Extracellular Biosynthesis of Monodispersed Gold Nanoparticles, their Characterization, Cytotoxicity Assay, Biodistribution and Conjugation with the Anticancer Drug Doxorubicin

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Abstract

Nanomaterials can be synthesized by chemical, physical and the more recent biological routes. Biological routes have many advantages as these are eco-friendly, cheap, non-toxic, do not flocculate, and are well dispersed in water. Gold nanoparticles have shown significant promise as drug carriers, and have been used in the past for targeted drug delivery applications. In this report, we for the first time, have produced monodispersed gold nanoparticles through a green-synthesis approach employing thermophilic fungus *Humicola* spp. Out of several microorganisms screened, *Humicola* spp. was found to reduce the precursor solution (HAuCl₄) at just 50°C, and lead to the formation of gold nanoparticles of 18-24 nm size with good dispersity, uniform spherical morphology and high stability. These nanoparticles were completely characterized by different standard techniques. Their biocompatibility, cytotoxicity and radiolabeling studies were carried out. Lastly, these nanoparticles were also conjugated with the anticancer drug Doxorubicin, for targeted drug delivery to liver (hepatic) cancer.

Keywords: Gold nanoparticles; Biosynthesis; Monodispersed; *Humicola* spp.; Biodistribution

Introduction

A variety of chemotherapeutic agents such as taxol [1,2], 5-fluorouracil [3], dexamethasone [4], vinblastine, vincristine, etc. are being used to deal with a variety of cancers, which remains to be the most dreadful disease till date. However, the use of chemotherapy not only kills the cancer cells, but also targets the normal living cells. The therapeutic agents used, invade the affected sites and then carry on to freely move through the circulatory system, affecting various other areas of the body and causing major side effects [5]. Thus, the need of the hour is to focus research upon the targeted delivery of drugs, so that minimum amount of drug would be sufficient to cure the disease, thus preventing wastage of valuable drugs and sparing the normal cells in the process [5].

In recent years, the use of nanoparticles in targeted drug delivery systems has gained immense popularity, as these can improve the uptake of poorly soluble drugs by the diseased cells, thus reducing the amount of drug to be used, and are also very site-specific [6,7]. Free drug, without being conjugated to nanoparticles, manages to enter the tumor cell by diffusion, but is immediately effluxed by the P-glycoprotein receptors present in the tumor cell, and thus a very insignificant amount of drug actually enters the diseased cell. Keeping this under consideration, researchers synthesized a conjugate of nanoparticle, drug and a target molecule, wherein the nanoparticles act as carriers and assist in receptor mediated endocytosis to achieve target specific drug delivery. Once the drug is conjugated with nanoparticles, the dimensions of the conjugate become favorable for easy uptake by the tumor cells via endocytosis [8,9].

It is now well known that cancer cells overexpress various receptors like hFR (folate receptors), EGFR (Epidermal Growth Factor Receptor), EpCAM (Epithelial Cell Adhesion Molecule), Asialoglycoprotein [10-13], etc. These receptors are being used as potential drug targets in cancer therapy, where they are recognized by their specific target molecules associated with the nano-drug conjugate. For example,

asialoglycoprotein receptors are present in abundance in liver and more importantly, are overexpressed in liver cancer. Pullulan is a compound which is known to have specific affinity for the asialoglycoprotein receptors. Thus, pullulan is being used in liver targeted drug delivery system by covalently coupling it with the drug Doxorubicin, which is most widely used for the treatment of liver (hepatic) cancer. The problems arising in free administration of this drug were related to multidrug resistance and acute renal- and cardiotoxicity, as the drug is fatal to normal tissues. Thus, doxorubicin coupled with nanoparticles and pullulan has been successful in treating hepatic cancer, with no side-effects, as only a minimum amount of dosage is required, which results in high intracellular concentrations of the drug in specific tumor cells only, thereby preventing the loss of this expensive and valuable drug [14].

The nanoparticles so far being used in targeted drug delivery systems are being synthesized by physical and chemical routes [15]. The chemical routes are toxic and require extremes of temperature, while the physical ones are expensive and laborious. Unlike these, biological routes for the synthesis of nanoparticles have recently been developed and yield nanoparticles at room temperature and physiological pH, with properties such as high stability, water dispersal, fluorescence, etc., which are very difficult to achieve by chemical and physical routes [16-22]. Moreover, biologically synthesized nanoparticles are naturally

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protein capped, which prevents their flocculation, thus eliminating the need of any external capping agent, which are usually toxic as is seen in chemical routes. Thus, the biosynthesis of nanoparticles is in complete synchronization with the environment and should be further developed in order to obtain nanoparticles of variable sizes, shapes and chemical compositions, which will find major applications in targeted drug delivery systems [23,24]. Gold nanoparticles are bio-compatible and non-toxic [25], and have already been reported to have tremendous potential as carriers of drugs.

The aim of the present study was to synthesize monodispersed nanoparticles, to be used in targeted drug delivery for treatment of cancers, with properties such as bio-compatibility, high stability, non-toxicity etc., which would further aid in its conjugation to the drug and target molecule. Moreover, we made significant attempts in order to obtain the nanoparticles in the size range of 10-50 nm, so that these inorganic particles may not block the glomerulus of the kidneys and may easily pass through the urine within a short period of time. This is the first report of its kind where nanoparticles have been synthesized by using a biological route employing *Humicola* spp., so that they may further be developed to find applications in targeted drug delivery systems.

Materials and Methods

Materials

Chloroauric acid (HAuCl₄) was obtained from Sigma Aldrich; malt extract, yeast extract, glucose and peptone were obtained from HiMedia and used as-received.

Isolation of the fungus *Humicola* spp. (AAH-SCH-1)

The fungus *Humicola* spp. was isolated from self heating compost samples that were collected from Pune district of Maharashtra, India. The hyphal tip and single spore isolation methods were employed for the isolation of fungus [26]. The fungus was identified by cultural, morphological and molecular methods.

Fungal growth and maintenance

The isolated fungus was maintained on MGYB [malt extract (0.3%), glucose (1%), yeast extract (0.3%), and peptone (0.5%)] agar slants. Stock cultures were maintained by sub-culturing at monthly intervals. After growing the fungus at pH 9 and 50°C for 4 days, the slants were preserved at 15°C. From an actively growing stock culture, subcultures were made on fresh slants, and after 4 days of incubation at pH 9 and 50°C, the same were used as the starting material for biosynthesis of gold nanoparticles.

Extracellular biosynthesis of gold nanoparticles by *Humicola* spp.

For the biosynthesis of gold nanoparticles, the fungus was grown in 250 mL Erlenmeyer flasks, containing 100 mL of MGYB medium. Sterile 10% sodium carbonate was used to adjust the pH of the medium to 9. After the pH of the medium was adjusted, the culture was grown with continuous shaking on a rotary shaker (200 rpm) at 50°C for 96 hr. After 96 hr of fermentation, mycelia were separated from the culture broth by centrifugation (5000 rpm) at 20°C for 20 min, and then were washed thrice with sterile distilled water under sterile conditions. The harvested mycelial mass (20 g of wet mycelia) was then resuspended in 100 mL of aqueous solution of 1mM HAuCl₄ in 250 mL Erlenmeyer flasks, and the same was put onto a shaker at 50°C (200 rpm). The reaction was carried out for a period of 96 hr and fungal biomass was separated by filter-

paper to collect biomass and filtrate in sterile conditions. Periodically, aliquots of the reaction solution were removed and subjected to UV-vis spectroscopy, to check the formation of nanoparticles extracellularly.

Characterization of biosynthesized gold nanoparticles

The optical properties of gold nanoparticles were examined by using UV-vis spectroscopy. The measurements were performed on a Shimadzu dual-beam spectrophotometer (model UV-1601 PC), operated at a resolution of 1 nm. The nanoparticles were also characterized by using Transmission Electron Microscopy (TEM) and X-ray Diffraction (XRD) measurements. The biosynthesized nanoparticles solution was drop casted on a carbon coated copper grid and analyzed using JEOL 1200EX instrument, operated at a voltage of 80 kV. The Selected Area Electron Diffraction (SAED) analysis was carried out on the same grid. HRTEM images were scanned on FEI Technai G2 system, operated at an accelerating voltage of 300 kV at room temperature. The XRD pattern was obtained on a Philips PW 1830 instrument operating at 40 kV, and at a current of 30 mA with Cu K_α radiation (λ=1.5404 Å). X-ray photoelectron spectra were recorded on VG MicroTech ESCA 3000 instrument. FTIR spectra of gold nanoparticle solution was recorded on Perkin Elmer spectrum one B in diffuse reflectance (DRS) mode at a resolution of 2 cm⁻¹. Energy Dispersive Analysis of X-rays (EDAX) was carried out on a Leica Stereoscan-440 SEM equipped with a Phoenix EDAX attachment. EDAX spectra were recorded in the spot-profile mode by focusing the electron beam onto a region on the surface coated with nanoparticles.

Cell Viability Assay

The effect of nanoparticles on the proliferation of NIH3T3 mouse embryonic fibroblast cell line and MDA-MB-231 human breast carcinoma cell line was assessed by MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Briefly, the cells (2×10⁴ for NIH3T3 and 1×10⁵ for MDA-MB-231) grown in 96-well plates were treated with varying concentrations of nanoparticles (0-1000 µg/ml) for 24 hr at 37°C. The cells were further incubated with MTT (0.5 mg/ml) at 37°C for 3 hr, followed by addition of 200 µl of isopropanol. The color intensity was measured at 570 nm using an Enzyme Linked Immunosorbent Assay (ELISA) reader. The experiments were performed in triplicates. The cell viability was plotted as percent of control [27].

Radiolabelling and biodistribution studies

Radiolabelling of gold nanoparticles with Tc-99m: Tc-99m-gold nanoparticles were prepared by dissolving 10 mg of gold nanoparticles in 1 ml of distilled water, followed by the addition of 100 µg of SnCl₂·2H₂O and the pH was adjusted to 6.5. Approximately Tc-99m (2mCi) was added to the content, mixed and incubated for 10-15min. The percent radiolabel was determined by using Instant Thin Layer Chromatography (ITLC) method [28].

Radiochemical Purity (RCP): The radiochemical purity of Tc-99m with gold nanoparticle was estimated by Instant Thin Layer Chromatography (ITLC), using silica gel coated fibre sheets. ITLC was performed using 100% acetone and 0.9% saline as the mobile phase. A measured amount of 2-3 µl of the radiolabelled complex was applied at a point 1 cm from one end of an ITLC-SG strip, and allowed to run for approximately 10 cm. Amount of reduced/hydrolyzed Tc-99m was determined using pyridine: acetic acid: water (3:5:1.5 v/v) as mobile phase and ITLC as the stationary phase, and the radioactivity distribution over the strip was determined with a well counter (ECIL). Radiochemical Purity (RCP) was calculated as the fraction of

radioactivity that remained at the origin and was designated as percent (%) RCP.

Biodistribution of radiolabelled nanoparticles: Male Sprague Dawley rat weighing 180-220 gm was selected for evaluating the localization of the labelled complex. Tc99m-gold nanoparticles of 14.8 MBq (400 μ Ci) were administered through the penile vein of rat. The biodistribution studies of labelled gold nanoparticles were evaluated after 45 min post injection.

Conjugation of anticancer drug doxorubicin with gold nanoparticles: Biosynthesized gold nanoparticles were conjugated with anticancer drug doxorubicin. As mentioned earlier, biosynthesized nanomaterials are capped by a protein layer secreted by the fungus in the reaction mixture, during the process of biosynthesis. Free amino as well as carboxylic groups are present in the capped protein layer. The free carboxyl group present on these proteins was targeted to couple with the free amino group present on the doxorubicin. Carboxyl group on the nanoparticles surface was estimated as described by Ansary et al. [29]. The gold nanoparticles were conjugated to doxorubicin, with the help of free carboxyl by using the activator 1-Ethyl-3-(3-Dimethylaminopropyl)-Carbodiimide (EDC). The coupling reaction was carried out in 5 ml reaction volume of 50 mM MES/HEPES buffer, containing 250 μ g of gold nanoparticles and 250 μ g of doxorubicin was added to 5 mM of EDC. Conjugation was performed at 30°C. After stirring for 12 h at room temperature, the reaction mixture was concentrated under high vacuum and further purification of the doxorubicin-gold conjugate was done by HPLC (Waters), using C₁₈ symmetry reverse phase column and gradient elution was carried out using Acetonitrile:Water (5%:95%), at a flow rate of 0.5 ml/min. A dual wavelength recorder set at 253 nm and 530 nm was used to detect the doxorubicin and gold nanoparticles, respectively, from the column. Fluorescence measurements were carried out using a Perkin Elmer LS-50B spectrofluorimeter.

Results and Discussion

Owing to the popularity gained by gold nanoparticles in the past due to their biocompatible nature, we directed our efforts in order to fabricate monodispersed, uniform gold nanoparticles, which could carry appreciable amounts of uniform quantities of drugs, and also pass easily through the kidneys thus, preventing any sort of toxicity or discomfort to the body. Using a thermophilic fungus *Humicola* spp., we have successfully synthesized and completely characterized monodispersed gold nanoparticles of 18-24 nm size, which are non-agglomerating due to natural capping by proteins secreted by the fungus are spherical in size, with remarkable shelf-life and high stability. Biocompatibility and cytotoxicity (MTT) assays show that our gold nanoparticles are not toxic to both normal (NIH3T3 mouse embryonic fibroblast) and cancer (MDA-MB-231 human breast carcinoma) cell lines, upto concentrations of 50 μ g/ml and thus, can fearlessly be used for conjugation with drug in targeted drug delivery systems.

These gold nanoparticles were radiolabeled with Technetium-99m to check their biodistribution, and it was found that major amounts reach the liver of the organism (rat). Keeping this in mind, we conjugated these gold nanoparticles with the anticancer drug Doxorubicin, which is widely used for the treatment of liver (hepatic) cancer. The asialoglycoprotein receptors are overexpressed on hepatic cancer tissues. The nano-drug conjugates until now require pullulan, which is a target molecule specific to the asialoglycoprotein receptors to help in binding to the liver tissues, after which the nanoparticles can lead to the endocytosis of drug into cancerous cells. Free drug would

otherwise have been effluxed by the P-glycoproteins persistent in the cancer cells.

On the other hand, the nanoparticles which we have fabricated have a natural protein coat secreted by the fungus. Thus, these may directly bind to various multi-receptors overexpressed on cancer cells, terminating the requirement of any targeting agents like pullulan, etc. As our nanoparticles may bind to multiple receptors, they can be used in targeted drug delivery for a wide variety of cancers.

Isolation of *Humicola* spp. from self heating compost

A total of 10 (AAH-SHC-1 to AAH-SHC-10) cultures were isolated from self heating compost collected from Pune (Maharashtra, India). These cultures were brought to pure form, and were maintained on alkaline MGYP agar slants. Out of 10 cultures screened for gold nanoparticles production, only AAH-SHC-1 was found to produce extracellular monodispersed gold nanoparticles.

The fungus *Humicola* spp. grown on alkaline MGYP medium produces fast growing colonies. Colonies which initially appear off-white and felt-like, turns grey with time due to commencement of sporulation of the surface, as seen on the upper side of petriplate (Figure 1a). Subsequently, the reverse side of colony turns brown and the agar substratum stains a deep brown or wine color (Figure 1b). The vegetative mycelium is hyaline, septate, thin walled, highly branched, and is differentiated into conidia on short lateral branches, functioning as undistinguished conidiophores. Conidia are attached at bulging conidiogenous cells. Conidia produced were single, apically positioned, globose or subglobose, brown and single celled. Conidia are attached terminally on the conidiogenous cells (Figure 1c-1f). Based on the above cultural and morphological features, the isolated strain has been identified as *Humicola* spp. (*Thermomyces lanuginosus*) [26]. To confirm these findings, molecular approaches were used to amplify ITS region of ribosomal DNA of the fungus. The ITS sequence analysis and homology alignment of isolate AAH-SHC-1, using BLAST and Clustal-W programme, respectively, revealed similarity with genus *Humicola* spp. (*Thermomyces lanuginosus*) (Genebank accession number JN600618). The phylogenetic studies also indicate that the closest relative of the fungus is *Thermomyces lanuginosus* (*Humicola* spp.)

Figure 1: Morphological features of the fungus *Humicola* spp. The fungal strain was grown on alkaline MGYP agar plate for microscopic observations. Fungal growth: front a) and back b) side of alkaline MGYP agar plate, c and d) Mycelia, e) Attachment of the conidia on conidiophore, f) Conidia.

Biosynthesis and characterization of gold nanoparticles by *Humicola* spp.

The fungus *Humicola* spp., when incubated with aqueous solution of 1 mM HAuCl_4 at temperature 50°C and pH 9 for 96 hr under shaking condition on a rotary shaker (200 rpm), resulted in the production of extracellular, monodispersed and water dispersible gold nanoparticles. Figure 2a shows two conical flasks with the fungal biomass, before (left side) and after (right side) exposure to 1 mM HAuCl_4 solution at 50°C for 96 hr. The change in color from pale yellow to vivid ruby red after incubation with fungus could clearly be seen in Figure 2a, which demonstrates the formation of Au nanoparticles by the fungal biomass. This change of color appears due to the surface plasmon resonance of Au nanoparticles. Biosynthesis of gold nanoparticles was monitored by UV-Vis spectroscopy, and it was observed that the surface plasmon band which occurs at ~530 nm corresponds to the surface plasmon resonance of gold nanoparticles [30]. The fact that the surface plasmon band in the gold nanoparticle solution remains close to 530 nm throughout the reaction period indicates that the particles are dispersed in the aqueous solution, with no evidence for aggregation. These gold nanoparticles were separated from the fungal biomass by filtration after completion of the reaction; this demonstrates the extracellular nature of the produced nanoparticles (Figure 2b). An absorption band centered at 270 nm, which can be attributed to the presence of protein secreted

by the fungus in the reaction mixture, can be clearly seen in the UV-Vis spectrum of the biosynthesized gold nanoparticles. These nanoparticles are thus stabilized in solution by the capping agent secreted by the fungus, which is likely to be a protein or set of proteins, which prevent aggregation and make the nanoparticles stable and water dispersible.

Morphology and size distribution profile of the biosynthesized nanoparticles was obtained by TEM analysis. These gold nanoparticles are monodispersed (Figure 3a and 3b), sized in between 18–24 nm, with average size approximately 20 nm (Figure 3d). These particles are very well dispersed in solution, which is due to the capping agent likely to be a protein. Figure 3c shows the Selected Area Electron Diffraction (SAED) pattern obtained from gold nanoparticles shown in figure 3a. The Scherrer ring pattern, characteristic of face centered cubic (fcc) gold is clearly observed, showing that the structures seen in TEM images are nanocrystalline in nature. The HRTEM micrograph showed that the inter planar distance is 2.35 Å, which exactly matches with the (111) plane of the gold nanoparticles.

XRD analysis in figure 4a showed the crystalline nature of nanoparticles. The presence of four prominent Bragg's reflection, corresponding to the (111), (200), (220) and (311) orientations, agree with those reported for gold nanoparticles [22], and interestingly the broadness of these reflection demonstrates that the formed particles are in the nanoscale dimension. XPS measurement revealed the chemical nature of the nanoparticles, as seen in figure 4b. The Au 4f spectrum could be resolved into two peaks ($\text{Au } 4f_{7/2}$ and $4f_{5/2}$) due to the spin-orbit coupling, with binding energy of 84.2 and 87.6 eV, respectively. The core level Binding Energy (BE) was aligned with 285 eV (binding energy of carbon). These binding energies are characteristic of metallic gold and agree with those reported for gold nanoparticles.

The fungus secretes biomolecules/proteins in reaction mixture, thus, formation and stabilization of nanoparticles occurs. To support these findings, FTIR analysis was performed and the results in figure 5a suggest two bands appearing at 1654 and 1541 cm^{-1} , assigned to amide I and II of proteins respectively [21]. Moreover, there is a possibility of stabilization of the gold nanoparticles by surface binding protein, either by free amino group or by cysteine residues present in the protein. EDAX spectrum in figure 5b represents signals from Au (2.15 and 9.70 keV), together with C, O and Si. The secreted biomolecules contribute C and O signals in spectrum, whereas Si signal likely appears from the glass substrate used for EDAX analysis.

Cell viability assay of gold nanoparticles

The effect of gold nanoparticles on the cell viability of NIH3T3 mouse embryonic fibroblast cell line and MDA-MB-231 human breast carcinoma cell line was assessed by MTT assay. The cells were treated with varying concentrations of gold nanoparticles (50 $\mu\text{g}/\text{ml}$, 250 $\mu\text{g}/\text{ml}$, 5000 $\mu\text{g}/\text{ml}$ and 1000 $\mu\text{g}/\text{ml}$) for 24 hr. There is no significant difference on cell viability at concentration of 50 $\mu\text{g}/\text{ml}$. The cell viability was reduced in a dose-dependent manner, in both the cell lines and the significant cytotoxicity of the nanoparticle was observed from concentrations of 250 $\mu\text{g}/\text{ml}$ and above, for both the cell lines. The cell viability was reduced by 52% in case of NIH3T3 and 71.17% for MDA-MB-231 cell line at 1000 $\mu\text{g}/\text{ml}$ concentration (Figure 6a).

Radiolabelling and biodistribution studies

Complex formation study: On the basis of chromatographic analysis, the radiolabeling efficiency was found to be consistently more than 99%. The optimal labeling efficiency was obtained with 100 μg of stannous chloride (the concentration of $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ was varied from 50-100 μg), and at pH 6.5.

Biodistribution and gamma scintigraphic imaging of Tc99m-gold nanoparticles: Localization and biodistribution study of Tc99m-gold nanoparticle in normal healthy rat over time was determined by gamma camera imaging, as shown in figure 6b. The study clearly indicates the biodistribution of the complex (Tc99m-gold nanoparticle) in above mentioned rat. These gold nanoparticles reach out to the liver, heart and kidneys, and pass out through the urine within 45 minutes.

The secreted protein in the reaction mixture provides dual function of formation and stabilization of nanoparticles. The free carboxyl group present on these proteins was targeted to couple with the free amino group present on doxorubicin. After successful conjugation of gold nanoparticles with doxorubicin, the conjugate was further purified by HPLC, at dual wavelength 253 nm and 530 nm. The purified conjugate shows maximum absorbance at both wavelengths, with retention time of 25.8 min (Figure 7a). There are no significant changes in fluorescence spectrum for doxorubicin and gold nanoparticles-doxorubicin conjugate after excitation at 480 nm (Figure 7b). Decrease in the peak intensity of doxorubicin after conjugation with gold nanoparticles is due to slight quenching of doxorubicin by gold nanoparticles.

Conclusion

In conclusion, we have isolated and purified a thermophilic fungus *Humicola* spp., and identified it on the basis of cultural, morphological and molecular techniques. This fungus produces extracellular, monodispersed, protein capped, water dispersible and highly stable gold nanoparticles, when reacted with aqueous solution of HAuCl_4 . To the best of our knowledge, this green biosynthesis approach for the production of monodispersed, uniform gold nanoparticles of 18-24 nm size, employing *Humicola* spp. is the first report of its kind. Since these nanoparticles are capped by natural proteins, they may directly bind to multiple-receptors such as LHRH, EGFR and EpCAM, without the involvement of any targeting agents. Also, our nanoparticles may bind to integrins and VEGFs, and help in the development of a novel anti-angiogenesis strategy, for the treatment of a wide range of solid tumors. Hence, these nanoparticles which were until now considered only as a carrier in targeted drug delivery systems may now be used directly as a drug. These may fulfill the emerging need for cheaper drugs in abundant amounts, with no side effects to the patients and non-hazardous to the environment.

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