

Facile synthesis of biocompatible gold nanoparticles from *Vites vinefera* and its cellular internalization against HBL-100 cells

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Abstract The remarkable health benefits of the chemical cocktails occluded within *Vites vinefera* (grapes) have been broadly used as dietary supplements and as natural pharmaceuticals in the treatment of various diseases including human cancer. Current discovery demonstrates the rapid formation of gold nanoparticles with the phytochemicals present in grapes, which serve a dual role as synergistic reducing agents to reduce gold salts into gold nanoparticles and also as stabilizers to provide a robust coating on the gold nanoparticles in a single step. Furthermore, the grape-generated gold nanoparticles (GAuNPs), have demonstrated remarkable in vitro stability on specific functionalization with peptides (GSH) and thiol-containing compounds (lipoic acid) followed by the induction of cell-specific response. In addition, the grape-generated gold nanoparticles (GAuNPs, GSH-GAuNPs, LA-GAuNPs) have demonstrated remarkable affinity towards human breast cancer cells (HBL-100) in

the present study. These studies thus signified the cellular internalization of GAuNPs and its conjugates by transmission electron microscopy through endocytosis into cancer cells. Notably, at higher concentration of gold nanoparticles conjugate, there was an asymmetric accumulation of gold nanoparticles in the periphery of the cell nucleus of the HBL-100 cells which was confirmed by fluorescence microscopy. Other than gold salts, no “manmade” chemicals are used in this truly biogenic, green nanotechnological process which thereby paves the way for outstanding opening for their application in molecular imaging and cancer therapy.

Keywords Green synthesis · Grape-gold nanoparticles · GSH-grape-gold nanoparticles · Lipoic acid-grape-gold nanoparticles · HBL-100 cells · Phytochemicals

1 Introduction

Cancer induction, growth, and progression are multistep events, and numerous studies have demonstrated that various dietary agents interfere with these stages of cancer of various organ sites thereby sharing great promise in our conquest to control human malignancies. Based on these encouraging observations, research efforts from across the globe have focused on identifying, characterizing, and providing scientific basis to the efficacy of various dietary phytonutrients in an effort to develop effective strategy to block malignancies. Fruits and vegetables represent untapped reservoir of various nutritive and nonnutritive phytochemicals with potential cancer chemopreventive activity. From the clue of “French paradox,” polyphenolics from grapes (*Vites vinefera*) and red wines attracted the attention of scientists to define their chemical composition

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and their properties for human health (Urpi et al. 2009). The reported evidences of beneficial health effects of phenolic compounds (Chacona et al. 2009) include inhibiting some degenerative diseases, such as cardiovascular diseases (Olas et al. 2008) and certain types of cancers (God et al. 2007) reducing plasma oxidation stress and slowing aging (Meyer et al. 1997). The antioxidative characteristics of phenolic compounds are mainly ascribed to their free-radical scavenging and metal-chelating properties, as well as their effects on cell signaling pathways and on gene expression (Soobrattee et al. 2005). Although grape polyphenols are widely used for the prevention and treatment of cancer, its therapeutic effects are always limited by severe adverse effects, such as the stability of biological activity in tissue, bioavailability in vivo, etc. (Chen and Dou 2008). There has been reported that the biological activity of polyphenols might depend on the form of their administration (Henning et al. 2005). To overcome these disadvantages and improve chemotherapeutic activity, researchers have focused on the development of nano-sized drug carriers (Forrest and Kwon 2008).

Nanoparticles have a specific capacity for drug loading, have efficient photoluminescence ability, and are therefore important materials in the targeted delivery of imaging agents and anticancer drugs. (Horcajada et al. 2010; Sajja et al. 2009). Drug carriers made up of nanoparticles (NP) are able to overcome biological barriers, accumulate preferentially in tumors, and specifically recognize single cancer cells for detection and treatment. As the nanorevolution in the realms of medical and technological applications unfolds, it is imperative to develop environmentally benign and biologically friendly green chemical processes (Studer et al. 2010). The utility of plant-based phytochemicals in the overall synthesis and the architecture of nanoparticles and various nanoparticle-embedded products are highly attractive as they bring an important symbiosis between natural or plant sciences and nanotechnology. Continuous demand for new anticancer drugs has stimulated chemotherapeutic research based on the use of metals since potential drugs developed in this way may be less toxic and more prone to exhibit antiproliferative activity against tumors (Shankar et al. 2004). The naturally grown plants which occlude phytochemicals may serve as long-lasting and environmentally benign reservoirs for the production of a myriad of gold nanoparticles. Recently, synthesis of Au and Ag nanoparticles using extracts of *Cinnamomum camphora* leaf (Huang et al. 2007), phyllanthin (Kasthuri et al. 2009), and Alfalfa sprouts (Gardea et al. 2003b) as a reducing and capping agent has been reported.

The utilities of NP strongly depend upon their physicochemical characteristics and their interaction with various surface moieties. Nanoparticles have to be surface modified

to make them stable, biodegradable, biocompatible, and with high specificity for preparation of bioconjugate and some functional groups, such as cyano, thiol (Yonezawa et al. 2006), glutathione (Basu and Pal 2007), and amino groups (Subramaniam et al. 2005; Aslam et al. 2004) which are known to have high affinity for gold can be used as capping agents for gold nanoparticles. Such systems can limit the release of encapsulated materials more effectively (Chen et al. 2007). A major advantage of using these short peptide motifs is that they home in to the tumor vasculature, which is less dependent on the variability of receptors expressed directly on the tumor cell surface (Ruoslahti 2000). Hence, by incorporating appropriate peptides and thiol-rich molecules into a linkage between carrier and drug, it is possible to develop rapid release without appreciably contributing to drug loss during circulation in the central blood compartment.

While the tremendous health benefits of chemical cocktails present within grapes is beyond doubt, the actual applications of the chemical reduction power of the myriad of chemicals present in herbs and spices is still in infancy. Therefore, we investigated the synergistic potentials of polyphenols, flavonoids, catechins, and various phytochemicals present grape extract for the reduction reactions of gold salts to produce AuNPs which have potential applications in the diagnosis and therapy of various deadly diseases including cancer. In this study, we synthesized a kind of novel gold nanoparticles conjugates using glutathione and lipoic acid as encapsulant materials for entrapment of grape polyphenols. The morphology, structure, and characteristic of the glutathione capped grape gold nanoparticles and lipoic acid capped grape gold nanoparticles were confirmed by UV–spectroscopy, scanning electron microscopy (SEM), transmission electron microscopy (TEM), and Fourier transform infrared (FTIR). We also extended our communication to study the application of gold nanoparticles as carriers of grape polyphenols by determining its cytotoxicity in vitro against human breast cancer (HBL-100) cell lines and such studies are missing up to date to the best of our knowledge.

2 Experimental

2.1 Synthesis of GAuNPs, GSH-GAuNPs, and LA-GAuNPs

Grapes were washed with distill water to remove any traces of contaminants. Grapes were cut into small pieces and added into a conical flask containing 100 ml of Millipore water and boiled for 10 min and filtered using Whatmann filter paper. To 10 ml of grape extracts, 10 ml of 1-mM aurochlorate was

added and heated to 75°C for 10 min. The color of the mixture changes from pale purple to dark purple. To the grape-gold colloid solution (25 mL), glutathione (20 mg) was added, and the mixture was stirred for 10–15 min. After the stirring was completed, the mixture was centrifuged at 4,500 rpm to separate the capped gold nanoparticles. The pellet obtained was resuspended in 1 mL of phosphate buffer (pH 7). A 600- μ mol portion of α -lipoic acid in 10 mL of NaOH 0.5 M solution was added to 25 mL of freshly prepared citrate-capped Au NPs under stirring at room temperature (20–23°C). After 24 h, AuNPs capped with dihydrolipoic acid (DHLA), obtained from α -lipoic acid reduction, were dialyzed against phosphate-buffered saline (PBS) for 48 h using a 10-kDa cut-off dialysis bag (Interchim®, France). The dialysis medium was changed once to fresh PBS after 24 h. The resulting Au at DHLANPs solution was stored in the dark at 4°C for a maximum period of 2 months. The gold nanoparticles thus formed were separated immediately using a 5- μ m filter and were characterized by UV–vis absorption spectroscopy FTIR and SEM analysis.

2.2 Characterization of GAuNPs, GSH-GAuNPs, and LA-GAuNPs

The stability and the identity of the grape-initiated gold nanoparticles (GAuNPs) were measured by recording UV absorbance after 30 min. The Plasmon resonance band at ~535 nm confirmed the retention of nanoparticulates in all the above mixtures. X-ray diffraction pattern of dry nanoparticles powder was obtained using Siemens D 5005 X-ray diffractometer with CuK α radiation (λ =0.1542 nm). The FTIR spectra were obtained on a Nicolet 5700 FTIR instrument with the sample as KBr pellets. The morphology of the nanoparticles was analyzed using the high-resolution images obtained with a JEOL 3010 transmission electron microscope and scanning electron microscope.

2.3 In vitro studies: HBL-100 cell culture and maintenance

The cells were maintained in Dulbecco's modified eagle's medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 12.5% horse serum, 2.5% fetal bovine serum, 50 U/mL penicillin, and 5 mg/mL streptomycin—at an incubator setting of 5% CO₂ and 37°C. All the experiments were carried out 24–48 h after cells were seeded. The cells were routinely harvested by trypsinization 0.25% when the cells approached sub-confluent stage and were placed on 25-cm culture flasks split into 1:6. Human breast cancer cell (HBL-100) cell line was purchased from NCCS Pune. GAuNPs was dissolved in PBS followed by ultra-sonification for five mins, and concentrations of 8 mg/10 ml were used for the various experiment.

2.4 Assay of in vitro cytotoxicity of GAuNPs, GSH-GAuNPs, and LA-GAuNPs in HBL-100 cells

The 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyltetrazolium bromide blue-indicator dye (MTT)-based assay is a simple nonradioactive colorimetric assay to measure cell cytotoxicity, proliferation, or viability. MTT reduction was examined according to the methods of Mosmann (1983), with modification. HBL-100 cells were used for the analysis of cytotoxicity in vitro. The cells (1×10^6 cells/mL) were placed into 96-well tissue-culture plates and incubated at 37°C. After 24 h, cells were treated with three different concentrations of GAuNPs, glutathione-capped grape gold nanoparticles (GSH-GAuNPs), and lipoic acid-capped-grape gold nanoparticles (LA-GAuNPs) (10, 50, 100, and 150 μ g/mL, respectively). Untreated cells were used as controls. Plates were incubated in a humidified 5% CO₂ balanced-air incubator at 37°C for 24, 48, and 72 h, respectively. Then, 10 μ L of 5 mg/mL MTT solution was added to each well, and the plates were incubated for another 4 h, and then the medium was discarded. Dimethyl sulfoxide (100 μ L) was added to each well, and the solution was vigorously mixed to dissolve tetrazolium dye. The absorbance of each well was measured by enzyme-linked immunosorbent assay reader (BioTek; Austria) at a test wavelength of 570 nm. Percentages of surviving cells to untreated controls were calculated by using the formula as % viability=[(At/As) \times 100] %, where At and As indicate the absorbance of the sample and control, respectively.

2.5 Determination of the integrity of cell membranes: the LDH leakage

The lactate dehydrogenase (LDH) assay is used to evaluate cell-membrane integrity because the release of this large (9–160 kDa) enzyme from the cytoplasmic compartment to the supernatant of cells is indicative of membrane damage. Based on the reduction of NAD by the action of LDH to form a tetrazolium dye, the amount of LDH was measured spectrophotometrically at 492 nm. The background absorbance measured at 660 nm was subtracted from the reading at 492 nm. After cells were exposed to different concentrations, GAuNPs, GSH-GAuNPs, and LA-GAuNPs (10, 50, 100, and 150 μ g/mL, respectively) for 48 h, the medium was collected, and the amount of LDH release into the medium and the total LDH were determined, respectively.

$$\text{LDH}(\%) = \frac{A_{\text{medium}}(492\text{nm} - 660\text{nm}) \times 100}{A_{\text{total}}(492\text{nm} - 660\text{nm})}$$

2.6 TEM analysis of surface characteristics of HBL-100 cells during internalization of GAuNPs, GSH-GAuNPs, and LA-GAuNPs

HBL-100 cells with a density of 1×10^6 cells/mL were grown in DMEM (Invitrogen) supplemented with 12.5% horse serum, 2.5% fetal bovine serum, 50 U/mL penicillin, and 5 mg/mL streptomycin—at an incubator setting of 5% CO₂ and 37°C. After trypsinization and centrifugation, cell pellets were resuspended in Hank's balanced salt solution for the exposure of 100 µg/mL of GAuNP's and further incubated for 24 h at 37°C. The medium was then aspirated, and the cell layer was rinsed three times with growth medium to remove any traces of uninternalized GAuNPs. About 0.5 mL of 0.1 M Trypsin-EDTA solution was added to each well to detach the cell layer from the plastic. Detached cells were dispersed in 4 mL of complete growth medium and gently pipette out of the well. The cell suspension was transferred into a centrifuge tube and centrifuged at approximately $125 \times g$ for 5 min. Supernatant was discarded, and cell pellet was embedded in paraffin, sectioned, and examined under transmission electron microscopy.

2.7 Assay of apoptosis induced by GAuNPs, GSH-GAuNPs, and LA-GAuNPs in HBL-100 cells DAPI staining assay

The fluorescent dye 4',6-diamidino-2-phenylindole (DAPI) was used to detect the nuclear fragmentation that is a characteristic of apoptotic cells. PC12 cells (5×10^3 cells/well in 12-well plates) were incubated at 37°C with GSH-GAuNPs and LA-GAuNPs (50 or 100 µg/mL) and then washed with PBS and fixed with 70% ethanol for 20 min. The fixed cells were washed with PBS and stained with the DNA-specific fluorochrome DAPI (1 µg/mL). Following 10 min of incubation, the cells were washed with PBS, and the plates were observed under a fluorescence microscope (Olympus Optical, Tokyo, Japan).

2.8 DNA fragmentation analysis

An apoptotic cell is characterized by its unique ladder of nucleotide fragments in DNA-agarose gel electrophoresis. HBL cells (5×10^3 cells/well in a 12-well plates) were lysed with lysis buffer (10 mM Tris-HCl, 5 mM EDTA, 200 mM NaCl, 0.2% SDS, and incubated at 60°C for 5 min); the sample was digested with 2.5 µL of proteinase K (more than $3 \mu\text{L}^{-1}$; Sigma) and 5 µL of RNase A ($1 \mu\text{L}^{-1}$) (Fermentas) and was further incubated at 50°C for 1 h. After centrifugation at $1,000 \times g$ for 15 min, the supernatants were extracted with an equal volume of phenol, chloroform, and isoamyl alcohol. The DNA was then mixed with 4 M

sodium chloride and 100% ethanol and stored at -70°C overnight. Each DNA sample was loaded onto a 1.8% Tris-boric acid-EDTA agarose gel and electrophoresed at 100 V for 30 min.

3 Results and discussion

The chemical inertness of gold has been used internally in humans for the past 50 years, from its use in teeth to implants to radioactive gold used in cancer treatment. The primary rationale for selecting gold nanoparticles is their biocompatibility, very high surface area (large amount of drugs can be loaded), ease of characterization, and surface modification (i.e., organic molecules such as drugs, peptides, antibodies, etc. can be easily conjugated to gold nanoparticles). Choosing the right ligand for nanoparticle synthesis is key in forming AuNPs with desirable properties. In this study, we chose the naturally occurring peptide ligand, GSH, and thiol-rich ligand, lipoic acid, because of the favorable properties such as the presence of thiol, carboxylic acid and amino groups, water solubility at relevant biological pH, biological compatibility, and ease of functionalization, thereby making water-soluble nanoparticles for biological applications.

The presence of carboxylic acid and amino groups on GSH ligand complexed with Au (I) has the potential advantage of being a pH-sensitive compound, which can adopt different conformational states and sizes depending on the pH of solution. Whetten et al. synthesized the Au NPs via sodium borohydride reduction of the mixture of tetrachloroauric acid and GSH in methanol–water (2:3) and obtained gold nanoparticles with most abundant component having a diameter of 0.9 nm. We have initiated to design such nanoparticles on the direct intervention of phytochemicals for the production of gold nanoparticles which may provide a new method and an important opportunity for improvement in breast cancer treatments, the most common form of cancer in women worldwide. Such nanoparticles coupled with the specific targeting agents have the ability to track and eliminate breast cancer cells. In this paper, we present grape-gold-based nanoparticles for breast cancer diagnosis and treatment for which we used human breast lymphoma cells.

3.1 Synthesis of the biocompatible nano gold from *V. vinefera*

Figure 1, a–c depicts the change in color of GAuNPs before and after capping. The color of the grape extract changed pink to wine red (1a), to blue upon addition of glutathione (1b), and to dark blue after capping with lipoic acid (1c). Synthetic conditions have been optimized for

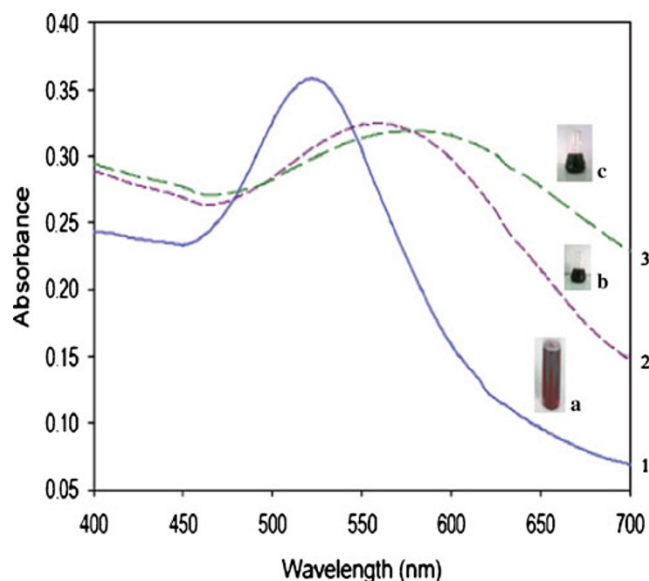


Fig. 1 UV-visible spectroscopic analysis of 1 GAuNPs, 2 GSH, and 3 lipoic acid-stabilized GAuNPs 1 the blue line indicates the peak at a wave length of 500–550 nm fir GAuNPs, 2 the dashed line indicates the peak at a wave length of 550–600 nm for GSH-GAuNPs, 3 the green dotted line indicates the peak at a wave length of 600–650 nm for LA-GAuNPs. The beakers *a*, *b*, and *c* imply the color change after the addition of auro chloric acid to grape extract, GSH, and lipoic acid *a* AUNPs synthesized from grape extract, *b* GAuNPs stabilized and capped with glutathione, *c* GAuNPs stabilized and capped with lipoic acid

the quantitative large-scale conversions of HAuCl_4 to the corresponding AuNPs using grape extract. The foremost phytochemicals present in grape extract consist of water-soluble catechins (catechin, epicatechin, epicatechin gallate, epigallocatechin, epigallocatechin gallate, etc.) and thearubigins which are oligomers of catechins of unknown structure. As the generation of AuNPs using grape extract involves aqueous media, the water-soluble phytochemicals of grape extract may be playing a major role in the overall reduction reactions of HAuCl_4 . Interestingly, the systematic investigation of Satish et al. (2009) granted the role of polyphenols (catechins and

theaflavins) for the generation and stabilization of AuNPs through independent experiments. Normally, thiols containing organic compounds are employed to stabilize AuNPs against agglomeration and strong interaction (Brust et al. 1994). It has been shown that all the catechins act as outstanding reducing agents to reduce the Au (III) to the corresponding gold nanoparticles (Satish et al. 2009). The nanoparticles thus generated were coated with GSH and lipoic acid stabilizing agent and showed significant stability. These experiments have decidedly confirmed that catechin and epigallocatechin gallate hand out dual roles as reduction and stabilizing agents, whereas epigallocatechin and epicatechin can be used only for the reduction of gold salts and require GSH and lipoic acid as an external stabilizing mediator. Thus, our study provided an evidence for the better stability by GSH and lipoic acid when coupled to biomolecules to obtain new delivery platforms (Roux et al. 2005).

3.2 UV-visible spectroscopy studies

The gold nanoparticles synthesized from grape extract were relatively monodisperse in colloidal solution, which was confirmed by a single peak in the absorbance spectra (Fig. 1). Gold nanoparticles exhibit some special optical properties such as Plasmon resonance, which is primarily a quantum phenomenon operative on the nanoscale. Absorption measurements indicated that the Plasmon resonance wavelength of GAuNPs was 535 nm. As shown in Fig. 1, the peaks 2 and 3 are shifted towards the higher wavelength after capping with glutathione (540–580 nm) and lipoic acid (560–620 nm), respectively. The λ_{max} shift in the absorbance spectra was mainly due to the surface modification of the gold nanoparticles (Figs. 2, 3). Fascinatingly, the surface Plasmon resonance, the major cause for the absorption, may be affected by surface modification with covalent coupling thereby increasing their size (Sheetal et al. 2008). The covalent coupling may also be due to the protective coating of the organic molecule, glutathione—a tripeptide (glutamic acid, cysteine, and glycine), which has

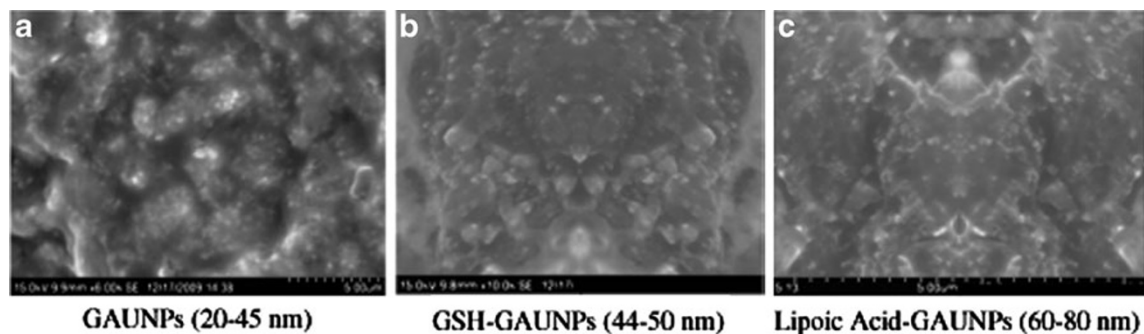


Fig. 2 SEM images of gold nanoparticles obtained using *a* GAuNPs, *b* GSH-GAuNPs, *c* lipoic acid-GAuNPs

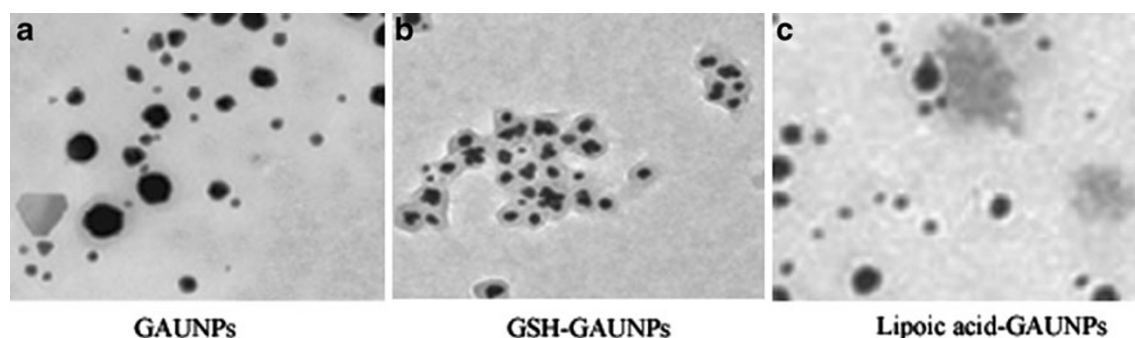


Fig. 3 TEM images of gold nanoparticles obtained using **a** GAuNPs, **b** GSH-GAuNPs, **c** lipoic acid-GAuNPs

many binding points for the gold nanoparticles (two carboxylic groups, one thiol group, and three amino groups). More effectively, the thiol group is involved in the attachment with the AuNP. In the case of lipoic acid-capped nanoparticles, the disulfides are reduced by polyphenols to two thiol groups ($-S-S- \rightarrow -SH+-SH-$), which are involved in the binding of lipoic acid to gold nanoparticles. And the coupling can be extended via either the carboxylic or the amino groups (of glutathione/lipoic acid). It is conceivable that the cocktail of phytochemicals in grapes along with nontoxic antioxidants lipoic acid and GSH are acting synergistically in stabilizing gold nanoparticles from any agglomeration in solution. A similar study by Gautham et al. (2009) created borohydride-reduced AuNPs capped with glutathione and lipoic acid that was covalently linked to horse radish peroxidase, provided some insight in the application of biosensors as tools for diagnostics.

3.3 Size, morphology, and stability properties

The techniques for the characterization of nanoparticle size and morphology are SEM (Bilati et al. 2005) and TEM

(Teixeira et al. 2005). Figures 4 and 5 indicate the size and morphology as observed under SEM and TEM on AuNPs synthesized using grapes as spherical in shape within the size range of 20–45 nm (Figs. 3a and 4a). Investigations on experiments using commercially available catechins have unambiguously confirmed that catechins are excellent reducing and stabilizing agents to reduce Au (III) to the corresponding gold nanoparticles (Satish et al. 2009). In addition, the effect of pH (Gardea et al. 2003b), time (Huang et al. 2007), temperature (Groning et al. 2004), and measurement of charges (Shankar et al. 2004a) may also play a major role in the determination of shape and size of nanoparticles. However, the presence of some phytochemicals (Brust et al. 1994) might render a minimum stability by failing to provide effective coating to shield the nanoparticles from agglomeration studies to GAuNPs. In order to capitalize on the reduction powers of such phytochemicals, we have utilized GSH, a tripeptide and lipoic acid, an antioxidant as naturally available stabilizing agent in our reactions. Thus, GSH-GAuNPs (Figs. 3b and 4b) and LA-GAuNPs (Figs. 3c and 4c) sizes as measured by SEM and TEM are in good agreement and are in the range of 40–80 nm suggesting that thiols and peptides are capped on grape phytochemically reduced gold nanoparticles. Such size distribution analysis of capped and non-capped GAuNPs confirms that particles are well dispersed. This extra stability rendered by GSH and lipoic acid-capped AUNP arises due to the chemical inertness by the complete coverage of the gold core by GSH ligands. Gold nanoparticles can be stabilized by anionic ligands such as carboxylic acid derivatives like citrate, tartrate, and lipoic acid (Peng et al. 2007). Earlier studies showed that glutathione used for capping gold quantum clusters (AUN-SG-m) ($-SG-$, glutathione thiolate) is one such group of compounds which has been well known for the stability of the AUNPs synthesized chemically (Habeeb and Pradeep 2007). Moreover, he confirmed in his results that the bigger clusters, $n > 25$, can be converted into AU25SG18 by adding excess GSH. In addition, the six free electrons present in the conduction band of nanoparticulate gold make

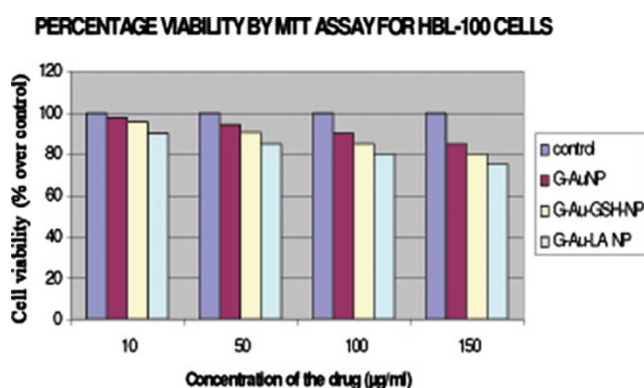


Fig. 4 Dose-dependent cytotoxicity of GAuNPs, GSH-GAuNPs, and LA-GAuNPs in HBL-100-cells after 24 h of exposure to different concentrations GAuNPs, GSH-GAuNPs, and LA-GAuNPs (10, 50, 100, and 150 µg/mL, respectively) using MTT assay. Values are expressed as percent over control

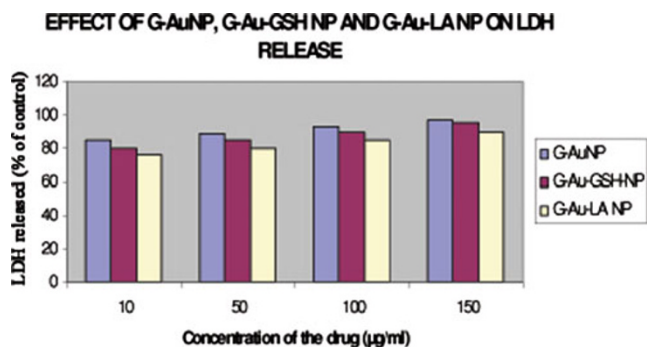


Fig. 5 LDH release LDH leakage after HBL-100 cells exposed to different concentrations GAuNPs, GSH-GAuNPs, and LA-GAuNPs (10, 50, 100, and 150 µg/mL, respectively)

them potential candidates to bind with thiols and amines. Therefore, by changing the size and shape of AuNPs, the SPB and scattering may be tuned for application in cellular imaging, drug delivery, and therapy.

3.4 In vitro uptake and localization characterization

Characterization of in vitro nanoparticle uptake and localization is intrinsically linked to cytotoxicological studies because uptake provides evidence of nanoparticle–cell

interaction, wherein the delicate intracellular machinery is exposed to nanoparticles. Compared to in vivo studies, in vitro studies benefit from being faster, lower cost, allowing greater control, and minimizing ethical concerns by reducing the number of laboratory animals required for testing. The most commonly used in vitro assessment techniques generally evaluate either viability (live/dead ratio) or toxicity mechanism. Herein, the major viability-based assays are organized into the categories of proliferation, necrosis, or apoptosis DNA damage detection techniques. In our present communication, the cytotoxicity of GAuNPs, GSH-GAuNPs, and LA-GAuNPs under in vitro conditions in HBL-100 cells was examined using two cytotoxicity markers, including MTT reduction, and LDH leakage is used to study the effects of gold nanoparticle conjugates on cellular viability. To check the cytotoxicity of cells treated with GAuNPs (10–150 µL), GSH-GAuNPs (10–150 µL), and LA-GAuNPs (10–150 µL) for 24 h, and a number of viable cells were enumerated by colorimetric MTT assay. After 24 h of post-treatment, HBL-100 cells showed excellent viability even up to 150 µL of GAuNPs, GSH-GAuNPs, and LA-GAuNPs (Fig. 5). Results of MTT assays clearly revealed the cytotoxic effect of GAuNPs in a dose-dependent manner for HBL-100 cell lines and LA-GAuNPs exerted slightly better cytotoxic effect towards HBL-100 cells in comparison

Fig. 6 Phase contrast microscopic pictures of HBL-100 cells **a** untreated and treated **b** with 500 µM GAuNPs, **c** GSH-GAuNPs, and **d** lipoic acid-GAuNPs

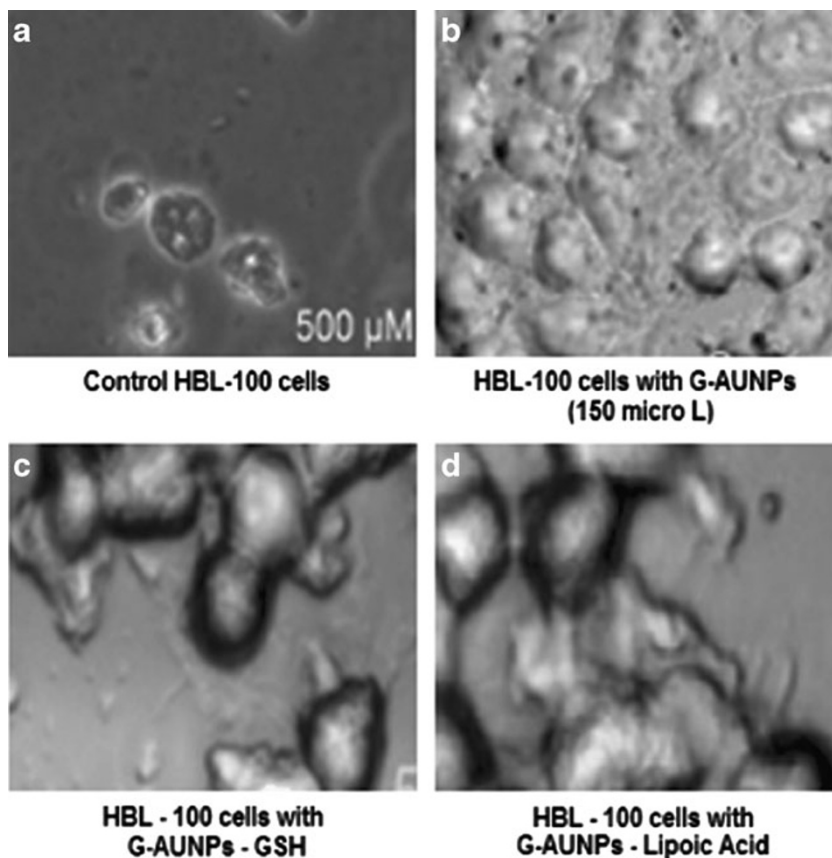
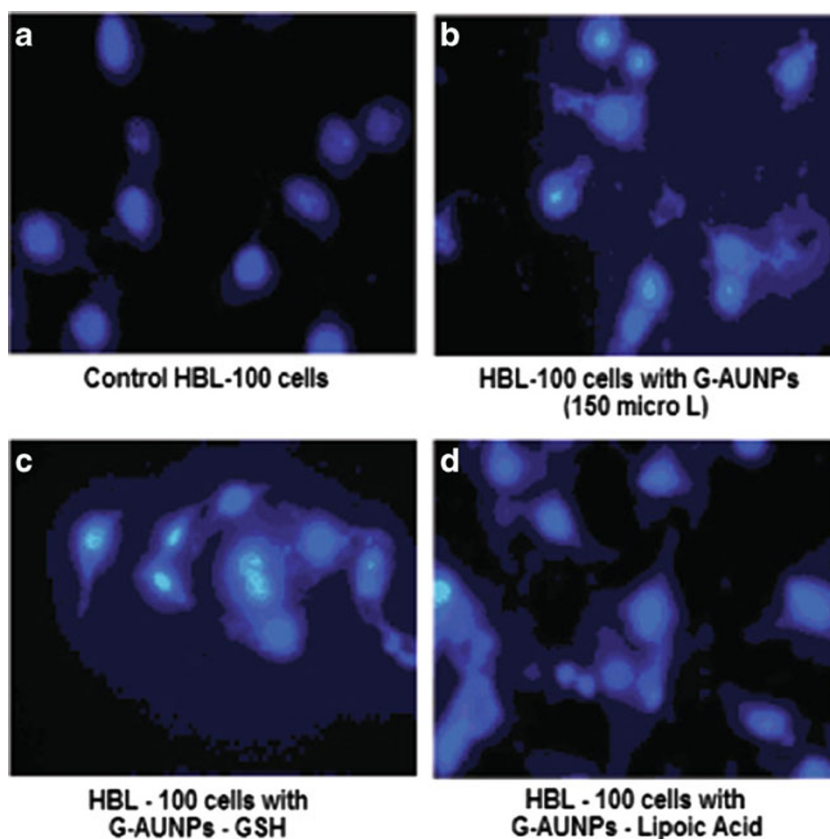


Fig. 7 Fluroscent microscopic pictures of HBL-100 cells **a** untreated and treated **b** with 500 μ M GAuNPs, **c** GSH-GAuNPs, and **d** liopic acid-GAuNPs



to GSH-GAuNPs. Although GAuNPs also showed cytotoxicity towards cancer cells, the effect was much less compared to GSH-GAuNPs and LA-GAuNPs. Treatment with 150 μ L or higher doses of GAuNPs and GSH-GAuNPs and LA-GAuNPs to HBL-100 cell increased LDH leakage into

culture media (Fig. 6), signifying an AUNP-induced compromise of plasma membrane integrity, and the IC₅₀ of AgNPs was 150 μ L. Henceforth, the release of LDH (Fig. 6) in our study is in agreement with the excellent viability of the cells treated with GAuNPs and GSH-GAuNPs and LA-GAuNPs as proven by MTT assay. It is also important to recognize that a vast majority of gold (I) and gold (III) compounds exhibit varying degrees of cytotoxicity to a variety of cells (Basset et al. 2003; Hamer 2007). GAuNPs pretreatment at a concentration of 150 μ L reduced the LDH leakage to a minimum, and this concentration is used in subsequent studies.

3.5 Induction of apoptosis by GAuNPs, GSH-GAuNPs, and LA-GAuNPs in HBL-100 cells

Surface reactivity, chemical composition, and large specific surface area have been deemed important properties in nanoparticle-mediated toxicity (Wallace et al. 2007). HBL-100 cells, after treatment with nanometer-sized GAuNPs, GSH-GAuNPs, and LA-GAuNPs, exhibited ultra structure and biochemical features that are characteristic of apoptosis, as shown by chromatin condensation and inter nucleosomal DNA fragmentation. The phase-contrast microscopic pictures of altered morphology of HBL-100 cells which is characteristic of apoptotic cell stage when

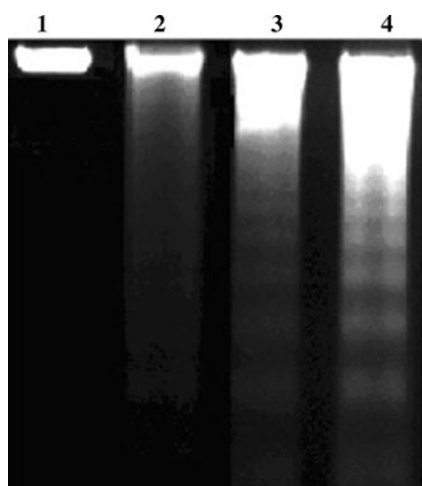
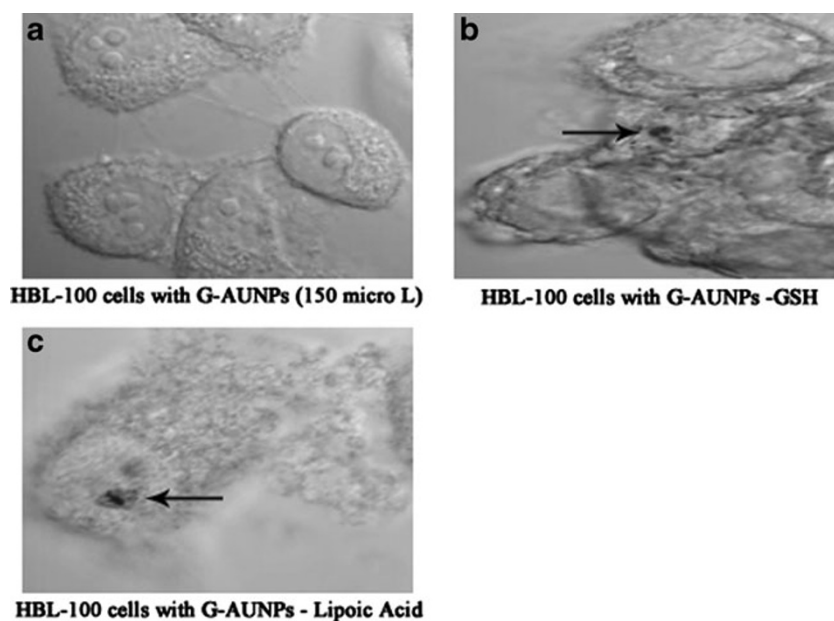


Fig. 8 HBL-100 cells were treated for 24 h with 500 μ M untreated cells (lane 1) GAuNPs (lane 2) GSH-GAuNPs (lane 3), and LA-GAuNPs (lane 4) for inter-nucleosomal DNA fragmentation analyzed by electrophoresis on a 1.6% Tris-Borate-EDTA agarose gel electrophoresis

Fig. 9 Confocal microscopic image of control HBL-100 cells (**a**); HBL-100 cells treated with 500 μ M of GSH-GAuNPs (**b**) arrows indicate agglomerated GAuNPs; **c** HBL-100 cells treated with 500 μ M of LA-GAuNPs for 14 h. Arrows indicate agglomerated AuNPs in a membrane-bound vesicle (probably perinuclear lysosome)

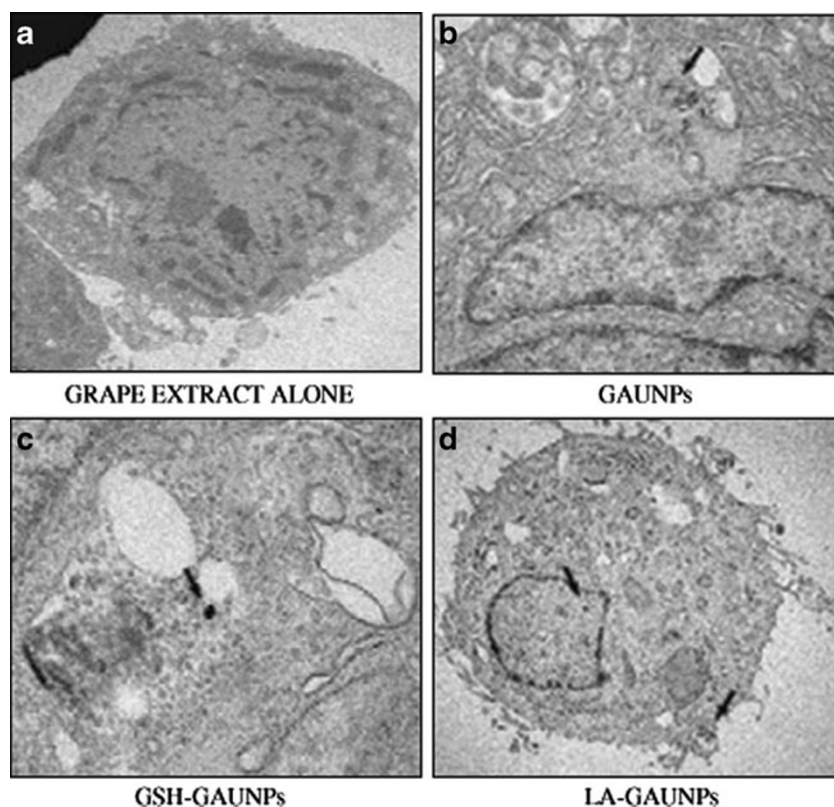


treated with GSH-GAuNPs, and LA-GAuNPs (40–80 nm) are shown in Fig. 7a–d. In addition, the nuclear fragmentation, a hallmark of cellular apoptosis, was clearly exhibited by fluorescent microscopic studies after DAPI staining of untreated and GSH-GAuNPs and LA-GAuNPs (40–80 nm)-treated HBL cells (Fig. 8a–d). A minimum of 200 cells were counted and classified as follows: (1) live

cells (normal nuclei: blue chromatin with organized structure); (2) stressed cells (bright-blue chromatin, which is highly condensed, margined, or fragmented).

Metal complexes have been extensively studied for their nuclease-like activity using the redox properties of the metal and dioxygen to produce reactive oxygen species to promote DNA cleavage by direct strand scission or base

Fig. 10 a–d TEM images of HBL-100 cells showing the internalization of GAuNPs depicting the arrival of a GAuNPs at the cells membrane, binding of the nanoparticles to surface receptors, membrane wrapping of the nanoparticles, and finally internalization into the cell nucleus



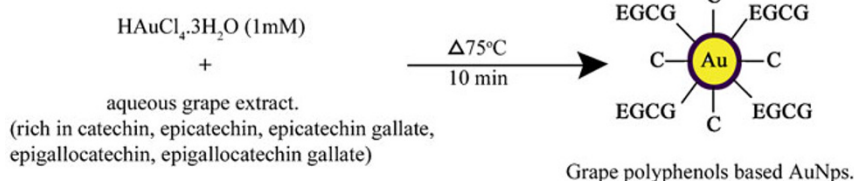
modification in cancer cells (Burrows and Muller 1998). A more current development in this area has been testing of metal nanoparticles such as gold and platinum nanoparticles for DNA degradation studies (Shen et al. 2009; López et al. 2010). Use of metal nanoparticles can be in particular advantageous in generating singlet oxygen [42 and 43] (Lipovsky et al. 2009; Portolés et al. 2010)

A recent report by Geddes and coworkers demonstrated that the presence of metal nanoparticles can enhance singlet oxygen generation (Zhang et al. 2008). The enhanced electromagnetic fields in proximity to metal nanoparticles are the basis for the increased absorption predicts the extent of absorption and the relative increase in singlet oxygen generation from photosensitizers (Barber et al. 1983; Yang et al. 1995)

A very recent study by Midander and coworkers reported the effect of metal nanoparticles inducing single-stranded breaks in the human lung cells (Midander et al. 2009). Previous studies illustrated the potent cytotoxic, genotoxic, and toxicological activities of nanoparticles in vivo (Midander et al. 2009; Chen et al. 2006) and in cultured cancer cell lines (Sengupta et al. 2007). However, a methodical study using GAuNPs on DNA degradation and cytotoxicity towards breast cancer cells are missing up to date to the finest of our information. Thus, 40–80-nm-sized GSH-GAuNPs and LA-GAuNPs (lanes 2 and 3) treated HBL-100 cells in our study displayed a ladder pattern of inter-nucleosomal DNA fragmentation on TBE-agarose gel electrophoresis in DNA ladder assay (Studer et al. 2010) as revealed in

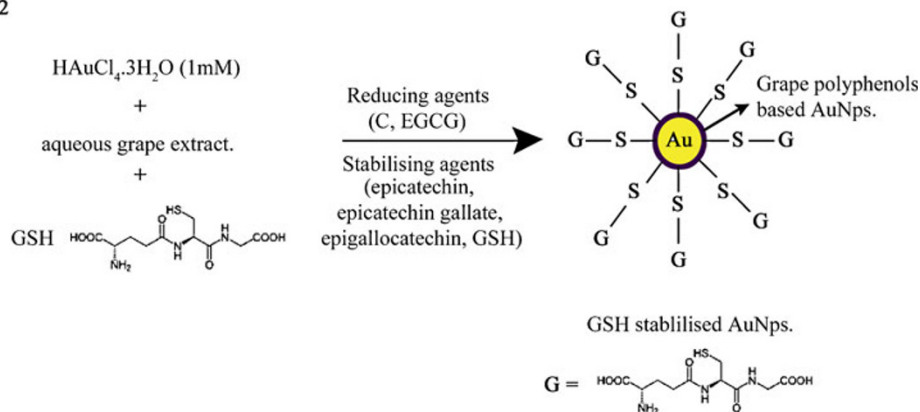
Fig. 11 Schematic representation of biosynthesis of grape-based gold nanoparticles (Step 1) and ligand (GSH and lipoic acid) stabilized grape gold nanoparticles (Steps 2 and 3)

Step: 1



C - catechin; EGCG- Epigallocatechin gallate (Water soluble phytochemicals)
 Plays the dual role as reducing and stabilising agents for AuNps.

Step: 2



Step: 3

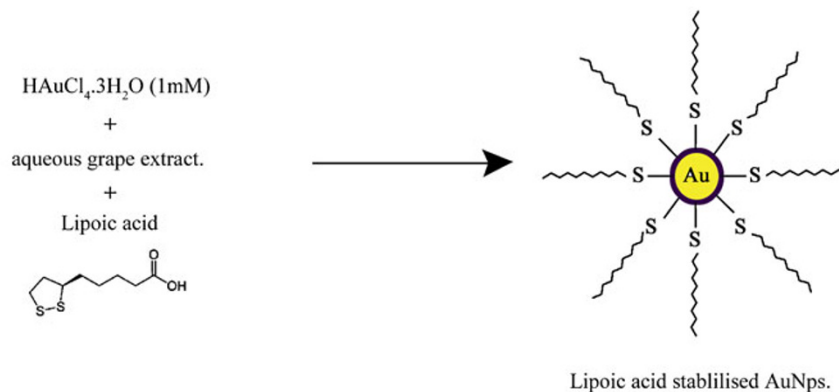


Fig. 9 which is also another characteristic of apoptosis. All these results confirm that treatment with GSH-GAuNPs and LA-GAuNPs induce apoptosis in human breast cancer cells compared to GAuNPs.

3.6 Cellular internalization studies of GAuNPs, GSH-GAuNPs, and LA-GAuNPs in HBL-100 cells

Membrane integrity is another cellular characteristic commonly used to determine viability during *in vitro* nanotoxicology experiments. Surprisingly, cancer cells are highly metabolic and porous in nature and are known to internalize solutes rapidly compared to normal cells (Sun et al. 2007). Do the cells internalize the particles or do the particles remain bound to the cell membrane? Results of cellular internalization studies of AuNPs solutions are keys to provide insights into their use in biomedicine. Their selective cell and nuclear targeting will provide new pathways for their site-specific delivery as diagnostic/therapeutic agents. To address these issues, confocal microscopic studies confirmed the uptake of GSH-GAuNPs and LA-GAuNPs inside the HBL-100 cells (Fig. 10a–c) with the presence of agglomerated gold nanoparticles; a similar observation was also reported by Stark and coworkers with copper nanoparticles for Hela cells (Studer et al. 2010).

Further, TEM images of breast tumor (HBL-100) cells treated with GSH-GAuNPs and LA-GAuNPs unequivocally validated our hypothesis. Significant internalization of GSH-GAuNPs and LA-GAuNPs via endocytosis within the HBL-100 cells was observed (Fig. 11). GSH-GAuNPs and LA-GAuNPs were detected within larger endocytic compartments of diverse morphology. These include peripherally both early and late endosomes and lysosomes. The internalization of nanoparticles within cells could occur via processes including phagocytosis, fluid-phase endocytosis, and receptor-mediated endocytosis. The viability of HBL-100 cells post-internalization suggests that the phytochemical coating and the size of the nanoparticles renders the nanoparticles nontoxic to cells. A number of studies have supported our study, demonstrating that phytochemicals have the ability to penetrate the cell membrane and internalize within the cellular matrix (Sun et al. 2007; Mizuno et al. 2007).

Therefore, we hypothesized that grape-derived phytochemicals and other antioxidants, if coated on gold nanoparticles, will show internalization within cancer cells. Such a harmless internalization of gold nanoparticles will provide new opportunities for probing cellular processes via nanoparticulate-mediated imaging.

The present investigation resulted in the development of environment-friendly green methodology to produce biologically benign gold nanoparticles stabilized with biologically

relevant thiol-rich antioxidants. Here, the gold nanoparticles are generated by reduction of gold precursor (a source of Au^{3+} ions) by a reducing agent (grape polyphenols) in the presence of stabilizers (GSH and Lipoic acid) that keeps nanoparticles apart, thus avoiding their aggregation. Although there are reports for the anticancer activity of phytochemically stabilized AUNPs, this is the first report to investigate the cytotoxicity of GSH and lipoic acid (in addition to phytochemicals) stabilized AUNPs. Herein, the major viability-based assays, such as proliferation, necrosis, or apoptosis and DNA damage detection of GSH-GAuNPs and LA-GAuNPs in HBL-100 cells have been proved as keys to provide insights into their use in biomedicine. In addition, GSH-GAuNPs and LA-GAuNPs selective cell and nuclear targeting compared to GAuNPs will provide new pathways for their site-specific delivery as diagnostic/therapeutic agents.

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