Drug Loaded Cyanobacterial Nano-formulation: Preparation, Characterization and Bioactivity Evaluation

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Abstract: Doxycycline is a tetracycline antibiotic, but it poorly treats intracellular infections. This problem can be overcome by preparing and using the drug with eco-friendly bio-nano-formulations, which directly act on a target (intracellular) site. Drug loaded Bio-Nano-formulations have antibacterial effect better than the drug alone. Also, low dose of the drug is more effective than its higher dose. In the current study, the bio-nano-formulation was prepared using silver nitrate and alginate by polyelectrolyte method. The biological source: Cyanobacteria acts as a carrier between drug and silver nano-particle. Particle size analysis depicted the size of the nano-capsule which was further characterized using different techniques – UV-VIS spectroscopy, XRD, Fourier transform infra-red spectroscopy (FTIR), Scanning electron microscopy (SEM). Spherical shape of the particle was analyzed using transmission electron microscopy (TEM). Drug release at controlled rate (in-vitro) at different time intervals was also studied. Small size and high surface area of these nanoformulation(s) help them to act on a definite target site. Bioactivity evaluation of the bio-nano-formulations against microbial pathogens and the comparative evaluation study (zone of inhibition ranging from 1.2-5.4 cm) helped in characterizing these particles / bio-formulations as effective antimicrobial agents. These nano-formulations prepared in-vitro may serve the purpose to meet the challenge of acting directly on the target site and acting as a good medicinal agent to treat intracellular infections

Keywords: Cyanobacterial polymer, silver nanoparticles, Doxycycline, antimicrobial activity



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1. Introduction

Doxycycline (drug) has a potent antibacterial activity against wide range of bacteria. But its use is limited to curb intracellular infectious disease due to poor cellular penetration [1]. Also, its high dose has potent side effects. Siver Nanoparticles penetrate deep inside a cell and act on a definite target site; these silver nanoparticles have broadspectrum antibacterial effect [2]. Combination of Doxycycline and silver nano-particle reduces the side effects and high levels/dose of the drug to be used. This conjugate also helps to enhance the antibacterial activity of the drug in comparison to the usage of the drug alone. This combination treats intracellular infectious diseases in a better way [3]. In the present study, Cyanobacterial polymer acts as a carrier between the drug and silver nano-particle. This conjugation of cyanobacterial polymer, silver nano-particle and doxycycline drug makes this nano-formulation a better vehicle to improve the delivery, stability and efficacy of the drug [1]. The conjugation helpsin the sustained release of the drug so that low dose of drug gives more effect for a longer period of time. Controlled release formulation(s) [CRFs] are emerging in the field of Nanotechnology. With the use of CRFs, the related side effects and the high dose of the drug are reduced to a significant level. Premature release of the drug before reaching the target site is also prevented by these CRFs. In the current study, doxycycline drug was chosen as the active ingredient for encapsulation in nanocapsules. Drug loaded silver- alginate polyelectrolyte nanocapsule was prepared using cyanobacteria which acted as a carrier. Alginate is an anionic biopolymer, two step procedures for alginate-silver nanocapsules preparation was followed in which first step involved the formation of pregel on addition of calcium chloride to sodium alginate and the second step involved formation of polyelectrolyte complex between carboxyl group of alginate and free group of silver. Major problem with the use of doxycycline drug is its inability to act on a specific intracellular target site. Its great demand prompted scientists for its encapsulation in nanocapsules formed using polyelectrolyte method for its controlled release with minimum side effects.

2. Material and Methods

2.1 Chemical synthesis of silver nanoparticles

1mM concentration (8.5 mg) of silver nitrate was dissolved in 50 ml of distilled water. Citrate of sodium solution (1% tri-sodium citrate) was used as a reducing agent. 5ml of citrate of sodium solution was added drop wise in silver nitrate solution at 85° C on continuous stirring condition. Pale yellow colour appeared after four minutes of incubation in sodium citrate solution [4]. Silver nanoparticles were sized by using particle size analyser (PSA) where the size of silver nanoparticles recorded was less than 100nm.

2.2 Cyanobacterial Culture (Bio-Polymer) Preparation

BG11 media was freshly prepared for the growth and maintenance of cyanobacteria. Working culture was prepared from the stock culture by using approx. 2-3 ml of a

three week old cyanobacterial culture (maintained in laboratory) asaninoculum in 50 ml of autoclaved BG 11 medium in 150 ml Erlenmeyer flasks. Cultivation was carried out at 27±2°C, under continuous illumination of 8 g mol/m² by cool fluorescence lamps. Bulk growth was observed after few weeks. The cultures were further transferred to 500ml flasks for large scale cultivation. These cultures were finally harvested after 4-6 weeks. The cells were separated from the medium by centrifugation (4000rpm/10min) followed by filtration with what manfilter paper. Finally, the biomass was lyophilized and stored atof the cvanobacterial 20°C[5]. One samples:B6 cyanobacterial sample (Dr. Namita Singh's culture collection, GJUS&T, Hisar) depicting high amounts of protein/ lipo-peptides/pigments in the extraction medium was used in the present study which was purified through regular sub-culturing and micrography...B6 sample: unidentified--(0.1g of the lyophilized biomass was dissolved in 5ml PBS buffer) was used with silver nanoparticles and doxycycline drug in the present study.

2.3Preparation of Alginate solution with cyanobacterial sample (B6)

3mM concentration of sodium alginate (70mg) was dissolved in 20ml distilled water and 0.03M concentration of calcium chloride solution (45mg in 10ml) was added drop wise on continuous stirring to sodium alginate solution. To the above mixture, after 10 minutes, PBS dissolved B6 cyanobacteria solution was added drop wise. These solutions were used in 5:1:4respectively. The sample was kept on continuous stirring for 2-3 hours [5].

2.4 Conjugation of silver nanoparticles with cyanobacterial (B6) – Alginate (linker) solution

Add 5ml of B6 cyanobacteria sample-linker solution to 5ml of silver nanoparticle solution on continuous stirring for 3 to 4 hours. The sample was further analysed by UV-visible spectroscopy analysis [5].

2.5 Drug loaded-nano-formulation

2mM concentration of drug doxycycline (0.05g in 5 ml) was added drop wise to the above conjugated solution (silver nanoparticles with cyanobacterial linker solution)at continuous stirring for 3-4 hours.

2.6 Test Micro-organisms

The gram positive and gram negative strains used in the present study: *Staphylococcus aureus* NCIM 5021 (gram positive), *E.coli* MTCC-723 (gram negative) were provided from NCIM (National Collection of Industrial Microorganisms) culture collection, Pune and Institute of Microbial Technology, Chandigarh, India respectively. These microbial strains were cultured on nutrient agar slants and were maintained at 30° C.

3. Characterization

Analytical Assays

UV-visible spectral analysis

One of the techniques to structurally characterize metal nanoparticles is to use UV-Visible spectroscopy technique. This technique helps in confirming the color change and thus the formation of silver nanoparticles. In the present study, double beam spectrophotometer (Shimadzu, Model: UV-2450) was used to record the UV-Vis photo spectra of AgNPs (Silver Nanoparticles) in the range of 200–800 nm with respect to the substrate placed in the reference beam.

Particle size analysis

The average particle size of the doxycycline loaded alginatesilver nanocapsules was determined using the Zetasizer Nano ZS.

FTIR Analysis

Nanoparticles (AgNPs) were subjected to FTIR spectroscopy by Fourier transform infrared spectrophotometer (IR Affinity-1, Shimadzu, Japan) in range of 4000-450 cm-1 as K Br pellet. The resulting spectrum represents the molecular absorption and transmissions, creating a molecular finger print of the sample. Like a fingerprint no two molecular structures produce the same infrared spectrum

SEM/ TEM Analysis

The shape of the silver nano particles was examined by SEM experiments. JoelJSM-6480LVSEMmachine was used to characterize mean particle size, morphology of nano particles. The powder sample and freeze dried sample of Ag NP solution was sonicated with distilled water, small drop of this sample was placed on glass slide and allowed to dry. A thin layer of platinum was coated to make the samples conductive. Joel JSM-6480LVSEM machine was operated at a vacuum of the order of 10^{-5} torr. The accelerating voltage of the microscope was kept in the range10-20kV. For TEM analysis, samples of the aqueous suspension of silver nanoparticles were prepared by placing a drop of the centrifuged suspension on carbon-coated copper grids and allowing water to evaporate. TEM observations were performed on an H-600 electron microscope (Hitachi, Japan) operated at an accelerating voltage of 200 kV.

XRD Analysis

For XRD analysis, the phase evolution of calcined powder as well as that of sintered samples were studied (Philips PAN analytical, The Netherland) using CuK α radiation. The generator voltage and current was set at 35KV and 25mA respectively. Silver samples were scanned in the 2 θ ranges 15 to 70°C range in continuous scan mode. The scan rate was 0.04o/sec. Phases present in the sample were identified with the search match facility available with Philips Expert high score software.

Antibacterial assay

The antimicrobial susceptibility and comparative tests of the Nano-formulation and drug loaded nano-formulation were evaluated using well diffusion method. Zone of inhibition was measured after overnight incubation at 30° C. The correlative study was made using doxycycline drug as a positive control.

Drug release Studies

Known quantity of encapsulated drug sample 200µlwas dissolved in 800µl PBS (buffer). This solution wasincubated at 37^{0} C under gentle agitation. At each specified time period (0.5, 1, 2, 3, 4, 5, 6, 8,10,12,16,20and 24hours) the sample was centrifuged and the supernatant was collected and analysed by Nano-drop spectrophotometerat260nm.All measurements were performed in triplicates (n=3) for each formulation. The percentage of drug release at each time point was calculated:[1]

 $Drugrelease(\%) = \frac{\text{Drug in solution (\%ml)}}{\text{Initial drug in particles (\%ml)}}$

Kinetics and Statistical Analysis

4. Results and Discussion

Silver nanoparticles were synthesized according to the chemical reduction method.



Silver nanoparticles solution of sample B6 Figure 1: Silver nanoparticles solution

The colourless transparent solution was converted to pale yellow solution after the addition of tri-sodium citrate or stabilizing agent. The occurrence of colour indicated the formation of silver nanoparticles which appeared due to excitation of the nano-particle Surface Plasmon by absorption of visible light involving color alteration [5, 6-8, 14].

4.1 UV-Visible Spectral Analysis

Absorption peak of Silver nano-particle synthesized by chemical reduction method using tri-sodium citrate and silver nitrate was recorded which gave absorption peak from

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215-430 nm. The absorption peak at 418 shows the formation of silver nanoparticles. Strong peak at 418 nm for silver nanoparticles was in accordance with the previous reports/ studies on assorted metal nano-particles [15]. In the present study conjugated sample gave absorption peak from 580-794nm and showed the excitation of electron and changes of functional group after conjugation.

Table 1: Peak values depicting UV-Visible absorption of	•
nanoparticles and drug loaded silver nano-formulation:	

Sample name	Wavelength
	<i>(nm)</i> .
Silver nanoparticles	215-430
Doxycycline drug	207-413
Strain B6 and silver nanoparticles	345-384
Strain B6 and silver nanoparticles	580-794
and doxycycline drug	

4.2 Particle size analysis & their antimicrobial activity

Particle size of Ag NPs formed was 85nm in diameter. Shape of the particles and spherical structure of the particles can be controlled experimentally. The result (table 2) indicated that the average particle size of the synthesized silver nanoparticles was highly influenced by the reaction. The nano size of material results in specific physicochemical characteristics different than those of their bulk materials or larger particles. This effect is mainly credited to high surface-area-to-volume ratio, which results in increased reactivity thus increasing the efficacy of silver nanoparticles to have a better contact with the microorganisms leading to better antibacterial activity [16]; hence, the nano scale materials are more advantageous than their bulk counterparts.It is well known that silver ion nanoparticles are highly toxic to microorganisms. Silver nanoparticles have been known to have inhibitory and bactericidal effects and thus we extend its application as an antibacterial agent.

The biological activity of silver based materials, depending on their structure and physicochemical properties, affects the interaction with the cytoplasmic membrane of bacteria and influences cell metabolism. In our study, the antimicrobial activity of nano-silver-containing cyanobacterial films was investigated against E.coli and Staphylococcus aureus. The Antibacterial activity was estimated by recording the diameter of zone of inhibition [9]. The differences in the antimicrobial activity against Gram negative (E.coli) and Gram positive (S.aureus) bacteria was induced due to composition of the cell wall of these bacterial strains and also on hydrophilic and hydrophobic character of E.coliandS.aureus respectively. The mechanism of the bactericidal effect of silver and silver nanoparticles though not very clearly understood could be either due to attachment of silver nanoparticles to the surface of the bacterial cell membrane disturbing permeability and respiratory function of the cell or due to interaction of silver nanoparticles with the thiol groups of many enzymes thus inactivating them or possibly due to formation of free radicals [17, 18]. It is also possible that silver nanoparticles not only interact with the surface of membrane, but can also penetrate inside the bacteria [10] or possibly by interaction with phosphorus containing compounds like DNA disturbing the replication process. It may be observed that silver nanoparticles have comparatively higher anti-bacterial activity against gram negative organism than gram positive, probably due to thinner peptidoglycan layer and presence of porins[11].

Two aspects are to be considered:

- Mean Width of Zone of inhibition (Diameter, cm)
- Microorganism used:
- Escherichia coli MTCC 723

Staphylococcus aureus NCIM 5021



Figure 2: Antimicrobial efficacy of [A] Drug alone [B] Silver Nanoparticles [C] B6 linked Silver Nanoparticles [D] Drug loaded Silver Nanoparticles [E] B6 Cyanobacterial Sample (Negative control) against *E.coli*.



Figure 3: Antimicrobial efficacy of [A] Drug alone [B] Silver Nanoparticles [C] B6 linked Silver Nanoparticles [D] Drug loaded Silver Nanoparticles [E] B6 Cyanobacterial (Negative control) sample against *S.aureus*.

Average size of the nano-formulation and drug loaded nano-formulation is stated in the table below:

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Fable 2: Peak val	ues depicting ave	rage size of r	nanoparticle	es
and dru	o loaded silver na	no-formulati	on	

and drug fouded sirver huno formulation		
Sample name	Size (r.nm)	
Silver nanoparticles	85	
Doxycycline	71	
Strain B6 and silver nanoparticles	266	
Strain B6 and silver nanoparticles	374	
and doxycycline		

Antimicrobial efficacy:

 Table 3: Samples showing zone of inhibition, diameter in cms

• mild			
Sample	Zone of Inhibition, Diameter(cm)		
	E.coli	S.aureus	
Silver Nanoparticle	2	1	
Doxycycline (Positive Control)	3.4	2.8	
B6	No zone of inhibition	No zone of inhibition	
B6 and silver nanoparticles	1.2	0.8	
B6 and AgNPs and drug	5.4	4	

Polymeric silver nanoparticle with drug show their particle size range more than the silver nanoparticle, instead of the large size of polymeric particle they give better antimicrobial effect. The polymeric particle retain the silver nano particle property and show their synergistic effect.

4.3 FTIR Analysis

The peaks in the region 3422.30 assigned to O-H stretching of alcohol and phenol compounds and aldehyde -C-H-stretching of alkanes. The peaks in the region 1616.60 to 1406.23 and 1300 to 650 corresponds to N-H(bond) of primary and secondary amide and -C-N-stretching vibration of amines or -C-O-stretching of alcohols ,ethers , carboxylic acids and anhydrides.





Figure 4: FTIR Analysis of (a) Cyano-(B6)+silver nanoparticles and (b) Cyano (B6) +silver nanoparticles+ Drug (doxycycline).

The peaks in the region between 3859.95 to 3422.35 assigned to O-H stretching of alcohol and phenol compounds and aldehyde –C-H- stretching of alkanes. The peaks in the region 1631.14 to 1335.19 and 1300 to 650 corresponds to N-H(bond) of primary and secondary amides and –C-N-stretching vibration of amines or –C-O- stretching of alcohols ,ethers , carboxylic acids and anhydrides [5].

Table 4:	Stretching and	vibration	of functional	group of
	Nano	formulati	ons	

Frequency (cm ⁻¹)	Functional group
3200-3400	Alcohol/phenolstretching
2800-3200	C-H structure
1615	Alkene stretching
1450	$-CH_3$ (bend)
1000-1300	Alcohol, ether esters, carboxylic acid, anhydrides
650-1000	Alkene (out-of-plane bend)

4.4 SEM Analysis

This sample has smooth surface which gives size of nanoparticle in the range of 133.1nm on 62.1° and 102.8nm on 121.0° . Spherical, hexagonal, triangular forms of nanoparticle indicated the reduction of silver ions to silver metal.



Figure 5: SEM data of Cyano-(B6)+silver nano-particle +Drug

4.5 XRD (X-Ray diffraction) Analysis

The synthesized silver nano structure confirmed by the characteristic peaks observed in the XRD image is shown in Figure 6. All diffraction peaks correspond to the characteristic face centered cubic (FCC) silver lines. These diffraction lines are observed at 2θ angle 20.2^{0} , 27.6^{0} , 32.0^{0} and 38.0^{0} respectively. XRD patterns were analyzed to determine peak intensity. A mixed phase of cubic and hexagonal structures of silver nanoparticles was shown and revealed by XRD. The crystallite size was determined from

X-ray line broadening using the Scherer's equation as follows:

 $D = 0.94 \lambda / \beta \cos \theta$

Where, D= crystallite size,

 λ = wavelength of the radiation,

 θ = Bragg's angle (diffraction angle)

 β = full width at half maximum of peak



Figure 6: XRD data of Doxycycline(drug) loaded cyanobacterial silver nano-particle

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4.6 TEM Analysis

TEM images show that the synthesized silver nanoparticles are poly-disperse .The shape of the nanoparticles are spherical with few exceptional as ellipsoidal, triangular, hexagonal. From Figure.7 it is found that increasing concentration in reaction mixture reduces the particle size and also their agglomeration tendency.



Figure 7: TEM data of drug loaded silver nano-particle

4.7 Drug release studies

The amount of the drug (unbound drug) in the supernatant was determined with the help of UV-Visible at 260 nm. Concentration and precentage of drug release were calculated using standard curve equation:

y = 0.024x - 0.001

 $x = \text{concentration} \; \mu g/ml$

y = absorbance

Drugrelease(%)

= <u>Drug in solution (%ml)</u> <u>Initial drug in particles (%ml)</u>

Initial drug concentration= 238 μ g/ml The standard drug release in μ g/ml at different interval:

Percentage (%)) release	of drug
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Time	% Release	
30 min	0.15	
1hr	0.22	
2hr	0.31	
3hr	0.415	
4hr	0.74	
5hr	2.07	
8hr	9.5	
10hr	14.7	
12hr	20.02	
16hr	24.08	
20hr	30.00	
24hr	34.01	

The doxycycline is the broad spectrum antibiotic drug. The encapsulated drug (doxycycline) with cyanobacterial polymeric nanoparticles showed 34% release of drug after 24 hr. Thus study demonstrated encapsulated cyanobacterial polymeric nanoparticles are good alternative as a carrier for delivery of drug at controlled rate.**[12-13]**.

5. Conclusion

The physical and chemical methods of metal-nano-particle synthesis are expensive and involve incorporation of toxic chemicals thus metal nano-particle formation using biological sources owing to their ease of availability, quicker synthesis and non-toxic nature find better application in metal-nano-particle synthesis. In the present study, the encapsulation of antibiotic drug (doxycycline) with Cyanobacterial polymeric (Cyano-B6) silver nanoparticles conjugate was characterized by antimicrobial activity and drug release studies. The diameter of zone of inhibition in the antibacterial assay of the drug loaded cyanobacterial polymericnano-particle conjugate was more than the cyanobacterial silver nano-particle conjugate alone. From the study it is suggested that the side effects of drug was minimized due to encapsulation of drug with cyanobacterial silver nano-particle polymer and also because the encapsulated cyanobacterial- drug conjugate gave same antibacterial activity even at lower concentrations. Also, it was observed that the conjugation of drug with cyanobacterial silver nano-particle polymer resulted in slow release of drug producing the same effect for longer time. This formulation was stabilized against various pathogens and hence may be applicable for medicinal use. So this formulated conjugate/ drug loaded silver nano-particle attached cyanobacterial formulation can find it's utility in various fields such as- biomedical, pharmaceuticals etc.

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