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In vitro characterization of physico-chemical properties, cytotoxicity, bioactivity of urea-crosslinked hyaluronic acid and sodium ascorbyl phosphate nasal powder formulation

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Abstract: An innovative lyophilized dry powder formulation consisting of urea-crosslinked hyaluronic acid (HA-CL) and sodium ascorbyl phosphate (SAP) -LYO HA-CL – SAP- was prepared and characterized *in vitro* for physico-chemical and biological properties. The aim was to understand if LYO HA-CL – SAP could be used as adjuvant treatment for nasal inflammatory diseases. LYO HA-CL – SAP was suitable for nasal delivery and showed to be not toxic on human nasal septum carcinoma-derived cells (RPMI 2650 cells) at the investigated concentrations. It displayed porous, polygonal particles with unimodal, narrow size distribution, mean geometric diameter of 328.3 ± 27.5 µm, that is appropriate for nasal deposition with no respirable fraction and 88.7% of particles with aerodynamic diameter > 14.1 µm. Additionally, the formulation showed wound healing ability on RPMI 2650 cells, and reduced interleukin-8 (IL-8) level in primary nasal epithelial cells pre-induced with lipopolysaccharide (LPS). Transport study across RPMI 2650 cells showed that HA-CL could act not only as carrier for SAP and active ingredient itself, but potentially also as mucoadhesive agent. In conclusion, these results suggest that HA-CL and SAP had anti-inflammatory

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Abbreviations: ALI: air-liquid interface; AR: allergic rhinitis; ATCC: American type cell culture collection; BEGM: bronchial epithelial growth medium; CRS: chronic rhinosinusitis; DMEM: Dulbecco modified eagle medium; DSC: differential scanning calorimetry; Dv10: 10% cumulative volume distribution; Dv50: 50% cumulative volume distribution, i.e. median particle size by volume; Dv90: 90% cumulative volume distribution; EC: 2-l glass expansion chamber; ELISA: enzyme-linked immuno assay; FBS: foetal bovine serum; HA: hyaluronic acid; HA-CL: urea-crosslinked hyaluronic acid; HBSS: Hank's balanced salt solution; HPLC: high-performance liquid chromatography; IC50: half maximal inhibitory concentration; IL-8: interleukin-8; LPS: lipopolysaccharide; LYO HA-CL – SAP: lyophilized formulation of urea-crosslinked hyaluronic acid and sodium ascorbyl phosphate; MC: modified expansion chamber; MEM: minimum essential medium; MTS: methyl tetrazolium salt; NGI: Next Generation Impactor; PBS: phosphate buffered saline; ROS: radical oxygen species; RPMI 2650: human nasal septum carcinoma-derived cells; SAP: sodium ascorbyl phosphate; SEM: scanning electron microscopy; TGA: thermal gravimetric analysis; UDS: unit dose system.

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activity and acted in combination to accelerate wound healing. Therefore, LYO HA-CL – SAP could be a potential adjuvant in nasal anti-inflammatory formulations.

Keywords: anti-inflammatory; hyaluronic acid; nasal diseases; sodium ascorbyl phosphate; urea-crosslinked hyaluronic acid; wound healing.

1. Introduction

The nose is an olfactory organ, and also represents the first tract of the respiratory system, with pivotal functions: filtration, warming and moistening of the inhaled air; drainage of paranasal sinuses; sneezing; heat regulation; nasopulmonary reflexes. Hence, nasal mucosal health and proper nasal airflow are essential for the accomplishment of nasal functions (Elad et al., 2008; Jones, 2001; Patel, 2017).

Every day, the nasal passages are exposed to several pathogens that could enhance inflammation, oxidative stress, wounding and infection (Helms and Miller, 2006; Hong et al., 2016). Allergic rhinitis (AR) is one of the most common chronic disease of the nasal mucosa, induced by an Immunoglobulin-E-dependent inflammation due to allergens' exposure (Greiner et al., 2011; Montoro et al., 2007; Small and Kim, 2011). AR can evolve in chronic rhinosinusitis (CRS) (Fokkens et al., 2012; Helms and Miller, 2006; Licari et al., 2017a; Johansson et al., 2003), and AR and CRS frequently coexist with asthma. Indeed, respiratory inflammation may start at one site and extend to another, as upper and lower airways are anatomically, histologically and immunologically linked (Jarvis et al., 2012; Licari et al., 2017a, b; Marseglia et al., 2011). Therefore, the treatment of AR and CRS also represents an integral part of the management of asthma (Licari et al., 2017a; Marseglia et al., 2011). Intranasal corticosteroids are the mainstay therapy for AR and CRS, exerting an anti-inflammatory action and thus relieving symptoms (Helms and Miller, 2006; Licari et al., 2017a). However, their long-term use may have side effects (Campbell, 2018; Fokkens et al., 2012; Licari et al., 2017a). To limit these complications and drug resistant refractory phenomena, adjunctive therapeutic agents with anti-inflammatory, antioxidant and wound healing activity -like resveratrol, alpha-tocopherol (vitamin E), curcumin, N-acetyl-L-cysteine, bromelain, quercetin, beta-carotene (provitamin A), vitamin C (ascorbic acid) and hyaluronic acid (HA)- are currently being investigated for their ability to restore and maintain nasal mucosal health and functions (Albano et al., 2016; Baumeister et al., 2009; Bozdemir et al., 2016; Casale et al., 2014, 2016; Cassandro et al., 2015; Chauhan et al., 2016; Ciofalo et al., 2017; Emiroglu et al., 2017; Helms and Miller, 2006; Hong et al., 2016; Horváth et al., 2016; Podoshin et al., 1991; Sagit et al., 2011; Testa et al., 2017). The present study focused on vitamin C and HA derivatives.

Vitamin C represents a major low molecular weight antioxidant of the respiratory tract lining fluid (Bastacky et al., 1995; van der Vliet et al., 1999; Wu et al., 1996). Vitamin C has been shown to scavenge radical oxygen species (ROS), thus protecting the surface of the airway epithelium (Mudway and Kelly, 2000) and intracellular DNA, lipids and proteins from oxidative stress (Li and Schellhorn, 2007). Oral vitamin C supplementation, in combination with other oral antioxidants and intranasal steroids, was found to be beneficial in the management of AR (Chauhan et al., 2016) and nasal polyposis (Sagit et al., 2011). Additionally, intranasal vitamin C was shown to decrease nasal secretions, blockage and edema in patients with AR (Podoshin et al., 1991).

High-molecular weight (≥ 1 MDa) hyaluronic acid (HA) is an important component of the normal airway secretions, produced by submucosal glands and by superficial airway epithelial cells

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(Basbaum and Finkbeiner, 1988; Fallacara et al., 2018a; Monzon et al., 2006). HA plays a central role in the homeostasis of the whole respiratory apparatus, influencing bio-mechanical forces, hydric balance, cellular functions, growth factors' activity and cytokines' behaviour (Casale, 2016; Fallacara et al., 2018a; Petrigni and Allegra, 2006). In the nasal epithelium, HA promotes mucociliary clearance and sustains mucosal surface healing (Gelardi et al., 2013a). Hence, intranasal exogenous HA may represent an anti-inflammatory, antioxidant, and immuno-modulating agent highly beneficial to treat the inflammatory components of AR (Fallacara et al., 2018a; Gelardi et al., 2013b, 2016), CRS (Casale et al., 2014; Cassandro et al., 2015) and other upper respiratory diseases like acute rhinosinusitis (Ciofalo et al., 2017a, b) and bacterial acute rhinopharyngitis (Varricchio et al., 2014). Additionally, intranasal HA enhances the regeneration and the morphofunctional recovery of nasal ciliated cells in patients undergoing surgery, thus promoting rhino-sinusal remodeling (Cassano et al., 2016). The major limitations of HA are its fluid nature and rapid degradation, leading to a short residence time in the nose. Therefore, HA can be crosslinked in order to synthetize novel derivatives with improved mechanical properties and bioactivity (Chen, 2012).

The combination of sodium ascorbyl phosphate -SAP, a pro-drug of vitamin C with improved physico-chemical stability- and urea-crosslinked hyaluronic acid -HA-CL, a novel biopolymer with promising bioactivity (Citernesi et al., WO/2015/007773 A1, 2015; Fallacara et al., 2017a, b)- has recently shown potential to treat and prevent lung diseases sustained by inflammation and oxidative stress (Fallacara et al., 2018b, submitted). Hence, the present work aimed to study if the combination of SAP and HA-CL could be beneficial as adjuvant therapy to treat inflammatory diseases of the upper airways. A novel nasal dry powder formulation containing SAP and HA-CL was developed using a freeze-dried technique, and characterized for its physico-chemical behaviour, *in vitro* cytotoxicity and bioactivity on human nasal epithelial cells.

2. Materials and Methods

2.1. Materials

Urea-crosslinked hyaluronic acid (HA-CL, molecular weight 2.0-4.0 MDa –raw material containing also pentylene glycol) was kindly donated by I.R.A. Srl (Istituto Ricerche Applicate, Usmate-Velate, Monza-Brianza, Italy). HA-CL was provided as raw material containing pentylene glycol, and it was used as supplied. Therefore, a 1% (w/v) 1:1 HA-CL – SAP solution contained also 0.75% (w/v) pentylene glycol. Sodium ascorbyl phosphate (SAP) was purchased from DSM Nutritional Products Ltd (Segrate, Milano, Italy). Phosphate buffered saline (PBS, pH = 7.4, 0.01 M) used for the physico-chemical characterization of the formulation was supplied by Sigma-Aldrich (Sydney, NSW, Australia). Water was purified by Milli-Q reverse Osmosis (Molsheim, France). Polyamide membrane filters 0.45 µm pore size were obtained from Sartorius Biolab Products (Goettingen, Germany), while wax foils were provided by Parafilm M, Bemis Company Inc. (Oshkosh, WI, USA).

The unit dose system (UDS) powder device was purchased from Pfeiffer, Aptar Pharma division (Radolfzell, Germany).

Human nasal septum carcinoma-derived cells (RPMI 2650) were purchased from the American Type Cell Culture Collection (ATCC, Manassas, VA, USA). Snapwell™ cell culture inserts (1.13 cm^2 surface area, polyester, 0.4 µm pore size) and 96-well plates were obtained from Corning Costar (Lowell, MA, USA). Disposable cytology brushes (Model BC-202D-2010) were provided by

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Olympus Australia Pty. Ltd. (Notting Hill, VIC, Australia). All other culture plastics were from Sarstedt (Adelaide, SA, Australia). Minimum essential medium (MEM), PBS for cell cultivation, Hank's balanced salt solution (HBSS), foetal bovine serum (FBS) and 0.25% trypsin−EDTA were all purchased from Gibco by Life Technologies (Sydney, NSW, Australia). Bronchial epithelial growth medium (BEGM) and SingleQuots™ kit were provided by Lonza (Walkersville, MD, USA). Lipopolysaccharide (LPS) from Escherichia coli, trans-retinoic acid, non-essential amino acids solution, 200 mM L-glutamine solution were purchased from Sigma-Aldrich (Sydney, NSW, Australia). CelLytic™ buffer was obtained from Invitrogen (Sydney, NSW, Australia). Methyl tetrazolium salt (MTS) reagent -CellTiter 96® Aqueous One Solution Cell Proliferation Assay- for cell viability test was purchased from Promega (Sydney, NSW, Australia). Bovine collagen was provided by Nutacon (Leimuiden, Netherlands). Rat collagen type I and enzyme-linked immuno assay (ELISA) kit for determination of the inflammation marker interleukin-8 (IL-8) were obtained from BD Bioscience (Sydney, NSW, Australia). All solvents were obtained from VWR Prolabo Chemicals (Milan, Italy) and were of HPLC grade.

2.2. Preparation of the freeze-dried formulation

HA-CL and SAP were dissolved into 50 mL of milli-Q water kept under constant magnetic stirring, at room temperature, overnight. A 1% (w/v) 1:1 HA-CL - SAP solution was obtained and placed in a -80°C freezer for 24 h. Then, the solution was lyophilized for 24 h, at -100°C, with a vacuum degree of 0.1 mbar, using an Alpha 2-4 LSC plus freeze dryer (Martin Christ, Osterode am Harz, Germany) equipped with a rotary vane Vacuubrand R26 pump (Wertheim, Germany). No cryoprotectant was added to the formulation. At the end of the lyophilisation process, the sample was stored in a desiccator for 24 h, manually grinded with a spatula and passed through a stainless steel sieve (300 µm pore-size).

2.3. Physico-chemical characterization

2.3.1. Chemical quantification of SAP by high-performance liquid chromatography

SAP was quantified using a high-performance liquid chromatography (HPLC) method that was adapted from literature (Foco et al., 2005). A Shimadzu Prominence UFLC system equipped with DGU-20A5R Prominence degassing unit, LC-20 AD solvent delivery unit, SIL-20A HT autosampler and SPD-20A UV/VIS detector was employed (Shimadzu Corporation, Tokyo, Japan). A volume of 50 µL of sample was injected using 40:60 (% v/v) acetonitrile:0.3 M phosphate buffer (pH = 4) as mobile phase, at a flow rate of 2.0 mL/min, in isocratic mode with a Luna NH_2 column (5 µm, 4.6 x 150 mm) (Phenomenex, Sydney, NSW, Australia). SAP retention time was 6 min. The concentration of SAP was measured at a wavelength of 258 nm from the peak area correlated with a predetermined standard curve over the range 0.1-1000 µg/mL ($R^2 = 1$).

2.3.2. Morphological analysis

Scanning electron microscopy (SEM) was used to investigate the morphology and surface properties of the lyophilized formulation containing HA-CL and SAP (LYO HA-CL – SAP). The analysis was carried out using a JCM-6000 benchtop scanning electron microscope (Jeol, Tokyo, Japan), operating at 10 kV. Prior to imaging, the sample was deposited on a carbon sticky tape, placed

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onto aluminium stubs, and gold-coated for 2 min using an automated sputter coater (Smart Coater, Jeol, Tokyo, Japan).

2.3.3. Particle size analysis

Particle diameter and particle size distribution of LYO HA-CL – SAP were determined by laser diffraction (Mastersizer 3000, Malvern, Worcestershire, UK). Approximately 10 mg of sample were dispersed in air using the Scirocco Aero-S dry dispersion unit (Malvern, Worcestershire, UK), with a feed pressure of 4 bars, a feed rate of 100% and a total time of analysis set at 3 s. Measurements were carried out in triplicate, with an obscuration value between 0.1% and 15%, and a reference refractive index of 1.33. The maximum particle size for 10, 50 and 90% of the cumulative volume distribution of the sample (defined as Dv10, 50 and 90, respectively) were used to describe particle size. The width of the particle size distribution was expressed by the Span index. Data were reported as mean ± standard deviation.

2.3.4. Thermal analysis

The thermal response of LYO HA-CL – SAP was assessed using differential scanning calorimetry (DSC - DSC823e; Mettler-Toledo, Schwerzenbach, Switzerland). Roughly 5 mg of sample were weighed, crimp-sealed in DSC standard 40 µL aluminium pans and heated at 10°C/min between -20 and 300°C. The endothermic and exothermic peaks were determined using STARe software V.11.0x (Mettler Toledo, Greifensee, Switzerland).

Additionally, the temperature stability and solvent evaporation of the freeze-dried formulation were investigated using the thermal gravimetric analysis (TGA - Mettler-Toledo, Switzerland). Approximately 5 mg of sample were placed onto aluminium crucible pans. The weight loss of the sample was evaluated over a temperature range of 20-400°C, with a scanning rate of 5°C/min, under constant nitrogen gas. Data were analyzed using STARe software V.11.0x (Mettler Toledo, Greifensee, Switzerland) and expressed as the percentage of weight loss with respect to initial sample weight.

2.3.5. In vitro SAP release study

Franz's diffusion cells (25 mm internal diameter, multi-station VB6 apparatus, PermeGear Inc., Hellertown, PA, USA) were used to study SAP release profile from LYO HA-CL – SAP, according to a previously published protocol (Ong et al., 2011). Briefly, polyamide membrane filters (0.45 µm pore size) were hydrated by sonication in PBS (pH = 7.4, 0.01 M) for 30 min, cut and placed between the receiver and donor compartments of the diffusion cells that were maintained at 37 ± 0.5°C. Samples were placed in the donor compartments in order to have ~ 5 mg of SAP on the surface of the membranes, which were sealed using a wax foil to prevent evaporation. The receiver compartments were filled with 23 mL of PBS continuously stirred at 150 rpm. At defined time points (0, 5, 10, 15, 20, 30, 45, 60, 90, 120 min), 0.5 mL of samples were withdrawn from the receptor compartments and replaced with equal volumes of pre-warmed PBS. After 120 min, each filter was washed with 5 mL of PBS and then sonicated with another 5 mL of PBS for 10 min. Samples were assayed for SAP concentration using HPLC. Experiments were performed in triplicate and data were expressed and plotted as mean ± standard deviation of the cumulative percentage of SAP released over time.

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2.3.6. Aerosol performance by cascade impaction

The aerodynamic performance of LYO HA-CL – SAP delivered using the UDS device was investigated using a British Pharmacopoeia Apparatus E – Next Generation Impactor (NGI -Westech W7; Westech Scientific Instruments, Upper Stondon, UK), equipped with a 2-L glass expansion chamber (EC), according to the Food and Drug Administration (FDA) guidance for industry, and as previously reported in literature (FDA CDER, 2003; Pozzoli et al., 2016a, b, 2017).

Considering that the human nose can accommodate about 10-25 mg of powder *per* nostril *per* shot (Elmowafy et al., 2014), 16 mg of LYO HA-CL – SAP (corresponding to ~ 5 mg of SAP) were loaded into the UDS device. Briefly, the device was connected to the inlet of the EC, that was assembled on the NGI. A rotary pump (Westech Scientific Instruments, Upper Stondon, UK) was connected to the NGI. The test was performed by actuating the device with an air flow of 15 L/min, calibrated using a flow meter (Model 4040, TSI Precision Measurement Instruments, Aachen, Germany), for 4 s. Each impactor stage was washed with the following volumes of deionized water: EC 25 mL; device, connection tube and first stage 10 mL; all other stages 5 mL. SAP was quantified using the HPLC method. Experiments were carried out in triplicate and data were expressed as mean ± standard deviation.

2.3.7. In-line geometric aerosol laser diffraction analysis

Laser diffraction (SprayTec^{TM}, Malvern Instruments, Worcestershire, UK) was used to measure the geometric particle size distribution of LYO HA-CL – SAP emitted from the UDS device (filled with 16 mg of formulation, corresponding to ~ 5 mg of SAP). The device was connected to the measurement cell at a fixed angle of 30°, with an extraction flow of 15 L/min. Three independent analyses were performed for 4 s, with an acquisition rate of 2.5 kHz. Results were expressed as mean ± standard deviation.

2.4. In vitro biological studies on nasal cell models

Cytotoxicity, wound healing activity, drug deposition and transport were investigated using RPMI 2650 human nasal cell line. Additionally, anti-inflammatory activity was explored using primary brushed nasal epithelial cells.

2.4.1. Cultivation of RPMI 2650 cell line

RPMI 2650 immortalized human nasal cell line were grown and passaged according to ATCC protocol and as previously described in literature (Bai et al., 2008; Kreft et al., 2015; Pozzoli, 2016a,b, 2017; Reichl and Becker, 2012). Briefly, cells between passages 17-25 were cultured in 75 cm^2 flasks containing MEM supplemented with 10% (v/v) FBS, 1% (v/v) non-essential amino acid solution and 2 mM L-glutamine, and maintained in a humidified atmosphere of 95% air, 5% CO_2, at 37°C.

Liquid covered cultures were obtained by seeding RPMI 2650 cells (5 x 10^4 cells/well) in a volume of 100 µL into 96-well plates, and were used to perform cell viability assay within 24 h from the seeding.

Additionally, an air-liquid interface (ALI) nasal model was established using Snapwell^{TM} cell culture inserts. In brief, inserts were coated with 250 µl of 1 µg/ml rat collagen type 1 solution in PBS and incubate overnight at 37°C to create the appropriate adherence of the cells to the membrane (Wengst and Reichl, 2010). 200 µl of RPMI 2650 nasal cells suspension (2.5 x 10^6 cell/ml) were

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seeded on Snapwell™ and after 24 h the medium from the apical compartment was withdrawn, resulting in an ALI model. The medium in the basolateral chamber was replaced 3 times per week. Wound healing and deposition/transport studies were performed 14 days after the seeding.

2.4.2. MTS cytotoxicity assay on RPMI 2650 cells

The *in vitro* cytotoxicity of LYO HA-CL – SAP was assessed on a liquid covered culture of RPMI 2650 cells. Sample solutions with increasing concentrations of the formulation were aseptically prepared, added to the cells and, after 3 days, a MTS assay was performed to measure cellular metabolic activity.

Briefly, 5 x 10^4 cells were seeded per well in a volume of 100 µL into 96-well plates. Cells were incubated overnight at 37°C in a humidified atmosphere with 5% CO_2. On the second day, 100 µL of pre-warmed solutions of LYO HA-CL – SAP were added to each well. The lyophilized formulation was investigated in a SAP concentration range from 8.0 mM to 60.9 nM. Background controls (medium) and untreated controls (untreated cells) were included in the experiment. Plates were incubated for 72 h at 37°C in a humidified atmosphere with 5% CO_2. Cells were then analysed for viability. Hence, 20 µL of MTS reagent were added to each well and the plates were incubated for another 3 h in the same conditions. Finally, the 96 well-plates were read at 490 nm using a SpectraMax microplate reader. The absorbance values were directly proportional to cell viability (%). Experiments were performed in triplicate. Data were expressed as mean ± standard deviation of the % cell viability relative to untreated control, and plotted against SAP concentration (nM) on a logarithmic scale.

2.4.3. Deposition/transport study on RPMI 2650 cells

The aerosol deposition of LYO HA-CL – SAP on RPMI 2650 cell surface was studied using a custom-built 3D printed modified expansion chamber (MC) (Pozzoli et al., 2016a). Prior to the experiment, three inserts were washed with pre-warmed HBSS and fitted into the upper hemisphere of the MC. As described above, a total dose of 16 mg of formulation (corresponding to ~ 5 mg of SAP, 1 shot/actuation) was delivered with the UDS device into the MC. The cell inserts were then removed from the MC and transferred into a 6-well plate, containing 1.5 mL/well of fresh pre-warmed HBSS. At defined time point (15, 30, 45, 60, 90, 120, 150, 180, 210, 240 min), 200 µL of samples were collected from the basal chamber and replaced with the same volume of fresh HBSS buffer. After 4 h, the apical surface of the epithelia was washed twice with 300 µL of HBSS in order to collect any remaining drug on the cells. At the end of the experiment, cells were washed with 300 µL of HBSS, scraped from the insert membrane, and lysed with 500 µL of CelLytic™ buffer in order to quantify intracellular SAP. Samples were centrifuged (10 min, 10,000 x g, 4°C) and the supernatants were analyzed for SAP concentration by HPLC. Experiments were performed in triplicate. The initial amount of SAP deposited on the cell layer was calculated as the sum of SAP mass transported, remaining on the epithelium and recovered inside the cells at the end of the experiment. Data were plotted as mean ± standard deviation of the cumulative percentage of SAP transported over time.

2.4.4. Physical injury and wound healing assay on RPMI 2650 cells

Wound healing properties of LYO HA-CL – SAP were evaluated on ALI RPMI 2650 cell layer. The cells were mechanically scraped using a sterile 200 µl pipette tip to form three homogenous and

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parallel scratches. After scraping, the wells were allocated into the upper hemisphere of the MC, and the formulation was subsequently deposited on the cell layers as described above. The healing of the layers was monitored via a time-lapse microscopy. Immediately after wounding and deposition, the Snapwells™ plates were transferred to the built-in chamber of a Nikon Eclipse Ti (Nikon, Tokyo, Japan) equipped with Clear State Solution humidifier (Clear State Solution, Mount Waverly, VIC, Australia) and CO_2 controller which provided a humidified CO_2 atmosphere at 37°C. Images of the scratches were captured by a CoolSNAP ES2 high resolution digital camera (Photometrics, Tucson, AZ, USA) every 20 min over 24 h. Images were analysed with FIJI (NIH), wound edges were manually selected and wound area was automatically calculated. Experiments were performed in triplicate. Data were expressed as mean ± standard deviation of the percentage of wound closed according to the following equation (**Eq. (1)**):

$$WC = \frac{W_i - W_t}{W_i} \times 100 \quad (1)$$

where WC is the wound closure (%), W_i is the initial area of wound and W_t is the area of the wound at certain time.

2.4.5. Sampling, expansion and ALI cultivation of brushed nasal epithelial cells

Nasal cells were obtained from 5 healthy volunteers between the ages of 20 and 30 through nasal brushing biopsies, and cultured according to methodologies as previously described (Müller et al., 2013). Brushings were performed only on volunteers without any upper respiratory tract disease and infection for at least 4 weeks prior to sampling. Briefly, a cytology brush was inserted approximately 5 cm into the volunteers' naris, and the inferior turbinate and the nasal epithelium were gently brushed. Strips of ciliated nasal epithelium were obtained, with each brushing containing up to 10,000 cells.

Prior to cell seeding, the microwell plates, flasks, and Snapwell™ inserts were coated for 1 h with 0.5 mL, 1 mL, and 0.1 mL of 0.3 mg/mL of bovine collagen, respectively, and were air-dried. The strips of ciliated nasal epithelium were grown on collagen-coated tissue culture 12-well plates in BEGM hormonally supplemented with SingleQuots™ for approximately 1 week, changing cell medium every other day. The confluent basal cells were then expanded onto a collagen coated 25 cm^2 flask with the BEGM replaced three times per week. When cultures reached ~ 80% confluency, the basal cells were detached with 0.25% trypsin−EDTA and seeded with a density of 200,000−250,000 cells per well onto collagen-coated Snapwell™ cell culture inserts. At this stage, the cells were fed with ALI medium made up of 1:1 BEGM:DMEM 4.5 g/L D-glucose including SingleQuots until 100% confluent (into 1-2 days). Primary human basal epithelial cells were then exposed to an ALI interface: to allow for cell differentiation, ALI medium supplemented with additional 100 nM all-trans-retinoic acid was placed only in the basolateral chamber (Hirst et al., 2014; Lee et al., 2005; Yoo et al., 2003). The medium was changed three times per week, and any apical surface liquid or mucus was removed. Cilia started to re-generate after 12 days from the establishment of the ALI conditions, and become visible under the microscope from day 21 ALI.

2.4.6. Pro-inflammatory IL-8 expression in brushed nasal epithelial cells

The expression of the pro-inflammatory cytokine IL-8 was evaluated using the ALI primary nasal

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cells treated with LYO HA-CL – SAP (150 µg/mL), before, after and without LPS exposure. The effect of the formulation on cells was compared with unstimulated and untreated cells (control cells, i.e. cells in culture medium, without LPS and formulations) and LPS-stimulated but untreated cells (positive control, i.e. cells exposed only to 10 ng/mL LPS).

Firstly, LYO HA-CL – SAP was evaluated for its ability to influence IL-8 production in non-inflamed nasal cells. Subsequently, cells were incubated at 37 °C, 5% CO_2, for 24 h, with a pre-warmed solution of LYO HA-CL – SAP. On the second day, cell culture medium was withdrawn, centrifuged (5 min, 13000 rpm, 4°C), and analyzed for IL-8 level using a human IL-8 ELISA kit, according to the manufacturer's protocol. The amount of IL-8 released in the samples was quantified using a standard calibration curve obtained with purified recombinant human IL-8 provided with the kit. The limit of detection was 3.1-200 pg/mL.

Then, to test the formulation ability to prevent inflammation, cells were exposed to a pre-warmed solution of LYO HA-CL – SAP, and incubated at 37 °C, 5% CO_2 for 24 h. Afterwards, cells were incubated again for another 24 h, at 37 °C, 5% CO_2, with 10 ng/mL LPS. Subsequently, cell culture supernatant was withdrawn, centrifuged (5 min, 13000 rpm, 4°C), and analyzed for IL-8 level as previously described.

Finally, the anti-inflammatory efficacy of LYO HA-CL – SAP was evaluated by its ability to reduce inflammation in primary ALI nasal cells after induction of IL-8 production by LPS. Hence, cells were incubated with 10 ng/mL LPS for 24 h at 37°C, in a humidified atmosphere at 5% CO_2. Subsequently, cells were exposed to a pre-warmed solution of LYO HA-CL – SAP, and after 24 h of incubation in the same conditions, cell culture supernatant was withdrawn, centrifuged (5 min, 13000 rpm, 4°C), and analyzed for IL-8 concentration as previously described.

2.5. Statistical analysis

All the data are presented as mean ± standard deviation of a minimum of three independent experiments. Statistical analysis was performed using GraphPad Prism software version 7.0b (GraphPad, San Diego, USA). Wound healing data were analyzed with unpaired *t*-test, while anti-inflammatory response data were analyzed with one-way analysis of variance (ANOVA) followed by Tukey *post hoc* test for multiple comparisons. Differences were considered statistically significant for $P < 0.05$ (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$).

3. Results and discussion

Previous studies showed that SAP and HA-CL, when delivered in combination, provide an enhanced effect in controlling lung inflammation and oxidative stress (Fallacara et al., 2018b, submitted). Additionally, emerging data showed the beneficial role in nasal inflammatory diseases for the precursors of these substances –vitamin C (Chauhan et al., 2016; Podoshin et al., 1991; Sagit et al., 2011) and HA (Casale et al., 2014, 2016; Cassandro et al., 2015; Ciofalo et al., 2017a, b; Garantziotis et al., 2016; Gelardi et al., 2013b, 2016; Varricchio et al., 2014). All these evidences strongly supported the present study to develop a novel formulation containing both SAP and HA-CL to enhance treatments for nasal diseases, like AR and CRS. A dry powder formulation was prepared, as powders, being solid dosage forms, are chemically and microbiologically more stable than liquids, and require simpler compositions in excipients (if any) (Tiozzo Fasiolo et al., 2018). Moreover, compared to nasal liquids, nasal powders undergo slower clearances from the nasal cavity, and can be delivered more efficiently by insufflation devices that permit the deposition on a larger surface of

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the nasal mucosa (Tiozzo Fasiolo et al., 2018). All this can improve drug diffusion, adsorption across the mucosa, and bioavailability (Tiozzo Fasiolo et al., 2018).

3.1. Physico-chemical characterization

3.1.1. Morphological analysis

In **Fig. 1** the morphology of LYO HA-CL – SAP observed by SEM is presented. The formulation was characterized by porous particles of polygonal shape, with size > 50 µm and < 600 µm, that is suitable for nasal delivery (Kippax et al., 2010).

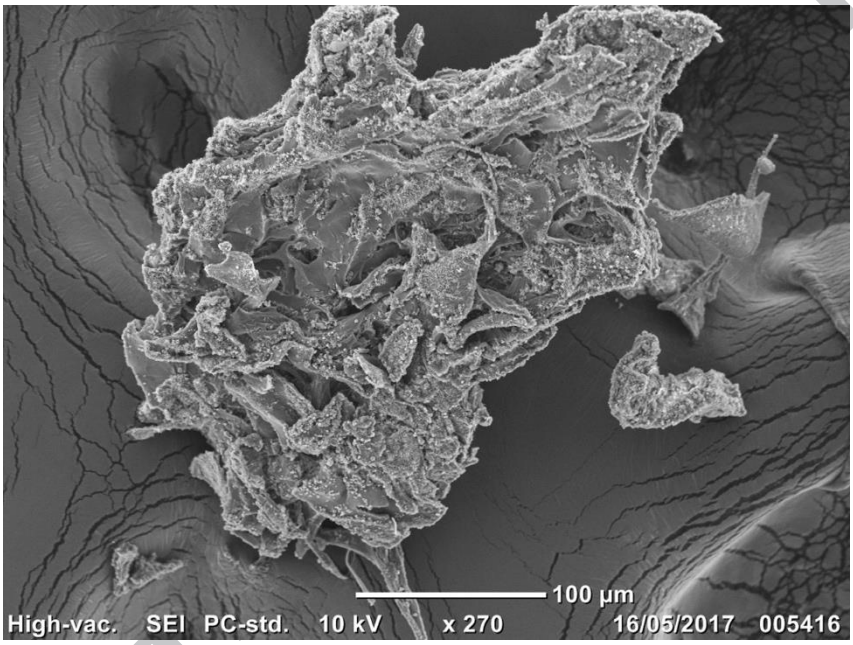

Fig. 1. SEM micrograph of LYO HA-CL – SAP.

3.1.2. Particle size analysis

Results of laser diffraction analysis of LYO HA-CL – SAP are shown in **Fig. 2**. A unimodal and narrow dispersion (Span index: 1.2 ± 0.0) was observed, with a Dv50 of 328.3 ± 27.5 µm. Moreover, 10% of LYO HA-CL – SAP particles had a diameter ≤ 179.0 ± 17.4 µm (Dv10), and 90% ≤ 566.7 ± 39.5 µm (Dv90). Hence, the freeze-dried formulation appeared to be suitable for nasal delivery as no particle fraction was found to be smaller than 10 µm (Ozsoy et al., 2009; Tiozzo Fasiolo et al., 2018).

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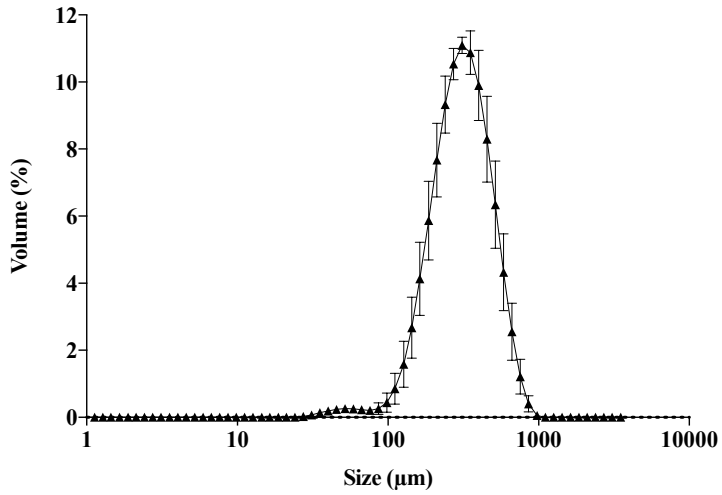

Fig. 2. Particle size distribution of LYO HA-CL – SAP. Data represent mean ± SD (n = 3).

3.1.3. Thermal analysis

The thermal behaviour and physical stability of LYO HA-CL – SAP were investigated using DSC and TGA, as shown in **Fig. 3**.

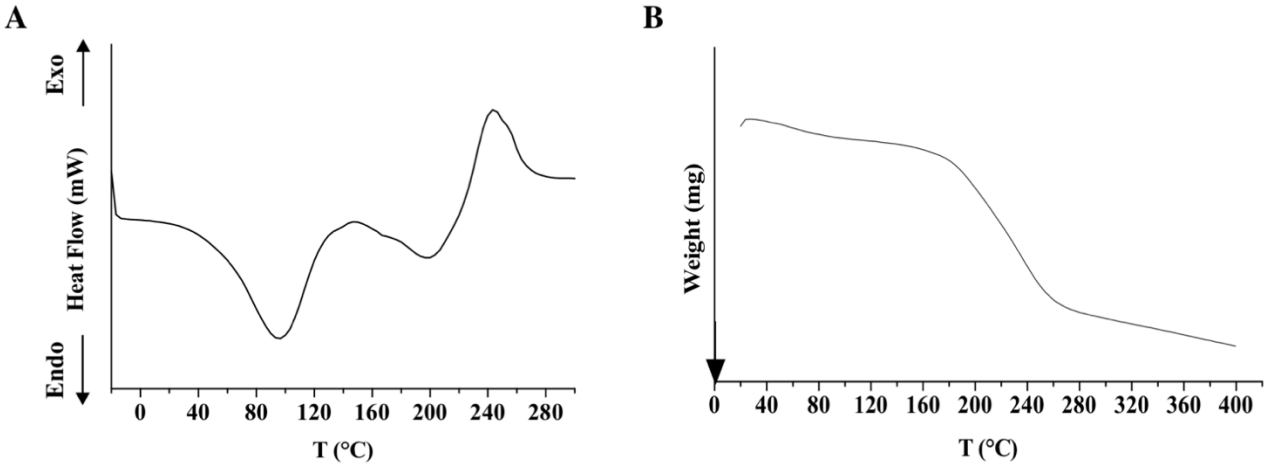

Fig. 3. DSC (A) and TGA (B) thermal profiles of LYO HA-CL – SAP.

The DSC thermal profile of LYO HA CL – SAP was characterized by two wide endothermic peaks: the first at 97°C, suggesting a dehydration process, and the second at 200°C, which was attributed to pentylene glycol evaporation (boiling range 198-200°C) (**Fig. 3A**). The thermal decomposition of LYO HA-CL – SAP and the formation of a carbonized residue occurred at 240°C, where a broad exothermic peak was observed. This DSC profile suggested that HA-CL and SAP formed a solid solution/amorphous solid dispersion in the freeze-dried formulation. Such result was in good agreement with previous observations reported for SAP raw material, HA-CL microspheres and other dry powder formulations containing both SAP and HA-CL (Fallacara et al., 2018c, submitted).

TGA thermograph of LYO HA-CL – SAP is displayed in **Fig. 3B**. Three distinct degradation stages could be observed, with the first stage (20-181°C, 6.7% w/w of weight loss) that is characteristic of water evaporation; the second (181-246°C, 22.5% w/w of weight loss, including also

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the evaporation of pentylene glycol) and the third stages (246-400°C, 12.8% w/w of weight loss) that are typical of a two-stages polysaccharide degradation.

Hence, the thermal decomposition of HA-CL and SAP occurred simultaneously, as showed by DSC and TGA curves, and confirmed by data previously obtained for HA-CL microspheres containing SAP (Fallacara et al., 2018c).

3.1.4. In vitro aerosolization performance

Particle size is a key parameter in defining the deposition pattern of formulations delivered to the nasal mucosa using nasal inhalers and pump sprays. To verify the absence of particles with aerodynamic diameter < 10 µm, that could reach the lower airways (Cheng et al., 2001; Doub et al., 2012), the FDA draft guidance for industry has suggested to perform impactor and laser diffraction analyses (FDA CDER, 2003). Hence, the aerodynamic efficiency of LYO HA-CL – SAP, delivered with the UDS nasal device, was investigated using the Apparatus E equipped with a FDA 2-L glass EC (FDA CDER, 2003). Additionally, SprayTec™ laser diffraction technique was employed to assess the geometric particle size distribution of the emitted dose from the UDS nasal device.

In **Table 1** the *in vitro* aerosolization performance of LYO HA-CL – SAP is presented. Results are presented as the percentage of the drug remaining in the device and deposited in the EC, connection tube, and each stage of the NGI, over the total theoretical amount of drug filled in the device. It was found that the LYO HA-CL – SAP formulation is suitable for nasal drug delivery, with 88.7% of the particles showing an aerodynamic diameter larger than 14.1 µm (78.3 ± 2.7% of the particles in the EC, plus 0.7 ± 0.5% in the connection tube and 9.7 ± 1.5% in the S1), while the remaining 11.3 ± 4.0% of SAP was recovered in the device. As no drug was detected in the lower stages of the NGI, there was no respirable fraction.

Table 1. Percentage of SAP recovered in each stage of the apparatus E equipped with the 2-l glass EC for nasal delivery. Data are presented as mean ± SD (n = 3).

	Amount of SAP recovered (%)	**Cut off diameter (µm)**
Device	11.3 ± 4.0	-
EC	78.3 ± 2.7	-
Connection tube	0.7 ± 0.5	-
S1	9.7 ± 1.5	> 14.1
S2-S8	-	< 14.1
Total recovery	100.0 ± 0.0	-

The aerosolization performance of the freeze-dried formulation was also characterised by SprayTec™ laser diffraction particle sizing. The SprayTec™ system ensures the characterization of the whole particle size distribution, and detects the dynamics of particle dispersion along with the device reproducibility, while cascade impaction provides information on the aerosol performance of the particles below 10 µm. **Table 2** shows the results of SprayTec™ analyses carried out on LYO HA-CL – SAP, which exhibited a geometric diameter that is suitable for nasal delivery. Indeed, the size distribution was unimodal and narrow (Span index: 1.8 ± 0.0), with the 90% of particles below 537.8 ± 28.8 µm and a median diameter of 243.0 ± 13.0 µm. No particles below 10 µm were detected.

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Table 2. Values of volumetric diameter, Span index and percentage of particles ≤ 10 μm obtained for LYO HA-CL – SAP with SprayTec™ system. Data represent mean ± SD (n = 3).

	LYO HA-CL - SAP
Dv(10)	100.5 ± 4.5
Dv(50)	243.0 ± 13.0
Dv(90)	537.8 ± 28.8
Span	1.8 ± 0.0
% V (≤ 10 μm)	0.0 ± 0.0

3.2. In vitro studies on nasal cell models

3.2.1. MTS cytotoxicity assay on RPMI 2650 cells

The *in vitro* cytotoxicity of LYO HA-CL **–** SAP was evaluated using the colorimetric MTS assay. RPMI 2650 cells were incubated for 72 h with solutions of the freeze-dried formulation with SAP concentration ranging from 8.0 mM to 60.9 nM. As displayed in **Fig. 4**, cells maintained their metabolic activity and were viable after the exposure to the solution with the highest concentration. Since the half maximal inhibitory concentration value (IC50) could not be determined across all the concentration interval examined, LYO HA-CL **–** SAP could be considered non-toxic for RPMI 2650 cells at the wide concentration range tested. Hence, this study showed for the first time that the novel HA-CL polymer could be safely used for nasal formulations. In fact, HA-CL maintained the excellent safety profile of high molecular weight native HA, which was previously found to be non-toxic even at high concentration -1% on RPMI 2650 cells (Horváth et al., 2016), 3% on adult volunteers affected by rhinosinusitis (Ciofalo et al., 2017a).

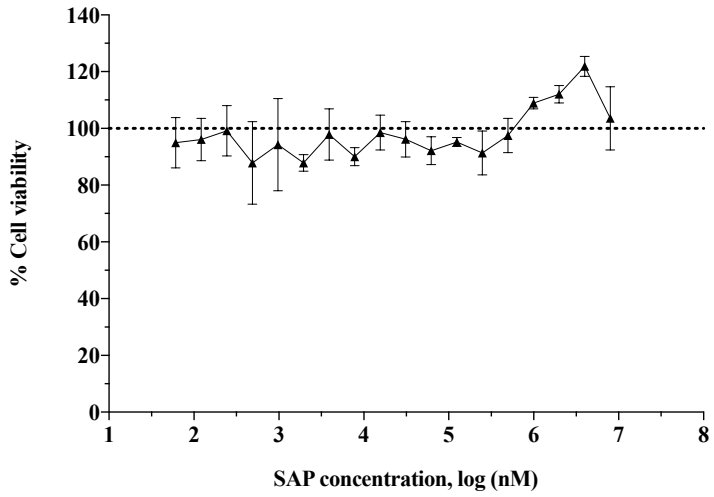

Fig. 4. Viability of RPMI 2650 cells evaluated using MTS assay after 72 h of treatment with LYO HA-CL – SAP. Data represent mean ± SD (n = 3).

3.2.2. Wound healing assay on RPMI 2650 cells

Factors such as allergies, prolonged/chronic inflammation, virus and bacterial infections can alter the nasal mucosa homeostasis and therefore, can damage or destruct the epithelium. In order to assess

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the ability of LYO HA-CL - SAP to improve epithelia damage, wound healing assays were performed. **Figure 5** shows images of the wound healing process without and with treatment (LYO HA-CL – SAP) at different time points (0, 1, 8 h). The freeze-dried powder formulation showed wound healing ability with significant improvement of the wound closure observed at 8 hours. Through image analysis, it was calculated that LYO HA-CL - SAP was able to close roughly the 62 ± 13 % of the wound after 8 hours. This closure was significantly different compared to untreated cells ($P < 0.0001$), where the wound closure was only 13 ± 4%. Hence, these results provided preliminary evidence that LYO HA-CL – SAP was able to promote the re-epithelialization of RPMI 2650 wounded cells, demonstrating its potential use as adjuvant in nasal anti-inflammatory treatments.

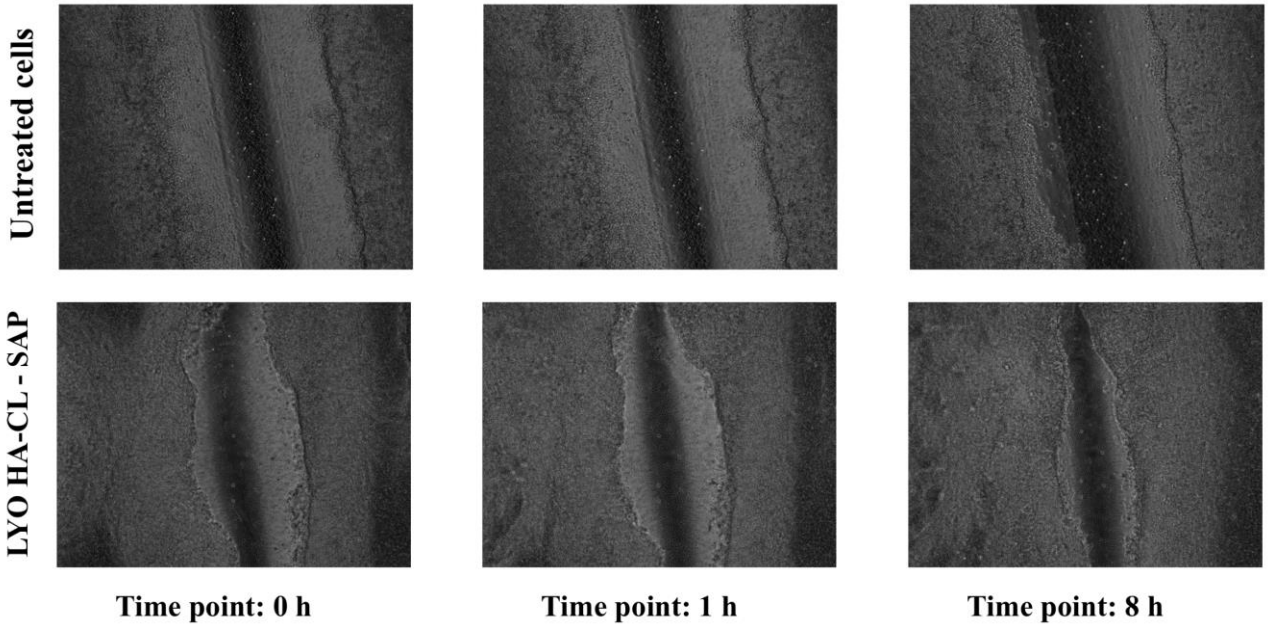

Fig. 5. Wound images of untreated cells and cells treated with LYO HA-CL – SAP at different time points (0, 1, 8 h).

3.2.3. Pro-inflammatory IL-8 expression in brushed nasal epithelial cells

IL-8 is a pro-inflammatory cytokine that is able to enhance allergic inflammation. It is highly expressed in many inflammatory diseases of the upper respiratory tract, including AR and CRS (Hu and Li, 2018; Lee et al., 2018). This *in vitro* study investigated the ability of the novel LYO HA-CL – SAP formulation in reducing the expression of IL-8 in primary ALI nasal cells, thus providing an anti-inflammatory activity.

Results showed that IL-8 production was not increased ($P > 0.05$) by the exposure of non-stimulated primary nasal cells to LYO HA-CL – SAP, with IL-8 levels of 15.2 ± 3.3 % in unstimulated and untreated cells and 19.2 ± 8.5 % in LYO HA-CL – SAP treated cells (LYO HA-CL – SAP), as showed in **Fig. 6**. On the contrary, cells exposed to 10 ng/mL LPS that remained untreated exhibited a significant inflammatory response ($P < 0.0001$), with an IL-8 concentration that was 6.5 times higher with respect to unstimulated and untreated cells (**Fig. 6**).

As depicted in **Fig. 6**, cell exposure to LYO HA-CL – SAP treatment 24 h prior to stimulation with 10 ng/mL LPS significantly reduced IL-8 production to 49.5 ± 9.7 % (LYO HA-CL – SAP + LPS), compared to LPS-stimulated but untreated cells (100.0 ± 0.0 %; $P < 0.0001$). Hence, LYO

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HA-CL – SAP was shown to potentially play a preventive anti-inflammatory action.

Additionally, the anti-inflammatory property of LYO HA-CL – SAP was investigated after stimulation of primary nasal cells with 10 ng/mL LPS 24 h prior to treatment exposure. IL-8 concentration was determined 24 h after the sample addition on the LPS-stimulated cells. LYO HA-CL – SAP showed the ability to reduce IL-8 release compared to LPS-stimulated but untreated cells: more specifically, LYO HA-CL – SAP decreased IL-8 concentration to 68.8 ± 4.6 % (LPS + LYO HA-CL – SAP) (**Fig. 6**). Therefore, LYO HA-CL – SAP was found to have a significant anti-inflammatory activity towards inflamed primary nasal cells ($P < 0.0001$) (**Fig. 6**). These results showed that the freeze-dried powder consisting of HA-CL and SAP could be a suitable formulation to reduce inflammation in upper airway diseases.

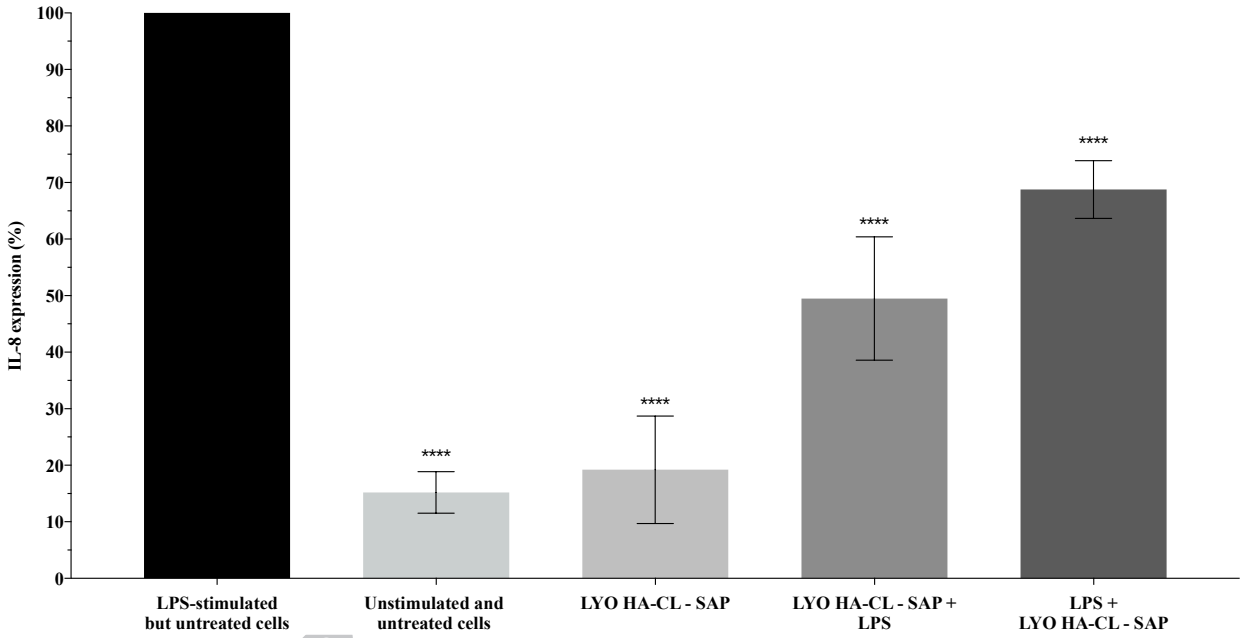

Fig. 6. Concentration of IL-8 inflammatory cytokine in primary brushed ALI nasal cells supernatant after exposure to: LYO HA CL – SAP; LYO HA CL – SAP then LPS (LYO HA CL – SAP + LPS); LPS and then LYO HA CL – SAP (LPS + LYO HA CL – SAP). IL-8 levels were assessed in comparison to those of LPS-stimulated but untreated cells (positive control, i.e. cells exposed only to 10 ng/mL LPS), and unstimulated and untreated cells (control cells). Data represent mean ± standard deviation (n = 5). Asterisks indicate significant difference from LPS-stimulated but untreated cells (****$P < 0.0001$).

3.3. In vitro release study: release by Franz's cells and transport across RPMI 2650 cells

Franz's cells were chosen to explore the dissolution and release properties of LYO HA-CL – SAP (**Fig. 7A**), as the thin layer of liquid on the cells membranes permits the progressive hydration of the formulation in a humid environment. This is more representative of the *in vivo* nasal mucus layer with respect to the larger volume of dissolution medium of other release methods. Furthermore, SAP permeation profile was also studied across RPMI 2650 cells grown in the ALI configuration, for 4 hours after LYO HA-CL - SAP deposition in the 3D MC (**Fig. 7B**).

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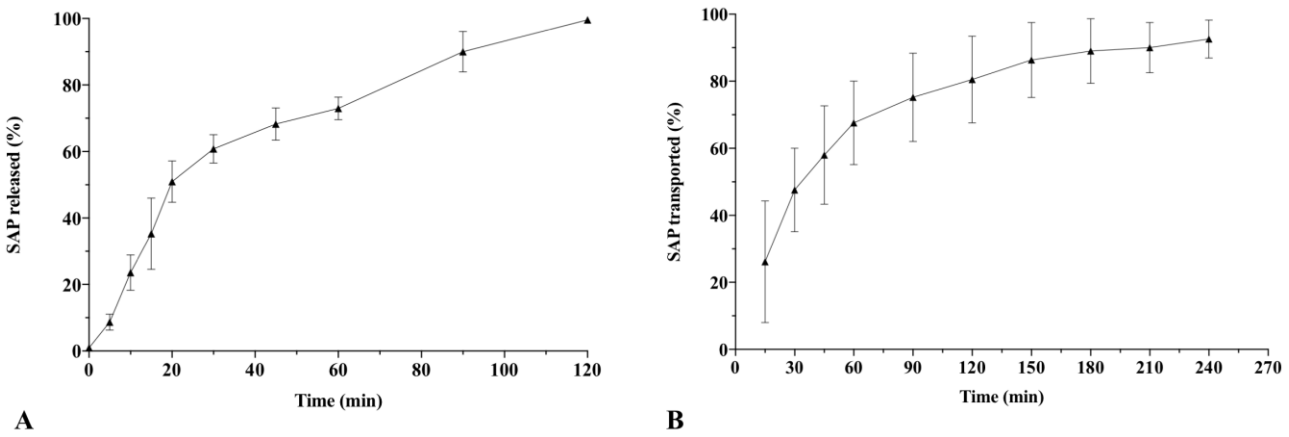

Fig. 7. *In vitro* release profiles of SAP from LYO HA-CL – SAP: release by Franz's cells (**A**) and transport across RPMI 2650 cells (**B**). Data represent mean ± SD (n = 3).

Due to its hydrophilicity, SAP rapidly diffused into the medium of the receptor compartment when LYO HA-CL – SAP was deposited on the filter membranes of Franz's cells. After 60 min, 73.0 ± 3.4% of the drug was released from the freeze-dried formulation, and after 120 min, the release was completed (**Fig. 7A**). These results could suggest a good *in vivo* dissolution of the formulation in the nasal epithelial lining fluid, enhancing drug transport, bioavailability, and therapeutic efficacy (Vasa et al., 2017).

Fig. 7B displays the percentage of SAP transported across RPMI 2650 epithelium as a function of time. During the first hour, LYO HA-CL – SAP exhibited marked permeation properties, with 67.6 ± 12.4% of SAP transported. Afterwards, a decreased permeation rate was observed. At the end of the 4 hours, almost all SAP was transported (92.6 ± 5.7%), as only the 7.4 ± 5.7% of SAP remained on the cells (**Fig. 7B**).

SAP release profile was extended when studied on RPMI 2650 cells (**Fig. 7B**) compared to Franz's cells (**Fig. 7A**). This delay was due to the low permeability of the simulated nasal epithelium, and so it was an effect of the cell barrier itself. Moreover, an enhanced mucoadhesive behaviour of hyaluronan on cell tissues could be hypothesized. In fact, HA-CL probably interacted with the mucus layer on the RPMI 2650 cells, absorbing water and thus becoming wet, swelled and gelled. Consequently, the formulation penetrated into the mucus, changed its structure and rheological properties that in turn slowed down drug release (Ugwoke et al., 2005). Hence, LYO HA-CL – SAP could overcome the drawback of a rapid clearance from the nasal cavity, since HA-CL could act not only as carrier for SAP and active ingredient itself, but also as mucoadhesive agent once dissolved in the nasal mucus layer to form a viscous solution/gel. This could potentially prolong the residence time of LYO HA-CL – SAP in the nasal cavity and thus the exposure of the nasal mucosa to the active ingredient (Horváth et al., 2016; Pires et al., 2009).

4. Conclusions

The present work aimed to develop a novel LYO HA-CL – SAP formulation for the treatment of nasal impairments sustained by inflammation. The dry powder formulation was characterized *in vitro* for its physico-chemical and biological properties. LYO HA-CL – SAP displayed suitable morphology, particle size distribution, mean diameter, thermal properties and *in vitro* aerosol performance for nasal drug delivery. The *in vitro* release/transport study performed on LYO

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HA-CL – SAP showed that HA-CL was able to act not only as carrier for SAP and active ingredient itself, but also as mucoadhesive agent. The formulation was shown to be not cytotoxic in the range of SAP concentrations explored (8.0 mM to 60.9 nM), and exhibited wound healing ability on RPMI 2650 cells and anti-inflammatory efficacy on primary nasal epithelial cells. These encouraging results showed that polymer and drug acted in combination to help reduce cell damage. Hence, this preliminary study has opened up potential perspectives for LYO HA-CL – SAP as innovative adjunctive treatment for nasal airway disorders like AR and CRS.

Disclosure

The authors declare no conflict of interest.

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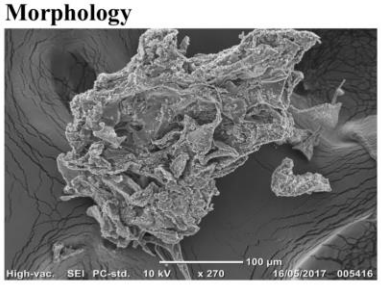
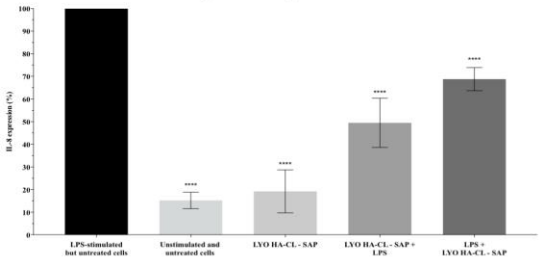
Lyophilized urea-crosslinked hyaluronic acid and sodium ascorbyl phosphate (LYO HA-CL – SAP): a nasal dry powder formulation

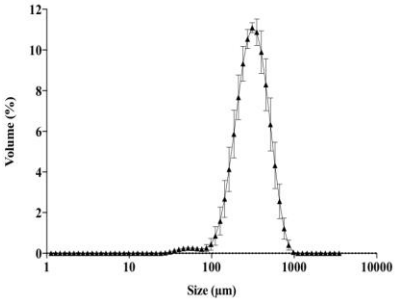
Morphology



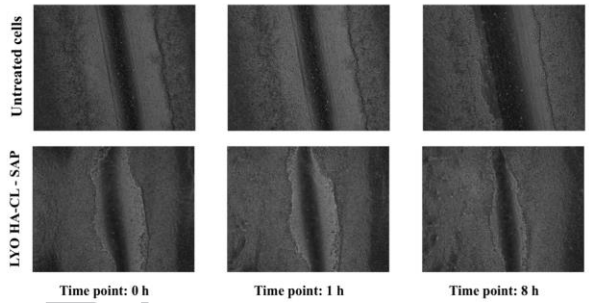
Anti-inflammatory activity



Particle size distribution



Wound healing activity



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