RESEARCH ARTICLE

In-vitro investigation of curcumin coated gold nanoparticles effect on human colorectal adenocarcinoma cell line

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ABSTRACT

Curcumin is a herbal supplement that has been mentioned for many biomedical applications. Several pieces of research demonstrated that curcumin could improve cancer chemotherapy, lagging the metastasis progress, and prevent healthy cells from radiation therapy damage. Through a simple and green synthesis procedure, stable gold nanoparticles were synthesized by natural phytomedicine curcumin. The curcumin-coated gold nanoparticles (Cur@AuNPs) are red and represent the distinct gold nanoparticles' plasmonic peak. The average diameter of the synthesized nanoparticles is 21.7. However, the hydrodynamic diameter was 45.1 nm. The cytotoxicity of Cur@AuNPs has been investigated through an MTT assay. 24-hour treatment of Cur@AuNPs could eradicate more than 30% of HT29 cancerous cells. The real-time polymerase chain reaction (PCR) technique was used to study the molecular change in apoptotic protein changes. The nanoparticle treatment increases the level of the pro-apoptotic protein (Bax, P53, and P21) and decreases the anti-apoptotic protein level (Bcl-2) in the treated cell population.

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INTRODUCTION

Death from colorectal cancers (CRC) is the second most common cause of cancer death in the United States [1]. Surgery, chemotherapy, and radiotherapy are the CRC regular treatments, which bring about side effects and risks for the normal tissues, cells, and immune system. For different therapies of malignancies, the development of nanomaterials and nanotechnology techniques provided much potential to increase the treatment efficacy and overcome the therapies' limitations.

One of the most common nanoparticles that have been developed for various colorectal cancer diagnostic and treatments are metal nanoparticles. Indeed, gold nanostructures, among different types of metal nanoparticles, are of great significance in nanomedicine because of distinct properties including, adjustable surface plasmon resonance (SPR) [2], simple surface modification [3], high energy adsorption [4], auspicious healing opportunities and low toxicity and biocompatibility [5].

Gold nanoparticles are known for their many applications in cancer nano hyperthermia. Various types of gold nanoparticles can absorb a variety of energy sources and can kill cancer cells in different ways [4, 6]. However, new drug delivery strategies have been offered by the application of gold nanomedicine, which has drawn lots of attention [7]. Owing to the high Z number, gold nanoparticles also have many applications in colorectal cancer radiotherapy treatment [8, 9]. Gold nanoparticles have shown high potential for photodynamic and photothermal treatments of colorectal cancers [8, 10].

It has been demonstrated that different phytomedicines could be applied for colorectal

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cancer treatments. However, there are many limitations on traditional medicine formulations application, which influence these drugs' efficacy. Some of them are drug insolubility, site-specificity, and decreased bioavailability [4, 8]. Curcumin, as a chemopreventive phytomedicine, shows very low blood solubility. As a result of curcumin's insolubility, its chemopreventive feature is not exercised if it is injected alone into the cancerous area, and its application in cancer therapy is restrained [11]. In the current study, we synthesized the curcumin coated gold nanoparticles through a one spot simple green synthesis route. After characterization through various techniques, the effect of nanoparticles on human colorectal cell line was investigated at the cellular and molecular levels.

EXPERIMENTAL

Chemical

Dimethyl sulfoxide (DMSO), Curcumin, Potassium carbonate (K_2CO_3), Nitric acid (ACS reagent, 65%), Hydrochloric acid (ACS reagent, 37%), and Trachloroauric acid trihydrate 99.5% ($HAuCl_4$.$3H_2O$), were purchased from Merck chemicals company (Darmstadt, Germany). Ltd. Fetal bovine serum (FBS), Trypsin (Biosera, England), RPMI-1640 culture medium (Biosera, UK), (GIBCO/BRL Invitrogen, Carlsbad, California), Penicillin/Streptomycin (Biosera, England) and Methylthiazolyl diphenyltetrazolium bromide (MTT, 98%, Sigma-Aldrich, USA) were applied without any additional change. HT-29 colon carcinoma cells were purchased from Pasteur Institute (Tehran, Iran). Deionized water (DIW) with a resistivity of 18.3 MΩ.cm was provided from a water purifying system (Milli-Q Plus 185, Millipore, Bedford, MA). DIW was applied for all aqueous solutions in the synthesis procedure. Glassware's were cleansed with Aqua Regia (a solution of hydrochloric acid and nitric acid, 3:1) and soaked thoroughly with DI water.

Synthesis of the Cur@AuNPs

The synthesis of Cur@AuNPs was completed as described in our previous studies [4, 5]. Briefly, a 20 mM solution of curcumin in DMSO was prepared. 15 mL of DIW was also poured into a round bottom flask. The pH of the water was adjusted by K_2CO_3 in the range of 9, 100 µl of curcumin solution was poured into the 15 ml of DIW under stirring. The colour of the yellow curcumin solution was turned red. After three minutes, 2.5 ml of $HAuCl_4$.$3H_2O$ (2.5 mM) was slowly poured into the curcumin solution. After three hours of stirring, the sample was held undisturbed in the dark for three days. According to our previous study, the nanoparticles were washed through a series of centrifugation and suspending the nanoparticles in DIW [10]. Also, a high concentration of the nanoparticles could be applied through centrifugation and decantation.

Characterizations of the Cur@AuNPs

Before each characterizations technique, the synthesized Cur@AuNPs were passed through a polyvinylidene fluoride (PVDF) filter (0.2μm). The size and morphology of the synthesized nanoparticles were characterized by a Transmission electron microscope (Zeiss EM 900, Germany). A double beam UV-visible absorption spectrophotometer (SPEKOL 2000, Analytik Jena, UK was applied for optical properties characterization of the nanoparticles. The Zeta potential analysis (Zeta-check, Microtrac, Germany), and hydrodynamic diameter (DLS, NANO-flex Particle Sizer Germany) of the nanoparticles were also studied. The concentration of gold ions in the nanoparticle samples was studied with inductively coupled plasma optical absorption spectroscopy (ICP-AES).

Cellular Studies

Cell investigations were performed on the colon carcinoma (HT-29) cell line provided by the Pasteur Institute (Iran). The RPMI-1640 cell growth medium applied for culturing the HT-29 cell line was supplemented with penicillin (100Uml−1), streptomycin (100µgml−1), and 10% FBS. A standard humidified atmosphere (95% air & 5% CO_2 at 37 °C) was imposed for the maintenance of the cells. For examining the Cur@AuNPs cytotoxicity, we seeded 10000 HT-29 cells into each well of a 96-well plate. Following 24h, a fresh culture medium was used for cell treatment, which carried a specific concentration of Cur@AuNPs. Then, they were incubated for an additional 24h. The MTT or Real-time PCR tests were conducted after 24 h incubation.

MTT assay

We incubated cells with various treatments with 100µl, 0.5mgml−1 of MTT for 2 to 4 hours. Then, it was replaced by 100µl of DMSO solvent. Lastly, the wells' absorption was recorded at 570nm

wavelength.

RNA extraction, cDNA synthesis and quantitative real-time PCR

The High Pure RNA Isolation Kit (Roche Life Science) was used for isolating the total RNA of cells with various treatments. Using PrimeScript First Strand cDNA Synthesis Kit (Takara), RNA was reverse-transcribed into cDNA. The specific primers were used for amplifying the extracted cDNA. SYBR Premix Ex Taq TM II (Takara Bio, Inc.) was used for performing quantitative real-time PCR in a Corbett Rotor-Gene 6000 thermocycler (Eppendrrof Biotech Company). The thermal profile for SYBR Green PCR was 95 °C for 15 s, tracked by 40 denaturation cycles for 5 s at 95 °C, and extension/annealing for 30 s at 60 °C. As the endogenous control, GAPDH was employed. The fold change in gene expressions was calculated using the comparative Ct technique.

Statistical analysis

Statistical data analysis was completed using one-way ANOVA. P-values <0.05 were regarded statistically significant. Data were given as mean ± SD.

RESULTS AND DISCUSSION

The synthesis of Cur@AuNPs was confirmed by observing the characterized plasmonic peak of gold nanoparticles at 526 nm (Fig. 1a). According to the supplementary data presented by Haiss et al. [12] for the determination of the size of gold nanoparticles from UV−Vis spectra, it was estimated that the average size of Cur@AuNPs was 18 nm in diameter. The spectra of the Cur@AuNPs are very different from curcumin spectra that have been reported in our previous study [5]. In another study by our team, we have investigated the stability of the Cur@AuNPs through UV-Vis Spectroscopy. It has demonstrated that the Cur@AuNPs are very stable nanoparticles in different physiological environments [4]. Similar gold nanoparticles that have been synthesized by phenolic compounds represent high stability in physiological environments [10].

Investigation of the hydrodynamic diameter of a particle is very common for nanoparticles size estimation. Based on our investigation, the hydrodynamic diameter of Cur@AuNPs was 45.1 nm (Fig. 2). The hydrodynamic diameter of a nanoparticle is usually higher than the actual nanoparticle diameter [13]. Also, it has been demonstrated that the intensity of light Rayleigh scattering is dependent on 10^6 order of magnitude [14]. In the case of metal nanoparticles especially gold nanoparticles DLS is a delicate device for obtaining the right value. Many unknown and unwanted factors could affect the results of the samples. Zheng et al. have reported some of the crucial factors for DLS measurement of gold nanoparticles which include concentration, scattering intensity, incident laser power and possible nanoparticles

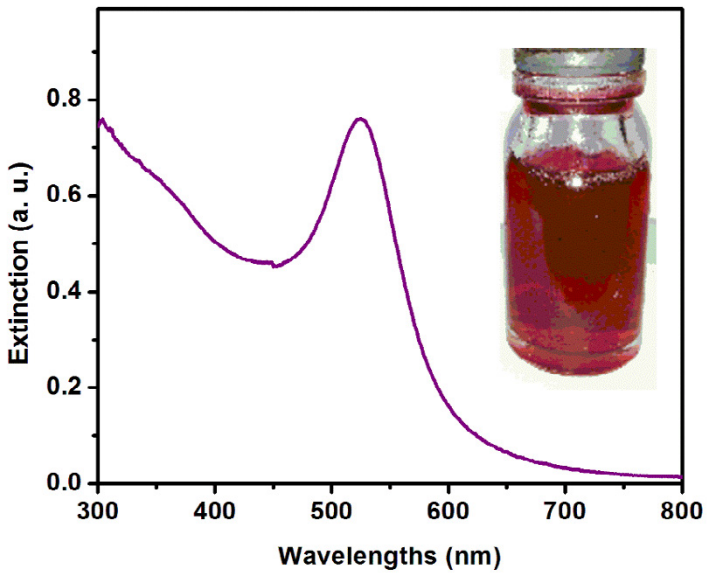

Fig. 1. UV-Vis spectrum and optical image of the Cur@AuNPs

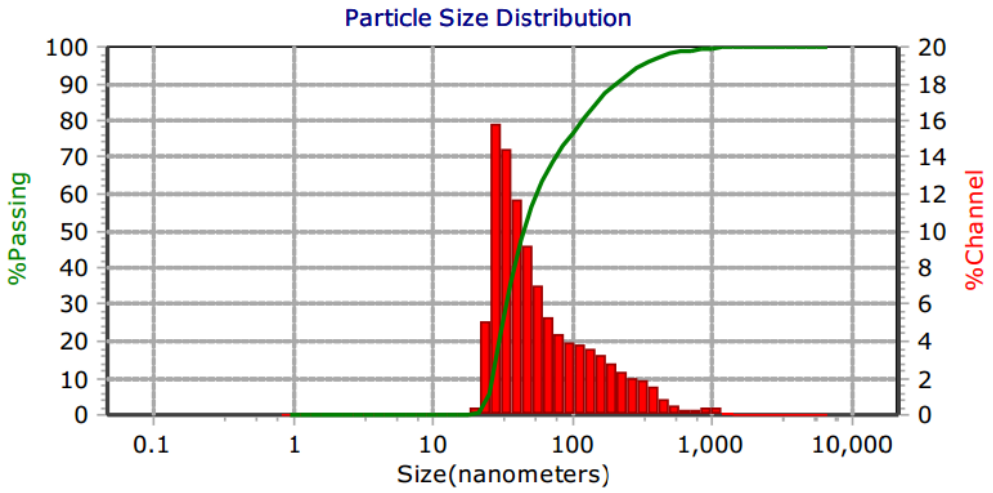

Fig. 2. The hydrodynamic analysis of Cur@AuNPs.

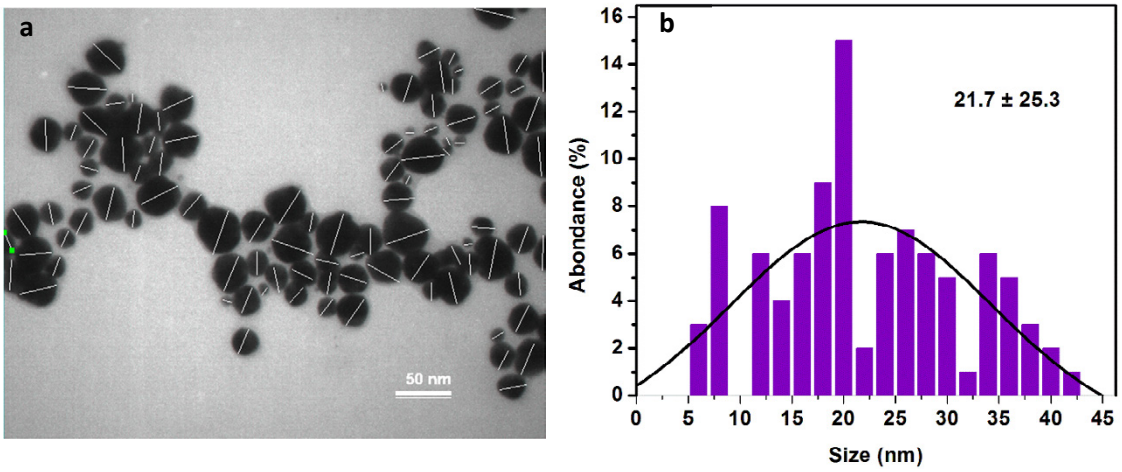

Fig. 3. investigation of size and morphology of Cur@AuNPs. The diameter of each nanoparticle has been demonstrated by digital micrographs in the TEM micrograph of Cur@AuNPs (a). The size distribution of the nanoparticles was calculated by Origin software which is 21.7±25.3 nm (b).

aggregation. They poured 100 nm particles into 20 nm particles solution in various proportions and analyzed the DLS diagram pattern. It has been demonstrated that a very low number of aggregate in gold nanoparticles solution could change the DLS diagram completely [14].

The obtained TEM micrographs of Cur@AuNPs showed that the average diameter of synthesized NPs is 21.7±25.3. The size that has been estimated by the UV-Vis spectrum is unacceptable. Consistency with UV–Vis spectra estimation. However, The TEM micrographs reveal that the nanoparticles are not very homogenous in size. However, all of the nanoparticles are almost spherical in shape (Fig. 3). It has been demonstrated that the green synthesized nanoparticles are very heterogeneous in size and shape [15]. Since the kinetics of metal ion reduction reactions by plant compounds are very slow, the synthesized nanoparticles by these compounds are likely to have a wide distribution size [15].

For synthesizing Cur@AuNPs, curcumin must be dissolved in DMSO, and it could be more diluted with water at higher pH with an increase in pH from 5 to 9, a redshift was observed in the absorbance spectra of Cur due to Cur de-protonation. It is necessary to increase the pH of Cur to release H molecules of its hydroxyl groups to reduce Au ions. $HAuCl_4$ should not be added

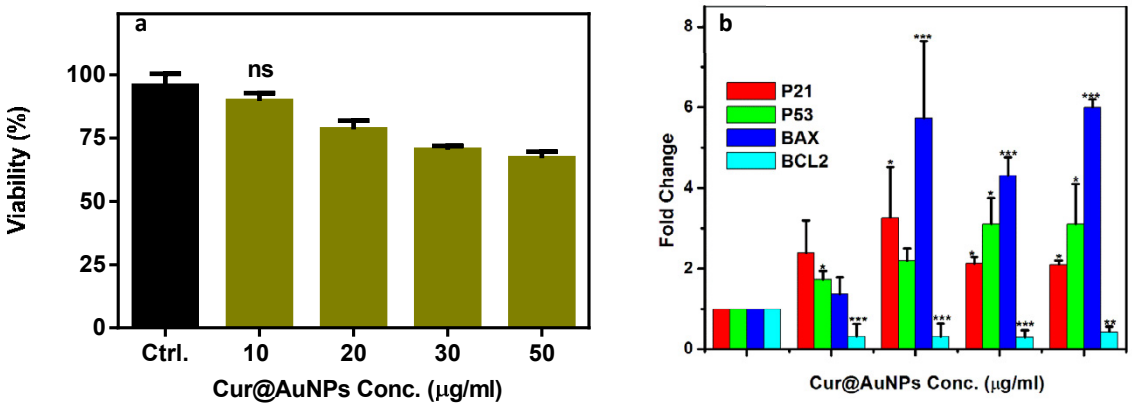

Fig. 4. The effect of Cur@AuNPs on cellular viability (a) and the expression levels of P_{21}, P_{53}, BAX, and BCL_2 genes (b).

later than 5 minutes since at a pH of 9, after 5 min, Cur degradation will accelerate. For the removal of non-reacted curcumin, Cur@AuNPs were washed through a series of centrifugation and decantation. It has been demonstrated that four rounds of washing through centrifugation and decantation can remove unreacted curcumin and Au ion [4].

To accurately understand the effects of nanoparticles on the human colorectal adenocarcinoma cell line, the cellular viability and the expression levels of P_{21}, P_{53}, BAX, and BCL_2 genes were performed. The uptake of Cur@AuNPs has been demonstrated in our previous study through ICP-OES [4]. The uptake of gold nanoparticles that have been synthesized by *Cornus mas* extract was also recorded in TEM micrographs [16]. The results of the MTT assay in the current study showed that Cur@AuNPs have a little toxic effect on the cell viability of colorectal cancer cells at the investigated concentrations (Fig. 4a). The biocompatibility of nanoparticles made with plant compounds has been studied in many studies. It has been demonstrated that metal nanoparticles that have been synthesized by natural phenolic compounds are not toxic in MTT assay in comparison with metal nanoparticles that have been synthesized by chemicals [5]. The low toxic effect of Cur@AuNPs has been demonstrated on various cell lines including the Human monocytic cells [17], peripheral blood mononuclear cells (PBMCs) [18], and murine colorectal carcinoma cells [4]. Few studies declare that the Cur@AuNPs could be toxic to cells such as 3T3 Fibroblasts [19], and breast cancerous cells [20, 21]. In the synthesis process of Cur@AuNPs, the quality and quantity of the washing procedure have a direct impact on cytotoxicity. Research groups who have reported high toxicity for Cur@AuNPs have generally not washed the nanoparticles well. Unwashed nanoparticles contain a high amount of gold ions, unreacted curcumin, or even dangerous substances such as DMSO that can cause cellular death [5]. In the current study, no cytotoxic effect was observed on the 10 μg/ml concentration. Also, a low cytotoxic effect was observed for other concentrations (Fig. 4a). Other important parameters such as the incubation time of nanoparticles [22] or the size of nanoparticles [7, 23] can also affect their cytotoxic effect. These parameters were not investigated in the current study.

The balance between pro-and anti-apoptotic constituents of the Bcl-2 class can ascertain the viability of the cells. Contrary to the cellular response, at the molecular level, Cur@AuNPs can trigger different responses in colorectal cancer cells. Mitochondrial dysfunction could trigger apoptotic cell death in colorectal cells. Bax and Bcl-2 regulation perform a key function in this apoptotic pathway [24]. The gene expression levels of apoptosis-related genes Bax and Bcl2 were found to be upregulated and downregulated, respectively (Fig. 4b). It can be concluded that the increase of pro-apoptotic proteins (Bax) and the decrease of anti-apoptotic proteins (Bcl-2) were observed in cells that have been treated by Cur@AuNPs. Similar results have been observed in human renal cell carcinoma for gold nanoparticles that have been synthesized from *Curcuma wenyujin* extract [25]. *Curcuma wenyujin* extract contains a high level of curcuminoids, however, there are a lot of other phyto ingredients in the extract that could interfere

with the results.

Also, it has been revealed that the expression of key tumour suppressors P_{53} and P_{21} was upregulated due to the cell treatment by various concentrations of Cur@AuNPs (Fig. 4b). P_{53} and P_{21} are tumour oncogene/suppressor genes involved in different cellular mechanisms such as DNA repair, apoptosis, cell cycle arrest and cell proliferation. Therefore, targeting the genes and corresponding signalling pathways has been considered a helpful strategy in cancer therapy. The anti-proliferative and apoptotic effects of curcumin in colorectal malignancies have been reported [26]. Similar effects have been observed for gold nanoparticles that have been synthesized by extract of *Mimosa pudica* [27]. Based on the provided results and literature, it could be considered that the Cur@AuNPs can cause low cell death and sensitize them to cell apoptosis.

Conclusion

Curcumin has been documented to possess some anticancer activities owing to modulating of cancer-related genes and signalling pathways. This phytomedicine represents significant chemical potential in the synthesis of metal nanoparticles. In the current study, curcumin coated gold nanoparticles have been synthesized through a simple and cost-effective one-pot synthesis procedure with an average diameter of 21.7 nm. The nanoparticles represent a low toxic effect on colorectal carcinoma cells in the MTT assay. However, it has been observed that the treatment of the nanoparticles could increase the regulation of apoptotic proteins such as Bax, P_{53} and P_{21} and decrease the regulation level of anti-apoptotic Bcl-2 protein.

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DECLARATION OF INTEREST

None.

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