SYNTHESIS OF PEG CAPPED METHOTREXATE LOADED SILVER NANOPARTICLES BY CHEMICAL REDUCTION METHOD

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

In
Biomedical Sciences and Engineering

By

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Declaration

It is hereby delared that this research study has been done for partial fullfillment of requirement for the degree of Master of Sciences in Biomedical Engineering. 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. Zarmina Muhammad** NUST201362085MSMME62413F Titled: **"Synthesis of PEG capped Methotrexate loaded silver nanoparticles by chemical reduction method"** be accepted in partial fulfillment of the requirements for the award of **MS Biomedical Sciences** degree. (Grade_____)

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

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list of Abbreviations

UV-Vis	Ultra violet visible absorption spectroscopy
FTIR	Fourier transform infrared spectroscopy
TEM	Transmission electron microscopy
MTX	Methotrexate
$AgNO_3$	Silver nitrate
PBS	Phosphate buffer Saline
$NaBH_4$	Sodium borohydride
K_2CO_3	Potassium carbonate
Rcf	Relative centrifugal Force
%	Percentage
Nm	Nanometer
mM	Millimolar

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Abstract

Background: Chemotherapeutics, the first line treatment, being used world wide each day by millions of people to combat cancer. Due to their specificity, insolubility, large molecular size and most importantly their adverse side effects these drugs were conjugated with silver nanoparticles. In this study, Methotrexate (MTX) an anticancerous drug was bound to silver nanoparticle by chemical reduction method and then capped with PEG-6000 to make it biocompatible. The invitro hemolytic activity of the conjugated nanoparticles (AgMTX) was checked before and after pegylation and also of pure drug.

Results: the synthesis, conjugation, size and shape of AgMTX was determined through FTIR, UV-Vis and TEM. Stability of nanoparticles against temperature and salt concentration was checked and particles were found to be stable. PEG-AgMTX showed significantly low hemolytic behavior in comparison to AgMTX and MTX.

Conclusion: Nanoconjugates showed more hemocompatibilty (caused less than 5 % Hemolysis) and enhanced biological activity as compared to parent drug and also the amount of drug required for its activity was reduced to less than 50%.

Chapter 1
Introduction

1.1 History

Nanotechnology is not something novel that came into existence some three, four decades before rather utilization of nanoparticles dates back to ancient civilization. the ancient Romans used to color glass with shades of yellow, mauve and yellow by using different concentrations of gold and silver(Daniel and Astruc 2004). In 4th century gold and silver nanoparticles were used for aesthetics in the famous Lycurgus cup that is now placed in the British museum(Barber and Freestone 1990). this cup show dual color behaviour that is deep wine red and opaque pea green in case of transmitted and refelcted light respectively and this is because of the surface plasmon resonance of gold andsilver nanocrystals dispersed on the glass matrix (Barber and Freestone 1990; Louis and Pluchery 2012). 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 due to presence od gold nanoparticles(Dreaden, Alkilany et al. 2012). in 9th century the islamic world developed a technique of obtaining nanoparticles by reduction of metal oxides upon heating at high temperature that were already deposited on ceramic surfaces(Padeletti and Fermo 2003; Daniel and Astruc 2004). in the 15th and 17th century this technique of glass coloring was refined 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), he reduced solution of chloroauric acid with carbon disulfide to obtain deep red colored gold nanoparticle solution "ruby fluids". Zsigmondy in 1906(Zsigmondy 1909), by reducing choloauric acid in the presence of formaldehyde to obtain monodisperse gold solutions(Overbeek 1984). in 1951, improved version of Zsigamody's method was introduced, the turkuvish method(Turkevich, Stevenson et al. 1951) that involves chloroauric acid reduction in the presence of sodium citrate to synthesize gold nanoparticles. this method was also used in the synthesis of silver nanoparticles. Thus It

clearly shows that the history of nanoparticles is quite too long but major developments and innovations of new methods took place in the last two to three decades. Richard Feynman, a Noble laureate first highlighted the idea of nanotechnology in December,1959, in a lecture at California Institute of Technology. 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 consolidating materials by atom or by molecules". in 1980, K. Eric Drexler worked on promotion of technological significance at nano level.

1.2 Nanoparticles: As Ideal drug delivery vehicles:

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 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).

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) .

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.

Cancer is one most common cause of death. 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(Society 2012).

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).

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).

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).

1.3 FDA approved nanomedicine:

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).

1.4 Nanoparticles: As nanomedicines

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. Further more 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).

Chapter 2
Literature review

2.1 Introduction

Nanoparticles are defined as particles or substances that have its size not more than100nm in its one dimension at either atomic or molecular state and even at macromolecular scale(Österberg, Persson et al. 1984; Dowling, Clift et al. 2004; Society and Engineering 2004). Nanoparticles have been of great importance from the last few decades is because of its extremely small size as it forms 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 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).

2.2 Silver nanoparticles and its applications

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).

2.3 Methods of preparation 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, $NaBH_4$(Lee, Jun et al. 2006; Song, Lee et al. 2009), 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.

2.4 Methotrexate: An anticancerous drug

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 bound to nanoparticles by many different ways including physical adsorption(Reszka, Beck et al. 1997), through covalent bonding(Torchilin, Levchenko et al. 2001; Zhang, Kohler et al. 2002) or through ionic bonding(Alexiou, Schmid et al. 2002; Zhang, Kohler et al. 2002). Methotrexate (MTX) is a folic acid analog used as a chemotherapeutic Agent(Ohata and Marmarou 1992; Bobo, Laske et al. 1994). As folate receptors(Duthie 2001) 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, and neuroendocrine carcinomas(Ohata and Marmarou 1992; Bobo, Laske et al. 1994; Sirotnak and Tolner 1999; Stella, Arpicco et al. 2000; Sudimack and Lee 2000).

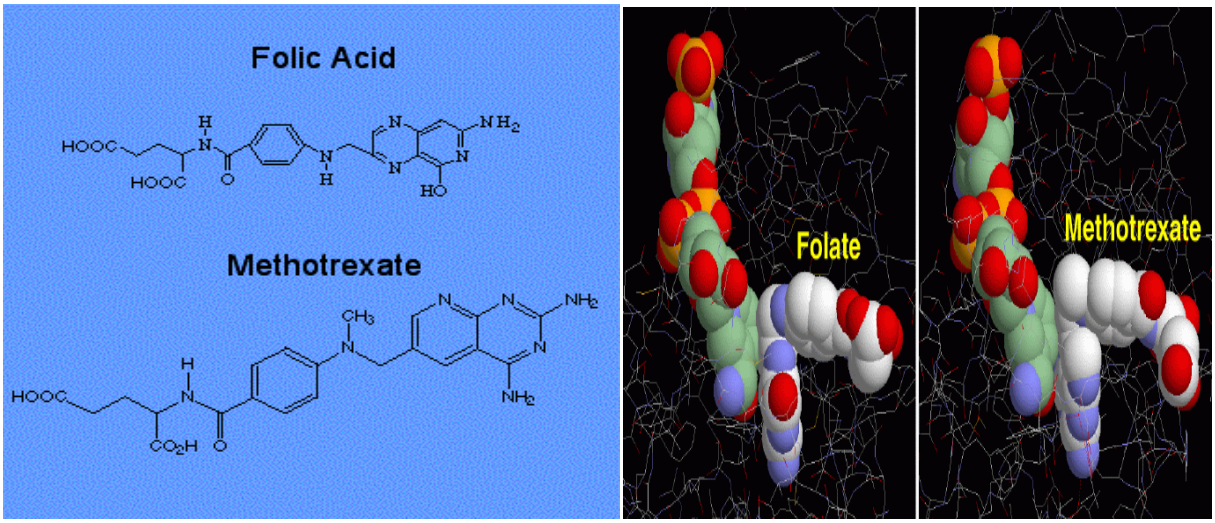

Figure 2.1: Strucutre of Methotrexate (Folate analogue of Methotrexate is also shown)

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, Laske et al. 1995; Rihova, Jelinkova et al. 2000; Di Stefano, Kratz et al. 2003; Chen, Tsai et al. 2007), also target cells develop resistance to MTX due to efflux o the drug by the cancerous cell membrance proteins into the extracellular environment(Banerjee, Mayer-Kuckuk et al. 2002). To overcome this problem conjugates of MTX with polymers such as PEG(poly ethylene glycol) and PGA (Poly glutamic acid) was produced, though these conjugates had higher rention value in the cell thus maintaining a higher concentration of MTX with in the cancerous cell(Piper, McCaleb et al. 1983; Riebeseel, Biedermann et al. 2002) but these large sized conjugates limit the drug administration only to target specific 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. In this study we developed MTX-nanoparticle conjugate by binding MTX to silver nanoparticles through physical adsorption.

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, Laske et al. 1995; Sirotnak and Tolner 1999; Stella, Arpicco 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 comparedto 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.

Chapter 3
Materials and Methods

3.1. Experimental

3.1.1. Materials and Instruments

Silver nitrate ($AgNO_3$), sodium borohydride ($NaBH_4$) and potassium carbonate (K_2CO_3) were purchased from Sigma Aldrich. Methotrexate (MTX) was used as drug. Deionized water was used throughout the experiment. Shimatzu UV-Vis 1800 spectrophotometer was used for recording UV-Vis spectra. Eyela FDU-1000 freeze dryer was used for freeze drying.

3.1.2. Synthesis of silver nanoparticles capped with MTX (AgMTX)

1 mM solution of $AgNO_3$ was prepared in deionized water. MTX is insoluble in deionized water but dissolves in the presence of carbonates and bicarbonates therefore 1mM solution of MTX was prepared in 1mM K_2CO_3 and 40 mM solution of $NaBH_4$ (prepared in chilled deionized water) was used as reducing agent.

1 mL of MTX and 1 mL of AgNO3 (1 mM) was taken and kept on shaking at 250 rpm for 20 min. Then 0.1 mL of NaBH4 was added drop wise. After addition of reducing agent, the color of the reaction mixture generally changed from transparent to brown, dark brown and ultimately to reddish brown. After 4 h shaking, the solution was characterized by UV–vis spectroscopy (Figure 4.1). Similarly different ratios of Ag, MTX and NaBH4 were tested (Additional file 2: Table S1 and Table S2) and their SPR peaks were recorded (Additional file Figure S1). From table S1 and S2 the optimum ratio for nanoparticles formation was found to be 20: 1.0: 2.0 (Ag: MTX: NaBH4). Using the same experimental conditions, the same ratio (20: 1.0: 2.0) was tried on a stirrer at 250 rpm and the results were reported in figure 4.2 (Additional file Table S3, Figure S2). The spectra obtained from shaker samples had high absorption peak as compared to stirrer samples and so shaker was chosen.

3.1.3. Bare silver nanoparticles (Ag)

As per already developed protocol for synthesis of bare nanoparticles(Naz, Shah et al. 2014) 100 mL of $AgNO_3$ was taken in a flask and kept on shaker. After 20 min of shaking 10 mL of 40 mM $NaBH_4$ was added to it as a result the color was completely changed from transparent to dark brown. The sample was left on shaker for 30 min at 250 rpm. Solid bare nanoparticles (Ag) were then collected *via* freeze drying procedure.

3.1.4. Freeze drying

Freeze drying is a dehydration technique in which the liquid samples are dried under vacuum and low pressure that has already been frozen. The main principle involved in the process of freeze drying is sublimation in which the frozen sample sublime to dried form under vacuum and highly reduced surrounding pressure(https://en.wikipedia.org/wiki/Freeze-drying ; GR.Nireesha 2013).

Solid silver nanoparticles (AgMTX) and bare silver nanoparticles (Ag) were obtained by freeze drying for FTIR and TEM characterizations and also for bioactivity screening. Bulk preparation of AgMTX and Ag was done and then it was poured in Petri plates, 25-30 mL each. Petri plates were then covered with parafilm and fine pores were made into it by a needle and were kept at -80 ^{o}C for 10 h. Petri plates were then placed in freeze dryer for 40-48 h at 15 pa.

After the samples were completely dried, the powdered material was transferred to a clean glass viol (already weighted) and the above mentioned procedure was repeated to obtain 5 mg of the sample.

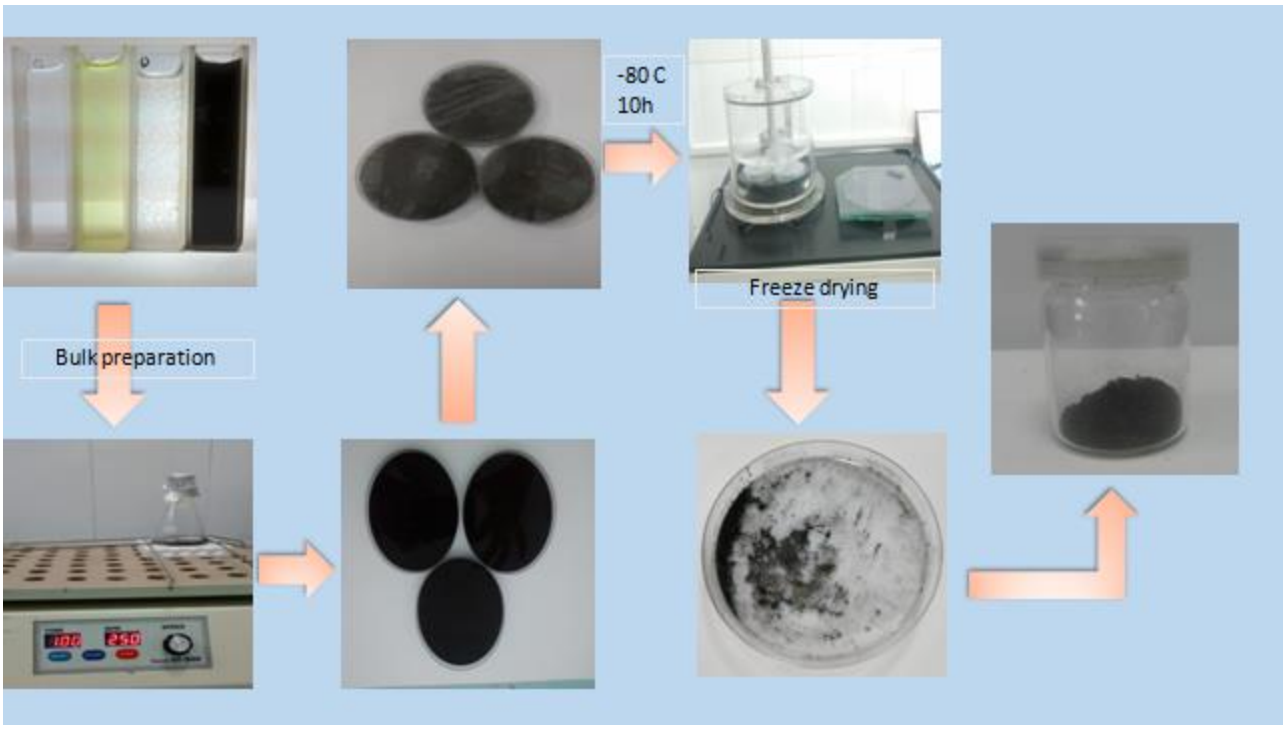

Figure 3.1: Schematic representation of Freeze drying process.

3.1.5. Coating of AgMTX with PEG-6000 (PEG-AgMTX)

5 mg of freeze dried AgMTX was dissolved in 50% ethanol/Deionized water to form AgMTX solution. This solution was kept on stirring for 2h and then its centrifugation was done at 14000 rcf for 1h. The supernatant was separated and UV-Vis spectra of both supernatant and the pellet was recorded. The pellet was then redissolved in ethanol and was poured in an already weighted Petri plate. Ethanol would get evaporated and pure AgMTX particles would be left behind. 5mg of this purified AgMTX was dissolved again in 50% ethanol/deionized water to form AgMTX solution. To this solution kept on stirring add drop wise PEG-6000 dissolved in deionized water. After 2h of stirring, centrifuge the sample again at 14000 rcf for 1h. Remove the supernatant and the pellet obtained is PEG-AgMTX. Record the UV-Vis spectrum of pellet.

3.2. Cell toxicity assay

To determine the Cytotoxic effects of newly synthesized molecules or test samples on cells in order to know the viability of cells by the end of experimental procedure, cell

toxicity assays are performed. The cytotoxicity assays performed for dertermining the toxicity of Ag, AgMTX and PEG-AgMTX are:

Hemolysis assay

3.2.1. Hemolysis assay

Cytotoxic effect of silver nanoparticles (Ag, AgMTX and PEG-AgMTX) was studied by carrying out its hemolytic activity. For this assay the blood of healthy donor was taken. 15 mL of the blood sample was collected and out of which 4 mL was taken and mixed with 8 mL of PBS (phosphate buffer saline solution). This mixture was then centrifuged at 10000 rpm for 10min. RBCs were collected in the pellet which was then washed three times with PBS for 3 min at 10000 rpm. The RBCs obtained so were diluted with PBS. The test samples were prepared by making stock solution of silver nanoparticles (Ag, AgMTX and PEG-AgMTX) using PBS as solvent(Lingheswar sadhasivam 2014). Various concentrations such as 1, 10, 100, 150 µg mL^{-1} were used for the hemolytic study. The RBC suspension diluted with PBS was taken as negative control and 0.5 %Triton X-100 as positive control(Fredrik Nederberg). The samples were kept for incubation at 37 ^{o}C for 1h, 4h and 24h. After incubation the samples were vortexed and then centrifuged at 10000 g for 5 min. 100 µl of supernatant was collected and poured into a 96 well plate(Fredrik Nederberg ; Julie Laloy1 2014). The absorbance was measured using a micro plate scanning spectrophotometer at 562 nm. Positive and negative controls induced 100 %and 0 % absorbance respectively.

The % hemolysis was calculated as

$$\text{Hemolysis (\%)} = \frac{\text{Absorbance of sample} - \text{Absorbance of negative control}}{\text{Absorbance of positive control} - \text{Absorbance of negative control}} * 100 \text{(Fredrik Nederberg ; Julie Laloy1 2014; Lingheswar sadhasivam 2014)}$$

3.3. Characterization of AgMTX

To determine different properties of AgMTX and PEG-AgMTX, it has to undergo various characterization techniques. The results of these techniques give information about the optical and structural properties of AgMTX.

3.3.1. Optical characterization

When AgMTX and PEG-AgMTX were put to the following characterization technique they showed their optical behavior related to their property and gives associated information. Those techniques are

UV-Vis absorption spectroscopy (UV-Vis)

Fourier transform infrared spectroscopy (FTIR).

3.3.2. Structural characterization

In order to determine the size, shape, surface morphology, structure and exact dimensions of AgMTX, the following characterization technique were performed.

Transmission electron microscopy (TEM).

3.4. UV-Vis absorption spectroscopy

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 (http://www2.chemistry.msu.edu/faculty/reusch/VirtTxtJml/Spectrpy/UV-Vis/uvspec.htm#uv1). 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(http://www.scribd.com/doc/21053651/Electronic-spectroscopy-UV-Visible).

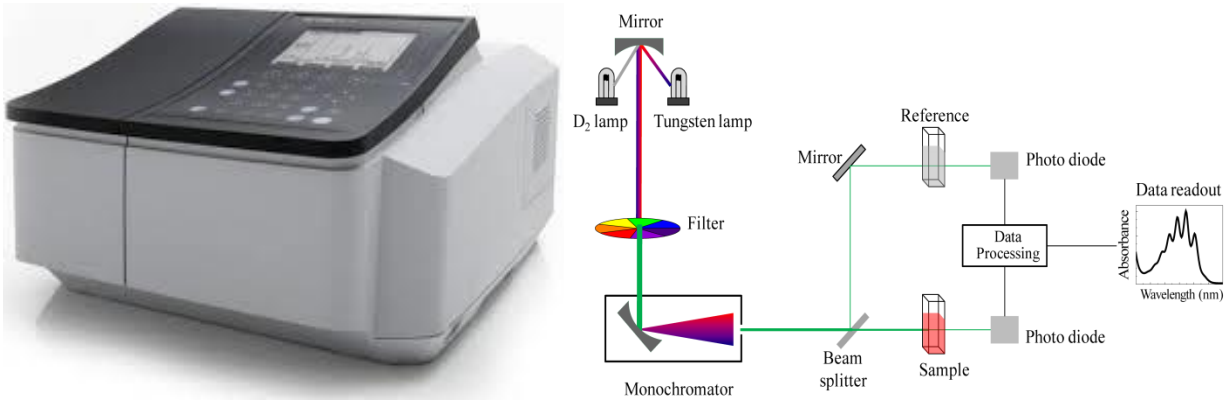

Figure 3.2: UV-Vis spectrophotometer and schematic diagram of UV-Vis spectrophotometer.

3.5. Fourier transform infrared spectroscopy (FTIR)

Fourier transform infrared spectroscopy (FTIR) is one the most commonly used tool for functional group detection in pure compounds, mixtures or their comparison. It is used to record an infrared spectrum of absorption, emission, photoconductivity or Raman scattering of a solid, liquid or gas(https://en.wikipedia.org/wiki/Fourier_transform_infrared_spectroscopy). FTIR is widely used for characterization of nanoparticles and studying the nature of surface adsorbents. As nanoparticles have large surface area and surface adsorbents modify the surface by generating different properties that occurs in the form of additional peaks in an FTIR spectra in comparison with the spectra having no adsorbents. So FTIR

spectroscopy can easily detect minute surface modifications(Samkaria and Sharma 2013). FTIR spectrophotometer is composed of complex electrical and mechanical system that detects minute changes in energy absorbed and convert it into an accurate recorded spectra(http://shodhganga.inflibnet.ac.in:8080/jspui/bitstream/10603/468/10/10_chapter4.pdf). For the analysis of broad range of materials including liquids, solids, fibers, powders, pastes and other many forms in either bulk or thin films, FTIR can be used. It not only gives us qualitative information but also analyse the material quantitatively as the size of the peak directly determine the amount of material present(http://en.wikipedia.org/wiki/Fourier_transform_infrared_spectroscopy#Conceptual_introduction ; mmrc.caltech.edu/FTIR/FTIRintro.pdf). FTIR Spectroscopy works on the principle that molecule absorbs frequencies that matches exactly the frequencies of the bonds type or group that vibrates the molecule also known as resonating frequency. The detection of this frequency absorption is done on the basis of atomic masses, shape of energy surfaces and its associated vibronic coupling.

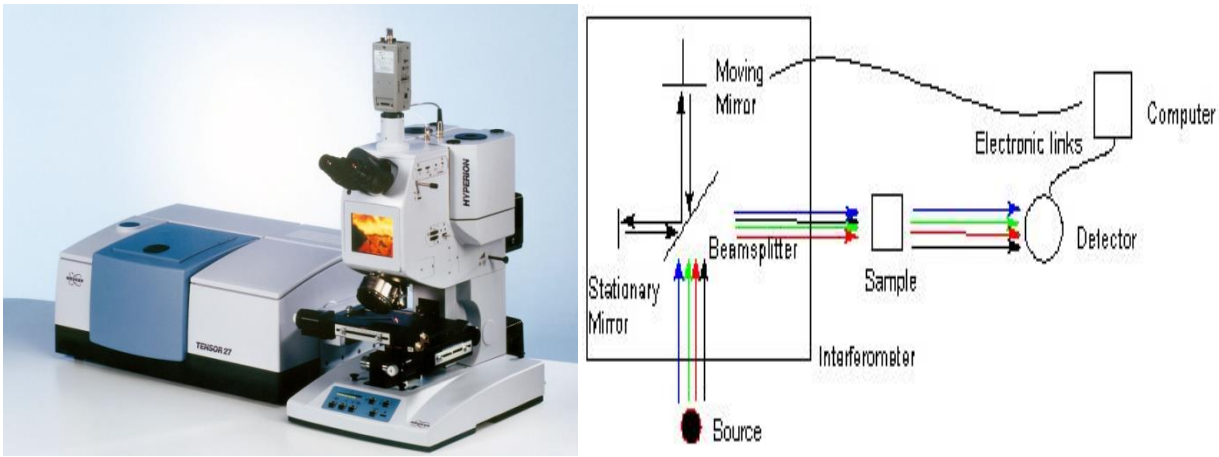

Figure 3.3: Schematic diagram of FTIR.

3.6. Transmission electron microscopy (TEM)

TEM is an important characterization tool for imaging directly nanoparticles to obtain particle size, size distribution, shape, topography, morphology, composition, crystallographic and other submicroscopic information quantitatively(https://en.wikipedia.org/wiki/Transmission_electron_microscopy ; 2012).

TEM uses high electron beam that has been transmitted to a very fine and thin sample to image and analyze the microstructure of sample with atomic scale resolution. A light source at the top of the microscope emits the electrons that travel through vacuum in the column of the microscope. These electrons are focused on electromagnetic lenses and then it travels through the sample. Depending on the sample's density some electrons are scattered and disappear from the beam. The unscattered electrons hit the fluorescent screen at the bottom of the microscope and a shadowed image is observed on a fluorescent screen with its different parts displayed according to their density in varied darkness(http://www.nobelprize.org/educational/physics/microscopes/tem/). TEM image has higher resolution that light-based imaging techniques by a factor of 1000(2012; Somsubhra Ghosh 2013). The image is either directly analyzed by the operator or is photographed *via* a digital camera. Samples are prepared for imaging by drying of nanoparticles on copper grid coated by thin layer of carbon(Hussain 2011; 2012). Those substances with higher electron densities than amorphous carbon are imaged easily and they mostly include metals, oxides and other polymeric materials such as nanoparticles, quantum dots, nanotubes etc(2012).

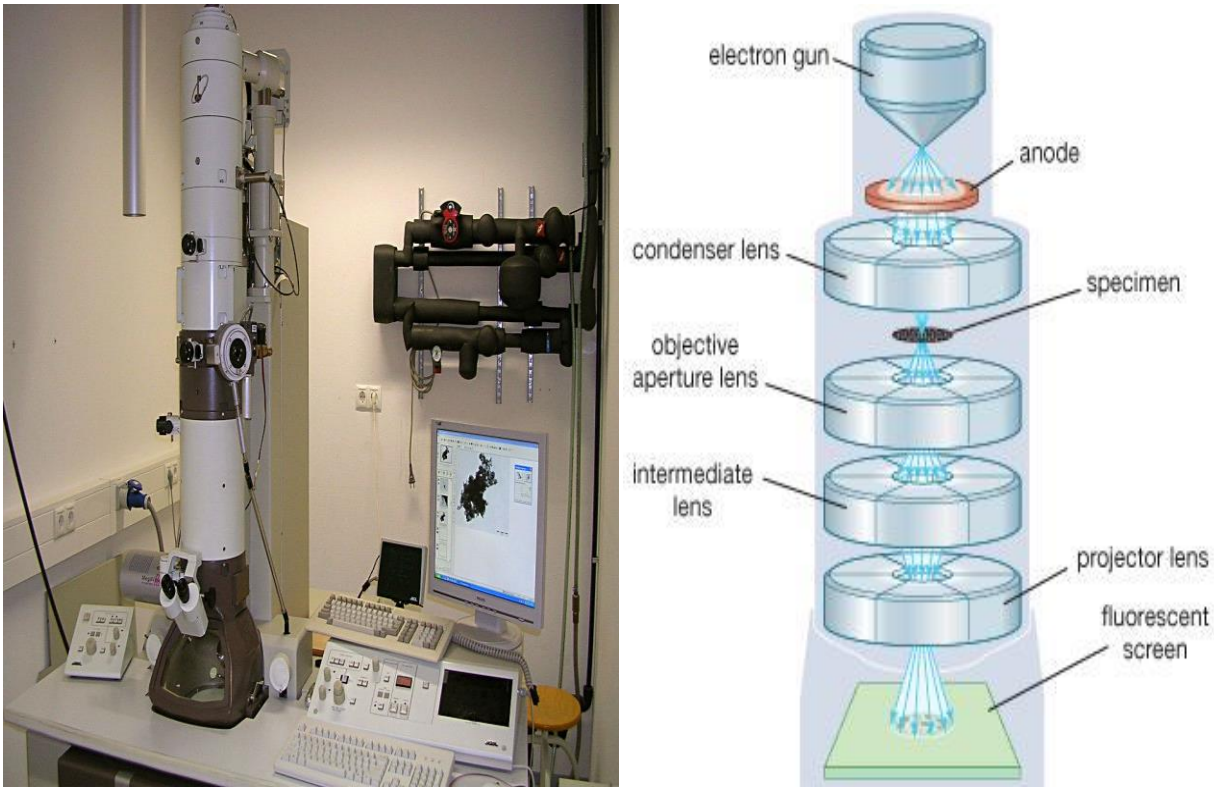

Figure 3.4: Schematic diagram of TEM.

Chapter 4
Results and Discussions

When MTX was added to the aqueous solution of $AgNO_3$, a change in color from transparent to light brown and then to dark brown, upon drop wise addition of $NaBH_4$ was observed that determines the formation of AgMTX (Figure 4.4).

4.1. UV-Vis absorption spectroscopy

Silver nanoparticles due to their surface plasmon resonance exhibit optical properties that depends on their size, shape and size distribution(VINITA ERNEST 2013)and for the structural characterization UV-Vis is the most widely used technique(Rashid, Bhuiyan et al. 2013). 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 $AgNO_3$ has no peak in this range (300-650 nm) that clearly determines that silver nanoparticles conjugated with drug has formed (Figure 4.1). Also the SPR peak of AgMTX lies within the UV-Vis absorption spectral range 350-600 nm(Sherry, Chang et al. 2005; Bhainsa and D'Souza 2006; Rashid, Bhuiyan et al. 2013) for silver nanoparticles that assures the formation of AgMTX.

UV-Vis absorption spectra of freeze dried sample of AgMTX showed surface Plasmon resonance peak at 386 nm while that of before freeze drying is at 397.56 nm. The blue shift observed in the two peaks (Figure 4.3) is due to the formation of aggregates of nanoparticles due to freeze drying as reported earlier.

4.2 Determination of encapsulation efficiency of AgMTX

UV-Vis spectra of supernatant and pellet obtained as a result of centrifugation of resuspended AgMTX are recorded to be 370 nm and 388 nm respectively (Figure 4.6).

The SPR peak of supernatant is more like the SPR peak of MTX, so in order to determine if there is any unbound drug removed in supernatant as a result of centrifugation different

molar concentration of MTX are prepared and its UV-Vis spectra is recorded and the SPR peak obtained was at 370nm but its absorbance increases with increasing molar concentration (Figure 4.5A). Different molar concentrations were plotted against their absorbance values and the slope obtained was 3.3906 (Figure 4.5B).

From the slope we determined the concentration of unbound drug removed in the supernatant. According to Lambert-Beer Law equation:

$$A = EcL$$

According to this equation the amount of unbound drug removed from 5 mg AgMTX was 0.25 mg. For calculating the % drug (MTX) loading in purified AgMTX the equation used was:

$$\text{Drug loading } (\%) = \frac{\text{Total amount of Drug - amount of Drug in supernatant}}{\text{Total amount of Drug}} * 100$$ (Muthukumar, Prabha

From this equation the amount of MTX bound to AgMTX was calculated to be 39.06 %.

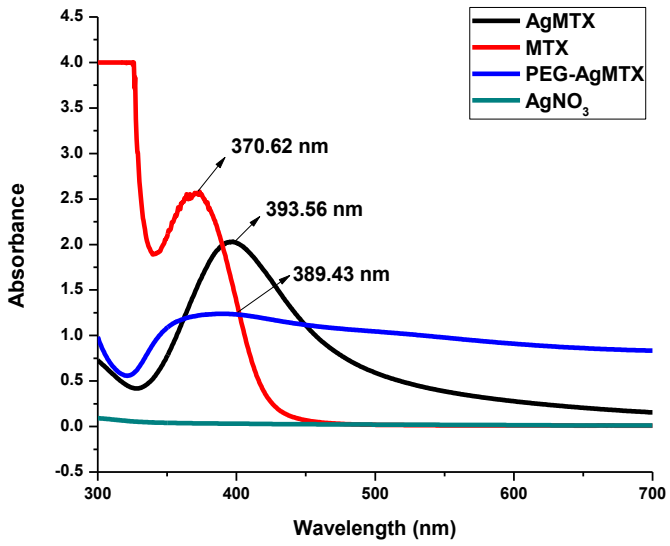

Figure 4.1 Comparative UV-Vis spectra of MTX, AgMTX, PEG-AgMTX and $AgNO_3$.

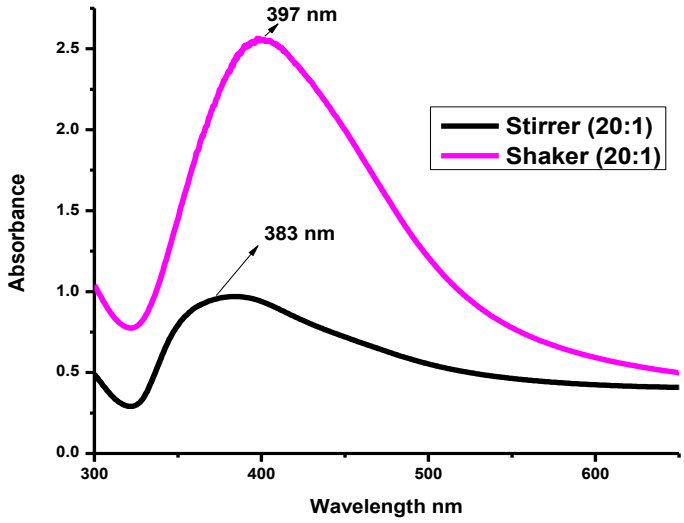

Figure 4.2 Optimizing the reaction by changing the amount and concentration of $NaBH_4$ and also by changing instrument.

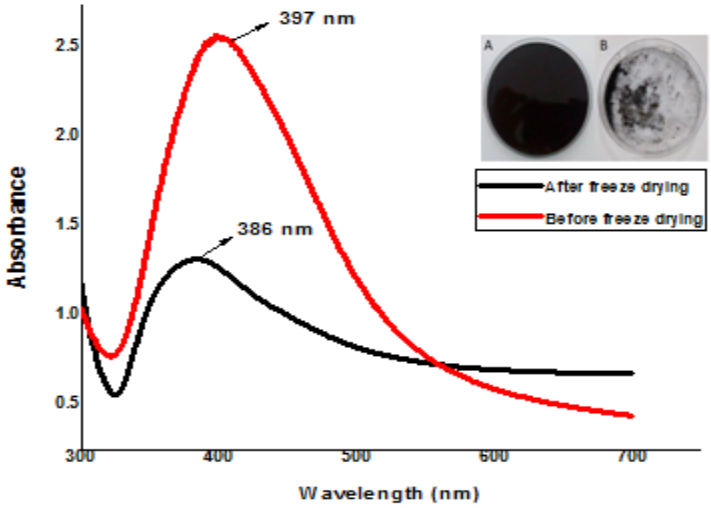

Figure 4.3 Comparison of SPR peak of AgMTX before freeze drying and after freeze drying (in the image shown 'A' represents before freeze drying and 'B' represents after freeze drying)

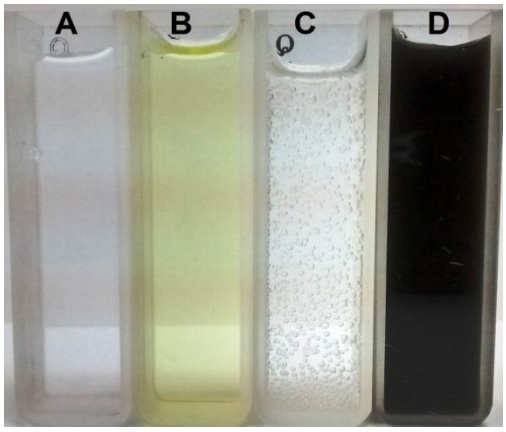

Figure 4.4 Optical recognition of (A) $AgNO_3$, (B) MTX, (C) $NaBH_4$ and (D) Ag-MTX.

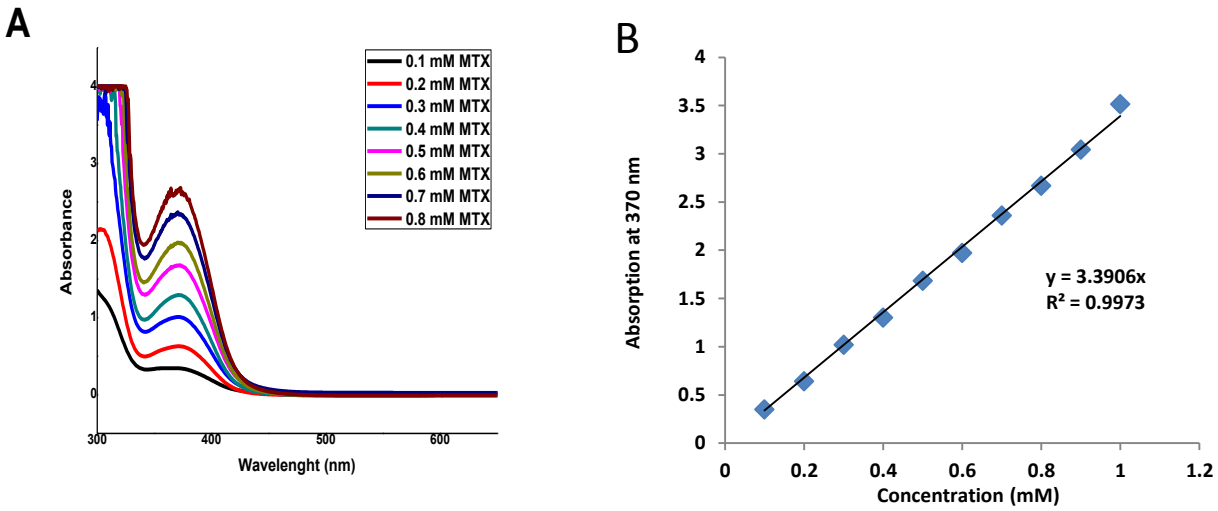

Figure 4.5 Different molar concentrations of MTX (mM): **A.** Increase in absorbance with increasing molar concentration, **B**. Determination of slope

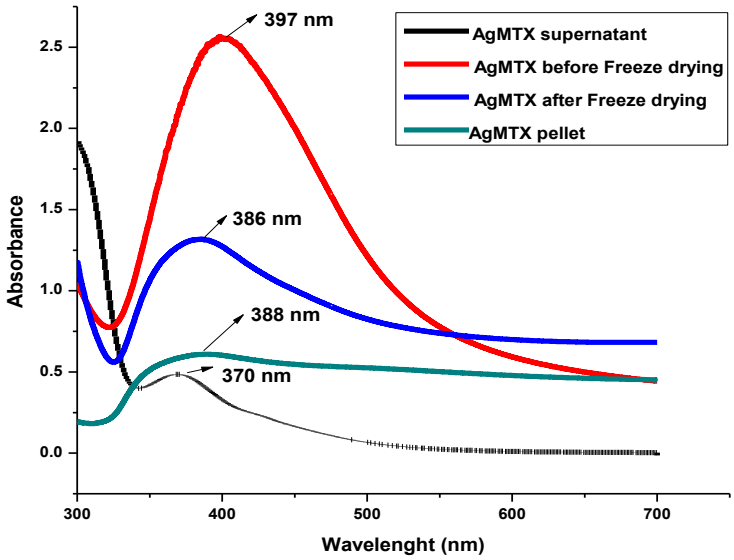

Figure 4.6 Comparison of SPR peak of AgMTX before freeze drying, after freeze drying and freeze drying centrifuged sample (pellet and supernatant).

4.3. Fourier transform infrared spectroscopy (FTIR)

Figure 4.7 show the FTIR spectrum of MTX and AgMTX in the range of 600-4000 cm^{-1}. The disappearance of peak at 1600cm^{-1} (due to carbonyl carbon) and appearance of new peak at 2300cm^{-1} (due to formation of Ag-O bond) indicated the chelation of carboxylic group of MTX with silver.

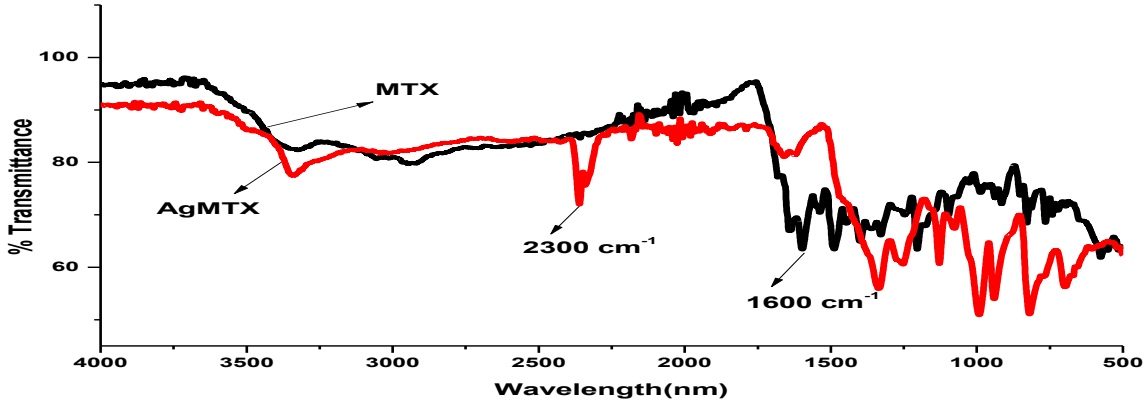

Figure 4.7 FTIR peaks of MTX, Ag, AgMTX and PEG-AgMTX.

4.4. Transmission electron microscopy (TEM)

Transmission electron microscopy finally confirms the formation of Methotrexate conjugated silver nanoparticles. The result of TEM is depicted in Figure 4.8. From the figure shown the average size of PEG-AgMTX was determined to be 12 nm.

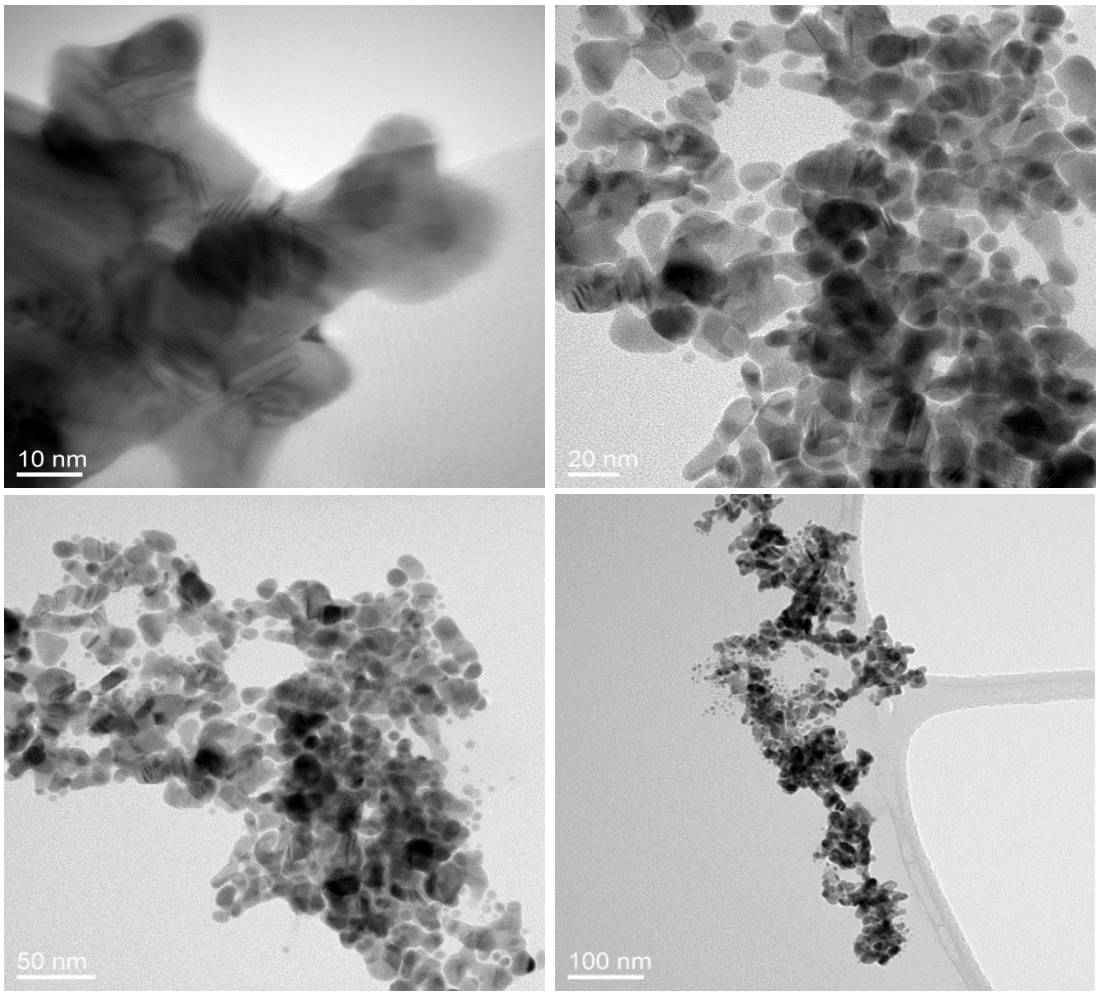

Figure 4.8 TEM images of AgMTX at 20 nm, 50 nm and 100 nm scale.

4.4. Haemolysis assay

Hemolysis assay gave a clear indication of the extent of toxicity caused by MTX, AgMTX and PEG-AgMTX of various concentrations (5, 10, 15, 25, 50, 75, and 100 ug/mL) to red blood cells quantitatively. The results obtained from this assay declared that PEG-AgMTX are more hemocompatible as compared to parent drug and to that of non PEGylated AgMTX (Figure 4.9). Hemolytic behavior of PEG-AgMTX increased gradually with increasing concentration and that the % hemolysis was less than 5 % that declares these particles to be hemocompatible and according to ISO/TR 7406 this ratio is considered as the safest value.

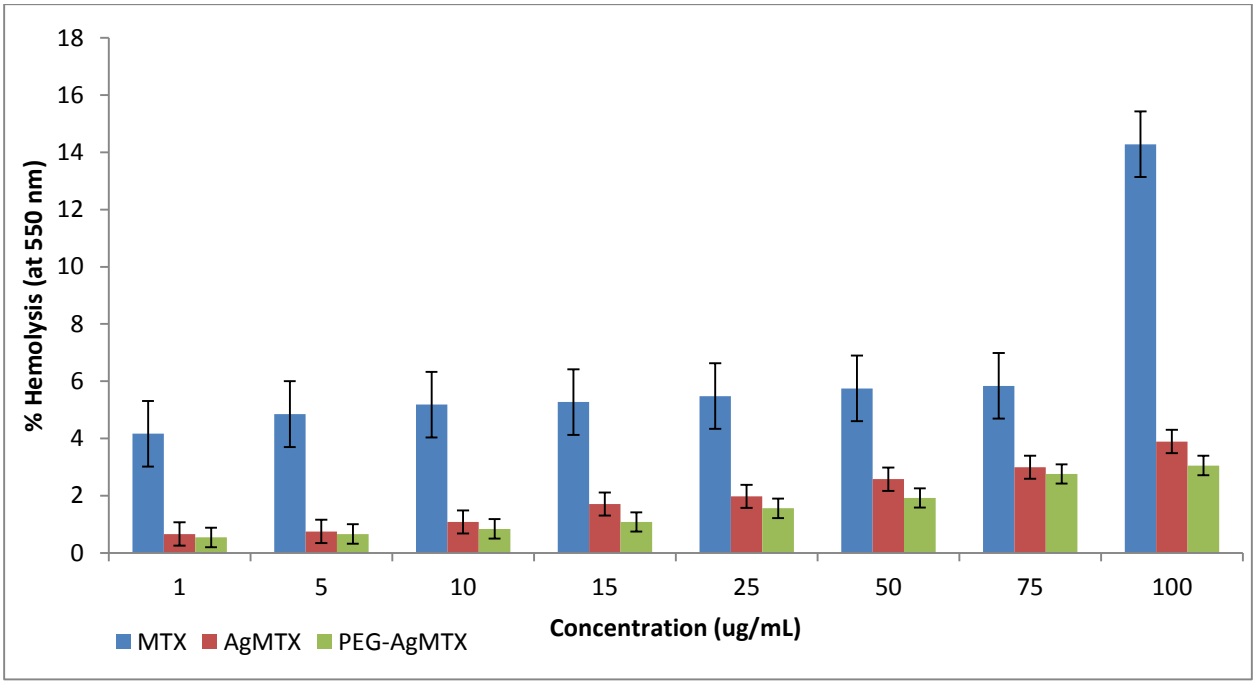

Figure 4.9 % Hemolysis of Red blood cells under the effect of MTX, AgMTX and PEG-AgMTX (Absorbance measured at 550 nm, n=3).

5. 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. PEG-AgMTX are not only biocompatible and serve as drug delivery vehicle for MTX but also shows relatively low hemolytic behavior i-e less than 5%, than the parent drug. 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. 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. This nanoformulation, PEG-AgMTX requires further invitro and invivo studies on animal models.

Supplementary material

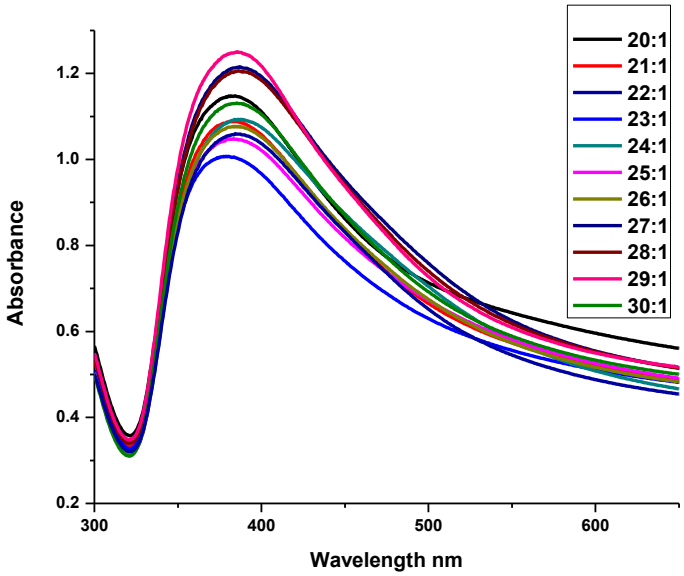

Figure S1 By changing the amount of $AgNO_3$, MTX (drug) and $NaBH_4$

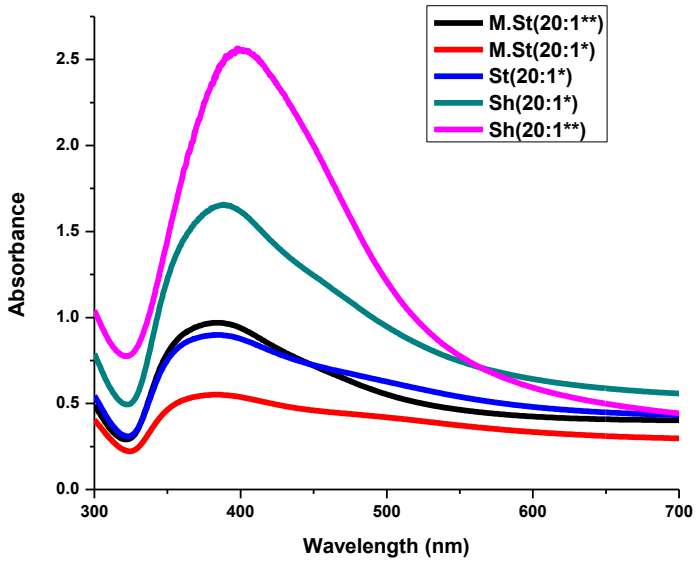

Figure S2 Optimizing the reaction by changing the amount and concentration of $NaBH_4$ and also by changing instrument

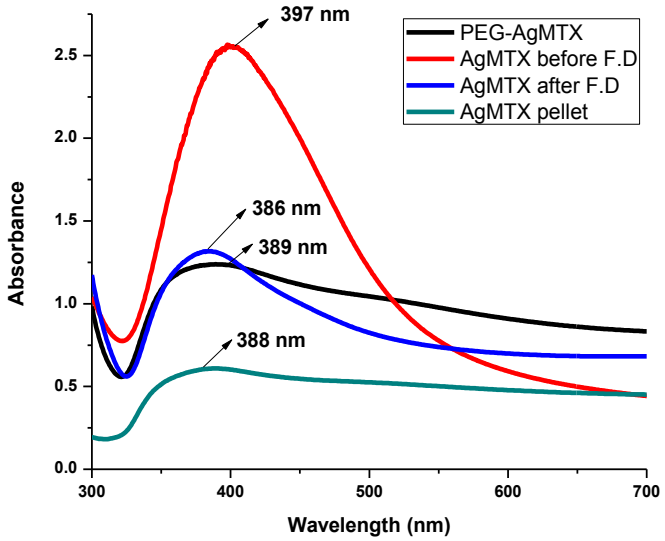

Figure S3 Comparative SPR peaks of AgMTX(before and after freeze drying), AgMTX pellet (by centrifugation of resuspended AgMTX) and PEG-AgMTX.

Table S1: Optimizing the reaction by changing the amount of $AgNO_3$, MTX (drug) and $NaBH_4$

Sample	**$AgNO_3$:MTX:$NaBH_4$ (mL)**	**λ^{max} /abs^{max} (nm)**
18:1	1.8: 1.0: 0.18	384/1.06
19:1	1.9: 1.0: 0.18	385/1.07
20:1	**2.0: 1.0: 0.20**	**384/1.15**
21:1	2.1: 0.1: 0.21	384/1.09
22:1	2.2: 0.1: 0.22	381/1.01
Conditions: $AgNO_3$ 1 mM, MTX 1 mM, $NaBH_4$ 4 mM		

Table S2 Optimizing the reaction by changing the amount of $AgNO_3$, MTX(drug) and $NaBH_4$

Sample	**$AgNO_3$:MTX:$NaBH_4$ (ml)**	**λ^{max} /abs^{max} (nm)**
20:1	**2.0:0.1:2.0**	**388.78/1.059**
21:1	2.1:0.1:2.1	384.96/1.086
22:1	2.2:0.1:2.2	380.19/1.015
23:1	2.3:0.1:2.3	384.96/1.070
24:1	2.4:0.1:2.4	386.871.109
25:1	2.5:0.1:2.5	384.01/1.046
26:1	2.6:0.1:2.6	383.05/1.093
27:1	2.7:0.1:2.7	386.87/1.227
28:1	2.8:0.1:2.8	386.87/1.219
29:1	2.9:0.1:2.9	384.97/1.262
30:1	3.0:0.1:3.0	384.97/1.153
Conditions: $AgNO_3$ 1mM, MTX 1mM, Na BH_4 4mM		

Table S3: Optimizing the reaction by changing the amount and concentration of $NaBH_4$ and also by changing instrument

Instrument	**Sample**	**$AgNO_3$:MTX: $NaBH_4$ (ml)**	**λ^{max} /abs^{max} (nm)**
Shaker	20:1*	2.0:0.1:2.0	388.78/1.059
Shaker	**20:1****	**2.0:0.1:0.2**	**397.37/2.571**
Stirrer	20:1*	2.0:0.1:2.0	384.96/0.905
multistirrer	20:1*	2.0:0.1:2.0	382.10/0.551
multistirrer	20:1**	2.0:0.1:0.2	383.62/0.984
Conditions: $AgNO_3$ 1mM,MTX 1mM and $NaBH_4$ (* represents 4mM and **represents 40mM)			

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Appendix

List of Publications

- **Conference Publication:**

1. Muhammad Fateh, Nasir Rashid, **Hafsah Akhtar**, Zarmina Muhammad, Syed Omer Gilani, and Umar Ansari. "Evaluation of LDA, QDA and decision trees for multifunctional controlled below elbow prosthetic limb using EMG signals." International Conference on Robotics and Emerging Allied Technologies in Engineering (iCREATE), 2014, pp. 115-117. IEEE, 2014.

- **Research Papers (Submitted)**

Zarmina Muhammad, Sundus Riaz, Syeda Sohaila Naz, Wajiha Ahmed, Sadia Malik, Abida Raza Rana, Nosheen Fatima Rana "Synthesis of PEG capped Methotrexate loaded silver nanoparticles by chemical reduction method" European Journal of Pharmaceutical Sciences. (Submitted, status: under review)