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Endophytic *Bacillus cereus* mediated synthesis of gold nanoparticles and their stabilization using biopolymer chitosan

Swetha Sunkar*, C. Valli Nachiyar and K. Renugadevi

Department of Biotechnology, Sathyabama University, Jeppiar Nagar, OMR, Chennai, Tamilnadu, India

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ABSTRACT

The growing use of gold nanoparticles and their conjugates in medical field for a number of purposes specially in cancer studies emphasizes on the method employed for their synthesis. The existing chemical methods of synthesis require high temperatures and also result in environmental pollution by the usage of toxic chemicals. The present study demonstrates the synthesis of gold nanoparticles using endophytic bacterium Bacillus cereus isolated from the medicinal plant Justicia beddomei. Initial synthesis of the nanoparticles was observed by the color change from pale yellow to pink which was later confirmed by the UV-vis spectra that showed a characteristic peak at 536 nm. The size of the synthesized gold nanoparticles obtained by TEM analysis revealed the size to be in the range of 16 – 40 nm. The three dimensional view of the gold nanoparticles was visualized by AFM analysis. EDX studies confirmed the presence of elemental gold in the nanoparticle solution. Further these gold nanoparticles were stabilized using a biopolymer chitosan to prevent aggregation of the nanoparticles. This functionalization of the nanoparticles is key step in their usability in various medical applications.

Keywords: Biosynthesis; *Bacillus cereus*; Gold nanoparticles; AFM; TEM

INTRODUCTION

Nanotechnology, an interdisciplinary research field involving chemistry, engineering, biology, and medicine, has great potential for early detection, accurate diagnosis, and personalized treatment of cancer [1]. Nanoparticles are typically smaller than several hundred nanometers in size, comparable to large biological molecules such as enzymes, receptors, and antibodies. With the size of about one hundred to ten thousand times smaller than human cells, these nanoparticles can offer unprecedented interactions with biomolecules both on the surface of and inside the cells, which may revolutionize cancer diagnosis and treatment. Multifunctionality is the key advantage of nanoparticles over traditional approaches. Targeting ligands, imaging labels, therapeutic drugs, and many other functional moieties can all be integrated into the nanoparticle conjugate to allow targeted molecular imaging and molecular therapy of cancer. Gold nanoparticles are unique in a sense because of its intriguing optical properties which can be exploited for both imaging and therapeutic applications [2].

Gold nanoparticles can be successfully synthesized by traditional chemical and physical methods. However, these methods strongly depend on severe reaction conditions; for example, aggressive agents like sodium borohydride, hydrazinium hydroxide, acetyltriethyl ammonium bromide, and harmful solvent system to environment and ecology, higher temperature and higher pressure [3]. For a safer environment, it is imperative to develop a clean synthetic approach using the concept of “green chemistry” to obtain nanomaterials targeted for different applications, especially in biomedical fields. In this direction, the synthesis of nanoparticles using biological systems has grabbed remarkable attention as they offer more facile and eco-friendly route to synthesis. Microbes have made a decent entry into this zone by producing metallic nanoparticles both extracellularly and intracellularly. Endophytes are one group of microorganisms that display the potential to synthesize metallic nanoparticles in a facile manner [4, 5].

The present study attempts to synthesize gold nanoparticles using the endophytic bacterium *Bacillus cereus* ABSW 10 (GenBank: HM998898.1) isolated from *Justicia beddomei* extracellularly as a one step process. The process of isolating and identifying the endophytic bacterium is described elsewhere and this bacterium was successful in synthesizing silver nanoparticles [4]. Hence, in the present investigation, this bacterium was employed in the synthesis of gold nanoparticles extracellularly as this step would facilitate the process of extraction.

EXPERIMENTAL SECTION

Chloroauric acid $HAuCl_4$ was procured from Sigma Aldrich, USA. The endophytic bacterium *Bacillus cereus* ADA was isolated from *Justicia beddomei* (*Adathoda beddomei*) is described elsewhere [4].

Biosynthesis of the Gold Nanoparticles

The endophytic bacterium *Bacillus cereus* was inoculated in 100 mL of Luria Broth medium and incubated for 36 hrs at 37±2 °C with shaking at 150 rpm. After incubation, the culture supernatant was collected by centrifugation at 10,000 rpm for 10 min followed by filtration. 10 mL of the supernatant is challenged with 20 ml of 1mM $HAucl_4$ and incubated under dark conditions for 48 hr. The formation of gold nanoparticles was observed by the change in color of the solution from pale yellow to pink.

Characterization of gold nanoparticles

Various spectrophotometric and microscopic analyses were carried out to characterize the gold nanoparticles. The formation of gold nanoparticles (AuNPs) was initially observed by the change in colour of the solution from pale yellow to pink. This was confirmed further from UV-vis spectroscopy of the reacting solution using spectrophotometer, in a 1 cm path quartz cell at a resolution of 1 nm from 250 to 800 nm. Additionally, morphology, size and distribution of the AuNPs were found various microscopic analyses namely Transmission Electron Microscope (TEM) and Atomic Force Microscopy (AFM). FTIR analysis was carried out to understand the possible biomolecules involved in the synthesis of nanoparticles. Elemental composition of the nanoparticles was carried out on an air-dried, carbon coated sample using an energy dispersive spectroscopy (EDX) attachment on a scanning electron microscope using the following instrumental conditions: accelerating voltage of 15 keV and counting time of 100 s.

Stabilization of gold nanoparticles using chitosan and their characterization

Chitosan solution (1%) (w/v) was prepared by dissolving 0.1 g chitosan powder in 10 ml 0.05 M acetic acid solution, and then it was mixed with AuNPs solution to prepare the chitosan - AuNP solution (1:1, v/v) [6]. The chitosan - AuNPs are characterized by TEM to determine the size of the coated to chitosan - AuNPs. Infra-red spectrophotometer (FTIR) Model: Perkin Elmer FT-IR 2000 was employed to verify the change of the functional groups of chitosan and Au-chitosan particles.

Cytotoxicity of Chitosan – AuNPs

The cell lines were procured from the Kings Institute of Preventive and Medicine, Guindy, Chennai and the cell lines were subcultured and maintained in CO_2 incubator at 37°C. 20% serum containing RPMI growth media was used for growing the cells. The cells were continuously monitored under inverted microscope for their confluence and to confirm the absence of the bacterial and fungal contaminants.

Cell lines were subcultured and 1x104 cells were transferred to the 96 wells and incubated at 37° C in CO_2 incubator for 2 days to form confluence. The spent media was removed and 150 µl of fresh media was added. 150 µl of Chitosan - AuNPs (1mg/ml) was added and serially diluted to get 1fold dilution in each wells till 6th dilution to get varying concentration from 75 µg to 1.14 µg. The plates were incubated at 37°C for 4 hrs in CO_2 incubator. After incubation the drug was removed and 20 µl of MTT solution (5mg/ml) and 180 µl of media were added to the wells. The plates were incubated at 37°C for 3.5 hrs in CO2 incubator. Without disturbing the cells the media was removed carefully. The insoluble formazon crystal was dissolved by adding DMSO and the Absorbance was read at 570 nm with reference filter at 630 nm. The percentage cytotoxicity was calculated and used for finding the IC_{50}. The untreated cells were considered as control.

$$\%\,Cytotoxicity = \frac{(Abs_{Control} - Abs_{Test})}{Abs_{Control}} \times 100$$

RESULTS AND DISCUSSION

The possible benefits of nanomaterials in various fields are well accepted in the recent times. In the biological context, recent reports focus on the effect of size, shape, bioavailability, uptake, and subcellular distribution of such nanomaterials. At the cellular level, gold nanoparticles have shown promising potential in catalysis, biological labeling, and sensing [7]. Because of its chemical inertness, gold has been used internally in humans for the past 50 years, from its use in teeth to implants to radioactive gold in cancer treatment [8, 9]. GNPs exhibit some special optical properties such as plasmon resonance, which is primarily a quantum phenomenon operative on the nanoscale. This means that GNPs make an interesting probe for studying some intricate biomolecular events including protein folding and also serve as specialized microscopic probes to study cancer cells, because GNPs selectively accumulate in tumor cells, showing bright scattering [10,11].

Incidentally, their use in human systems demands their biocompatibility with the same and hence their mechanism of synthesis is crucial at this juncture. Various existing physical and chemical methods often pose the problems like the use of toxic chemicals under severe conditions. A facile green route to their synthesis is provided by biological sources where the plants and microorganisms display enormous potential as nanosynthesizers. In this line, the present study successfully demonstrated the facile synthesis of gold nanoparticles using an endopytic *Bacillus cereus*.

Biosynthesis of gold nanoparticles

The main focus of the study was to synthesize gold nanoparticles (AuNPs) extracellulary as the use of biomass would demand an additional step of ultrasonication for their release into the surrounding liquid media. The formation of extracellular AuNPs was initially observed by the change in colour of the supernatant from pale yellow to pink (Fig. 1 inset) after challenging with chloroauric acid.

This change in colour was due to the collective coherent oscillation of conduction electrons at the surface of the gold nanoparticles when these particles interact with the oscillating electric field of the incident light, a phenomenon called surface plasmon resonance (SPR). This change in color indicates the reduction of $AuCl_4^-$. Similarly, gold nanoparticles were synthesized by *Klebsiella pneumoniae* [12], and *Pseudomonas aeruginosa* [13] which was initially observed by the change in color from pale yellow to pink.

Characterization of Gold nanoparticles

The preliminary information on the formation of AuNPs was initially gathered by observing the colour change in the reaction medium from pale yellow to pink. It was further confirmed by various instrumental analyses that authenticate the formation and delve into the size and shape of these nanoparticles.

UV-Vis spectrophotometry

The most commonly used method for confirming the presence of nanoparticles is UV-Vis spectroscopy. UV-Vis absorption measurements in the range 350-600 nm can provide an insight about their size, distribution and surface properties and also give information about the optical properties of the AuNPs. The surface plasmon bands for the AuNPs usually ranges between 510 and 560 nm in aqueous solution depending upon the function of their morphology, since plasmon bands are very sensitive to the length and sharpness of the tips of nanomaterials. The wavelength of peak absorption depends upon several factors such as particle size, dielectric constant of surrounding media and the inter-particle distance.

The UV-Vis spectrum of the AuNPs obtained displayed a clear distinct peak at 536 nm (Fig. 1). The results obtained in the present study very well adhere to the standard SPR peaks wavelengths [14]. The clear single peak obtained in the present study was observed at 536 nm which indicates the spherical nature of the AuNPs.

It is well known that spherical nanoparticles of Au should exhibit single-surface plasmon bands whereas anisotropic particles should exhibit two or three bands, corresponding to the quadrupole and higher multipole plasmon excitations [15, 16]. It was stated earlier that spherical nanoparticles have strong absorption at 520 nm with almost no absorption after 600 nm with only a single SPR band in the absorption spectra [17].

The methodology demonstrated in the above study required no harsh chemicals or conditions and hence satisfies the "green approach" towards the synthesis of nanoparticles. The reduction of gold was not instantaneous, but the gold nanoparticles are found to be very stable in the colloidal suspension. Extracellular biosynthesis of gold nanoparticles was achieved by an easy biological procedure using *Klebsiella pneumoniae* as the reducing agent [12]. Even marine bacteria are exploited towards their capability of gold nanoparticles (AuNPs) production. Stable, monodisperse AuNP formation with around 10 nm dimension occured upon exposure of $HAuCl_4$ solution to whole

cells of a novel strain of *Marinobacter pelagius* [18]. The use of endophytes for synthesizing gold nanoparticles was initiated by Vijay Verma [19] who used the extracts of endophytic *Aspergillus clavatus* for the synthesis and stabilization of gold nanoparticles.

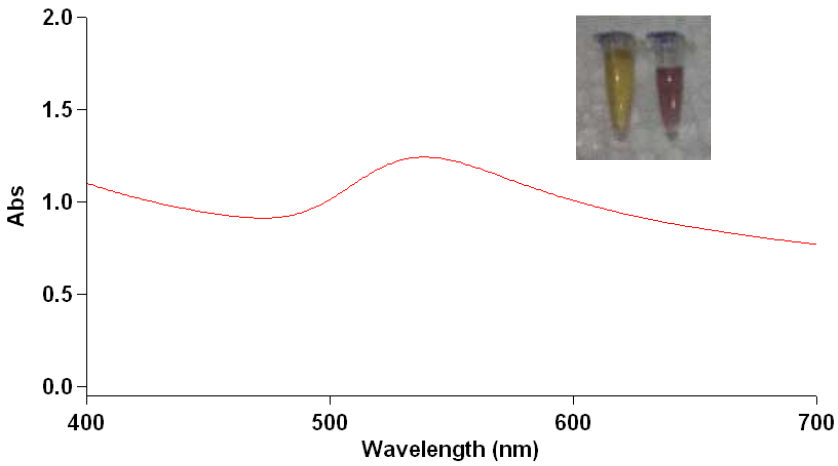

Fig. 1. UV-Vis spectrum of the gold nanoparticles (Inset: Color change of the nanoparticles from pale yellow to pink)

Morphological studies –TEM, EDX and AFM analysis

The morphology and size of the nanoparticles plays an important role in their functional properties. Hence the size and shape of these AuNPs were identified by TEM analysis (Fig. 2a). The TEM image revealed the AuNPs to be smooth and spherical in shape without much aggregation. The size was observed to be in the range of 16 - 37 nm. Among the various types of gold nanoparticles, the spherical form is the most widely used. As gold nanospheres can be easily prepared by reducing the gold ion and controlling the particle size, it is possible to carry out the production of gold nanoparticles with relative ease and subsequently to use them not only in a variety of medical diagnoses but also as therapeutic and drug delivery systems [20]. The presence of elemental gold in the biologically synthesized nanoparticles was confirmed by EDX analysis (Fig. 2b) where optical absorption peaks were observed approximately at 2 keV, confirming the presence of gold in the solution.

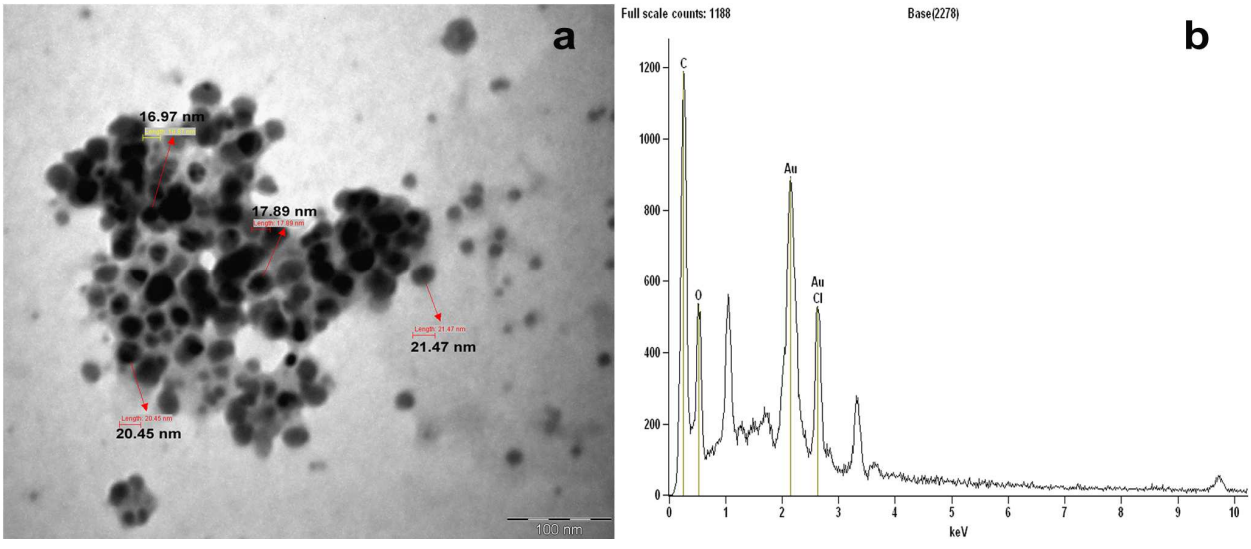

Fig. 2. a) TEM micrograph of biogenic AuNPs b) EDX spectrum of the biogenic AuNPs

Microscope images are essential in research and development projects and can be critical when troubleshooting quality control issues. Using the AFM, individual particles and groups of particles can be resolved. The AFM offers visualization in three dimensions. Resolution in the vertical, or Z, axis is limited by the vibration environment of the instrument: whereas resolution in the horizontal, or X-Y, axis is limited by the diameter of tip utilized for scanning. For individual particles, size information (length, width, and height) and other physical properties (such as morphology and surface texture) can be measured. AFM has several advantages over TEM for characterizing

nanoparticles. Images from an AFM represent data in three dimensions, so that it is possible to measure the height of the nanoparticles quantitatively. With an AFM, images can be measured in all environments; ambient air, liquids and vacuums. The AuNPs were analysed using AFM to understand their morphology and size as this instrumental method would facilitate the 3D visualisation of the particles.

The images of the biogenic AuNPs (2D and 3D) were both run on the same area of the sample. Briefly, the AFM topography of the AuNPs (Fig. 3) shows the structure of gold nanoparticles to be spherical. The surface appeared to be smooth and the average roughness of the particles was calculated and was obtained to be 9 indicating the relative smoothness of the nanoparticles. The overall size of the nanoparticles was in the range of 16 - 45 nm which is similar to the result obtained in TEM analysis. The histogram analysis of the size distribution of the nanoparticles also indicated that most of the nanoparticles are within the range of 30 – 80 nm.

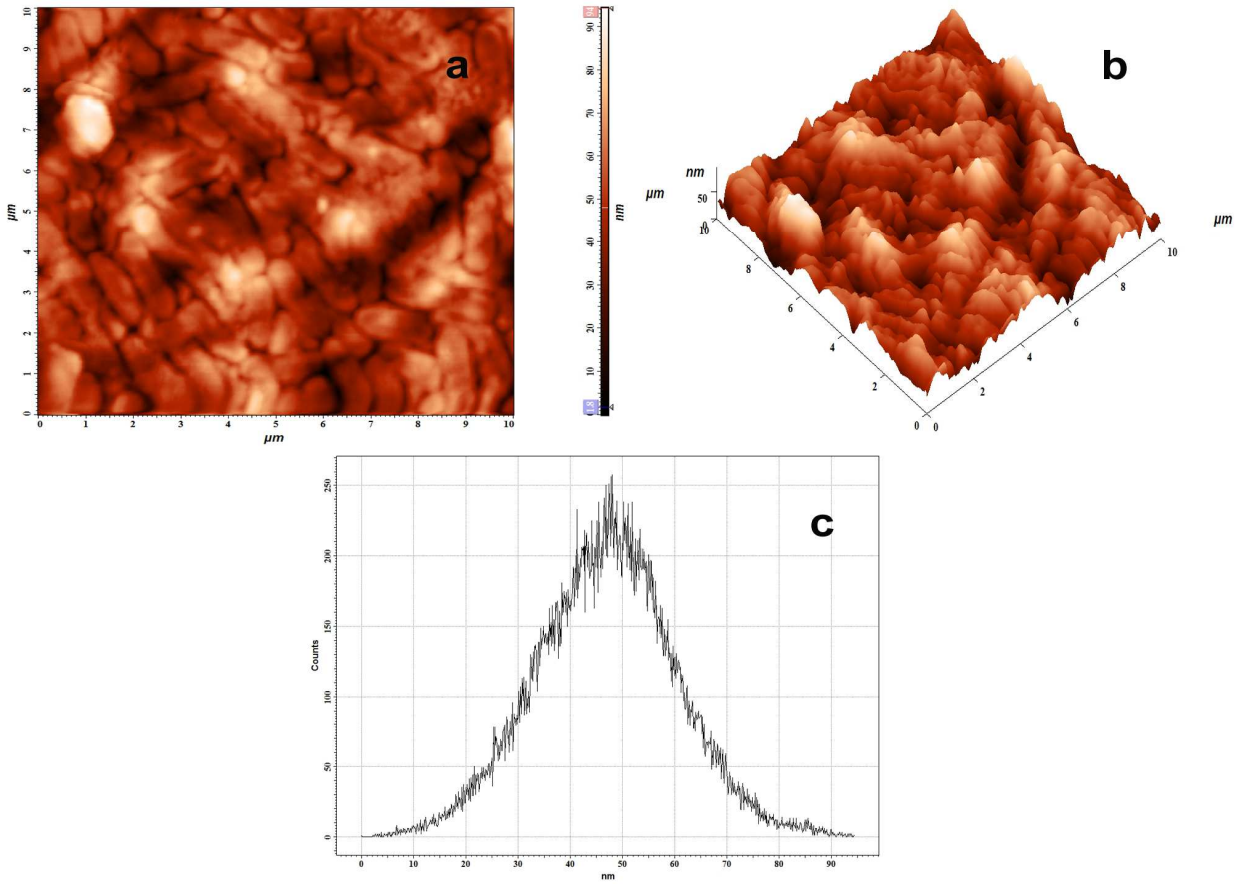

Fig. 3. AFM images of AuNPs A) 2D image of AuNPs B) 3D image of AuNPs C) Histrogram showing the size distribution

FTIR spectra of gold nanoparticles

The nature of possible biomolecules involved in the synthesis and stabilisation of nanoparticles was identified by FTIR spectra. Many reports have stated that biological synthesis of nanoparticles is facilitated by the proteins produced by the microorganisms as revealed by the FTIR spectra. The singular advantages of FTIR over other techniques are that spectra can be obtained for proteins in a wide range of environments, requiring less time and sample, and direct correlations between the IR amide I band frequencies and the secondary structure components can be found [21]. The FTIR spectrum of the biogenic AuNPs is given in Figure 4.

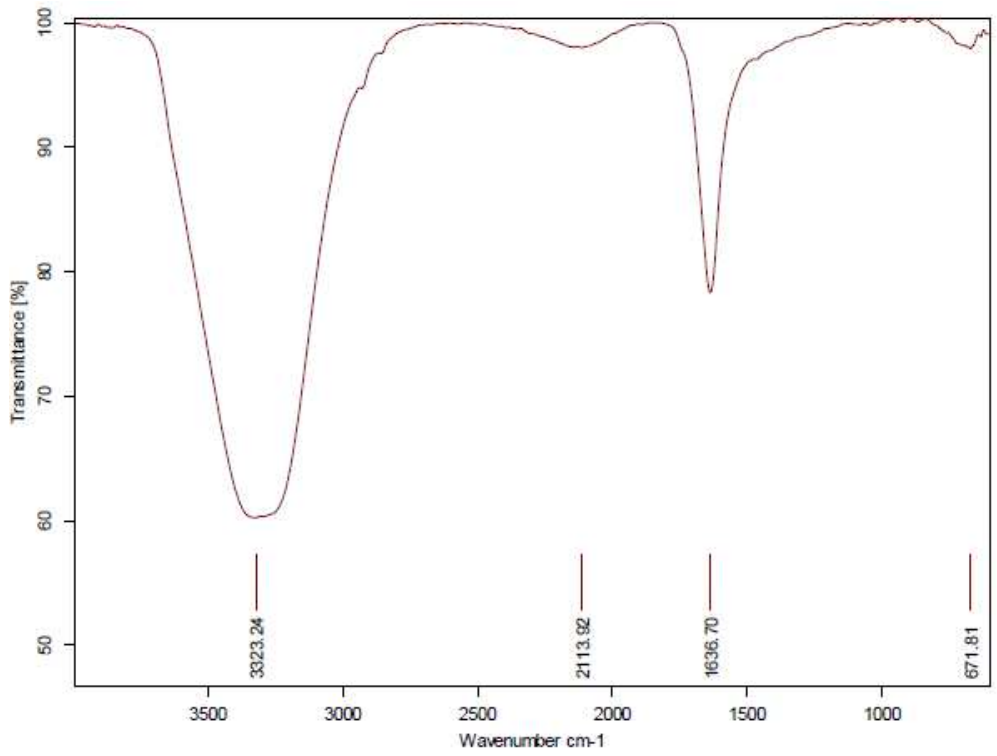

Fig. 4. FTIR spectra of the biogenic AuNPs

The spectra documented two very prominent peaks at 3325 cm^{-1} and 1632 cm^{-1} with small peaks at around 600 – 700 cm^{-1}. The peak at 3325 cm^{-1} corresponds to amide A absorption band which is characteristic of N-H stretching vibrations. The peaks at 1632 cm^{-1} correspond to C=O stretching vibrations. This spectra confers to the standard peaks of FTIR spectra of the proteins and peptides [22 – 25] thus confirming the presence of extracellular proteins that may be responsible for the synthesis of gold nanoparticles. It has been documented earlier that the extracellular proteins perform dual functions of formation and stabilization of silver nanoparticles in the aqueous medium [26]. These proteins function as capping agents as the carbonyl group from the aminoacid residues show stronger ability to bind to metals [27].

The IR spectral data of high polymers are usually interpreted in terms of the vibrations of a structural repeat unit [22, 23, 28]. The polypeptide and protein repeat units give rise to nine characteristic IR absorption bands, namely, amide A, B, and I−VII. Of these, the amide I and II bands are the two most prominent vibrational bands of the protein backbone [23, 29-30]. The most sensitive spectral region to the protein secondary structural components is the amide I band (1700−1600 cm^{-1}), which is due almost entirely to the C=O stretch vibrations of the peptide linkages (approximately 80%). The frequencies of the amide I band components are found to be correlated closely to the each secondary structural element of the proteins. The amide II band, in contrast, derives mainly from in-plane NH bending (40−60% of the potential energy) and from the CN stretching vibration (18−40%) showing much less protein conformational sensitivity than its amide I counterpart [23]. Gold nanoparticles can bind to proteins through free amine groups or carboxylate groups in the protein. The presence of the intense peak at C=O stretching mode indicates the presence of carboxylic groups in the material bound to gold nanoparticles [31].

Stabilization of AuNPs using biopolymer Chitosan and characterization

It is well known that metallic nanoparticles have the tendency to aggregate in solution due to their small size. Moreover, upon extraction in powder form, the colloidal particles (which are held apart by electrostatic interactions while in solution) agglomerate and lose their characteristic properties [32]. Therefore, one of the effective ways to avoid agglomeration of the particles is to use stabilizing agents or protective agents such as thiols, surfactants and polymers. It is well established that AuNPs are biocompatible and non-toxic [33]. Due to the presence of negative charge on the surface of AuNPs, they are highly reactive, which helps to modify the surface of AuNPs using several biomolecules. The stabilizing molecules can control the particle size as well as prevent the agglomeration of metal particles. Ideally, this polymeric material should be biocompatible and have reactive functional groups for further attachment of biomolecules.

One such natural polymer is chitosan that offers distinctive advantages such as good biocompatibility, nontoxicity, remarkable affinity to proteins and excellent gel-forming ability and hence can be used to prepare metal nanocomposites [34-35]. The suitability and performance of chitosan for drug delivery applications have been investigated earlier [36]. It is used in controlled drug delivery application as a component mucoadhesive dosage forms and rapid release dosage forms in improved peptide delivery and for gene delivery. In view of the advantages posed by chitosan in biological systems, the present study focuses on the stabilization of biogenic AuNPs using chitosan. Chitosan stabilized biogenic AuNPs have been characterized using the standard instruments namely TEM and FTIR analysis to confirm the process of coating chitosan onto the gold nanoparticles.

UV-vis spectrophotometry and TEM analysis of Chitosan - AuNPs

The colour of the AuNPs solution after stabilizing with chitosan was dark pink in color which is corroborating with the results obtained by Jin et al. [37]. The UV–vis absorption spectrum of Chitosan – AuNPs was collected from 300 nm to 800 nm (Fig. 5a). The UV- Vis spectra recorded for the Chitosan - AuNPs showed a slight shift in the peak from 536 nm to 500 nm except which there was not much variation thus confirming the presence of stable gold nanoparticles.
The TEM micrographs (Fig. 5b) revealed the AuNPs with a region around them indicating that the chitosan has coated the AuNPs in a uniform manner thereby preventing the aggregation of the nanoparticles and stabilizing them. The size of Chitosan-AuNPs was more compared to that of naked AuNPs which points out that these AuNPs were coated with chitosan.

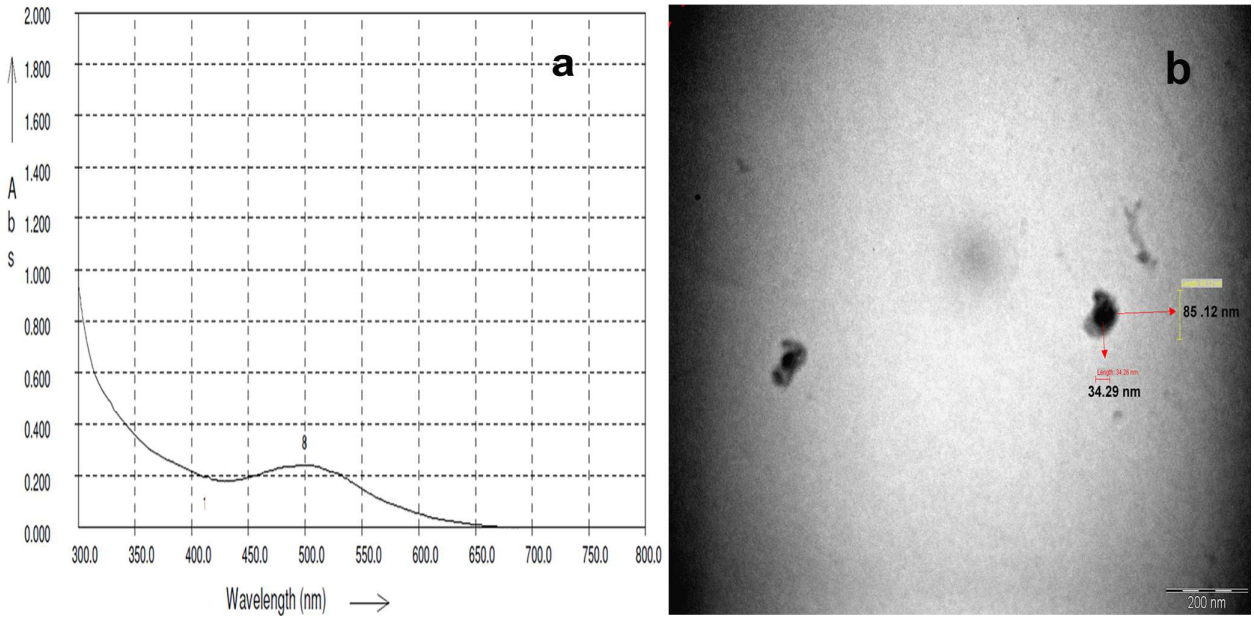

Fig. 5. a) UV-Vis spectra of Chitosan – AuNPs b) TEM image of chitosan stabilized AuNPs

FTIR analysis
Chemical modification of the AuNPs was studied by Fourier-transform infrared spectroscopy. Figure 6 shows the FTIR spectrum of chitosan and Chitosan - AuNPs.

The characteristic peaks of chitosan were obtained at 3375 cm^{-1}, 2138 cm^{-1}, 1651 cm^{-1}, 794 cm^{-1} and 495 cm^{-1} represent the $–NH_2$, –OH and $–CH_2$, and $–CH_3$ aliphatic groups. Absorption bands at 1651 cm^{-1} represent the amino group bending vibrations. The amino group has a characteristic absorption band in the region of 3400 – 3500 cm^{-1} which is masked by the broad spectrum band from the –OH group. The absorption band at 1657 cm^{-1} is attributed to the $–CONH_2$ group of chitosan. Similar peaks were also observed in the chitosan stabilized gold nanoparticles at 3399 cm^{-1}, 2131 cm^{-1} representing the $–NH_2$, –OH and $–CH_2$, and $–CH_3$ aliphatic groups. The peak obtained at 1653 cm^{-1} represents the amino group bending vibrations and few other smaller peaks at 817 cm^{-1}, 568 cm^{-1}, 547 cm^{-1}, 516 cm^{-1} with a reduction in the peak at 2138 cm^{-1}, broadening of peak at 1653 cm^{-1} and disappearance of the peak at 794 cm^{-1}. This spectrum clearly indicates that the AuNPs are coated with chitosan.

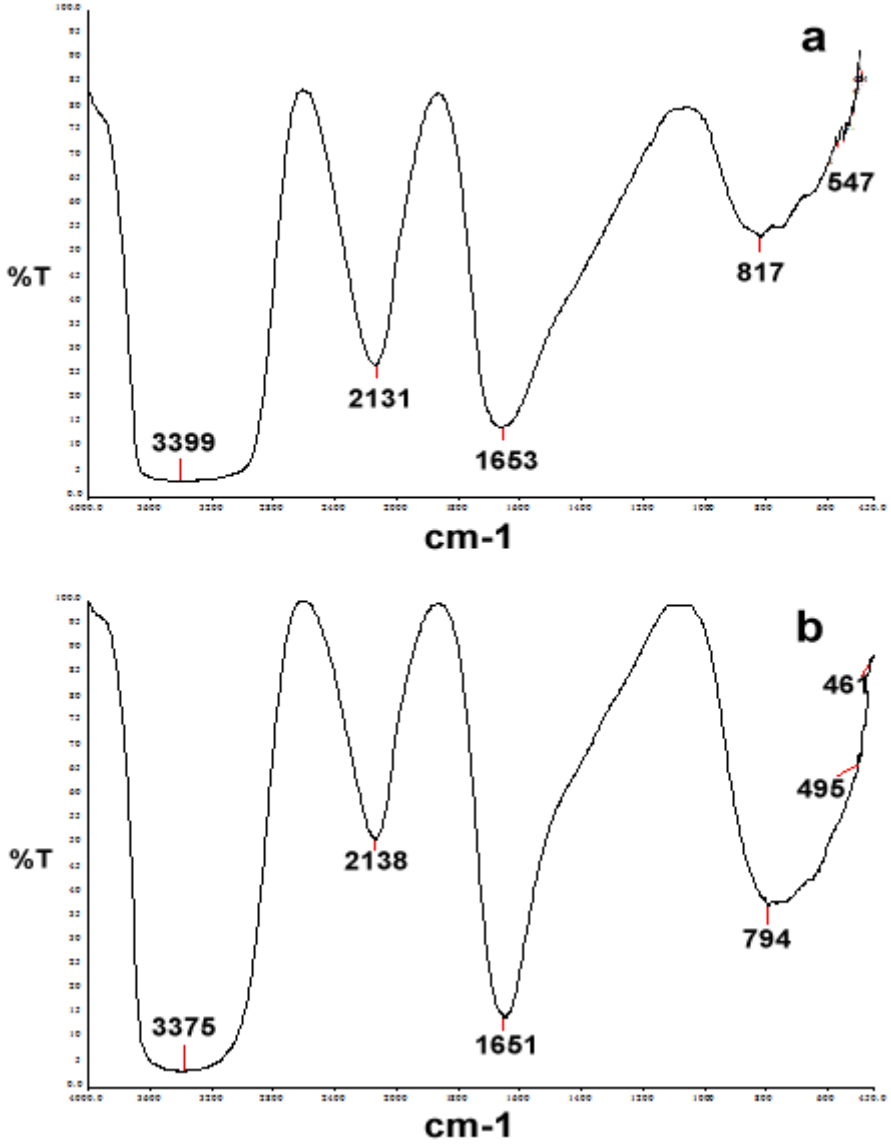

Fig. 6. FTIR spectrum A) Chitosan B) Chitosan – AuNPs

Cytotoxicity of Chitosan – AuNPs

A preliminary study on the cytotoxicity of chitosan - stabilized AuNPs was carried out using the MTT assay with 4 hrs of incubation time (Fig 7).

It was noticed that the % cytotoxicity showed a gradual increase in both the cell lines (Vero and Hep2) with an in the concentration of Chitosan - AuNPs. The IC_{50} value for Vero and Hep2 cells was found to be 27µg/ml and 38µg/ml respectively. Maximum cytotoxicity was observed to be 73 and 78 % for Vero and Hep2 at a concentration of 75 µg/ml. While significant toxicity was not noticed till ~10 µg/ml, there has been noteworthy increase in the toxicity after this concentration. This study putforths the potential of the Chitosan – AuNPs in medical field, but a detailed study on the effect of these nanoparticles at increased concentrations for a prolonged period of time is required to understand their efficacy and efficiency in cancer studies.

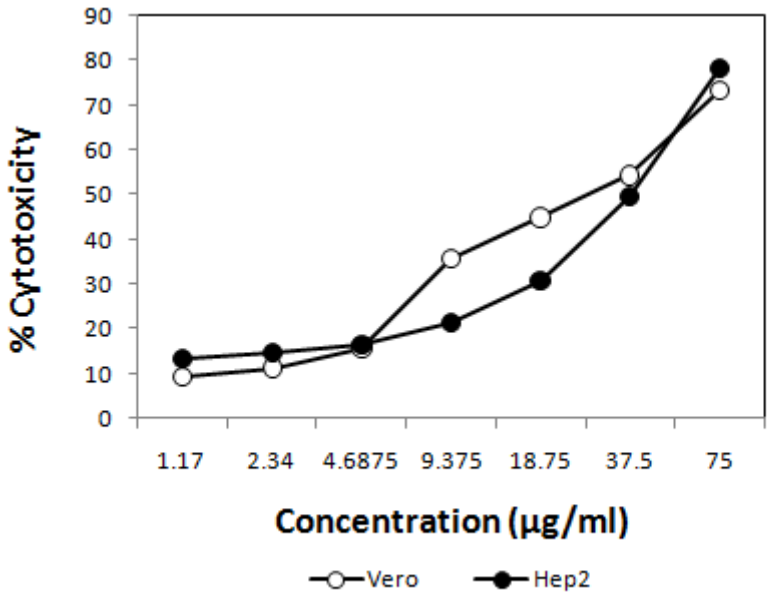

Fig. 7. Cytotoxicity of Chitosan – AuNPs against Vero and Hep2 cell lines

CONCLUSION

Micron and nanosized particles are sophisticated technologies that were developed to answer specific demands in the field of drug delivery. It was demonstrated that several gold compounds were nanoparticles have been designed and tested for medical applications. The present study demonstrated the development of gold nanoparticles in a "green" way using an endophytic bacterium *Bacillus cereus* in one step. Their size was in the range of 30-80 nm with a smooth surface which was well convinced by instrumental analyses. These nanoparticles have been further stabilised with chitosan as it has been proved biocompatible and safe. TEM and FTIR confirmed the presence of chitosan as a coating over the gold nanoparticles. This study establishes the fact that biogenic gold nanoparticles synthesised in a facile manner can be well used in various fields of medicine as these were appropriately stabilised by chitosan.

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REFERENCES

[1] W Cai, X Chen. *Small*, **2007**, 3, 1840 - 1854.
[2] W Cai, T Gao, H Hong, J Sun. *Nanotech Sci Applications*., **2008,** 1, 17-32.
[3] UK Parida, BK Bindhani, P Nayak. *World J Nano Sci Eng*., **2011,** 1, 93 -98.
[4] S Sunkar and V Nachiyar. *Global J Medical Res*., **2012,** 12, 43-49.
[5] S Sunkar and V Nachiyar. *Asian J Microbiol Biotech Environ Sci*., **2013,** 15, 495 -502.
[6] MH Xue, Q Xu, Mi Zhou, Jun-Jie Zhu. *Electrochem Comm*., **2006,** 8, 1468 -1474.
[7] B Wang, K Chen, S Jiang, Francüois Reincke, Weijun Tong, Dayang Wang, and Changyou Gao. *Biomacromol*., **2006,** 7, 1203-1209.
[8] LQ Wu, K Lee, X Wang, DS English, W Losert, GF Payne. *Langmuir*., **2005,** 21, 3641-3646.
[9] Y Yonezawa, I Kawabata, T Sato. *Ber. Bunsen-Ges. Phys. Chem*., **1996,** 100, 39-45.
[10] N Ishizuki, K Torigoe, K Esumi, K Meguro. *Colloids Surf*., **1991,** 55, 15-21.
[11] a) M Ly Arrascue, H Maldonado Garcia, O Horna, E Guibal. *Hydrometallurgy*. **2003,** 71, 191. (b) E Guibal. Prog. *Polym. Sci*., **2005,** 30, 71.
[12] C Malarkodi, S Rajeshkumar, M Vanaja, K Paulkumar, G Gnanajobitha and G Annadurai. *J Nanostruct Chem*., **2013,** 3, 30.
[13] MI Husseiny, MA El-Aziz, Y Badr, MA Mahmoud. *Spectrochim Acta A Mol Biomol Spectrosc*., **2007,** 67, 1003-1006.

[14] http://www.cytodiagnostics.com/gold_nanoparticle_properties.php; http://www.sigmaaldrich.com/materials-science/nanomaterials/gold-nanoparticles.html
[15] TK Murphy, AM Sau, CJ Gole, J Orendorff, L Gao, SE Gou, TH Li. *J Pharm Biomed*., **2005,** 38, 186-190.
[16] W Xu, Y Wang, R Xu, S Liang, G Zhang, D Yin. *J. Mat. Sci*., **2007,** 42, 6942-6945.
[17] G Mie. *Ann. Phys*., **1908,** 25, 377-445.
[18] N Sharma, AK Pinnaka, M Raje, A Fnu, MS Bhattacharyya, AR Choudhury. *Microb Cell Fact*., **2012,** 20, 86.
[19] VC Verma, SK Singh, Ravindra Solanki and Satya Prakash. *Nanoscale Res Lett*., **2011,** 6, 16.
[20] G Han, P Ghosh, M De, VM Rotello. *Nanobiotech*., **2007,** 3, 40-45.
[21] J Kong and S Yu. *Acta Biochimica et Biophysica Sinica*., **2007,** 39 (8), 549-559.
[22] A Elliot and EJ Ambrose. *Nature*., **1950,** 165, 921-922.
[23] S Krimm and J Bandekar. *Adv Protein Chem*, **1986,** 38, 181-364.
[24] J Banker. *Biochim Biophys Acta*., **1992,** 1120, 123-143.
[25] T Miyazawa, T Shimanouchi, S Mizushima. *J Chem Phys*., **1956,** 24, 408.
[26] K Mallikarjun, G Narsimha, GR Dillip, B Praveen, B Shreedhar, S Lakshmi, VS Reddy, DP Raju. *Digest J Nanomat Biostruct*., **2011,** 6 (1), 181-186.
[27] R Sathyavati, MB Krishna, SV Rao, R Saritha, DN Rao. *Adv Sci Lett*., **2010,** 3, 138-143.
[28] CY Liang, S Krimm, GBBM Sutherland. *J Chem Phys*., **1956,** 25, 534-549.
[29] H Susi and DM Byler. *Methods Enzymol*., **1986,** 130, 290-311.
[30] WK Surewicz and HH Mantsch. *Biochim Biophys Acta.*, **1988,** 952, 115-130.
[31] D Philip. *Spectrochim Acta Part A*., **2009,** 73, 374-381.
[32] KB Narayanan and N Sakthivel. *Mat. Lett*., **2008,** 68, 4588-4590.
[33] JF Hainfeld, DN Slatkin, HM Smilowits. *Phys. Med. Biol*., **2004,** 49, N309-315.
[34] A Tiwari, Polysachharides: Development, Properties and Application, Nova Science Publishers, Inc, New York, USA. **2010**, 552
[35] A Tiwari, SR Dhakate. *Int J Biol Macromol*., **2009,** 44, 408-412.
[36] DR Bhumkar, HM Joshi, M Sastry, VB Pokharkar. *Pharm Res.,* **2007,** 24, 1415-1426.
[37] Y Jin, Z Li, L Hu, X Shi, W Guan, Y Du. *Carbohydrate Polymers.,* **2013**, 91, 152