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Chitosan coated niosomes for nose-to-brain delivery of clonazepam: Formulation, stability and permeability studies.

Giulia Nerli [a], Sandra Robla [b], Marta Bartalesi [a], Cristina Luceri [c], Mario D'Ambrosio [c], Noemi Csaba [b,*], Francesca Maestrelli [a,*]

[a] Department of Chemistry "U. Schiff" via U. Schiff, 6 Sesto Fiorentino, Florence, Italy
[b] Centre for Research in Molecular Medicine and Chronic Diseases (CiMUS) and Department of Pharmacology, Pharmacy and Pharmaceutical Technology, University of Santiago de Compostela, Spain
[c] Department of Neurosciences, Psychology, Drug Research and Child Health (NEUROFARBA) University of Florence, Florence, Italy

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ABSTRACT

Crossing the Blood Brain Barrier constitutes a challenge in drug administration to the brain. In this context, nose-to-brain delivery is explored as an alternative route in the treatment of central nervous system disorders, and nanotechnology constitutes a promising tool for drug delivery to the brain. In this work, we explored niosomes and chitosan-coated niosomes (chitosomes) as possible tools for nose-to-brain delivery of clonazepam. The formulations have been optimised using different chitosan concentrations and different preparation methods as Thin Layer Evaporation-paddle (TLE-P), Evaporation (E), and Solvent Displacement Technique (SDT). The most suitable formulations were loaded with clonazepam (CLZ) and a full physicochemical characterization was performed. Chitosomes presented a size of around 200 nm, PDI < 0.3, a positive surface charge, spherical shape and a CLZ encapsulation above 60%. Chitosomes were stable for 12 weeks under storage conditions at 4ºC, in simulated nasal fluid for 24 h as well as after a lyophilization-sonication process. A CLZ release of 50% was also achieved after 4 h in this media. The mucoadhesive properties of chitosomes were also confirmed, with a 1.5-fold reduction of CLZ toxicity after encapsulation and a 10-fold increase of its permeability.

1. Introduction

The major drawback of peripheral administration routes is the limited accessibility of the drug molecules or active ingredient to the brain due to the Blood Brain Barrier (BBB) which severely restricts the entry of almost all the drug molecules, proteins, and other large molecules, protecting the brain from any potential threat. The intranasal (IN) route has emerged as an attractive alternative to parenteral administration routes, offering direct nose-to-brain delivery via different mechanisms such as the olfactory nerve, the perineural drug transport and the drug transport across the brain-blood-barrier from the systemic circulation. This nose-to-brain administration pathway is associated with enhanced safety, increased patient compliance (as it is a comfortable and non-invasive route), rapid onset of action, as well as minimal systemic exposure (Ahmad et al., 2017; Lochhead & Thorne, 2012). In the last years, nose-to-brain delivery was explored as a promising approach in the treatment of central nervous system (CNS) disorders (Agrawal et al., 2018) and the IN the delivery route could represent a breakthrough in the treatment of neurological diseases since it presents a valid strategy to avoid bioavailability issues due to anatomical barriers (Erdő et al., 2018; Kumar et al., 2018), and in particular, in the treatment of epileptic seizure emergencies by benzodiazepines (Kapoor et al., 2016) These disorders require daily use of medicines and therefore, an easily administered formulation with a fast onset of action is desirable, especially in the treatment of seizures and other symptoms like panic attacks and anxiety disorders. Commercial IN formulations of diazepam (Valtoco®) and midazolam (Nayzilam®), have been recently approved (Boddu & Kumari, 2020), however, among the different benzodiazepines, clonazepam (CLZ) offers the advantage of a longer duration of action (Nardi & Perna, 2006; Rey et al., 1999) A nose-to-brain delivery of CLZ could be an interesting alternative to the oral or parenteral administration that could improve patient compliance. However, when developing an effective nose-to-brain formulation, several factors must be considered. For example, the short time of absorption due to the rapid mucociliary clearance, the limited volume available for administration (up to 150 μl per nostril), the difficult permeation thought the mucus

* Corresponding authors.
E-mail addresses: noemi.csaba@usc.es (N. Csaba), francesca.maestrelli@unifi.it (F. Maestrelli).

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barrier, and the risk of enzymatic degradation (Karavasili & Fatouros, 2016; Putri et al., 2017). To overcome these issues, the use of mucoadhesive and permeation enhancer polymers (i.e. chitosan) have been suggested to increase the residence time of the formulations and improving drug permeation (Sonaje et al., 2012). It has also been shown that the use of nanocarriers as niosomes could provide increased permeation, sustained release and protection of the drug from enzymatic degradation. Niosomes are nanoscale vesicles mainly composed of non-ionic surfactants and cholesterol that present high biocompatibility and biodegradable characteristics (Bragagni et al., 2012). Due to their structure, niosomes can entrap lipophilic or hydrophilic drugs and can deliver them to their target site in a controlled and/or sustained manner. In addition, their surface can be modified to obtain properties like optimal surface charge or specific targeting. Previous studies have successfully obtained niosomes functionalized with N-palmitoyl glucosamine (NPG), a specific ligand to enhance drug accumulation in the brain from the systemic circulation, demonstrating good permeation enhancement (Bragagni et al., 2014; Maestrelli et al., 2019). Moreover, chitosan coating could modulate niosomes properties, conferring positive surface charge and consequently improving the interaction with the negatively charged nasal mucosa. Chitosan is a well-known natural polymer, biocompatible and with already reported properties of enhancing drug permeation (Mura et al., 2022).The use of chitosan-based formulations is a promising approach for nose-to-brain drug delivery since the extended residence time and intimate contact with nasal epithelium improve the drug uptake from the nasal cavity (Aderibigbe & Naki, 2019). Chitosan coated nanoparticles should have specific physicochemical characteristics as size of <300 nm, since higher dimensions may further reduce mucosal penetration and thus avoid their interaction with the underlying epithelial cells (Feng et al., 2018). A recent study demonstrated that nanocarriers smaller than 300 nm were easily transported transcellular through olfactory neurons to the brain via the various endocytic pathways of sustentacular or neuronal cells in the olfactory membrane (Patil, 2021). Other authors evidenced that chitosan coated liposomes and niosomes were a promising and non-invasive brain delivery system in vivo, able to maximize drug efficacy and reduce dosage-related effects (Salade et al., 2018). Moreover also the drug deposition pattern in the nasal cavity, linked to physiochemical parameters of the formulation, applicators employed and the mode of the application, is an important factor in nose-to-brain drug targeting as evidenced by several Authors (Gao et al., 2020; Guellec et al., 2021; Maaz et al., 2021). In this light, colloidal systems such as chitosomes are highly versatile since they can be administered both such as liquid and dry powdered formulation, taking into account all the parameters, also dependents by the type of the used applicator, such as formulation stability, fluid viscosity and surface tension or particle size of the powders.

The aim of this work has been the development of a chitosan coated niosomes, named chitosomes, for the nose-to-brain delivery of CLZ. Niosomes and chitosomes have been prepared at different chitosan concentrations and with different preparation methods. The selected formulations were fully characterised in terms of drug entrapment, drug release, mucoadhesion, morphology, particle size, polydispersion index and zeta potential. Stability studies have been performed in simulated nasal fluid (SNF) and in phosphate buffer solution (PBS) pH 7.4 on fresh and freeze-dried chitosomes after reconstitution. Cytotoxicity and transport studies of loaded chitosomes have been performed in Caco-2 cell lines.

2. Materials and methods

2.1. Materials

Solulan™C24 (SOL) (Poly-24-oxyethylene cholesteryl ether) was a gift by Lubrizol (Cleveland, OH, USA). Cholesterol (CH), Sorbitan Stearate (Span 60, HLB 4.7), low molecular weight chitosan (50−90 kDa) (CS), Triton X-100 (TX) and Clonazepam (5-(2-chlorophenyl)-7 -nitro-3H-1,4-benzodiazepin-2(1H)-one) (CLZ) and mucin type III were purchased from Sigma-Aldrich (Milan, Italy). N-palmitoyl glucosamine (NPG) was synthesized according to Bragagni et al. (2012), and carefully characterized as previously described (Bragagni et al. 2014). Briefly, glucosamine was added under stirring to triethanolamine and DMSO. The palmitic acid N-hydroxy succinimide was dissolved in $CHCl_3$ and added to the mixture, under nitrogen atmosphere. The product was precipitated by immersion in an ice bath, filtrated, washed, dried and stored at 4 °C protected from the light. For simulated nasal fluid preparation, sodium chloride (NaCl), potassium chloride (KCl) and calcium chloride hydrate ($CaCl_2{\cdot}2H2O$) were purchased from Sigma-Aldrich (MO, USA). All other reagents were of analytical grade or higher.

2.2. Preparation of colloidal systems

Niosomes (NIO) and chitosomes (CHITO) were prepared by three different techniques using a previously optimized composition of Span 60 (8 mg/ml); CH (5.73 mg/ml); SOL (5.33 mg/ml) and NPG (1.58 mg/ml) (Maestrelli et al., 2019). The three different methods were: thin layer evaporation-paddle, evaporation, and solvent displacement technique.

The thin layer evaporation-paddle (TLE-P) stirring was a previously developed technique for NIO preparation. Briefly, the lipidic phase was dissolved in chloroform ($CHCl_3$) and completely removed by rotary evaporation under vacuum to form a thin layer on the flask wall. Then, the layer was hydrated with 20 mL of hydrophilic phase (PBS pH 7.4,) under stirring by a paddle at 2000 rpm for 30 min, heating in a water bath at 65 °C. The suspensions were centrifuged at 4000 rpm for 15 min (HERMLE Z 200 A centrifuge). The supernatant was collected, and 10 mL were subjected to sonication in an ice bath with a Sonoplus HD2200 ultrasonic homogenizer (Bandelin Electronic GmbH, Berlin, Germany) equipped with a KE76 probe (power up to 200 Watts and horn frequency of 20 kHz, 20,000 cycles per second) at 50% power for 5 min for 2 cycles. This method was also used for optimize CS concentration. Briefly, to obtain CHITO, a solution of CS in Acetic Acid 1% v/v (adjusted with NaOH 1 M at pH 4.5) at different concentrations (0.5, 1.0, 2.0 and 3.0 mg/mL) was filtered (pore size 0.45 μm) and drained on NIO suspension.

In the Evaporation (E) method, the lipidic phase dissolved in $CHCl_3$ was submitted to rotary evaporation at 37 °C for 5 min obtaining an oily solution. Then the hydrophilic phase was added, and the mixture was submitted to 4 cycles, each one composed of 2 min of stirring by vortex and 2 min of heating at 65 °C. The suspensions were centrifuged at 4000 rpm for 5 min (EPPENDORF 5430 R centrifuge). Subsequently, the supernatant was collected, and 3 mL were subjected to sonication in an ice bath with a Branson ultrasonic Digital Sonifier 450 (Emerson, USA) equipped with a 102C probe with a 1/2″ Dia. tapped Bio Horn cell disruptor accessory and a 1/8″ tapered Microtip (power up to 400 Watts and horn frequency of 20 kHz, 20,000 cycles per second) at 10% power for 2 min for 2 cycles. Then a solution of CS in Acetic Acid 1% v/v (adjusted with NaOH 1 M at pH 4.5) in water, was filtered (pore size 0.45 μm) and drained on NIO suspension, dealing to the formation of CHITO.

Finally, with the Solvent Displacement Technique (SDT) CHITO were obtained with a one-step method. Briefly, the dissolution of the lipidic phase in CHCl3 was poured over a CS solution and kept under stirring conditions. The suspensions were evaporated using rotary evaporation (R-300 Buchi®, 150 rpm, 220 mbar, during 10 min at 37 °C), resuspended in PBS 7.4 and centrifuged at 4000 rpm for 5 min (EPPENDORF 5430 R centrifuge). In all method was assessed the complete organic solvent removal. Methods are summarized in Table 1S in Supplementary Materials. Once selected the best preparation method, drug loaded CHITO were prepared by adding 0.1 mg/ml of CLZ directly in the lipophilic phase and dissolved in $CHCl_3$.

2.2.1. Analytical method for CLZ determination

Drug was analytically quantified by HPLC equipped with an Elite LaChrom L-2400 UV–vis detector (Merck Hitachi Darmstadt, Germany), following a method described in a previous work (Mura et al., 2016). Briefly, the analysis was carried out with an isocratic method using a mobile phase of acetonitrile (CH_3CN)/water 30/70 v/v. The column was an Agilent Zorbax CN (4.6 mm x 150 mm, 5 μm), the UV detection was carried out at 310 nm. The injection volume was 20 μL, the flow rate was 0.9 mL/min, the temperature was maintained at 40±1.0 °C. In these conditions the CLZ retention time was 7.23 ± 0.01 min. The method was validated for linearity in a range 4.0504 – 8.1008 μg/mL (y = 215613x-11,451; r2 = 0.9985), with a limit of quantification (LOQ) of 0.27 μg/mL and limit of detection (LOD) of 0.08 μg/mL.

2.2.2. Characterization of colloidal systems

Particle size, polydispersion index (PDI) and the zeta potential (Z-pot) of colloidal suspensions were measured by Dynamic Light Scattering (DLS) using a Zetasizer Pro Red Label (Malvern Instrument, Worcestershire, UK) at an angle of 90 in 0.01 m width cells at 25±1 °C. Colloidal suspensions were properly diluted with distilled water to avoid scattering phenomena and each sample was analysed in triplicate. Particle size was also analysed by Nanoparticle Tracking Analysis (NTA) using a Nanosight NS3000 (Malvern analytical Ltd., Malvern, UK). For NTA analysis, formulations were diluted 1:100 to keep the concentration within the instrument measuring range.

Mucoadhesion test was performed in order to evaluate the capability of colloidal systems to interact with mucin and adhere with the nasal mucosa. The test was performed by measuring particle size and zeta-potential of colloidal systems in the presence of mucin, according to Bonferoni et al. (2010) with some modifications (Akel et al., 2021; Piazzini et al., 2017). Briefly, an equal volume of colloidal suspension and an aqueous solution of mucin (1% w/v) were mixed, vortexed for 1 min and stored in a water bath at 37 °C for 1 h. Mean diameter and Z-pot were evaluated before and after the incubation period by DLS. CHITO and NIO were both subjected to analysis to underline the difference of the chitosan coating on their performance. Changes in size and charge were evaluated and correlated to a positive or negative interaction between vesicles and mucin. The experiment was performed in triplicate.

Colloidal systems were morphologically analysed by scanning transmission electron microscopy (STEM), scanning electron microscopy (FESEM) (FESEM Ultra Plus, ZEISS, Germany) and cryo-transmission electron microscope (Cryo-EM). For FESEM analysis, 10 μL of the diluted sample was placed on a silicon wafer and kept in the desiccator overnight and then, the samples were sputter coated with iridium in an argon atmosphere. For STEM studies, 10 μL of the diluted samples were placed on copper grids with carbon films and then washed drop-by-drop with water. The grids were air dried overnight in the desiccator for drying. Cryo-EM micrographs were obtained with an in-house Cryo-TEM Glacios (Thermo Fisher Scientific©) equipped with a X-FEG high-brightness gun at 200 kV and a Falcon 3 Electron Counting detector. For sample preparation, 3μL of samples were loaded on a Quantifoil© holey carbon film R2.2 300 Cu Mesh grid and mounted into a VitRobot© Mark IV. Thin layer of sample was ensured by blotting excess liquid for 5 s with blotting force 4. Vitrification was then obtained by quickly plunge-freezing the grid in liquid ethane. Image collections were performed using the software EPU (Thermo Fisher Scientific©) at 36kx magnification with a pixel size of 0.4 nm. Electron dose was 7.54 e-/A2/*sec* and total exposure dose was 30 e-/A2.

Drug entrapment was determined by direct method, removing non-entrapped drug by dialysis at 4 °C. 3 cm of a dialysis membrane (with, previously hydrated (MW cut-off 14,000, area 19.8 cm^2 Sigma-Aldrich) was filled with 1 mL of the samples and kept for 24 h in 5 mL of PBS pH 7.4 at 4 °C to remove the free CLZ. A 0.5 mL aliquot of CHITO suspension was withdrawn, diluted with 0.5 mL of a 10% w/v solution of Triton X-100 to destroy the CHITO, and analysed by HPLC UV/VIS as previously described. Drug entrapment was calculated as drug loading (DL%) and entrapment efficiency (EE%) according to Eqs. (1) and (2):

$$DL\% = (mgentrappeddrug)/(mgCHITO)x100 \quad (1)$$

$$EE\% = (concentrationofentrappeddrug)/(totaldrugconcentration)x100 \quad (2)$$

2.3. Stability studies

Stability studies were conducted on empty CHITO for 24 h in PBS and in simulated nasal fluid (SNF) prepared with 7.45 mg/mL NaCl, 1.29 mg/mL KCl and 0.32 mg/mL $CaCl_2 \cdot 2H_2O$ adjusted pH 6.5 (Wang et al., 2019). CLZ loaded CHITO suspensions were stored at 37 °C for 24 h and analysed in terms of size and PDI every hour, while drug entrapment was analysed at the end of the experiment. For storage stability, CLZ loaded CHITO suspensions were stored 4 °C in PBS for 12 weeks and analysed in terms of size and PDI every week, while drug entrapment was also analysed at the end of the experiment.

2.4. In vitro drug release studies

Drug release in vitro was assessed using the dialysis bag method. 3 mL of the CHITO suspension or a solution containing the same concentration of CLZ (0.1 mg/mL) was placed in a cellulose acetate dialysis bag hydrated (MW cut-off 1400, Sigma-Aldrich), immersed in 50 mL of SNF, and incubated at 37 °C under electromagnetic stirring. At predetermined interval points (5, 10, 15, 30, 60, 120, 180, 240 and 1440 min), a volume of receptor solution was withdrawn and refilled with an equivalent volume of fresh SNF and the concentration of CLZ was determined using HPLC-UV/VIS. At the end of the experiment CHITO suspension was withdrawn from the dialysis bag, diluted 1:1 v: v with a 10% w/v solution of Triton X-100 and analysed by HPLC UV/VIS to calculate EE% as previously described. The used experimental conditions assure the maintenance of sink conditions. The experiment was performed in triplicate.

In order to investigate the drug release mechanism, the release data obtained were assessed with different models:

$$Zero - orderM_t = M_0 + k_0 t \quad (3)$$

$$First - orderlogM_t = logM_0 + k_1 2.303t \quad (4)$$

$$HiguchiM_t = M_0 + k_H t^{1/2} \quad (5)$$

$$Korsmeyer - -PeppasM_t = k_{KP} t^n \quad (6)$$

M_t = initial amount of drug
M_0 = total amount of drug release at time t
k_0 = zero-order release constant
k_1 = first-order release constant
k_H = Higuchi constant
k_{KP} = Korsmeyer–Peppas constant
n = exponent characterizing the release mechanism

Based on the highest correlation coefficient (r2) value, the model that best represented this experiment was chosen.

2.5. Nanoparticle freeze-drying

Freeze-drying is an optimal technique to improve colloidal systems long term stability but a critical step is the formulation reconstitution. For this reason, CHITO were freeze-dried (Labconco Corp., USA), in the presence or not of cryoprotective agents. Trehalose and sorbitol were selected since are usually used in nasal formulations and have been added to CHITO at two different concentrations: 2% and 20%. Samples were frozen at −80 °C and then lyophilized following an initial drying cycle from −40 to 20 °C (increasing 10 °C/step, during 18 h, 12 h, 5 h, 2

h and 2 h) followed by a secondary drying cycle (22 °C, for 3 h, −0,0266 mbar under vacuum conditions). After the freeze-drying process, the original sample (with and without cryoprotectant) was reconstituted with the original volume of distilled water and subjected to sonication in an ice bath with a Sonoplus HD2200 ultrasonic homogenizer (Bandelin Electronic GmbH, Berlin, Germany) equipped with KE76 probe at 50% power for 5 min for 5 cycles. This procedure was carried out to ensure the complete redispersion of powder. Each sample has been analysed before and after sonication in terms of particle size, PDI and Z-pot.

2.6. Cytotoxicity and transport studies

Caco-2 (human colorectal adenocarcinoma) cell line coming from the American Type Culture Collection (ATCC, Rockville, MD, USA) was cultured in Dulbecco's modified Eagle's medium (DMEM, Euroclone, Milan, Italy), with 20% foetal bovine serum (FBS), 1% L-glutamine and 1% penicillin-streptomycin (Carlo Erba, Milan, Italy). The cells were maintained at 37 °C in a humidified incubator in an atmosphere containing 5% CO_2. Viability analyses were performed by a Cell Titer 96® Aqueous One Solution Cell proliferation (3-(4,5-dimethylthiazol-2-yl) 5-(3-carboxymethoxyphenyl) -2-(4-sulfophenyl)-2H-tetrazolium) inner salt (MTS) assay kit (Promega Corporation, Madison, WI, USA). This is a colorimetric method based on MTS reduction by viable cells in a coloured product (formazan). Briefly, Caco-2 cells were seeded in 96-well culture plates at a density of 5×10^3 cells/well. At approximately 80% of confluency, cells were incubated for 2 h with different dilutions (1:10, 1:50, 1:100) of the CHITO formulations and of a CLZ solution in dimethyl sulfoxide (DMSO) at the same drug concentration. The optical density of the formed chromogenic product was measured at 490 nm with a Multilabel Counter (Victor3 Wallac 1240, Perkin Elmer, Waltham, MA, USA). Relative cell viability was expressed as a percentage of viable cells compared to the untreated control group. For transport studies, cells were seeded at 2 × 105 cells/well in 12-well PET Trans well plates (1.13 cm^2 growth surface area and pore size 0.4 μm, Greiner Bio-One, Milan, Italy) and allowed to differentiate for 21 days. Caco-2 cells were used as epithelial model barrier also in accordance with other Authors (Dukovski et al., 2019; Esim et al., 2020; Wang et al., 2019). Culture medium was added to apical (AP) and basolateral (BL) side and replaced every second day for the first week and daily thereafter. The cell layer integrity was checked by the Lucifer Yellow (LY, Sigma Aldrich, Milan, Italy) permeability assay. LY was diluted in the transport buffer (Hank's Balanced Salt Solution (HBSS/HEPES, 1 M)) and added to the AP compartment at a final concentration of 100 μM. After 1 h incubation at 37 °C, the HBSS in the BL chamber was collected, and LY concentration determined by a fluorescence plate reader (Victor3 Wallac 1240, Perkin Elmer, Waltham, MA, USA) at 485 nm excitation and 530 nm emission. The AP-to-BL% permeability was calculated by Eq. 7:

$$Permeability(\%) = (FluorescenceinBL - blank)/(FluorescenceinAP - blank)x100 \quad (7)$$

The critical maximum LY flux, index of leaky monolayers, was fixed at 5% of the initial amount.

Transport studies were carried out as previously reported (Abbas et al., 2022). Briefly, the cell culture medium was replaced by the HBSS/HEPES transport buffer, previously heated at 37 °C. Caco-2 cells were then exposed for 2 h with CHITO formulations or CLZ solution at the same drug concentration, suitably diluted, in the AP chamber. At given time intervals, 0.3 mL of medium was withdrawn from the BL side and for CLZ assay by HPLC and replaced with the same volume of fresh HBSS/HEPES. At the end of the experiment, the cell layer integrity was rechecked by the LY permeability assay, as described above. The apparent permeability coefficient (Papp) was calculated according to Eq. 8:

$$Papp = dCBLx1/AxCAP \quad (8)$$

where CBL is the CLZ concentration (mg/mL) in the basolateral (BL) compartment as a function of time t (s), A is the surface area of the Transwell membrane (1.13 cm^2) and CAP the initial CLZ concentration in the AP (donor) chamber (mg/mL).

2.7. Statistical analysis

Statistical analysis of data was carried out by one-way analysis of variance (ANOVA), testing differences between groups by Student-Newman-Keuls comparison post hoc test (GraphPad Prism version 6.0 Software, San Diego, CA, USA). P-values <0.05 or <0.01 were considered significant.

3. Results

3.1. Preparation and characterization of colloidal systems

Empty NIO and CHITO suspensions were prepared with the TLE-P method in order to choose the ideal CS concentration. Unloaded vesicles were prepared at different concentrations of CS: 0.5, 1.0, 2.0 and 3.0 mg/mL. The results are reported in Table 1.

As shown, increasing CS concentration particle size and PDI increase. The optimal concentration resulted 2 mg/mL, so this concentration of CS was selected for further studies. Once the CS concentration was selected, NIO and CHITO were prepared by three different methods, as described in 2.2., in order to evidence the effect of different preparation steps and conditions on vesicles characteristics. The characteristics of obtained vesicles are reported in Table 2.

Niosomes (NIO) prepared by TLE-P, and E methods were analysed also before CS coating, to evaluate how and how much CS could affect CHITO features. Mean particle size and PDI were adequate in all the cases, but Z-pot was much higher using the E method as compared with TLE-P or SDT. Based on these results, the Evaporation (E) method was selected to continue the study. Nanoparticle Tracking Analysis (NTA) performed on CHITO in water and in SNF confirmed DLS results. The results are reported as Supplementary materials in Figure S1 Particles size distribution (mean particle size ± standard deviation) by NTA of CHITO in SNF and in Video S1: NTA of CHITO in SNF.

In order to evidence the mucoadhesion capacity of the vesicles, the interaction with mucin was analysed by DLS before and after an incubation period of 1 h at 37 °C with a solution of mucin 1% and the results are summarized in Fig. 1.

CHITO morphology was observed with FESEM, STEM, and cryo-EM analysis microscopy techniques, and the results are reported in Fig. 2A, B, and C, respectively. The vesicles presented a spherical and homogeneous shape and a mean diameter which confirmed DLS values (around 200 nm). A slight reduction of the size could be attributed to the drying process during sample preparation.

Drug loaded formulations were prepared with the optimized formulation by adding 0.1 mg/ml of CLZ directly in the lipophilic phase with the E method and using as coating solution CS at 2 mg/mL in Acetic Acid 1% v/v (adjusted with NaOH 1 M at pH 4.5). The CLZ-loaded CHITO were characterised in terms of particle size, PDI, Z-pot., entrapment efficiency (EE%) and drug loading (DL%). The results were 203.1 ± 3.3 nm of particle size, 0.355 ± 0.021 of PDI, +31.9 ± 1.72 of

Table 1
Effect of different CS concentrations on colloidal formulations. Data represents mean ± standard deviation (S.D.) $n \geq 3$.

[CS] (mg/ml)	Size (nm) ± S.D.	PDI	Z-pot (mV)± S.D.
0.0	152.70 ± 1.4	0.245 ± 0.007	−15.9 ± 2.3
0.5	238.70 ± 5.7	0.328 ± 0.040	+23.5 ± 1.5
1.0	240.60 ± 3.1	0.329 ± 0.047	+28.3 ± 1.6
2.0	222.00 ± 3.1	0.207 ± 0.012	+29.8 ± 1.7
3.0	305.20 ± 3.2	0.343 ± 0.004	+31.2 ± 1.4

Table 2
Effect of different preparation methods, Thin Layer Evaporation-paddle (TLE-P), Evaporation (E), and Solvent Displacement Technique (SDT). Data represents mean ± standard deviation (S.D.) $n \geq 3$.

	Size (nm) ± S.D.	PDI (mV) ± S.D.	Z-pot (mV) ± S.D.
NIO (TLE-P)	152.7 ± 1.4	0.245 ± 0.007	−15.9 ± 2.3
CHITO (TLE-P)	222.0 ± 3.1	0.207 ± 0.040	+29.8 ± 1.7
NIO (E)	127.4 ± 6.2	0.256 ± 0.047	−22.3 ± 1.3
CHITO (E)	206.7 ± 2.0	0.270 ± 0.012	+37.0 ± 1.2
CHITO (SDT)	189.1 ± 29.4	0.240 ± 0.004	+33.3 ± 1.4

Z-pot., 60.59 ± 10.03 of EE% and 0.042 ± 0.007 DL%.

Since the presence of CLZ produced particles with dimension and charge according to the empty vesicles and good EE% and DL% the following experiments have been performed on both empty and loaded CHITO.

3.2. Stability studies

Stability studies were conducted on empty and CLZ loaded CHITO at 4 °C for 12 weeks, in PBS pH 7.4 and analysed in terms of size and PDI. No difference was observed between empty and loaded CHITO. Based on the obtained data, the formulations were stable, as reported, as an example, for empty CHITO in Fig. 3. In fact, after 12 weeks the size was still acceptable (d = 221.5 ± 25.58 nm), there was a slight increase in the dimension but without a statistically significant difference. Even PDI did not show statistically significant differences among all the tested periods. After 12 weeks, the EE% of loaded CHITO was tested and exhibited a minimum drug leakage (original EE% 60,95± 10.03 vs = 55.83 ± 7.92 after study).

Visual inspection has been performed and it was not noticed the appearance of sediment or mould. Stability studies of both empty and loaded CHITO were also conducted at 37 °C in PBS and SNF in order to simulate the stability in vivo behavior. Any difference was observed between empty and loaded CHITO and the results are shown for empty CHITO in Supplementary Materials in Fig. 2S.

3.3. In-vitro release studies

CLZ release from CHITO was analysed with the dialysis bag method at 37 °C for 4 h. The results are reported in Fig. 4. As shown, most 45.96% (corresponding to 9.45 mg/L) of the drug is released from the formulation within 4 h while from CLZ suspensions just the 25.75% of drug was dissolved.

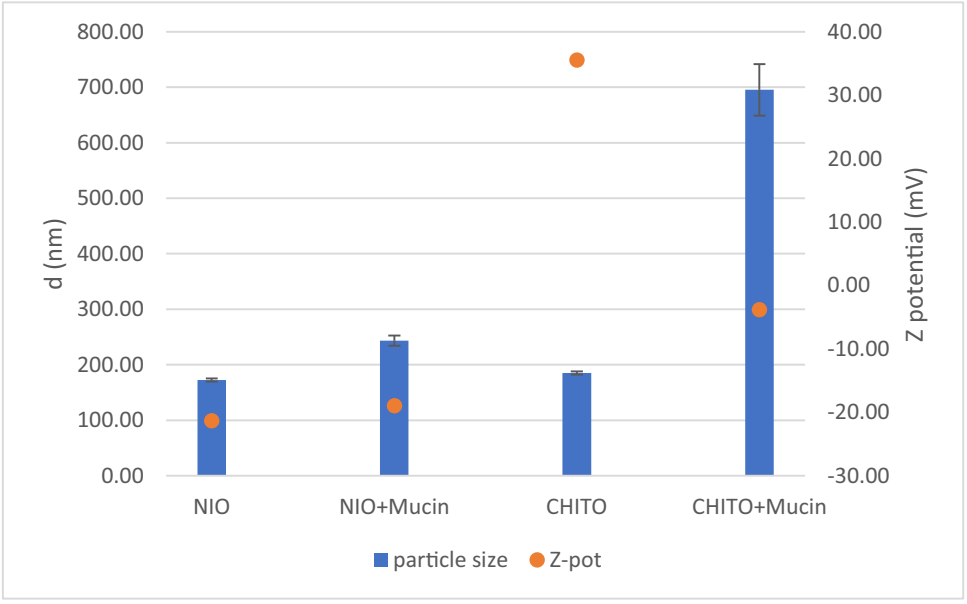

Fig. 1. Nanoparticles interaction with mucin, particle size (■) and Zpot. (●). Data represents mean ± standard deviation (S.D.) $n \geq 3$.

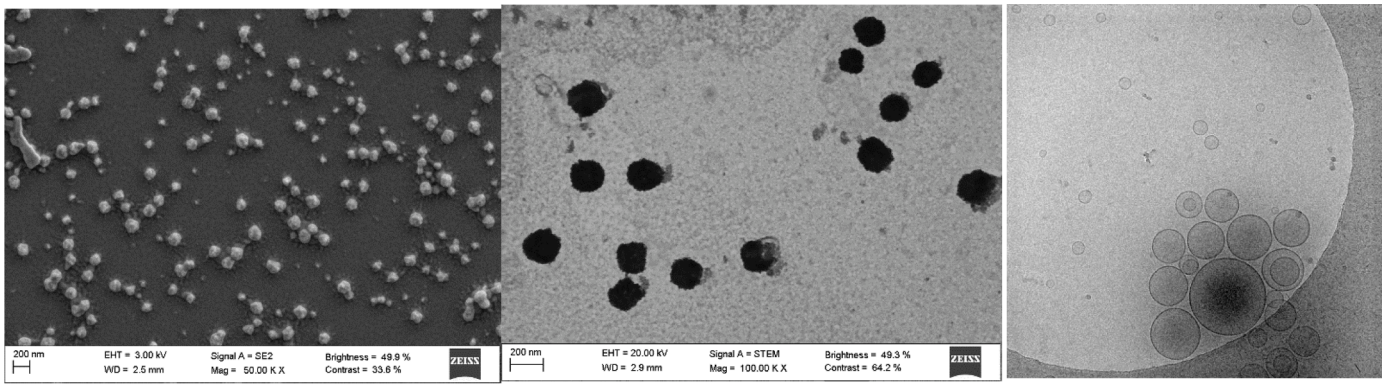

Fig. 2. Optimized CHITO micrographs obtained by (A) STEM, (B) FESEM and (C) Cryo-EM analysis.

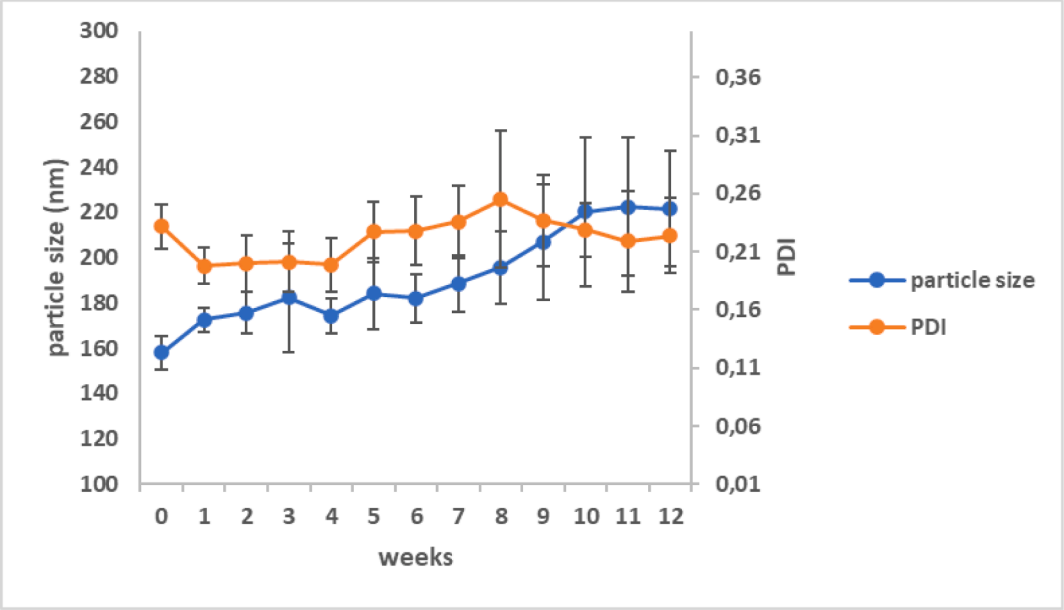

Fig. 3. Particle size and PDI variation of CHITO during stability studies conducted in PBS at 4 °C for 12 weeks. Data represents mean ± standard deviation (S.D.) $n \geq 3$.

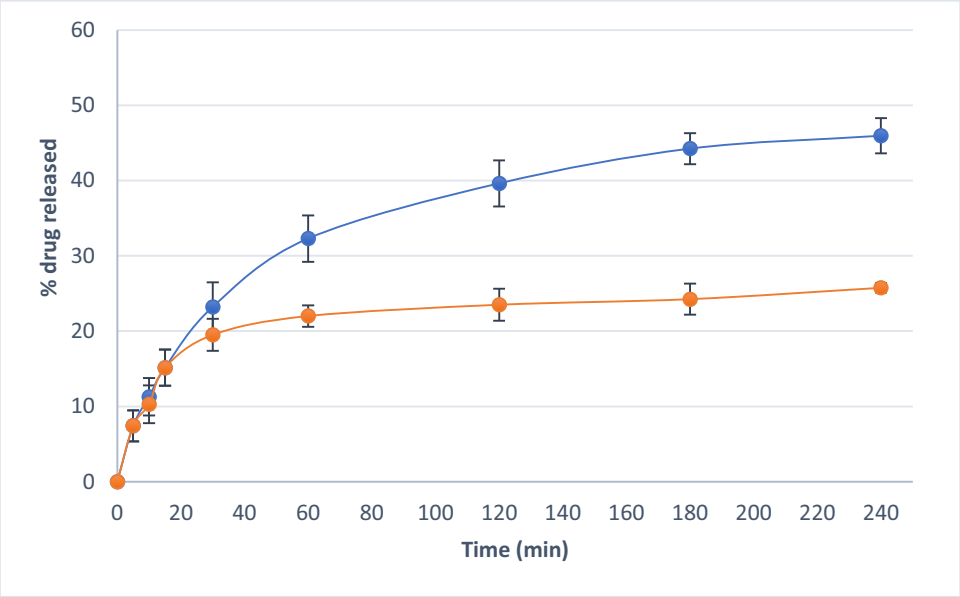

Fig. 4. CLZ drug release profile from CHITO (blue line) and from a suspension (orange line). Data represents mean ± standard deviation (S.D.) $n \geq 3$.

From the formulation it was observed a biphasic release. During the first 30 min a burst effect was observed, attributed to the unloaded drug that is released from the CHITO immediately; after that the release of the CLZ was sustained and controlled until the 4th hour, thus a plateau was reached. The release kinetics is represented by the Higuchi model, in which the best fit was detected ($r^2 = 0{,}9546$) The remaining 50% of CLZ was found in the CHITO at the end of the analysis after 24 h.

3.4. Nanoparticle freeze-drying

An optimized freeze-drying process improves the long-term physical stability of nanosuspension in drug delivery applications. A dry powder product is easy to store and handle, allowing for extemporaneous preparation of the nanosuspension. In order to prevent aggregation during the freeze-drying process cryoprotectants are generally added, but, since often higher concentration are required, it may affect the osmotic pressure and isotonicity of the solution. Freeze-drying and reconstitution studies were conducted on empty and CLZ loaded CHITO, in absence and in presence of two different cryoprotectants (trehalose and sorbitol) at two concentrations (2 and 20%) and the results are reported as Supplementary Material in Table 2S.

3.5. Cytotoxicity and transport studies

An MTS assay was performed on the Caco-2 cell line to evaluate the possible cytotoxicity of loaded CHITO. Cells were exposed to different concentrations of a CLZ solution and loaded CHITO (at the same concentration of CLZ: 800 μM) for 2 h. Test samples were diluted (1:10; 1:50; 1:100) with HBSS/HEPES (Fig. 5).

The cellular permeation through Caco-2 cells was performed to

investigate if the developed nano-formulation could improve the drug permeation in comparison with a CLZ solution at the same concentration. The dilution 1:50 (CLZ concentration 0.002 mg/mL) was selected for transport studies. Lucifer Yellow (LY) concentrations tested in the basolateral (BL) compartment at the end of the experiments were always lower than 3%, indicating the maintenance of integrity of the cell layer, which confirmed the good tolerability of the tested formulations.

As observed in Fig. 6, CHITO formulation increased the CLZ apparent permeability (Papp) by almost 10-fold with respect to the corresponding CLZ solution in the first 30 min of the experiment. Then, after 60 min CHITO showed a slightly reduction of permeability that was maintained after 120 min.

4. Discussion

Aim of this study has been the preparation and the in vitro characterization of chitosan coated niosomes for the nose-to-brain delivery of clonazepam. Size range is a key parameter to evaluate when developing a nose-to-brain delivery since nano-carriers smaller than 300 nm are easily transported transcellular through olfactory neurons to the brain Md et al., (2013). Also, PDI is an important parameter to evaluate the non-uniformity of particles size distribution. Successful formulation of safe, stable, and efficient nanocarriers requires the preparation of homogenous (monodisperse) populations of a certain size (Danaei et al., 2018). In drug delivery applications a PDI of 0.3 and below is considered to be acceptable and indicates a homogenous population of surfactant vesicles. On the other, since this formulation was designed to maintain an intimate contact between the drug and the nasal mucosa, thus promoting drug absorption, a higher positive zeta potential is important, not only for a great stability but also for a good mucoadhesion.

As expected, NIO prepared without CS showed a negative surface charge and became positive with the addition of CS. This phenomenon can be attributed to the presence of the positively charged amine groups of CS, proving that nanovesicles were successfully coated. The significant inversion to positive Z-pot from a negative Z-pot because of the cationic nature of CS was confirmed also by other authors (AbuElfadl et al., 2021). On the other hand, charge variation was not statistically significant with increasing amounts of CS, as noticed also by other Authors (Abbas et al., 2022). For these reasons, the CS concentration of 2 mg/ml was selected as optimal, for this formulation, considering particle charge, size and polydispersity index (PDI) and similar results were obtained for niosomes prepared with different composition and method, coated with chitosan by other Authors (AbuElfadl et al., 2021). Since the preparation method can also affect the properties of the colloidal systems, three different methods have been evaluated and the evaporation method was selected since with this method a higher surface charge was obtained. In fact, in this method, a gentler and quicker evaporation of the organic solvent, ensures a concentrated solution of CS that probably leads to a better interaction between negatively charged niosomes and positively CS and produces a more effective coating with a higher particle charge. It may suggest that gentler and quicker evaporation of the organic solvent could ensure a more concentrated solution and consequently a more charged colloidal suspension. In the liquid form, charged molecules are freer to move due to the state of matter and CS coating is more effective due to the interaction between the negatively charged NIO and the positively charged CS. In the TLE-P method, the organic solvent was completely removed, and components appeared as a uniform solid layer on the glass flask, leading to a more structured and stiff structure that reduces charge interactions. Surface charge is a fundamental parameter to consider in nasal formulations, since a higher surface charge leads to a stronger bond between formulation and mucin, fundamental to provide the time necessary for the absorption of the active principle via the nasal mucosa. The strong bond between CHITO with respect to NIO formulation and mucin, was observed by the increase of CHITO size (Fig. 1A) after 1 h of incubation at 37 °C with a solution of mucin 1%, thus confirming the mucoadhesive properties of this formulation Also, the increase of the size could be related with the decrease of the Brownian movement of the particles, because mucin increased SNF viscosity (Robla et al., 2021). The development of a mucin layer around the vesicles was further confirmed by the changes in the Z-pot. (Fig. 1B). In fact, NIO were negatively charged before and after the treatment with mucin, meaning that there was no interaction between them. On the other hand, CHITO had a positive charge (around +37 mV), as expected due to the presence of amine groups on the polymer. The addition of the mucin, negative for the presence of sialic acids in its structure, determined a decrease of the potential that could be attributed to the coating of nanovesicles. Akel et al., 2021 has observed the same trend with SLN and PLGA nanoparticles: chitosan coated nano-carriers showed a significant decrease of surface charge after the embedding with mucin as a consequence of the formation of ionic bonds (Akel et al., 2021). CLZ loaded CHITO were then prepared evidencing good characteristics in terms of particle size, PDI, Z-potential, entrapment efficiency (60.59 ± 10.03%) and drug loading (0.042 ± 0.007%).

During the storage at 4 °C the developed colloidal suspension demonstrated good stability studies for 12 weeks as well at 37 °C in simulated nasal fluid at 37 °C in terms of particle size and PDI. Particle size was maintained in presence of SNF at 37 °C, meaning that the formulation preserves its characteristics for at least 24 h. Interestingly, the sample analysed in PBS did not show the same behavior, probably due to the different composition and ionic strength. Altogether, these results highlight the suitable biological stability of CHITO for the nasal environment, and their excellent long-term storage stability.

Drug release studies evidenced an improvement of drug solubility due to the particle size reduction and a controlled release for the 50% of the entrapped drug. Similar results were observed for microemulsion systems by other Authors, even if in this case, 100% of release was obtained, due to the presence of oil in the formulation (Abdel-Bar et al., 2013). Lyophilization was performed in absence and in presence of trehalose and sorbitol at 2 and 20%

The formulations were not suitable for reconstitution after simple resuspension without sonication process, even if in presence of cryoprotectants. In fact, particle size was greatly increased, probably due to particle aggregation as confirmed also by PDI values. Cryoprotectant presence, independently from type of cryoprotectant and concentration, reduce the particles aggregation but particle size results higher than 300 nm and PDI>0.3, indicative of low homogeneity of the colloidal suspension in terms of size. On the other hand, when the sample was subjected to sonication, independently from cryoprotectant presence and concentration a size<300 nm and a PDI<0.3 was obtained. Also, in absence of cryoprotectant, after the sonication step, CHITO regained their initial characteristics; it is therefore possible to assume that lyophilized CHITO required a suitable amount of energy to completely re-hydrate. On the other hand, cryoprotectant presence, that can modify osmotic pressure and formulation isotonicity, can be avoided since, in this case, is not useful. Drug entrapment was maintained after

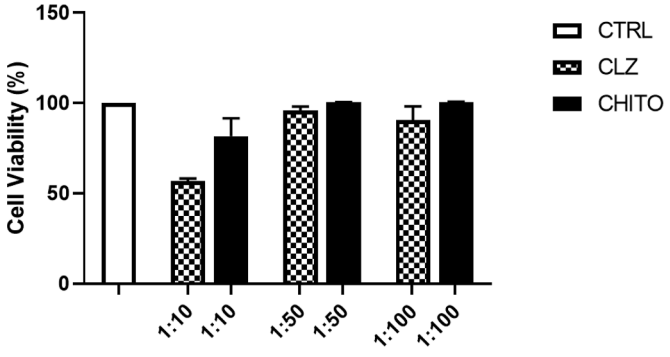

Fig. 5. Cell viability assay on Caco-2 cell line at different concentration of CLZ alone or included in CHITO at 0.01 mg/mL (1:10) 0.002 mg/mL (1:50) and 0.001 mg/mL (1:100). Data represents mean ± standard deviation (S.D.) $n \geq 3$.

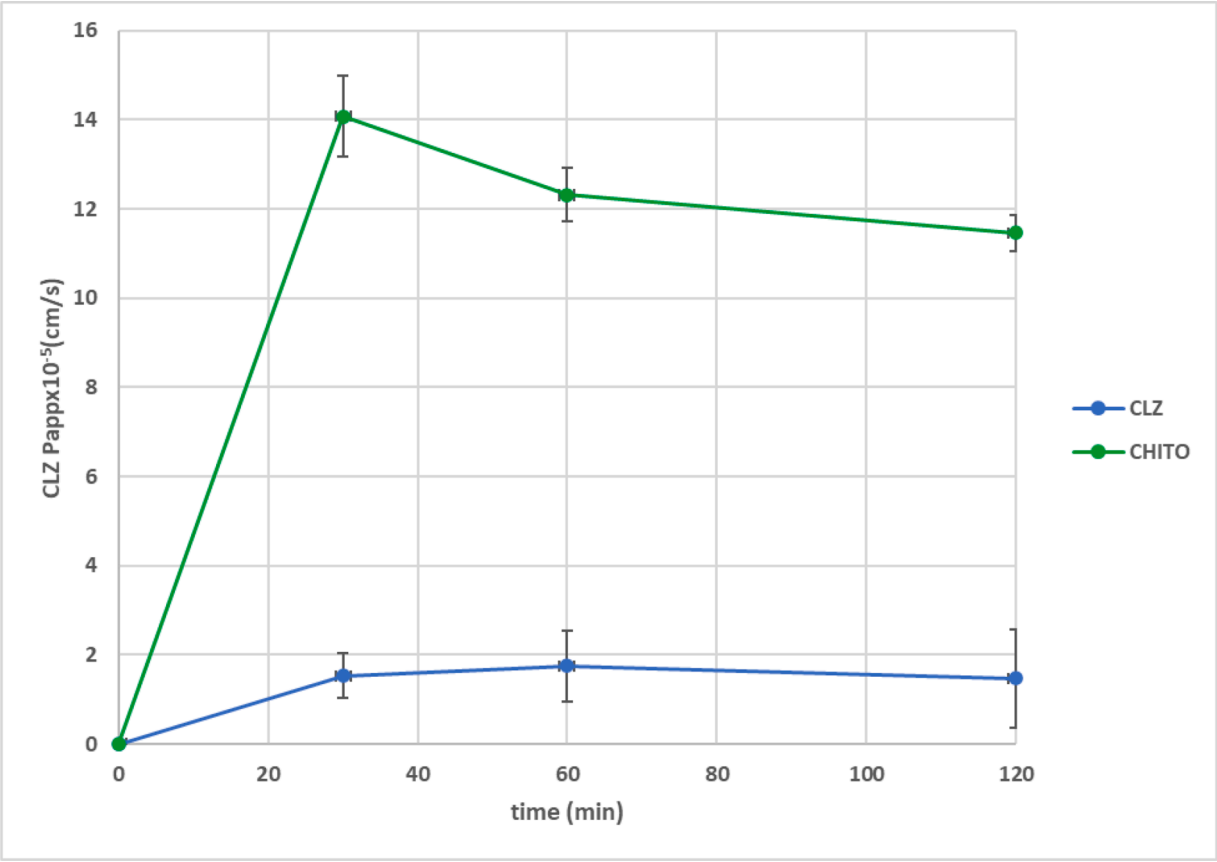

Fig. 6. Permeability assay (Papp) studies of clonazepam in HBSS/HEPES. Data represents mean ± standard deviation (S.D.) $n \geq 3$.

reconstitution and any difference was observed between empty and loaded CHITO, probably due to the lipophilicity of drug that is placed in the lipidic layer and not in the aqueous compartment, as observed also by other Authors (Guimarães et al., 2019).

Toxicity studies performed with Caco-2, showed, for the higher dilutions (1:50 and 1:100) almost the same cell viability (100%) as the untreated control group, indicating the lack of cytotoxicity. On the other hand, the most concentrated sample of CHITO (1:10) still exhibited a good cell viability (around 80%), in contrast with the corresponding CLZ solution that showed a reduction up to 55%. These data highlight the possible reduction of CLZ cytotoxicity when encapsulated into CHITO, due to a camouflage of the drug inside the niosomal particles, as observed also by other Authors (AbuElfadl et al., 2021).

Permeation studies evidenced an improvement of 10-fold permeation of CLZ in CHITO in comparison with a CLZ solution. _ In a previous study we observed a slight reduction of the expression of the tight junction protein 1 (ZO-1) following the exposure to a formulation with chitosan after 2 h (Piazzini et al., 2019). In this study we also used a cellular model that mimics what happens centrally, which was different to what happens in the intestine. The data suggests that ZO-1 regulation is transient but also modest, which maintained intestinal integrity and did not allow LY passage at the end of the experiment. Moreover, in the intestinal epithelium there are however also mechanisms that mediate the passage of formulations with chitosan The data evidenced that the drug formulation in chitosomes improves both its dissolution and permeability compared to the pure drug thus suggesting an improving in biovailability.

In conclusion, the results indicate a good capability of the developed formulation to be an effective nose-to-brain delivery system for clonazepam, even if further ex-vivo and in-vivo studies are required to support this hypothesis.

Author contributions

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CRediT authorship contribution statement

Giulia Nerli: Conceptualization, Methodology, Software, Investigation, Writing – original draft, Visualization. **Sandra Robla:** Methodology, Validation, Writing – review & editing. **Marta Bartalesi:** Software, Formal analysis. **Cristina Luceri:** Resources, Data curation, Supervision, Funding acquisition. **Mario D'Ambrosio:** Validation, Formal analysis. **Noemi Csaba:** Conceptualization, Resources, Writing – review & editing, Supervision, Funding acquisition. **Francesca Maestrelli:** Conceptualization, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1 Representative image of the particles size distribution by Nanoparticle Tracking Analysis of chitosomes in simulated nasal fluid, with the mean particle size ± standard deviation; Figure S2 Particle size variation of CHITO maintained in SNF and in PBS at 37 °C for 24 h. Data represents mean ± standard deviation (S.D.) $n \geq 3$ Video S1: Nanoparticle Tracking Analysis of chitosomes in simulated nasal fluid.; Table S1: Summary of methods Table S2: DLS analysis on CHITO before and after lyophilization process with and without trehalose and sorbitol (2% and 20%).

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