Complexation as an approach to entrap cationic drugs into cationic nanoparticles administered intranasally for Alzheimer’s disease management: preparation and detection in rat brain

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Abstract

Objective: Complexation was investigated as an approach to enhance the entrapment of the cationic neurotherapeutic drug, galantamine hydrobromide (GH) into cationic chitosan nanoparticles (CS-NPs) for Alzheimer’s disease management intranasally. Biodegradable CS-NPs were selected due to their low production cost and simple preparation. The effects of complexation on CS-NPs physicochemical properties and uptake in rat brain were examined.

Methods: Placebo CS-NPs were prepared by ionic gelation, and the parameters affecting their physicochemical properties were screened. The complex formed between GH and chitosan was detected by the FT-IR study. GH/chitosan complex nanoparticles (GH-CX-NPs) were prepared by ionic gelation, and characterized in terms of particle size, zeta potential, entrapment efficiency, in vitro release and stability for 4 and 25°C for 3 months. Both placebo CS-NPs and GH-CX-NPs were visualized by transmission electron microscopy. Rhodamine-labeled GH-CX-NPs were prepared, administered to male Wistar rats intranasally, and their delivery to different brain regions was detected 1 h after administration using fluorescence microscopy and software-aided image processing.

Results: Optimized placebo CS-NPs and GH-CX-NPs had a diameter 182 and 190 nm, and a zeta potential of +40.4 and +31.6 mV, respectively. GH encapsulation efficiency and loading capacity were 23.34 and 9.86%, respectively. GH/chitosan complexation prolonged GH release (58.07% ± 6.67 after 72 h), improved formulation stability at 4°C in terms of drug leakage and particle size, and showed insignificant effects on the physicochemical properties of the optimized placebo CS-NPs (p > 0.05). Rhodamine-labeled GH-CX-NPs were detected in the olfactory bulb, hippocampus, orbitofrontal and parietal cortices.

Conclusion: Complexation is a promising approach to enhance the entrapment of cationic GH into the CS-NPs. It has insignificant effect on the physicochemical properties of CS-NPs. GH-CX-NPs were successfully delivered to different brain regions shortly after intranasal administration suggesting their potential as a delivery system for Alzheimer’s disease management.

Keywords

Alzheimer’s disease, brain delivery, chitosan nanoparticles, complexation, galantamine, intranasal route

History

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Introduction

Alzheimer’s disease (AD) is defined as an irreversible progressive neurodegenerative disease that gradually and intensively impairs cognition and memory, mainly in the geriatric population. Several theories have been proposed in trying to understand the etiology and factors behind AD progression, such as reduced acetylcholine (ACh) levels, deposition of β-amyloid plaques and neurofibrillary tangles in brain, excessive metal ion accumulation and oxidative stress. However, none of these theories succeeded to offer a full comprehension of AD etiology.

Since the announcement of the first diagnosed AD case by Dr. Alois Alzheimer in 1906, management of AD still represents a challenge for both physicians and caregivers. At this very moment, there are two classes of therapeutic agents approved for managing AD: cholinesterase inhibitors (ChEIs) and N-methyl-d-aspartate (NMDA) receptor antagonists. The approved treatments provide only symptomatic improvement of AD but with little or no effect on the progression of the disease. In addition, these treatments generally have severe peripheral side effects due to a lack of brain targeting. Besides, studies have shown that only a fraction of these drugs is able to cross restrictive blood–brain barrier (BBB). Polymeric nanoparticles, among nanotechnology-based drug delivery systems, have drawn...
a great interest in the past 40 years. Nanoparticles offer not only brain drug delivery, which impressively decreases the peripheral side effects, but also help overcoming the BBB by crossing it through different underlying transport mechanisms such as opening the tight junctions, endocytosis by the endothelial cells followed by drug release within the cells, and transcytosis through the endothelial cell layer.

To provide a successful AD regimen treatment, patient compliance and treatment persistence should be considered, especially knowing that AD patient is one of the most difficult hard-to-handle patients. Therefore, a careful consideration of the dosage form type is unavoidable. The available dosage forms of the approved drugs are mostly oral tablets, capsules and solutions, in addition to the approved rivastigmine transdermal patch.

Since the discovery of intranasal route as a CNS delivery approach by Frey in 1989, several studies investigated the efficacy of such route to bypass the BBB and deliver various drugs to the brain. Compared to other routes of administration, intranasal route offers the following advantages: minimizing the systemic side effects, avoidance of first pass metabolism, ease of self-administration, dose adjustment capability, non-invasiveness and enhanced patient compliance.

To provide further opportunity for drugs applied intranasally to reach the brain through the nasal mucosa, it is beneficial to use mucoadhesive nanoparticles that increase the contact time between the formulation and nasal mucosa, decrease the mucociliary clearance and minimize the peripheral side effects and frequency of dosing. Among several polymeric nanoparticles, chitosan nanoparticles (CS-NPs) gained the attention as biodegradable and biocompatible nanoparticles with good stability and low toxicity. They can be prepared by simple and cheap preparation methods such as ionic gelation.

Due to the cationic nature of chitosan polymer under the conditions of ionic gelation, the entrapment of cationic drugs into CS-NPs with reasonable efficiency is quite challenging. On the other hand, from an industrial perspective, the production cost and ease of manufacture of CS-NPs prepared by ionic gelation should not be overlooked. CS-NPs are not only simply and rapidly prepared, but also chitosan is considered of a relatively low cost compared to the other biodegradable polymers. Therefore, pharmaceutical researchers are encouraged to find an approach to improve the entrapment of cationic neurotherapeutics into CS-NPs.

Chitosan itself is prepared by partial alkaline hydrolysis of the acetamide groups of chitin; a natural polysaccharide found mainly in the structure of the exoskeleton of crustaceans. Chitosan is able to adhere to nasal mucosal surface for extended time due to its hydrophilic cationic amine groups that bind electrostatically to the negatively charged sialic acid moiety of the nasal mucosa. Chitosan not only delays the mucociliary clearance but also transiently opens the tight junctions between the epithelial cells, thus enhancing the drug permeation to the brain and cerebrospinal fluid.

Galantamine hydrobromide (GH) is one of the four major FDA approved ChEIs for management of mild to moderate AD. GH provides only a symptomatic treatment by increasing the levels of ACh through inhibiting the acetylcholinesterase enzyme in the central nervous system (CNS) competitively and reversibly, which enhances patient’s memory and cognition. It also enhances the sensitivity of the nicotinic ACh receptors and demonstrates an ability to prevent β-amloid aggregation. GH is currently marketed in the form of oral tablets, capsules and solution. Although GH exhibits a high oral bioavailability of 90–100%, its administration, either orally or by injection, is accompanied by severe side effects such as nausea, vomiting, diarrhea, insomnia and muscle tremors, which hinder the patient compliance and might lead to treatment discontinuation. Therefore, developing a system to deliver GH directly to the brain is of a great importance.

The current work aimed to investigate the potential of complexation as an approach to enhance the entrapment of GH into CS-NPs prepared by ionic gelation. The parameters affecting the physicochemical characteristics of placebo CS-NPs prepared by ionic gelation were optimized. GH/chitosan complex nanoparticles were prepared. The effect of the complexation interaction on physicochemical properties of nanoparticles was studies. Fluorescent GH/chitosan complex nanoparticles were used to detect their uptake into different brain region 1 h after intranasal administration to rats.

**Materials and methods**

**Materials**

Galantamine hydrobromide (GH) was purchased from Shaanxi Jintai Biological Engineering Co., Ltd (Xi’an City, China). Medium molecular weight chitosan (Mw = 200 kDa, degree of acetylation >90%) was purchased from Carl Roth GmbH Co. (Karlsruhe, Germany). Sodium tripolyphosphate (TPP) and rhodamine B isothiocyanate (RBITC) were purchased from Sigma-Aldrich Chemie GmbH (Munich, Germany). Fluorescent mounting Media was purchased from KPL, Inc. (Gaithersburg, MD). Methanol HPLC grade was purchased from Fisher Scientific UK Ltd. (Loughborough, UK). Concentrated hydrochloric acid and glacial acetic acid were purchased from S.D. Fine Chemicals, Ltd. (Mumbai, India). Sodium hydroxide was purchased from Chemajet Chemical Co. (Alexandria, Egypt). Polysorbate 80 (Tween 80) was purchased from El-Nasr Pharmaceutical Chemicals Co. (Qaliubiya, Egypt). All chemicals were of analytical grade.

**Methods**

**Preparation of placebo CS-NPs**

Chitosan nanoparticles (CS-NPs) were prepared according to the ionic gelation method, developed by Calvo et al. It is based on the crosslinking between the positively charged chitosan and the negatively charged TPP molecules. Chitosan solution was prepared by dissolving an accurately weighed amount of chitosin in 1% v/v glacial acetic acid using a magnetic stirrer (Daikin Scientific Co., Seoul, Korea). After dissolution, the pH was adjusted to 4.5 using 5N NaOH using the pH-meter. Finally, chitosan solution was filtered to remove any undissolved particles using Whatman Filter Papers (Whatman Co., London, UK). The TPP aqueous solution was prepared simply by dissolving an accurately weighed amount of TPP into distilled water using a magnetic stirrer. Then, TPP solution was filtered and used without any pH adjustment.

Placebo CS-NPs were prepared upon flush addition (i.e. addition at once rather than drop-wise) of 4 ml TPP aqueous solution to 10 ml chitosan solution into a conical flask, under stirring rate of 1200 rpm. The stirring was continued for 30 min, at room temperature, to ensure the formation of a stable colloidal dispersion. Different formulations were prepared by mixing different concentrations of chitosan and TPP solutions (Table 1).

**Study of some factors affecting the particle size of the prepared placebo nanoparticles**

Different factors have been investigated to study their effect on the particle size and polydispersity index (PDI) of the prepared CS-NPs. Based on preliminary trials, all of these experiments...
were done at the same CS:TPP volume ratio of 2.5:1 and at room temperature (25 °C). Upon studying each factor, the other factors were kept constant.

**Effect of method of addition of TPP**

An aqueous solution of 1 mg/ml TPP and a 2 mg/ml chitosan solution in 1% v/v glacial acetic acid (pH = 4.5) were prepared as previously mentioned. Two placebo NPs samples were prepared. The first sample was prepared by adding 4 ml TPP solution dropwise to 10 ml chitosan solution, using a syringe needle (gauge no. 27), by a rate of 0.4 ml/min under magnetic stirring at 1200 rpm for 30 min. The second sample was prepared by flush addition (at once addition) of 4 ml TPP to the chitosan solution, using a graduated pipette, and the procedure was continued as previously mentioned.

**Effect of stirring rate**

An aqueous solution of 1 mg/ml TPP and a 2 mg/ml chitosan solution in 1% v/v glacial acetic acid (pH = 4.5) were prepared as previously mentioned. Three placebo NPs samples were prepared by flush addition of 4 ml TPP to 10 ml chitosan solution. The samples varied only in the applied stirring rate (600, 900 and 1200 rpm).

**Effect of the pH of chitosan solution**

Four chitosan solutions were prepared which varied at their pH (4, 4.5, 5, 5.5) using 5 N NaOH. For comparison, a control chitosan solution was prepared without any pH adjustment prior to TPP solution addition. Five NPs samples were prepared by the flush addition of 4 ml TPP to 10 ml of each of chitosan solution under magnetic stirring at 1200 rpm for 30 min.

**Effect of chitosan:TPP mass ratio**

Four different chitosan solutions (0.5, 1, 1.5, 2 mg/ml) and 4 different TPP solutions (0.5, 1, 1.5, 2 mg/ml) were prepared. By combining 10 ml of each of the chitosan solutions with 4 ml of each of the TPP solution by flush addition, 16 formulations were obtained. The formulations were stirred for 30 min at 1200 rpm. Each formulation was characterized as ‘clear solution’, ‘opalescent colloidal dispersion’, or ‘aggregates’ by visual observation. The formulations were set aside for 30 min to observe the formation of aggregates. In addition, the formation of an opalescent colloidal dispersion was confirmed by observing Tyndall light scattering phenomenon using a red laser pointer. The detailed composition of each formulation is illustrated in Table 1.

**Effect of concentration of polysorbate 80**

Polysorbate 80 (Twee80) is usually added during CS-NPs preparation as a stabilizer to prevent the formation of aggregates. Tween 80 was mixed with the chitosan solution in concentrations 0.1, 0.25 or 0.5 %w/v, prior to the addition of TPP solution. The preparations were characterized for their particle size, PDI and zeta potential when freshly prepared, a week later, and after 3 months storage at 4 °C.

**Preparation of galantamine hydrobromide-loaded chitosan nanoparticles**

**Method A**

Five different GH-loaded formulations were prepared by dissolving an accurately weighed amount of GH (4, 5, 7, 10 and 20 mg) into the 10 ml chitosan solution, along with Tween 80, before the flush addition of 4 ml TPP solution. The dispersion was stirred for 30 min at 1200 rpm at room temperature (25 °C).

**Method B: chitosan–GH complex formation**

Galantamine hydrobromide (GH) was added to chitosan solution in 1% v/v acetic acid, adjusted at pH 4.5. The final GH concentrations were 25 and 35%. The solutions were stirred over a magnetic stirrer for 24 h at room temperature. After 24 h, appropriate concentrations of Tween 80 and TPP solution were added. The dispersion of GH/chitosan complex nanoparticles was stirred for 30 min at 1200 rpm, and then characterized as stated in the following sections.

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Table 1. Effect of the concentration of chitosan and TPP solutions, and chitosan:TPP mass ratio on the particle size, polydispersity and zeta potential of chitosan nanoparticles.

| Formulation | Chitosan concentration (mg/ml) | TPP concentration (mg/ml) | Mass ratio (CS:TPP) | Visual observation* | Mean diameter (nm ± SD) | Mean PDI (PDI ± SD) | Zeta potential (mV ± SD) |
|-------------|-------------------------------|---------------------------|---------------------|---------------------|------------------------|------------------|------------------------|
| F1-A        | 0.5                           | 0.5                       | 2.5:1               | ×                   | N/A                    | N/A              | N/A                    |
| F1-B        | 0.5                           | 1                         | 1.25:1              |                      | N/A                    | N/A              | N/A                    |
| F1-C        | 0.5                           | 1.5                       | 0.83:1              |                      | N/A                    | N/A              | N/A                    |
| F1-D        | 0.5                           | 2                         | 0.625:1             |                      | N/A                    | N/A              | N/A                    |
| F2-A        | 1                              | 0.5                       | 2.5:1               | ×                   | 200 ± 2.254            | 0.187 ± 0.007    | 36.1 ± 7.21            |
| F2-B        | 1                              | 1                         | 2.5:1               | ✓                   | 217.2 ± 2.831          | 0.266 ± 0.003    | 40 ± 12.2              |
| F2-C        | 1                              | 1.5                       | 1.67:1              |                      | N/A                    | N/A              | N/A                    |
| F2-D        | 1                              | 2                         | 1.25:1              |                      | N/A                    | N/A              | N/A                    |
| F3-A        | 1.5                            | 0.5                       | 7.5:1               | ×                   | N/A                    | N/A              | N/A                    |
| F3-B        | 1.5                            | 1                         | 3.75:1              | ✓                   | 252.2 ± 2.274          | 0.280 ± 0.009    | 44.3 ± 8.91            |
| F3-C        | 1.5                            | 1.5                       | 2.5:1               |                      | 182 ± 2.507            | 0.264 ± 0.012    | 40.4 ± 4.92            |
| F3-D        | 1.5                            | 2                         | 1.875:1             |                      | N/A                    | N/A              | N/A                    |
| F4-A        | 2                              | 0.5                       | 10:1                | ×                   | N/A                    | N/A              | N/A                    |
| F4-B        | 2                              | 1                         | 5:1                 | ✓                   | N/A                    | N/A              | N/A                    |
| F4-C        | 2                              | 1.5                       | 3.33:1              | ✓                   | N/A                    | N/A              | N/A                    |
| F4-D        | 2                              | 2                         | 2.5:1               |                      | N/A                    | N/A              | N/A                    |

CS, chitosan; N/A, not determined; TPP, tripolyphosphate; PDI, polydispersity index.

*Amount added = 10 ml.

*Amount added = 4 ml.

*Aggregates (%), Opalescent colloidal dispersion (✓), Clear Solution (×).
Physicochemical characterization of the prepared placebo CS-NPs and GH/chitosan complex nanoparticles

Particle size, polydispersity index and zeta potential

Mean particle diameter, PDI and zeta potential were determined using a Zetasizer Nano ZS (Malvern Instruments Ltd, Malvern, UK). The mean particle diameter and PDI were measured based on the dynamic light scattering (DLS) technique. Particle size measurements were intensity-based and were performed at 25°C with an angle detection of 173°. The sample volume used for the particle size analysis was kept constant (1 ml). The samples were measured with a preceding dilution factor of 3. All the measurements were done in triplicates and the means and standard deviations were calculated. The zeta potential of the prepared formulations was determined based on an electrophoretic light scattering technique.

Entrapment efficiency, loading capacity and process yield

The entrapment efficiency (EE) and loading capacity (LC) of the GH-loaded NPs were calculated by separation of the formed NPs from the medium by centrifugation at 15000rpm, 4°C for 45 min. The amount of free (unentrapped) GH in the supernatant was measured spectrophotometrically at λmax 289 nm using a UV–Visible spectrophotometer (Shimadzu Corporation, Kyoto, Japan). The EE and LC of GH-loaded NPs were calculated using Equations (1) and (2), respectively. All the measurements were performed in triplicate (n = 3).

\[
\begin{align*}
\%EE &= \frac{\text{Total drug} - \text{Free drug}}{\text{Total drug}} \times 100 \\
\%LC &= \frac{\text{Total drug} - \text{Free drug}}{\text{Nanoparticles weight}} \times 100
\end{align*}
\]

The process yield of the NPs was calculated using Equation (3). The prepared NPs were separated from the aqueous medium by centrifugation of 1 ml sample at 15000rpm, 4°C for 45 min. The supernatant was discarded, and then the pellet was dried by incubation at 40°C till obtaining constant weight (W1). The sum of the initial dry weight of starting materials contained in 1 ml NPs sample was assigned as W2.

\[
\%\text{Process Yield} = \frac{W_1}{W_2} \times 100
\]

Fourier transform-infrared spectrometry

Fourier transform-infrared (FT-IR) spectra for pure chitosan, pure GH and freeze-dried GH/chitosan complex were detected using a Perkin-Elmer FT-IR spectrometer (Perkin-Elmer, London, UK). A small amount of each sample was mixed with KBr and compressed into tablets. The FT-IR spectra were obtained in the spectral region of 350–4000 cm⁻¹.

Visualization by transmission electron microscopy

The shape and size of the placebo CS-NPs and GH/chitosan complex nanoparticles were examined using the transmission electron microscope (TEM) (JEM-1010, Joel Ltd., Tokyo, Japan). The carbon film-covered copper grid was dipped into the sample suspension in filtered distilled water for 30 s. Then, the grid was placed onto a filter paper to remove any remaining suspension. Then, the grid was placed on a drop of sodium phosphotungstate for 10 s. A filter paper was used to remove the excess solution and finally the grid was left to air dry before examination by TEM.

Differential scanning calorimetry analysis

Differential scanning calorimetry (DSC) analysis of the pure GH, pure chitosan, dried placebo CS-NPs and dried GH/chitosan complex nanoparticles was carried out using a Differential Scanning Calorimeter-6 calorimeter (Perkin-Elmer, UK) calibrated with indium standard. Five milligrams sample was placed onto a standard aluminum pan with pierced lid, and heated from 35 to 400°C at a heating rate of 5°C/min under continuous purging of nitrogen (20 ml/min). The heat flow as a function of temperature was recorded for all samples.

In vitro drug release study

Galantamine hydrobromide (GH/chitosan complex nanoparticles were resuspended in filtered distilled water (pH 6.5–7) by gentle vortexing to a final nanoparticles concentration of 1 mg/ml. The nanoparticles dispersion was divided onto glass vials (2 ml each). The vials were then incubated at 37°C in a shaking water bath (100 rpm). At pre-determined time intervals, the corresponding vial was taken out, centrifuged at 15000 rpm for 45 min at 4°C. The pellet was discarded, and the supernatant content of GH was quantified spectrophotometrically at λmax of 289 nm. Placebo nanoparticles had also been prepared as blank and treated similarly to the GH-loaded nanoparticles to subtract their absorbance at each corresponding time interval. To ensure the sink condition, GH solubility in distilled water was determined before conducting the release study.

Data obtained from the in vitro release study were fitted into different release kinetic equations including zero-order, first-order, Higuchi, Korsmeyer–Peppas, Weibull and Hixon–Crowell models using the Add-In ‘DDSolver’ software.

Mucoadhesion study

The mucoadhesion of GH/chitosan complex nanoparticles with commercially available mucin was studied using the mucin-particle method. Equal volumes of GH-loaded CS-NPs (2 mg/ml) and type II mucin solution in distilled water (2 mg/ml) were vortex mixed for 10 s, and then incubated at 37°C for 0, 30 and 60 min. At each time interval, the suspensions were centrifuged at 15000 rpm for 45 min at 4°C. The free (unadsorbed) mucin in the supernatant was determined spectrophotometrically at λmax of 289 nm. Placebo CS-NPs, suspended in filtered distilled water without mucin addition, were incubated under the same temperature, centrifuged and their supernatant absorbance at λmax 258 nm was subtracted from the test samples to eliminate any possible interference. For comparison, the mucoadhesiveness of the optimized placebo CS-NPs was also determined using the same technique.

The percent mucin binding efficiency of nanoparticles was calculated from Equation (4).

\[
\%\text{Mucin binding efficiency} = \frac{\text{Total mucin} - \text{Free mucin}}{\text{Total mucin}} \times 100
\]

Calibration curve of mucin type II in distilled water was constructed by measuring the absorbance of mucin serial dilutions (0.2–0.33 mg/ml) spectrophotometrically at λmax 258 nm.

Assessment of storage stability of drug-loaded nanoparticles

The stability of the selected GH/chitosan complex nanoparticles, as dispersion in distilled water, was assessed according to the protocol described by Wilson et al. Samples were kept into rubber-capped glass vials and stored at room temperature (about
25 °C), and in a refrigerator (4 °C) over a period of 3 months. At 0, 1, 2 and 3 months, samples were evaluated for their drug content and particle size. In addition, any changes in the physical appearance of the dispersion were noted.

Detection of GH/chitosan complex nanoparticles into different rat brain regions qualitatively

Synthesis of RBiTc-labeled chitosan

Rhodamine B isothiocyanate (RBiTc)-labeled chitosan was first synthesized based on the method described by Ma et al. Chitosan (200 mg) was dissolved in 50 ml of 1% w/v acetic acid, and then 50 ml of anhydrous methanol was added and stirred for 3 h. An accurately weighed 13 mg of RBiTc was dissolved in 7 ml anhydrous methanol, and then added drop-wise to chitosan solution with continuous stirring at 300 rpm. Stirring was continued for 18 h in the dark. RBiTc-labeled chitosan was precipitated by adding 1 ml of 5 N NaOH, and then washed several times with deionized water till no free RBiTc can be detected in the wash solution. At the end, RBiTc-labeled chitosan was freeze-dried.

Preparation of RBiTc-labeled GH/chitosan complex nanoparticles

An accurately weighed 7 mg of GH was dissolved in RBiTc-labeled chitosan solution (2 mg/ml, pH 4.5) and stirred for 24 h in the dark at room temperature. Appropriate concentrations of Tween 80 and TPP solution (1.5 mg/ml) were added and the dispersion was stirred for 30 min at 1200 rpm. Particle size, zeta potential, mucoadhesiveness and in vitro drug release of RBiTc-labeled GH/chitosan complex nanoparticles were determined and compared to those of the selected GH/chitosan complex nanoparticles.

Rhodamine B isothiocyanate (RBiTc)-labeled nanoparticles were resuspended in 0.9% w/v saline to a final nanoparticles concentration of 10 mg/100 μl. Before administration to rats, the nanoparticles were visualized using a fluorescence microscope (Olympus BX51, Olympus Co., Tokyo, Japan) with a rhodamine filter set to ensure that the RBiTc amount attached to chitosan is sufficient.

Administration of RBiTc-labeled GH/chitosan complex nanoparticles intranasally to rats

All animal procedures in this study were conducted according to the published guidelines by the Ethical Committee for Care and Use of Laboratory Animals (Faculty of Pharmacy & Drug Manufacturing, Pharos University in Alexandria) and approved by the Research Ethics Committee of Faculty of Pharmacy & Drug Manufacturing, Pharos University in Alexandria. The conducted research study adhered to the “Principles of Laboratory Animal Care” (NIH publication #8523, revised in 1985). Male Wistar rats (260–270 g) were used. The animals were maintained under standard diurnal conditions and allowed access to food and water ad libitum. The animals were divided into two groups: six rats each; a control group and a test group. Before administration, all rats were anesthetized with intraperitoneal injection of thiopental at a dose of 50 mg/kg. The rats were placed and kept in a supine position with their noses at a 90° upright angle to maximize absorption through the nasal mucosa. The control group received 0.9% w/v saline (50 μl), while the test group received 50 μl of the RBiTc-labeled GH/chitosan complex nanoparticles (equivalent to 780 μg GH; dose = 3 mg/kg body weight); both by intranasal administration (25 μl per nostril). According to a study published by Migliore et al., bolus administration of volumes > 5 μl per nostril, or administering 5 μl in both nostrils simultaneously, resulted in respiratory distress. Therefore, a volume of 5 μl was administered in alternating nostrils, and an absorption time (5 min) was kept between successive increments. Doses were administered using 5–50 μl micropipette tip (Accumax Co., India). The micropipette tip was inserted ~5 mm inside the rat’s nostril to facilitate reaching the deeper olfactory epithelial layers. After administration, the rats remained in a supine position for 1 h, after which they were euthanized by cervical dislocation.

Brain preparation for microscopy and image processing

After the rats had been decapitated, the brain removal from the skull and the subsequent dissection were performed according to the procedures described by Spijker. To remove the brain along with the olfactory bulb, the skull was cut at the mid temporal line along the occipital, frontal and nasal regions. Thereafter, the occipital, frontal and nasal bones were lifted up to expose the brain. The brain and olfactory bulbs were very cautiously removed and transferred to a metal plate placed on ice. The olfactory bulb was cut off directly and frozen at −30 °C.

To dissect the hippocampus, the cerebellum was first removed. Then, the cortex was folded backward from the hippocampus using a forceps. Finally, the corpus callosum was separated from the hippocampus, and the latter was dissected out and frozen at −30 °C. The cortex was placed back and coronal sections were taken from the orbitofrontal and the parietal cortices, and both were frozen immediately as previously mentioned. Protection from light was maintained throughout the experiment. The frozen brain regions and olfactory bulbs were sectioned using a cryostat (CM1850, Leica Biosystems Co., Wetzlar, Germany) into 10 μm thick sections at −30 °C. The sections were placed onto glass slides, a glycerol-based mounting medium was added to retard photobleaching and preserve the tissues, and finally the covers were placed.

The sections were examined to localize clusters of fluorescence using a fluorescence microscope with a rhodamine filter set at 100 × magnification power. The control sections were also examined for comparison. Images were photographed using a digital camera (Olympus C-5060, Olympus Co., Tokyo, Japan). ImageJ 1.46r software (National Institute of Health, Bethesda, MD) was used to analyze the images, measure the integrated density and area, and finally quantify the fluorescence intensity expressed as the corrected total fluorescence (CTF) using Equation (5). The fluorescence intensity for each image was normalized by subtracting the background fluorescence.

\[
\text{CTF} = \text{Integrated density} - \frac{\text{Image area} \times \text{Mean fluorescence of background readings}}{\text{background readings}}
\]

Statistical analysis

All reported data are represented as mean ± SD. Statistical significance was checked by student’s t-test and considered to be granted at p < 0.05 unless otherwise indicated.

Results and discussion

Optimization of placebo chitosan nanoparticles

It is believed that the diameter of nanoparticles administered intranasally strongly influences their internalization via endocytic pathways. In general, nanoparticles of a diameter <200 nm are internalized by clathrin-mediated endocytosis efficiently. Therefore, different factors were thoroughly investigated in the current study to obtain nanoparticles with a diameter < 200 nm.

Chitosan nanoparticles (CS-NPs) are formed through the ionic interaction between the positively charged amino groups of
chitosan and the negatively charged phosphate groups of TPP. The preliminary trials showed that several factors affect the formation of CS-NPs in terms of particle size, PDI, zeta potential and process yield. Hence, these factors were studied carefully, one factor at a time, starting from the preliminary ones and ending with the basic ones. Once a factor has been optimized, its optimum value is used in all the subsequent trials.

**Effect of the method of addition of TPP**

The effect of the method of TPP addition to the chitosan solution on the particle size and PDI of the formed nanoparticles is shown in Figure 1(A). It is obvious that flush addition of TPP solution led to the formation of nanoparticles with smaller diameter and more uniform sample i.e. smaller PDI. This observation was in agreement with Dong et al. as they reported that the formation of CS-NPs involves two processes that are sequential but likely to proceed in parallel though. First, TPP anions have to be dispersed into chitosan molecules, and then the crosslinking between the protonated amine groups and TPP anions occurs. As the CS/TPP interaction is almost instantaneous, rapid and uniform mixing favors the fast and uniform dispersion of TPP anions within the chitosan molecules. Thus, CS-NPs with smaller size and narrower PDI could be obtained. Therefore, flush addition of TPP coupled with proper mixing will ensure the balance between TPP dispersion and the subsequent crosslinking with the chitosan molecules. Consequently, the flush addition technique was selected for the subsequent experiments.

**Effect of stirring rate**

The effect of stirring rate on the particle size and PDI was investigated. The results showed that increasing the stirring rate from 600 to 1200 rpm decreased the particle size markedly and led to more homogenous dispersion too, i.e. lower PDI (Figure 1B). However, during the preliminary trials, it was found that increasing the stirring rate to 1800 rpm increased the particle size and PDI (data not shown). It is inferred that an optimum stirring rate is required to allow the dispersion of TPP in chitosan solution and increasing the shear force helps improving the monodispersity. On the other hand, the intense stirring (1800 rpm) might diminish the repulsion between particles, leading to their aggregation. Thus, a stirring rate of 1200 rpm was selected to be used in the subsequent experiments.

**Effect of pH of chitosan solution**

Investigating the effect of pH of chitosan solution on the particle size and polydispersity showed a non-simple relationship with both of them. Similar results were obtained by Abdel-Hafez.
The pH was varied over the range from 4 to 5.5 (4, 4.5, 5 and 5.5). The results showed that adjusting the pH of chitosan solution from 4 to 5 produced colloidal dispersions i.e. nanoparticles but with variable size and polydispersity (Figure 1C). On the other hand, when the pH was 5.5, aggregates and microparticles were formed.

To explain the behavior of chitosan under different pH values, its protonation should be considered. Chitosan is a weak polyelectrolyte with a pKa around 6.5. As previously reported by Shu and Zhu, increasing the pH of chitosan solution from 4.7 to 8 decreased the protonation degree of chitosan from 100% to 0%. Although adjusting the pH at 5 produced samples with high monodispersity, the particle size was larger. Therefore, pH 4.5 was selected as it showed the smallest particle size and a reasonable PDI.

Effect of the concentration of chitosan solution, TPP solution and chitosan:TPP mass ratio

It is worth mentioning that formation of chitosan/TPP nanoparticles was only possible for some specific concentrations of chitosan and TPP, despite keeping the mass ratio constant. Based on the preliminary trials, the concentration of chitosan and TPP solutions should be lower than 3 and 2 mg/ml, respectively, to avoid the formation of aggregates.

When chitosan is dissolved in acidic medium, the amino groups of chitosan get protonated, exerting intermolecular repulsion. However, intermolecular hydrogen bonding exists concurrently between chitosan molecules. These two opposite interactions remain in equilibrium only below a certain chitosan concentration (2 mg/ml as our obtained results showed).

Above this concentration (2 mg/ml), chitosan molecules approach each other much more, increasing the intermolecular hydrogen bonding and overcoming the intermolecular repulsion. Therefore, the number of chitosan molecules involved in the formation of a single particle increases, which gradually leads to the formation of microparticles that appear as a flocculent precipitate.

To specify the optimum concentrations of chitosan and TPP solutions, 16 formulations were prepared using all the possible combinations between four concentrations of chitosan and TPP solutions (0.5, 1, 1.5 and 2 mg/ml; Table 1). The appearance of the dispersion was observed, which can be categorized into: clear solution, colloidal dispersion and aggregates. The combinations that produced clear solutions or aggregates were rejected, while the colloidal dispersions were characterized for their particle size, PDI and zeta potential.

Considering the optimum TPP concentration that is required to achieve crosslinking with chitosan molecules, 0.5 mg/ml TPP failed to obtain crosslinking with chitosan in all concentrations. On the other hand, a TPP concentration of 2 mg/ml led to the formation of aggregates when combined with every chitosan concentration, regardless of the chitosan/TPP mass ratio (Figure 1D). Therefore, a TPP concentration of 1–1.5 mg/ml was regarded as critical.

With respect to chitosan/TPP mass ratio, it was noted that a mass ratio lower than 2.5:1 led to the formation of microparticles and aggregates. This might be explained as there was too many TPP molecules that not only led to chitosan crosslinking, but also helped inretchain crosslinking, which produced the aggregates. On the other hand, a mass ratio higher than 5:1 failed to produce nanoparticles, as the TPP molecules available were not adequate enough to help the crosslinking of chitosan. These findings are in agreement with the work of Alam et al.

Based on the previous screening, the four formulations, that manifested colloidal dispersions, were selected to be characterized for their particle size, PDI and zeta potential (F2-B, F3-B, F4-B and F4-C; Table 1).

Considering the particle size as the prior selection criteria, F4-C nanoparticles formulation has the smallest diameter (182 ± 2.507 nm) and a low PDI (0.264 ± 0.012). In addition, F4-C nanoparticles have a high zeta potential (+ 40.4 ± 4.92 mV) which improves their stability in solution through the repulsion between nanoparticles. Therefore, F4-C was selected as the optimum placebo CS-NPs formulation, and used in the subsequent experiments.

Effect of concentration of polysorbate 80

Polysorbate 80 (Tweens 80) is usually added to CS-NPs dispersions to prevent their aggregation and help their resuspendibility. Tweens 80 was added in different concentrations (0.1, 0.25 and 0.5 %w/v). When freshly prepared, Tweens 80, in all concentrations, had a statistically insignificant effect on the particle size, PDI and zeta potential of F4-C nanoparticles (p > 0.05; Supplementary Table S1).

Furthermore, the ability of Tweens 80 to preserve the particle size and PDI upon short-term (a week) and long-term (3 months) storage was estimated. While 0.1 and 0.25 %w/v Tweens 80 were able to preserve the monodispersity of F4-C nanoparticles for a week after preparation, they failed to prevent particle growth, leading to a substantial diameter increase (Figure 2). In contrast, Tweens 80 in a concentration of 0.5 %w/v preserved the monodispersity of the nanoparticles over long-term storage and the diameter increase was statistically insignificant (p > 0.1; Figure 2). Therefore, 0.5 %w/v Tweens 80 was selected to be used in subsequent experiments.

Preparation of galantamine hydrobromide-loaded chitosan nanoparticles

Based on the previous results, formula F4-C was selected as the optimum placebo CS-NPs formulation, and loaded with GH in different CS:GH ratios. Two preparation methods (A and B) were compared and their effects on the %EE, %LC, process yield, particle size, PDI and zeta potential were determined.

Method (A)

Five different chitosan:GH (CS:GH) ratios were prepared (1:1, 2:1, 3:1, 4:1 and 5:1) by method (A). It was found that decreasing the ratio of CS:GH from 5:1 to 3:1 increased the %EE and %LC from 4.8% to 9.3% and from 1.23% to 3.27%, respectively. On the other hand, continuing decreasing CS:GH ratio to 2:1 and 1:1 intensely decreased the %EE and %LC (Table 2). It was greatly observed that there was a non-simple relationship between CS:GH ratio and %EE.

It has been reported that GH has a pKa value of 8.2. At pH 4.5 of the preparation medium, GH would be positively charged. Therefore, upon addition of negatively charged TPP over positively charged chitosan and GH, there might be a competition between chitosan and GH molecules over TPP molecules. That might explain why increasing the GH amount was accompanied by decreasing the process yield.

Although all GH-loaded formulations showed an increase in particle size and PDI, it was marginally significant (p > 0.1) when compared to those of placebo F4-C nanoparticles (182 ± 2.507 nm and 0.264 ± 0.012, respectively). On the other hand, GH-loaded formulations showed a slight increase in zeta potential when compared to that of F4-C formulation (+ 40.4 ± 4.92 mV).

As shown in Table 2, generally the encapsulation efficiency of GH in CS-NPs prepared by method A was intensely low, regardless of CS:GH ratio. This could be due to the probable...
repulsion between the positively charged chitosan and GH molecules at pH 4.5, which hindered the ability of GH to get entrapped into the nanoparticles. This finding agreed with the study conducted by Janes et al. trying to encapsulate doxorubicin in CS-NPs. As doxorubicin has a pK$_a$ of 8.2, which is similar to that of GH, repulsion existed between doxorubicin and chitosan molecules, which hindered the encapsulation efficiency to only 8–13%. Accordingly, it is concluded that the entrapment efficiency is highly influenced by the ionic nature of the drug. Therefore, improving the entrapment of the cationic GH into the cationic CS NPs requires overcoming the repulsive forces between them.

**Method (B): chitosan–GH complex formation**

Based on the previous facts and results, encapsulation of GH into the positively charged CS-NPs was really challenging. According to the study conducted by Janes et al., the complexation of doxorubicin with chitosan prior to the formation of nanoparticles led to a significant increase in the encapsulation efficiency of doxorubicin. Therefore, in trying to increase the encapsulation efficiency, we investigated the possibility of forming a complex between chitosan and GH molecules prior to the formation of the nanoparticles (Method B). Since the two CS:GH ratios 3:1 and 4:1 resulted in the highest %EE (9.3 and 7%, respectively), they were selected to investigate the complexation possibility.

CX-NP1 and CX-NP2 were prepared by stirring the corresponding quantity of GH (5 or 7 mg) into 10 ml of 2 mg/ml chitosan solution for 24 h prior to the addition of Tween 80 and TPP solution. A noticeable increase in the %EE was observed with both formulations (16.9 and 23.34% for 4:1 and 3:1 CS:GH ratios, respectively). The complexation between GH and chitosan apparently led to overcoming the repulsive forces between them, thus improving the %EE.

Although the particle size and PDI of CX-NP2 were insignificantly different from those of GH-NP3 (Table 2), surprisingly the zeta potential of CX-NP2 decreased markedly from +43.4 ± 5.21 mV (GH-NP3) to +31.6 ± 9.75 mV. Since the positive charge of CS-NPs is due to the deacetylated amino groups, it was thought that some of these amino groups might have been involved in the complexation with GH, which decreased the zeta potential of CX-NP2 to that extent.

**FT-IR spectrometry**

Fourier transform-infrared (FT-IR) spectrometry was used as a tool to detect if there is any interaction between chitosan and GH molecules. The FT-IR spectra of pure chitosan, pure GH and chitosan/GH complex are revealed in Figure 3(A). The chitosan FT-IR spectrum shows a characteristic band at 3413 cm$^{-1}$ for the stretching vibration of O–H bond overlapping with the stretching vibration of N–H bond. According to Traykova et al., the FT-IR spectrum of GH is very characteristic. It shows several bands, of which the most characteristic are: the stretching O–H bond vibration of the enol group (C=O–H) at 3559 cm$^{-1}$ and the band which results from overlapping of the stretching vibration of physically adsorbed hydrogen bonded water and hydrogen bonded N–H

Table 2. Composition and properties of different galantamine hydrobromide-loaded chitosan nanoparticles prepared by methods (A) and (B).

| Preparation method | Formula | CS amount added (mg) | TPP amount added (mg) | GH amount added (mg) | CS:GH ratio | %EE | %LC | Process yield (%) | Diameter (nm ± SD) | PDI ± SD | Zeta potential (mV) |
|--------------------|---------|----------------------|-----------------------|---------------------|-------------|-----|-----|--------------------|------------------|----------|---------------------|
| Method A           | GH-NP1  | 20                   | 6                     | 4                   | 5:1         | 4.80| 1.23| 51.3              | 209 ± 0.325      | 0.261 ± 0.002   | 40.9 ± 6.2 |
|                    | GH-NP2  | 20                   | 6                     | 5                   | 4:1         | 7.00| 2.55| 44.2              | 211 ± 1.401      | 0.265 ± 0.013   | 43.1 ± 9.6 |
|                    | GH-NP3  | 20                   | 6                     | 7                   | 3:1         | 9.30| 3.27| 60.3              | 224 ± 1.752      | 0.269 ± 0.008   | 43.4 ± 5.21 |
|                    | GH-NP4  | 20                   | 6                     | 10                  | 2:1         | 4.40| 2.34| 52.2              | 227 ± 0.306      | 0.279 ± 0.010   | 41.9 ± 7.5  |
|                    | GH-NP5  | 20                   | 6                     | 20                  | 1:1         | 0.50| 0.62| 35.2              | 230 ± 2.601      | 0.301 ± 0.007   | 43.3 ± 4.52 |
| Method B           | CX-NP1  | 20                   | 6                     | 5                   | 4:1         | 16.90|5.63|48.7              | N/A              | N/A       | N/A                 |
|                    | CX-NP2  | 20                   | 6                     | 7                   | 3:1         | 23.34|9.86|43.0              | 190 ± 1.159      | 0.276 ± 0.006   | 31.6 ± 9.75 |

CS, chitosan; EE, entrapment efficiency; GH, galantamine hydrobromide; LC, loading capacity; N/A, not determined; PDI, polydispersity index; TPP, tripolyphosphate. 

![Figure 2. The effect of tween 80 concentration on the particle size (left) and PDI (right) of F4-C chitosan nanoparticles when freshly prepared, after 1 week, and 3 months storage at 4°C (Mean ± SD, n = 3). PDI, polydispersity index.](image-url)
bonds in the $\equiv$N$^+$–H cation at 3433 cm$^{-1}$. These findings were consistent with the previously reported results by Bastida et al. and Li et al.$^{32,33}$

Regarding the FT-IR spectrum of CS–GH complex, the most characteristic bands are: the extremely broad band that appears at 3428 cm$^{-1}$ which is attributed to the hydrogen bonding, and the intense N–H bending vibration band at 1569 cm$^{-1}$. Therefore, it is evident that there is a possible hydrogen bonding between the –OH group of GH and –NH group of chitosan. This finding can explain the reason standing behind the increased %EE upon preparing the nanoparticles using method B. A hypothetical structure of a nanoparticle showing the hydrogen bonding complexation between chitosan and GH molecules is shown in Figure 3(B).

Visualization by TEM

Transmission electron microscopic (TEM) photographs of the placebo F4-C CS-NPs and the GH-loaded CX-NP2 nanoparticles are shown in Figure 4. In general, the nanoparticles were uniform and their shape ranged from perfectly spherical to subspherical. They were well-separated from each other, and no aggregation was observed suggesting stabilization by their positively charged surfaces. The particle size of F4-C nanoparticles was found in the range 38.3–52 nm, while that of CX-NP2 was found in the range 48.3–68.3 nm. Therefore, it can be concluded that the size of CX-NP2 increased as a result of GH encapsulation. This finding is in agreement with Fazil et al.$^{34}$ results. In addition, GH encapsulation was manifested as a heavier density white color inside the CS-NPs (Figure 4B and C).

Upon comparing the particle size measurements obtained by DLS and TEM analyses, there is an obvious discrepancy between them for both the placebo F4-C and GH-loaded CX-NP2 nanoparticles. Size measurements obtained by TEM are much smaller than those obtained by DLS technique. This is presumably because sample preparation for TEM imaging involves drying, while DLS determines the “apparent” nanoparticle size, i.e., the hydrodynamic diameter, which includes the aqueous layers surrounding the nanoparticles.$^{35}$

Differential scanning calorimetry analysis

Differential scanning calorimetric (DSC) thermograms of pure chitosan, pure GH, placebo F4-C CS-NPs and GH-loaded CX-NP2 nanoparticles are presented in Figure 5. The DSC thermogram of chitosan polymer shows an endothermic peak at 67.8 °C, which is accredited to the loss of bound and absorbed water. A corresponding endothermic peak appeared in the DSC thermograms of placebo F4-C CS-NPs and GH-loaded CX-NP2 nanoparticles at 68.1 and 65.2 °C, respectively. The slight differences in the position and peak area of these endothermic peaks from that of pure chitosan might be due to the different
holding capacities and variant water–polymer interaction strengths.\(^3^6\)

Chitosan DSC thermogram shows an exothermic broad peak at 299.2°C, which corresponds to its degradation, and it disappeared in both thermograms of placebo F4-C CS-NPs and GH-loaded CX-NP2 nanoparticles. Instead, a new exothermic peak is observed at 210 and 212.1°C in the thermograms of placebo F4-C CS-NPs and GH-loaded CX-NP2 nanoparticles, respectively. It is probable to be owing to the breakdown of the ionic interaction between the polycationic chitosan and polyanionic TPP molecules.\(^3^6\)

Regarding the DSC thermogram of GH, it is characterized by a small endothermic peak at 266.6°C, which could be due to its melting point, overlapping with a sharp and prominent exothermic peak at 272°C.\(^3^7\) This peak completely disappeared in the thermogram of the GH-loaded CX-NP2 nanoparticles, which verifies the GH encapsulation into CS-NPs and its existence in an amorphous state.

**In vitro drug release study**

Distilled water was selected as a release medium instead of phosphate buffered saline (PBS) to disperse the nanoparticles nicely as the phosphate ions in PBS led to nanoparticles aggregation.\(^3^8\) To maintain the sink condition through the release study, GH solubility in distilled water was determined and found to be 25.2 mg/ml. Figure 6(A) shows the in vitro release profile of GH from CX-NP2 nanoparticles. It is apparent that there was an initial burst effect (14.08 ± 2.19%) within the first 30 min, followed by a prolonged slow release that reached only 58.07 ± 6.67% after 72 h. Usually, the burst drug release at the beginning is due to those GH molecules entrapped near the nanoparticle surface, which diffuse faster and easier.\(^3^9\) The markedly slow release that followed the burst release could be due to, besides the presence of GH in a polymeric matrix, the hydrogen bonding interaction between GH and chitosan molecules, which needs a prolonged time to break down and allow the drug to diffuse outside the nanoparticles.\(^2^9\) The prolonged release is expected to be advantageous in vivo as the nanoparticles would not exhibit a premature drug release before reaching their target: the brain. Nevertheless, it should be considered that the release obtained in vitro might not simulate the in vivo formulation behavior.

The release kinetics of GH from CX-NP2 nanoparticles were characterized by fitting the in vitro release data to six different release models (zero-order, first-order, Higuchi, Korsmeyer–Peppas, Weibull and Hixon–Crowell).\(^3^9\) By comparing the coefficient of determination (R\(^2\)) obtained in each model, the model that best fits the release data is Korsmeyer–Peppas model, characterized by the highest R\(^2\) value (0.990; Supplementary Table S2).

Korsmeyer–Peppas model is represented by the following equation:

\[
\frac{M_t}{M_\infty} = Kt^n
\]

where \(\frac{M_t}{M_\infty}\) is a fraction of drug released at time t, K is the release rate constant and n is the release exponent. The obtained n value (0.315) indicates that the mechanism of GH release from CX-NP2 nanoparticles was Fickian diffusion.\(^3^9\)

**Mucoadhesion study**

Mucoadhesiveness is a glycoprotein that represents the major component of the mucus coating the nasal mucosal cells.\(^4^0\) Therefore, measuring the binding efficiency of the GH-loaded CX-NP2 nanoparticles to mucoadhesiveness was necessary to predict the residence time of the nanoparticles upon intranasal administration.
of CS-NPs was measured as the percent of mucin (2 mg/ml) that was adsorbed on the nanoparticles surface (2 mg/ml) in different time intervals.

Figure 6(B) shows the adsorption kinetics of mucin (2 mg/ml) on GH-loaded CX-NP2 chitosan nanoparticles (2 mg/ml) at 37°C (Mean ± SD, n = 3). It was clearly observed that more than 80% of mucin adhered to the surface of the nanoparticles instantaneously upon their mixing through the electrostatic interaction between the positively charged surface of CS-NPs and the negatively charged sialic acid groups characterizing the mucin molecules. Nevertheless, the mucoadhesion of nanoparticles to mucin persisted over 60 min (with a negligible decline that might arise from dissociation of the electrostatic interaction over time)\(^\text{41}\). Moreover, the mucoadhesiveness of placebo CS-NPs was insignificantly different from that of CX-NP2 (\(p > 0.05\); data not shown). Thus, it can be concluded that the complexation reaction between GH and chitosan did not affect the mucoadhesiveness of CX-NP2. Therefore, CX-NP2 nanoparticles have a remarkable mucoadhesiveness that would help their adherence to nasal mucosa upon \textit{in vivo} administration and decrease their mucociliary clearance.

**Assessment of storage stability of drug-loaded nanoparticles**

As the physicochemical properties of the prepared CX-NP2 nanoparticles affect their \textit{in vivo} performance and efficacy, their ability to preserve their particle size, polydispersity and drug content over storage was studied.

Nanoparticles dispersion stored for 3 months at 4°C exhibited only 6.09 ± 0.013% drug leakage and a slight particle size increase, and both changes were considered insignificant (\(p > 0.05\)). However, storage at 25°C for 3 months has noticeably affected not only the GH content (14.78 ± 0.042% drug leakage), but also the particle size (about 65% diameter increase; Figure 7A and B). These results are in agreement with previously reported data\(^\text{15}\).

Regarding the dispersion polydispersity, numerous aggregates were visually observable in the nanoparticles dispersion stored at 25°C, but not in those stored at 4°C. This observation was confirmed by the significant increase in the PDI under storage at 25°C for 3 months (Figure 7C).

The significant drug leakage, coupled with the increased particle size and polydispersity, all recorded under storage at 25°C, can be owed to the destructive aggregation of CX-NP2 nanoparticles at elevated temperatures. This aggregation not only increased the particle size, but also it might have destabilized the nanoparticles and affected the hydrogen bonding that holds the GH with chitosan moieties. Therefore, it appears that the optimal conditions for storage of CX-NP2 nanoparticles aqueous dispersion are in a tight container at 4°C.

**Detection of GH/chitosan complex nanoparticles into different rat brain regions qualitatively**

The use of fluorescent-labeled nanoparticles is one of the most rapid and simple methods used to study the uptake of nanoparticles within the brain sections. Fluorescence microscope is used to visualize these labeled nanoparticles with high sensitivity. Afterwards, the photographed images could be analyzed using software such as ImageJ to measure the fluorescence intensity and compare images belonging to different brain regions.

Rhodamine B isothiocyanate (RBITC) was used to label the selected CX-NP2 nanoparticles (RBITC-CX-NP2) by the formation of a covalent bond between the amino group in chitosan and...
the reactive isothiocyanate group in RBITC. Therefore, obtaining fluorescence would be necessarily due to the delivery of the whole CX-NP2 rather than leakage of RBITC molecules. The particle size, zeta potential, mucoadhesion and GH in vitro release of RBITC-CX-NP2 were insignificantly different from those of CX-NP2 ($p > 0.05$; data not shown).

It has been reported that the progress of AD through the brain is not random at all. In contrast, the pattern of AD propagation follows a specific, yet slow, pathway. The deposition of β-amyloid plaques and neurofibrillary tangles usually leads to brain atrophy. According to the severity of the brain atrophy, AD stages could be classified into early, moderate and severe. As concluded by some studies, the first brain region that undergoes damage is the hippocampus, and then the disease propagates out to medial, parietal and frontal regions, eventually affecting all cortex regions. On the other hand, Sepulcre et al. argued that the deposition of β-amyloid plaques is initiated in the “amygdala–orbitofrontal–hippocampal” formation simultaneously, and then it extends to the posterior, medial and lateral temporal pole, the medial parietal cortex and the medial and lateral prefrontal cortex. The damage of each brain region is responsible for the manifestation of specific symptom(s). For instance, the hippocampal damage results in short-term memory loss, while the parietal cortex atrophy leads to reading difficulties and...

Figure 8. Fluorescence microscopic uptake study of RBITC-labeled GH/chitosan complex nanoparticles (RBITC-CX-NP2) into different rat brain regions after 1 h incubation following intranasal administration. (A) Dorsal, ventral and sagittal views of an adult rat brain indicating the location of the examined brain regions; (B) Microscopic brain images of [1] the brain orbitofrontal cortex of a control rat administered 50 μl 0.9% w/v saline, and [2 → 5] different brain regions of a rat administered 50 μl RBITC-CX-NP2 nanoparticles [100 × magnification]; (C) Comparison of the corrected total fluorescence values of different brain regions to the control brain (Mean ± SD, n = 6). The increase in fluorescence intensity is significant ($p < 0.05$). (Please refer to online version for interpretation of references to color.)
topographical disorientation. Therefore, representative regions of rat brain have been selected to examine if RBITC-CX-NP2 nanoparticles could be detected in them 1 h after intranasal administration (Figure 8A).

It has been reported that transfer of nanoparticles from nasal cavity to the brain involves crossing the nasal epithelial cells to peripheral neuronal components of olfactory bulb and trigeminal nerve, with a subsequent spreading of the transported substances to distant CNS locations. Therefore, olfactory bulb sections were also examined for the presence of RBITC-CX-NP2 nanoparticles.

Figure 8(B) shows photomicrographs of various brain sections 1 h after administering 50 μl RBITC-CX-NP2 nanoparticles intranasally, compared to the corresponding control rat brain. A comparison of the mean CTF values calculated using ImageJ 1.46r image processing, using six photographs for each section, is shown in Figure 8(C). It is evident that there are distinguishable patterns of red fluorescence in all brain regions when compared to the control brain (student’s t-test, p < 0.05).

It can be inferred that the prepared RBITC-CX-NP2 managed to be delivered to the brain after intranasal administration. Nanoparticles could have been transported across the olfactory and respiratory epithelia. Intracellular transport across the olfactory epithelium leads to the olfactory bulb, while intracellular transport across the respiratory epithelium ends in trigeminal nerve fibers. Moreover, RBITC-CX-NP2 could have been transported extracellularly across nasal epithelia, reaching the lamina propria and absorbed into blood vessels ending in systemic circulation, or absorbed in lymphatic vessels draining into cervical lymph nodes. Upon reaching the olfactory neurons or trigeminal nerve fibers, RBITC-CX-NP2 may have spread intracellularly (via synapses) or extracellularly (by diffusion) to different front (olfactory bulb and orbitofrontal cortex), middle (hippocampus) and back (parietal cortex) brain regions.

It is apparent that the administration of RBITC-CX-NP2 to the nasal mucosa could have led to their cellular internalization by different endocytic pathways. Endocytosis types include macropinocytosis, clathrin-mediated, clathrin-independent, caveolin-mediated, caveolin-independent, in addition to phagocytosis. In a valuable study, Rejman et al. investigated the effect of particle size on the involved cellular internalization pathway. They found that <200 nm nanoparticles were internalized via clathrin-coated pits. However, caveolea-mediated endocytosis became more noticeable as the nanoparticles increased in size (200–1000 nm). Based on the average size of the RBITC-CX-NP2, it is thought that the clathrin-independent endocytosis could be the predominant endocytic mechanism.

Conclusion

It is concluded that complexation is a promising approach to overcome the repulsive forces between GH, a cationic drug, and CS-NPs, cationic nanoparticles. GH/chitosan complexation prior to nanoparticles formation by ionic gelation enhanced the entrapment efficiency and loading capacity compared to direct entrapment method. Furthermore, the complexation prolonged GH release, improved the formulation stability at 4°C, and insignificantly affected the physicochemical properties of optimized CS-NPs. In addition, RBITC-CX-NP2 nanoparticles were detected in the olfactory bulb, hippocampus, orbitofrontal and parietal cortices 1 h after intranasal administration, proving that the complexation interaction between GH and chitosan has not impaired the potential of CS-NPs as intranasal brain delivery system for Alzheimer’s disease management.

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Supplementary material available online
Supplementary Tables S1 and S2.