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Synthesis and Characterization of Gold Nanoparticles Loaded with 5-Fluorouracil



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ABSTRACT

5-Fluorouracil-1-acetic acid (5-FUA) and amino polyethylene glycol-undecyl mercaptane (NH₂-PEG-AlkSH) were prepared and covalently conjugated through amide linkage. Gold nanoparticles (GNPs) were synthesized by citrate reduction method with a resulting gold core diameter of ~17 nm as determined by Transmission Electron Microscope (TEM) and a characteristic surface Plasmon band at about 522 nm as detected by UV-Vis spectroscopy. 5-Fluorouracil conjugated polyethylene glycol was used to functionalize the surface of GNPs. The antitumor activity of 5-fluorouracil loaded on gold nanoparticles was demonstrated *in vitro* against human breast cancer cell line (MCF-7) using sulphorhodamine B (SRB) assay and the results showed an improvement in the drug anticancer activity upon loading on GNPs compared to free drug.



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1. INTRODUCTION

5-Fluorouracil (5-FU) is widely used in the treatment of a great variety of solid tumors like colorectal cancer, pancreatic cancer and breast cancer. It is an antimetabolite fluoro-pyrimidine analog which acts by inhibiting thymidylate synthase or by being incorporated into RNA to disrupt normal cell functions. 5-FU has low bioavailability and serious side effects as myelosuppression, gastrointestinal, haematological, neurological and dermatological toxicities which may cause significant limitations to its clinical uses [1]. Nano size 5-FU delivery systems have been developed to prolong the duration of drug activity and to overcome toxicity problems [2-4]. Gold nanoparticles (GNPs) of various sizes and shapes have been used for biomedical applications [5], including drug delivery [6], cancer therapy and tumor imaging [7, 8]. GNPs have high surface area to volume ratio which provides dense loading of functionalities including targeting ligands and therapeutic materials [9]. Also they can be easily detected and characterized due to the presence of the characteristic surface plasmon resonance bands [10]. GNPs have been synthesized by several methods which are based on the reduction of chloroauric acid in the presence of stabilizing agent [11]. In this study, the synthesis of heterobifunctional polyethylene glycol with a thioalkane part on one terminal and amino group on the other for conjugation with 5-fluorouracil-1-acetic acid (5-FUA) was demonstrated. The synthesis of gold nanoparticles by citrate reduction method and the modification of their surface with thioalkylated polyethylene glycol were reported. Therefore, thioalkane part forms self-assembled monolayer [12] and polyethylene glycol prevents unspecific opsonins from recognizing GNPs, increases their circulation time [13] and so allows them to reach the tumor by Enhanced Permeation and Retention effect (EPR) [14]. The anticancer activity of 5-FU loaded on GNPs has been investigated against breast cancer cell line and compared with that of free 5-FU and plain GNPs.

2. MATERIALS AND METHODS

2.1. Materials and Instruments

11-Bromoundecene, *N*-hydroxysuccinimide (NHS) and dry toluene were purchased from Alfa Aesar Company. 5-Fluorouracil, *N,N'*-dicyclohexylcarbodiimide (DCC), thioacetic acid, chloroacetic acid, *p*-toluenesulfonyl chloride (*p*-TsCl), polyethylene glycol (PEG, Mw 4000), triphenylphosphine (PPh₃), sodium azide (NaN₃), diethyl ether, *N,N*-dimethyl formamide (DMF),

tetrahydrofuran (THF), silica gel, chloroauric acid trihydrate (393.833 Mw), trisodium citrate dihydrate (294.1 Mw) and 2,2'-azobisisobutyronitrile (AIBN) were purchased from Sigma Aldrich Company. Other chemicals and solvents were of analytical grade.

^1H -NMR and C^{13} -NMR spectra were recorded using Bruker-400 spectrometer. Fourier transform infrared (FTIR) spectra were obtained on a Nicolet 6700 FTIR spectrometer. Ultraviolet-Visible light absorption spectra were carried out using Shimadzu 1601 UV/Vis Spectrophotometer. The size distributions and the zeta potentials of GNPs were analyzed by photon correlation spectroscopy using a Zetasizer (Malvern nano ZS). The morphology of GNPs was recorded using Transmission Electron Microscope (a Tecnai G2 Spirit TWIN operating at an acceleration voltage of 120 kV). The cell viability was measured by ELISA microplate reader at wavelength 540 nm (Tecan Sunrise).

2.2. Synthesis of 5-fluorouacil conjugated polyethylene glycol (scheme1)

2.2.1. Synthesis of 11-bromoundecyl thioacetate (1)

11-Bromoundecyl thioacetate was synthesized according to the method reported by Moldt et al [15]. A round-bottom flask was charged with dry toluene (50 mL) and purged with nitrogen for one hr under stirring. 11-bromoundecene (1 mL, 1.06 g, 4.55 mmol) was added under a nitrogen atmosphere, followed by thioacetic acid (1.85 mL, 1.97 g, 25.9 mmol) and AIBN (9.65 mL, 0.14 M solution in toluene). The reaction mixture was refluxed for three hrs under nitrogen and then allowed to cool to room temperature. The mixture was washed several times with a saturated aqueous NaHCO_3 solution, followed by water. The organic phase was dried with MgSO_4 and evaporated under reduced pressure. The crude product was purified by column chromatography on silica gel using a mixture of hexane and dichloromethane (4:1, v/v) as an eluent to give the pure product as colorless oil. ^1H -NMR (DMSO-d_6 , δ in ppm): 3.50 (t, 2H, BrCH_2 -), 2.82 (t, 2H, $-\text{CH}_2\text{S}-$), 2.31 (s, 3H, $\text{CH}_3\text{COS}-$), 1.83 -1.26 (alkane backbone).

2.2.2. Synthesis of monotosyl polyethylene glycol (2)

Monotosyl-PEG was prepared as reported by Dinesh et al [16]. To a chilled ($0\text{ }^\circ\text{C}$) solution of the PEG (5.0 g, 1.25 mmol) in dry methylene chloride (100 mL), freshly prepared Ag_2O

(0.434 g, 1.87 mmol) and KI (0.83 mg, 0.5 mmol) were added. The mixture was stirred then *p*-TsCl (250 mg, 1.31 mmol) was added in one portion. After two hrs, the mixture was filtered and the filtrate was evaporated under reduced pressure to give a colorless oily product. Monotosyl-PEG was obtained by precipitating the oily product using cold ether. ¹H-NMR (CDCl₃, δ in ppm): 7.72 (d, Ar-H), 7.11 (d, Ar-H), 3.77-3.59 (PEG backbone), 2.30 (s, 3H, CH₃ tosyl).

2.2.3. Synthesis of monotosyl polyethylene glycol-undecyl thioacetate (3)

Monotosyl PEG undecyl thioacetate was synthesized according to the method reported by Prime et al [17]. Monotosyl PEG (2g, 0.481 mmol) was melted using an oil bath under nitrogen atmosphere, NaOH (192 mg, 4.81 mmol) was added to the molten polymer and the mixture was heated at 110 °C for 30 minutes. 11-Bromoundecyl thioacetate (0.5 mL, 1.92 mmol) was added and the reaction mixture was kept at the same temperature under stirring overnight. At the end of reaction, the mixture was dissolved in dichloromethane, filtered to remove inorganic salts (NaOH and NaBr). The filtrate was reduced to a small volume by rotary evaporator and precipitated by dropping it into cold diethyl ether. The product was collected by filtration. ¹H-NMR (DMSO-d₆, δ in ppm): 7.51 (d, Ar-H), 7.12 (d, Ar-H), 3.70-3.53 (PEG backbone), 3.26 (t, 2H, CH₂-S), 2.32 (s, 3H, CH₃COS-), 1.78-1.27 (alkane backbone).

2.2.4. Synthesis of α-azide polyethylene glycol-undecyl thioacetate (4)

α-Azide PEG undecyl thioacetate was synthesized as described by Mahou et al [18]. Monotosyl PEG-undecyl thioacetate (1 g, 0.299 mmol), NaN₃ (74 mg, 1.14 mmol) were dissolved in dry DMF (30 mL) and the mixture was stirred overnight at 90 °C under nitrogen atmosphere. After cooling down to room temperature, the mixture was filtered and DMF was removed under vacuum. The crude product was dissolved in 50 mL dichloromethane then washed twice with brine (solution of sodium chloride) and water. The organic phase was dried over MgSO₄, reduced to a small volume by rotary evaporator and finally precipitated by dropping it into cold diethyl ether. The product was collected by filtration. ¹H-NMR (DMSO-d₆, δ in ppm): 3.70-3.54 (PEG backbone), 3.2 (t, 2H, CH₂-S), 2.30 (s, 3H, CH₃COS-), 2.02-1.28 (alkane backbone), 1.6 (2H, CH₂N₃). The spectra did not show any aromatic signals.

2.2.5. Synthesis of α -amine polyethylene glycol-undecyl mercaptan (5)

As reported by Mahou et al [18]. To a solution of α -azide polyethylene glycol-undecyl thioacetate (0.30 g, 0.07 mmol) in dry MeOH (50 mL), PPh₃ (84.7 mg, 0.322 mmol) was added and the reaction mixture was heated to reflux overnight under nitrogen atmosphere. After cooling down to room temperature, the solvent was removed by rotary evaporator. The resulting solid was dissolved in dichloromethane and the solution was added drop-wise into cold diethyl ether. The product was collected by filtration. ¹H-NMR (DMSO-d₆, δ in ppm): 3.70-3.53 (PEG backbone), 3.21 (2H, CH₂-NH₂), 2.70 (2H, CH₂-SH), 1.63-1.27 (alkane backbone).

2.2.6. Synthesis of 5-fluorouracil-1-acetic acid

5-Fluorouracil-1-acetic acid was synthesized according to the method reported by Tada [19]. 5-Fluorouracil (300 mg, 2.3 mmol) was dissolved in water (2 mL) containing potassium hydroxide (257 mg, 4.59 mmol). Chloroacetic acid (217 mg, 2.3 mmol) was added to the solution and the mixture was stirred at 100 °C for two hrs, keeping pH of the solution at 10 by KOH solution. After the reaction solution was cooled to room temperature, HCl was added to adjust pH at 2 and the resulting precipitate was filtrated. The product was re-dissolved in a saturated potassium bicarbonate solution and re-precipitated with HCl to give 5-FUA. ¹H-NMR (DMSO-d₆, δ in ppm): 11.5 (brs, COOH), 8.04 (s, CH=C, 5FU), 4.4 (s, 2H, CH₂COOH).

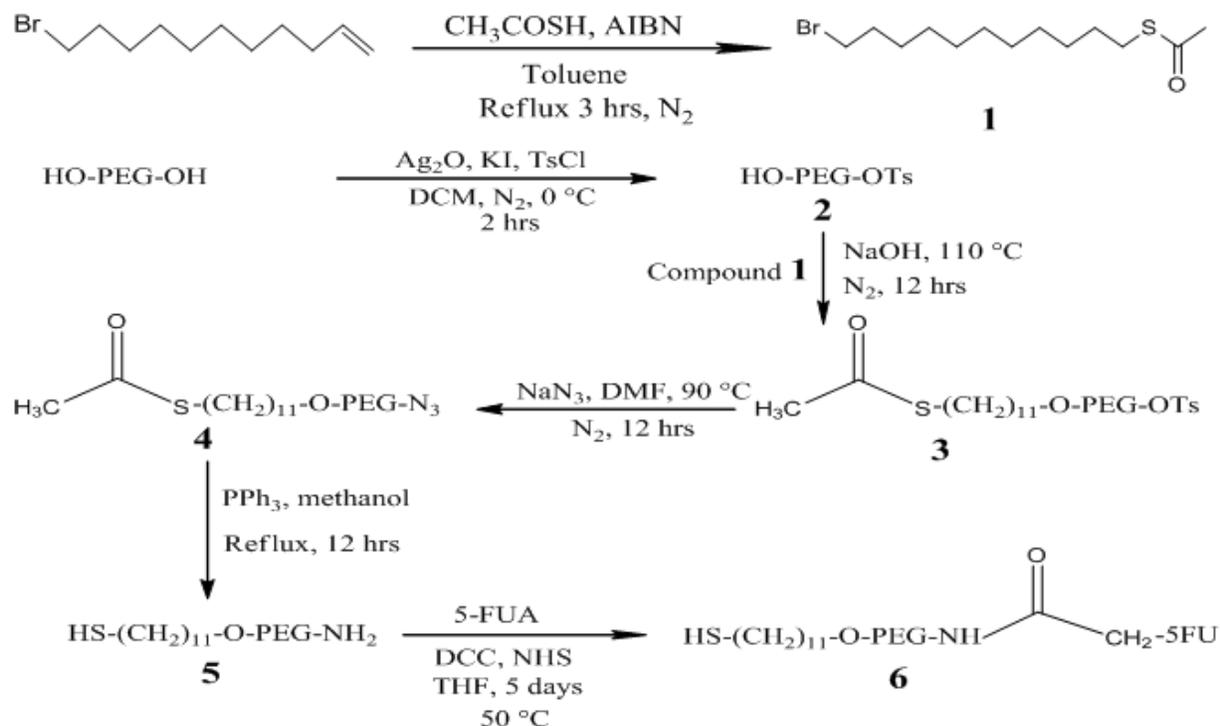
2.2.7. Conjugation of amino polyethylene glycol-undecyl mercaptan with 5-fluorouracil-1-acetic acid (6)

The amine terminated polymer was conjugated with 5-fluorouracil-1-acetic acid according to the method reported by Ouchi et al [20]. 5-Fluorouracil-1-acetic acid (26,6 mg, 0.142 mmol) and *N*-hydroxysuccinimide (32.6 mg, 0.283 mmol) were dissolved in tetrahydrofuran, DCC (58.4 mg, 0.283 mmol) was added to the solution on ice and the mixture was stirred at room temperature to activate the carboxyl group of 5 fluorouracil-1-acetic acid [21]. After one day the polymer (5) (300 mg, 0.07 mmol) was dissolved in dioxan and added to the mixture then stirred at 50 °C for five days. Then the precipitated dicyclohexylurea was filtered off, the solvent was evaporated under vacuum. The obtained product was dissolved in dichloromethane, precipitated in acetone

and collected by filtration. $^1\text{H-NMR}$ (CDCl_3 , δ in ppm): 8.03 (s, $\text{CH}=\text{C}$, 5-FU), 4.4 (s, CH_2CO), 3.73-3.64 (PEG backbone), 2.60 ($\text{CH}_2\text{-SH}$), 1.63-1.29 (alkane backbone).

C^{13} NMR (CDCl_3 , δ in ppm): 171, 160.8, 157.1, 77.28, 76.97, 76.65, 72.55, 70.58, 68.46, 66.26, 63.73, 62.61, 61.71, 49.31, 33.79, 29.63, 25.58, 24.84

The peaks from the IR (cm^{-1}) analysis were as follows: 3322 (N-H), 1715 ($\text{C}=\text{O}$, 5-FU), 1623 ($\text{C}=\text{O}$, amide).

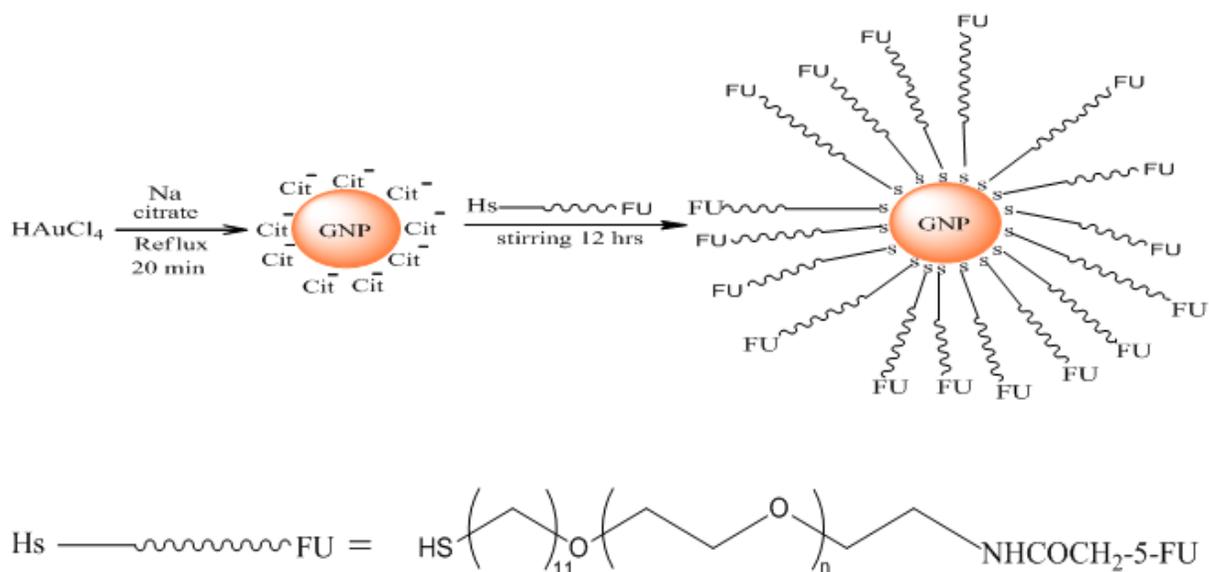


Scheme 1: Synthesis of 5-fluorouracil conjugated polymer

2.3. Synthesis of gold nanoparticles functionalized with 5-FU conjugated polymer

To synthesize gold nanoparticles, aqueous solution of chloroauric acid (100 mL of 0.01% w/v) was heated to reflux and stirred in 250 mL round-bottom flask using electro-mantle then 1 mL of 10% trisodium citrate solution was added rapidly. The boiling and stirring were continued for 15 minutes. Finally, the solution was removed from the mantle and allowed to cool to room temperature with continuous stirring yielding citrate-coated GNPs. For functionalization of GNPs, 5-FU conjugated polyethylene glycol-undecyl mercaptane was added to GNPs solution and stirred overnight at room temperature to allow a complete exchange of the citrate anions

with the modified polymer (scheme 2). In order to remove free polymers, the colloidal gold solutions were centrifuged at 10,000 rpm for 30 minutes. Supernatant was decanted to leave the GNPs pellets at the bottom of the centrifuge tube. The pellets were re-dispersed to its original volume with deionized water using sonicator. This washing process was repeated again to remove any unattached polymer.



Scheme 2: Representation of GNPs loaded with 5-Fluorouracil

2.4. Cytotoxicity evaluation

Cytotoxicity of GNPs, 5-FU and GNPs loaded with 5-FU was estimated by cell culture method using human breast cancer cell line (MCF-7) which was purchased from The Holding Company for Biological Products and Vaccines, Cairo. The Cells were grown in RPMI-1640 media supplemented with 10% fetal bovine serum. The Cell viability was carried out in Genetic Engineering Center, Al-Azhar University.

Cellular viability was detected by sulphorhodamine B (SRB) assay as described by Houghton et al [22]. Briefly, the cells were added to 96 well culture plate then incubated at 37 °C in 5% CO₂ incubator for one day to allow adherence of cells to the surface of the plate. GNPs, 5-Fu and GNPs loaded with 5-Fu were added to the cells and further incubated for two days with constant supply of 5% CO₂. The remaining cells were fixed with trichloroacetic acid for one hr at 4 °C. The plate was washed with distilled water several times, dried before adding SRB solution and

kept at dark place for 30 minutes at room temperature. Unbound dye was removed by washing the plate with 1% acetic acid then the plate was dried in air. Finally, the bound SRB was extracted and measured using ELISA reader. The Cell viability was calculated by dividing the absorbance of test sample on the absorbance of control.

3. RESULTS AND DISCUSSION

The citrate reduction method is considered as the most representative and popularly used procedure to synthesize GNPs, because of its simplicity, reproducibility and the loose shell of citrates on the nanoparticle surfaces is easily replaced by other desired ligands with valuable function. Gold salt solution was yellow at first, after addition of sodium citrate to the solution; a bluish color appeared, indicating the formation of gold nuclei. A few minutes later, the solution turned wine red due to the formation of the nanoparticles which have characteristic surface plasmon resonance peak at about 522 nm (figure 1). The adsorbed citrate anions are able to prevent the close contact between individual particles due to the electrostatic repulsion forces between similarly charged particles. The zeta potential value for GNPs capped with citrate was measured to be -58 mV. From the TEM image, the GNPs are approximately spherical and fairly monodisperse with an average diameter of 17.6 nm which was confirmed by zetasizer (figure 2). 5-FU conjugated polyethylene glycol was used to functionalize the surface of GNPs, an excess amount of 5-FU conjugated polymer (80 µg/mL) was added to nanoparticle dispersion to allow a complete coating of the particle surface with thiolated polymer, and unattached polymer was then removed by centrifuge tubes. The amount of 5-FU conjugated polymer bound to GNPs was estimated by using UV-Vis spectroscopy, the absorption maximum for 5-FU conjugated polymer was found to be 260 nm (figure 3). The percentage loading was calculated with equation 1:

$$\% \text{ loading} = \frac{A-B}{A} \% \quad (1)$$

Where A is the absorbance of total 5-FU conjugated Polymer and B is the absorbance of free 5-FU conjugated polymer in the supernatant after centrifugation. The maximum binding occurred was 53%. The zeta potential of GNPs capped with modified polymer was increased to -11 mV which could be related to the removal of most citrate anions adsorbed on the particles surface during the synthesis process. The TEM measurements showed that there is no significant

difference in size between GNPs capped with citrate or polymer, as the TEM measures the gold core only at the acceleration voltage used. However, zetasizer measurement showed an increase in the average diameter of GNPs capped with modified polymer to 28.2 nm (figure 2), as the zetasizer measures equivalent hydrodynamic radius and is affected by polymer capping [23, 24].

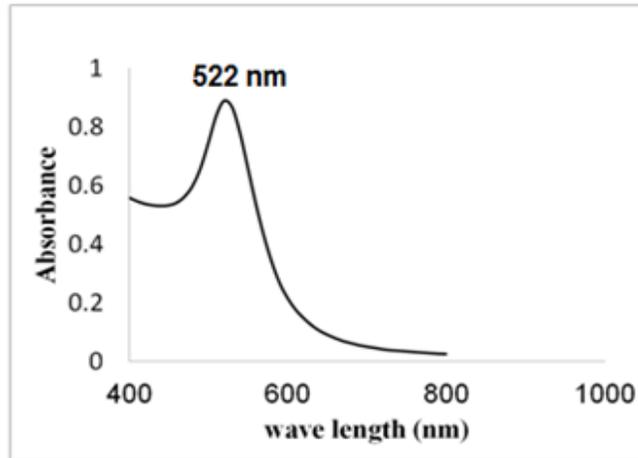


Figure 1: UV-Vis spectra of gold nanoparticles

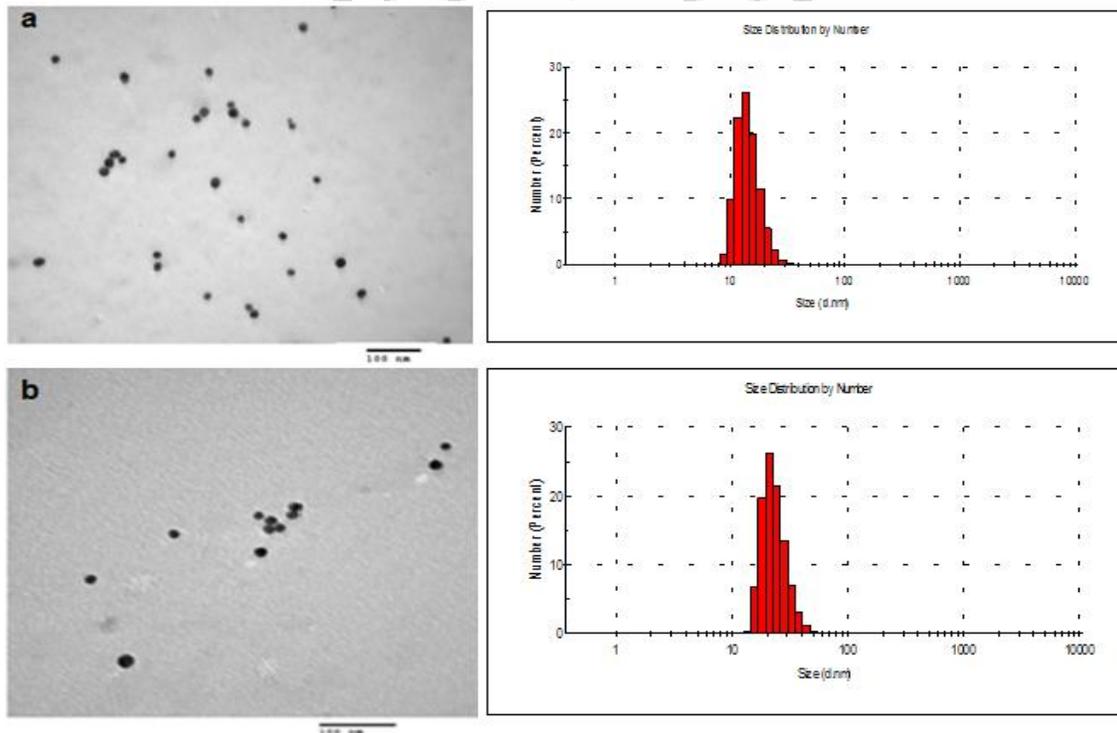


Figure 2: TEM images and corresponding histograms for (a) citrate-capped and (b) thiolated polymer capped GNPs

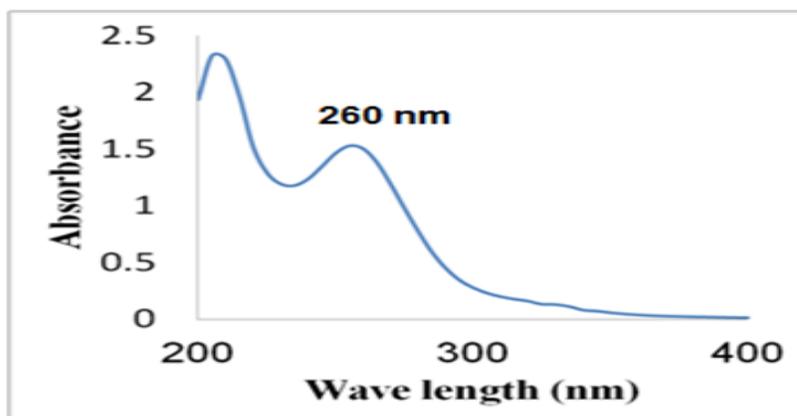


Figure 3: UV spectra of 5-FU conjugated polymer in phosphate buffer at pH 7.4

SRB assay using human breast cancer cell line was performed to evaluate the cytotoxicity of the GNPs, 5-FU and 5-FU loaded on GNPs. After incubation time of 48 hrs, viable cells have the ability to bind with a dye which was extracted and measured spectrophotometrically at 540nm using ELISA reader. The obtained values were correlated to the number of living cells (figure 4). The results showed that GNPs capped with citrate exhibited cell viability close to 90%, suggesting that plain GNPs had no significant cytotoxic effects. On the other hand, free 5-FU demonstrated cell viability of 62%, which indicate that it has minor cytotoxic effects. In contrast, the cell viability decreased significantly to 27% when incubated with GNPs loaded with 5-FU. This represents more than double the effectiveness of free 5-FU. The improvement of activity could be due to the enhanced intracellular diffusion by enhanced permeation and retention effect.

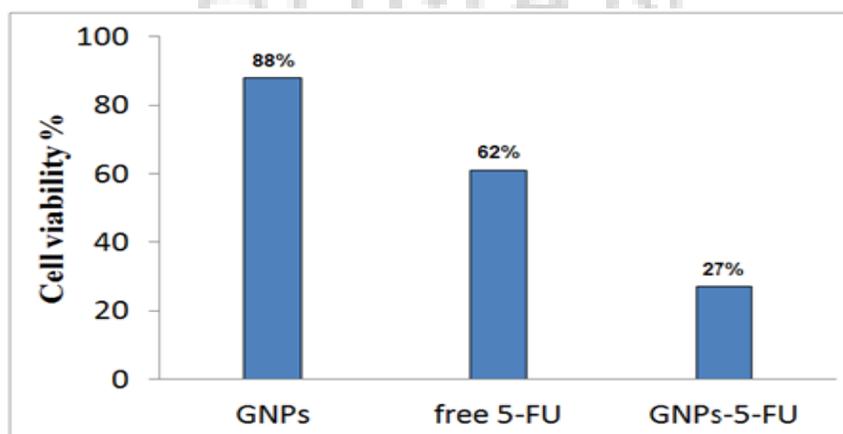


Figure 4: SRB assay showing the percentage viability of cells incubated with GNPs, 5-FU and 5-FU loaded on GNPs

4. CONCLUSION

In this study, 5-FU was considered as a modern antineoplastic agent. 5-FU was loaded on GNPs surface through modified polyethylene glycol spacer, the anticancer activity of such system against breast cancer cell line was increased compared to free drug and plain GNPs. This results showed the significance and applicability of using cytotoxic drugs loaded on GNPs to reduce their side effects and increase their effectiveness.

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