World Applied Sciences Journal 32 (6): 1153-1158, 2014
ISSN 1818-4952

DOI: 10.5829/idosi.wasj.2014.32.06.14535

Green Synthesis of Chitosan Based Gold Nanoparticles Using Leaf Extracts of *Terminalia catappa* L. And Study of Their Effects on Cancerous Cells

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Abstract: Nanoparticles are considered to be the pre-eminent component of the rapidly advancing field of nanotechnology. However, developments in the biologically inspired synthesis of nanoparticles are still in their infancy and consequently attracting the attention of material scientists throughout the world. In this research the ethanol extract of *Terminalia catappa* leaf and used for green synthesis of gold nanoparticles (AuNPs). AuNPs were characterized using by UV-visible spectroscopy, XRD, TEM. The absorbance spectrum of AuNPs were demonstrated 546 nm, XRD analysis indicated crystalline geometry in nanoparticles. Transmission electron microscopy was revealed the size of spherical gold nanoparticles to be in the range of 20-40 nm. We also evaluated the potential of biogenic AuNPs to probe SUDHL-4 cell lines by conjugating them with cancer cell surface-specific protein. The protein conjugated gold particles were found to bind specifically to the surface protein of the cells. Cell exhibit significant uptake of Bombesin AuNPs, internalizing them through a highly specific receptor mediated endocytosis pathway.

Key words: Green Synthesis · *Terminalia catappa* · Gold nanoparticles · SUDHL-4 cell lines

INTRODUCTION

Currently, there is growing need to develop eco-friendly and body benign nanoparticle synthesis processes without use of toxic chemicals in the synthesis protocols to avoid adverse effects in biomedical applications. Obviously, researchers in this field paid their attention towards the use of biological systems for the synthesis of biocompatible metal and semiconductor nanostructures. Some well-known examples of bio-organisms synthesizing inorganic materials include magnetotactic bacteria (synthesizing magnetite nanoparticles) diatoms (synthesizing siliceous materials) and *S*-layer bacteria (producing gypsum and calcium carbonate layers) [1-6].

Many biotechnological applications such as remediation of toxic metals employ microorganisms such as bacteria [7] and yeast [8]. Nair and Pradeep have synthesized nanocrystals of gold, silver and their alloys by reaction of the corresponding metal ions within cells of lactic acid bacteria present in buttermilk [9]. The bacteria [10] and algae [11] are exploited for synthesis of gold nanoparticle.

Bombesin is proved to have numerous pharmacological effects, such as control of gastrin release, modulation of gastrointestinal secretions, smooth muscle motility and the amount of food intake [12-14]. Recently, it has been shown to play a major role in cell proliferation, tumor growth and inflammation [15]. There is substantial evidence indicating increased BBN/GRP (Gastric releasing peptide) receptor expression in prostate, small-cell lung, ovarian, breast, pancreatic, gastric, renal cell and thyroid cancers, suggesting their use as specific markers for targeting [16]. Since BBN have high affinity, conjugating AuNps with these peptides should allow for greater binding of these particles to the tumor sites, thus making them suitable for early detection of tumors by imaging techniques [17-19].

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In our earlier reports, synthesis of gold nanoparticles has been shown by the reduction of aqueous $HAuCl_4$ using extracts from *Terminalia catappa*. There is still much scope for improvement in bio-based methods for metal nanoparticle synthesis, particularly in relation to improving the monodispersity of the nanoparticles and modulating their size and shape, as well as in reducing the time required for nanoparticle synthesis. This work focused on conjugation of Bombesin to gold nanoparticles and study of their target specificity. The target specificity of gold nanoparticles coated with Bombesin, specific protein was tested using cancer cell lines. Cell exhibit significant uptake of Bombesin gold nanoparticles, internalizing them through a highly specific receptor mediated endocytosis pathway.

MATERIALS AND METHODS

Plant Material Source: *Terminalia catappa* leaf was procured from the earthen pots at Green house of Botanical Garden, Department of Botany, Bermpur University and Odisha, India. Hydrochloro auric acid ($HAuCl_4$) was obtained from Hindustan scientific research Pvt. Ltd. 7-amino acid Bombesin (BBN) was obtained from Biogenix India. Minimum essential medium (MEM with nonessential amino acids, powdered), were obtained from Sigma Chemical Company (St. Louis, MO). Bovine calf serum, phenol red (sodium salt) and lyophilized trypsin were obtained from Biogenix India.

Cell Culture: A SUDHL-4 cell line was maintained in School of Biotechnology, KIIT University, Odisha, India. It was grown in medium (*i.e*. RPMI-1640) supplemented with 10% foetal calf serum in a humidified incubator at 37°C along with 5% CO_2 .

Preparation Of Leaf Extracts: The air dried leaf and stem were milled to get a coarse powder. About 100g of dry powder was extracted with petroleum ether at room temperature using soxhlet apparatus for 8 hrs. or the extraction was continued until the liquid was clear. The extracts were then filtered and concentrated to a dry mass under vacuum.The marc left after petroleum ether extraction was air dried and then extracted with solvents ethanol as done earlier and the extracts were similarly filtered and concentrated under vacuum.

Synthesis of AuNPs: 100 ml of plant or leaf extract were collected in a 500 ml beaker which was kept on magnetic stirrer f or stirring at 200 rpm at room temperature, to this stirring solution 10 ml of 1mM auric acid solution was added drop wise. The ratio of extract and auric acid solution in above procedure is 10:1, similarly 5:1 and 1:1 ratios were followed for optimization of protocol [20].

Synthesis of Chitosan Stabilized AuNPs (C-AuNPs): To a 10 ml vial, 0.0225 g of chitosan was added and dissolved in 6 ml of 2% acitic acid and deionized water. This chitosan solution was heated up to 90°C-100°C and stirred continuously until dissolved. To the dissolved chitosan solution, 100µl of 0.1 M $HAuCl_4$ solution was added resulting in a pale yellow color solution. To this solution, 20ml of *Terminalia catappa* extract solution was added with continuous stirring. The resulting solution slowly turned into purplish-wine color representing the formation AuNPs. The mixture was cooled to room temperature.

The Reaction:

Au^{+3} (aq) + Reducing Agent (aq) + Stabilizer →Au°
[$HauCl_4$; $NaAuCl_4$] Plant Extract Chitosan AuNPs

Conjugation of Thioctic acid-BBN (TA-BBN) to C-AuNps: Based on the studies done by Kattumuri [21], 0.885 µM of Thioctic acid- BBN conjugate (1 mg in 1 ml of methanol) was added to 1 ml of filtered C-AuNps and stirred for 60 hrs. The product, thus formed was further filtered and purified by washing several times with mixture of methanol and water to remove all traces of unconjugated reactants. The purified Bombesin-conjugated gold nanoparticles (BBN-AuNPs) solution was vacuum dried and re-dissolved in PBS (without Ca^{2+} and Mg^{2+}). This conjugate was used to study its target specificity towards on SUDHL-4 cell lines.

MEASUREMENTS

UV-Vis Spectroscopy: Ultraviolet-visible spectroscopy (UV-1601 pc shimadzu spectrophotometer) or ultraviolet-Visible spectrophotometer (UV-Vis) refers to absorption spectroscopy in the UV-Visible spectral region.

X-ray Diffraction: It was done by using BEDE D-3 system with Cu Kα radiation at a generator or voltage of 40 kV and a generator current of 100 mA. The samples were scanned from scanned from $2\theta = 1 - 100°$ at a scanning rate of 2°/min.

Transmission Electron Microscope (TEM) Analysis: Transmission electron microscope (TEM) (Philips CM-10) is a microscopy technique whereby a beam of electrons is transmitted through an ultra-thin specimen, interacting with the specimen as it passes through. An image was formed from the interaction of the electrons transmitted through the specimen; the image was magnified and focused onto an imaging device.

RESULTS AND DISCUSSIONS

Uv-visible Spectrophotometer Analysis: The AuNPs were synthesized by incubating $HauCl_4$ solution with various proportions of *Terminalia catappa* leaf extract. Incubation of increasing concentrations of *Terminalia catappa* leaf extract with solution led to a change in the plasmon resonance band that eventually resulted in the appearance of different colors, from pale yellow to mauve red, after 24 hours (Figure 1a). The first was that the transverse plasmon resonance band that appeared at 540 nm shifted towards a higher wave length, confirming a red shift along with amplified absorbance intensity. Earlier reports showed that the stability of chitosan-AuNPs and BBN-AuNps were measured by a characteristic absorbance at 546 nm and 549 which has been supported by spectrophotometer [22-25].

XRD: Structural characterization has been performed using XRD analysis and the typical XRD pattern for gold nanoparticles is shown in Figure 2. In addition to these three peaks there are some unidentified peaks appeared in the XRD pattern. The characteristic peaks corresponding to (111), (200), (220) of Au are located at $2\theta = 38.29°$, 44.43° and 64.68°, respectively. The result indicates that the sample is composed of crystalline gold [26].

Transmission Electron Microscopy: Figure 2 represents a transmission electron micrograph of AuNPs. It was found that Hexagonal, triangular and spherical nanoparticles could be seen in the transmission electron micrographs. It was observed that gold nanoparticles of 20-40 nm in size could be seen in the transmission electron micrographs (Figure 3). The edge length of the nanoparticles was also found to decrease with an increased amount of *Terminalia catappa* leaf extract and for the synthesis of BBN-AuNps showed a uniform size distribution of 20nm [27].

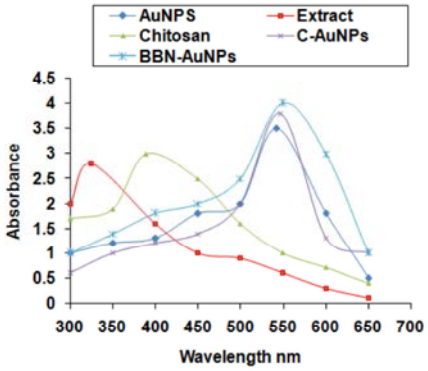

Fig. 1: UV-Visible absorption spectrum of *Terminalia catappa* leaf extract, AuNPs, Chitosan, C-AuNPs, and BBN-AuNps

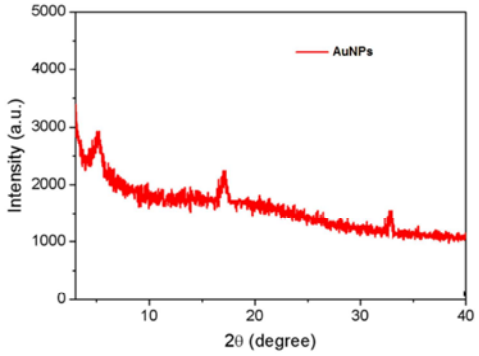

Fig. 2: XRD of AuNPs

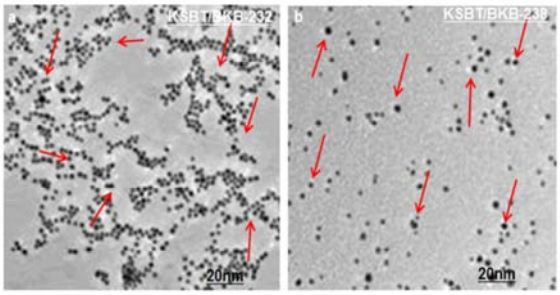

Fig. 3: TEM of AuNPs and BBN-AuNps

Effect of Aunps on Cell Proliferation of SUDHL-4 Cells: To determine the effect of AuNPs on the colony-forming ability of cancer cells, To determine the IC50 value (the concentration required to kill half the cells), a broader range of concentrations were used in the same cell line and the IC50 obtained was 200 µM (Figure 4) [28]. This data clearly indicates a decrease of clonogenic cell survival in cells with AuNP exposure. These results clearly demonstrate that the phytochemicals within these herbs provide a nontoxic coating on AuNps and corroborate the results of the internalization studies

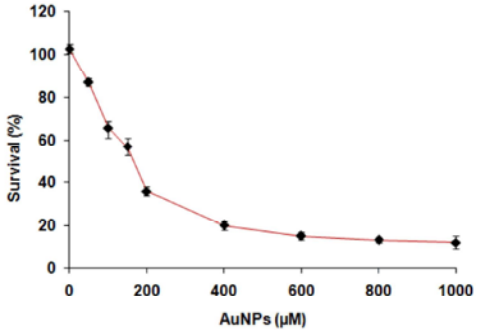

Fig. 4: Percentage survival of clonogenic cell growth after treatment with increasing concentrations of AuNP for 72 h.

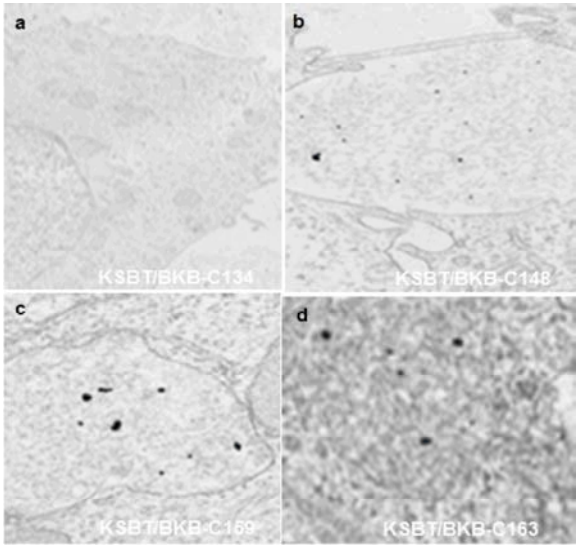

Fig. 5 a, b, c, d: Different stages of the cellular uptake process of BBN-AuNps. TEM images of SUDHL-4 depicting the arrival of a BBN-AuNps at the cell membrane, binding of the nanoparticles to surface receptors, membrane wrapping of the NPs, and finally internalization into the cell nucleus, respectively

discussed above. It is also important to recognize that a vast majority of exhibit varying degrees of cytotoxicity to a variety of cells (Figure 4). The lack of any noticeable toxicity of AuNps provides new opportunities for the safe application in molecular imaging and therapy.

Cellular Interactions of Bombesin Conjugated Gold Nanoparticles: To determine whether BBN-AuNps can be used to label BBN receptors over expressed in SUDHL-4 cancer cells, cultures of confluent SUDHL-4 were exposed for 4 hrs to BBN-AuNps. These incubated samples are analyzed under TEM to verify whether the BBN-AuNps are bound to the GRP receptors present in SUDHL-4 cells. Figures 5 a,b,c,d clearly show binding of BBN-AuNps to the plasma membrane of SUDHL-4 cells by forming clathrin pits and then those pits delivering the nanoparticles into the nucleus via endosomes [29].

CONCLUSION

We report here the biogenic synthesis of AuNPs employing a novel source, *i.e*. a *Terminalia catappa* leaf extract, which was found to be simple, economically viable and environment friendly. We also infer from our study that the shape and size of the nanoparticles formed govern the characteristic features of their spectra. This research resulted in the synthesis of non-toxic, biologically friendly AuNPs that are conjugated with highly specific biomolecules such as Bombesin. In addition, the conjugated AuNps were tested for their target specificity towards cancerous cells. Our results show that Bombesin conjugated gold nanoparticles can enter SUDHL-4 cells via BBN receptor-mediated mechanism.

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