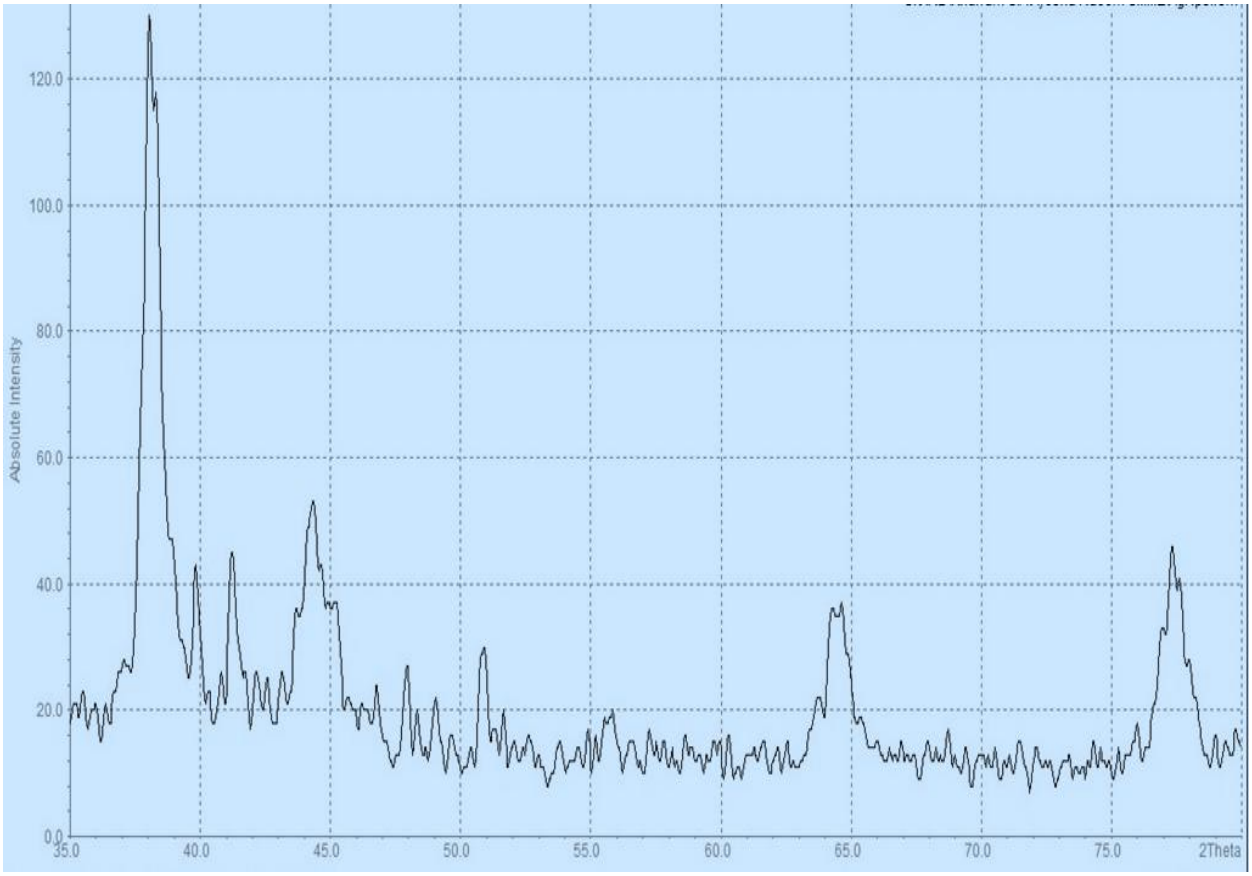

Figure 4.3. X-ray diffraction pattern of Ag nanoparticles.

4.2.3 Particle Size and Area Distribution

The particle size as determined by the transmission electron microscopy (Figure 4.4) was further analyzed and area distribution of the nanoparticles was calculated by using image j software. Analysis was performed on a selected area. 8–bit binary image containing the best fit ellipse (cf. Edit . Selection . Fit Ellipse) of each measured particle (gray levels: Ellipses: 0; Background: 255) was analyzd and a histogram of particle size distribution was obtained, as shown in Figure 4.5. The results of this analysis corresponded with the TEM results, as the mean particle was determined to be 12.6 microns.

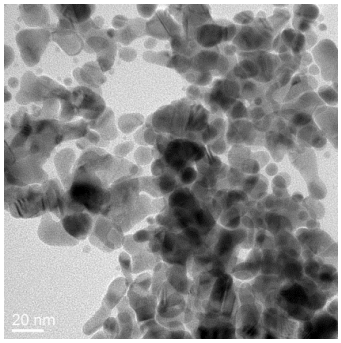

Figure 4.4 TEM images of AgMTX at 20 nm scale (Taken from Muhammad et al, 2016)

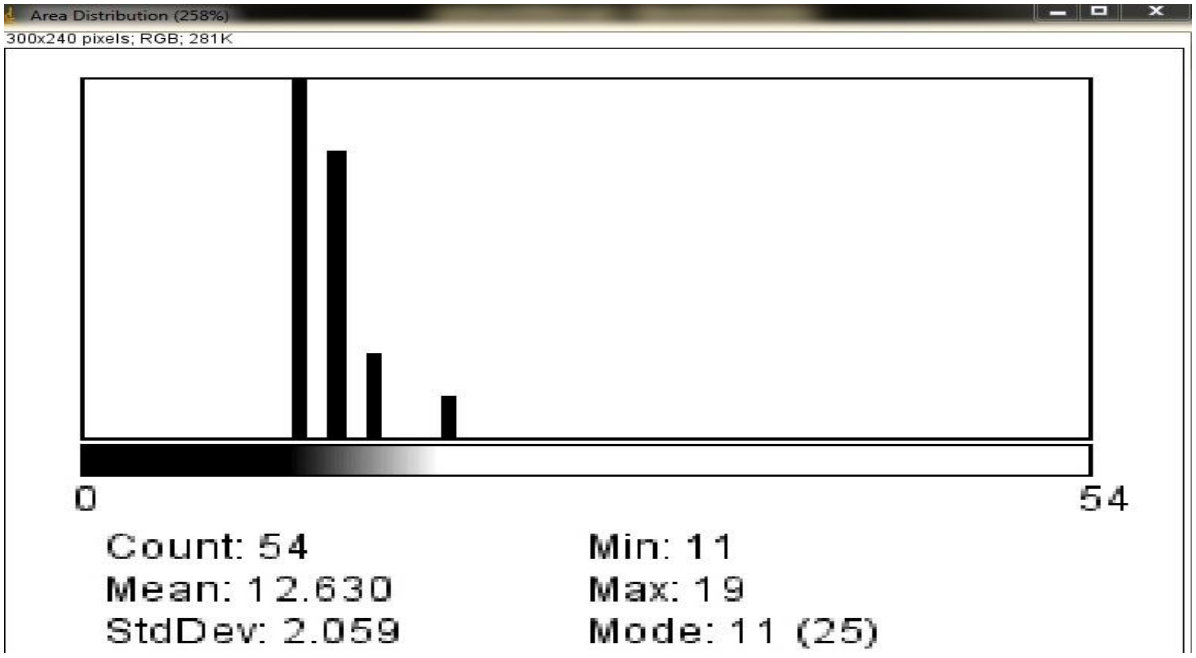

Figure 4.5 Histogram showing the Area Distribution of Ag-MTX Nanoparticles

4.2.4 Surface Charge or Zeta Potential

Zeta potential can be used to gain further insights into the stability of the obtained colloidal AgNPs. The magnitude of zeta potential gives an insinuation of potential stability of colloid. It should be noted that the particles with zeta potential values more positive than +30 mV or more negative than −30 mV are considered to be stable. In contrast, the colloids are least stable at isoelectric point, where the zeta potential is zero.

Zeta potential (surface charge) of PEG Ag MTX nanoparticles was evaluated by Dynamic Light Scattering (DLS) using Nanotrac Wave II (Microtrac® Systems). The zeta potential measures the potential difference between the surface charges and those of opposite sign deriving from the medium that are arranged around the particle. More positive or negative is the zeta potential, larger is the colloidal stability. The zeta potential of silver nanoparticles was found to be -30mV, which shows that our nanoparticle formulation is very stable.

4.2.5 Drug Release Efficiency

Since the release behavior of Ag-MTX nanoparticles at the desired site is of a great importance for formulating an ideal cancer-targeted drug delivery system, in-vitro release studies were performed. The drug release analysis of nanoparticles was carried out using a UV spectrophotometer with empty nanoparticle solution used as control, over a time duration of 48h. The results were tabulated and a cumulative drug release percentage graph was obtained using graphpad prism 6, as shown in figure 4.6. This graph shows little burst effect, with a prolonged sustained release of methotrexate from nanoparticles for 48 h. This is very helpful to eliminate the side effects of methotrexate that are associated with its high doses and low retention time in the body (Cooper and Harirforoosh, 2014).

The in-vitro release studies also demonstrated that free MTX was released faster than PEG capped Ag-MTX nanoparticles at the maintained pH values for blood. The delay of MTX release from PEG capped Ag-MTX nanoparticles was due to the binding of MTX with the silver NPs, which accordingly improved the release profile of MTX and prolonged its half-life compared to free MTX. The fast release of free MTX was based on the fact that unbound MTX has a higher solubility. However, the initial fast release of MTX from nanoparticles s was due to weak linkages between MTX and silver nanoparticles, which occurred in some of the unbound or

loosely bound nanoparticles. This increased half life of MTX in nanoparticles increases drug bioavailability to cancer cells, and leads to high therapeutic efficacy compared to normal cells.

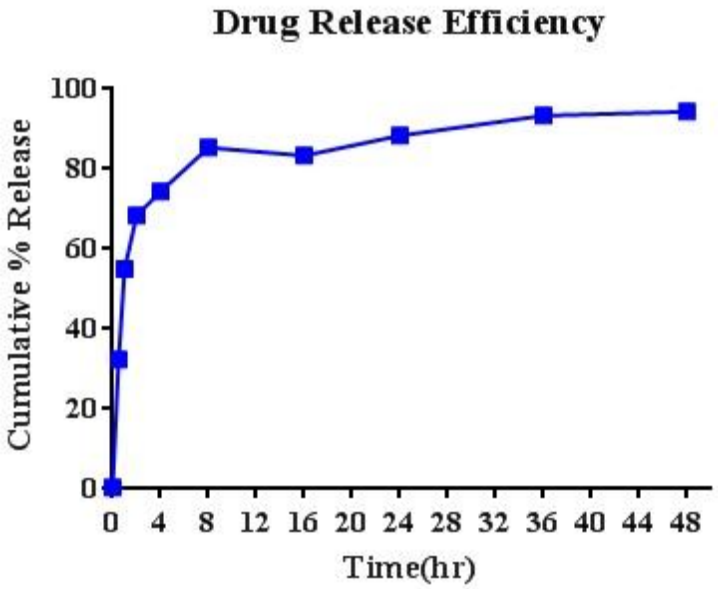

Figure 4.6 Graph showing %age Cumulative Release of MTX from Nanoparticles

4.3 Cytotoxicity assay

In order to assess the cytotoxic potential of the MTX nanoparticles, cell viability (%) was checked against MCF-7 breast cancer cell line (Figure 4.7). The pegylated nanoparticles showed higher anticancer activity and the IC50 for PEG-Ag-MTX (258.6 µg/ml) was less than that of MTX (512.7 µg/ml). The increased anticancer activity reveals the efficient drug delivery mediated by pegylation and additional toxicity offered by incorporated silver.

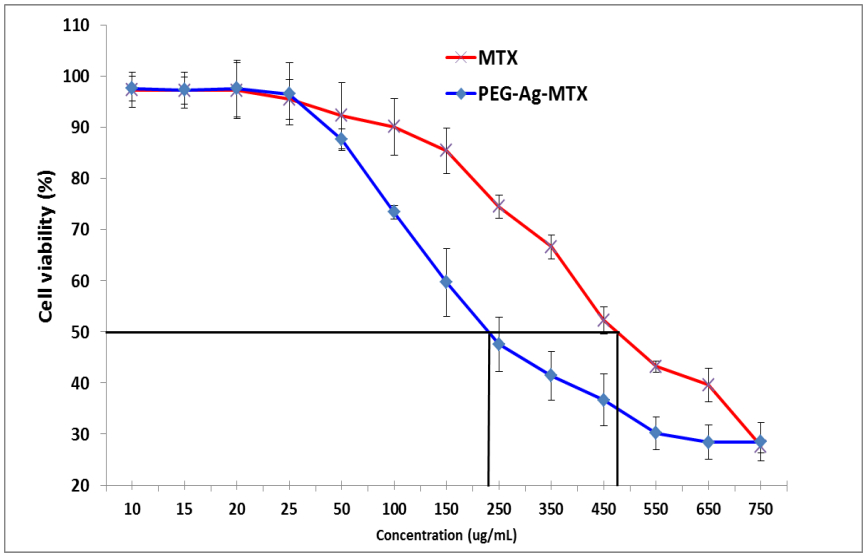

Figure 4.7 MCF-7 cell line (%) viability to varying concentration of MTX and PEG-Ag-MTX

4.4 Biocompatibility assay

To examine the effect of PEG-Ag-MTX and MTX on normal cells, cell viability of Human Corneal Epithelial Cells (HCEC) was checked by exposing them to varying concentration of MTX and its nanoparticles (Figure 4.8). Although no significant difference was found between drug and nanoparticles, the pegylated nanoparticles may offer higher specificity for tumors in- vivo as they get accumulated specifically in the tumor stroma and inner microenvironment.

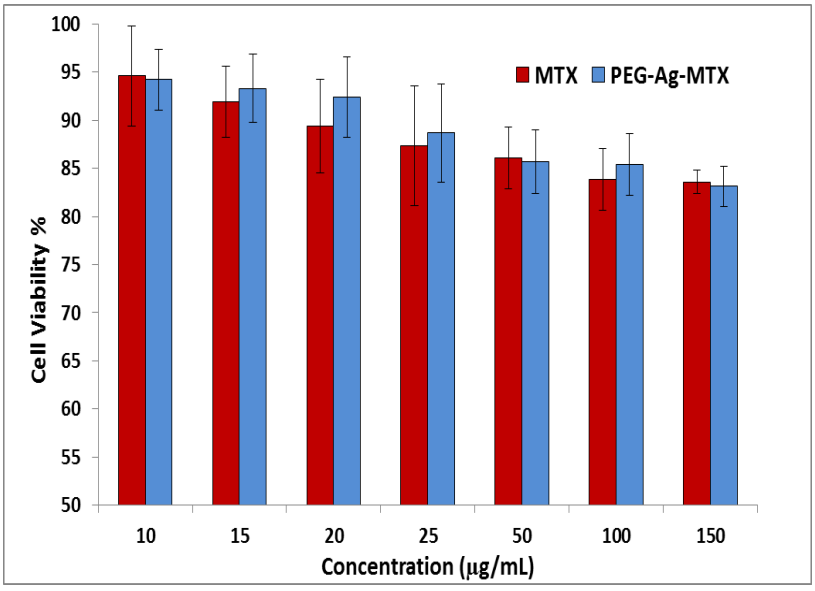

Figure 4.8 Cell viability of HCEC cell line to varying concentration of MTX and PEG-Ag-MTX

4.5 Development of Chemically Induced Tumor Model in Mice

4.5.1 Induction of Tumors

The mice in group 2, 3 and 4 were used for the development of tumors by a two-stage tumor induction protocol using DMBA and croton oil as initiator and promoter respectively. After a period of 4-5 weeks, visible tumor masses ranging from a single spherical lobe to multiple lobes, were observed, as shown in Figure 4.9. The digital vernier callipers was used to measure tumor diameters in millimeters, which were recorded periodically over the entire duration of tumor induction and its treatment with nanoparticles.

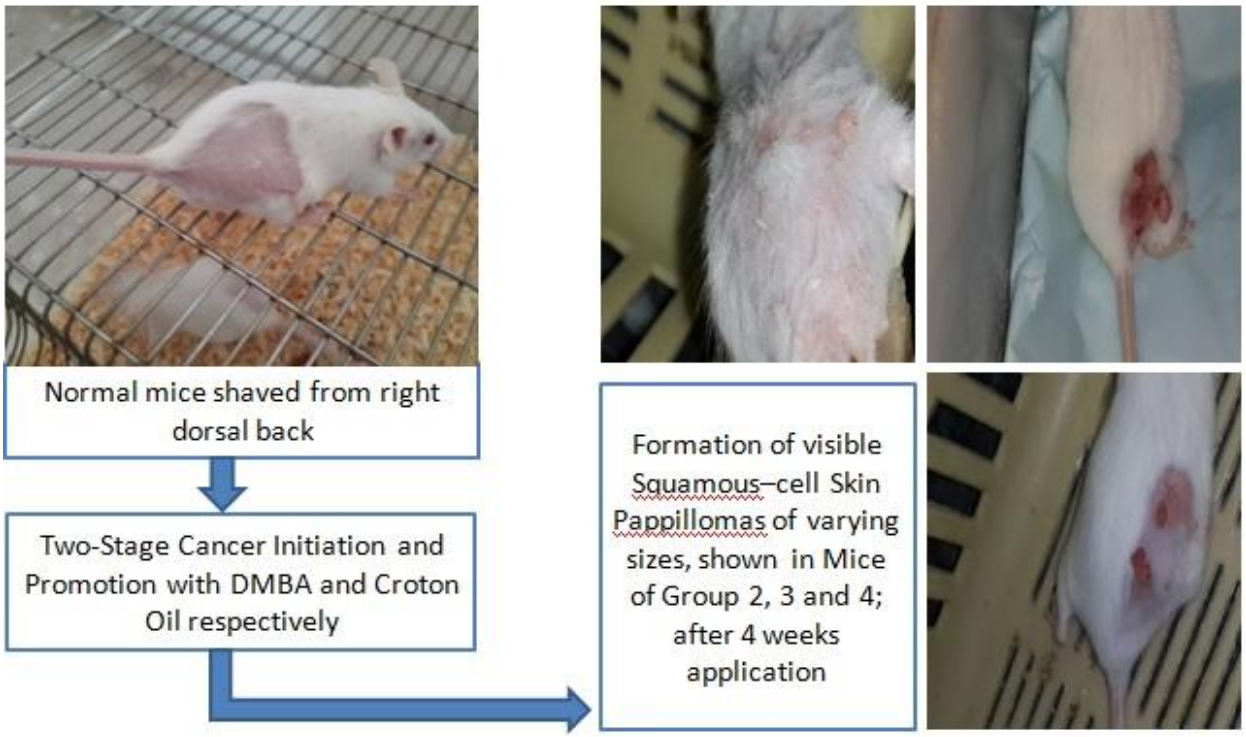

Figure 4.9 Development of Two-Stage Tumor Model in Balb/c Mice

4.5.2 Confirmation of Tumors by Histological Examination

Histological examination of skin biopsies from tumor sites showed marked progression of cancer cells and the type of tumor idientified was Squamous cell skin pappilloma with visible hyperkeratosis, as shown in Figure 4.10.

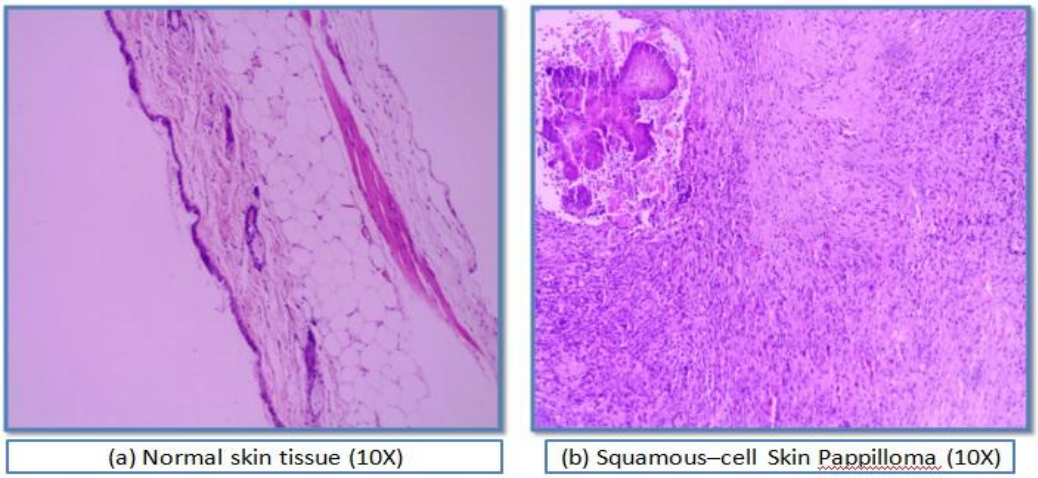

Figure 4.10 Micrographs for Histological Examination of (a) Normal (b) Skin Pappilloma Tissues

4.5.3 Evaluation of Tumors

Tumors ranging in size from 1.9mm to 6.4mm diameter were obtained, including single-lobed as well as multi-lobed tumors. The diameter of tumors was used to find its radius. For the calculation of tumor volume, the tumors were considered as spherical , and the formula $V = 4/3\pi r^3$ was used. Tumor incidence rate was found to be approx. 55% in the mice. Average latent period was determined to be 4 weeks.

4.6 *In-vivo* Treatment of Tumors with Ag-MTX Nanoparticles

4.6.1 Tumor Regression and Survival Rate Analysis

4.6.1.1 Negative Control Group

The Group 2 was assigned as the negative control group. The mice in this group were left untreated through the rest of the experiment, and the effect on their tumor size was noted for comparison. The tumor volme measurements in these mice showed that their tumors kept on

increasing in size for a significant time, and some even turned into life endargering growth, leading to the death of these mice. The survival rate in these mice gradually decreased over the period of time and eventually it resulted in the death of all cancerous mice, hence the tumors proved to be life-long and could not be cured in these mice.

4.6.1.2 Positive Control Group

Group 3 was used as the positive control group. The mice in this group were given an intravenous injection of free Methotrexate drug for the duration of 5 weeks. The effect on weight, tumor size and volume was noted throughout the experiment. It was observed that the size of tumor in these mice progressively decreased, however it also lead to a decrease in their weights and their abnormally low appetite. Among many other side effects exhibited by treatment with methotrexate drug, the most prominent were the unusually low body weights in mice, dark coloured faeces and irritability. Some mice even exhibited hypersensitivity reactions to the drug. Moreover, the mice in this group also displayed high mortality rate over the duration of 5 weeks treatment, i.e., almost 60% of the mice in this group succumbed to the tumors and could not survive the duration of treatment.

4.6.1.3 PEG-Ag MTX Nanoparticles Treatment Group

Group 4 was allocated as the PEG-Ag MTX Nanoparticles treatment group. These mice were given an intravenous injection of PEG-Ag MTX Nanoparticles in their tail veins for a duration of 5 weeks. The reduction in the size and volume of the tumor was noted after regular intervals for these mice. The results indicated that these mice showed relatively highest reduction in their tumor volumes, and the most unusal observation was that all the mice in this group survived the duration of treatment and more. All the mice in this group remained healthy after treatment with PEG-Ag MTX Nanoparticles, and showed almost no considerable side effects. All these mice were healthy and no mortality was observed among these.

The comparative results of tumor regression and the survival rate of the mice in group 2, 3 and 4 are shown in Figure 4.11 and Figure 4.12 respectively.

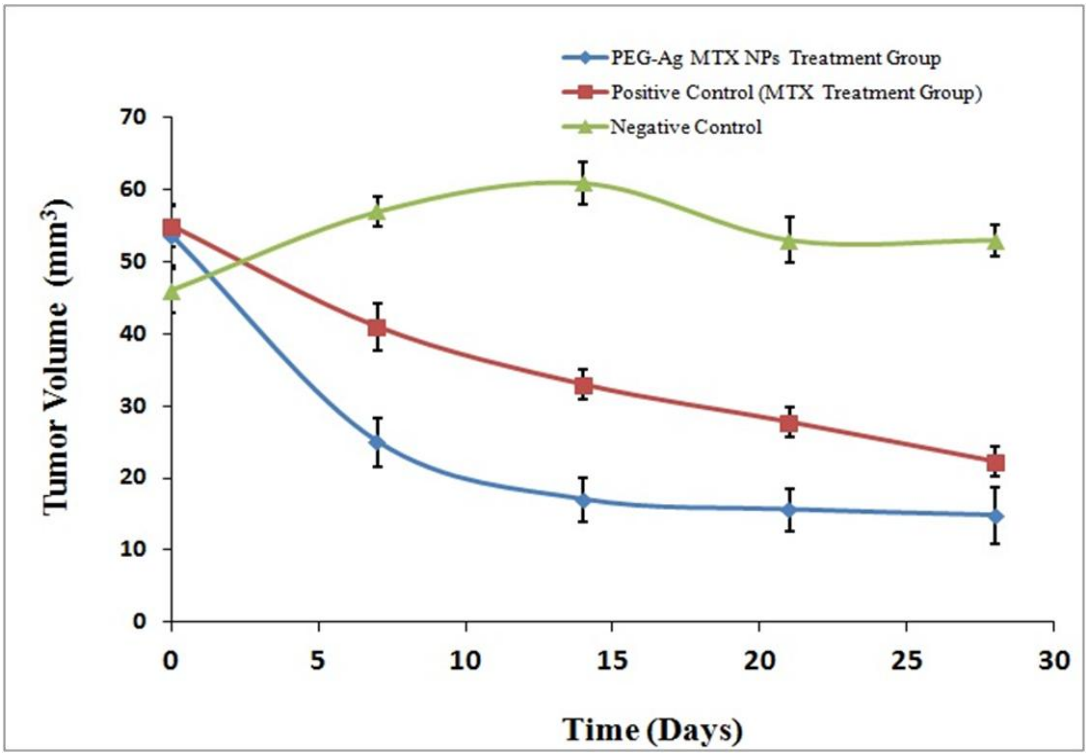

Figure 4.11 Graph showing Reduction in Tumor Volume of Mice in Group 2,3 and 4 over a Period of Time

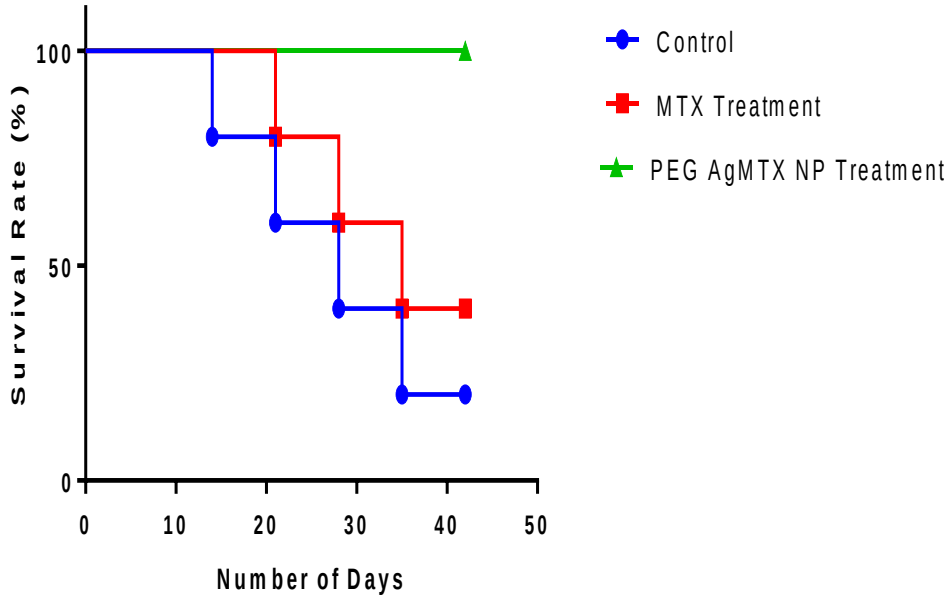

Figure 4.12 Keplen-Mayer Graph showing the Survival Rate of Mice in 3 Groups

4.6.2 Histological Examination

After the required treatment was carried out, the skin biopsy from the tumor tissue was obtained to perform histological examination for each group. The histological examination showed the highest reduction in tumor load for nanoparticles treated group, as shown in Figure 4.13. These results prove our findings that PEG-Ag MTX Nanoparticles are very effective means to eliminate the tumor from skin cells in-vivo.

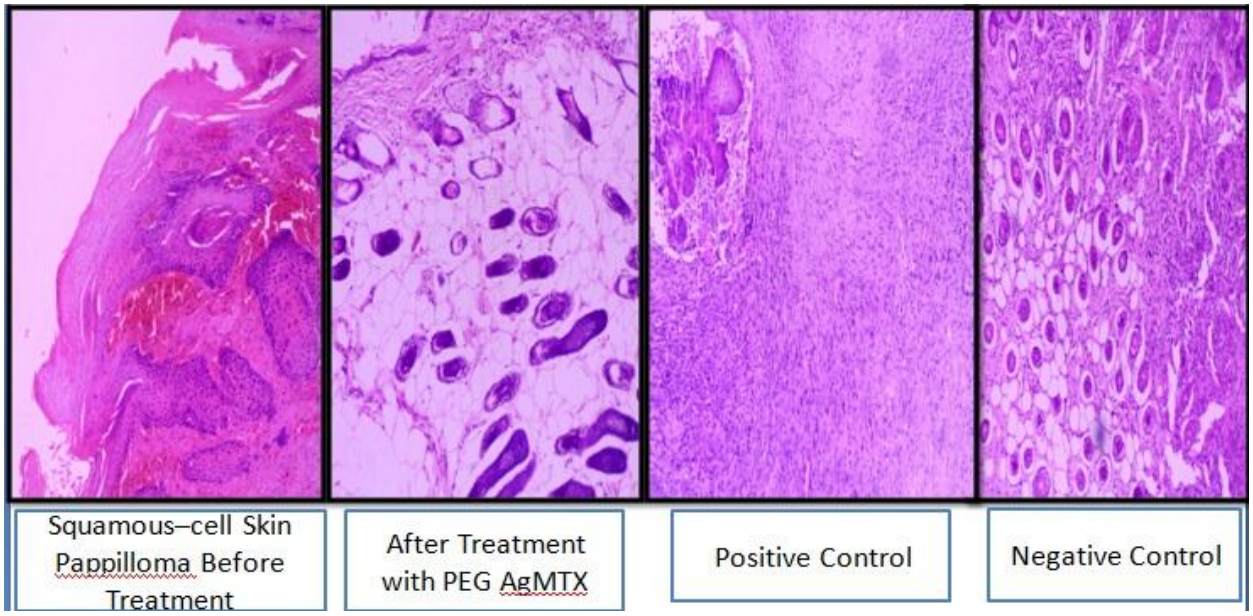

Figure 4.13 Slides showing Reduction in Tumor Volume of Mice in Group 2,3 and 4 under 40X Magnification of the microscope

CONCLUSION

The main objective was to design a nanoformulation that acts as vector for carrying an anticancerous drug MTX, which would be more biocompatible than the drug alone. PEG coated silver nanoparticles are biocompatible entities and are widely used in targeted drug delivery systems. Most importantly PEG-AgMTX contains lower amount of drug with enhanced activity than when the drug is given alone thus reducing the side effects of MTX to a much higher level. The IC50 value for anticancer activity of PEG-Ag-MTX nanoparticles was less than that of the methotrexate alone showing them more effficient than the original drug. It is also notable that the hemolytic activity of these particles was also significantly decreased (previous result). Pegylation reduces opsonization process in which nanoparticles are directed to the liver by help of macrophages. In long run, Pegylation of Nanoparticles may also improve the circulation half time of the MTX. Tailoring of PEG-AgMTX would make them more targets specific with minimal exposure to normal cells with enhanced activity so as small amount of drug causes more toxicity in cancerous cells. It is therefore worthy to investigate *in-vivo* antitumor activity as well the biodistribution of these nanoparticles.

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