Cellular uptake and cytotoxic effects of broccoli phytochemicals based gold nanoparticles (B-AuNPs): Enhanced cancer therapeutic efficacy

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ABSTRACT

The interaction of phytochemicals from Broccoli with gold salt results in dual reduction and surface capping to produce well-defined stable and biocompatible gold nanoparticles (B-AuNPs). The core sizes of B-AuNPs is 15±2 nm as measured from TEM techniques which was also corroborated by CPS technique. Hydrodynamic diameter of B-AuNPs is 100±5 nm, suggesting that cocktail of broccoli phytochemicals are capped on gold nanoparticles. The highly -ve zeta potential of -29.0 mV indicates that the particles are sufficiently stable. The effect of B-AuNPs on various cancer cell lines showed significant cytotoxic effect. The cellular localization of B-AuNPs against cancer cell lines was evaluated using dark field optical microscopy and TEM image analysis. Cytotoxic results and tumor cell affinity suggest that the unique synergistic cocktail effect of B-AuNPs may provide new opportunities for generating biocompatible AuNPs for applications in molecular imaging and therapy.

Keywords: broccoli gold nanoparticles, isothiocyantes, glucosinolates, cell internalization, cytotoxicity

1 INTRODUCTION

A wealth of epidemiological studies indicate that consumption of large quantities of fruits and vegetables, particularly cruciferous vegetables (e.g., cabbage, kale, Brussels sprouts), is associated with a reduced incidence of cancer [1-2]. Among various vegetables, broccoli (*Brassica oleracea*) certainly deserves attention. Broccoli belongs to the Cruciferous family and is originated in the Mediterranean regions and distributed in Europe and US. Broccoli is rich in micronutrient: carotene, vitamin C, folic acid, fibers and comprises of phytochemicals such as glucosinolates (Gls), polyphenols [3]. It has been speculated that the isothiocyanates (ITCs), obtained from *myrosinase* hydrolysis of GLs, are in great part responsible for the protective effects of Brassica vegetable [4]. Discovery of phytochemicals that possess pharmacological efficacies from Natural Products continues to be an important area of research in pharmaceutical sciences.

Recently, application of Nanotechnology in biology and medicine has received considerable attention because tumor specfiic nanoparticles with dual imaging and therpaeutic characteristics can be created [5]. Their unique size-dependent properties make these nanoparticles superior and indispensable in the development of sophisticated nanomedicines for the detection and therpay of various human diseases and disorders. Therefore, an empirical approach is proposed to find effective combination of cocktail of phytochemicals for cancer treatment. We hypothesized that cocktail of cancer fighting phytochemicals can be embedded on the surface of gold nanoparticles serving effective storage and delivery of antitumor agents at the target sites. The versatile surface chemistry of gold makes them very attractive for conjugation of phytochemical cocktails onto their surface. In this context, the biosynthesis of gold nanoparticles by plant extracts draw attention due to their target oriented bioefficacy, non toxicity, stability and cost effectiveness. Katti et al have poineered on the utility of green nanotechnological processes for the biosynthesis of AuNPs by plant extracts of Soybean [6], tea [7, 8], Cinnamon [9], and Cumin [10]. The potential of phytochemicals in broccoli as source of anticancer molecules for the synthesis of AuNPs is not yet explored. In this study, we report a rapid and nontoxic route for the synthesis of stable gold nanoparticles from broccoli extract by simple mixing of gold salt with the phytochemical cocktail. We also report detailed *in vitro* anticancer activity of B-AuNPs against various cancer cell lines followed by cell internalization studies.

2 EXPERIMENTAL SECTION

All chemicals used in the synthesis of gold nanoparticles (AuNPs) were procured from Alfa-Aesar and Sigma, USA. All cell culture materials were obtained from Sigma, and Gibco BRL, USA. All cancer cell lines were obtained from ATCC, USA.

2.1 Prepration of broccoli extract

Broccoli (*Brassica oleracea*) purchased from a local grocery shop were used in all the experiments. Broccoli

(100 gm) was washed with DI water and dried at 50°C. The dry powder was stored at room temperature and was used for subsequent gold nanoparticle synthesis.

2.2 Synthesis and charecterization of broccoli gold nanoparticles

To a 20 mL vial, 6 ml doubly deionized (DI) water was added, followed by the addition of 60 mg broccoli powder. The mixture was stirred continously at 80°C for 10 min then 90 µ of 0.1 M $NaAuCl_4$ solution was added. The color of the mixture turned to ruby-red, indicating the formation of gold nanoparticles and were characterized by UV-Vis absorption spectroscopy, differential centrifugal sedimentation, and Transmission Electron Microscopy (TEM) analysis.

Gold metal content of B-AuNPs were estimated by furnace atomic absorption spectroscopy (fAAS) using a standard curve spanning 0–100 micrograms/L [11].

2.3 *In vitro* stability sudies

In vitro stability studies of B-AuNPs were performed by mixing gold nanoparticles to aqueous solutions of 10% NaCl, 0.5% cysteine, 0.2 M histidine, 0.5% HSA, 0.5% BSA, pH5, pH7 and pH9 buffer. The stability was measured by UV absorbance over a period of 2h, 24h and 7 days.

2.4 *In vitro* Cytotoxicity assay

The *in vitro* cytotoxicity evaluation of B-AuNPs was performed as described by the manufacture protocol (Promega, USA). Briefly, $1x10^5$/ml human cancer cells at the exponential growth phase were seeded in each well of 96 well plate and were incubated at 37°C for 24h in a CO_2 incubator. Cells were treated with different dilution of test samples (25-200 µg/ml) and incubated for 24h. MTT dye was added and incubated for 4h. The reaction was terminated by adding solubilizing buffer and plates were read by micro plate reader at 570 nm wavelength.

2.5 Cellular uptake studies

The PC-3 and MDA-MB-231 cells ($5x10^5$ cells) were seeded into 6 well plates in RPMI/DMEM medium respectively and allowed to adhere for 24h in CO_2 incubator at 37°C. The cells were treated with B-AuNPs and inbubated at 37°C for 12h. The TEM grid was prepared by the described method [12] and viewed with a JEOL 1400 TEM.

The cell internalization was also confirmed by cytoviva study where the cells ($3x10^5$ cells) were seeded into 6 well plate in RPMI medium and incubated at 37°C for 24h. The cells were treated with B-AuNPs were added to cells and incubated for 12h. The slides were prepared by using DAPI nuclear dye and observed with CytoViva dark field microscope.

2.6 Statistical analysis

All experimental data were given as mean±SE. Statistical analysis was carried out using the one-way analysis of variances (ANOVA) using Graph Pad Prism software. Probability values were found to be equal to or less than 0.05 ($p<0.05$).

3 RESULTS AND DISCUSSION

In this manuscript, we are presenting the synthesis, characterization and *in vitro* cytotoxicity and cell internalization study of B-AuNPs. The phytochemicals present in broccoli extract contains active functional groups, such as hydroxyl, aldehyde and carboxyl units, which may play pivotal roles in providing synergistic chemical property for the reduction of $NaAuCl_4$ salt into gold nanoparticles.

3.1 Characterization and size distribution of gold nanoparticles

B-AuNPs were characterized by various instrumentation techniques. The synthesis of B-AuNPs was confirmed by UV-visible spectrometry by recording their peak at 535 nm (Figure 1). Physiochemical properties, such as size, charge, and morphology of B-AuNPs, were determined by three independent techniques: TEM, CPS and hydrodynamic measurement. The core sizes of B-AuNPs is 15±2 nm as measured from TEM and CPS technique (Figure 1). TEM measurements on B-AuNPs show that the particles are spherical in shape within the size range of 15±2 nm. Size distribution analysis of B-AuNPs confirms that particles are well dispersed (Table 1). Hydrodynamic diameter of B-AuNPs is 100±5 nm, suggesting that broccoli phytochemicals are capped on gold nanoparticles. Zeta potential (ζ) provides crucial information on the stability of nanoparticle dispersion. The negative zeta potential of -29.0 mV for B-AuNPs indicates that the particles repel each other and there is no tendency for the particles to aggregate. The content of gold metal in 1 mg of dry B-AuNPs powder was estimated to be 0.480 mg/ml by fAAS.

The stability of B-AuNPs was evaluated by monitoring the plasmon (λmax) in 0.5% cysteine, 0.2 M histidine, 0.5% HSA, 0.5% BSA, and 10% NaCl solutions over 30 min. The stability of B-AuNPs has also been checked at pH 5, 7, and 9 buffer. Our results from these *in vitro* stability studies have confirmed that the gold nanoparticles are highly robust and thus demonstrate excellent *in vitro* stability in biological fluids and physiological pH. For various biomedical applications that require lower concentrations of gold, it is important that

dilutions of nanoparticle solutions should not alter their physiochemical and biotherapeutic properties.

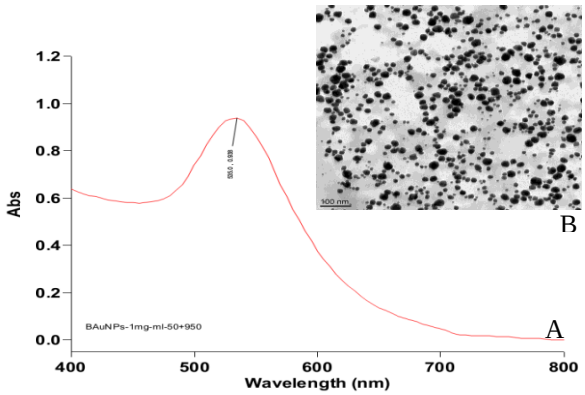

Figure 1: (A) UV-visible absorption spectra and (B) TEM images of B-AuNPs

Instrumentation	B-AuNPs
CPS: Core size	20 nm
TEM: Core size	15±2 nm
Zeta size: hydrodynamic size	100±5 nm
Zeta potential	-29.0 mV
AAS (Au content)	0.48 mg/ml AuNPs

Table 1: Physiochemical data of B-AuNPs

3.2 *In vitro* cytotoxicity Studies

In order to study cytotoxic effect in tumor cells, we selected weakly invasive breast cancer cell lines: T47D; highly invasive breast cancer cell lines: MDA-MB-231 and SKBR3; prostate cancer cell line (PC-3) and multiple myeloma (U266) cell line [13]. The cytotoxicity of B-AuNPs and broccoli extract under *in vitro* conditions against various cancer cells was examined in terms of their effect on cell proliferation by the MTT assay. In this assay, only cells that are viable after 24h exposure to the sample are capable of metabolizing a dye efficiently and produce purple-colored crystals, which are dissolved in a detergent and analyzed by micro placte reader. IC50 values of B-AuNPs against various cancer cell lines were as follows: MDA-MB-231: 160 µg/ml, PC-3: 150 µg/ml, U266: 125 µg/ml, SkBr3: 80 µg/ml and T47D: 22 µg/ml. The cytotoxic effect of test samples were significantly ($p<0.01$) different from untreated controls. Growth inhibition by B-AuNPs was concentration dependent. These results clearly demonstrate that the coating of broccoli phytochemicals on gold nanoparticles inhibit the growth of cancer cells and showed antiproliferative activity. The order of cytotoxic activity of B-AuNPs in our experiment was as follows: T47D> SkBr3>U266> PC-3> MDA-MB-231.

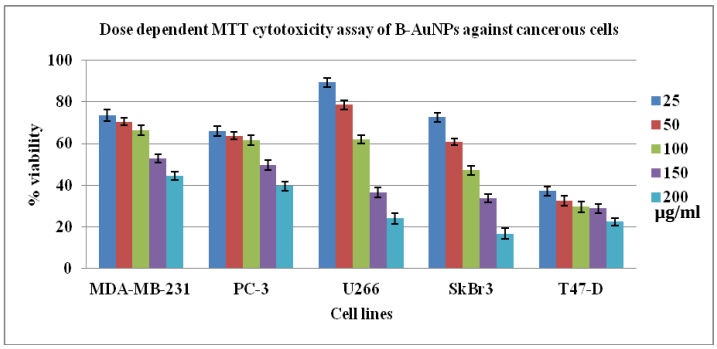

Figure 2: Dose dependent MTT cytotoxicity assay of B-AuNPs against cancerous cells at 24h treatment

3.3 Cellular internalization study

Interaction of phytochemical conjugated gold nanoparticles with tumor cells provide insights into cellular uptake and such information will enhance the scope of broccoli gold nanoparticles in biomedicine. Cancer cells are highly metabolic and porous in nature and are known to internalize solutes rapidly compared to normal cells [14]. We have evaluated the endocytosis of B-AuNPs in breast and prostate cancer cell lines using dark field optical microscopy and transmission electron microscopic techniques. It is well-known that the internalization of nanoparticles strongly depends on their physical characteristics including size, shape, and charge. Dark field microscopy images showed the internalization of B-AuNPs into the cells breast (T47D and MDA-MB-231) and prostate (PC-3) cancer cell lines at 25 and 50 µg/ml and incubation time was 8h and 18h. B-AuNPs showed efficient internalization at 18h as compared to incubation time 8h. We also confirmed the cell internalization effect by TEM analysis. TEM images of breast and prostate cancer cells treated with B-AuNPs unequivocally validated our hypothesis that B-AuNPs internalize through receptor mediated endocytosis (Image 1). The internalization of nanoparticles within cells could occur via several processes, including phagocytosis, fluid-phase endocytosis, and receptor-mediated endocytosis. However, significant internalization of B-AuNPs in breast and prostate cancer cells observed in our studies, strongly suggests that internalization of B-AuNPs does not happen through charge-mediated endocytosis. Further studies on the precise mechansim of endocytosis of B-AuNPs within various tumor cells are underway.

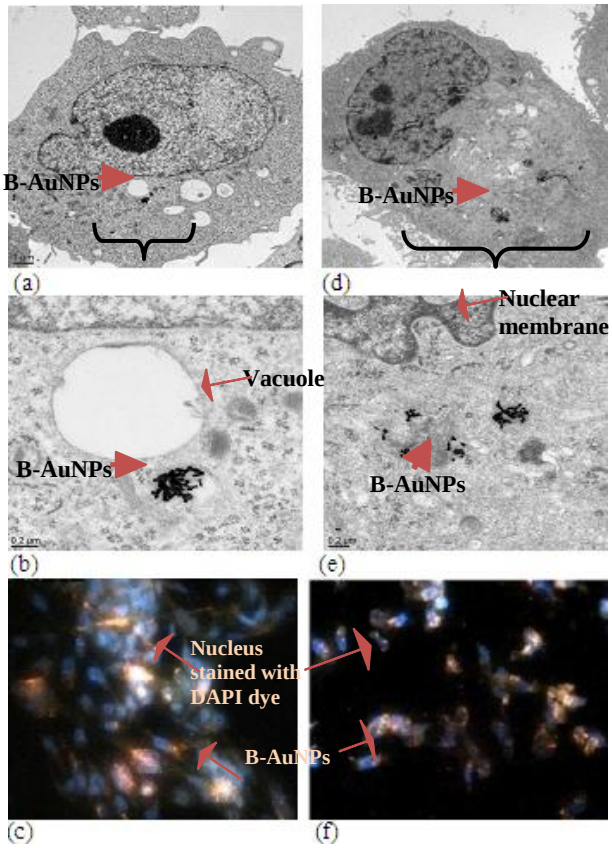

Image 1: Cellular internalization of B-AuNPs against (a, b) PC-3 cell line via TEM, (c) PC-3 cell line via dark field microscopic, (d, e) MDA-MB-231cell line via TEM, (f) MDA-MB-231 cell line via dark field microscopic

3.4 Role of Broccoli Phytochemicals

The chemical roles of different phytochemicals in broccoli responsible for the production of B-AuNPs are still not fully understood, but we believe that water-soluble antioxidant constituents of broccoli may be playing a major role in the overall reduction process of $NaAuCl_4$ to the corresponding gold nanoparticles. The main phytochemicals present in broccoli dry powder consist of vitamin C, folic acid, flavonoids and glucosinolates and their isothiocyantes derivatives. In order to understand the critical roles of various phytochemicals present in broccoli on the overall reduction of $NaAuCl_4$ to the corresponding gold nanoparticles, we have performed a series of independent experiments using commercially available chemicals that are present in broccoli extract. Results of these experiments using those chemical compounds have unambiguously confirmed that Glucosinolates are primary reducing agent to reduce Au (III) to gold nanoparticles, whereas polyphenols result in excellent coating to afford optimum *in vitro* (and presumably *in vivo*) stability. Results from these experiments have revealed that the synergistic effect of all the phytoconstituents present in broccoli extract show significant cytotoxicity against various cancer cell lines.

4 CONCLUSION

Our studies indicate that gold nanoparticles conjugated with broccoli-based phytochemicals show excellent internalization in prostate (PC-3) and breast (MDA-MB-231 and T47D) cancer cells. This unique synergistic cocktail effect of B-AuNPs may provide new opportunities for generating AuNPs for applications in nanoparticulate-mediated imaging and therapy.

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