



Formulation and *in vitro* Characterization of Donepezil-loaded Chitosan Nanoparticles

Muhammad Wahab Amjad^{1*} and Nawaf Mohamed Alotaibi¹

¹Department of Pharmaceutics, Faculty of Pharmacy, Northern Border University,
Rafha, Saudi Arabia.

Authors' contributions

This work was carried out in collaboration between both authors. Author MWA designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author NMA managed the analyses of the study and literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

Millions of people are affected globally by alzheimer's disease and it is regarded as a dangerous progressive medical and socio-economic burden. The drug delivery to brain is hindered due to the presence of blood brain barrier. Nanoparticle mediated drug delivery is a promising approach in this regard. Chitosan is a hydrophilic polysaccharide polymer of N-acetylglycosamine and glucosamine. Owing to its biodegradability, nontoxicity and biocompatibility it is regarded as a safe excipient. The aim of the study was to fabricate donepezil-loaded sustained release chitosan nanoparticles as a simple way to deliver nano-drugs to the brain. The nanoparticles were fabricated using ionic gelation method using different concentrations of Sodium tripolyphosphate (TPP) and chitosan. The fabricated nanoparticles were assessed for particle size, zeta potential, encapsulation efficiency and *in vitro* drug release. The effect of sonication time on the particle size of nanoparticles was also studied. The nanoparticles exhibited mean particle size (between 135-1487 nm) and zeta potential (between +3.9-+38mV) depending on chitosan and TPP concentration used. The rise in the sonication time from 25 to 125 sec exhibited a decrease in particle size. The encapsulation efficiency was found to be in the range of 39.1-74.4%. Sustained and slow release of donepezil at a constant rate was exhibited from nanoparticles. The nanoparticles show potential to deliver donepezil to brain with enhanced encapsulation efficiency.

*Corresponding author: E-mail: mwbamjad@yahoo.com; Muhammad.Hussain@nbu.edu.sa;

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1. INTRODUCTION

Neurodegenerative diseases comprise a range of disorders which progressively damages the cognition and memory of patient in the elderly people. 24.3 million Global population has been affected by Alzheimer's and it is regarded as a harsh medical and socio-economic problem around the world [1]. It is the most prevalent neurodegenerative disease over the past decades. The disease's etiology is still uncertain however few aspects which are considered to be crucial in its pathogenesis comprise reduced acetylcholine levels, abnormal proteins and their unwanted buildup and oxidative stress [2]. The presence of blood brain barrier (BBB) is one of the main factors that hampers the drug development and discovery of new compounds in the prevention and treatment of Alzheimer's as it hinders the drug delivery to brain [3]. To attain efficacious AD treatment, the blood-brain barrier's (BBB) role has to be considered. It is a specific biochemical, structural and physiological barrier [4]. It only allows specific molecules transport that is vital for the function of brain. Almost 100% of large and more than 98% of small drug molecules are precluded from brain drug delivery [5]. Owing to these factors CNS is one of the most complex microenvironments of the body. Drug delivery system (DDS) shows promise in the treatment of CNS diseases because it displays numerous benefits such as drug delivery to a specific site, protection of drug from clearance by immune and circulatory systems, changing the physicochemical characteristics of drugs, reduction of doses and control of drug release [6–8]. They make DDS an attractive option for treating AD.

The current interest in the development brain drug delivery has resulted in the advancement of numerous colloidal systems such as dendrimers, [9] solid lipid nanoparticles, [10] polymeric nanoparticles (NPs) [11] and liposomes [12]. Amongst the several delivery systems used polymeric NPs have shown promise owing to their potential in opening tight junctions (Tj) of BBB, they efficiently prolong the release of drug and protect against enzymatic-mediated degradation. Nanoparticles composed of hydrophilic polymers such as chitosan possess benefits including extended circulation and nanoparticles <200 nm particle size evade opsonisation [13]. Chitosan is a hydrophilic polysaccharide polymer of N-acetylglycosamine and glucosamine. Owing to its biodegradability,

nontoxicity and biocompatibility it is regarded as a safe excipient [14]. Furthermore its mucoadhesive property and cationic nature enhance its cellular uptake via ionic interaction. It has found applications in biomedical applications and drug delivery such as in bandages and contact lenses, [15] artificial membranes [16] healing dressings and ointments [17].

Cholinesterase inhibitors are drugs which stop acetylcholine or butyrylcholine breakdown. This upsurges their concentration in the synaptic cleft that can attach to nicotinic, muscarinic and other receptors. They are used as Alzheimer's drugs. Common side effects of these drugs comprise nausea, vomiting, loss of appetite, loose stools etc. Donepezil is used in the treatment of Alzheimer's [18]. Its quick absorption leads to high first pass effect and multi-dosing contributes to cardio toxicity. Therefore advanced drug delivery systems have been developed to avoid multi-dosing and reduce the first pass effect. Though owing to its poor BBB penetration, it is usually essential to take higher drug doses which leads to cholinergic unwanted effects. Donepezil was selected as a model drug to load onto nanoparticles as fewer studies are available.

The aim of the study was to fabricate donepezil-loaded sustained release chitosan nanoparticles as a simple way to deliver nano-drugs to the brain.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Donepezil hydrochloride, CS (medium molecular weight), sodium tripolyphosphate (TPP), acetonitrile, sodium hydroxide, ortho phosphoric acid, methyl tert-butyl ether, potassium dihydrogen phosphate, dialysis tubing cellulose membrane with molecular weight cut-off of 12,000-14,000 daltons and glacial acetic acid were purchased from sigma-aldrich.

2.2 Chitosan Nanoparticles Preparation

Ionic gelation method was used to prepare donepezil-loaded CS NPs [19]. Measured weights of CS for all six formulations were dissolved in 1% (v/v) glacial acetic acid. To the above solution, 10 mg donepezil each was added under persistent magnetic stirring, followed by drop wise addition of aqueous TPP

solutions to the respective formulations. Afterwards, the solutions were magnetically stirred for a period of 30 min and later sonicated by the help of probe sonicator. To eliminate un-encapsulated donepezil and excessive amounts of TPP the suspensions of nanoparticle were centrifuged at 13,000 rpm for 30 min at 4°C using an ultracentrifuge. The resultant pellets were dispersed in deionized water. Lastly, NPs were freeze-dried for 24 h and then stored as powder.

2.3 Measurement of Particle Size and Zeta Potential Using Dynamic Light Scattering Technique

The average particle size (hydrodynamic diameter) and polydispersity index (PDI) of CS NPs were determined using a particle size analyzer (PSS Nicomp ZLS Z3000 Particle sizing system) The samples (1ml each) of nanoparticles suspension were placed in quartz cuvettes for the determination of particle size. The experiments were carried out in triplicates. The measurements of zeta potential were made using the particle analyzer at a temperature 25°C. The samples were diluted with deionized water [20].

2.4 Measurement of Encapsulation Efficiency

The nanoparticles were extracted from water using ultracentrifugation at 13000 rpm for 45 min at 4°C. The supernatants were collected and assessed for the presence of donepezil residue using a UV-Vis spectrophotometer [21]. Indirect method was used to measure the encapsulation efficiency (EE) by measuring the level of free donepezil in the supernatant post ultracentrifugation and the following equation was used to calculate it:

$$EE = \frac{\text{Amount of total drug} - \text{Amount of free drug in supernatant}}{\text{Amount of total drug}} \times 100$$

2.5 Investigation of *in Vitro* Donepezil Release

A dialysis method was used to investigate the *in vitro* release profile of donepezil-loaded CS NPs. The nanoparticles (2 ml each containing 2 mg drug) were placed in respective dialysis bags (cellophane, molecular weight cut-off 12,000–14,000), tied and placed into phosphate buffer (20 ml, 0.1 M at pH 7.4) kept at 37°C under the influence of constant magnetic stirring. At

designated time periods, aliquots were taken from the media of release and substituted with the equivalent volume of fresh phosphate buffer. The samples were assayed spectrometrically to detect the quantity of drug present [22].

2.6 Data Analysis

All data were analyzed by means of descriptive and inferential statistical analysis using Microsoft Excel.

3. RESULTS AND DISCUSSION

We developed a nanoparticulate drug delivery system comprising of a natural hydrophilic polymer chitosan having numerous advantages such as easy fabrication of NP by minor agitation without the use of high temperature or organic solvents and obtaining a cationic charge on the surface of nanoparticles thereby enhancing the cell uptake and imparting muco-adhesive characteristic.

3.1 Development and Characterization of Donepezil-loaded CS NPs

Ionotropic gelation method was used to prepare CS NPs [23]. The NPs were prepared by introducing gelation and monitoring its interaction with TPP which resulted in the reduction of CS's aqueous solubility. This drug delivery system is based on intra- and inter-molecular bonds generated among positive charges of CS amino groups and TPP thereby resulting in successful nano-particulate formation. The ratio of CS to TPP is rate-limiting step and governs the particle size distribution of nanoparticles [24]. To fabricate NPs below an average particle of 200 nm we explored the influence of CS/TPP ratio on nanoparticulate formation. The maximum concentration of TPP used was 4 mg/ml while that of CS was up to 6 mg/ml. The average particle size, PDI and zeta potential were investigated and the results are shown in Table 1. The findings revealed that the particle size depends on the concentrations of both TPP and CS such that only a certain ratio of CS/TPP could fabricate the NPs with small particle size.

3.2 Effect of CS Concentration on the Particle Size and PDI of NPs

The influence of CS concentration (0.2-0.6%) on the particle size of NPs was assessed. At constant TPP concentration (0.2%) and a rise in

the concentration of CS from 0.2% to 0.6% exhibited a reduction in average particle size with encouraging PDI. On increasing the concentration of CS above 0.6% an opalescent suspension was formed resulting in aggregation. Latest findings revealed that at low CS concentration (0.6%) a low viscosity gelation medium is formed leading to a reduction in the dispersion of liquid phase, thereby enhancing the formation of smaller sized particles [25].

3.3 Effect of TPP Concentration on the Particle Size and PDI of NPs

The influence of the concentration of TPP (0.2 & 0.4%) on the particle size was investigated. An increase in the particle size was observed on increase in the concentration of TPP. TPP at concentrations of 0.2 and 0.4% with 0.2 and 0.6% CS concentrations yielded the particle size of NPs below 200 nm. Furthermore, TPP at a concentration of 0.4% with 0.6% CS concentration exhibited a massive rise in particle size leading to the formation of microparticles. At a TPP concentration higher than 0.4% highly opalescent suspension was formed whose particles precipitated down on storage. Zeta potential is an indicator of the nanoparticle stability. A higher electric charge on nanoparticle surface will avert their aggregation in buffer solution owing to potent repellent forces among particles.

3.4 Effect of Sonication Time on the Particle Size of NPs

In the formation of small sized CS NPs sonication time played a vital part. At a sonication time of 2 min, smallest nanoparticles (135 ± 6 nm) were formed. The formation of acoustic cavitations during ultrasonication is the main cause for reduction in particle size. Chitosan molecules due to high shear force caused by acoustic cavitations break the particles into smaller ones. The rise in the sonication time from 25 to 125 sec exhibited a

decrease in particle size (Fig. 1). Sonicating the particles for more than 2 min did not exhibit more reduction in particle size.

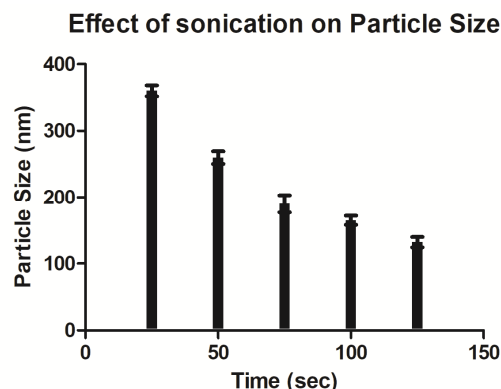


Fig. 1. Effect of sonication time on the particle size of NPs

3.5 Measurement of Particle Size and Zeta Potential

With different concentrations of CS and TPP six formulations were prepared. The average particle sizes of the prepared CS NPs ranged from 135 ± 6 to 1487 ± 9 nm. An increase in CS concentration resulted in a decrease in particle size and a rise in the value of zeta potential. At 0.2% TPP concentration the cross-linking with CS (0.6%) was high which lead to the formation of compact structured particles. Moreover, the degree of neutralization of charged amino acid was also enhanced resulting in positive net charge on the NPs. Owing to compact structure the prepared NPs at that concentration exhibited smaller particle size.

The prepared CS NPs exhibited zeta potential in the range of +3.9 to +38 mV. As the CS concentration increased the zeta potential also rose because of high level of amino group protonation in the CS molecule with high cationic charge resulting in higher value of zeta potential.

Table 1. Average particle size, PDI and zeta potential of different combinations of CS NPs

| Formulation | TPP (%) | CS (%) | Particle size (nm) | Polydispersity index (PDI) | Zeta potential (mV) |
|-------------|---------|--------|--------------------|----------------------------|---------------------|
| CST1 | 0.2 | 0.2 | 390 ± 3 | 0.37 ± 0.11 | +7 ± 2.1 |
| CST2 | 0.2 | 0.4 | 240 ± 8 | 0.43 ± 0.29 | +23 ± 2.3 |
| CST3 | 0.2 | 0.6 | 135 ± 6 | 0.33 ± 0.04 | +38 ± 1.9 |
| CST4 | 0.4 | 0.2 | 185 ± 11 | 0.45 ± 0.08 | +27 ± 1.8 |
| CST5 | 0.4 | 0.4 | 1203 ± 6 | 0.46 ± 0.06 | +6.2 ± 2.1 |
| CST6 | 0.4 | 0.6 | 1487 ± 9 | 0.36 ± 0.06 | +3.9 ± 1.8 |

The optimal CS/TPP concentration was recognized as 0.2% TPP with 0.6% of CS (CST3) exhibiting a particle size of 135 ± 6 nm as displayed in Table 1. The zeta potential of donepezil-loaded CS NPs (CST3) was found to be 38 ± 1 mV indicating promising stability.

The percentage encapsulation efficiency (EE) of donepezil-loaded CS NPs was in the range of 39.1 ± 2.1 to $74.4 \pm 0.9\%$ (Table 2). The rise in the concentration of CS from 0.2 to 0.6% whilst keeping the concentration of TPP constant at 0.2% resulted in a surge in EE. Among all six formulations CST3 turned out to be optimal on the basis of average particle size, zeta potential ($>+30$ mV) and EE of 70.4 ± 0.8 . Hence CST3 was used to perform subsequent studies.

Table 2. The percentage EE and appearance of donepezil-loaded CS NPs

| Formulation | Entrapment efficiency EE (%) | Appearance |
|-------------|------------------------------|-------------------|
| CST1 | 51.7 ± 0.9 | Opalescent |
| CST2 | 62.6 ± 1.2 | Opalescent |
| CST3 | 70.4 ± 0.8 | Opalescent |
| CST4 | 74.4 ± 0.9 | Highly opalescent |
| CST5 | 51.7 ± 1.9 | Highly opalescent |
| CST6 | 39.1 ± 2.1 | Highly opalescent |

3.6 *In vitro* Drug Release Experiment

The cumulative donepezil release from CS NPs (CST3) and free donepezil solution was investigated at pH 7.4 in phosphate buffer as displayed in Fig. 2. The release of drug was discovered to be 100% in 2 h from blank donepezil solution whereas it was found to be $67.8 \pm 1.6\%$ in 24 h from CST3.

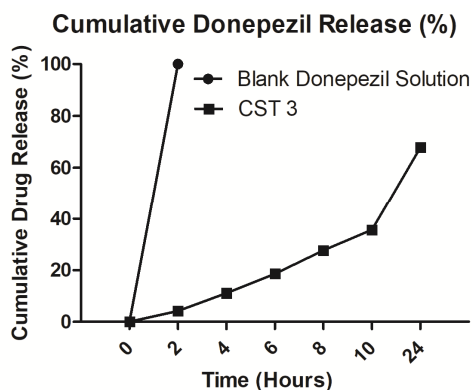


Fig. 2. Cumulative donepezil release from CST3 and blank donepezil solution

The drug release profile of donepezil-loaded CS NPs exhibited an early burst release of 20% in the first hour followed by a sustained release of 38% drug in 24 h. The initial burst effect occurred owing to the molecular dissociation of drug that was loosely attached on CS NPs surface. The latter phase comprised a sustained and slow release of donepezil at a constant rate from NPs.

4. CONCLUSION

This experiment shows that ionic gelation technique can be employed in the loading of hydrophilic drugs to NPs fabricate them at a size lower than 200 nm. The CS/TPP concentration and the time of sonication greatly affect the particle size of CS NPs. The CS NPs (CST3) composed of 0.2% TPP and 0.6% CS were chosen as the optimized formulation as it yielded higher drug encapsulation and smaller particle size. The CS NPs released the drug in a sustained manner for 24h.

5. LIMITATIONS OF THE STUDY

More *in vitro* and *in vivo* studies are needed to endorse the targeting efficacy of CS NPs across BBB in the treatment of Alzheimer's.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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