Preparation of novel anisotropic gold nanoplatform as NIR absorbing agents for photothermal therapy of liver cancer and enhanced ultrasound contrast imaging

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Abstract
Nanoparticles (NPs) with different shape, size, architecture and composition were studied for their application as photo-thermal agents in the area of cancer nanomedicine. Out of them, gold nanoparticles (Au NPs) depending on their \textit{in vivo} biocompatibility provide a simple thermal ablation platform. However, fabrication of these Au NPs showing appropriate properties for photo-thermal function requires complex routes utilizing hazardous chemicals as capping agents which may cause \textit{in vivo} concerns. In this study, the fabricated Au NPs utilizing biosynthetic approach having near-infrared (NIR) absorbance assisting photo-thermal treatment could be a possible alternative. Herein, anisotropic Au NPs were fabricated utilizing an aqueous extract of \textit{Ceratonia siliqua (carob)} which acts as both stabilizing and reducing agent. The biosynthesized Au NPs were exposed to density-gradient centrifugation for the optimization of NIR absorption in 800 to 1000 nm wavelength range. Colloidal Au NPs showed outstanding contrast enrichment for ultrasound imaging, and also Au nanoplates were obtained by density gradient centrifugation can function as a NIR absorbing agent for efficient photothermal killing of Hep-G2 liver tumor cells \textit{in vitro} with negligible cytotoxicity to active cells. Furthermore, the present approach recommends an innovative way for treating theranostic cancer.

Introduction
Nanotechnology is found to have a huge advancement in biological sciences. The treatment and diagnosis in cancer field has extended through the incorporation of different arsenals as polymeric micelle delivery vehicles [1], metallic NPs for radio-thermal [2–5], and photo-thermal treatment [6], iron–oxide NPs for a magnetic nanoparticle mediated intracellular hyperthermia [7], quantum dots (QD) for fluorescent diagnostic imaging [8], contrast agents for computerized tomography (CT) and Magnetic resonance imaging (MRI) [9–11] etc. Since many years, numerous developments and contributions in using NPs for the photo-thermal treatment were being reported [12–15]. Photo-thermal treatment involves the utility of NIR light with highest tissue penetrability [16] which could be absorbed by the NPs for the production of heat in order to eliminate cancerous cells located in the vicinity. Numerous NPs as Polypyrrole Nanoparticles [12], gold/gold sulfide (CS-GGS) NPs [13], gold nanorods (Au NRs) [14] Au-Cu$_2$–Se [17] etc. were prepared and documented to show photo-thermal effect. These types of NPs are engineered specifically via careful reaction methods so that they show excellent stability and intense absorptivity in NIR region. The commonly used reactants in these reaction methods are hazardous and its residue if existed in nanodrug can result in systemic toxic effects [18, 19]. For instance, in Au NPs fabrication, Cetrimonium bromide (CTAB), which has widely known cytotoxic effect, is utilized as a stabilizing agent for the facilitation of its directional fabrication procedure [19, 20]. Hence, there is a great desire for a completely safe and aqueous method of fabricating NPs for its utilization \textit{in-vivo}. Bio-synthetic approach has emerged in the last ten years as an easy and cheap alternative for fabricating metallic NPs of silver and gold [21]. Various plant chemical reagents such as \textit{Cinnamomum tsoi} plant extract [22], soybeans [23], lemon grass...
extract [24] garlic extract [25], tyrosine [26] etc., were utilized for the fabrication of composite or metallic gold NPs. NPs with dissimilar shape and size fabricated by altering the reaction method parameters through these approaches displayed good biocompatibility and stability [25, 27]. For instance, Shankar and his co-workers have revealed that on altering the quantity of lemon grass extract, the anisotropic NPs displaying NIR absorbance could be fabricated [24]. In the current study, NIR light absorbing Au NPs are indicated for their utility in glass coatings as Infra-red blockers in the applications of architecture. Although, applying such NPs in biological uses such as photo-thermal treatment, NPs absorbance must be highest in wave length range (800 to 1000 nm) where the absorptivity of tissue is high [16]. Most of the studies on biofabricated Au NPs mainly focus on its synthesis and stabilization characteristics, with limitations on its utility in biology. In this study, utilizing the carob extract, anisotropic Au NPs are fabricated and then stabilized without using any extra capping agents. In accordance to the US department of agriculture, coco is one among the substances which has maximum value of Oxygen Radical Absorbance Capacity (ORAC) [28]. A higher value of ORAC indicates the intense reducing ability by favoring the selection of carob to synthesize Au NPs from the solution containing Au ions. The proceeding parameters that produce NPs having maximum optical absorbance were investigated. A procedure was designed to distinguish anisotropic NPs from a mixture of anisotropic and spherical particles utilizing a technique of density gradient centrifugation in order to obtain optimum NIR absorbance. The obtained NPs were studied for the ultrasound imaging and photothermal therapy of liver cancer treatment.

Materials and methods

Materials
Chloroauric Acid (HAuCl₄), Dulbecco’s Modified Eagle Medium (DMEM), fetal bovine serum (FBS), streptomycin, penicillin, Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Potassium bromide (KBr, FT-IR grade, >99% trace metals basis) and sucrose were obtained from sigma Aldrich, Shanghai and unprocessed carob powder was obtained from the local market (shanghai). All other chemicals and reagents were utilized without any additional processing and purification. All the utilized magnetic stir bars and glass wares are washed using Aqua regia before use.

Preparation of carob extract powder solution
Before the synthesis of NPs, we prepared the solution of carob extract powder. For preparing the extract solution, raw carob powder weighing 1g was mixed with deionized (DI) water of 200 ml and mixed for 20 min at a temperature of 80 °C in order to remove its hydrophilic constituents. The resultant mixture was then centrifuged at 3000 rcf for 15 min at a temperature of 4 °C. Later, the supernatant from the above mixture was separated and then allowed to dry at a temperature of 80 °C to obtain the carob extract powder. The resultant powder was dissolved in the DI water for obtaining the final reductant at a concentration of 10 mg/ml. The final obtained mixture was then autoclaved and preserved in an optimal refrigerated environment for future utilization.

Fabrication of Au NPs
The solution of chloroauric acid was prepared using Milli Q water and placing on a water bath under constant stirring. Various quantities of carob extract powder solution (500, 400, 300 and 200 μl) were mixed with 500 μl of 1mM chloroauric acid solution and continued the reaction process for 15 min under optimal temperature conditions (90, 75, 60, 45 and 30 °C). Once the obtained reaction solution was brought to ambient temperature, stirring was allowed to continue throughout the night for completing the reaction process resulting in a purple-coloured crude suspension of NPs.

Anisotropic Au NP separation
For the separation of anisotropic NPs from the crude suspension of NPs, a density gradient centrifugation process based on sucrose was utilized. Falcon tubes (50 ml) were utilized for preparing sucrose gradients through layering sucrose solution of 5 ml in reducing concentration conditions (90% to 10%) in the falcon tubes. Then, 5 ml crude NP solution was added to the above sucrose gradient and then subjected to centrifugation for 80 min at 5000 rcf at a temperature of 4 °C. Following the centrifugation, each sucrose layer was carefully separated and cleaned repeatedly through centrifugation for 10 min at 17000 rcf. The NPs were re-dispersed in Milli Q water followed by sonication of the probe for future utilization.

Characterization of the NPs
Surface Plasmon Resonance (SPR) of separated anisotropic NPs and the as-synthesized crude NP suspension was evaluated utilizing UV–vis near infrared spectrophotometer (UV–vis instrument, Specord S600, Analytic
Jena, Germany) to verify the Au NPs absorbance at 350 to 1200 nm wavelength range. The morphology and size of the separated anisotropic Au NPs which displayed maximum NIR absorbance was assessed utilizing Transmission electron microscopy (TEM, JEM 2100, Jeol Ltd, Japan) operated at acceleration voltage of 200 kV and Field-emission scanning electron microscopy (FE-SEM, Carl Zeiss Supra 55VP) operated at an accelerating voltage of 5 kV. The TEM samples were prepared by drop casting dilute NP solution on the surface of copper grid (400 mesh), whereas for FE-SEM, the samples were prepared by dropping NPs solution directly on the foil of aluminum located on a stub. TEM analysis was performed to observe the crystallite planes and compute the d-spacing of NP. Crystalline nature of fabricated NPs was assessed utilizing an x-ray diffraction (XRD, Bruker D8 Advance diffractometer) instrument equipped with CuKα radiation at λ = 1.54056 Å. A drop of Au NPs solution was placed on the glass substrate which yields a thick film for spectroscopic analysis ranging from 5 to 80°, at 0.02° step size. In addition, standard ICPDS database was utilized to perform the phase identification. The stability and surface charge of the NPs were evaluated utilizing a Zeta potential instrument by examining its zeta potential. The functional groups on the surface of NPs were identified by performing Fourier Transform Infrared (JASCO ATR-FTIR 4100 instrument) spectroscopy in a 400 to 4400 cm⁻¹ wavelength range. The preparation of samples was carried out by blending and pelletization of 2 mg Au NPs with 175 mg Potassium bromide (KBr). For additional confirmation of the functional groups present on NPs, x-ray Photoelectron Spectroscopy (XPS, ESCA-3000 VG Scientific UK instrument) assessments were performed utilizing Al Kα radiation source (1468.6 eV). For XPS assessments, preparation of samples was carried out by placing a drop of NP dispersion on the glass cover slip and air dried.

In vitro ultrasonic scanning

In-vitro ultrasonic scanning of spherical Au nanospheres was carried out using Esaote Mylab 90 scanner. Usually, the normal saline with various concentrations of colloidal Au NPs (0 to 0.2 mg ml⁻¹) was added into a 2 ml Eppendorf tube, later the tube was immersed in a water tank. Ultrasonic scanning was performed by utilizing La435 linear array ultrasound sensor probe with various mechanical indices (MIs) and frequencies. Ultrasonic gel or coupling agent was coated on the sensor to prevent the air background. The videos and images were documented as digital files for the replay and subsequent examination.

Cell culture

Liver cell lines (Hep-G2 cell lines) were obtained from the Department of Oncology, Shanghai Cancer Center and were grown in DMEM (HyClone) provided with streptomycin-penicillin solution (100 μg ml⁻¹, 100 U ml⁻¹) and 10% of fetal bovine serum (FBS) in a humidified environment at 5% CO₂ at 37 °C. These cells were seeded at a density of 1 × 10⁴ cells well⁻¹ into each well of 96-well microplates consisting 100 ml DMEM provided with FBS (10%) for a day at 37°C temperature in a humidified environment at 5% CO₂ and cultured throughout the night prior to further investigations.

Evaluation of cell viability and in vitro cytotoxicity

In-vitro cytotoxic effect of the Au nanoplates over Hep-G2 cell lines was examined through MTT assay. In particular, Hep-G2 cell lines were added into 96-well microplates with a cell density of 1 × 10⁴ cells well⁻¹ consisting standard culture medium and grown at 37 °C temperature in a humidified environment with 5% CO₂ for a day prior to the exposure of above materials. Later, Hep-G2 cell lines were subjected to incubation in culture medium consisting various Au nanoplates concentrations (200, 100, 50, 20 and 10 μg ml⁻¹) for additional 24 h. In the meantime, microplate wells comprising the cell medium alone are prepared as controls. Following the administration, MTT assay was selected as a marker for determining cell viability through mitochondrial depended reduction of tetrazolium salt to formazan. Furthermore, 20 μl MTT solution of 5 mg ml⁻¹ concentration was injected into individual well and the microplates were kept for incubation for additional 4 h at 37 °C temperature in humidified environment with 5% CO₂. The formed supernatant solution was removed and later DMSO (150 μl/well) was added for dissolving the insoluble formazan crystals completely. Absorbance of individual well was estimated utilizing Multi scan ELISA microplate reader.

Measurement of the photothermal performance

To estimate the photo-thermal conversion efficiency of the Au nanoplates, 2 ml aqueous suspension of microspheres at various concentrations (50 to 300 μg ml⁻¹) was added into a quartz cuvette and irradiated utilizing NIR laser for 15 min at 1 W cm⁻² power density. For monitoring the real-time temperature, FLIR A300 thermal imager was equipped with thermal scanning camera was utilized.
Photothermal cytotoxicity
Photo-thermal cytotoxic effect of colloidal Au nanoplates was determined utilizing Hep-G2 cells. To measure the photo-thermal cytotoxic effect of Hep-G2 cells, the cells were incubated with various concentrations of Au nanoplates (200, 150, 100, 50, 25 and 0 μg ml⁻¹) for 1 h in humidified environment at 37°C with 5% CO₂. Lastly, the complete system was irradiated under NIR laser at 1 W cm⁻² power density for 10 min and then incubated for additional 1 h. Then, the cell viability was determined utilizing MTT analysis.

Statistical significance
All the obtained results were examined using SPSS software. Data expressed as mean ± standard deviation and the obtained values were considered significant at P > 0.05. Statistical assessments were conducted using ANOVA.

Results and discussion
A characteristic feature of Au NPs is the absorption spectra displayed in UV–vis NIR region due to the SPR phenomenon [29], which differs with shape and size of NPs [30, 31]. On adding a reducing agent, the precursor solution color has gradually turned into black from yellow and later into purple red. The UV–vis NIR spectra of fabricated Au NPs obtained by adding 500 μl solution of carob extract powder to the HAuCl₄ solution of 1 mM concentration at 37 °C is shown in figure 1. The change of yellow coloured precursor solution into black colour indicates the first nucleation step, where the small unstable Au seeds were produced because of the Au ions reduction initiated by the solution of carob extract powder. As the size of the seed grows for the formation of stable NPs, the solution turns to purple red from black which results in distinctive SPR absorbance at a wavelength of 535 nm representing the Au NPs. Even after repetitive centrifugation and cleaning, the Au NPs were found to be stable. Hence, the carob extract has dual function of reducing Au ions and provides stabilizing effects to synthesized NPs. This stabilization effect was suggested by the polyphenols of reductant such as carob which was authenticated by the data of FT-IR analysis in the resultant section.

Furthermore, reaction process parameters such as precursor concentration, temperature and carob extract amount were changed in order to evaluate their impact on size and morphology of Au NPs (figure 2). Usually, for spherically shaped NPs, growth in the size of particle results in a red shift of the SPR in noticeable wavelength range of electromagnetic spectra. Alternatively, change in Au NPs shape far from its lower free isotropic energy state (i.e. spherical morphology) results in the advancement of a longitudinal SPR peak in the NIR or far-Vis region [31]. Hence, for investigating the synthesized NPs, absorption spectrum was utilized for optimizing the method to fabricate NIR absorbing Au NPs for photo-thermal treatment.

Figure 2(A) displays the deviation in the spectral absorbance of Au NPs with different amounts of reducing agent. With decrease in the quantity of reducing agent, the NIR absorbance peak owing to the longitudinal SPR was red-shifted with increase in intensity. Moreover, similar outcomes were reported previously for the fabrication of Au NPs utilizing garlic [25] and lemon grass extracts [27]. This effect was due to the low rate of fabricating NPs than the rate of its yielding, sintering anisotropic particles. In the current study, the carob extract contains reducing agents in higher quantities in comparison to the capping agents, which utilized at minimal concentrations produces numerous unstable small NPs. These small particles then combine with each other to produce large and stable anisotropic NPs. However, maximum carob extract concentrations result in smaller and stable spherically shaped NPs. Furthermore, 400 μl of extract volume yields spherical NPs along with few
triangular, hexagonal plates with an optimum optical absorbance at a wave length of 800 nm and thus selected to optimize further (figure 2(B)).

Figure 2(C) displayed variation in the absorption spectrum of fabricated NPs with increase in the reaction temperature using 400 μl of extract solution. In addition, at a temperature of 30 °C, a characteristic SPR absorption peak at a wavelength of 535 nm in visible region was noticed along with a weak absorbance in NIR wavelength region. With an increase in temperature of the mixture up to 45 °C, a comparatively noticeable maxima in near-infrared wavelength region was observed and corresponding TEM images exhibited few spherical nanoparticles along with several uniformly size triangular nanoplates (figure 2(D)). Furthermore, increasing the reaction temperature results in the blue-shift of NIR absorption with decrease in the absorption intensity. Upon raising the reaction temperature, the elevated reduction rate and stabilization might exceed the coalescence rate of small unstable NPs and thus produces a large number of steadied small anisotropic NPs as demonstrated by blue shifting of near-infrared absorption. Therefore, at 400 μl extract solution, the temperature of reaction was adjusted to 45 °C to obtain maximum NIR absorption.

Figure 2(E) shows the absorption profile of the NPs achieved following the reduction utilizing various precursor concentrations of HAuCl₄. At precursor concentrations of 0.5 and 0.25 mM, low intensity SPR
absorption peak in the NIR region at a wavelength of 535 nm was noticed, which indicates the spherical Au NPs formation in low concentrations. With increase in the precursor concentration to 1 mM, a proportion of the anisotropic NPs were developed having typical absorbance at 700 to 1000 nm in the NIR region. These anisotropic NPs were formed when the excess residue precursor was reduced; however, these were not well stabilized, as the stabilizing agent’s concentration was limited. As the precursor concentration was further increased to 3 mM, the precursor molecule number per unit volume was also increased in comparison to the reductant concentration and thus preventing the total reduction reaction. Therefore, an optimum concentration of the chloroauric acid precursor (1 mM), reaction temperature of 45 °C and 400 μl carob extract were selected for the fabrication of anisotropic NPs for future experimentations.

Sucrose density gradient centrifugation was used to enhance the concentration of anisotropic NPs and also to obtain NPs containing high NIR absorption intensity. As per TEM images represented in figure 3, it was noticed that concentration of the spherically shaped NPs was reduced significantly after sucrose-based density gradient centrifugation. From TEM analysis of mixture before density gradient centrifugation, it was observed that anisotropic Au NPs proportion has particle size ranging from 100 nm to 150 nm (figure 3(A)). To further prove the enhancement in anisotropic NPs proportion, HR-TEM of NPs achieved in 40%−50% (figure 3(C)) and 0%−10% (figure 3(B)) sucrose layers was also performed. Enhancement of anisotropic NPs concentration over the spherical shaped NPs after density gradient centrifugation was explained clearly in the figure 3.

XRD pattern of the anisotropic Au NPs displayed the metallic gold distinctive peaks as represented in figure 4(A). The discrete peaks present in the X-RD pattern shows that carob-reduced Au NPs were extremely crystalline. Furthermore, widening of peak indicates the smaller nano-crystalline size of fabricated NPs. In contrast, the carob displayed a clear amorphous XRD pattern. The highly crystalline nature of bio-synthesized particles was further evaluated using HR-TEM for various anisotropic gold structures. Figure 4(A) inset represents the HR-TEM images that show well-ordered crystallite planes having d-spacing of 2.27 Å and also
with other study findings [32], indicating that the green approach has enabled the fabrication of crystallite Au NPs.

Figure 4(B) shows the FTIR analysis of the particles which was carried out for determining the presence of functional groups in the biofabricated Au NPs. After comparing FTIR spectra of Au NPs and carob powder, the presence of three characteristic overlapping peaks was evident. The wide peak ranging in between 3000 to 4000 cm$^{-1}$ mostly corresponded to stretching vibrations of hydroxyl functional group (−OH). Furthermore, the peak at 2900 cm$^{-1}$ corresponded to stretching vibrations of long chained hydrocarbon groups, stretching vibrations of the carbonyl functional group was represented between 1600 to 1700 cm$^{-1}$ and peak at 1063 cm$^{-1}$ present in the region of fingerprint corresponds to groups with aromatic rings. The above all functional groups are typical characteristic of carob which was also published previously [33]. The carob reduced Au NPs displayed FTIR spectrum bands that have overlapped along with carob powder. The band present at 1063 cm$^{-1}$ wavelength and the band absent at 2900 cm$^{-1}$ wavelength in the Au NPs indicated clearly that polyphenols which behaved as stabilizers formed the major constituents but not long chained hydrocarbon groups. The corresponding polyphenolic carbonyl group signatures were present in Au NPs like a band between 1600 to 1700 cm$^{-1}$ which confirms our hypothesis. This coating of polyphenols on NPs generated a higher value of zeta potential as $-50 \pm 8$ mV that measures its higher colloidal stability present in the aqueous systems.

XPS analysis was carried out for further confirmation of the polyphenol coating present on the NPs. The Au core levels, namely, 4f$5/2$ and 4f$7/2$ depicts the resolved spectra at 87.3 and 85.6 eV respectively which correlates with reported values of Au as represented in figure 5(A) [27, 34]. The spectra displayed for C1s and O1s was represented in figures 5(B) and (C) respectively. The presence of O1s peak at 530 eV and C1s peak at 283.2 eV could be because of carbonyl groups and aromatic hydrocarbon groups present in the polyphenols that stabilized Au NPs [27, 35]. Higher intensity peak of O and C present in the XPS pattern, further confirms the outcomes of the FTIR analysis.

Further, the contrast enrichment for ultrasound imaging studies were performed by using Au nanocolloid prepared using 400 μl reducing agent and 1 mM concentration of HAuCl$_4$ precursor at 45 °C. To investigate the uses of colloidal Au NPs as an ultrasonic scanning contrast agent, the contrasting efficiency of ultrasound was evaluated systematically in the normal saline. The in-vitro ultrasonic scanning of colloidal Au NPs was examined utilizing Mylab 90 scanner. Based on earlier investigations [36–38], the constant frequency and MI were predetermined at 9MHz and 0.060 correspondingly and the ultrasonic scanning of Au NPs was studied, as represented in figure 6. When the sphere concentration increases (0.04 mg ml$^{-1}$ to 0.20 mg ml$^{-1}$), the ultrasonic signals intensity was also elevated at concentrations of 0.04 mg ml$^{-1}$ to 0.10 mg ml$^{-1}$ but the signal intensity was moderately dropped off at 0.02 mg ml$^{-1}$ (maximum concentration) which was assigned to the aggregation induced by the high concentration. The optimal images were noticed at 0.06 to 0.01 mg ml$^{-1}$ concentrations, utilizing Au NPs. Additionally, the elevation of ultrasonic signals with increase in loading of Au NPs was noticed. These findings showed that existence of Au NPs may enhance impedance and increase the noticeable scattering, which results in high intensity scattering signals and subsequently ultrasonic scanning [39]. Therefore, the colloidal Au NPs indicated higher ability as an effective contrasting agent in ultrasonic scanning.

Later, the photo-thermal conversion performance and NIR absorption of the Au nanoplates obtained with 40% to 50% of sucrose layer after density gradient centrifugation of nanocolloid obtained after treating 400 μl of
reducing agent with 1mM gold salt at 45 °C temperature have been studied. As represented in figure 7, colloidal gold displayed a wide absorbance spectrum that ranged from NIR to visible regions. Furthermore, colloidal Au displayed increased absorbance at a long wavelength region ranging from 600 to 1000 nm, indicating that the colloidal Au might be appropriate for photo-thermal irradiation under NIR laser. Consequently, we further investigated the NIR irradiation-induced photo-thermal activity of Au nanoplates. The Au nanoplates were initially suspended in the PBS solution and later irradiated under NIR laser (808 nm) for 15 min at 1 W cm⁻² power density. The pure PBS served as control. Even though, the temperatures of samples elevated with extended duration of NIR irradiation, the temperature elevation ratio of individual samples were in the following order: control < Au/PDA (figure 7(A)). This order suggested that the Au NPs increased the photo-thermal conversion efficiency. Moreover, Au nanoplates showed a concentration-based photo-thermal property as displayed in figure 7(B). On increasing the concentration of Au nanoplates in the solution of PBS, temperature of the solution was elevated. Later, we further estimated the photo-thermal conversion performance (η) of the Au nanoplates. On turning the irradiation under NIR laser for 15 min, the Au nanoplates at a concentration of 200 μg ml⁻¹ increased by 13.5 °C. Later the temperature was decreased slowly to the initial point in 15 min. As demonstrated in figure 7(C), the η value of Au nanoplates was estimated as 19.88% [40-43].
Our findings demonstrated clearly that these Au nanoplates showed good photo-thermal characteristics, which renders them as a promising photo-thermal agent.

Before using the Au nanoplates in various applications, their cytotoxic effect was determined through MTT assay. As represented in figure 8, the Au nanoplates has no significant cytotoxic effect over the cells. When the Au nanoplates concentration was increased to 200 μg ml⁻¹, the cell viability was observed to be 88.9% after incubating for 24 h. This indicates that Au nanoplates had a minimal cytotoxic effect towards the living cells.

Based on the biocompatibility and superior photo-thermal effect of Au nanoplates, we further investigated their application in photo-thermal treatment to the in-vitro cancerous cells. In order to achieve this, Hep-G2 cell lines were chosen as a model for in-vitro studies. Incubation of Hep-G2 cells was carried out along with Au nanoplates for about 1 h at 37 °C and later irradiated under NIR laser (808 nm) for 10 min at 1 W cm⁻² power density. Prior to the irradiation, propidium iodide (PI)/ calcine acetoxyethyl ester (calcine-AM) were utilized for staining the dead (red) cells and live (green) cells correspondingly. Likewise, the viability of cells could be detected directly using confocal laser imaging microscopy. As observed in figure 9, the Hep-G2 cell lines displayed an intense green fluorescence of the calcine-AM dye prior to irradiation, demonstrating viability of major Hep-G2 cell lines, whereas these cells exhibited red fluorescence after irradiation, indicating the great

Figure 7. (A) Temperature raise of Au nanoplates at a concentration of 200 μg ml⁻¹, (B) at different concentrations. (C) photothermal response of Au nanoplates in aqueous solution.

Figure 8. In vitro cytotoxicity of colloidal Au nanoplates towards Hep-G2 cells after the incubation for 12 h and 24 h at 37 °C.
efficiency of Au nanoplates in killing the cancerous cells with the help of the near-infrared laser irradiation. Furthermore, we investigated quantitatively the effect of Au nanoplates concentration on photo-thermal therapy of cells. As showed in figure 10(A), concentration of Au nanoplates has a minimal effect on the viability of cells without irradiation. The viability of cells was predominantly decreased as displayed in figure 10(B). Increase in the microscopic spheres concentration results in decrease in the viability of cells. Particularly, 4.6% of Hep-G2 cell lines could only remain active at a 200 μg ml$^{-1}$ of concentration.

Conclusions

In conclusion, anisotropic Au NPs were fabricated utilizing an aqueous extract of carob as both stabilizing and reducing agent. Colloidal Au NPs showed very good contrast enrichment for ultrasound imaging, and also Au nanoplates were obtained by density gradient centrifugation can function as a NIR absorbing agent for efficient photothermal killing of Hep-G2 liver tumor cells in-vitro with negligible cytotoxicity to normal cells. Furthermore, the present approach suggests an innovative way for treating theranostic cancer.

Figure 9. Fluorescence microscopic images of liver cancer cell lines incubated with colloidal Au nanoplates for 1 h (A) before the laser irradiation and (B) after the laser irradiation. (808 nm, 1 W cm$^{-2}$ for 10 min). All the cells were undergone for staining with PI (red fluorescence, dead cells) and calcian AM (green fluorescence, live cells).

Figure 10. Cell viability of (A) Hep-G2 cells after incubating with increased Au nanoplates concentrations without laser irradiation (B) Hep-G2 cells administrated with various Au nanoplates concentrations after performing laser irradiation.
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