



Formulation and Evaluation of Azathioprine Loaded Silver Nanoparticles for The Treatment of Rheumatoid Arthritis

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ABSTRACT

The present study deals with formulation and development of silver nanoparticles using green approach based on polysaccharides as reducing and stabilizing agent. In this study chitosan stabilized silver nanoparticles were prepared and azathioprine was conjugated with silver nanoparticles to treat the inflammation in rheumatoid arthritis. The azathioprine loaded silver nanoparticles were characterized by UV-Visible Spectrophotometry, FT-IR, Scanning electron microscopy and Zeta potentiometer. The absorption maxima of the silver nanoparticles was 420nm. SEM images of Azathioprine Silver nanoparticles showed spherical particles in the range of 180nm to 220nm. An *in vitro* drug release study was carried out and percentage drug release was found to be 67.34% at the end of 24 hours for formulation F2. *In vitro* toxicity of azathioprine loaded silver nanoparticles was studied in 3T3 NIH fibroblast cell line. The formulation plays a dual role, to target the diseased site and to release the drug in a controlled manner and produces synergistic effect to the inflammatory sites.

Keywords: Azathioprine, Chitosan, Silver nanoparticles, Rheumatoid arthritis.

1. INTRODUCTION:

An ideal drug delivery system should deliver the drug at a rate dictated by the needs of the body over a specified period of treatment.¹ To reach a desired target; drug can be chemically modified using prodrug approach or use a carrier system. There are two general classes of carrier system: a. soluble carrier systems (With these systems the drug is conjugated to the carrier). b. Particulate Carrier systems (Here the drug is either surface bound or entrapped within the carrier Ex: liposomes, microspheres and nanoparticles.)²

Nanoparticles are small colloidal particles that are made up of non-biodegradable and biodegradable polymers and their diameter is around 1nm to 1000nm. Nanoparticles possess large surface area and their surface to mass ratio is extremely high compared to other particles. The nanoparticles are able to bind, adsorb and carry other

compounds such as drugs, probes and proteins due to large surface area. Nanoparticulate carrier system permits entrapment /encapsulation of therapeutics without modification, as it requisite for polymer drug conjugates. Both metallic and polymeric nanoparticles are used to encapsulate drugs within the solid core. The use of metals can yield multifunctional nanoparticles whereby both therapeutic delivery and imaging are facilitated.³

Metallic nanoparticles have fascinated scientists for over a century and are now heavily utilized in biomedical sciences and engineering. Silver nanoparticles (AgNPs) have emerged as an arch product from the field of nanotechnology. Silver has gained interest over the years because of distinctive properties, such as good conductivity, chemical stability, catalytic and antibacterial activity.⁴

Nano Silver may have different shapes, such as spheres, rods, cubes etc., at nano scale, the silver particles exhibit deviating physico-chemical properties and biological

activities compared with the regular metal. This is due to the higher surface area per mass, allowing a large amount of atoms to interact with their surroundings. Due to the properties of silver at the nanoscale, nano-silver is now a days an increasing number of consumer and medicinal products.

Silver NPs have become efficient vehicles to store and deliver medicines. They are used for the controlled release of drugs, and albumin-drug complexes, where NPs acts as a carrier of drugs and liberate them on a selective basis, at the right speed and in the intended environment within organism. They can be used as magic "bullets" that go directly to cells of a particular tissue.⁵

Silver has been now recognized as a developing therapeutic molecule and will surely extend its use as a drug carrier. Silver nanoparticles can be used for both active and passive targeting of drugs. Silver nanoparticles have recently emerged as an attractive candidate for delivery of various payloads into their targets. Use of nanoparticles as a carrier has become widespread, but yet another aspect of nanoparticles that needs attention is their resplendent property of action as a drug by itself.⁷

In this study green synthesis of AgNPs was done using nontoxic chitosan as both reducing agent and stabilizing agent. Chitosan was chosen because of their potential properties. It is derivative of polysaccharide chitin, has large number of amino group and hydroxyl groups in chitosan chains offering unique physicochemical properties including the polycationic, chelation, film forming etc., Chitosan played a role of reductant and the reduction of Ag⁺ was coupled to the oxidation of the hydroxyls groups in molecular CS and its hydrolyzates. The reducing ability of chitosan depends also on concentration and reaction temperature.⁸

Azathioprine (AZA) is primarily an immune suppressive agent, used mainly in allotransplantation procedures. It is also used in systemic anti-inflammatory states, such as rheumatoid arthritis, lupus erythematosus, polymyosites and Crohn's disease. It is chemically known as 6-[(1-methyl-4-nitroimidazol-5-yl) thiol] purine and is official in the United States Pharmacopeia and British Pharmacopeia. Azathioprine has been used in the treatment of rheumatoid arthritis since 1964. Azathioprine has been shown to be an effective agent in the treatment of active rheumatoid arthritis (RA). The main objective of the study is to use chitosan as reducing and stabilizing agent for reducing silver nitrate to silver nanoparticles. Then the prepared silver nanoparticles were conjugated with azathioprine for the treatment of Rheumatoid arthritis.^{9, 10, 11}

2. MATERIALS AND METHODS

Preparation of Chitosan Stabilized Silver nanoparticles:¹²

0.1% chitosan solution was preheated at 80°C±2°C for 30 minutes. Aqueous solution of 0.1M Silver nitrate was added to the above solution and kept on magnetic stirrer for 15 minutes to achieve uniform mixing. Beaker containing the solution was transferred into a microwave oven.

The microwave power was kept constant at 840Watts and any colour change was observed at 30 seconds interval. Time required for reduction of silver nitrate in chitosan solution to obtain silver nanoparticles was optimized. Yellowish brown colour confirms the formation of silver nanoparticles and analyzed by UV- Visible Spectroscopy. Colloidal silver nanoparticles exhibit absorption at wavelength from 380-420nm due to Mie scattering Theory.

Separation of Silver Pellets:

The centrifugal force was used to separate silver pellets. The centrifugal force was applied at 5000, 10000, 15000 rpm for different time periods. The speed and time intervals were optimized.

Formulation of Azathioprine Loaded AgNPs:¹³

10mg, 20mg, 30mg, 40mg, 50 mg of Azathioprine was accurately weighed and added to the dispersion of Silver nanoparticles. The mixture of Azathioprine and AgNPs solution was then incubated for 24 hours under stirring. Then the resulting solution was centrifuged at 10,000 rpm for 60 minutes. The pellets thus obtained was separated from supernant solution and redispersed in distilled water for further characterization. The free drug present in the supernant was analyzed by UV-Visible spectroscopy.

Drug loading Efficiency:¹⁴

Surface adsorption of Azathioprine on the silver nanoparticles was determined by measuring the amount of free drug present in the supernant.

Drug loading efficiency of Nanoparticles is calculated by the following formula,

$$\text{Loading efficiency} = \frac{\text{Total amount of Drug} - \text{Free drug}}{\text{Total amount of drug}} \times 100$$

Total amount of drug

In-vitro drug release characteristics:

In vitro drug release was carried out using an open ended tube method. One end of the open ended tube was tied with cellophane paper previously soaked in glycerin for about 20 minutes which acts as a semi permeable membrane. The open ended tube was fixed in a stand and end tied with cellophane was immersed in 100 ml of Phosphate buffer pH 7.4 at 37°C ± 0.5°C and stirred at 50rpm. Azathioprine loaded AgNPs was transferred into the tube. Drug release was assessed by withdrawing 5 ml of the sample at 15-minute interval for first hour and one hour intervals for 8 hours and finally at 24th hour. The withdrawn (5ml) samples were transferred into 100ml

standard flasks and made upto volume with Phosphate buffer pH7.4. The resulting solutions were analyzed by measuring the absorbance at 280nm using UV Visible spectrophotometer.

CHARACTERIZATION:

Scanning electron microscopy:

The particle size of the optimized nanoparticles was measured and photographed using scanning electron microscope. The nano suspension of AgNPs was diluted 10 fold with millipore water and transferred to a glass slide which was cut in the diameter of 20×20mm. The slide was mounted on an aluminum stub using double sided carbon tape. The solution was slowly evaporated at room temperature. The completely dried sample was coated with gold by sputter coating unit at 10 Pascal vacuum for 10 seconds to a thickness of 100 Å using HITACHI evaporator. The image was captured on SEM mode at desired magnification.

Fourier Transformation Infrared Spectroscopy:

FTIR spectrum of Azathioprine and Azathioprine loaded silver nanoparticles were recorded using Nicolet Fourier transformation Infrared spectroscopy (FT-IR) combined to PC (with spectrum 2000 analysis software) in the range of 4000 cm⁻¹ to 400 cm⁻¹. The pellet was placed in the light path and the spectra were analyzed.

Zeta Potential:

The zeta potential of optimized Silver nanoparticles were determined by Malvern Zetasizer.

Stability Studies: ¹⁵

In order to study the stability of the AgNPs, Physical appearance, absorption maxima and zeta potential of the prepared AgNPs were measured. Azathioprine Loaded silver nanoparticles were stored in amber colour bottles, covered with aluminum foil and maintained at accelerated condition 40°C±2°C/75%±2% RH for a period of 3 months. The samples withdrawn at the end of every month were tested for colour change, absorbance and zeta potential.

3. RESULTS And DISCUSSION

Colour change of Optimized Concentration of Silver Nitrate during microwave irradiation:

Colour change is an important criterion for reduction of silver nitrate to silver nanoparticles. Chitosan solution was clear & Silver nitrate is soluble in water and remained transparent. While adding 0.1M silver nitrate to the 0.1% chitosan solution, the colour change was noted (Fig:1). Silver nanoparticles were formed at end of 2minutes 30 seconds.



Figure 1: Colour change of AgNPs at 30seconds interval during Microwave irradiation

Absorbance of Silver Nanoparticles under Microwave irradiation technique:

The Chitosan reduced silver nanoparticles were analyzed by UV-Visible Spectrophotometer by taking samples at 30 seconds interval. At the end of 2 minutes 30 seconds colour turns into yellowish brown and λ_{max} was 425nm (Figure 2). It shows the formation of stable silver nanoparticles.

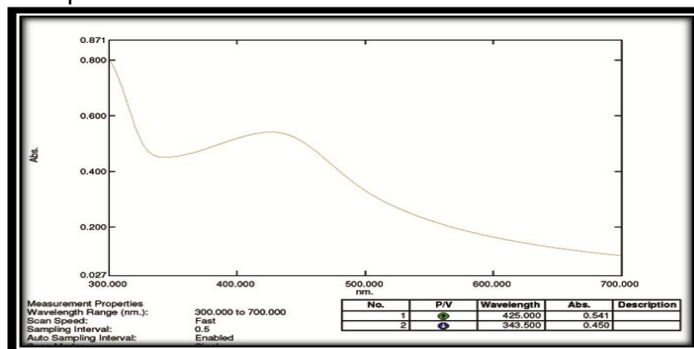


Figure 2: λ_{max} of Silver Nanoparticles using Microwave Technique

Drug Loading Efficiency:

S. No.	Formulation	Amount of Drug	% Drug Loading
1.	F1	10	78.3 %
2.	F2	20	81.95 %
3.	F3	30	54.45 %
4.	F4	40	47.73 %
5.	F5	50	41.0 %

Table: 1 Optimization of drug loading efficiency

The principle of drug loading on Silver Nanoparticles is surface adsorption. Increasing quantity of drug was added to Silver nanoparticles. When 10mg was added the percentage drug loading was 78.3%. When 20mg was added the percentage drug loading was 81.95%. When the drug quantity was increased further the percentage drug loading decreases. This indicates the saturation of the surface. The percentage drug loading efficiency is shown in table: 1.

Scanning Electron Microscopy:

The external morphology and shape of AgNPs was identified by SEM image. The size of AgNPs was 128nm & the size of Azathioprine loaded AgNPs was found to be in the range of 180 to 238 nm. The drug loaded at the

surface of AgNPs was confirmed by SEM image shown in Figure 3.

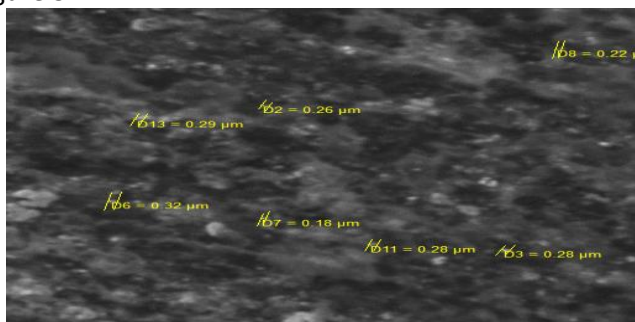


Figure 3: SEM image of Azathioprine loaded Silver nanoparticles

Separation of Silver pellets:
The silver pellets were removed using centrifuge at 15000rpm for 90minutes.

Zeta Potential of Azathioprine loaded Silver nanoparticles:

The Zeta potential of azathioprine loaded AgNPs was found to be -29.06. Hence the formulation stability was found to be "good". Zeta potential of Azathioprine AgNPs are shown in Figure 4.

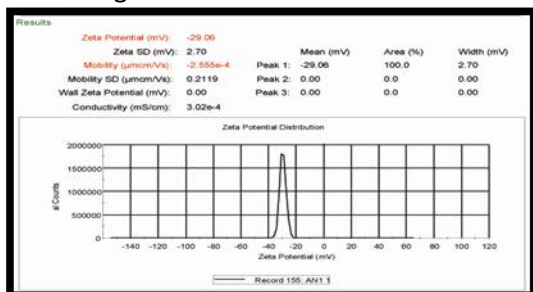


Figure 4 Zeta Potential of Azathioprine loaded Silver nanoparticles

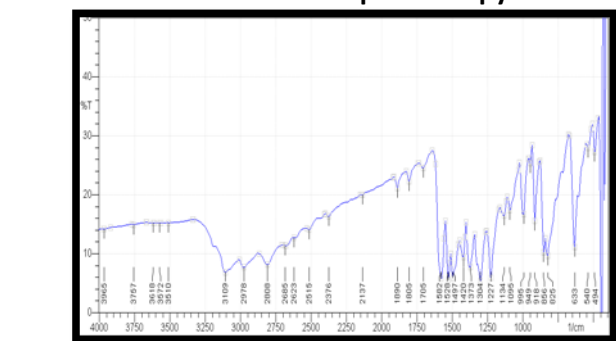


Figure 5: FTIR Spectrum of Azathioprine

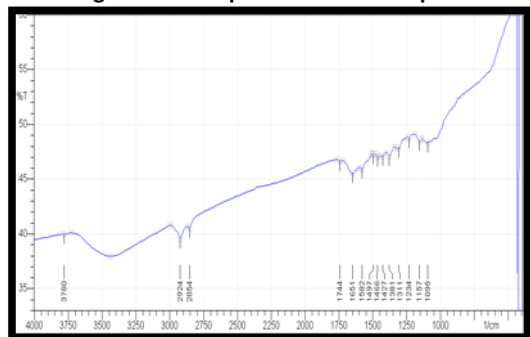


Figure 6: FTIR Spectrum of Chitosan

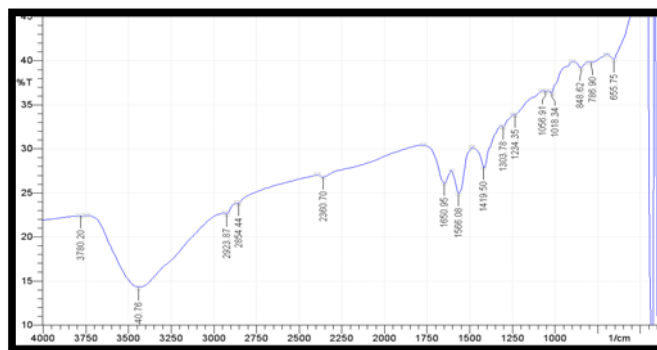


Figure 7: FTIR Spectrum of Azathioprine Loaded AgNPs

FTIR spectra of Azathioprine, Chitosan and Azathioprine Loaded AgNPs are shown in the Figures 5, 6, 7. FTIR spectrum of chitosan shows a broad peak of O-H Stretching at 3440cm^{-1} which is due superposition of O-H band over the weak N-H band. In Azathioprine AgNPs, there is no significant change of peaks compared with Azathioprine. However a broad peak was observed at 3440cm^{-1} which may be due to the presence of Chitosan in the formulation.

In vitro Drug Release Studies:

In vitro drug release of optimized formulation F2 was carried out in Phosphate buffer pH 7.4. The Percentage cumulative drug release is shown in Table 2 & Figure 8.

S. No.	Time in Hours	Cumulative Percentage drug release*
1.	0	0
2.	1	13 ± 0.308
3.	2	15.23 ± 0.676
4.	3	17.68 ± 0.9699
5.	4	18.80 ± 0.4596
6.	5	21.08 ± 0.9616
7.	6	25.79 ± 0.7848
8.	7	27.94 ± 0.1697
21.	8	29.06 ± 0.9885
22.	24	67.22 ± 0.2828

*Mean±SD (n=3)

Table 2: *In Vitro* drug release of optimized formulation

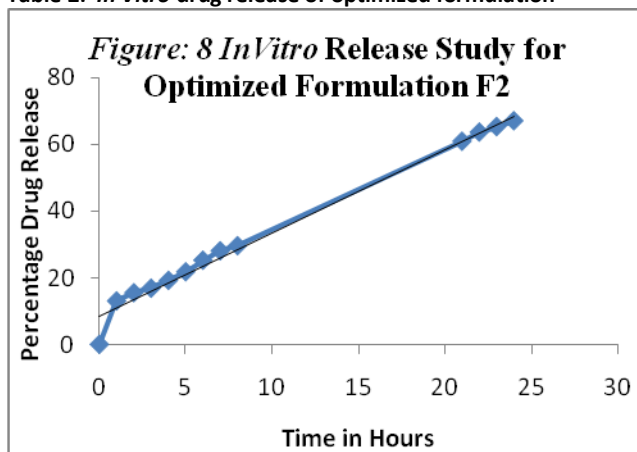


Figure: 8 *In Vitro* Release Study for Optimized Formulation F2

The drug release of Azathioprine loaded AgNPs (F2) was fitted into various kinetic models to find out the mechanism of drug release. Among this the highest correlation coefficient was found to fit the First order equation in which the regression value is $R^2 = 0.995$. The slope of Korsmeyer peppas equation plot is more than 0.5 indicating the diffusion is anomalous diffusion (non-fickian).¹⁶

Stability Studies of Azathioprine AgNPs:

The Azathioprine AgNPs at accelerated condition ($40^\circ\text{C}\pm 2^\circ\text{C}/75\%\pm 2\% \text{ RH}$) were found to be stable and it was confirmed by UV –Visible Spectrophotometer and Zeta Potentiometer. The Stability study data is shown in the table: 3

Parameters	Initial	After 1 st month	After 2 nd month	After 3 rd month
Colour	Yellowish Brown Colour	Yellowish Brown Colour	Yellowish Brown Colour	Dark yellowish brown colour
Absorption maxima(nm)	425	431	442	456
Zeta Potential	-29.06	-28.51	-28.12	-27.13

Table: 3 Stability studies of Azathioprine AgNPs

5. CONCLUSION

On microwave irradiation technique the Stable silver nanoparticles were prepared by optimizing microwave time .At the end the 2 minutes 30 seconds colour change was noted and silver nanoparticles were formed. The optimized centrifugal speed of 15,000rpm for 90 minutes was used to separate silver pellets. Maximum drug loading efficiency was determined by centrifugation as analyzed by UV Spectroscopy & the optimized formulation was found to be F2. Scanning electron microscopy showed AgNPs were uniform in size and spherical shape. The size range of Azathioprine AgNPs was found to be 180 to 220 nm. *In vitro drug* release of the optimized formulation F2 was found to be 67.22% at the end of 24 hours. The stability studies at the end of 3 months showed no change in physical appearance, Absorption maxima and zeta potential. This formulation will produce synergetic effect to treat the inflammation in rheumatoid arthritis efficiently and release the drug in controlled manner to target the inflammatory site.

6. ACKNOWLEDGEMENTS

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Conflict of Interest: None Declared

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