Research Article

Development and characterization of Methotrexate loaded PEGylated gold nanoparticles

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ABSTRACT

Cancer is a disease characterized by uncontrolled multiplication and spread of abnormal forms of the body's specific cells. The aim of these studies was to synthesize, characterize and evaluate the efficacy of PEGylated gold nanoparticles. Gold nanoparticles were synthesized by chemical reduction method. The anti-cancer drug Methotrexate (MTX) was encapsulated by the AuNPs by non covalent adsorption. The particle size, PDI, zeta potential, % drug bound, % drug content of optimized PEGylated gold nanoparticles were found to be 19.79 nm, 0.703, -6.73, 78.38 % ± 0.75 and 97.03 % ± 2.904 respectively. The proficiency of MTX loaded PEGylated gold nanoparticles in inhibiting the proliferation of breast cancer cells MCF-7 as compared to the free drug and Methotrexate loaded gold nanoparticles is demonstrated based on MTT assay. Notably, MTX loaded PEGylated gold nanoparticles was found to have 30% higher cytotoxicity on MCF-7 cells compared with an equivalent dose (0.5 µg/µL) of free MTX and Methotrexate loaded gold nanoparticles. Taken together, all results unveil that MTX loaded PEGylated gold nanoparticles could be more effective than free drug and Methotrexate loaded gold nanoparticles for cancer treatment.

Keywords: Cancer, Methotrexate, gold nanoparticle, PEGylation, Cell viability

1. INTRODUCTION

Cancer rests as a prominent reason of mortality globally, interpreting for 8.2 million cancer associated deaths in 2012. Once a year cancer cases are anticipated to increase from 14 million in 2012 to 22 within the next two decades, agreeing to be the digits stated by World Health Organization (WHO) [1].

Cancer is an ailment regarded as uncontrolled proliferation and spread of unusual forms of the body's specific cells. Each day, cells in your body split, mature and die. Furthermost, cells divide and grow in an organized way. Nevertheless, sometimes cells grow out of hand [2].

It would be necessary to improve chemotherapeutics that can either passively or actively target cancerous cells. Passive targeting exploits the specific features of tumour anatomy that let Nano-carriers to accumulate in the tumour by the enhanced permeability and retention (EPR) effect. Dynamic approaches accomplish this by conjugating Nano carriers comprising chemotherapeutics with molecules that bind to over expressed antigens or receptors on the target cells [3].

Gold nanoparticles (AuNPs) could successfully be utilized as carriers for chemotherapeutic drugs in cancer therapy [4]. Gold nanoparticles are also known as colloidal gold. Colloidal gold is a suspension of sub-micrometre-sized particles

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of gold in a fluid, usually water. Hence, the colloidal is either an intense red color (for particles less than 100 nm) or a purple color (for larger particles) [5].

The optimal size of nanocarriers for attacking tumors should range from 10 nm to 200 nm and premodified with caution charges on the nanocarrier's surface. Without positively or negatively charged modifications on the shell surface, those nanoscale carriers would be rapidly cleared by the reticuloendothelial system. Thus, the most common surface coating agent for protecting and reducing reticuloendothelial system-mediated clearance is to decorate nanocarrier surface with polyethylene glycol (PEG)[4, 6].

PEGylation is the process of both covalent and non-covalent attachment which can improve the safety and efficiency of many therapeutic agents. It produces alterations in the physiochemical properties including changes in conformation, electrostatic binding, hydrophobicity etc. These physical and chemical changes increase systemic retention of the therapeutic agent. Also, it can influence the binding affinity of the therapeutic moiety to the cell receptors and can alter the absorption and distribution patterns. PEGylation of gold nanoparticles is frequently accomplished by incubation of a reactive derivative of PEG with the target molecule [7, 8].

Literature report suggests that non covalent drug loading is prevalent compared to covalent loading and release the drug easily in extracellular acidic cancerous medium [9].

The citrate reduction method is well known for its simple procedure, yielding stable and reproducible AuNPs of narrow size distribution. This method was pioneered by Turkevich et al. to synthesize 20-nm AuNPs through reduction of HAuCl4 via trisodium citrate [10, 11].

2. MATERIALS AND METHODS

2.1. Materials

Methotrexate was provided as a gift sample by sun Pharmaceuticals, Baroda; Chloroauric acid was purchased from Indian platinum, Mumbai. Trisodium citrate and Polyethylene glycol 400 was purchased from suvidhinath laboratories pvt. Ltd,Vadodara. All other ingredients were supplied by S.D.fine-Chem ltd, Mumbai. All the samples were prepared in deionized water.

2.2. Methods

2.2.1. Synthesis of PEGylated gold nanoparticles

Dilute 2 mL 10mM of Chloroauric acid stock solution to 1mM by deionized water to make up the 20 ml solution. That 20 ml of 1.0 mM HAuCl4 transfer in 50 ml conical flask clamped to a ring stand and place it on a hot plate. When solution starts boiling (up to 100° C), slowly add 2 ml of 1% trisodium citrate dehydrate to above flask. The colour of the solution changed instantly from pale yellow to colourless to light purple to dark blue in colour after approximately 70 sec. Stirring and heating of the solution was maintained for 35 min after the addition of sodium citrate. The heat was then removed and the solution was stirred upon cooling to room temperature. After cooling ruby red coloured gold nanoparticles were observed and PEG 400 was added for surface modification. The solution was stirred for 1 hr to obtain PEGylated gold nanoparticles.

2.2.2. Loading of Methotrexate over PEGylated gold nanoparticles

Methotrexate was added to dispersion of gold nanoparticles. The solution was then incubated for 24 hrs at room temperature [10, 11]. The amount of ingredients and PEG 400 used is given in Table 1.

Table 1. List of ingredient with quantity for GNP and PGNP

Ingredients	PGNP	GNP
Chloroauric acid	20 ml (1mM)	20 ml (1mM)
Trisodium citrate	2ml (1%)	2ml (1%)
PEG 400	1.42 ml (1:200)	-
Methotrexate	5mg	5mg

2.3. Characterization of Gold nanoparticles

2.3.1. Physical observation

Gold nanoparticles appear in different color that depends on the size and shape of the particles. The color change of resulted gold nanoparticle formulations was observed. Literature reports suggests that colloidal gold suspension is typically either an intense red color for particles less than 100 nm or a dirty yellowish to blue color for larger particles [10].

2.3.2. Particle size measurement

Particle size and particle size distribution of gold nanoparticles were determined using Malvern Zetasizer by filling sample in an insulating sample cell. Particle size distribution is determined from the velocity distribution of particles suspended in dispersing medium, using the principle of dynamic light scattering. The measurement is based on the particle diffusion due to Brownian motion, which is related to particle size. Particle size was then calculated from the translational diffusion coefficient using the Strokes-Einstein equation by in-built software.

2.3.3. Transmission electron microscopy (TEM)

TEM (Philips Tecnai 20 G2, FEI) was used to carry out morphological investigation of gold nanoparticle after leading into pre-monomer mixture by operating at 100 kV accelerating voltage capable of point-to-point resolution. Sample was stained with 0.5 % aqueous solution of phosphotungstic acid and directly positioned on the copper electron microscopy grids. Using different combination of bright-field imaging at increasing magnification, the form and size of the gold nanoparticle was revealed.

2.3.4. Polydispersity index (PDI)

Polydispersity index (PDI) or heterogeneity index, is a measure of the distribution of molecular mass in a given sample. It determines size range of particles in the system. It is expressed in terms of Polydispersity index (PDI) which is measured by equation:

$$\text{PDI} = \frac{\text{Number of particles having particle size} > 100\text{ nm}}{\text{Number of particles having particle size} < 100\text{ nm}}$$

2.3.5. Zeta potential determination

Malvern Zetasizer determines Zeta Potential by measuring the response of charged particles to an electric field. Malvern Zetasizer utilizes a high frequency AC electric field to oscillate the charged particles. The measurement is based on electrophoretic mobility (μm/s) of the particles, which is converted to the zeta potential by in-built software based on Helmholtz-smoluchowski equation. Malvern Zetasizer measures the potential ranged from -120 to 120 mV. For measurement of zeta potential 1 ml of each formulation were diluted with deionised water (10 ml).

2.3.6. Viscosity

The viscosity was determined using rotational viscosity measuring device coupled with concentric cylinders (Brookfield viscometer DVII plus pro, Brookfield engineering laboratory, U.S.A.). Viscosity measurement was done at 50 rpm and 100 rpm using spindle no. 62 at room temperature. Experiment was performed in triplicate and fresh sample was taken each time.

2.3.7. % Drug Bound

A calculated amount of MTX was added to a dispersion of AuNPs. The solution was then incubated for 24 h at room temperature and then centrifuged at 12,000 rpm for 35 min. The pellets thus obtained after centrifugation were separated from the supernatant solution and redispersed in deionized water prior for further characterization. The free MTX present in the supernatant was determined by measurements of its UV absorbance and the percentage loading of MTX on AuNPs was estimated by following formula [11]

$$\%\ \text{Bound drug} = \frac{\text{Total amount of MTX added} - \text{Amount of MTX in supernatant}}{\text{Total amount of MTX added X } 100}$$

2.3.8. % Drug content

1 ml dispersion of gold nanoparticles was taken into 10ml volumetric flask and volume was made up with deionised water. It was sonicated for 5 min in bath sonicator. Solution was filtered through cellulose Whatman filter paper (0.45 µ) and filtrate was analysed spectrophotometrically at λmax 303 nm using UV spectrophotometer (UV-1800, Shimadzu, Japan) after suitable dilution with water. Percentage drug content was calculated using calibration curve of drug prepared previously [12, 13].

2.3.9. In vitro drug release study

In vitro drug release study was carried out using cellulose acetate membrane diffusion cell in 50 ml of phosphate buffer pH 7.4 and acidic buffer pH 4 and pH 5 at 50 rpm at temperature 37 ± 0.5˚C. Nano-dispersion containing 5mg of drug is filled in diffusion cell and assembly was set. A 5 ml of sample was withdrawn at predetermined time intervals of each hour and replaced by an equal volume of dissolution medium. Samples were analysed using UV-visible spectrophotometer at λmax 303nm.

2.3.10. % Cell Cytotoxicity by MTT assay

Human breast cancer cell line MCF-7 was procured from the Cell Repository of National Centre for Cell Science, Pune, India and was cultured in Minimum Essential Eagle's Medium (MEM), 10 % fetal bovine serum (Gibco, USA). The cultures were maintained in a humidified atmosphere of 5 % CO_2 at 37° C in an incubator.

Cell cultures were removed from culture flasks by enzymatic digestion by trypsin and PBS. The cell suspension is centrifuged for 15 min at 1500 rpm. The cells were then resuspended in a 5 ml culture medium and the cell suspension is adjusted at a density of 1×10^5cells/ml. Using a micro pipette, dispense 50 µl of a cell suspension, 40 µl MEM culture medium, 10 µl FBS that makes total 100 µl volume into the peripheral wells of a 96-well tissue culture micro titre plate. Incubate cells for 24 h (5 % CO2, 37 °C, > 90 % humidity) so that cells form a half-confluent monolayer. This incubation period ensures cell recovery, and adherence and progression to exponential growth phase. After 24 h incubation, aspirate culture medium from the cells per well, add 50 µl of treatment medium containing the appropriate concentration of sample extract, or the negative control, or the Positive control, or nothing but blank, 40 µl MEM culture medium, 10 µl FBS. Culture medium should be used as blank and Incubate cells for 24 h (5 % CO2, 37 °C, > 90 % humidity).After 24 h treatment, carefully remove the culture medium from the plates. This is an important step, because reductive chemicals in the extract can also reduce MTT, causing false negative results. 100 µl of the MTT solution is then added to each test well and the plates were further incubated for 2 h in the incubator at 37 °C. Then the MTT solution is decanted and 100 µl of DMSO was added in each well. Sway this plate and subsequently transfer it to a micro plate reader equipped with a 450-630 nm filter to read the absorbance [14].

3. RESULTS AND DISCUSSION

3.1. Physical observations

There was no colour change observed for both batches GNP and PGNP and it was shown in Figure 1. Ruby red colour was observed for gold nanoparticles before and after pegylation it reflects that particles size is less then 30nm before and after pegylation.

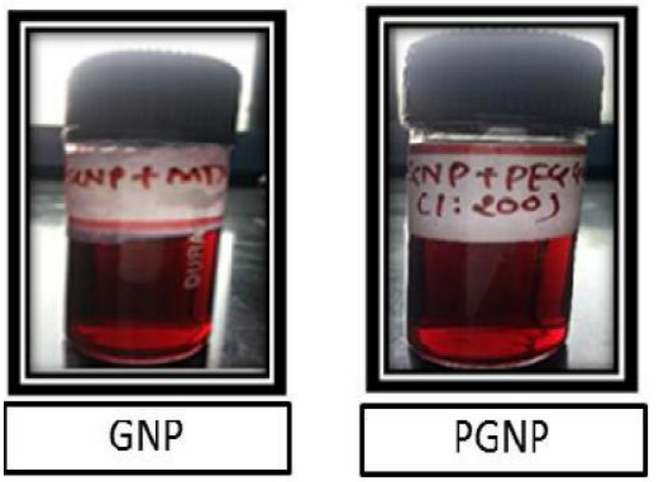

Figure 1. Color of simple and PEGylated Gold nanoparticles

3.2. Particle size measurement and Polydispersity index (PDI)

Particle size and polydispersity index (PDI) of both the batches GNP and PGNP were determined using Malvern Zetasizer. GNP batches showed the particle size of 19.79 nm and PDI of 0.536. Whereas PGNP batches shown the particle size of about 28.96 nm and PDI of 0.703.

The size of nanoparticles is the most important physicochemical factor influencing the tumour targeting efficiency. It was reported that smaller gold nanoparticles (20-40 nm) coated with PEG had longer blood circulation time than larger gold nanoparticles (80 nm**).** It was also reported that 13 nm sized PEG-coated gold nanoparticles were seen to induce acute inflammation and apoptosis in the liver [15]. The 20-nm PEG coated gold nanoparticles demonstrated the lowest uptake by reticuloendothelial cells and the slowest clearance from the body. Furthermore, smaller particles have a better chance of extravasation from blood vessels into extravascular fluid space. Hence, to improve targeted delivery of Gold nanoparticles, it is desirable that their size was between 15 to 50 nm [16].

3.3. Zeta potential

Zeta potential of both the batches GNP and PGNP were determined using Malvern Zetasizer. In the formulation GNP, zeta potential was found to be -24.8 mV, whereas formulation PGNP zeta potential was found to be the -6.73.

The PEGylated Gold nanoparticles have a negative charge definite by Zeta potential measurements. A surface charge value of −24.8 mV in the case of GNP (without PEGylation) was drops up to a −6.73 mV of PGNP which coated with short-chain PEGs (PEG400). The value of the negative surface charge gradually decreases by the PEG chain length used in the preparation of PEGylated Gold nanoparticles. The PEG molecules bounded to gold nanoparticles usually form hydrogen bonds with the water molecules increasing the hydrophilicity of these nanoparticles and preventing their agglomeration through steric repulsion.

TEM (Philips Tecnai 20 G2, FEI) was used to carry out morphological investigation of PEGylated gold nanoparticle. TEM image of Formulation PGNP was shown in Figure 2. The shape of Gold nanoparticles was found to be spherical and the particle size was found to be 20 nm. There was no agglomeration observed. So it was a strong proof that the main mechanism responsible for colloidal stability was the electrostatic repulsion [17].

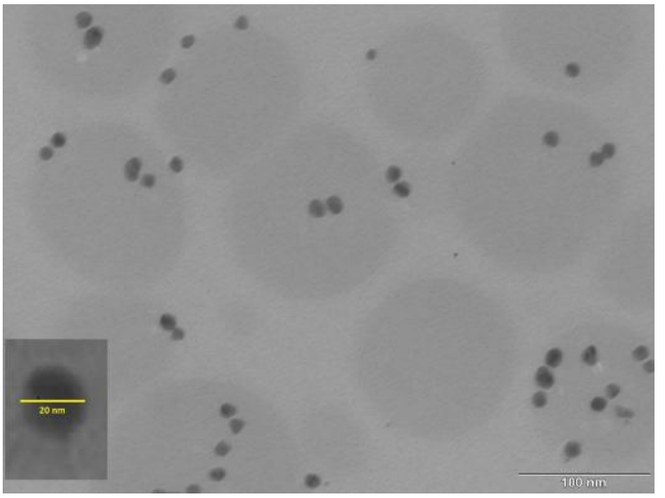

Figure 2. TEM images of PEGylated Gold nanoparticles

3.4. Viscosity

The viscosity of PGNP batch was determined using rotational viscosity measuring device coupled with concentric cylinders (Brookfield viscometer DVII plus pro, Brookfield engineering laboratory, U.S.A.) and found to be 10.16 ± 0.05 cp and 10.43 ± 0.11 cp at 50 rpm and 100 rpm respectively using spindle no.62. As the formulation contain 80% of the water and it showed low viscosity so, it can be confirmed that the viscosity of the batch PGNP accepted for the parenteral administration [6].

3.5. % Drug Bound and % Drug Content

The % drug bound and % drug content of batch PGNP was found to be 78.38 % ± 1.05 and 97.03 ± 2.904 respectively.

3.6. In vitro drug release study

Acidic extracellular pH is a major feature of tumor tissue, which lies at the pH below 5.5, where extracellular acidification being primarily consi-

dered to be due to lactate secretion from anaerobic glycolysis. An acidic extracellular pH activates secreted lysosomal enzymes that have an optimal pH in the acidic range. In addition to lactate, CO_2 from the pentose phosphate pathway is an alternative source of acidity, showing that hypoxia and extracellular acidity are, while being independent from each other, deeply associated with the cellular microenvironment [18]. Thus pH 4 and pH 5 selected as diffusion media. Where normal colon cell had a pH 7.4 so it also selected as diffusion media.

The *in-vitro* release profile of MTX from the PEGylated Gold nanoparticles is shown in Figure 3. The release was performed at phosphate buffer pH 4, pH 5, and phosphate buffer pH 7.4 at 37 °C. The MTX release from the PEGylated Gold nanoparticles exhibited 99.31 %, 94.87 % release in pH 4, pH 5 and only 64.05 % release in pH 7.4 within 48 hours.

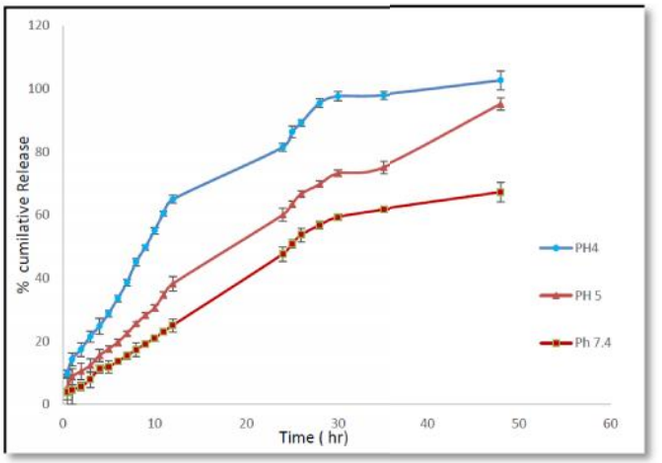

Figure 3. Percentage in vitro drug release of PGNP batch

From the above results, it was found that the release of MTX from PEGylated nanoparticles at pH 7.4 was low. At pH 4, drug release was found higher and thus it cause damage to tumour cells as cancer cells having acidic environment as compared to normal cells.

The release of MTX from PEGylated Gold nanoparticles showed a sustained release as a result of ester linkage between MTX and PEG. PEG plays a crucial role in drug release due to high chain mobility in an aqueous environment and having a large excluded volume, the PEG molecules can inhibit MTX release from PEGylated gold nanoparticles. Moreover, a high value of pH may promote ester hydrolysis, thus release of drug demonstrates pH dependence [19].

3.7. % Cell Viability by MTT assay

The MCF-7 cell lines were hired to test the MTX-PEG-AuNPs as MTX carriers *in vitro*. Ngos et al had already reported that reference gold nanoparticles have no cytotoxic effect so we have not kept it in this MTT study. The cells were individually incubated with an two equal dose of MTX-PGNP, MTX-GNP, and free MTX for comparison. All the samples were incubated for 24 hr, after which, the cell toxicity were determined using MTT assays. The results which presented as mean cytotoxicity from performed experiments in triplicate which shown in Figure 4.

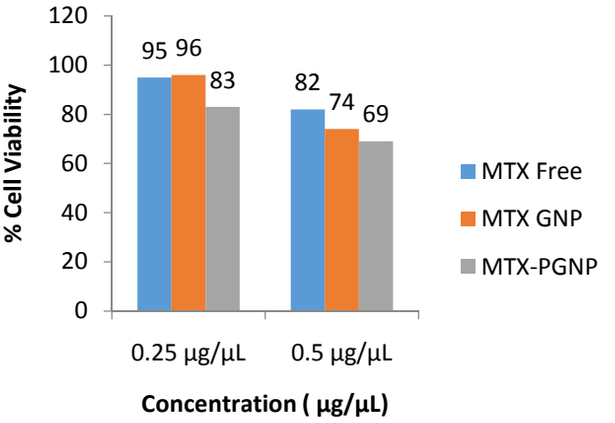

Figure 4. The Cell viability of the free MTX, MTX-GNP and MTX-PGNP with the concentration of 0.25µg/µL and 0.5 µg/µL for 24 h in MCF-7 cell line

As shown in Figure-4 free MTX, MTX-GNP and MTX-PGNP with the concentration of 0.25µg/µL demonstrated % Cell viability of 95%, 96% and 83%, respectively. Which show that they had minor cytotoxic effects on MCF-7 cells. Moreover, free MTX, MTX-GNP and MTX-PGNP with the concentration of 0.5 µg/µl demonstrated survival rates of 82%, 74% and 69%, respectively. Which show that MTX-PGNP with the concentration of 0.5 µg/µl had major cytotoxic effects on MCF-7

cells. It is evident that MTX-PGNP had a significantly greater cytotoxic effect on the MCF-7 cell lines than equal doses of MTX-GNP, free MTX. From the literature report it is suspected that the higher cytotoxicity was due to two reasons: first, small sizes gold nanoparticles resulted in higher surface area, leading to more quantity of MTX conjugation. Second, small particle size resulted in the easier internalization of MTX-PGNP in the cellular environment [20].

4. CONCLUSION

The study demonstrates the successful development of gold nanoparticles which have been functionalized with unmodified PEG molecules. The results presented spherical PEGylated gold nanoparticles with the particles size was 20 nm. The release of MTX from PEGylated Gold nanoparticles was sustained and found to be 99.31 %, 94.87 %, and 64.05 % within 48 hours in phosphate buffer pH 4, pH 5, and phosphate buffer saline pH 7.4 respectively. MTX loaded PEGylated gold nanoparticle were more cytotoxic to MCF-7 cell at the concentration of 0.5 µg/µL. The results demonstrated that the MTX-AuNPs of small size less then 20nm exhibited considerably high cytotoxic effects in human adenocarcinoma cell lines compared to the effects of equal doses of free MTX; these results suggest that anti-cancer drug-conjugated AuNPs have an enhanced therapeutic effect

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