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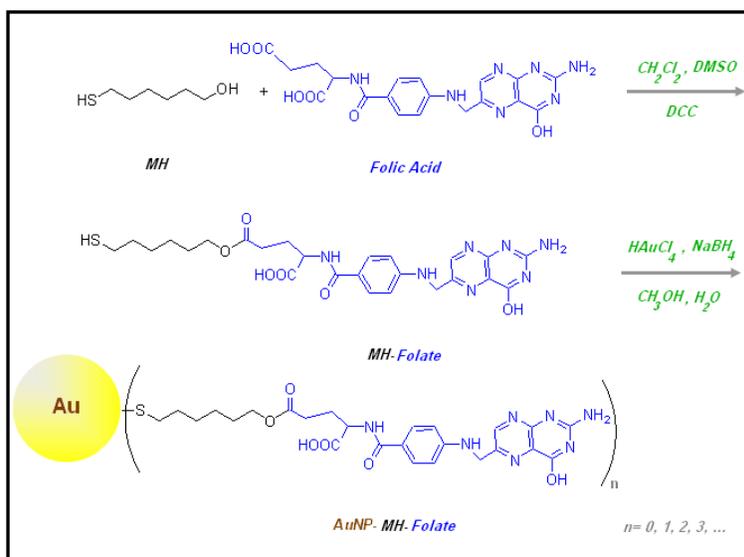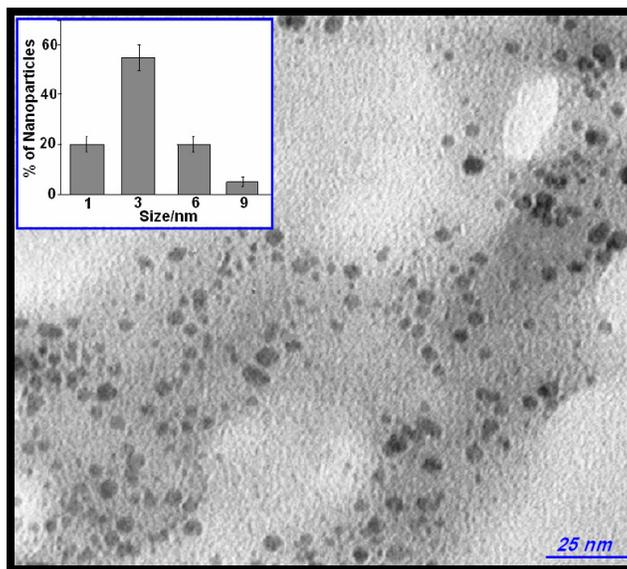
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# Gold Nanoparticles Conjugated with Folic Acid using Mercaptohexanol as the Linker

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Abstract:

Nano-conjugation (also known as nano-coupling) is one of the important procedures to build nanotechnology platforms. We have designed a new nano-conjugate made of folic acid and gold nanoparticle (AuNP). This nano-conjugate has application for selective targeting of the folate receptor that is overexpressed on the surface of tumor cells. For this purpose, we conjugated 6-mercapto-1-hexanol, as a bifunctional linker, to folic acid through its (–OH) group with a (-O-CO-) linkage formation. Then, we made new (-SH) terminated product to react with $HAuCl_4$ in the presence of sodium borohydride and it was bound to the AuNP surface through its thiol group.
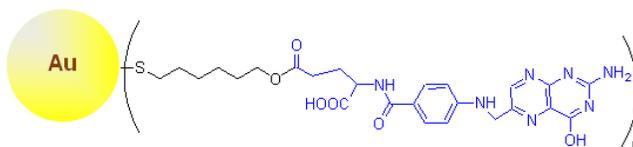
Finally, we evaluated the specific interaction between the folic acid and AuNP by the corresponding observed characteristic bands in the ultraviolet-visible (UV-vis) and Fourier transform infrared spectroscopy (FTIR) spectra. Transmission electron microscopic (TEM) images reveal the spherical AuNPs formation induced by the bifunctional linker. For such a new synthesized nanoconjugate, metallic peso-cubic structure (alpha=beta=gamma=90º) with lattice constants of a=1.348 nm, b=1.348 nm, and c=0.725 nm and (110), (011), (221), (321), (060), and (004) crystal planes were confirmed through powder X-ray diffraction. We estimated the average size of the conjugated nanoparticles to be about 3 nm by TEM. The Elemental analysis and atomic absorption showed around 70 % organic molecules on the surface of AuNPs. The procedure presented in this report may be applied to a variety of conjugations of interest in nanoscience and nanotechnology.

Keywords: 6-mercapto-1-hexano, Cancer Cell Targeting, Conjugated Nanoparticle, Folate, Folate Receptor, Folic Acid, Gold Nanoparticle, Nano-Conjugation, Nanotechnology

1. Introduction

The emphasis of this report is nano-conjugation (also known as nano-coupling) of gold nanoparticles with folic acid. Nano-conjugation is a procedure which links a nanoparticle

to other nanoparticles or molecules, macromolecules and/or biomolecules through non-covalent bonding. The non-covalent coupling of a nano-material with other nano-materials and molecular entities has found applications in many branches of nanoscience and nanotechnology. It specialty has an important application for *in vivo* targeted nano-drug delivery.[1] The nano-conjugates of gold nanoparticle with folic acid can be used for nano-cancer treatment through hyperthermia.[2] In this project we chose the gold nanoparticle due to its unique properties.[1] We report here conjugation of folic acid to gold nanoparticles (nano-conjugate) through a linker as reported below. These nanoconjugates can be utilized to deliver the gold nanoparticle to cancer cells through the folate-receptor. This is achieved since cancer cells over express the folate-receptor about hundred times more than the expression level within healthy cells. The folate-receptor expression in healthy cells is not accessible to intra venous administered treatments because of the localization of the receptor on the apical membrane of the epithelial cells. Conversely, the localization of the folate-receptor on cancer cells within malignant tumors is accessible to intra venous administered treatments, due to the defects in the tumor vasculature. [1]

1.1. Gold Nanoparticles: Generally, the nanostructures could possess interesting size-dependent magnetic, optical, electrical or electrochemical properties. Gold nanoparticles (AuNPs) have received wide interest in recent years because of their unique physical and chemical properties. AuNPs have attracted attention as a new material for medical therapeutics, optical imaging, radiation therapy, photo thermal ablation of unwanted cell and tissue, and catalyst. Production of AuNPs goes back to Michael Faraday in the mid-1800s. Faraday was the first to prepare stable AuNPs sols. Since the work of Faraday efforts have been made to produce AuNPs of various sizes, but we still lack precision in producing truly mono-dispersed AuNPs. Presently different methods exist to prepare AuNPs in different shapes and sizes, but the difficulty in producing monodisperse AuNPs is still a challenge.

The usual synthesis technique that is used to produce AuNP involves chemical reduction of metal ions to metal atoms in the presence of a reducing agent, which helps the reaction to reduce the metal ions and form nanoparticles. A polymeric stabilizer can play an important role during the synthetic process to prevent the nanaoparticles from agglomeration in order to suppress the growth rate.

1.2. Folic Acid: Folic acid is also named pteroylglutamic acid and has the closed chemical formula $C_{19}H_{19}N_7O_6$ (Mw=441.4 Da) and open chemical structure as shown in Figure 1.
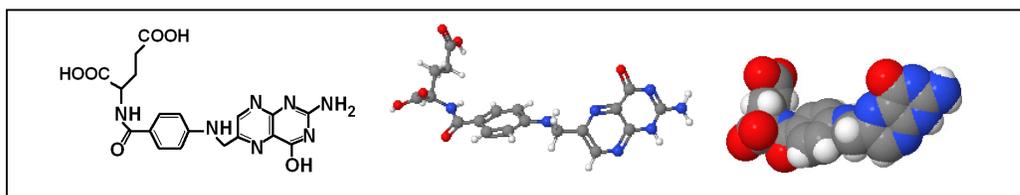

Figure 1. The molecular structure of folic acid

Folic acid and its salt folate (pteroylglutamate) are water-soluble. Folic acid or folate (folic acid salt) is an important vitamin required for the healthy functioning of all cells. Folate is brought into, both, healthy and cancerous cells by the folate-receptor.

Folate is necessary for DNA nucleotide synthesis and cell division. A cancer cell will require much more folate than a healthy cell.[3] Folate receptor is used to transport folate into the cytosol of the cell for the synthesis of thymine by dihydrofolate reductase. [4] The presence of the folate receptor (FR) on a cell surface is regulated by the cell function. Cancer cells overexpress the FR because of their vast requirement for folate .[5-7] It has been proposed that because of the low expression level of FR in healthy cells and its overexpression in cancerous tissues, folate behaves as a suitable targeting agent.[8-12]

Since folic acid in nano-cancer therapy is important, it is necessary to investigate its conjugation with nanotechnologies drug delivery platforms which is the subject of this report. When a nanotechnology platform, such as AuNP, is deposited at the tumor site a variety of methods to eradicate the cancer cells can be used. They include thermal ablation in the case of AuNP, drug release or delivery, or even coating the cancer cells in a high affinity antigen, which the body's immune system can detect and mount a defense against. Due to its promising characteristics of non-immunogenic, specificity for cancer, folic acid (and folate) is a front-runner as a targeting system for many cancer treatments.

1.3. Cancer Treatment through Nanotechnology: There are many chemical compounds, of either natural origin or synthetic, which are effective as anticancer drugs. However, they cannot be used in conventional cancer therapy (chemotherapy) because of their toxicity to normal and healthy cells.[13] The limiting factor in the current cancer therapy is balancing acceptable damage resulted from these chemicals to, both, healthy and cancerous tissues. To overcome this problem and at the same time provide a broader range of therapeutic agents, such medicines may be delivered through a targeted system. Towards this goal, many researchers have been working on finding methods of increasing the efficacy of therapeutic agents by targeted therapy.

Nanotechnology-based targeting of cancer cells is one such promising approach to meet this goal. In this method, therapeutic agents attached to a nanoparticle, like AuNP, can specifically target a binding site on the surface of cancer cells.[14] Recently, a number of materials have been introduced and applied as targeting agents for therapeutic purposes. Folic acid is one such targeting agent. [15] Cancer cells display more FRs on their cell membrane. Accordingly, one should be able to target cancer cells by taking advantage of a cancer cell's appetite for folate.

On the other hands, nanotechnology [16] offers the opportunity to produce metallic nanoparticles holding many capabilities in cancer therapy. The synthesis of metallic nanoparticles has been the focus of extensive research over the past decade due to their particular properties. [17, 18] Among these metallic nanoparticles, AuNP is of great interest for developing a unique system with high potential for biological applications such as nanophotothermolysis. [19,20] When short laser pulses irradiate AuNPs, a process named nanophotothermolysis occurs. In this process, AuNPs heat up quickly. It has been determined that the absorbed light by AuNP converts to heat on the picosecond time

scale.[21] AuNPs are the most promising candidates for nanophotothermolysis since they are strong absorbers, photostable, nontoxic, and have adjustable optical properties.[22] The absorption maximum of AuNP is tunable from the mid visible region into the infrared region based on the size, shape and material.[23] AuNPs strongly absorb laser irradiation. This absorbed energy transforms quickly into heat, which could cause fatal damage to cancer cells through local overheating effects. Accordingly, AuNPs are potentially very practical and efficient photothermal agents in therapeutic applications, especially in cancer treatment.[1,24]

With respect to the capabilities of AuNPs and through active targeting by folic acid (or folate), nanotechnology holds tremendous potential for the delivery of precisely targeted AuNPs that will reduce the collateral cell and tissue damage. The conjugation of AuNP with folic acid as a new system for the targeting of cancer cells is the main purpose of the present work. AuNPs cannot conjugate to folic acid directly and it is necessary to use a suitable linker, which has the capability to conjugate with them from two separate ends, simultaneously. To achieve this goal, in an earlier project we selected 4-Aminothiophenol (4Atp) as an aromatic linker between AuNP and folic acid, the synthesis of which was described in an earlier publication.[25] In the present report we have selected 6-mercapto-1-hexanol ($C_6H_{14}OS$) as the linear linker between AuNP and folic acid. The procedure for the synthesis of this new nano-conjugate and its characteristics are reported below.

2. Conjugation Procedure

We synthesized gold nanoparticles (AuNPs) according to the standard wet chemical methods[26] using sodium borohydride as a reducing agent. Folate-conjugated AuNPs were prepared using 6-mercapto-1-hexanol (MH) as the linker as shown in Figure 2. MH as a bidentate spacer attaches to folate via its –OH group and forms Folate■MH conjugation with orange color. Our preference for the choice of (-O-CO-) bond formation in the attachment of folate to AuNP is its stronger bond as compared to other linkers such as thioesters.[26] Ester and thioester hydrolysis may be problematic in the physiologic transfer of drug to the target cells. After production of Folate■MH conjugation, it is easy to attach Folate■MH to AuNP because of free –SH group at the other side of such a conjugation. In practice, MH has been selected for binding to AuNP because it is –SH terminated and its oxidation in the attachment to AuNP is well known.[27,28] Folate■MH reacts with chlorauric acid (HAuCl₄) in aqueous methanol solution at room temperature to form dark brown Folate■MH stabilized gold nanoparticles (Folate■MH■AuNP).
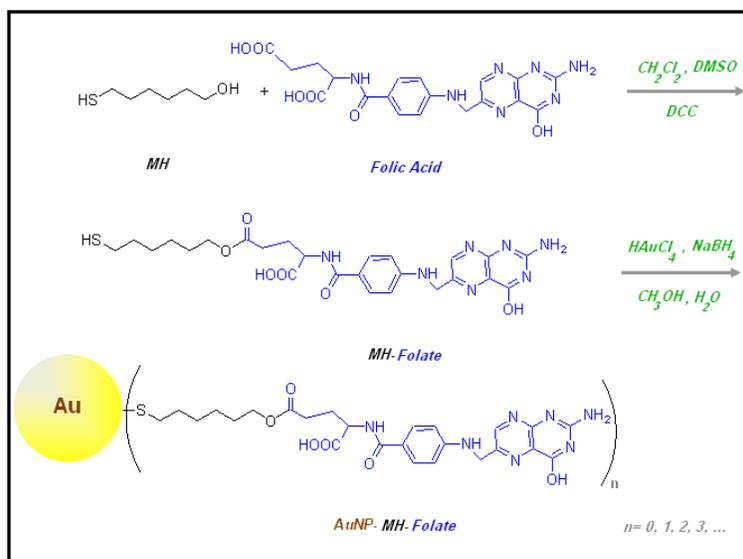
Figure 2: Synthesis route for nano-conjugation of AuNP to folic acid using 6-mercapto-

1-hexanol as the linker.

Because of the bonding procedure mentioned above we obtained the folate conjugated gold nanoparticles (Folate∎MH∎AuNP for short) which is a clean colloid with dark brown color. It was then separated and precipitated through centrifugation. We clearly identified the formation of such conjugations from their ultraviolet-visible (UV-visible) and Fourier transform infrared spectroscopy (FTIR) spectra.

3. Experimental Methods

3.1. Materials: All the chemical compounds used in this research were acquired from Merck, Germany and Fluka, Switzerland. Hydrogen tetrachloroaurate (III) trihydrate ($HAuCl_4 \cdot 3H_2O$, 99.5 % purity), 6-mercapto-1-hexanol (C_6H_7NS, 95% purity), Sodium borohydride ($NaBH_4$, 99.99% purity), and N, N'-dicyclohexylcarbodiimide ($C_{13}H_{22}N_2$, >99% purity) were purchased from Merck. and Folic acid ($C_{19}H_{19}N_7O_6$, 97 % purity) was obtained by Fluka, Switzerland.

3.2. Preparation of Folate Conjugated with 6-mercapto-1-hexanol (Folate∎MH): A solution of folic acid (22 mg, 0.05 mM) in *DMSO* (5 mL) was added to a solution of 6-mercapto-1-hexanol (*MH* , 6.7 mg, 0.05 mM) in CH_2Cl_2 (10 mL). The yellow mixture that resulted was vigorously stirred for 20 min. After that, a solution of *N, N'*-dicyclohexylcarbodiimide (*DCC*) (11 mg, 0.05 mM) in CH_2Cl_2 (10 mL) was added to the yellow mixture drop-by-drop while stirring. The stirring was continued for ten hours and a yellow-color mixture was obtained. The final yellow solid product was separated by a 4000 rpm centrifuge. It was subsequently washed with CH_2Cl_2. To obtain more purification, the mixture was washed with deionized water. After drying, the final product (*Folate∎MH*) was obtained as an orange powder.

3.3. Preparation of Folate∎MH Conjugated with Gold Nanoparticles (Folate∎MH∎AuNP): A solution of $HAuCl_4\cdot 3H_2O$ (40 mg, 0.1 mM) in CH_3OH (10 mL) was added to a stirred solution of *Folate∎MH* (33.42 mg, 0.6 mM) in CH_3OH (10 mL). After 20 minutes of stirring, a freshly prepared solution of sodium borohydride (57 mg, 1.5 mM) in deionized water (5 mL) was added to the vigorously stirred reaction mixture drop-wise in a 15 min period. The reaction mixture turned deep brown indicating the formation of *AuNP*s. The stirring was continued for one hour. Finally, the conjugates of *AuNP* with *Folate∎MH* were separated from methanol by precipitation under centrifuge at 10,000 *rpm* and purified by successive washing with CH_2Cl_2 and deionized water. Ultrasound sonication was performed to disperse the nanoparticles into the base material thoroughly. Subsequently, centrifuging and washing with deionized water was carried out to reach additional purification. We obtained the *Folate∎MH∎AuNP* nanoconjugate as a deep brown powder after drying and the results were stored for further characterization.

3.4. Characterization Techniques: UV-visible (UV-vis) absorption spectroscopic measurements were recorded on a single beam UV-vis spectrometer, Agilent 8453, using quartz cells of 1 cm path length and methanol as the reference solvent at room temperature. Also, Fourier Transform Infra Red (FTIR) measurements were recorded on a Shimadzu FT-IR 4300 instrument using KBr pellets at room temperature. Transmission electron microscopic (TEM) images of the nanoparticles were taken with a LEO 912AB instrument operated at an accelerating voltage of 120 kV with line resolution of 0.3 nm at room temperature. The samples for TEM measurements were prepared by placing a droplet of the colloidal solution onto a carbon-coated copper grid and allowing it to dry in air naturally. X-ray diffraction (XRD) was carried out with a Bruker D8 ADVANCE X-ray Diffractometer, using the wavelength of 0.15406 nm (Cu Kα) radiations at room temperature. Based on the TEM images we determined the size distributions of the final product by counting at least 300 particles. The elemental analyses for carbon, hydrogen, nitrogen, sulfur and oxygen were performed using a Perkin-Elmer CHNS-O analyzer. We determined the gold percentage in the Folate∎MH∎AuNP by Shimadzu model AA-670 atomic absorption spectrophotometer.

4. Analysis of Results

Figure 3 shows the Infra Red spectrum of Folate∎MH as compared with the spectrum of neat MH. According to this Figure, the infrared spectrum of Folate∎MH shows the characteristic bands at 3450, 3350, and 1720 cm^{-1} corresponding to amine and ester groups respectively. The weak SH stretching vibration is appeared in 2560 cm^{-1} which confirm the ester bond formation rather than thioester bond formation.
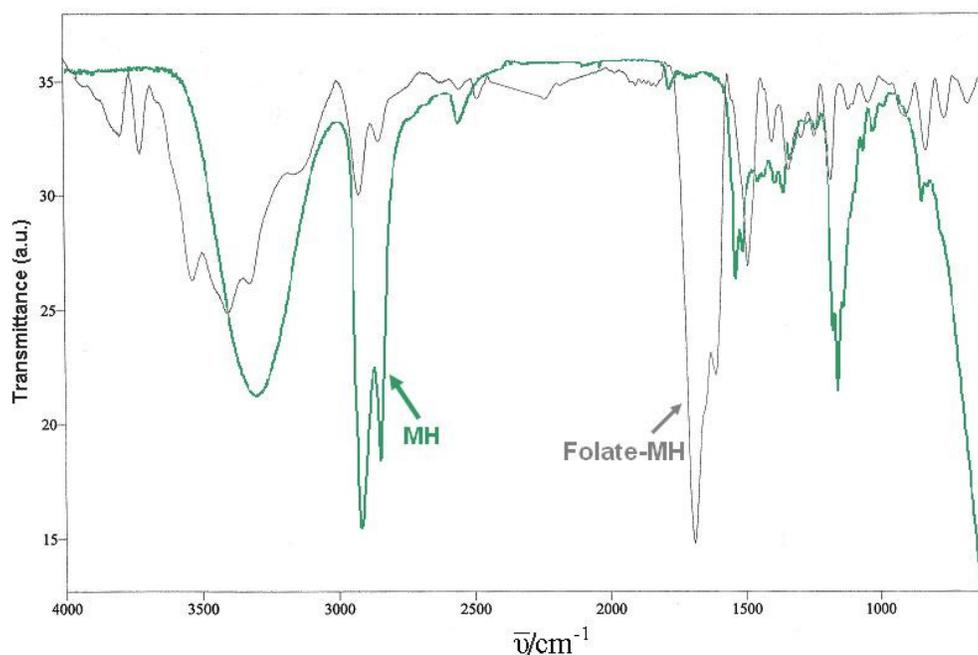
Figure 3. Infra Red spectra of Folate■MH in comparison with MH.

Figure 4 shows the UV-visible spectra of the synthesized Folate■MH and Folate■MH■AuNP conjugations.
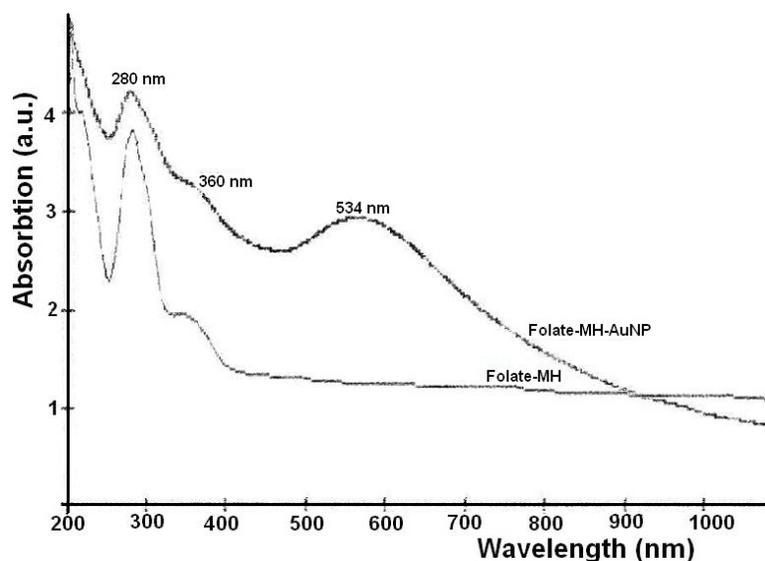

Figure 4. UV-Visible absorption spectra of Folate■MH and Folate■MH■AuNP .

In the spectrum of Folate■MH , the absorption maxima at 280 and 360 nm can be used for confirming the covalent attachment of the folate with MH, according to the literature. [29,30] AuNPs possess the characteristic surface plasmon absorption at 520 nm in the UV-visible absorption spectrum. This characteristic absorption band in the assemblies of AuNPs interlinked by various molecules exhibit a peak between 520 and 620 nm.[31]

The propensity for intermolecular hydrogen bonding in the assemblies of AuNPs interlinked by different ligands, resultant broadening and red-shifting of the plasmon absorption peak are expected. Also, λ_{max} of these assemblies (AuNPs-Ligands) is dependent on the particle size. [31] Accordingly, a significant broadening of the gold surface plasmon bands or the appearance of a red-shifted absorption band due to coupling of the individual surface plasmon of nanoparticles in the aggregated structures will be observed if the interlinked nanostructures are formed. Moreover, the appearance of surface plasmon bands around 534 nm in the spectrum of Folate■MH■AuNP confirms the formation of stable gold nanoparticles.[31,17]

Elemental analysis of Folate■MH gave: C, 53.67; H, 5.83; N, 17.63 and S, 5.50% which similar with calculated analysis data C, 53.85; H, 5.56; N, 17.59 and S, 5.74%. However, elemental analysis of Folate■MH■AuNPs showed approximately 71.5% of theoretical data for carbon and hydrogen. Atomic absorption analysis confirmed by 32% Au, which correlate with elemental analysis of organic moiety of this conjugation. The C:H and S:H ratios in Folate■MH and Folate■MH■AuNP s are well correlate.

We report the transmission electron microscopic (TEM) image of the synthesized Folate■MH■AuNP in Figure 5. Also, the size histograms of the Folate■MH■AuNP s are determined by counting at least 300 particles. The nanoparticles' shape is nearly spherical and the micrographs clearly indicate the formation of nearly monodispersed nanoparticles with an average diameter of 3 nm. As a result, nanoconjugates attached to folic acid through a bifunctional MH linker have a great advantage from point of view of size which is very considerable for biological tests . [1,16,32]
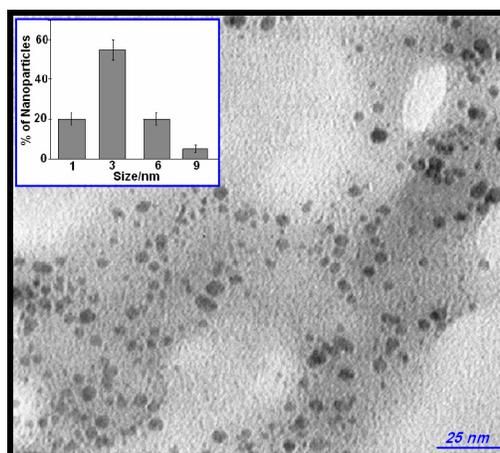

Figure 5. TEM photograph of Au nanoparticles in Folate■MH■AuNP .
The insert histogram is for the size distribution of Au nanoparticles.

As shown in Figure 6, the crystalline nature of the nanoconjugate is confirmed through the X-ray diffraction (XRD) analysis, where (110), (011), (221), (321), (060), and (004) crystal planes of metallic peso-cubic structure (alpha=beta=gamma=90º) are identified with a lattice constants of a=1.348 nm, b=1.348 nm, and c=0.725 nm.
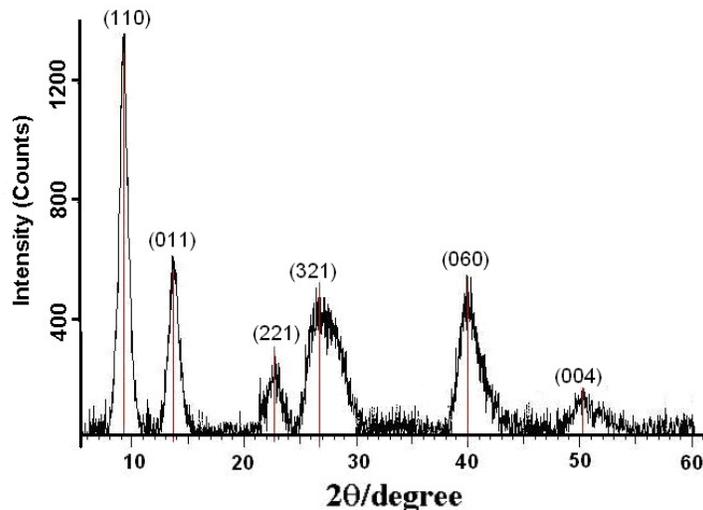
Figure 6. XRD pattern of Au nanoparticles in Folate▪MH▪AuNP .

5. Conclusions

We report the synthesis and characteristics of a new Folate-conjugated gold nanoparticles using 6-mercapto-1-hexanol (MH) as a linker. UV-visible and FTIR spectroscopy confirms the attachment of folic acid to the gold nanoparticles. We confirm the crystalline nature of the final product by XRD spectroscopy. Using the data obtained from elemental analysis techniques such as CHNS-O Analysis and Au Atomic Absorption Spectrometry, the conjugation of organic chains (Folate▪MH) with AuNPs were confirmed.

This new synthesized complex material contains folate, it can be applied to selective targeting of folate receptor positive cancerous cells, which overexpress folate receptor on their surface. Significant absorption of this new synthesized complex makes it a promising material for using in the area of cancer therapy and thermal ablation of tumors. Further investigations in cellular targeting by these new folate-conjugated gold nanoparticles are in progress in our laboratories.

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