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Development of Eudragit® Nanoparticles for Intranasal Drug Delivery: Preliminary Technological and Toxicological Evaluation

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Abstract: Intranasal administration has assumed in the last years an increasing value as an alternative strategy for the systemic adsorption of drugs, as an alternative to oral and parenteral routes thanks to the high vascularized nasal mucosa. Nevertheless, different drug features may restrict its absorption through the nasal mucosa with an insufficient diffusion to the systemic circulation. Several technological strategies are under investigation to improve drug absorption during nasal formulation design and production. The use of bioadhesive polymers can be considered a valid approach to pursue the aforementioned goal. Based on this consideration, Eudragit® Retard RS100 and RL100 resins were selected as positively charged copolymers to prepare polymeric NPs with potential mucoadhesive properties suitable for intranasal application. NPs were produced by the Quasi-emulsion Solvent Evaporation (QESD) method and loaded with diclofenac acid (DIC) or its epolamine salt (DIEP). Preliminary investigations were performed to obtain the optimized blank formulation and drugs loaded NPs evaluating different parameters that can affect particles size and polydispersity. The optimized formulations unloaded and loaded with DIC and DIEP were further evaluated for their thermotropic behavior by differential scanning calorimetry. Mucoadhesive evaluation was assessed by measuring variation in zeta potential and by turbidimetric assay after incubation of particles with mucin in simulated nasal fluid (SNF) at 37 °C at different time points (0, 1 and 24h) compared to the pure suspensions. Stability of DIC and DIEP loaded NPs was also evaluated in SNF to predict potential aggregation phenomena after nasal administration. Finally, in vivo experiments showed absence of toxicity on the nasal mucosa of mice.

Keywords: drug delivery; diclofenac; diclofenac epolamine; nasal mucosa; mucoadhesion; ciliotoxicity

1. Introduction

The intranasal (IN) administration of drugs often appears as a valid alternative to oral route, that can experience limitations such as poor absorption in the gastrointestinal

tract and hepatic first-pass effect, or the impossibility to administer the drug to unconscious patients, like in many emergency situations [1].

Due to the relatively large surface of nasal mucosa and the high level of vascularization, a drug administered intranasally can be quickly absorbed and distributed in the systemic circulation, thus offering a valid tool for the therapy of keen conditions, such as seizure or acute pain [2]. Furthermore, IN is a non-invasive way of administration, therefore encountering the favorable compliance of children and elderly patients.

IN administration is currently proposed for a topical effect on the nasal mucosa (e.g., therapy of inflammation, allergies or nasal congestion) but also to exert a systemic effect, such as with hormones, analgesic and anti-inflammatory drugs, cardiovascular and antiviral agents [3]. More recently, the 'nose-to-brain' (N2B) route is under intense investigation as a strategy to overcome the blood–brain barrier and convey drugs directly in some brain districts [4,5].

IN administration has been associated in the very last years with innovative drug delivery technologies. Micro- and nanosized drug delivery systems (DDS) can in fact modulate the rate or time of drug release in the nasal mucosa, thus definitely controlling drug local effect or its levels in the systemic circulation [6].

Among the clinical conditions in which a IN administration could support the therapy are inflammatory states, often associated with pain. Non-steroidal anti-inflammatory drug (NSAIDs) are undoubtedly the pharmacological class of drugs more used, especially in self-therapy. Most of these drugs are however also associated to gastrolesivity after oral administration, thus requiring technological approaches to modulate the time and site of release in the gastro-intestinal tract. Therefore, IN route could be an interesting alternative for this drug class, to reach both goals of a quick adsorption and a reduced irritant effect on the stomach mucosa [7]. A nasal spray based on ketorolac tromethamine even reached the market [8].

Diclofenac (2-[2-(2,6-dichloroanilino)phenyl]acetic acid; DIC) is a phenylacetic derivative of propionic acid. It is a non-steroidal anti-inflammatory drug (NSAID), largely used, in different dosage forms, with the aim to reduce joint inflammations and pain [9]. It is characterized by a low water solubility, and it is thus often applied as a soluble sodium of potassium salt [10,11].

Diclofenac epolamine (DIEP) (diclofenac N-(2-hydroxyethyl)-pirrolidine), is a salt form very soluble in water and polar solvents. High concentrations of DIEP aqueous solutions show a tenside-like behavior [12]. Solubility and surfactant properties of DIEP enhance its permeability through the biological membranes [13].

The relatively high gastro-intestinal toxicity tolerability of DIC, in comparison with most other NSAIDs [14], makes it a valid pharmacological tool. Unfortunately, it shows an unpleasant pharmacokinetic profile, including a short plasma lifespan (1.1 h), that limits the clinical use [10,15]. Encapsulation of DIC and its salts in nano- and microparticle systems that allow a prolonged release could therefore be a valid strategic approach to improve their efficacy [16–21].

In this study we evaluated new colloidal systems for the IN delivery of DIC and DIEP. Eudragit® RS100 (RS) and RL100 (RL) are copolymers of poly(ethylacrylate, methyl-methacrylate and chlorotrimethyl-ammonioethyl methacrylate), containing an amount of quaternary ammonium groups between 4.5 and 6.8% and 8.8 and 12% for RS and RL, respectively. Both are insoluble at physiological pH values and capable of swelling [22], thus representing good materials for the dispersions of drugs [23,24].

They are commonly used for the coating of solid oral dosage forms, to achieve a modified drug release; however, recent works suggested the suitability of these resins for producing other drug controlled-release formulations and delivery systems. For instance, in our lab Eudragit® Retard NP suspensions have been investigated as carrier systems for the ophthalmic administration of some NSAIDs [25–28]. They demonstrated a good physical stability and technological properties, such as a high homogenous size

distribution, along with a positive surface charge that can facilitate the adhesion to mucosal surfaces, allowing a slow and prolonged drug release pattern.

RL/RS nanoparticles (EUD NPs) were produced by a modification of the Quasi-emulsion Solvent Diffusion (QESD) method, previously applied by some of us for the production of NPs [26,27], and adapted here to produce larger NPs suitable for the intranasal administration of the chosen drugs. Particles with size less than that of the olfactory axons could drive the release of the encapsulated drugs towards the N2B pathway [29,30], instead than promoting their permanence on the nasal mucosa and drug systemic adsorption [31].

A preliminary investigation was performed to obtain optimized blank formulations evaluating three different copolymer concentrations and two stirring speeds during the preparation procedure. When EUD NPs were loaded with DIC and DIEP, the effect of different drug-to-polymer ratios was assessed upon particle mean size and polydispersity.

The optimized formulations, both unloaded and loaded with DIC and DIEP, were further evaluated for their thermotropic behavior using differential scanning calorimetry (DSC). Mucoadhesive properties were assessed by incubating the particle suspensions with mucin in simulated nasal fluid (SNF), compared to the pure drug suspensions. Stability of DIC- and DIEP-loaded NPs was also evaluated in SNF containing mucin to predict any potential aggregation phenomena after nasal administration. Finally, a preliminary evaluation of potential toxicity of the prepared systems on mouse nasal mucosa was performed in vivo.

2. Materials and Methods

2.1. Materials

Eudragit® Retard RL100 (RL) and RS100 (RS) resins were produced by Evonik Industries AG (Essen, Germany) and were kindly provided by Rofarma srl, Milan, Italy; the surfactant agent Tween 80 was a Fluka product, bought from Merck KGaA (Darmstadt, Germany), as wells as diclofenac (acid form, DIC) and the solvents used in the study. Diclofenac epolamine (DIEP) was purchased from Dipharma Francis srl (Baranzate, Italy). Mucin (mucin from porcine stomach type II) was purchased from Sigma-Aldrich (Milan, Italy).

2.2. Solubility Studies

Preliminary studies were carried out to assess the solubility limit of DIC and DIEP in ethanol. To 5 mL of solvent, progressive amounts of the two drugs were added at room temperature under magnetic stirring for 1 h, until a further addition of a drug aliquot remained insoluble [32]. A solubility of 25 mg/mL and 350 mg/mL was measured for DIC acid and DIEP, respectively.

2.3. Preparation of Nanoparticles

RS/RL NPs, either blank or loaded with DIC or DIEP were prepared by the QESD method [26] (Table 1). Different concentrations of the copolymer blend, and the drug when required, were dissolved in ethanol. This organic phase was poured into 20 mL water containing a stabilizer (0.02% Tween 80), by dripping for 6 min through a thin Teflon tube connected to a syringe under intense homogenization using an Ultra-Turrax T18 apparatus (IKA GmbH, Königswinter, Germany) equipped with a G10 dispersion accessory, at 5000 rpm for 15 min.

Table 1. Production conditions and features of blank Eudragit NPs.

EUD NPs Batch Code	Copolymer(s) Concentration (% w/v)	RL:RS Weight Ratio	EtOH Volume (mL)	Water Volume (mL)	Tween 80 (% w/v)	Stirring Speed (rpm)	Z-Ave (nm) ± SD	PdI ± SD
EUD1A	1	1:1	3	20	0.02	5000	286.5 ± 0.31	0.322 ± 0.11
EUD1B	1	1:1	3	20	0.02	15,000	341.5 ± 0.11	0.311 ± 0.02
EUD2A	2	1:1	3	20	0.02	5000	302.0 ± 0.22	0.304 ± 0.05
EUD2B	2	1:1	3	20	0.02	15,000	380.6 ± 0.03	0.234 ± 0.24
EUD3A	3	1:1	3	20	0.02	5000	290.9 ± 0.22	0.119 ± 0.06
EUD3B	3	1:1	3	20	0.02	15,000	350.5 ± 0.21	0.401 ± 0.09

The quasi-emulsion formed was left to evaporate for 24 h under magnetic stirring at room temperature. To optimize the working time, alternatively a rotavapor was used under vacuum at 40 °C for 2 h. The evaporation of the organic solvent and the simultaneous counter-diffusion of the aqueous phase in the dispersed droplets of the polymer solution, leads to the precipitation of the polymer and the formation of the NPs.

2.4. Nanoparticle Characterization

The mean particle size (Z-ave), polydispersity index (PdI) and zeta potential (ZP) of the produced systems were determined at 25 °C by Photon Correlation Spectroscopy (PCS), using a Zetasizer Nano S90 instrument (Malvern Instruments, Malvern, UK) at a detection angle of 90 °C, with a 4mW He-Ne laser operating at 633 nm. NP suspensions were diluted tenfold with bidistilled water before the analysis; the reported values are the mean ± SD from 90 measurements (three consecutive sets of 10 measurements, made in triplicate). The ZP values were calculated by the software of the same instrument, using the Smoluchowski equation and including the average values of electrophoretic nanoparticle mobility. ZP values are reported as the mean of three sets of up to 100 measurements.

2.5. Mucoadhesive Evaluation of Unloaded and DIC- or DIEP-Loaded NPs

Unloaded EUD particles (EUD3A) and loaded with DIC or DIEP (EUD3A_DIC4 and EUD3A_DIEP3, respectively) were evaluated for their mucoadhesive properties. An in vitro method based on the evaluation of two parameters (zeta potential and turbidimetry) was used to assess the mucoadhesive properties of EUD3A, EUD3A_DIC4, and EUD3A_DIEP3.

Briefly, mucin (0.1%, w/v) was suspended in SNF (2.192 g NaCl, 0.145 g $CaCl_2$ and 0.745 g KCl in 250 mL of double distilled water; pH 5) and stirred overnight to allow its complete dispersion. The interaction between EUD3A, EUD3A_DIC4 and EUD3A_DIEP3 and mucin was determined by mixing equal volumes of mucin dispersion and each system for 15 min at 25 °C. After 0, 1 and 24 h of incubation at 37 °C, turbidimetric and zeta potential measurements were performed by comparing the absorbances at 650 nm by UV–Vis spectrophotometry and the difference in zeta potential values, respectively, of the pure suspension and each dispersion with mucin.

2.6. Stability Assessment of Unloaded and DIC- and DIEP-Loaded EUD NPs in Simulated Nasal Fluid

The stability of EUD3A, EUD3A_DIC4 and EUD3A_DIEP3 was determined after incubation of each particle's suspension in SNF containing mucin (0.1%, w/v) at 37 °C. After 1 and 24 h the mean size and PdI of the nanoparticle suspensions were measured by the Zetasizer NanoZS90.

2.7. Differential Scanning Calorimetry (DSC)

Thermal analyses were carried out using a Mettler Toledo DSC 1 STARe system equipped with a Poly-Science temperature controller (PolyScience, Niles, IL, USA). The detection system was a HSS8 high sensitivity sensor (120 gold–gold/palladium–palladium thermocouples) and the ceramic sensor (Mettler Full Range; FRS5) with 56 thermocouples. A pure indium sample was used to calibrate the instrument. Each DSC scan had an accuracy of ±0.4 °C and a reproducibility and resolution of 0.1 °C. To determine the thermotropic behavior, about 5 mg of each sample (RS100, RL100, RS/RL physical mixture (p.m.), DIC, DIEP, RS/RL-DIC p.m., RS/RL-DIEP p.m., EUD3A, EUD3A_DIEP3, EUD3A_DIC4), was sealed in a 40-μL aluminum pan; an empty pan was used as a reference. In the case of EUD NP specimens, the NP suspensions were lyophilized for 24 h (Edwards Modulyo, Thermo Fisher Scientific Italia, Rodano, Italy), after a quick freezing in liquid nitrogen. Each sample was submitted to a heating cycle between 25 and 210 °C, at a scan rate of 5 °C/min. The thermal behavior was extrapolated using the software provided (Mettler STARe Evaluation software system, version 13.00, installed on an Optiplex3020 DELL PC).

2.8. Toxicological Studies

2.8.1. Animals

CD-1 mice (Envigo, Varese, Italy) weighing 20–25 g at the beginning of the experimental procedure were used. Animals were housed in the Centro Stabulazione Animali da Laboratorio (University of Florence) and used at least 1 week after their arrival.

Ten mice were housed per cage (size 26 × 41 cm); animals were fed a standard laboratory diet and tap water ad libitum and kept at 23 ± 1 °C with a 12 h light/dark cycle (light at 7 a.m.). All animal manipulations were carried out according to the Directive 2010/63/EU of the European Parliament and of the European Union Council (22 September 2010) on the protection of animals used for scientific purposes. The ethical policy of the University of Florence complies with the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health (NIH Publication No. 85-23, revised 1996; University of Florence assurance number: A5278-01). Formal approval to conduct the experiments described was obtained from the Italian Ministry of Health (No. 498/2017) and from the Animal Subjects Review Board of the University of Florence. Experiments involving animals have been reported according to ARRIVE guidelines [33]. All efforts were made to minimize animal suffering and to reduce the number of animals used. The animals were randomly divided into the various groups and treated with the products under examination. Of each product (EUD NP suspensions or neat drug suspensions in PBS, pH 7.4), 25 μL per nostril were administered daily for 7 days.

2.8.2. Tissue Preparation

At the end of the treatments, mice were sacrificed by decapitation, the head was quickly dissected to isolate the anterior part of the snout, including the nasal cavities. This was fixed by immersion in 10% paraformaldehyde in PBS, pH 7.4, for 24 h followed by bone tissue decalcification in 10% EDTA for 3 weeks. Finally, the specimens were carefully rinsed in tap water for 24 h, dehydrated in graded ethanol and embedded in paraffin. Sections 5 μm thick were cut, dewaxed and stained with hematoxylin and eosin (H&E). The effects of the different treatments on the nasal mucosa were evaluated paying attention to: (i) loss of cilia at the surface epithelium, (ii) signs of mucus hyperproduction by goblet cells and muciparous glands, (iii) occurrence of inflammatory infiltrate.

2.9. Statistical Analysis

For the statistical analysis of NPs during mucoadhesive study in mucin dispersion, Two-way ANOVA was performed. Multiple comparisons were performed according to Sidak's multiple comparisons test. Analyses were performed using Prism 8 (GraphPad Software, Inc., La Jolla, CA, USA), applying $p < 0.05$ as the minimum level of significance.

3. Results and Discussion

3.1. Preparation of Blank Eudragit NPs

The EUD NP batches reported in Table 1 were produced by the QESD method [25–28] to assess the effect of formulation variables on their technological properties.

In particular, the effect of copolymers concentration and the stirring speed during the preparation were evaluated.

PCS analysis revealed mean particle size in the range between 285 and 380 nm and a narrow width of distribution (PdI ≤ 0.25). Among the experimental variables tested, copolymer concentration did not seem to affect the particles size response; conversely, a higher stirring speed was associated to the formation of relatively larger NPs, although the differences between a 5000 rpm speed (producing NPs of about 300 nm) and the 15,000 rpm one (with NP sizes between 350 and 380 nm) were very limited. In the QESD method, the stirring speed influences the velocity by which the dropping organic phase, which contains the polymeric material, is dispersed into the aqueous one forming a 'quasi-emulsion' of polymeric droplets. The formation and following precipitation of the NPs from these droplets derives from the counter-diffusion of the organic solvent towards the aqueous phase and of the water inside the polymer organic solution [25,26]. Therefore, experimental evidences would support the idea that at a lower stirring speed, the velocity of counter-diffusion of the two liquid phases prevailed on the dispersion of the organic (polymeric) phase in the aqueous one, with a quicker formation of relatively smaller particles.

Based on the above trials, the polymeric systems EUD3A, that resulted to be the most homogeneous formulation (PdI 0.119 ± 0.06) with a mean size < 300 nm was selected and loaded with the chosen active compounds, namely DIC and DIEP, producing the NP batches EUD3A-DIC and 3A-DIEP, respectively (Table 2). On this system, the effects of different drug-to-polymer ratios (from 1:2 to 1:4 for DIC; from 1:2 and 1:3 for DIEP) on NPs mean size and PdI were further evaluated.

Table 2. Composition, mean size (Z-Ave) and polydispersity index (PdI) of DIC-loaded EUD NPs (EUD3A-DIC) and DIEP-loaded EUD NPs (EUD3A-DIEP).

Batch	Drug-to-Polymer Weight Ratio	% Polymer (w/v)	Z-AVE (nm) ± SD	PdI ± SD
EUD3A-DIC2	1:2	3	706.3 ± 0.21	0.993 ± 0.03
EUD3A-DIC3	1:3	3	196.6 ± 0.12	0.345 ± 0.12
EUD3A-DIC4	1:4	3	244.2 ± 0.13	0.258 ± 0.11
EUD3A-DIEP2	1:2	3	1908.1 ± 0.51	0.375 ± 0.05
EUD3A-DIEP3	1:3	3	1434.3 ± 0.34	0.365 ± 0.32

Both the actives showed a certain difficulty to be encapsulated in the polymer matrix; in fact, DIC shows a sparingly solubility in ethanol (25 mg/mL), whereas DIEP, although is much more soluble in the organic solvent, when came into contact with the aqueous phase produced some stiffing material; for this reason, DIEP-to-polymer 1:4 ratio was not further considered (Table 2).

Furthermore, lower polymer concentrations in the organic phase were used to allow incorporating higher drug amounts. Results showed that the drug in the form of a free acid or as a salt significantly affect the physico-chemical properties of EUD-NPs: the DIC-loaded systems produced with a 1:3 or 1:4 drug-to-polymer ratio (EUD3A_DIC3 and EUD3A_DIC4) had mean sizes around 300 nm, with a good size homogeneity (PdI < 0.4). In the presence of a lower drug/polymer ration (EUD3A-DIC2) larger nanoparticles were

produced, with a heterogenous particle population. Using DIEP, particles with a size of 1434.3 ± 0.34 (EUD3A_DIEP3) and 1908.1 ± 0.51 (EUD3A_DIEP2) were obtained, always associated to a relatively low PdI value (PdI < 0.4).

Formulations EUD3A_DIC4 and EUD3A_DIEP3 with notable dimensional differences were selected for further technological evaluations. Moreover, despite the low homogeneity of the EUD3A_DIEP3 batch, compared to EUD3A_DIC4, it could be an appropriate formulation for a local mucosal interaction after nasal administration, since particles with a size less than 500 nm could follow the trigeminal and/or olfactory pathways after IN instillation [30].

3.2. Mucoadhesive Evaluation of Optimized Free and Drug-Loaded EUD NPs

Several features may hamper drug absorption through the nasal mucosa, such as molecular weight and permeability. The amount of drug transported from nose to the systemic circulation can be quite variable, ranging from almost 100% to less than 1% of the administered dose [34].

Therefore, several strategies are under investigation to improve drug absorption after intranasal application [35]. The use of bioadhesive polymer is considered a convenient method to enhance drug permanence to epithelial surface and improve its diffusion to the systemic pathway, when needed.

Polymers can be considered "mucoadhesive" if they possess a number of hydrophilic groups able to attach to mucus or the cell membrane by different ways, such as forming hydrogen bonding or by hydrophobic or electrostatic interactions. Polymers with hydrophilic groups can also swell in water and thus expose the maximum number of adhesive sites available for interaction with the biological compartments [36].

The presence of quaternary ammonium groups imparts a positive charge to RS and RL copolymers, promoting their interaction with anionic drugs [26,28] or the surface of some target tissues. The application of RL and RS copolymers for their cationic charge and mucoadhesive properties to provide a direct contact of NPs with the gastrointestinal tract (GIT) mucosa has been investigated, which may increase the likelihood of cellular uptake by endocytosis and increase the transit time of NPs in the GIT, leading to an improved therapeutic efficacy [37].

Herein, a preliminary evaluation of interaction with mucin, which is one of the main components of the nasal mucus layer, was carried out in order to assess the potential application of EUD NPs free or drug-loaded for IN administration.

ZP measurement is a common method to investigate the mucoadhesive properties of several biopolymers and evaluate the interactions of NPs with mucin. As shown in Figure 1, EUD3A and EUD3A-DIC4 present a strong positive surface charge that was significantly converted toward lower values after incubation with mucin. These results suggest that electrostatic interactions occurred between the positively and negatively charged entities. Significant interactions occurred also for EUD3A_DIEP3 and mucin, despite EUD3A_DIEP is characterized by a negative charge, probably due to the partial absorption of the drug on particles surface.

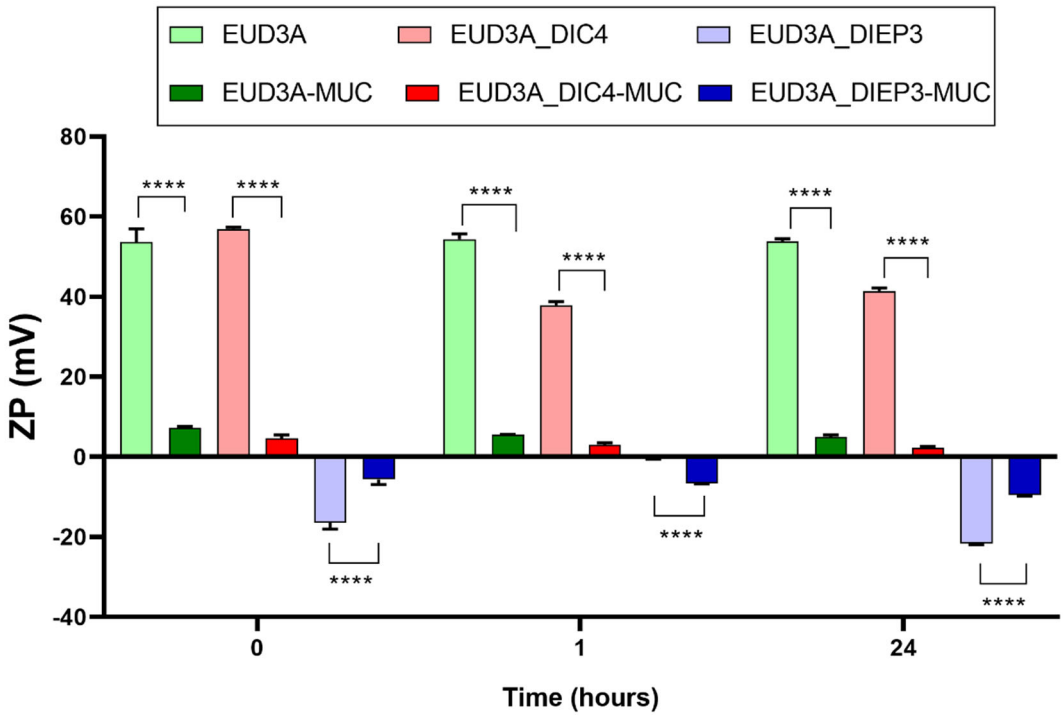

Figure 1. NPs zeta potential values (ZP) before (EUD3A, EUD3A_DIC4, EUD3A_DIEP3) and after (EUD3A_MUC; EUD3A_DIC4-MUC; EUD3A_DIEP3-MUC) 0, 1, and 24 h of incubation with mucin at 37 °C. Significance was set as **** $p \leq 0.0001$.

The principal process in the delivery and absorption of a drug by the nasal cavity route is through the mucus. Mucin is a protein that has the potential to bind with solutes and thus affect the diffusion process.

Mucus is a viscoelastic gel that lines the lumen of the respiratory tissue. The sialic acid and sulphate content are very high in most of the moist mucosal epithelial interfaces, thereby imparting a pronounced negative charge [38]. Increasing the residence time of drug-loaded NPs in the packed layer of mucus enhances the chances to cross the mucus barrier and promote drug systemic diffusion [39]. Bioadhesive and penetrating NP can interact with the mucus layer through electrostatic interactions, hydrogen bonding, or simple van der Waal's forces. Herein, the mucoadhesion study was carried out in SNF (pH 5 at 37 °C) to reproduce the nasal environment. In this condition, mucin suffers a conformational change from a random coil to a rod-like structure by exposing hydrophobic regions, which were folded and sequestered in the inner part at neutral pH. This is a favorable condition for the interactions between mucin and other entities [40,41].

The positive charge of the copolymers may promote an electrostatic interaction with mucin, as previously observed by other authors [42]. Therefore, polymeric nanoparticles produced with EUD copolymers blend develop a positive surface charge, as stated by their ZP values, resulting in a higher adhesiveness to mucosal surfaces.

In the study of Dos Santos Chaves et al., the mucoadhesion resulting from the combined effects of nanocapsules produced with RS100, Eudragit® S100 or poly(ε-caprolactone) (PCL) was investigated after incorporation into different vehicles (suspension, hydrogel, and powder) and application on different mucosal surfaces. Authors found that RS nanocapsules had higher adhesiveness when compared to Eudragit S100 or PCL nanocapsules, regardless of mucosal surface. This better performance was observed independently on the vehicle in which the nanocapsules were dispersed (suspension, powder, hydrogel) [42].

NPs mucoadhesion evaluation was furtherly confirmed by a turbidimetric assessment, as reported in Figure 2.

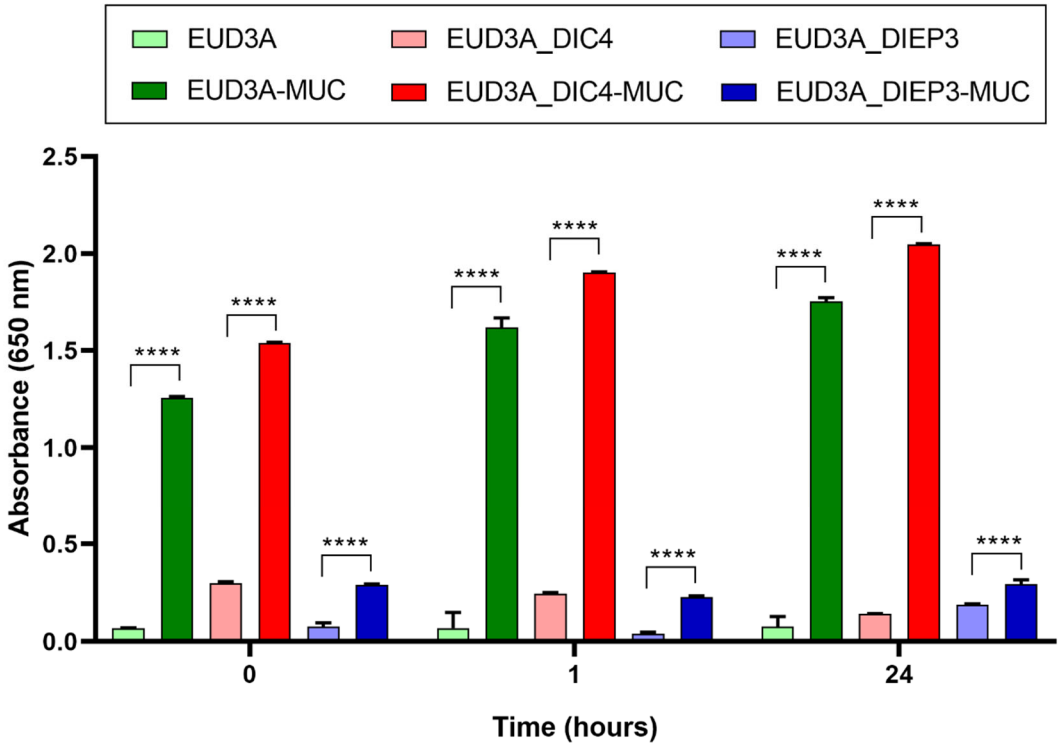

Figure 2. In vitro assessment of EUD3A, EUD3A_DIC4, EUD3A_DIEP3-mucin interactions by turbidimetric assay at 650 nm. Significance was set as **** $p \leq 0.0001$.

According with ZP results, turbidimetric measurements confirmed the strong interactions between EUD3A, EUD3A_DIC4, and EUD3A_DIEP3 and mucin at all time points considered. Absorbance measurement was reported to give a rough estimation of particle–mucin interaction [43] as the results of the adsorption of mucin around the surface of the particles. This phenomenon induces particles aggregation that can be detected as an increase in UV-VIS-absorbance [44].

Overall, significant results in the turbidity (650 nm) and ZP values were observed even if EUD3A and EUD3A_DIC4 revealed higher variations after incubation with mucin compared with the pure suspensions for both parameters.

3.3. Stability of EUD3A, EUD3A_DIC4 and EUD3A_DIEP3 in Simulated Nasal Fluid

NP interactions with the cellular compartment is a complex phenomenon governed by different critical parameters such as particles properties (i.e., shape, size, charge, etc.) and phenomena such as particle interaction with cellular microenvironment [45]. In order to investigate the NPs behavior after IN administration is of outmost importance to evaluate the particle stability in the nasal environment. Accordingly, stability study was performed after incubation of EUD3A, EUD3A_DIC4, and EUD3A_DIEP3 in SNF containing mucin (0.1%, w/v) at 37 °C after 1 and 24 h investigating the effect of protein and ions on the stability of NPs intended for nasal delivery.

Experimental results (Figure 3) revealed a variation in particle size after incubation with a mucin dispersion in SNF compared with the particle suspensions alone (EUD3A, 290.9 ± 0.22 nm; EUD3A_DIC4, 244.2 ± 0.13; EUD3A_DIEP3, 1434.3 ± 0.34). This trend was particularly evident for EUD3A and EUD3A_DIC4 after 1 and 24 h of incubation, while EUD3A_DIEP3 remained almost unchanged. Similarly, PdI values were influenced by mucin presence, since all formulations appeared heterogeneous with the following trend: EUD3A > EUD3A_DIC4 > EUD3A_DIEP3 compared with the native systems after both time interval considered.

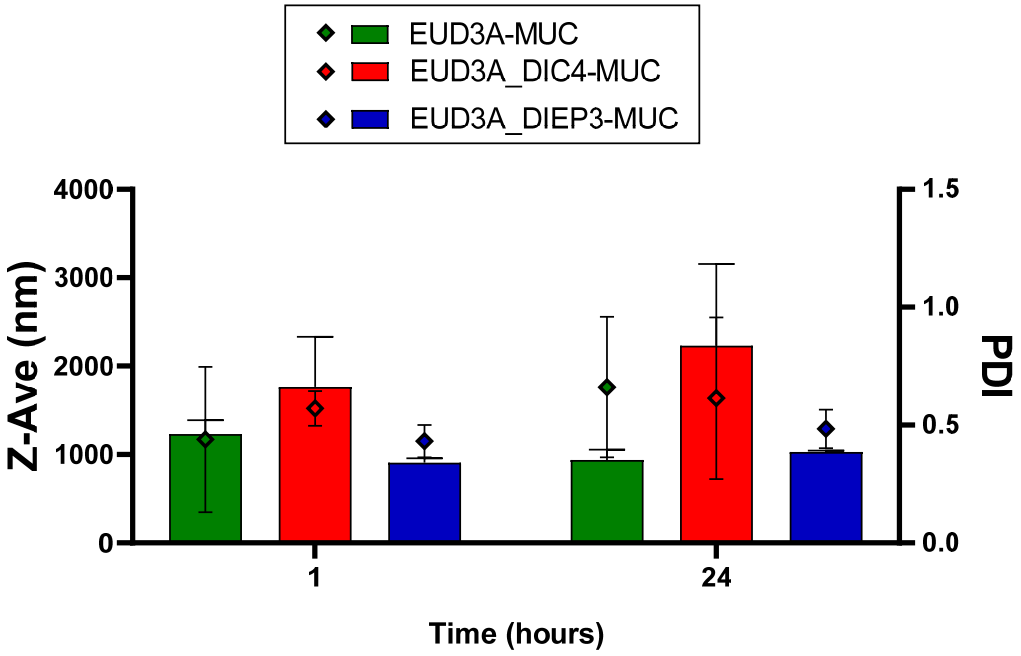

Figure 3. EUD3A, EUD3A_DIC4, and EUD3A_DIEP3 mean size (Z-Ave, bars) and PdI (symbols) after 1 and 24 h of incubation with mucin at 37 °C (EUD3A-MUC, EUD3A_DIC4-MUC, and EUD3A_DIEP3-MUC).

These findings corroborate the mucoadhesive evaluation confirming that the interaction of EUD_NPs with mucin is stronger for EUD3A and EUD3A_DIC4, compared to EUD3A_DIEP3.

Moreover, the increased particle size up to 1–2 μm for all systems once in contact with the mucin dispersion could assure their absorption on the nasal mucosa avoiding possible particle transport through nerve pathways located in the mucosal epithelia [31].

The application of particles with "bioadhesive" properties would permit to enhance their residence time once delivered in the nasal mucosa, control the rate of clearance from the nasal cavity, thereby giving drugs a longer time to be available at the absorptive surface and facilitate drug fit with mucous pores.

3.4. Thermotropic Evaluation

DSC is one of the most used techniques for the characterization of nanomaterials and for detection of material phase transitions [46]. DSC provides qualitative and quantitative information about the thermal properties of solid materials can measure the thermal and physical property changes of materials correlated to temperature [47]. Furthermore, DSC is commonly used in nanotechnology to evaluate any interactions between components and to characterize drug encapsulation within colloidal matrices.

As shown in Figure 4, DIEP thermogram showed an endothermic peak at 103.94 °C with an onset temperature of 95.60 °C, typical of its melting temperature [48]. The thermal curve of DIC showed the typical melting peak of the drug at 175.38 °C with the onset temperature of 170.43 °C. Both curves revealed that DIC as free acid or in the salt form are highly crystalline. No characteristic peak of both drugs was observed in the DSC runs of EUD3A_DIC4 and EUD3A_DIEP3 formulations, which is probably because the drug was encapsulated in the NP matrix, as described in the literature and the amount absorbed on the surface was not detectable [49].

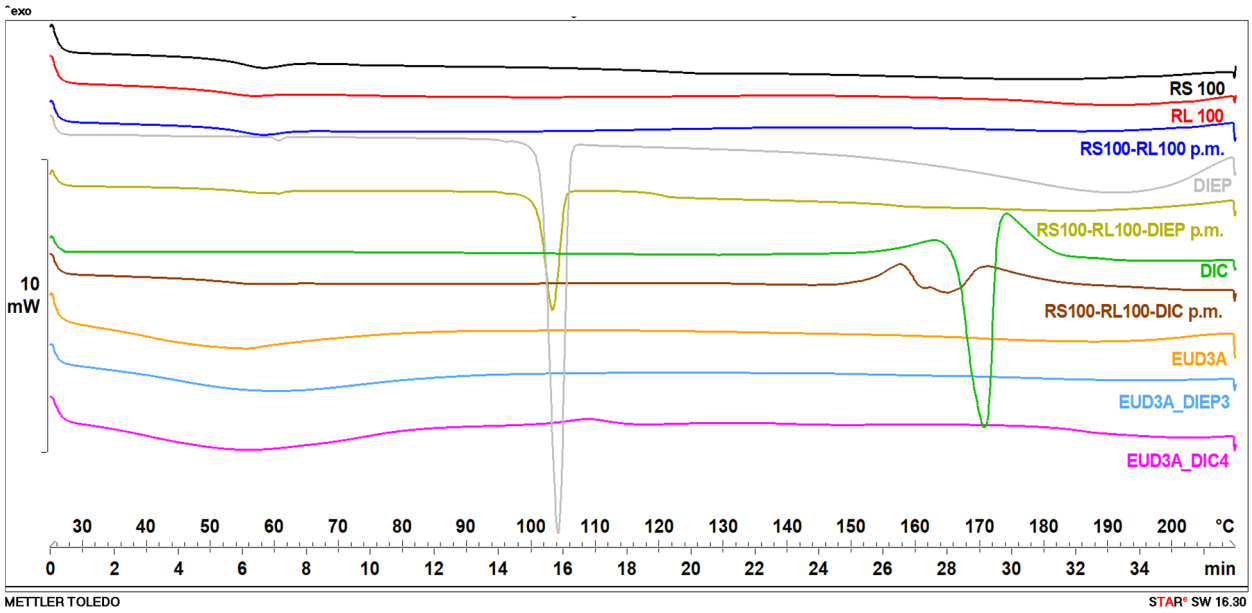

Figure 4. DSC thermograms of RS, RL1, RS/RL physical mixture (p.m.), DIC, DIEP, RS/RL-DIC p.m., RS/RL-DIEP p.m., EUD3A, EUD3A_DIEP3, and EUD3A_DIC4.

No interaction between DIC or DIEP and the copolymer blend was found as shown in the curves of RS/RL-DIC and RS/RL-DIEP physical mixtures (p.m.), in which the melting peaks of both drugs can be still identified.

Based on these encouraging preliminary technological findings and aiming at EUD3A_DIC4 and EUD3A_DIEP3 intranasal application, both formulations were furtherly investigated to evaluate their potential toxicity on mouse nasal mucosa, as described in the following paragraph.

3.5. In Vivo Toxicity Study on Nasal Mucosa

The investigated nanocarriers were subjected to a toxicity study to evaluate their safety towards nasal tissues [50,51]. A first control experiment was performed to investigate the potential adverse effects on mouse nasal mucosa of unloaded EUD NPs (EUD3A). NP suspension (50 µL) was administered intranasally for 1 week. The results of the histological analysis are shown in Figure 5.

Administration of EUD3A had no effect on the normal morphology of the nasal mucosa. A subsequent experiment was performed to investigate the effects on mouse nasal mucosa of EUD3A_DIC4, EUD3A_DIEP3 in comparison with native DIC and DIEP. Suspensions of the different formulations (50 µL) were administered intranasally (30 mg/kg b.wt. dose) for 1 week. The results of the histological analysis revealed that administration of EUD3A_DIC4, EUD3A_DIEP3 had little effects on the surface columnar epithelium and muciparous glands of the nasal mucosa, the only detected abnormalities being clusters of mucus and shed epithelial cells in the nasal cavities and sporadic dilatation of mucosal blood vessels. Administration of EUD3A_DIC4, EUD3A_DIEP3, or free DIEP did not result in appreciable abnormalities of the different histological components of the nasal mucosa.

Conversely, DIC-induced secretory activation of muciparous glands, as judged by reduced size of their adenomeres and occurrence of clusters of mucus embedding shed epithelial cells within the nasal cavities, as well as rarefication of cilia in the columnar surface epithelium. Moreover, DIC also caused prominent and diffuse dilatation of blood vessels in the mucosal lamina propria.

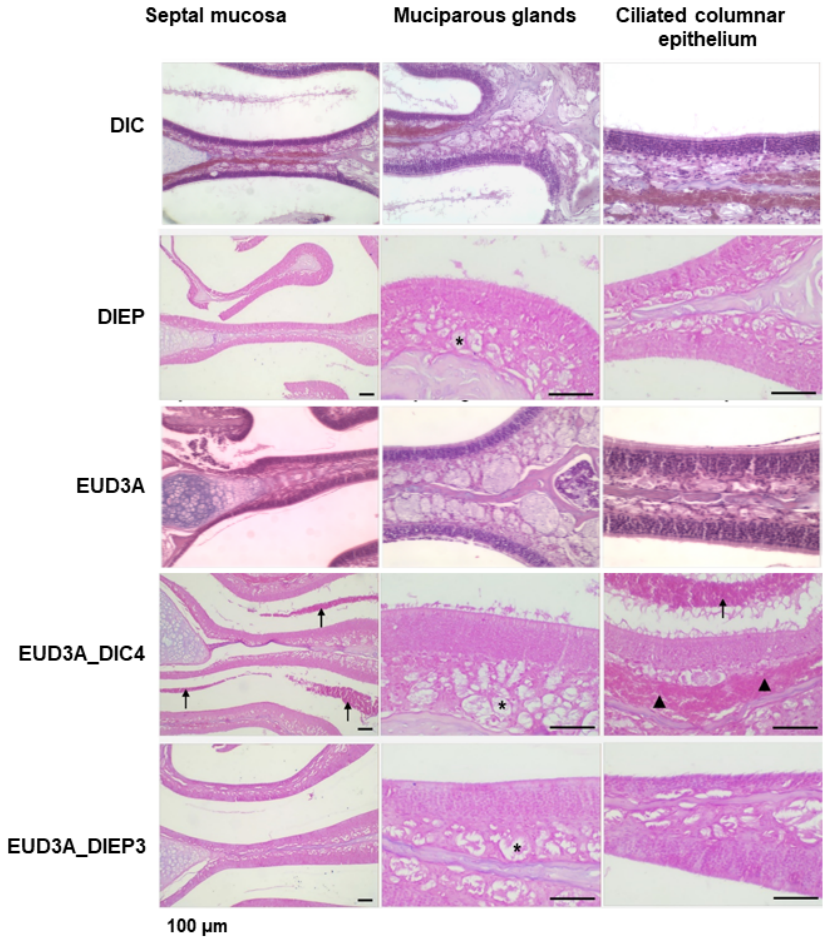

Figure 5. Histological features of the nasal mucosa of mice administered with the control formulation of unloaded EUD NPs (EUD3A); neat diclofenac (DIC) and diclofenac epolamine (DIEP); and EUD NPs with loaded with DIC or DIEP (EUD3A_DIC4 and EUD3A_DIEP4). Hematoxylin and eosin; bars = 100 µm.

Specifically, the septal mucosa showed normal features in the mice given EUD3A, while it evidenced marked blood vessel dilatation (arrowheads) in those given DIC. The muciparous glands (central panels, asterisks) showed normal size and appearance upon treatment with EUD3A, while they are reduced in size, as occurs during secretory activation, upon treatment with DIC; membranes of mucus and shed epithelial cells can also be seen within the nasal cavities (arrows). The ciliated columnar epithelium showed normal features upon treatment with EUD3A, while it displayed rarefication of cilia upon treatment with DIC.

The septal mucosa shows normal features in the mice given EUD3A_DIEP3 and DIEP, while it shows clusters of mucus and shed epithelial cells within the nasal cavities (arrows) in those given EUD3A_DIC4. The muciparous glands (central panels, asterisks) show substantially normal features and size with all the treatments. The ciliated columnar epithelium (right panels) also shows normal features with all the treatments. Occasionally, marked blood vessel dilatation (arrowheads) was detected in the nasal mucosa of mice given EUD3A_DIC4.

Overall, both formulations do not cause any irritation or toxicity compared to the free DIC, this might be due to the encapsulation of the drug within the polymeric system, thereby reducing its direct irritant effect on the mucosal tissue, and can be considered to be biocompatible for nasal administration.

4. Conclusions

The goal of this study was to produce and investigate polymeric NPs based on Eudragit® Retard resins, loaded with two different forms of the NSAID diclofenac, as a free acid or its epolamine salt, to achieve physico-chemical characteristics suitable for the administration via the nasal route. NPs were formulated using a QESD method, obtaining nanocarriers with dimensions ranging from 300 nm to few micrometers. Stored under different temperature conditions they proved to possess a relatively good stability over time; similarly, their incubation with simulated nasal fluid (SNF) at 37 °C did not show relevant signs of physical alteration.

Mucoadhesion studies revealed the occurrence of interactions between particles and mucin after incubation at 37 °C for 1 and 24 h that could enhance the particle residence time in the nasal mucosa and improve drug absorption and diffusion into the systemic circulation.

Stability study of EUD3A_DIC4 and EUD3A_DIEP3, performed in SNF containing mucin, indicated a certain particle instability due to aggregation phenomena, confirming the interaction between these particles and mucin. The apparent increase in particle size observed upon contact with the mucin dispersion could ensure particles absorption on the nasal mucosa, reducing drug clearance from the nasal cavity and thereby promoting drug diffusion into the systemic district.

Toxicity studies on mice ciliary tissue, while evidencing a discrete ciliotoxicity at the mucosal level exerted by the neat active compounds, indicated that the drug-loaded carrier formulations can be considered biocompatible for nasal administration and a good candidate for further biological investigations.

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