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# Synthesis, Characterisation of Azathioprine Loaded Chitosan based Nanoparticles


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**ABSTRACT**

*Chitosan (CS) nanoparticles were prepared by ionic gelation technology, and then used for trapping Azathioprine. Combination drugs were encapsulated into CS nanoparticles as a result of electrostatic interactions, which was confirmed by Fourier transform spectroscopy (FTIR). Efficient encapsulation efficiency (EE) and loading capacity (LC) were achieved which were correlated to their initial drug concentration. The drug free CS nanoparticles are roughly spherical in shape with a size distribution range of 39.4 to 59.4 nm in diameter, drug loaded CS nanoparticles is in the range of 700 to 806 nm. The zeta potential of the chitosan nanoparticles is 42.7 mV, while zeta potential of the Azathioprine loaded chitosan nanoparticles is 50.6 mV. The drug loading capacity of Azathioprine loaded chitosan nanoparticles was 32.2%.*


**KEYWORDS : Nanoparticles, Chitosan, Azathioprine**

## Introduction

The potential use of nanoparticles as drug carriers has been represented over the last few years as an important challenge, since nanoparticles have been designed to improve the pharmacological and therapeutic effects in terms of reducing their toxic side effects [1, 2]. Nanoparticles as drug carriers not only allow the continuous and controlled release of therapeutic drugs to maintain drug levels within a desired level but also include localizing and specifically targeting the drugs to their intended tissues and cells, thereby decreasing drug doses and improving patient compliance [3]. Recently, chitosan has attracted a great attention in pharmaceutical and biomedical fields because of its advantageous biological properties, such as biodegradability, biocompatibility, and nontoxicity [4, 5]. Chitosan is a cationic polysaccharide which is obtained by partial deacetylation of chitin. In contrast to other polymers, chitosan is a hydrophilic polymer with positive charge, which reveals a special characteristic to chitosan from the technological point of view [6]. The process for production of chitosan nanoparticles depends on the approach on ionic gelation, where nanoparticles are formed by means of electrostatic inter actions between the positively charged chitosan chains and polyanions employed as cross-linkers like tripolyphosphate (TPP). Tripolyphosphate (TPP) has been popular because it is nontoxic, has the ability to gel quickly, and interacts electrostatically with cationic chitosan. Recently, there has been a substantial interest on chitosan nanoparticles since production process is simple and mild. Chitosan nanoparticles, which are launched as promising carriers for controlled-release drug delivery, are exploited extensively in the pharmaceutical industry [7, 8, 9, 10].

Azathioprine is a purine antimetabolite drug and pro-drug of 6-mercaptopurine. It has more marked immune-suppressive effect than the anti-neoplastic effect. Azathioprine (Aza), or 6-[(1-methyl-4-nitro-1H-imidazol-5-yl)sulfanyl]-7H-purine, is an immunosuppressive drug administered to prevent the rejection of new organs following a transplant operation [11]. Because there is always a chance that the body will try to reject new donor tissue, Aza helps to prevent this rejection by suppressing the body's immune or defense system. It is also used in some auto-immune illnesses (e.g., rheumatoid arthritis and pemphigus), inflammatory bowel diseases (e.g., Crohn's disease and ulcerative colitis) and multiple sclerosis. Side effects are uncommon, but may include nausea, fatigue, hair loss and rash. Because Aza suppresses the bone marrow, patients will be more susceptible to infection. Unfortunately, its use is limited due to its associated high toxicity [12,13]. Azathioprine has been used in the treatment of auto-immune diseases, such as rheumatoid arthritis, or in combination with cyclosporine and corticoids to prevent rejection after transplantation, inflammatory bowel diseases and multiple sclerosis. However, Azathioprine may cause some serious adverse effects, such as bone marrow suppression, acute and chronic hepatotoxicity, and interstitial pneumonitis. Therefore, it's necessary to make some attempts to reduce its toxic and side effects and improve the specificity and selectivity. Hence, in the present study, we aimed in synthesis and characterisation of TPP-crosslinked Azathioprine encapsulated chitosan nanoparticles.

## Materials and Methods

Chitosan, was purchased from Sigma-Aldrich (Low Molecular Weight). Sodium tripolyphosphate (TPP) (purity: 85%), Azathioprine (purity: 99%), Tween 80 was purchased from Sigma-Aldrich Chemical Co. Ltd. All other reagents were in analytical grade.

**Preparation of Chitosan Nanoparticles.**
Chitosan nanoparticles were produced based on ionic gelation of TPP with chitosan[10]. Chitosan was dissolved in 1% (v/v) acetic acid solution to make up chitosan concentrations at 1.00 (mg/mL). Tween 80 (Sigma, Germany) (0.5% (v/v)), as a resuspending agent, was added to chitosan solutions in order to prevent particle aggregation, and then chitosan solutions were adjusted to pH 4.6–4.8 with 1N NaOH. TPP was dissolved in distilled water to maintain TPP solutions of 0.50 (mg/mL). All solutions were filtered through whatmann filter paper. Prepared chitosan solution were flushed mixed with TPP solutions with a volumetric ratio of 2.5: 1 (v/v) (chitosan : TPP) under magnetic stirring at room temperature. The formation of chitosan-TPP nanoparticles started via the TPP-initiated ionic gelation mechanism. Nanoparticles were purified by centrifugation at 12000 g for 30min. Supernatants were discarded and resuspended in water, and the chitosan nanoparticles were then freeze-dried before further use or analysis.

**Dynamic Light Scattering (DLS) Analysis.**
Particle size distribution of chitosan nanoparticles was analyzed through DLS with Zetasizer Nano S (Malvern, UK). The analysis was performed in triplicate at a temperature of 25°C.

**Preparation of Azathioprine Encapsulated Chitosan Nanoparticles.**
Chitosan was dissolved in 1%(v/v) acetic acid solution to maintain chitosan concentration at 0.75 (mg/mL). Prepared chitosan solutions were mixed with Azathioprine solutions (Aza dissolved in DMF), and 1.0mg/mL Aza containing chitosan solutions were maintained. Tween 80 (Sigma, Germany) (0.5% (v/v)) was added to chitosan solutions, and pH was adjusted at 4.6–4.8. Prepared Aza-containing chitosan solution were flushed mixed with 0.5mg/mL TPP solutions with a ratio of volume ratio of (2.5: 1) (v/v) (chitosan: TPP). The nanoparticle suspension was gently stirred for 30min at room temperature to allow excess Azathioprine adsorption on the nanoparticles to reach isothermal equilibrium. Aza encapsulated chitosan nanoparticles were centrifuged at 12000 g for 30min, resuspended in water and freeze-dried used for further analyses. Prepared Aza encapsulated chitosan nanoparticles were analyzed by Zetasizer Nano S (Malvern,UK) in order to determine mean average particle size distributions.

**Scanning Electron Microscopy (SEM).**
The morphological characteristics of both chitosan and Azathioprine encapsulated chitosan nanoparticles were examined by scanning electron microscope (SEM) (FEI, Nova 600 Nano SEM). One drop of dilute chitosan nanoparticles' solution was dropped on a carbon film and let air-dried before viewing.

**Fourier Transform Infrared (FTIR) Spectra Studies.**
FTIR spectra of chitosan and Azathioprine encapsulated chitosan nanoparticles were recorded on KBr pellets with a FT-IR spectrophotometer (Thermo Scientific Nicolet iS10, USA)

**Evaluation of Encapsulation.**
Encapsulation efficiencies of prepared chitosan nanoparticles were determined by LCMS-MS.

**Results and Discussion**
In the present study, we focused on chitosan nanoparticles which have smart features for drug delivery concerning cancer treatments. Description of such kind of system should have some important properties: (i) obtained spontaneously under exceptionally mild conditions without involving high temperatures and organic solvents, (ii) has a valuable drug loading capacity and provides a continuous and sustainable release of the encapsulated drug for several days, and (iii) has a pH-sensitive behavior. The properties basically concern optimizing general conditions of chitosan nanoparticle production and the feasibility of drug entrapment and release with regard to cancer treatment applications. In terms of appropriate localized drug delivery by means of tumor treatment, chitosan and Azathioprine encapsulated chitosan nanoparticles were produced.

**Chitosan Nanoparticle Production Conditions.**
Chitosan's ability of quick gelling on contact with polyanions relies on the formation of inter- and intramolecular crosslinkages mediated by polyanions [14]. The preparation of chitosan nanoparticles is based on an ionic gelation interaction between positively charged chitosan and negatively charged tripolyphosphate (TPP) at room temperature immediately [15,16]. TPP is a multivalent anion that possesses negative charges; chitosan in acidic solution has amino groups that can undergo protonation. During the preparation process, TPP electrostatically attracted to the NH3+ groups in chitosan to produce ionically crosslinked chitosan nanoparticles [8; 17]. Size and size distribution of the chitosan nanoparticles depend largely on concentration of chitosan and TPP solutions. For the success of chitosan with nanosized scale, the concentration of chitosan and TPP should be controlled at a suitable range [18]. The mean size and size distribution of each batch of chitosan nanoparticle suspension were analyzed using the Zetasizer analysis. Previously it had been shown that the appearance of the solution changed when a certain amount of TPP ions was added to the chitosan solution, from a clear to opalescent solution that indicated a change of the physical states of the chitosan to form nanoparticles, then microparticles, and eventually aggregates [19].

Chitosan (1mg/ml) and TPP (0.5mg/ml) was maintained at a volumetric ratio of 2.5:1, Tween 80 (Sigma, Germany) (0.5% (v/v)), as a resuspending agent, was added to chitosan solutions in order to prevent particle aggregation, and then chitosan solutions were adjusted to pH 4.6–4.8 with 1N NaOH. The preparation of CS nanoparticles was based on the ionic interaction of a positively charged CS solution and negatively charged TPP solution [20]. The charge density of both CS and TPP solution has a great effect on the ionic interaction. The results concluded that 1 mg/mL chitosan and 0.5mg/mL TPP solutions with 2.5 : 1 (chitosan : TPP) volume ratio in the presence of 0.5% Tween 80 succeeded in the formation of minimum particle size diameter (50.1 ± 5.1 nm) of chitosan nanoparticles.

It is well known that particle size plays an important role on mucosal and epithelial tissue uptake of nanoparticles and on the alternation of pharmacokinetics by affecting the tissue distribution and clearance [4]. With regard to all these data, minimum mean diameter size chitosan nanoparticles (50.1 ± 5.1 nm) were selected for further studies.

The mean size and size distribution of each batch of chitosan nanoparticle suspension were analyzed using the Zetasizer analysis.

**Azathioprine loaded chitosan based nanoparticles**
Azathioprine encapsulated chitosan nanoparticles were prepared by ionic crosslinking method between TPP and chitosan solutions having 1.0 mg/mL Azathioprine concentrations. Azathioprine encapsulated chitosan nanoparticles formed instantaneously when polyanionic TPP is added to readily mixed chitosan–Azathioprine solutions. The results concluded that 1 mg/mL chitosan, 1 mg/ml of Azathioprine and 0.5 mg/mL TPP solutions with 2.5 : 1 (chitosan : TPP) volume ratio in the presence of 0.5% Tween 80 succeeded in the formation of particle size diameter (806 ± 10.5 nm) of chitosan nanoparticles.

**Characterisation of Azathioprine loaded chitosan based nanoparticles**
**Morphology of nanoparticles**
Spherical nanoparticles were obtained for both drug free and drug loaded samples and the encapsulation of Azathioprine slightly increased the diameter of the nanoparticles as shown from the SEM images of drug free and CS nanoparticles co-loaded with Azathioprine (Fig 1 and 2) and the statistical data on particle size and size distribution . The drug free CS nanoparticles are roughly spherical in shape with a size distribution range of 39.4-59.4 nm in diameter and the mean diameter of the particles is about 50.1 ± 10.1 nm. Compared to what was observed with the drug free CS nanoparticles, significant difference can be noticed in the size of drug loaded particles. The statistical diameter of the combination drug loaded CS nanoparticles was in the range of 700-806 nm with a mean diameter of 806 nm.

**Zeta potential of combination drug loaded CS nanoparticles**
Zeta potential and particle size are two important characteristics of nanoparticles; the stability of nanoparticles in aqueous is strongly correlated to its zeta potential. The higher the absolute zeta potential the more stable the nanoparticles due to the stronger repellent interaction between each other. In addition, it was reported that the charge density of nanoparticles plays an important role in its binding with negatively charged cancer cell membrane [21]. Therefore, positive charged nanoparticles are ideal options for the preparation of drug delivery systems in the therapy of cancer related diseases.

Zeta potential decreases with the increase of Azathioprine concentration, while the particle size of the combination drug loaded nanoparticles increases with the Azathioprine loading. The zeta potential of the chitosan nanoparticles is 42.7 mV, while zeta potential of the Azathioprine loaded chitosan nanoparticles is 50.6 mV.

### Analysis of chitosan and Azathioprine loaded chitosan based nanoparticles by FT-IR

The FTIR spectra of pure Azathioprine, CS and CS nanoparticles co-loaded with Azathioprine was analysed. The intense characteristic peaks at 1578, 1357, 1295, 829, 857 and 605.97cm−1 were detected due to the vibration of NH stretch (amide II and amide III) C–H groups and aromatic ring in the structure of Azathioprine. The intense peaks at 1591 and 1420 cm−1 confirmed the presence of amide I and amide II in the chemical structure of CS. After ionic crosslinking with mixture of TPP and drugs, the characteristic peak at 1591 cm−1 in the CS spectra shifted to 1540.61 cm−1, while the peaks at 1420 cm−1 shifted to 1406.80 cm−1 due to the strong ionic interaction between positively charged CS and TPP solution. In addition, most of the intense characteristic peaks of Azathioprine were not observed at the same position in the drug loaded nanoparticles, indicating the intense interaction between the drugs and CS.

### Encapsulation efficiency and loading capacity of nanoparticles

During preparation of Azathioprine loaded chitosan nanoparticles, 100 mg of Azathioprine dissolved in 100 ml of chitosan and mixed with 40 ml of TPP, after lyophillization 80 mg of nanoparticles were yielded. By LC-MS/MS Studies, it was found that Azathioprine Concentration in Azathioprine loaded chitosan nanoparticles was 32200 ng/mL. 32.200 mg of Azathioprine was present in every 100mg of nanoparticles [22].

The drug loading capacity of Azathioprine loaded chitosan nanoparticles was found to be 32.2%.

**Fig 1. SEM images of drug free Chitosan nanoparticles**

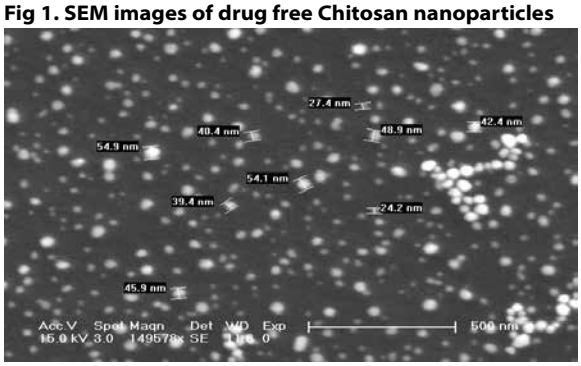
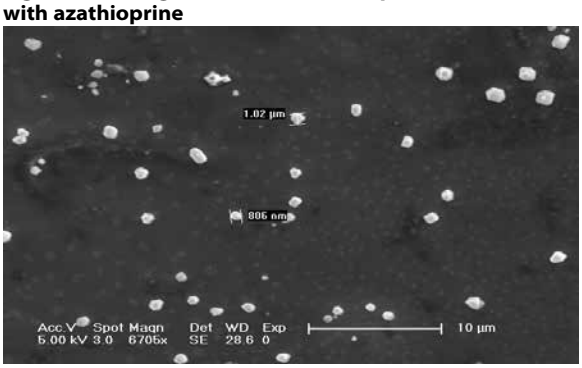



**Fig 2. SEM images of Chitosan nanoparticles co-loaded with azathioprine**




### Conclusions

In this work, CS nanoparticles co-loaded with Azathioprine were successfully fabricated by ionic gelation. Azathioprine encapsulated chitosan nanoparticles not only would offer several advantages over conventional drug therapies but also expected to overcome side effects regarding to dosing and toxicity. However, further optimization studies including stabilization and targeting should be performed both *in vitro* and *in vivo*.

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