Research Article

Bio-functionalized Gold Nanoparticles: A Potent Probe for Profound Antibacterial Efficiency through Drug Delivery System

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ABSTRACT

In the present manuscript, we reported extracellular biosynthesis of gold nanoparticles through extract of leaves from *Dictyota dichotoma*. Biosynthesized nanoparticles were characterized using UV– Vis spectroscopy, HRTEM, EDX and FTIR spectroscopy. The polyshaped gold nanoparticles of size 8±21 nm were synthesized. The antibacterial activities were observed by the agar well diffusion method for the action of Streptomycin and Gentamycin and their formulation with biocapped gold nanoparticles. The profound efficacies were supported by the increase in fold area of inhibition against the tested bacteria. The appropriate cause of this divergence has also been discussed due to suitable binding of biogenic gold nanoparticles with drugs and supports these findings to overcome the conflict of previously reported data.

Key words: *Dictyota dichotoma*, Antibacterial efficacy, Biomimetic, Drug delivery systems, Gold nanoparticles, Streptomycin.

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INTRODUCTION

The ocean is considered as an integrated biogeocenosis linked to the relative homogeneous and constantly moving mass of seawater.[1] Oceans contain a variety of marine plants, which are unique resources that provide a diverse array of for biological synthesis of nanoparticles. A nanometer (nm) is one thousand millionth of a meter, 10^{-9}. Thus, Nanotechnology is the ability to measure, design and manipulate at the atomic, molecular and supramolecular levels on a scale of about 1 to 100 nm in an effort to understand, create, use material structures, devices and systems with fundamentally new properties and functions.[2] Nanoparticles of wide range of materials can be prepared by a variety of methods. A number of methods including chemical methods,[3] electrochemical reduction,[4] photochemical reduction[5] and physical method such as heat evaporation[6] have been used for the synthesis of silver and gold nanoparticles. The traditional and most widely used methods for synthesis of metallic nanoparticles use wet-chemical procedures. To prevent the agglomeration of metallic nanoparticles, a stabilizing agent such as sodium dodecyl benzyl sulphate or polyvinyl pyrrolidone is also added to the reaction mixture.[7]

There is an increasing need to develop high-yield, lowcost, nontoxic and environmentally benign procedures for synthesis of metallic nanoparticles. Therefore, the biological approach for synthesis of nanoparticles becomes important. Of note, both unicellular and multicellular organisms have been known to produce intracellular or extracellular inorganic materials. Since the first evidence of the capability of living systems

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to reduce metal ions to zerovalent form developed to explore the bioreduction potential of different micro-organism and plants.[8] In particular, there is great interest in the development of nanoparticle-based vectors that decrease the toxicity of free drugs and ensure targeted delivery directly to tumour cells.[9] Conjugates of nanoparticles with antibodies have been used for selective photo thermal killing of micro-organism.[10]

An important aspect of nanotechnology which has big challenge is the development of metal nanoparticles synthesis that should exhibit completely new or improved properties as compared to the larger particles of the bulk material with specific characteristics such as size, distribution and morphology.[11] Resistance of bacteria to bactericides and antibiotics is a subject of immense interest due to the development of resistant strains. Some antimicrobial agent act as irritant and hence novel ways of formulating biocidal materials is an upcoming field of attraction.[12] Thus, the present finding was aimed for the development of macroalgae assisted synthesis of gold nanoparticles and its formulation with drug as a probe for profound antibacterial efficiency.

MATERIALS AND METHODS

Preparation of the reaction solutions

Carefully weighed 10^{-3} M $HAuCl_4$ aqueous solution was used for the biological synthesis of AuNPs. The marine macroalgae filtrate was used as reducing and stabilising agent. In a typical synthesis of gold (Au) nanoparticles for the reduction of Au^{3+} ions, 20 mL of macroalgae filtrate was added to 80 mL of 10^{-3} M $HAuCl_4$ solution in a 250 ml Borosil flask and kept on a rotary shaker (120 rpm) at 30°C.[13] The change of colour from light yellow or light brown to light or dark ruby red was the confirmatory feature to proceed for characterization.

Characterization of gold nanoparticles

The reduction of metal ions was routinely monitored by visual inspection of the solution as well as by measuring the UV-Vis spectra of the solution by periodic sampling of aliquots (0.2 mL) of the aqueous component. The UV-Vis spectroscopy measurements were recorded on a Shimadzu dual-beam spectrophotometer (Shimadzu, UV 2500) operated at a resolution of 1 nm between 300 to 700 nm in a 10-mm-path-length quartz cuvette. To study the size, morphology and pattern of the biosynthesized gold nanoparticles high resolution TEM analysis was carried on carbon coated copper grids. The films on the grids were allowed to dry prior to measurement on a TEM (JEOL-2100F) operated at an accelerating voltage of 200 Kv. The EDX analysis was performed to monitor the elements present in the bio-conjugated nanomaterial. The size and the distribution of the prepared gold nanoparticles were analyzed by photon correlation spectroscopy using a Zetasizer PALS Zeta Potential Analyzer ver. 3.54. The FT-IR was done to examine surface characterization of various chemical and conformational changes of nanoparticles samples. The freeze dried sample powder of gold nanoparticles was analyzed using FTIR (Shimadzu 8400S). Infrared spectra were recorded in the region of 500 to 4500 cm^{-1}.

Preparation of antibiotic coated gold nanoparticles and test bacteria

The drugs coated gold nanoparticles were prepared by mixing nanoparticles (10 cm^3 of 1mM AuNPs) with (10 cm^3 of 1mM) of drugs diluted to 50 ml of double distilled water and stirred effectively for 2 hr. This is marked as the standard sample. Antibiotic protected gold nanoparticle was prepared to study the function of nanoparticle on the microbial activities. The two different drugs used in this study were Streptomycin and Gentamycin. The bacterial isolates used for this study were procured from the Microbial Type Culture Collection (MTCC). They are Staphylococcus *aureus* (MTCC – 737), *Pseudomonas aeruginosa (*MTCC – 424) and *E. coli* (MTCC – 739).

Microbial assay

Bacterial sensitivity to antibiotics coated with bio-conjugated nanoparticles was studied using a disk diffusion assay. The drugs coated gold nanoparticles were placed on agar plates and the plates were left for 1 hr at 25°C to allow a period of pre-incubation diffusion in order to minimize the effects of variation in time between the applications of different solutions. The plates were incubated, at 37°C for 24 hr and observed for antibacterial activity by determining the diameters of the zones of inhibition for each bacterial culture. Three parallel tests were run with the standard for avoiding any errors.

Assessment of increase in fold area

The increase in fold area was assessed by calculating the mean surface area of the inhibition zone of pure drugs and drugs coated gold nanoparticles. The fold increase area of different tested bacteria was calculated by the equation $(B^2 - A^2)/A^2$,[14] where A and B were zones of inhibition for drugs and drugs coated gold nanoparticles, respectively.

RESULTS

Figure 1 showed the UV–vis spectra recorded from the aqueous solution of 1 mM $HAuCl_4$ as a function of the reaction time using 5% *Dictyota dichotoma* leaf broth. The maximum absorbance was observed at ca. 548 nm and the intensity steadily increased to saturation as a function of the reaction time. Colloidal solutions of gold nanoparticles showed very intense ruby-red colour, which was absent in the bulk material as well as for individual atoms. The reduction of gold ions and the formation of stable nanoparticles occurred after an hour of reaction, making it one of the biomimetic methods to produce Au nanostructures.

The TEM image showed that the particle size were highly polydispersed in nature. There were very few mixtures of plate (triangles, pentagons and hexagons) present (Figure 2A). Representative TEM images and corresponding size distribution histogram of gold nanoparticles were nearly spherical and more dominant than triangular and hexagonal shapes with a wide spectrum of particle size distribution ranging from 9 to 20nm (Figure 2B). Large quantities of small number of nanoparticle with the average diameter of 13 nm were formed. The high-resolution TEM (HRTEM) images displayed clear lattice fringes on the particle surfaces.

The EDX profiling of sample was investigated for confirmed synthesis. It showed the presence of strong gold signal of the biologically synthesized gold particles. However, the signal of copper peak was also strong, most likely due to background from the supporting copper grid (Figure 3). These signals are the fingerprints of the elements in the sample and can be used as a source of quantitative chemical and electrical structural information.

FTIR analysis was conducted for the functional group analysis of the synthesized gold nanoparticles (Figure 4). The absorbance bands were observed in the region of 500–4500 cm^{-1}. The AuNPs showed stronger absorption band at 2107, 1649, 1427, 1325 and 1031cm^{-1}.

Due to well-developed surface chemistry, chemical stability and appropriate smaller size of gold

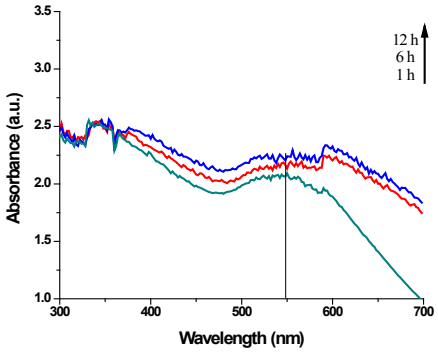

Figure 1: UV-Vis spectra of gold nanoparticles recorded after the reaction of 1 mM HAuCl4 solution with 5% aqueous leaves extract of *Dictyota dichotoma* for 12 h.

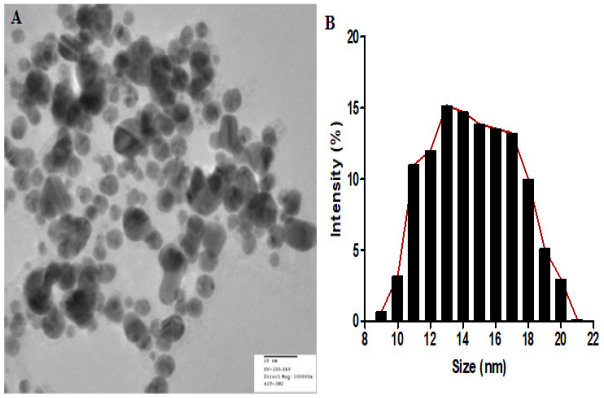

Figure 2: (A) TEM micrograph recorded from a drop-coated film of gold aqueous solution formed by the reaction of 1 mM HAuCl4 and 5% *Dictyota dichotoma* leaf broth The scale bar corresponds to 20 nm. (B) Gold nanoparticles size distribution histogram.

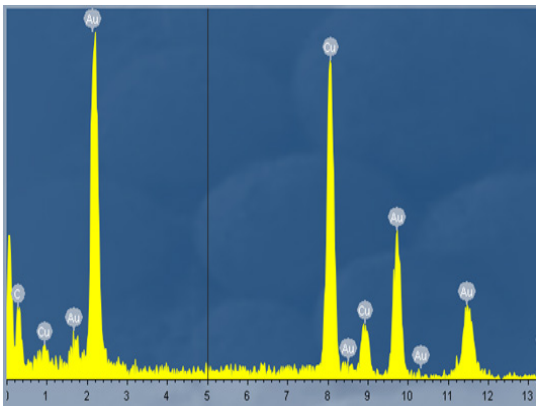

Figure 3: EDX spectral analysis of gold clusters formed by the reaction of 1 mM $HAuCl_4$

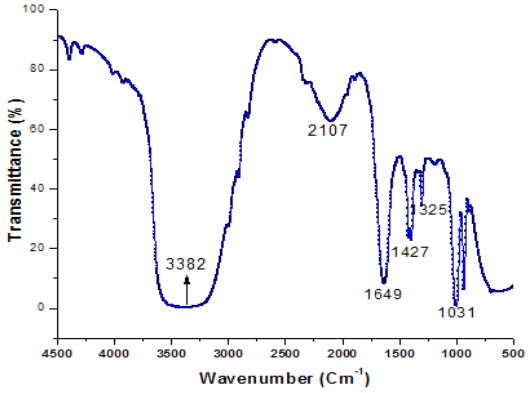

Figure 4: FTIR spectra of gold nanoparticles synthesized by the reduction of gold chloride.

nanoparticles, it was interesting to further extend the work towards biological investigations in order to establish that AuNPs could act as an effective drug carrier. In view of this regard, a comparative investigation was done on studying the microbial efficacy of drugs formulated with nanoparticles. For *in vitro* antibacterial activity, streptomycin and gentamycin along with their nanoformulation with gold nanoparticle were widely tested against resistant strains of *Staphylococcus aureus*, *E. coli* and *Pseudomonas aeruginosa*. The zone of inhibition for streptomycin formulated gold colloids for *Staphylococcus aureus* was more significant than gentamycin nanocolloids. Whereas, the zone of inhibition for both nanoformulated drugs against gram negative bacteria, *E. coli* (S ~30 mm and Gm~24 mm) and *Pseudomonas aeruginosa* (~20 mm and ~16 mm) were highly significant than its pure form (Figure 5 and Figure 6).

The increase in fold area was calculated based on zone of inhibition produced by drugs and its nanoformulation against the test bacteria (Table 1). In case of the drug streptomycin, it was observed that gram negative bacteria *E. coli* and *Pseudomonas aeruginosa* had the highest increase in the fold area (1.77 and 1.04) where as gram positive bacteria *Staphylococcus aureus* showed the lowest fold area (0.77).

It was understood that the gentamycin drug coated gold colloid was little effective for Gram negative organisms. In case of the drug gentamycin, it was observed that gram negative bacteria *E. coli* had the highest increase in the fold area (1.25), it was much higher in contrast with *Pseudomonas aeruginosa* (0.77). The gram positive bacteria *Staphylococcus aureus* showed the fold area (0.96) for the same drug, (Table 2). The gold nanoparticles did not produce any adverse or side effects on the microbial activities whereas a positive effect was only exerted over the system, i.e. while carrying the drugs, say for drug delivery, there was no negative effect imparted on the system due to the presence of gold. This property of gold nanoparticles as an efficient drug carrier was exploited for drug delivery systems.

DISCUSSION

The AuNPs synthesized using macroalgae showed characteristic peaks between 500-550 nm respectively, which is the confirmatory feature for gold nanoparticles formation due to the excitation of surface plasmon vibrations in gold nanoparticles.[15] Our findings were also supported by Chandran *et al.* (2006)[16] who reported that flat gold nanoparticles absorb the wavelength in the NIR region of the electromagnetic

Table 1: Zone of inhibition of Streptomycin formulated gold nanoparticles against bacteria.

Micro-organisms	Streptomycin (mm, mean)[a]		Increase in fold area[b]
	Std (A)	Au@Strep (B)	
Staphylococcus aureus	12	16	0.77
E. coli	18	30	1.77
P. aeruginosa	14	20	1.04

Table 2: Zone of inhibition of Gentamycin drug coated gold nanoparticles against bacteria.

Micro-organisms	Gentamycin (mm, mean)*		Increase in fold area[b]
	Std (A)	Au@Genta (B)	
Staphylococcus aureus	10	14	0.96
E. coli	16	24	1.25
P. aeruginosa	12	16	0.77

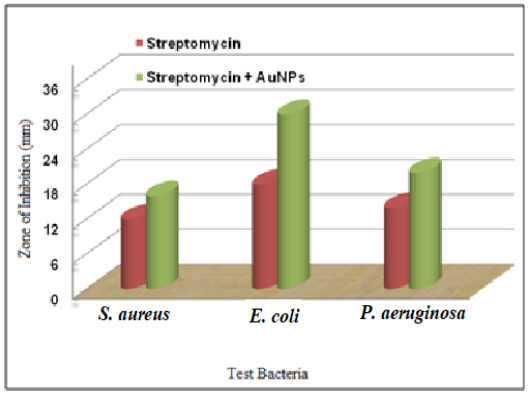

Figure 5: Graphical representation of zone of inhibition for streptomycin drug and drug mixed with AuNPs against the test bacteria

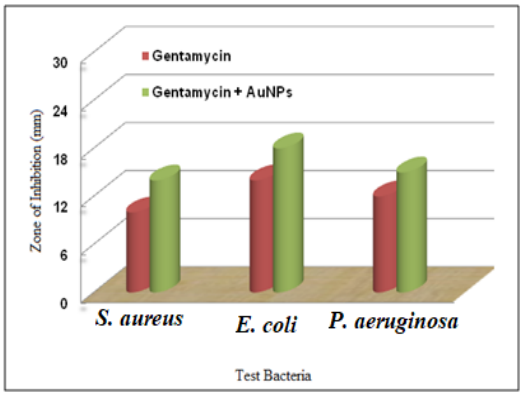

Figure 6: Graphical representation of zone of inhibition for gentamycin drug and drug mixed with AuNPs against the test bacteria.

spectrum, which corresponds to the longitudinal surface plasmon absorption. The stable synthesis of gold nanoparticle with narrow size distribution was obtained from macroalgae leaves extract, which was further confirmed through UV-visible spectroscopy and atomic force microscopy.[5] The findings were in agreement with other reports on the complete formation of nanoparticles synthesized using biological source.[17] This was the fastest method of biosynthesis in comparison to the earlier conventional studies with other micro-organism.[18] The anisotropic gold nanoparticles of different size were observed through electron microscopy. Representative TEM images and corresponding size distribution histogram of AuNPs were nearly spherical. The similar result was also observed by Rai *et al.* (2006)[19] in case of gold nanotriangle synthesis using lemongrass extract. Song *et al.* (2009)[8] has recently reported that control of the shape and size of metallic nanoparticles is very much required to enable the tuning of their optical, electronic, magnetic and catalytic properties. Singh *et al.* (2014)[13] have also confirmed that elemental silver and gold can be seen, which indicated the reduction of silver and gold ions to elemental silver and gold.

The probable pathway of biosynthesis was studied Shankar *et al.* (2004)[20] using Fourier transformed infrared spectroscopy. FTIR measurements were carried out to identify the potential biomolecules in macroalgae leaf responsible for reduction, capping and efficient stabilization of the bioreduced gold nanoparticles. From the results obtained and observations made it is clear that the biomolecules responsible for reduction and capping are different in gold nanoparticles. It is well-known that proteins can bind to Au nanoparticles through the free amine groups or carboxylate ion of amino acid residue in it.[21] Algal pigments, a kind of carotenoids rich in hydroxyl groups, could also have participated in the gold reduction.[22] These pigments have reductive properties and are released to solution by diffusion.[23] The increase in fold area was calculated based on zone of inhibition helped in comparing the efficiency of drug formulated with gold nanoparticles. The results showed that streptomycin and gentamycin nanoformulated drug were highly effective against *E. coli*. Both the drug and its formulations were least effective against gram positive bacteria. This could be explained based on the nature of the material present in cell wall. Thus, an easier permeability could be achieved in the case of Gram negative organisms.[24] We compared our findings with Singh *et al.* (2013)[10] and found that nanoparticle when formulated with drugs at same concentration was found to be more effective against the pathogens. This could be explained based on a single nanoparticle surrounded by a number of drug moieties and hence these drugs capped nanoparticle act as a single group against the microbial organisms. Despite the fact that the mechanism of interaction between nanoparticles and the constituents of the outer membrane of micro-organisms are still unanswered, it might be that the particles interact with the building elements of the outer membrane causing structural changes, degradation and finally cell death. In our opinion, the mechanism(s) of possible enhancement of the antibacterial activity of conjugates is still an open question and needs further study.

CONCLUSION

A critical need in the field of nanotechnology is the development of a reliable and ecofriendly process for synthesis of metallic nanoparticles. The synthesize of highly stable gold nanoparticles was confirmed through UV-visible spectroscopy. The broad size distribution in the size range of 8-20 nm in diameter and shape of the gold nanoparticle were further characterized using TEM. The EDX analysis confirmed the presence of elemental gold by the sharp signals. The probable pathway of biosynthesis was confirmed through Fourier transformed infrared (FTIR) spectroscopy. The drug formulated gold colloids were studied against both gram positive and gram negative micro-organisms. The study confirmed that fabricating gold nanoparticles with targeting agent such as conjugating a receptor specific biomolecules and a florescent probe would act as an efficient drug carrier and this could be exploited for drug delivery systems in the near future.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

HRTEM: High-resolution transmission electron microscopy; **EDX:** Energy-dispersive X-ray; **FTIR:** Fourier-transform infrared spectroscopy; **MTCC:** Microbial Type Culture Collection; **NIR:** Near Infrared.

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