Pharmaceutical Development of Sodium Hyaluronate Respirable Dry Powders for Targeted Drug Delivery

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Summary

Background: CD44 is the major hyaluronic acid binding receptor involved in pathological conditions, including tumour progression and metastasis formation. It has been reported that CD44 is a protein over-expressed in non-small cell lung tumours.

The aim of this work was to produce sodium hyaluronate (HA) dry powders using a particle engineering approach based on spray drying technique in view of an efficacious platform capable to deliver chemotherapeutics to the lungs.

Methods: Different dry powder formulations were obtained by spray-drying (SD) water/ethanol mixtures containing HA and a selected adjuvant. HA dry powders were characterized in terms of morphology and *in vitro* respirability. Biocompatibility of excipients and formulations was investigated using A459 cell culture and the MTT test.

Results: Pure HA SD powders showed a poor aerodynamic performance. Three surfactants were included in the formulations (stearylamine, cetostearyl alcohol and stearyl alcohol) at three different proportions (10%, 5% and 1%). Microparticles of HA had a spherical shape with smooth surface; stearylamine led to wrinkled particles whereas cetostearyl and stearyl alcohol kept a roundish shape and some holes were evident onto the surface. Formulations containing 5% of stearylamine showed the best aerodynamic performance (emitted dose of 89% and FPF of 52% of nominal dose).

MTT results ranked the biocompatibility of the excipients: stearylamine < cetostearyl alcohol < stearyl alcohol.

Conclusions: Dry powders suitable for lung delivery were produced by co-spray-drying hyaluronate and surfactants. These might be employed as a platform to directly target tumour cells with antineoplastic drugs.

Introduction

Under physiological conditions, pulmonary HA is involved in many functions such as the stabilization of the connective tissue and organization of the extracellular matrix, water homeostasis [1], regulation of the inflammatory response and tissue modelling [2],cell migration and phagocytosis [3]. Various functions are related to CD44 receptor, such as in cell detachment, cancer development, and inflammation [4]. CD44 receptor belongs to the family of cell adhesion molecules (CAMs) specifically involved in the control of cell behaviour by mediating contact between cells or between cells and the extracellular matrix. This regulation is essential for maintaining tissue integrity [5]. Recent studies showed that the CD44 protein is over-expressed in many cancer tissues as in non-small cells lung cancer. Consequently, these tumour cells show enhanced binding and internalization of HA so that a higher concentration of HA in cancer cells forms a less dense matrix, thus enhancing invasive ability into other tissues [6].

The aim of this work was the production of flowable and high respirable dry powders with a high HA content. To date, no inhaled drug delivery formulation has been produced as dry powder due the difficulty in micronizing this polymer. A particle engineering approach based on spray drying technique was employed to produce and optimize the HA powder characteristics. In particular the purpose was to obtain dry powders that could represent a cancer-targeted platform to deliver drugs to the lungs. The pharmaceutical development explored different surfactants in increasing amounts, evaluating their capacity to interact positively with the polymer through specific characterization.

Materials and Methods

Materials

Sodium hyaluronate (HA) (PrymalHyal 50, average MW=29504 Da) was purchased from Soliance (France). Stearylamine, thiazolyl blue tetrazolium bromide (MTT), sodium dodecyl sulphate (SDS), N,N-dimethylformamyde (DMF), RPMI-1640, Fetal Bovine Serum (FBS), L-glutamine, gentamicin were supplied by Sigma–Aldrich (Sigma Chemical Co., St. Louis, MO, USA). Stearyl alcohol and cetostearyl alcohol were provided by ACEF (Fiorenzuola d'Arda, Italy). All chemicals used were of analytical grade and water was purified by Elix® Essential (Merck Millipore, USA). A549 alveolar epithelial cells were obtained from American Type Cell Culture; tissue culture

flasks (75 cm^2 with ventilated caps) and 96-well plates were from Costar (through Fisher Scientific, Leicestershire, UK). Phosphate buffered saline (PBS) tablets were purchased from Oxoid (Basingstoke,UK).

Methods

Spray-drying process

Engineered sodium hyaluronate powders were manufactured using a mini Spray-Dryer Büchi B-290 (Büchi Laboratoriums-Technik, Swiss). HA was dissolved in purified water at room temperature under stirring at 50 rpm and this solution was added to the required amount of ethanol. When a surfactant was incorporated in the formulation (1, 5 and 10% w/w), it was added in the ethanol phase according to the amount required.
The solutions were sprayed using the following parameters: inlet temperature 90 °C, drying air flow rate 750 l/h, solution feed rate of 3.0 ml/min and nozzle diameter of 0.7 mm. Under these conditions an outlet temperature of 45-52 °C was measured. A dehumidifier B-296 was used to control and limit the air humidity during the process. Spray-dried powders were kept in the collector for at least 24 hours in order to allow electrostatic charge time to dissipate.

Scanning Electron Microscopy

Scanning electron microscopy (SEM, Zeiss SUPRA 40, Oberkochen, Germany) was employed to investigate particle morphology and surface characteristic of the powders produced by spray-drying. The microscope was operated under high vacuum conditions with an accelerating 1.5 kV voltage, at different magnifications. Powders were deposited on adhesive black carbon tabs pre-mounted on aluminium stubs and imaged without undergoing any metallization process.

In vitro aerodynamic assessment

A Fast Screening Impactor (FSI, Copley UK) employs a two-stage separation process: a coarse fraction collector (CFC) that captures particles with an aerodynamic diameter larger than 5 µm and a fine fraction collector (FFC) that collects particles with an aerodynamic diameter below 5 µm. The respirable fraction (RF% m/m) was calculated by the ratio between the amount of hyaluronate in the FFC and the total amount of hyaluronate recovered. The emitted dose (ED) was quantified by HPLC. The entire system was connected to a vacuum pump (Erweka GmbH, Germany) to draw particles over the system.
In particular, 5.0 mg of powder were loaded into a size 3, hard HPMC capsule (Vcaps® DPI, Capsugel, Colmar, France). A single capsule was discharged inside the impactor for each aerodynamic test. A medium resistance version of the single dose RS01 inhaler was provided by Plastiape Spa (Italy) and chosen to aerosolize the spray-dried powders. According to current USP guidelines the flow rate used (60 L/min) during each tests was adjusted with a Critical Flow Controller TPK (Copley Scientific, Nottingham, UK) in order to produce a pressure drop of 4 kPa over the inhaler. RS01 was activated for 4 seconds so that a volume of 4 L of air is withdrawn from the inhaler.

MTT assay

For MTT assay, cells were seeded in 96-well plates (Costar Corning, UK) at a density of 10,000 cells per well (in 200 µl cell culture medium; CCM) and incubated for 24 h to allow the cells to attach and form a monolayer.
Biocompatibility of raw materials and three formulations (HA:adjuvant 90:10) were tested over 24h. Substances were dissolved/dispersed in a medium:ethanol mixture (95:5) and incubated at 37°C for at least 1 h before addition to the cells. All materials were tested over 9 different concentrations following a serial quarter log dilution and CCM:ethanol (95:5) mixture was used as a negative control.
After 24h of incubation, cells were washed with PBS and then 200 µl of CCM was added. Finally, 50 µl of MTT solution (2.5 mg/ml in PBS) were added to each well and the plate was incubated for 4 h in a humidified incubator. Subsequently, the CCM was removed and cells were lysed and any formazan crystals generated were solubilised with 100 µl of a surfactant solution comprising 10% SDS in DMF:water (1:1). Plates were incubated overnight at 37 °C before the absorbance of solubilised formazan was measured at 570 nm using a SpectraMax microplate reader (Molecular Devices, UK) The cell viability was expressed as a percentage of negative control (100% metabolic activity). LC50 values were obtained by plotting $\log_{10}$ concentration of adjuvant vs % cellular viability using GraphPad Prism 6 (GraphPad Software, USA). All assays were performed in triplicates.

Results

Powders have been produced with a good yield (>50%) and the HA content was in accordance with the theoretical value of each dispersion.

SEM images (Figure 1) showed that spray-dried powders containing different type of adjuvant exhibited significant differences in morphology. Moreover, the increase of the concentration of the adjuvant contributed to modify the particle shape as well.

Figure 1/A shows that powders containing only HA had a spherical shape with some concavities and a smooth surface. Increasing sodium hyaluronate concentration, the particle size is increased, but the morphology was kept similar. These samples showed agglomerates which led to very cohesive powders with a poor flowability during the aerodynamic tests as shown with FSI.

Powders with stearylamine (Figure 1/B, C and D) showed a different shape compared to spray-dried powder containing HA alone. They had an irregular wrinkled shape according to the amount of excipient added in the formulation. Moreover, reducing stearylamine content, the particles were inclined to dry in a round shape, as previously observed with HA alone.

Powders containing HA:cetostearyl alcohol in different ratios had a roundish shape (Figure 1/E, F and G). Particles were spongy like and the number and size of holes were proportional to the cetostearyl concentration. The same behaviour was highlighted using stearyl alcohol (Figure 1/H, I and L).

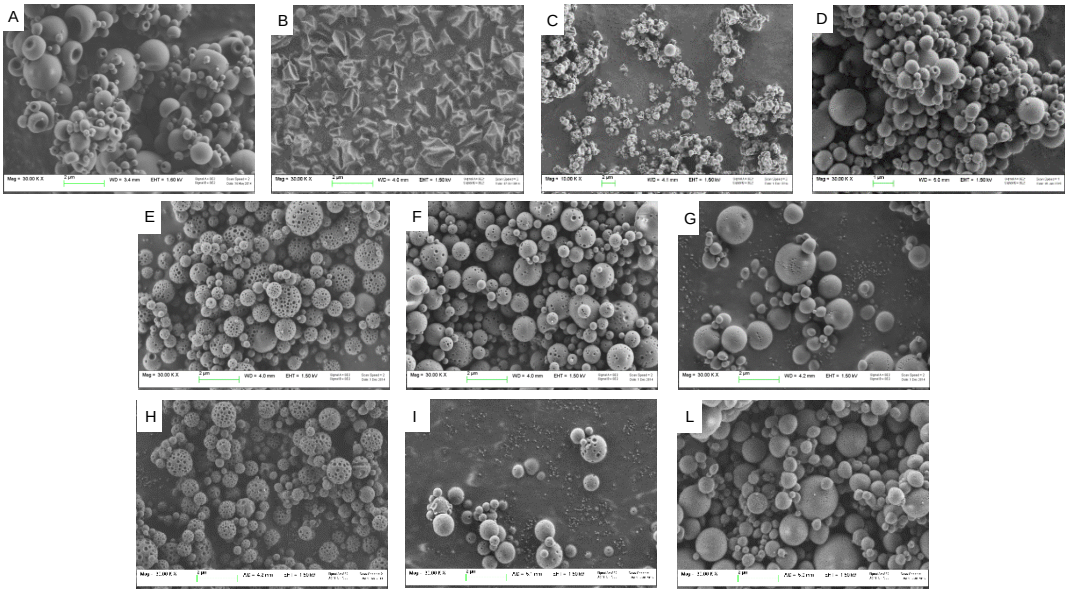

Figure 1. SEM images of spray-dried powders: pure HA particles (A); HA:stearylamine particles in the ratio 90:10 (B), 95:5 (C) and 99:1 (D); HA: cetostearyl alcohol particles in the ratio 90:10 (E), 95:5 (F) and 99:1 (G); HA: stearyl alcohol particles in the ratio 90:10 (H), 95:5 (I) and 99:1 (L).

As far as the aerodynamic behaviour is concerned, the SD pure HA powder was very cohesive, presented many agglomerates and had a very poor flowability. In particular, 60% of the loaded powder was retained inside the capsule, the capsule chamber and mouthpiece (Figure 2). This behaviour is due to the high cohesive forces among microparticles leading to a low respirable fraction, around 25%.

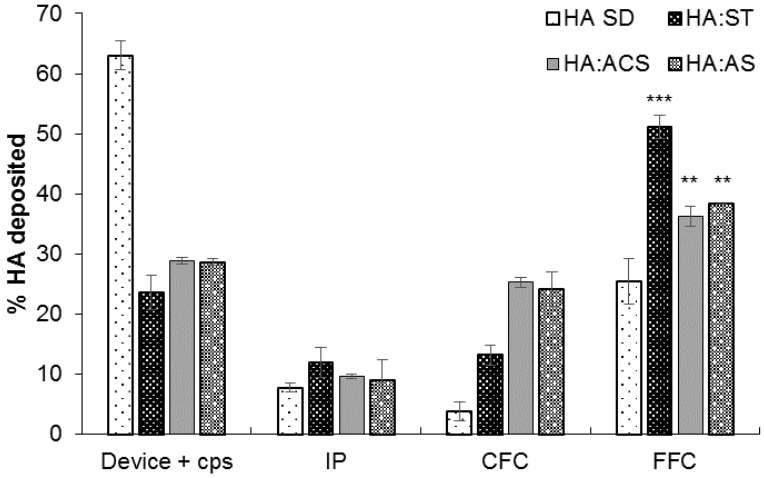

Figure 2. Fast Screening Impactor deposition of HA pure spray-dried powder (HA SD), HA:stearylamine (HA:SA), HA:cetostearyl alcohol (HA:ACS) and HA:stearyl alcohol (HA:AS) at fixed ratio HA:adjuvant (95:5) aerosolized with RS01 (n=3; mean ± sd). cps=capsule, IP= Induction port, CFC=coarse fraction collector, FFC = fine fraction collector. P value obtained comparing % FFC of HA SD with powders containing surfactants (* p<0.001, ** p<0.01).**

Hence, in order to enhance the flowability, to decrease the particle cohesivity and to increase the respirability of HA powder, the addition of different types of adjuvant was investigated. The addition of a surfactant improved the aerodynamic properties compared to the pure HA spray dried powders (p value < 0.01), decreasing the powder entrapped in the device. In all the cases, the emitted dose was higher than 70%, reaching 90% when stearylamine was added as adjuvant.

Among the selected excipients, stearylamine also afforded particles with the highest respirable fraction, improving the microparticles deaggregation during aerosolization. A reduction of the surfactant amount led to a decrease of the powder emission. In all cases, the best results in term of respirable fraction were achieved using at least 5% of adjuvant; when the adjuvant was added at 1%, the powders did not properly exit from the device.

The presence of these adjuvant modified the surface characteristic and particle shape leading to an improvement of the aerodynamic behaviour due to the decrease of particle cohesion.

Table I. Comparison of LC50 values (inhibition of cell viability) after 24 h exposure in A549 cells. The data represent the mean (95% C.I.) of n=3 individual experiments.

Samples	LC_{50} (95% Confidence Interval) (µg/ml) 24h incubation
HA:stearylamine (90:10)	3.85 (3.09-4.79)
Stearylamine	1.81 (1.51-2.18)
HA:cetostearyl alcohol (90:10)	15.59 (14.42-16.86)
Cetostearyl alcohol	13.18 (11.53-15.06)
HA:stearyl alcohol (90:10)	19.42 (17.17-21.97)
Stearyl alcohol	24.65 (18.37-33.07)

The *in vitro* biocompatibility was tested for both formulations and adjuvants on the widely used human alveolar epithelial cell line, A549. To date, there are no toxicological data available for the employed materials delivered by inhalation. HA did not display any cytotoxicity after 24 h of exposure over the concentration range tested. The effect of excipient by itself on A549 cells was similar to the effect of the formulation containing excipients (Table I) leading us to conclude that the cytotoxicity observed was due to the presence of the excipient and not due to HA. Moreover, based on MTT LC50 (Table I), A549 cells were most sensitive to stearylamine (LC_{50} = 1.81 µg/ml) and least affected by stearyl alcohol (LC_{50} = 24.65 µg/ml) with the same trend was observed for the formulations containing these excipients.

Conclusion

Powders containing sodium hyaluronate were designed as a platform to preferentially deliver drugs to the lung. Critical powder quality properties have been characterized as well as a safety investigation in which *in vitro* biocompatibility was tested using an alveolar cell line. HA-stearylamine proved to be the most suitable powder for aerosolization, although they were the most toxic ones.

References

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