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Chitosan coated nanostructured lipid carriers for brain delivery of proteins by intranasal administration

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ABSTRACT

The remarkable increase in the prevalence of neurodegenerative diseases has become a serious public health problem. Considering the lack of effective treatments to address these diseases and the difficulties in accessing the brain due to the blood–brain barrier (BBB), to attain a successful strategy to improve drug delivery to the brain, the administration route becomes a point of interest. The intranasal route provides a non-invasive method to bypass the BBB. Moreover, the development of new technologies for the protection and delivery of peptides is an interesting approach to consider. Thus, in this work, a suitable chitosan coated nanostructured lipid carrier (CS-NLC) formulation with the capacity to reach the brain after being intranasally administered was successfully developed and optimized. The optimal formulation displayed a particle size of 114 nm with a positive surface charge of +28 mV. The *in vitro* assays demonstrated the biocompatibility of the nanocarrier and its cellular uptake by 16HBE14o- cells. Furthermore, no haemagglutination or haemolysis processes were observed when the particles were incubated with erythrocytes, and no toxicity signals appeared in the nasal mucosa of mice after the administration of CS-NLCs. Finally, the biodistribution study of CS-NLC-DiR demonstrated an efficient brain delivery of the particles after intranasal administration. In conclusion, CS-NLC can be considered to be a safe and effective nanocarrier for nose-to-brain drug delivery; however, to obtain a higher concentration of the drug in the brain following intranasal administration, further modifications are warranted in the CS-NLC formulation.

1. Introduction

In the last decade, there has been a remarkable increase in the prevalence of neurodegenerative diseases (NDs), which have become a serious public health problem. Currently, there is no cure for most NDs, and current therapies are focused on modifying the disease progression and symptoms, presenting insufficient or null effects on the improvement of the disease [1]. A notable obstacle to identifying an adequate therapy is the presence of the blood–brain barrier (BBB), which limits the effective delivery and distribution of therapeutic agents to the central nervous system (CNS) [2]. This limitation is observed due to the function of the BBB in maintaining CNS homeostasis and preventing the free diffusion and penetration of most drugs and other foreign components from the bloodstream to the brain [3].

Considering these factors, the scientific community is making enormous efforts in the development of new successful treatment options to improve drug delivery to the brain by means of invasive or non-invasive ways. Through intracerebroventricular and intraparenchymal administration routes, drugs are administered directly into the brain, thereby avoiding the BBB. Therefore, high drug concentrations can access the target site. However, both methods are invasive techniques in which the drug diffusion from the injection site is not easy [4]. Intraperitoneal, intravenous or subcutaneous routes are much simpler techniques that are used as a less invasive alternative. Nevertheless, after parenteral administration, most of the drugs present serious difficulties in crossing the BBB, and hence, to obtain therapeutics levels in the brain, the administration of high doses is required, which may result in adverse systemic effects [5,6]. Lastly, recent research describes

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several studies that propose intranasal (i.n.) administration route as a non-invasive way to transport drugs directly to the CNS through the olfactory and trigeminal nerve pathways; thus, it presents the capacity to bypass the BBB [7]. Furthermore, clinical trials in humans have demonstrated that intranasal administration offers a successful alternative to deliver drugs to the brain. In this regard, *Reger et al.* confirmed that intranasally administered insulin improves verbal memory in Alzheimer disease patients without systemic side effects [8].

Currently, there have been enormous efforts to identify new treatments to address CNS disorders. Accordingly, neurotrophic factors have become an interesting therapy due to their ability to provide neuroprotective, neurorestorative and stimulating effects on diseased neurons. However, it is interesting to mention that after their *in vivo* application, these peptides present important shortcomings, such as a short circulation half-life, a rapid degradation rate, or a poor ability to cross the BBB due to their unsuitable molecular weight, lipophilicity or surface charge [9]. Hence, new technologies for brain drug delivery have been investigated in the last few years to provide new strategies to overcome the mentioned limitations [2]. In this sense, nanometric drug delivery systems could be considered possible tools to protect drugs against degradation in the nasal cavity as well as to stimulate nose to brain drug delivery [10]. Among these substances, nanostructured lipid carriers (NLCs), which are the improved second-generation derived from solid lipid nanoparticles, represent an attractive system for this purpose. NLCs are usually composed of biodegradable and biocompatible lipid components primarily obtained from natural sources. NLCs also offer high drug entrapment efficiencies and high stability, and they have a well-established safety profile and toxicological data [11–13]. However, NLCs' major drawbacks after i.n. administration are a low residence time in the nasal cavity and incomplete drug absorption due to mucociliary clearance [14]. With the aim of solving these challenges, lipid formulations enable the possibility of surface charge modification with various cationic substances, such as chitosan (CS), with interesting characteristics. Several studies have demonstrated the excellent mucoadhesive properties of this cationic polysaccharide, enhancing the penetration across epithelial mucus and prolonging the retention time in the nasal cavity [15–17].

Accordingly, the aim of our study was to design and optimize a CS-NLC formulation to obtain mucoadhesive and positively charged nanoparticles with a particle size of approximately 100 nm for promoting the delivery of drugs to the brain after intranasal administration. *In vitro* tests were undertaken in 16HBE14o- cells to determine the cytotoxicity and cell uptake capability of our formulation. The interaction of CS-NLC with erythrocytes was analyzed by haemolysis and haemaglutination assays. Additionally, the nasal toxicity of the nanoparticles was also evaluated *in vivo* in C57 mice. Finally, CS-NLCs were loaded with the near infrared dye, DiR, and administered intranasally to nude mice to explore the possibility of brain targeting by nose-to-brain delivery using fluorescence imaging (FLI) monitoring.

2. Materials and methods

2.1. Materials

Precirol ATO®5 (Glycerol distearate), Dynasan 114® (Trimyristin) and Miglyol® (Caprylic/Capric Triglyceride) were donated by Gattefosé (France), Oxi-Med Expres S.A (Spain) and Sasol Germany GmbH, respectively. Tween 80, Lutrol® F-68 (Poloxamer 188), sodium citrate and 3.7% paraformaldehyde were purchased from Panreac (Spain). Protasan UP CL 113Chitosan was obtained from NovaMatrix (Norway). Trehalose dihydrate, NileRed, Cell Counting Kit-8 (CCK-8), Bovine serum albumin, Vitrogen 100, Human fibronectin, Hepes, Glucose, $Na_2HPO_4{\cdot}7H_2O$, Phenol Red, Polyvinilpyrrolidone, EGTA, EDTA and citric acid were bought from Sigma–Aldrich (Spain). DiR DiIC18 (7) (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindotricarbocyanineiodide) was purchased from Molecular Probes© by Life Technologies (Spain). hIGF-I was obtained from Peprotech (UK). The 16HBE14o- cell line was bought from the Dr. Gruenert Laboratory (University of California, San Francisco). MEM, LHC basal medium, foetal bovine serum, L-glutamine, penicillin/streptomycin and PBS pH 7,4 (1×) were purchased from Gibco© by Life Technologies (Spain). Sodium chloride was obtained from Labkem (Spain), K_2HPO_4, KH_2PO_4 and KCl were obtained from Scharlau (Spain) and 4′,6-diamidino-2-phenylindole (DAPI) was bought from SouthernBiotech (USA).

2.2. NLC preparation and optimization

Precirol ATO5 (melting point: 56 °C) or Dynasan 114 (melting point: 56 °C) and Miglyol (liquid at room temperature (RT)) were chosen to form lipid matrix. The lipid phase was melted 5 °C above its melting point until a clear and homogeneous phase was obtained. The aqueous solution was composed of various percentages of Tween 80 and Poloxamer 188 to obtain a final volume of 4 ml, and the solution was warmed in a water bath. The hot surfactant phase was then added to the melted oily phase and was sonicated for 60 s at 50 W (Branson® sonifier 250). The nanoemulsion was maintained under magnetic stirring during 15 min at RT and stored at 4 °C for 12 h overnight to allow the re-crystallisation of the lipid for NLC-formation. On the following day, the nanoparticle dispersion was centrifuged in an Amicon filter (Amicon, "Ultracel-100k") at 2500 rpm (MIXTASEL, P Selecta) for 15 min, the nanoparticles were washed three times with milli Q water and were finally lyophilized during 42 h (LyoBeta 15, Telstar, Spain). Prior to the lyophilization of the resultant NLC dispersion, a solution of a cryoprotectant (trehalose (15% w/w)) was added to the collected nanoparticles.

Chitosan coated NLCs were prepared as described above but followed by a chitosan coating process. The nanoparticle dispersion was added dropwise to an equal volume (4 ml) of a chitosan solution (0.5%, w/v) kept under continuous agitation at RT, and the suspension was maintained under these conditions for 20 min to allow the coating of the nanoparticles. After this step, the CS-NLC dispersion was centrifuged and lyophilized as previously mentioned. To optimize the formulation, various lipid and surfactant percentages, described in Table 1, were tested.

Finally, the neurotrophic factor human insulin-like growth factor-I (hIGF-I) was loaded in the CS-NLCs at a concentration of 0.5% (w/w) to assess the suitability of the nanoparticles to encapsulate a therapeutic candidate for ND applications. Additionally, the lipophilic dye NileRed and the near infrared dye DiR were incorporated into the NLC (CS-NLC-NileRed and CS-NLC-DiR), both at a concentration of 0.5% (w/w), for cellular uptake assays and to determine the biodistribution profile. These formulations were elaborated as described above but included the corresponding neurotrophic factor or dye, depending on the formulation, in the lipid phase prior to the sonication process.

2.2.1. Nanoparticle characterization: size, zeta potential, morphology and encapsulation efficiency (EE%)

The mean diameter (*Z*-average diameter) and size distribution were measured by Dynamic Light Scattering, and the zeta potential was determined through Laser Doppler Micro-Electrophoresis (Malvern® Zetasizer Nano ZS, Model Zen 3600; Malvern Instruments Ltd.). Three replicate analyses were performed for each formulation, and the data are presented as the mean ± S.D. Nanoparticle surface characteristics and morphology were exam-