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ORIGINAL ARTICLE

Novel synthesis of Streptomycin and Nystatin conjugated gold nanoparticles with potent antimicrobial activity

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ABSTRACT
Due to a sharp increase in case of fungi and multidrug-resistant bacteria worldwide, there is a great demand for the development of a new generation of antibiotics and fungicide agents to counter them. We have produced an effective antibacterial and antifungal agent as a substitute to the conventional drug discovery route by modifying presented commercial antibiotic (streptomycin) and fungicide (Nystatin), which is conjugated upon the surface of Gnps. In this study, bactericidal and fungicidal efficiency of gold nanoparticles (Gnps) conjugated with streptomycin and nystatin were evaluated. The conjugation of nanoparticles was confirmed by different characterization techniques such as UV-Visible spectroscopy, FTIR, Zeta potential, XRD and scanning electron microscopic studies. These antibiotics conjugated Gnps confirmed increased fungicidal and bactericidal action in the standard agar-disc diffusion assay. MIC (minimum inhibitory concentration) of the antibiotic and fungicide beside with their conjugated Gnps forms were determined in five bacterial and two fungal strains. Among them, in their conjugated form, streptomycin and nystatin showed significant reduction in the MIC values. Consequently, our results suggested that Gnps conjugated antibiotics and fungicides are more effective and could have significant therapeutic consequences.
Keywords:- chemical synthesis, Gnps, streptomycin, MIC, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT).

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INTRODUCTION

Bacteria have initiated the process of developing resistance mechanisms against various synthetic antibiotics since the discovery of the first antibiotics, Penicillin, in 1928. This is obvious from the existence of multiple resistant bacterial strains in the early 1930s and 1940s. In addition, extensive and irrational use of antibiotics worldwide has resulted to the creation of antibiotic resistant bacterial strains. A number of bacterial strains are reported to be resistant against multiple antibiotics, known as MDR (multi-drug resistant) bacterial strains [1].

An alternative strategy has to be used with the already well studied drug combined it with other molecules to improve antimicrobial activity to win the fight against new strains of bacteria. Nano materials have got tremendous applications in biotechnology over the past century [2].

Functionalized nanoparticles have been used to conjugate various drugs with the development of nanotechnology. GNPS was found to be least toxic to humans and thus widely used for this purpose among the various nanoparticles. In most cases, the conjugation took place through functionalized gold particles, where amino acids, glutathione, polyethylene glycol, etc. were used as functional agents [3]. But in order to prevent these agents and their potential impacts on biological system, they have been omitted, by directly conjugating GNPS with the antibiotics [4].

Conjugated GNPS were also used with antibodies and antibiotics for targeted photothermal killing of bacteria and protozoa [5, 6]. A stable vancomycin conjugated gold nanoparticles are produced and

showed a substantial increase in anti-bacterial activity as compared to free antibiotic activity [7]. A comparable results were recorded for Au/ SiO core/ shell nanoparticles conjugated with ciprofloxacin [8]. Our primary focus in this research is to assess the stability of gold nanoparticles conjugated antibiotics in comparison with their corresponding free form. Gold nanoparticles conjugated streptomycin and nystatin were screened for their efficacy on seven distinct microbial systems, the *Escherichia coli*, *Pseudomonas aeroginosa, Bacillus cereus, Klebsiella pneumoniae*, *Staphylococcus aureus, and two fungus Aspergillus niger and Candida albicans* for their efficacy. Their effectiveness has been better than their corresponding free forms at room temperature. Thus, in developing countries, where storage of medicine in rural regions is a large issue, these studies will have a tremendous impact.

MATERIAL AND METHODS

Tri-sodium citrate, Chloroauric acid, streptomycin and Nystatin were obtained from Hi-media Pvt. Ltd. All other chemicals and solvents used were of analytical grade.

Source and Maintenance of bacterial culture

Bacterial strains namely *Bacillus cereus* MCC 2039, *Pseudomonas aeroginosa* MCC 2080, *Esherichia coli* MCC 2246, *Staphylococcus aureus* MCC 2408 and *Klebsiella pneumoniae* MCC 2252 and fungal strains namely *Aspergillus niger* MCC 2180 and *Candida albicans* MCC 2162 , were obtained from the Culture Collection Center National Centre for Cell Science, Pune. The cultures were maintained by repeated subculturing on nutrient agar and potato dextrose slants. For every experiment 24 hour old bacterial cultures and 48 hour old fungal culture were used.

Preparation of GNPs

Gold nanoparticles (GNPs) were prepared by the reduction of chloroauric acid (H[AuCl4]) by tri sodium citrate. The normal reduction process was performed according to the turkevich method [4].

Preparation of conjugates

Antibiotic and fungicide conjugated gold nanoparticles were synthesized by mixing antibiotic, fungicide solution and gold nanoparticles in the ratio of 1:1 according to one pot synthesis method [9].

UV-VIS spectral analysis

Initially, the formation and stability of conjugated Gnps in aqueous solutions was characterized by a Multimode microplate reader (Thermo Fischer Scientific Varioskan Flash) within a wavelength range of 200–800 nm.

Zeta potential

A surface charge of synthesized Gnps was analyzed using a zeta potential analyzer (Beckman coulter DELSA Nano). The measurement of zeta potential is based on the direction and velocity of particles under the influence of known electric field [11].

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Scanning Electron Microscopy

The elemental and structural composition of the chemically synthesized gold nanoparticles was identified by Scanning Electron Microscopy (SEM, JEOL). For this lyophilized samples of gold nanocolloids were taken.

X-Ray diffraction studies

To peep into the crystallinity and the lattice properties of the Gnps, XRD at 2θ range from 20 to 80° using X-ray diffractometer (Rigaku, Smart Lab, Japan) equipped with Cu/Kα radiation source using Ni as filter at a setting of 30 KV/30 mA. The sample was prepared under high vaccum using freeze drying process.

Anti-microbial Activity

This assay was carried out using standard agar disks diffusion method. The bacteria namely *Escherichia coli*, *Pseudomonas aeroginosa*, *Bacillus cereus*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and two fungus *Aspergillus niger* and *Candida albicans* strains were grown at 37°C and 28°C overnight up to a turbidity of 0.5 Mac Farland level (10^8 CFU per ml) on nutrient Broth and potato dextrose broth media respectively [12]. Discs with diameter of 0.56 cm2 were dipped in conc. of 10 µg/ml solution of conjugated and placed on the culture lawn made on nutrient agar and potato dextrose agar, equi-distant from each other. Plates were then incubated at 37°C and 28° for overnight in case of bacterial culture and 48 hrs for fungal cultures respectively. Antibacterial and antifungal activities were evaluated by measuring the zone of inhibition. Throughout the experiment sterile water was regarded as a negative control whereas fungicide and antibiotic as positive control.

MIC Study

A loop full of bacterial and fungal strains from the slant was inoculated into nutrient broth and potato dextrose broth respectively and incubated for bacteria at 37 ° C for 24 hours and for fungi at 28 ° C for 2 days. The culture together with the test sample with the concentration range from 200µg-1.8µg in a final volume of 100 µL was transferred into each of three wells of a 96-well plate and incubated at 37°C and 28°C for 24 and 48 hrs respectively. After that, 5 µL of 4 mg/mL INT was added into each well. They was incubated at room temperature for 30 min. Plates were then observed for colour change. For positive control and for negative control streptomycin sulfate, nystatin(10µg/ml) and culture with solvent only were used respectively.. The same was done with streptomycin and nystatin for determination of MIC values against bacterial and fungal cultures [14].

RESULT

UV visible

The different reducing agent can be used for synthesizing gold nanoparticles like sodium borohydride and citrate. Here we have used tri-sodium citrate as a reducing agent. The primary colour of the solution mixture of tri sodium citrate and aqueous $HAuCl_4$ was colorless and light yellow respectively. The intensity of the color of reaction mixture changes to ruby red after 3h of reaction. This shows that there action medium behaves with time kinetics. The formation of Gnps was indicated by the colour change of the solution. At 530nm, the surface Plasmon resonance of free Gnps was obtained and the color turned from red wine to purple with a SPR at 550nm and larger particles were produced, when the antibiotics were conjugated with free gold nanoparticle [Fig 1]. Resulting this a red shift was analysed in surface Plasmon resonance by gold nanoparticles conjugated with antibiotics. This result also corresponds to gold nanoparticles which was synthesized from sodium borohydride [4].

Zeta potential measurement

Zeta potential values has revealed information regarding the surface charge and stability of biosynthesized Gnps. It was very much clear from the data obtainted that value of zeta potential of conjugated Gnps and free Gnps was same i.e., -20.80mV [Fig.2]. It indicates that the capping molecules present on the surface of GNPs are mainly comprised of negatively charged groups and also responsible for moderate stability of the nanoparticles which is not being influenced in conjugated Gnps [15].

Scanning Electron Microscopy (SEM)

SEM micrographs revealed the size and structural patterns of gold nanocolloids. The scanning electron microscopy (SEM) images of the synthesized gold nanoparticles are reported in [Fig 3]. which shows dispersed and nearly spherical nanoparticles with average diameter in the range from 50nm these results are similar to the gold nanoparticles synthesized from sodium borohydride [16]. It is very much clear from the data obtained that value of SEM micrograph of conjugated Gnps are some what different from the free Gnps as the conjugated gold nanoparticles are some what larger than that of free Gnps as seen in the Fig 3.

XRD

It is necessary to know the exact crystal structure of the formed gold particles, and this can be accomplished by measuring the sample XRD spectrum. The XRD peaks at 38.2°, 44.5°, 64.7° and 77.7°, respectively, can be indicated as (111), (200), (220) and (311) FCC (face-centered cubic) reflections of metallic gold (JCPDS no. 04-0784) shows the gold nanoparticles hence synthesized contains pure crystalline gold [Fig. 4]. It is very much clear from the data obtained that the conjugated Gnps are also composed of pure crystalline gold and of FCC structure. The most intense peak is of (111) as compared with other plane, hence it is predominating orientation, and that the synthesized Gnps are crystalline in nature. In previous almost same results have been analysed in AuNPs synthesized with tri sodium citrate [17].

Antimicrobial activity

After characterizing gold nanoparticles involving various techniques, gold nanoparticles were synthesized at room temperature and conjugation of antibiotic conjugated nanoparticles, were tested for antibacterial and antifungal activity against Gram-positive *(Staphylococcus aureus, Bacillus cereus*), Gram-negative (*Klebsiella pneumonia, Pseudomonas aeruginosa, Escherichia coli,*) bacteria and *Candida albicans* & *Aspergillus niger* fungus. All antibacterial and anti fungal activity tests were done three times. Streptomycin and nystatin were taken as positive control 10µg/ml concentration. Zone of inhibition were measured for all and it was found that the conjugated Gnps have significant antimicrobial and antifungal activity as compared to antibiotics and free Gnps [Table 1]. This result also corresponds to gold nanoparticles which was synthesized from leaf extract of sodium borohydride [16].

MIC

MIC (Minimal inhibitory concentration), lowest dilution at which the growth of the micro-organisms is inhibited, was evaluated for antibiotic, fungicide, Gnps and their conjugate in each bacterial and fungal strains. MIC (Minimal inhibitory concentration) for every conjugated Gnps with antibiotic and fungicide decreased significantly with respect to their free forms [Table 2]. These results are similar to the Gnps synthesized from sodium borohydride [18].

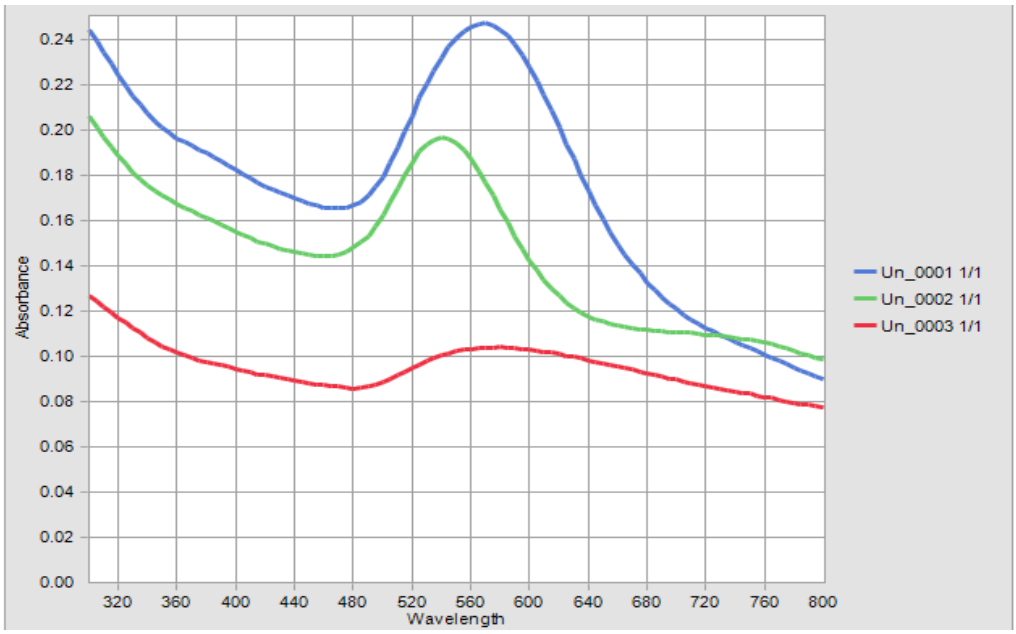


Fig. 1. Showing UV –visible absorption spectra

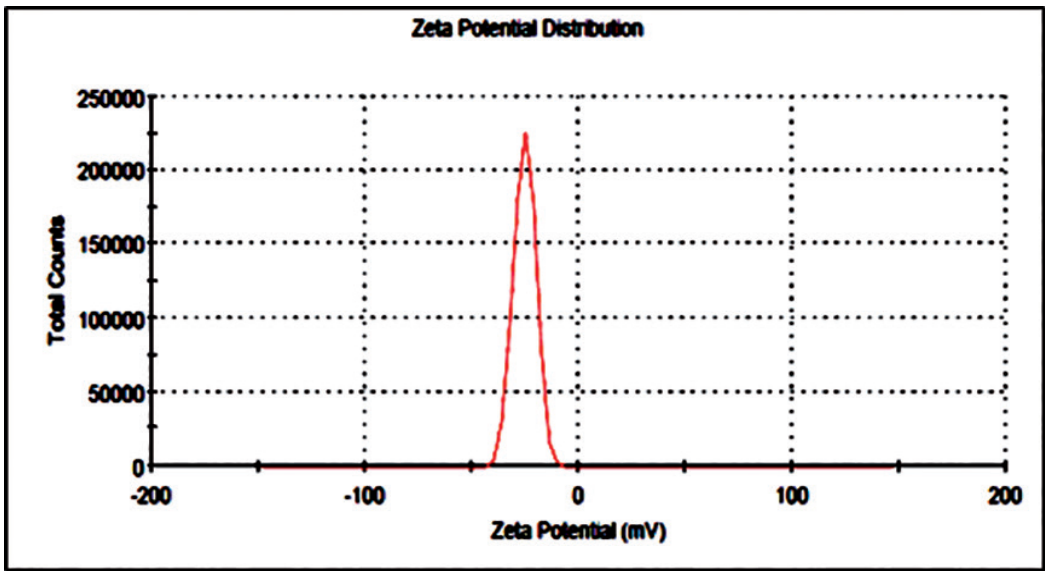
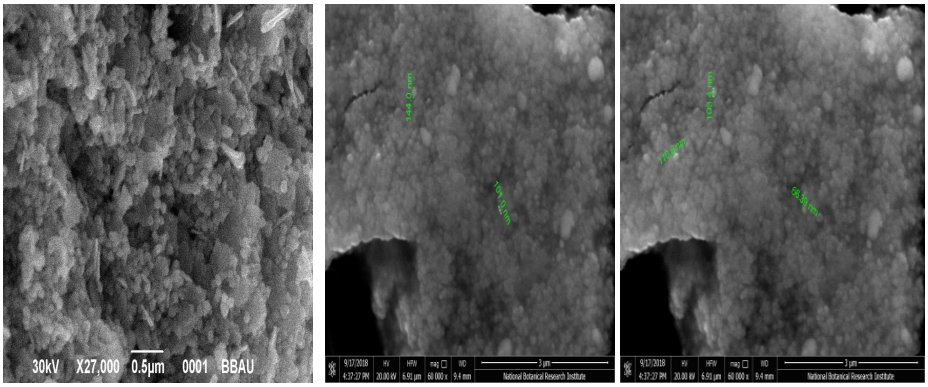


Fig 2 Zeta potential measurements



a. b. c.

Fig 3 Showing SEM micrographs of the gold nanocolloids A. Free Gnps B. Conjugated with streptomycin C. Conjugated with Nystatin

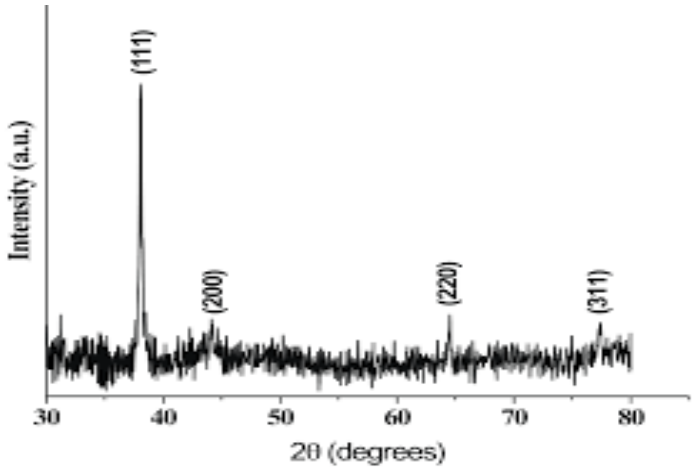


Fig 4 Showing XRD pattern of gold nanoparticles

Table 1:- Represents the zone of inhibition for free antibiotics and antibiotics conjugated with GNPs

Microbial strain	Positive control	GNPs	Conjugate
E.coli	36	34	32
B.cereus	32	30	29
S.aureus	34	30	33
K.pneumonia	33	31	30
P.aeruginosa	35	33	32
A.niger	22	15	13
C.albicans	20	14	12

Table 2:- Represents minimal inhibitory concentrations (MIC) for free antibiotic along with their respective GNPs conjugated form in five bacterial and two fungal strains

Microbial strain	Positive control	GNPs	Conjugate
E.coli	0.5	2.1	2.0
B.cereus	2.0	2.8	2.7
S.aureus	0.8	2.6	2.1
K.pneumonia	1.0	2.8	2.6
P.aeruginosa	0.6	2.7	2.1
A.niger	1.2	1.8	1.6
C.albicans	1.6	1.6	1.4

DISCUSSION

In the present work, the stability as well as the efficiency of Gnps conjugated with antibiotics and fungicides have been evaluated on various bacterial and fungal strains. Some nanoparticles are modified with functionalizing agents to combine different drugs for effective therapeutic use. The addition of functionalizing agents may sometimes interact with biological system so biodegradable agents are now being considered. For this a unique system was detected where addition of such agent can be avoided. Gnps conjugation with antibiotics and fungicide was evident through various techniques of characterization. Although there is no proof of the interaction mode between Gnp and antibiotics, it seems possible that antibiotics are adsorbed on the surface of the nanoparticles. The nanoparticles conjugated with streptomycin and nystatin show significant anti-microbial effects. The adsorption may be responsible for the reducing property of antibiotics. In addition, multiple antibiotics adsorbed on the surface of nanoparticles in different ways resulting in a specific structure, as observed in different studies [17]. The increase in antibiotic stability may be due to the close association between antibiotics and Gnp, which in turn increased antibiotic bond strength. Antibiotic conjugation may further enhance antibiotic concentration within the cells. This can be done by stronger nanoparticles providing the antibiotics. Another possibility of increased activity may be due to the presence of a powerful reduction agent, the tri-sodium citrate. To resolve this possibility, Gnps was washed three times after preparation had been conjugated. Thus in this situation, the stability of Gnps conjugated with antibiotics and fungicides significantly increased at room temperature. This is the most significant observation, added to recent research as these conjugated Gnps will not face any temperature abuse type conditions which is common in the case of drugs in rural areas.

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