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Research paper

# Engineering hydrophobically modified chitosan for enhancing the dispersion of respirable microparticles of levofloxacin

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ABSTRACT

The potential of amphiphilic chitosan formed by grafting octanoyl chains on the chitosan backbone for pulmonary delivery of levofloxacin has been studied. The success of polymer synthesis was confirmed using FT-IR and NMR, whilst antimicrobial activity was assessed against *Pseudomonas aeruginosa*. Highly dispersible dry powders for delivery as aerosols were prepared with different amounts of chitosan and octanoyl chitosan to study the effect of hydrophobic modification and varying concentration of polymer on aerosolization of drug. Powders were prepared by spray-drying from an aqueous solution containing levofloxacin and chitosan/amphiphilic octanoyl chitosan. L-leucine was also used to assess its effect on aerosolization. Following spray-drying, the resultant powders were characterized using scanning electron microscopy, laser diffraction, dynamic light scattering, HPLC, differential scanning calorimetry, thermogravimetric analysis and X-ray powder diffraction. The in vitro aerosolization profile was determined using a Next Generation Impactor, whilst in vitro antimicrobial assessment was performed using MIC assay. Microparticles of chitosan have the property of mucoadhesion leading to potential increased residence time in the pulmonary mucus, making it important to test the toxicity of these formulations. *In-vitro* cytotoxicity evaluation using MTT assay was performed on A549 cell line to determine the toxicity of formulations and hence feasibility of use. The MTT assay confirmed that the polymers and the formulations were non-cytotoxic. Hydrophobically modifying chitosan showed significantly lower MIC (4-fold) than the commercial chitosan against *P. aeruginosa*. The powders generated were of suitable aerodynamic size for inhalation having a mass median aerodynamic diameter less than 4.5 μm for formulations containing octanoyl chitosan. These highly dispersible powders have minimal moisture adsorption and hence an emitted dose of more than 90% and a fine particle fraction (FPF) of 52%. Powders with non-modified chitosan showed lower dispersibility, with an emitted dose of 72% and FPF of 20%, as a result of high moisture adsorption onto the chitosan matrix leading to cohesiveness and subsequently decreased dispersibility.



## 1. Introduction

Chronic respiratory infections are difficult to eradicate leading to high rates of morbidity and mortality and high healthcare costs [1]. Lower respiratory tract infections (LRTIs) are responsible for the highest number of deaths in low-income countries, placing a considerable strain on their health economies [2]. LRTIs lead to 3.2 million deaths annually worldwide, accounting for 6.7% of the global disease burden, and they were the 3rd leading cause of death in 2011 [2]. A major concern with conventional antibiotic treatment of LRTIs is the requirement of a high dose to be delivered for effective eradication of the organism [1]. Hence, in the past decade increasing attention has been given to developing systems for delivery of antibiotics by means of inhalation directly to the respiratory epithelium [3,4].

There are very few antibiotic formulations marketed for the treatment of pulmonary infections, e.g. Tobramycin and Aztreonam inhalation solutions for nebulization. However, much research is being conducted into engineering inhalable antibiotic carrying nanoscale carriers such as liposomes [5,6], polymeric nanoparticles [7,8], solid lipid nanoparticles [9,10] and polymer

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nanoparticle aggregate particles (PNAPs) [11], in particular to target the biofilm pulmonary infections associated with cystic fibrosis (CF) or chronic obstructive pulmonary disease (COPD).

CF and COPD are characterized by the presence of a thick viscoelastic mucus layer which has mesh with average spacing of 100–400 nm, and is responsible for failure of many treatment modalities using the pulmonary route for local and systemic delivery [12,13]. *Pseudomonas aeruginosa* has been reported to be the major causal pathogen associated with approximately 80% of CF cases [14]. Another hindrance to antibiotic penetration is the bacterial generated multicellular surface associated biofilm composite of exopolymeric substances (EPS), such as polysaccharide, proteins and extracellular DNA which forms a strong, sticky network housing bacterial cells and serves as a barrier delaying penetration of antibiotics [14,15]. Polymer/lipid nanoscale systems have been proposed to be more effective than conventional antibiotic formulations as they can penetrate the biofilm due to a decrease in direct interactions between EPS and antibiotic [12,14]. Carrier-associated delivery of antibiotic also aids in decreasing the degradation caused by antibiotic-inactivating enzymes, such as β-lactamase present in the biofilms secreted by *P. aeruginosa* [14]. However, delivery of nanoparticle systems directly to the pulmonary region is problematic due to their instability resulting from particle–particle interaction, and poor deposition properties within the airways, resulting in loss during exhalation [16]. Tsapis et al. [17] have described a strategy whereby nanoparticles are incorporated into microscale structures, embracing the advantages of both nanoscale and micrometre-scale particles, enabling efficient delivery using a simple dry powder inhalation (DPI) system. Microparticles formed by aggregated nanoparticles of mass median aerodynamic diameter (MMAD) 1–5 μm, when inhaled, deposit efficiently in the peripheral lung at the site of pulmonary infection. Exposure to the humid/moist conditions of the lungs results in liberation of nanoparticles. On reaching the vicinity of the biofilm colonies, the matrix/polymer dissolves and releases the antibiotic leading to high localized exposure [18,12].

Drugs employed in inhalation products require a size less than approximately 5 μm. This is usually achieved by micronization/jet milling [19]. Powders produced by such high energy processes exhibit strong inter-particulate attractions, leading to agglomeration. Moreover, micronization does not permit precise control of particle morphology, size distribution, particle density or surface composition leading to variations, and produces defects and/or amorphous regions on crystal surfaces, resulting in altered aerosolization properties and posing considerable formulation challenges [20–22]. This has led to alternative ways of controlling particle size distribution, particle porosity, surface roughness, particle density, etc. Spray-drying has emerged as an attractive, one-step technique for producing powders suitable for pulmonary delivery [23]. This method also provides potential for incorporating a wide range of excipients into the spray-drying feedstock, including dispersibility enhancers, such as L-leucine [4,24–26]; drug-release modifiers, such as glyceryl behenate [27], hydroxypropyl cellulose [28] and poly lactic acid poly lactides [29,30], providing a convenient means of manipulating the properties of the end product [31].

Chitosan, a naturally occurring linear copolymer of β-(1 → 4)-2-acetamido-D-glucose and β-(1 → 4)-2-amino-D-glucose processed by partial deacetylation of chitin has been extensively investigated for transmucosal drug delivery, for instance for pulmonary, nasal and vaginal mucosal administration [32–34] due to its low toxicity, biodegradation, biocompatibility, mucoadhesiveness and enhancement of transcellular permeation [4,16,30]. Additionally, the potential benefits of chitosan and its derivatives include the following: immune enhancing effects [35], hypercholesterolemic effects [36], antimicrobial properties and antitumor properties [37]. Furthermore, the highly reactive primary amino groups and primary/secondary hydroxyl groups of chitosan provide opportunities for derivatization [38,39]. For instance, polymeric amphiphiles formed by hydrophobic modification of chitosan, following acylation using fatty acid chains, such as stearoyl, octanoyl and palmitoyl, have been widely investigated due to the potential of forming nanosized micelles for drug and gene delivery [40]. Acylated chitosan has also been proposed to possess anti-microbial properties against the gram-negative bacteria *P. aeruginosa* and *Escherichia coli*, which are commonly associated with lung infections [41].

Varied strategies have been employed to engineer particles with improved flowability and aerosolization of dry powders and subsequently powder performance. These include modification of particle surface [42,43], porous low density particles [44] and inclusion of coarser carrier particles and excipients. Current excipients used for pulmonary delivery are confined to classes such as sugars viz. lactose, trehalose, and mannose and amino acids viz. L-leucine, trileucine and phenylalanine [45,46]. These carrier/excipients help to reduce the interparticulate forces between drug molecules, alter density of particles, improve surface activity and subsequently reduce the dependence of FPF on flow rate and inhaler type hence improving aerosolization [46]. L-leucine a hydrophobic amino acid in particular has been shown to possess the capability of migration to the surface of the particle droplet in the rapid atomization phase of spray-drying hence preventing water from being entrapped onto the surface producing pitted particles on drying, which have a reduced contact area and consequently reduced cohesion.

However, previous studies have demonstrated that L-leucine enhances the growth of bacterial associated biofilms in *P. aeruginosa* models and hence limiting its use in CF and COPD making it absolutely necessary to find a replacement dispersibility enhancer for dry powders [47]. None of the previous literature studies have shown the dispersibility enhancement effects of hydrophobically modified chitosan. In this study, we have hydrophobically modified chitosan and investigated the effects on the aerosolization of the fluoroquinolone antibiotic levofloxacin, and the antimicrobial activity of polymer and formulations. It would be interesting to study the effect of hydrophobic modification of chitosan on the dispersibility of the dry powders as L-leucine is a model dispersibility enhancer which is a hydrophobic amino acid. These formulations, optimized for levofloxacin content, were prepared by spray-drying, and were investigated for their thermal, physicochemical, antimicrobial, aerosolization and toxicological properties in order to explore the potential of this formulation approach to enhancing the delivery of levofloxacin as a therapeutic inhalation aerosol for the treatment of lung infections.

## 2. Materials and methods

### 2.1. Materials

Levofloxacin ⩾98.0% purity, octanoyl chloride 99% purity and L-leucine ⩾98.0% purity were purchased from Sigma–Aldrich Life Sciences (Poole, UK); (1 → 4) 2-amino-2-deoxy-β-D-glucose (Chitooligosaccharide) 1–3 K was purchased from Kitto Life Co. Ltd (Kyongki, Korea); Spectra/Por® pre-wetted dialysis tubing with Molecular Weight Cut off (MWCO) 1 kDa; sodium bicarbonate 99.7% purity and HPLC grade methanol, absolute ethanol, acetonitrile, trifluoroacetic acid (TFA), acetone and chloroform were purchased from Fisher Scientific Ltd. (Loughborough, UK).

### 2.2. Synthesis of water soluble N,O-octanoyl chitosan 1–3 K

N,O-octanoyl chitosan was synthesized as shown in Fig. 1. Chitosan oligosaccharide 1–3 K (10.0 g) was dispersed in 100 mL

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of methanol by drop-wise addition with magnetic stirring (multi-position magnetic stirring, RO 10 power IKA WERKE, Germany). 20 mL octanoyl chloride mixed with 80 mL methanol was added drop-wise and allowed to react, with magnetic stirring, at 60 °C for 30 min, after which it was stirred at room temperature for 10 h.

The solution was poured into acetone and product precipitated. This precipitate was washed with acetone at 50–60 °C and filtered through a Whatmann® qualitative 110 nm filter paper. The precipitate was then dissolved in water, and chloroform was added to remove traces of unreacted octanoyl chloride which fractionated into the chloroform layer. This was repeated three times to ensure the product was free from octanoyl chloride. The product was re-precipitated from water at room temperature by addition of acetone, then dissolved in absolute ethanol to remove unreacted chitosan, after which it was rotary evaporated using Hei-Vap (Heidolph Instruments, Gmbh, UK) at 80 °C under vacuum until a dry powder was obtained. This powder was then dissolved in water and dialysed against deionized water at room temperature for 48 h using a Spectra/Por® dialysis tubing membrane with cut off of 1 kDa. The final product was obtained by re-precipitation from acetone.

### 2.3. Characterization of N,O-octanoyl chitosan 1–3 K

#### 2.3.1. Proton nuclear magnetic resonance ($^1$H NMR)

High resolution $^1$H NMR was recorded on a Bruker AVANCE 500 spectrometer to confirm the macromolecular structure of native chitosan and octanoyl chitosan, and to ascertain the success of derivatization. The degree of deacetylation of native chitosan was also calculated as per the $^1$H NMR spectra. The spectrum was obtained at 298 K using 16 $^1$H scans per sample and at a resonance frequency of 500.13 MHz for $^1$H. The polymer solution was prepared at a concentration of 1% in Deuterium oxide ($D_2O$) for acquiring the spectrum for chitosan, and $d_6$-dimethyl sulfoxide ($d_6$-DMSO) for octanoyl chitosan.

#### 2.3.2. Fourier Transform Infrared Spectroscopy (FT-IR)

FT-IR was performed to establish the chemical structure of the starting and synthesized polymer and hence determine the success of derivatization. FT-IR spectra were analysed by placing the sample on a diamond crystal of an ATR polarization assembly of Spectrum™ 100 spectrophotometer (PerkinElmer, UK) and scanning in the spectral region of 4000–6500 cm$^{-1}$.

#### 2.3.3. X-ray powder diffraction (XRPD)

The long range order of the molecules and hence crystallinity of chitosan, octanoyl chitosan and spray-dried microparticles was determined at ambient temperature using wide angle X-ray diffraction with a 5th generation Rigaku Miniflex 600 X-ray powder diffractometer equipped with a 600 W X-ray tube, a copper anode operating in reflectance mode at wavelength $k_\alpha$ $\lambda$ 1.5418 Å, voltage of 40 kV and current 15 mA. Polymer samples were mounted perpendicular to the horizontal axis and then rotated to obtain a scanning range of 2–50° 2 theta at a step size of 0.010°/min and step rate of 2 step/min at 25 °C and hence a scanning time of 24.05 min per sample. Spray-dried microparticles were mounted perpendicular to the horizontal axis and then rotated to obtain a scanning range of 2–70° 2 theta at a step size of 0.010°/min and step rate of 2 step/min at 25 °C and hence a scanning time of 32.5 min per sample. The relative long range order (crystallinity) v/s non-order (amorphous) was assessed by comparison of the data obtained by XRPD of native and acylated chitosan and spray-dried v/s physically mixed microparticles.

#### 2.3.4. In vitro antimicrobial assessment by MIC studies

The minimum inhibitory concentrations (MICs) of water-soluble chitosan, octanoyl chitosan and microparticles were determined by the broth dilution method in 96-well microplates (BD biosciences Falcon™, UK). Three sensitive strains of *P. aeruginosa* were used for the experiment namely 10663, 8620 and 599. Briefly *P. aeruginosa* strains were grown in iso-sensitest broth (ISB) at 37 °C. Bacterial suspension was prepared in broth and diluted to obtain an optical density (OD) of 1 at 600 nm which corresponds to $1 \times 10^8$ CFU/mL of broth, after which a 200 X dilution with the same broth was performed to obtain $5 \times 10^5$ CFU/mL, which was used in the determination of MIC.

Aqueous solutions of polymers were prepared to obtain a standard concentration of 80 mg/mL chitosan. Further dilutions were performed in broth to obtain concentrations ranging from 40 mg/mL to 0.625 mg/mL in a final volume of 100 µL in each well by serial dilution. Standard aqueous solutions of the drug levofloxacin and spray-dried powders were prepared as per the drug loading for each formulation to obtain a concentration of 64 µg/mL of levofloxacin. Further dilutions were performed in broth to obtain concentrations ranging from 32 to 0.006 µg/mL in a final volume of 100 µL in each well. To these wells, 100 µL of the *P. aeruginosa* strains ($5 \times 10^5$ CFU/mL concentration in ISB) was added and the plates then closed and packed into a plastic bag before incubation for 12 h at 37 °C. The MIC endpoints for all the tested materials were determined by visual inspection where no turbidity corresponded to inhibition of growth of organism.

#### 2.3.5. In vitro cell toxicity of polymers and spray-dried microparticles (MTT assay)

The toxicity profiles of samples were evaluated over 24 h in adenocarcinomic human alveolar basal epithelial cell line, A549. The cells (passage no. 45–49) were cultured in 96-well plates with 100 µL Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% foetal bovine serum (FBS)/1% antibiotic/antimycotic solution (complete medium) for 24 h in a humidified 5%

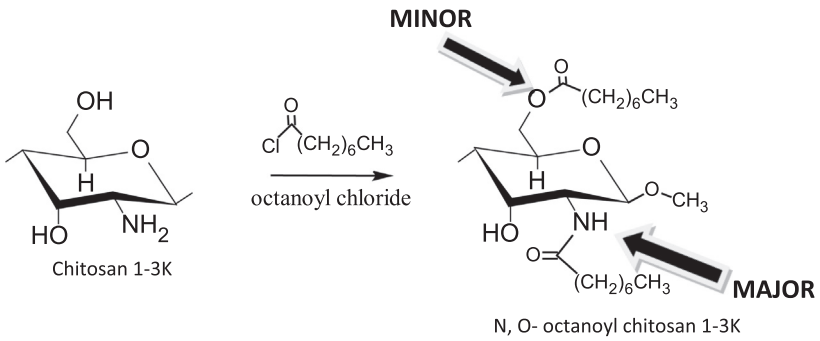


**Fig. 1.** Chitosan derivatization with octanoyl chloride.

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$CO_2$/95% incubator at 37 °C. 100 μL of freshly prepared dispersions in the complete medium was added to the wells to achieve an appropriate concentration (0–5 mg/mL; $n = 3$), and 10% dimethyl sulfoxide (DMSO) was used as a positive control, and incubated for a further 24 h, followed by addition of 40 μg/mL MTT solution (5 mg/mL in PBS, pH 7.4) to each well. After further incubation for 2 h, the medium was carefully removed and any formazan crystals generated were solubilized with 100 μL DMSO. Thereafter, the optical density (OD) was measured at 570 nm (SpectraMAX 190, Molecular Devices). The relative cell viability (%) was then calculated using Eq. (1)):

$$\text{Cell viability } (\%) = \frac{\text{OD of treated cells}}{\text{OD of untreated cells}} \times 100 \quad (1)$$

### 2.4. Preparation of formulations

#### 2.4.1. Spray-dried powders

Formulations for spray-drying were prepared by dissolving individual compounds weighed accurately (SD was ±0.2 mg) on a high accuracy analytical balance in deionized water, then mixing solutions of the drug, polymer and excipients together and spray-drying. The composition of the formulations is shown in Table 1. Levofloxacin raw material was spray-dried to serve as a control. The formulations were spray-dried using a lab-scale nanospray dryer Büchi B-90 (Büchi Labortechnik AG, Switzerland) equipped with a piezoelectrically driven droplet atomizing technology. The inlet temperature was 120 °C, with 100% aspiration, pump flow rate 20 mL/h, gas flow rate 133 L/min, 5.5 μm mesh nozzle and instrument pressure 47 mbar. Spray-dried powders were immediately transferred to a glass vial and kept in a desiccator to prevent crystallization. Spray-dried levofloxacin:octanoyl chitosan formulations were also stored at room temperature at ambient humidity to study the effect of moisture on the spray-dried powder. Each preparation was prepared 3 times and spray-dried to indicate the reproducibility of the preparations.

#### 2.4.2. Physical mixtures of powders

Powder blends were prepared by physically mixing levofloxacin and the different excipients at the ratios outlined in Table 1, using a mortar and pestle for 15 min. In vitro aerosolization studies and XRPD were performed on these samples alongside spray-dried powders.

### 2.5. Characterization of spray-dried powder

#### 2.5.1. Spray-drying yield, % yield and drug content

The yield of the spray-dried powders was determined gravimetrically and calculated as a percentage of the initial weights of formulation components. The levofloxacin content of the powders was measured in triplicate using high performance liquid chromatography (HPLC) (Section 2.5.5), and expressed as a percentage of nominal content using Eq. (2).

$$\text{Percentage drug content} = \frac{\text{Drug content of levofloxacin obtained by HPLC}}{\text{Theoretical drug content of spray} - \text{dried powder}} \times 100 \quad (2)$$

#### 2.5.2. Particle size distribution and zeta potential measurements

The volume median diameter (VMD) and the particle size distribution (PSD) for all the spray-dried powders and the respective raw materials were determined using a Sympatec laser diffraction particle sizer (Sympatec Gmbh System-Partikel-Technik, Germany). A RODOS/L dispersing unit, equipped with a microdosing device ASPIROS/L was used. A feed rate of 50 mm/s was applied and analysis was performed at varying pressures of 4 bar and 2 bar to ensure deagglomeration of powders. Six replicate readings were performed on a single sample and results were expressed as VMD and Span; a common dimensionless parameter used for the quantification of distribution width based on the 10th, 50th and 90th percentile of cumulative distribution calculated using Eq. (3):

$$\text{Span} = \text{Dv90} - \text{Dv10}/\text{Dv50} \quad (3)$$

The hydrodynamic diameter and zeta potential of the dispersions formed following dissolution of the spray-dried powders in deionized water were measured by dynamic light scattering (DLS) using the Malvern ZetaSizer Nano ZS (Malvern Instruments Inc., UK) using 0.5% (w/v) dispersion of the respective formulations at 25 °C.

#### 2.5.3. Crystallinity, thermal properties and water content of spray-dried powders

*2.5.3.1. Differential scanning calorimetry (DSC).* Thermal analysis was performed on accurately weighed 5 mg samples using hermetically sealed aluminium pans (Tzero pans), a Q series™ DSC autosampler and TA DSC Q2000 (TA instruments-Waters LLC, New Castle, DE, USA). The melting point ($T_m$) of samples was recorded under a 20 mL/min dry nitrogen gas purge at a flow rate of 50 mL/min. The samples were heated at a rate of 20 °C/min from 20 °C to 200 °C. Calibration of the instrument was performed routinely using indium as the calibrant. At least three scans were performed to ensure reproducibility.

*2.5.3.2. Thermogravimetric analysis (TGA).* Residual water content of spray-dried powders was performed using a Pyris 6 TGA (Perkin Elmer, Cambridgeshire, UK). 5–10 mg samples in open ceramic pans were placed on the balance and heated from 20 °C to 500 °C at a rate of 10 °C/min then held for 1 min at 500 °C. The moisture content of the samples was calculated from the weight change between 20 °C and 130 °C.

#### 2.5.4. Scanning electron microscopy (SEM)

The shape and surface morphology of the spray-dried powders was examined using scanning electron microscopy (SEM) (FEI Quanta 200F SEM; Eindhoven, Netherlands). Samples were prepared by placing a small amount of freshly prepared spray-dried powder on to 12.5 mm aluminium specimen pin stubs covered with double-sided adhesive black carbon tabs (Agor Scientific, UK) then sputter-coated with gold (Qu0rum Q150R; Quorum Technologies Ltd., Sussex, UK) prior to observation under SEM.

#### 2.5.5. High-performance liquid chromatography (HPLC) of levofloxacin

Levofloxacin was quantified using reversed-phase HPLC (Agilent 1260 infinity series; Agilent Technologies UK Ltd., Berkshire, UK). 20 μL samples were run at room temperature with a mobile phase comprising 0.1% TFA in HPLC grade water at pH 2.0: HPLC grade acetonitrile (85:15) and flow rate 1 mL/min using a 15 cm × 4.6 mm, 5 μm Supelco $C_8$ column (Supelco, Bellefonte, PA, USA) with UV detection at 294 nm. Levofloxacin was quantified by peak area measurements at a retention time of 6.25 min. A standard curve for levofloxacin was constructed before each sample analysis in triplicates. The standard curve was linear from 0.25 to 16 μg/mL, and was used to derive the standard equation for calculation of concentration of levofloxacin in experimental samples. The limit of detection and limit of quantification of levofloxacin were calculated as per the International Conference on Harmonization guidelines using formulae [48]:

$$\text{LOD} = 3.3\ \text{SD}/s$$
$$\text{LOQ} = 10\ \text{SD}/s \quad (4)$$

where SD is the standard deviation of the *Y*-intercept and s = slope of calibration curve.

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**Table 1**
Composition of formulations investigated.

| Spray-dried powders | Levofloxacin (mg) | N,O-octanoyl chitosan (1–3 K) (mg) | Chitosan (1–3 K) (mg) | L-Leucine (mg) | Final volume of feedstock (mL) |
|---|---|---|---|---|---|
| Levofloxacin | 400 | – | – | – | 100 |
| Levofloxacin:octanoyl chitosan (4:1) | 400 | 100 | – | – | 125 |
| Levofloxacin:octanoyl chitosan (2:1) | 400 | 200 | – | | 150 |
| Levofloxacin:octanoyl chitosan (1:1) | 400 | 400 | – | – | 200 |
| Levofloxacin:chitosan (4:1) | 400 | – | 100 | – | 125 |
| Levofloxacin:chitosan (2:1) | 400 | – | 200 | – | 150 |
| Levofloxacin:chitosan (1:1) | 400 | – | 400 | – | 200 |
| Levofloxacin:L-leucine (4:1) | 400 | – | – | 100 | 110 |
| Levofloxacin:octanoyl chitosan:L-leucine (4:0.1:0.25) | 400 | 100 | – | 25 | 127.5 |
| Final concentration of individual compounds mixed for spray-drying | 4 mg/mL | 4 mg/mL | 4 mg/mL | 10 mg/mL | |

### 2.6. In vitro aerosol analysis using the Next Generation Impactor (NGI)

The aerosolization properties of the spray-dried formulations and physically mixed formulations were investigated using an NGI (Copley Scientific Ltd., Nottingham, UK). Powder aliquots (10 mg) were loaded manually into size 3 HPMC capsules and placed into a Cyclohaler® (Pharmachemie, UK) that was attached to the NGI via a stainless steel USP throat. The capsule was pierced and the liberated powder drawn through the NGI at a flow rate of 60 L/min for 6 s, using a low capacity pump (Model LCP5; Copley Scientific Ltd., Nottingham, UK). Stages 1–7 of the NGI were coated with 1% silicon oil (Sigma–Aldrich, UK) in N-hexane (Fisher scientific, Loughborough, UK) to prevent particle re-entrainment. A 75 mm terminal micro-orifice filter was placed on Stage 8. Under these conditions, the effective cut-off diameters are as follows: Stage 1-8.06 μm; Stage 2-4.46 μm; Stage 3-2.26 μm; Stage 4-1.66 μm, Stage 5-0.94 μm; Stage 6-0.55 μm; Stage 7-0.34 μm, with Stage 8 having a terminal filter (Copley Scientific, UK). After aerosolization, each stage was rinsed with 10 mL of ultrapure water (HPLC grade) and the drug content was quantified by reversed-phase HPLC after diluting each stage to achieve appropriate concentration as per the linearity of the calibration curve. Each experiment was performed in triplicate. The particle size distribution of the spray-dried and physically mixed powders was compared, determining fine particle fraction (FPF) as the amount of drug deposited on Stages 2–8 as a percentage of the total drug collected representative of the fraction of aerosolized particles having cut-off diameter lower than 5 μm. Mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) were also calculated from the plot of log cumulative mass percent deposited on each stage of the NGI versus the cut-off diameter of that stage.

### 2.7. Statistical analysis

The values obtained from different experiments were statistically compared to control spray-dried powders by using One-way Analysis of Variance (ANOVA) and Tukey's post hoc test. A $p$ value of less than 0.05 was considered significant for all experiments. A significance level of $p$ less than 0.02 has also been considered for a few experiments to show the high significance of the outcome of results.

## 3. Results and discussion

### 3.1. N,O-octanoyl chitosan 1–3 K characteristics

#### 3.1.1. Synthesized polymer yields

The yield of N,O-octanoyl chitosan obtained after synthesis as analysed by gravimetry and expressed as a percentage was 72.5%.

#### 3.1.2. FT-IR analysis

The FT-IR spectra for chitosan 1–3 K and N,O-octanoyl chitosan 1–3 K are shown in Fig. 2. The spectrum for Chitosan 1–3 K has characteristic absorption peaks at *ca* 1636 $cm^{-1}$ attributed to carbonyl stretching of secondary amine (amide I band), at 1594.4 $cm^{-1}$ indicative of the N—H bending vibrations of non-acylated 2-aminoglucose primary amine and 1540.4 $cm^{-1}$ indicative of the N—H bending vibration of secondary amine (amide II band) i.e. 2-acetamidoglucose. The presence of both 2-aminoglucose and 2-acetamidoglucose units was confirmed by the presence of bands at 1636, 1594.4 and 1540.4 $cm^{-1}$.

On acylation of chitosan to form N,O-octanoyl chitosan, the vibrational band attributed to the free amino group of chitosan shifted to lower frequency and higher intensity at 1511 $cm^{-1}$, attributed to the formation of N-octanoyl and hence intermolecular hydrogen bonding between the carbonyl of the octanoyl group and the hydrophilic groups on the polymer backbone. C=O absorption by the octanoyl group is more defined and prominent, with increased intensity of the band at 1611 $cm^{-1}$, compared to the band at 1636 $cm^{-1}$ for non-modified chitosan. The increase in intensity and prominence of vibrational bands at 2885 $cm^{-1}$ of octanoyl chitosan is attributed to absorption by the $—CH_2$ chain of octanoyl substituted at the primary amine of 2-aminoglucose. Furthermore, a relatively weak vibrational band was observed at 1748 $cm^{-1}$ indicative of O-acylation. FT-IR confirmed octanoylation of chitosan with more prominence of N-octanoylation as compared to weak O-octanoylation and hence formation of N,O-octanoyl chitosan.

#### 3.1.3. $^1H$ NMR (proton nuclear magnetic resonance)

The $^1H$ NMR spectra of chitosan in $D_2O$ and N,O-octanoyl chitosan in $d_6$-DMSO are shown in Figs. 3 and 4 respectively. The chemical shift at 1.9–2.1 ppm represents three protons (—NH—CO—$C\underline{H}_3$) of N-acetyl glucosamine (GlcNAc). The chemical shifts of the non-anomeric protons connected to the ring skeleton of glucosyl residue having similar electron densities show a partially overlapping behaviour to produce a broad signal around 3.5–4.0 ppm. The —C$\underline{H}$—OH, HOHC—C$\underline{H}$—$CH_2$ and —$C\underline{H}_2$—OH protons appear at around 3.75 ppm, whereas the chemical shifts of —C$\underline{H}$—$CH_2$ and $C\underline{H}_2$—OH appear at 3.59 ppm in both spectra. The anomeric protons of the chitosan backbone, due to their neighbouring glycosidic and ring oxygen, appear at higher chemical shifts compared to non-anomeric protons, *i.e.* around 2.8–3.2 ppm. The degree of deacetylation of commercial chitosan calculated from the $^1H$ NMR spectra was found to be 86.42%.

Other than the native chitosan chemical shifts, the spectra of N,O-octanoyl chitosan have three peaks at chemical shifts 0.85, 1.25 and 1.8 ppm which confirms the success of acylation. The chemical shift seen at 0.85 ppm in the $^1H$ NMR spectrum of N,O-octanoyl chitosan (Fig.4) can be assigned to methyl protons —$CH_2$-—$C\underline{H}_3$ of the octanoyl group. This peak becomes more prominent

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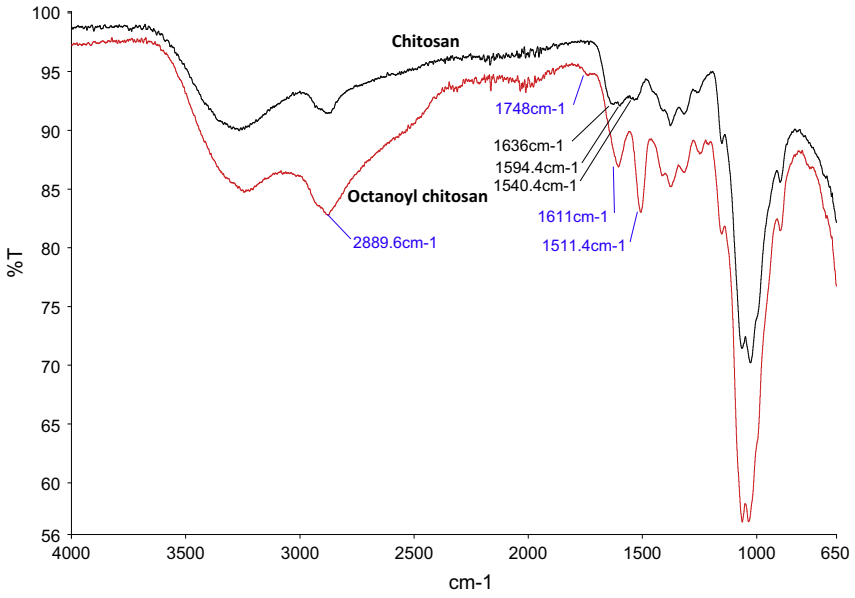


**Fig. 2.** FT-IR spectra of chitosan and N,O-octanoyl chitosan. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

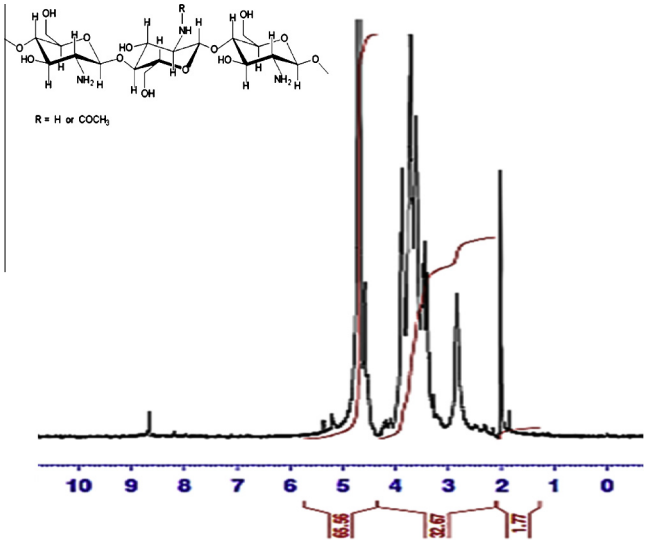


**Fig. 3.** NMR spectrum of chitosan 1–3 K. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

with an increase in degree of acylation [40], and hence the low intensity of the peak can be attributed to low substitution of the octanoyl groups onto the chitosan backbone. The chemical shift at 1.25 ppm can be attributed to the —C$\underline{H}_2$—CH$_3$, and that appearing at 1.8 ppm to —CO—C$\underline{H}_2$—CH$_3$ of the octanoyl groups. The peak at 1.8 ppm was seen to overlap with the peak of three methyl protons (—NH—CO—C$\underline{H}_3$) of N-acetyl glucosamine and gives a triplet.

### 3.1.4. XRPD (X-ray powder diffraction)

XRPD was performed to characterize the crystallographic structure of the polymers, raw materials, spray-dried microparticles and physically mixed powders. The XRPD diffractogram of chitosan (Fig. 5) shows a broad diffusive halo at around 21° indicative of the amorphous nature of chitosan. The XRPD diffractogram of synthesized N,O-octanoyl chitosan (Fig. 5) shows a similar halo around the same 2$\Theta$, however, the "hump" was seen to be split into two peaks as encountered earlier in the literature, confirming the identity of successful derivatization [49]. It is assumed that the attachment of an octanoyl group onto the chitosan backbone leads to the formation of sharper peaks with generation of new peaks at 19.6° and 25.71° which forms a more stable organization and hence enhances the stability of the polymer. XRPD analysis of spray-dried levofloxacin (Fig. 6) showed sharp diffraction peaks at 2$\Theta$ of 6.91, 10.0, 13.6, 16.35, 20.08 and 26.93 characteristic of high degree of crystallinity of the drug. The diffraction pattern was similar to that produced by unprocessed raw material (Fig. 7). Spray-dried powders containing chitosan and octanoyl chitosan showed the presence of broad diffuse peaks indicative of the long range disorder and hence amorphous nature of the formulations (Fig. 6). However, formulations spray-dried without chitosan (levofloxacin:L-leucine (4:1)) showed narrow diffraction peaks indicative of crystalline nature of the powders. Physical mixtures at the same molar mass as the spray-dried formulations (Fig. 7) showed narrow diffraction peaks, indicating that spray-drying in the presence of chitosan or hydrophobically-modified chitosan, leads to transformation of the powders into an amorphous state.

### 3.1.5. In vitro antimicrobial activity of polymers and spray-dried microparticles

MIC data for chitosan and synthesized N,O-octanoyl chitosan against three planktonic strains of *P. aeruginosa* namely 10663, 8620 and 599 are shown in Table 2. N,O-octanoyl chitosan showed a 4-fold decrease in MIC compared to non-modified chitosan, hence, showing higher anti-pseudomonal activity due to its increased hydrophobic character. Optimized levofloxacin spray-dried powders also possessed an in vitro antibacterial activity (Table 2). Similar or a 1-fold less MIC was seen for the spray-dried powders with octanoyl chitosan, wherein, the slight decrease in MIC could be attributed to an additive anti-pseudomonal activity of octanoyl chitosan as shown in Table 2.

### 3.1.6. In vitro cytotoxicity of the polymers and spray-dried microparticles

The MTT assay is a reliable method to test the cytotoxicity of polymers. Hence, A549 lung epithelial cell viability was investigated by exposure to various concentrations of polymers within

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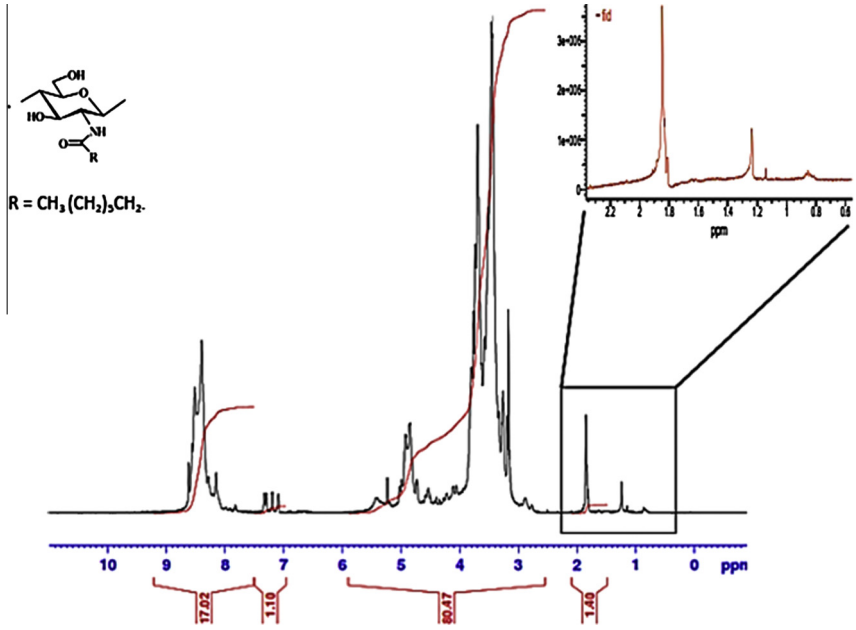


**Fig. 4.** NMR spectrum of N,O-octanoyl chitosan 1–3 K. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

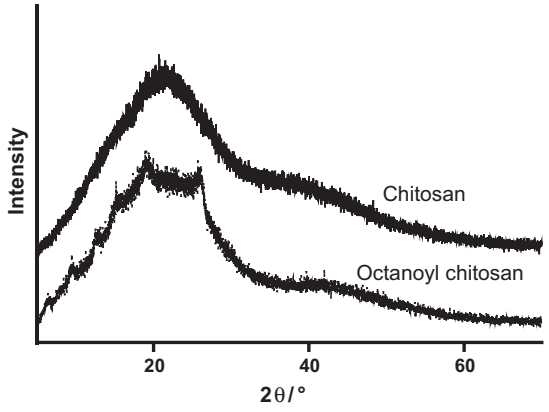


**Fig. 5.** X-ray diffractograms of chitosan 1–3 K and N,O-octanoyl chitosan 1–3 K.

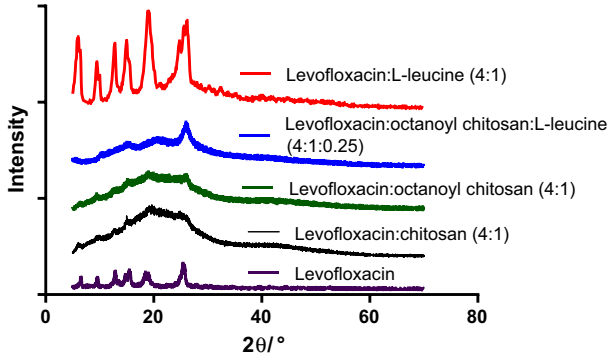


**Fig. 6.** X-ray diffractograms of spray-dried formulations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the range of 0–2.5 mg/mL (Fig. 8). Increasing the hydrophobicity of chitosan, by derivatization did not significantly change ($p < 0.05$) cytotoxicity to control non-modified chitosan. A cell viability of 86.71 ± 13.20% and 83.39 ± 11.24% was seen for chitosan and N,O-octanoyl chitosan respectively at a concentration of 2.5 mg/mL, as compared to the positive control 10% DMSO which showed viability of only 7%. Hence, it can be deduced that the synthesized polymer was non-toxic and would be predicted to not cause harm to the lung epithelia under the conditions and timescale of this study. The spray-dried formulations, i.e. spray-dried levofloxacin:octanoyl chitosan:L-leucine (4:1:0.25) cell viability of 88.52%, spray-dried levofloxacin:octanoyl chitosan (4:1) cell viability of 91.7%, spray-dried levofloxacin:octanoyl chitosan (2:1) cell viability of 93.74% (not shown) and spray-dried levofloxacin:octanoyl chitosan (1:1) cell viability of 85.93% (not shown) indicate that up to concentrations of 0.3125 mg/mL formulations were well tolerated and not significantly different from that of spray-dried levofloxacin with a cell viability 89.66% at a concentration of 0.3125 mg/mL ($p < 0.05$; Fig. 9). However, the viability decreased at 1.25 mg/mL concentrations but still showed survival greater than 50%. Consequently, it may be concluded that the spray-dried formulations, with potential for use as dry powders inhalations to treat pulmonary infections were non-toxic under the conditions and timescale of study.

### 3.2. Spray-dried powder characteristics

#### 3.2.1. Drug content, % yield, size and size distribution

With the innovative electrostatic collector of the Büchi B-90 nanospray dryer, yields in the range 51–79% of theoretical were produced, dependent on composition (Table 3), with formulations containing chitosan and hydrophobically-modified chitosan giving statistically greater yields ($p < 0.02$) than spray-dried levofloxacin alone, indicating better deposition of formulations containing chitosan in the electrostatic collector of the spray dryer. The limit of detection and quantification for levofloxacin using HPLC-UV was found to be 0.08 mcg and 0.25 mcg respectively. The levofloxacin content of spray-dried powders, determined by HPLC, ranged from 77% to 118% of predicted amount (Table 3). Data for size distributions of the spray-dried powders, which were unimodal, are shown in Table 3. The VMD for all the spray-dried powders was between 2.5 and 4.6 μm, i.e. of a size appropriate for ensuring greatest

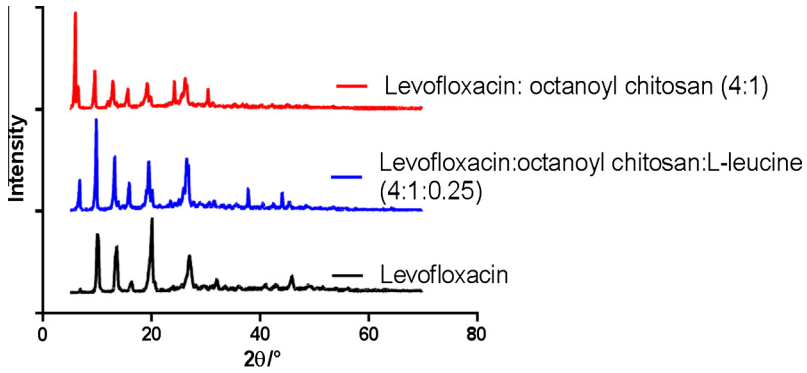


**Fig. 7.** X-ray diffractograms of levofloxacin raw material and physically mixed formulations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**Table 2**
Minimum inhibitory concentration against *P. aeruginosa* (*P.A.*) ($n = 3$).

| Polymer | Minimum inhibitory concentration | | |
|---|---|---|---|
| | *P.A.* 10663 | *P.A.* 8620 | *P.A.* 599 |
| Chitosan 1–3 K | 20 mg/mL | 40 mg/mL | 20 mg/mL |
| N,O-octanoyl chitosan 1–3 K | 5 mg/mL | 10 mg/mL | 5 mg/mL |
| Raw material levofloxacin | 0.25 μg/mL | 0.25 μg/mL | 0.5 μg/mL |
| Spray-dried levofloxacin | 0.25 μg/mL | 0.25 μg/mL | 0.5 μg/mL |
| Spray-dried levofloxacin:octanoyl chitosan (4:1) | 0.25 μg/mL | 0.125 μg/mL | 0.5 μg/mL |
| Spray-dried levofloxacin:octanoyl chitosan (2:1) | 0.125 μg/mL | 0.125 μg/mL | 0.5 μg/mL |
| Spray-dried levofloxacin:octanoyl chitosan (1:1) | 0.125 μg/mL | 0.125 μg/mL | 0.5 μg/mL |
| Spray-dried levofloxacin:octanoyl chitosan:L-leucine (4:1:0.25) | 0.125 μg/mL | 0.125 μg/mL | 0.5 μg/mL |

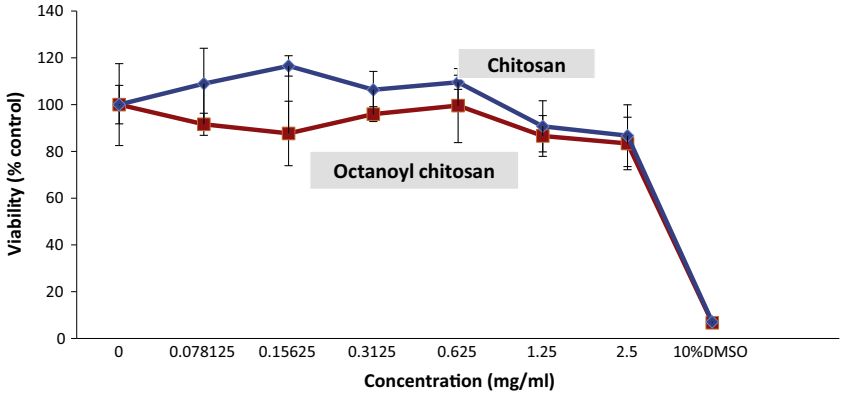


**Fig. 8.** MTT assay-cell viability data of chitosan 1–3 K and N,O-octanoyl chitosan 1–3 K ($n = 3 \pm$ SD). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

probability of delivery to the lungs. Following dispersion of powders in deionized water the size was found to be in the nanometric range as analysed by the DLS with a small net positive charge indicative of their formation of nanometric dispersion on exposure to the moist surrounding of the airway epithelium where they will be deposited.

*3.2.2. Scanning electron microscopy (SEM)*

SEM micrographs of the spray-dried powders and raw materials are shown in Fig. 10. Levofloxacin (Fig. 10A) comprised irregular, columnar, crystals having a wide size distribution. The columnar elongated shape presents a relatively large contact area between particles, likely to lead to cohesiveness and poor dispersibility. Spray-dried levofloxacin (Fig. 10B) comprised spherical particles with a relatively smooth surface and size in good agreement with that measured by laser diffraction (Table 3). Spherical particles have decreased contact area leading to decreased cohesiveness and better dispersibility and aerosolization [50,51]. SEM micrographs of spray-dried powders of levofloxacin:octanoyl chitosan (Fig. 10C–E) and levofloxacin:chitosan (Fig. 10F, G and H) revealed spherical particles, but with a less smooth, corrugated surface morphology and diameters in good agreement with that measured by laser diffraction (Table 3). Corrugations on the surface may further decrease the contact area between particles, reducing cohesiveness and increasing dispersibility [52]. SEM micrographs of the spray-dried levofloxacin:L-leucine formulation (Fig. 10I) which contains about 20%w/w L-leucine, showed fractured, hollow-surfaced spheres, with curved plates of nanometric thickness which have been reported earlier by various authors. These have spaces which produce an open powder which may increase dispersibility of cohesive material by increasing median geometric diameter and subsequently decreasing particle density [51,53,54]. The levofloxacin:octanoyl chitosan:L-leucine (Fig. 10J) particles contain only 5% w/w L-leucine, and hollow surfaced curved plates are not evident.

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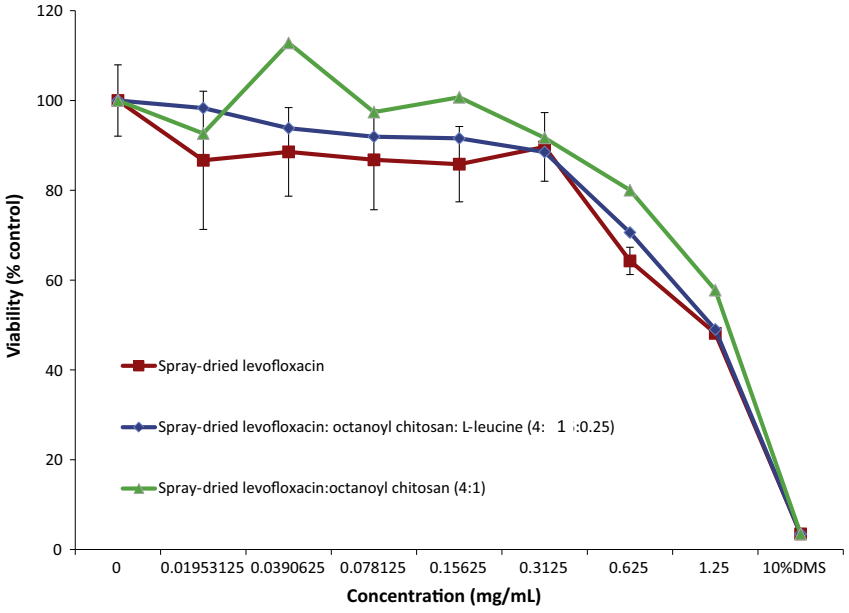


**Fig. 9.** MTT assay-cell viability data of spray-dried formulations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**Table 3**
Physical characteristics of spray-dried powders (mean ± SD; $n = 3$).

| Sample | Yield (%) | Drug content (%) | Size–DLS (nm) | $z$-potential DLS (mv) | VMD (μm) | | Span | | TGA mass loss (%) |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | 4 bar | 2 bar | 4 bar | 2 bar | |
| Levofloxacin raw material | – | – | – | – | 8.80 ± 2.06 | 9.63 ± 2.37 | 2.33 | 2.66 | 2.34 ± 0.750 |
| Chitosan | – | – | – | – | 32.29 ± 3.96 | 38.06 ± 4.53 | 1.94 | 1.65 | 6.50 ± 2.440 |
| Octanoyl chitosan | – | – | – | – | 28.57 ± 3.09 | 37.78 ± 3.10 | 2.06 | 1.64 | 15.75 ± 3.675 |
| Spray-dried – levofloxacin | 51.03 ± 5.68 | 93.04 ± 0.27 | – | – | 2.89 ± 0.36 | 3.23 ± 0.87 | 1.63 | 2.05 | 1.74 ± 0.880 |
| Spray-dried – levofloxacin:octanoyl chitosan (4:1) | 76.52** ± 1.26 | 79.13 ± 0.14 | 137.26 ± 1.34 | +2.58 ± 0.08 | 2.66 ± 0.68 | 2.83 ± 0.69 | 1.62 | 2.72 | 6.94 ± 0.800 |
| Spray-dried – levofloxacin:octanoyl chitosan (2:1) | 71.33** ± 1.59 | 78.71 ± 0.16 | 183.7 ± 1.59 | +1.70 ± 0.11 | 2.50 ± 0.41 | 2.61 ± 0.55 | 1.67 | 2.01 | 10.82 ± 0.74 |
| Spray-dried – levofloxacin:octanoyl chitosan (1:1) | 75.73** ± 2.02 | 85.22 ± 3.88 | 178.8 ± 1.86 | +1.65 ± 0.41 | 2.53 ± 0.69 | 2.70 ± 0.73 | 1.99 | 2.25 | 11.37 ± 0.75 |
| Spray-dried – levofloxacin:chitosan (4:1) | 65.44** ± 5.09 | 83.69 ± 0.79 | 472.5 ± 2.52 | +3.69 ± 0.21 | 3.03 ± 1.57 | 3.23 ± 1.61 | 2.78 | 2.62 | 23.99* ± 1.96 |
| Spray-dried – levofloxacin:chitosan (2:1) | 65.92** ± 5.74 | 77.13 ± 0.38 | 487.5 ± 3.06 | +4.01 ± 0.55 | 4.21 ± 1.40 | 4.54 ± 1.55 | 3.26 | 1.54 | 21.45* ± 1.64 |
| Spray-dried – levofloxacin:chitosan (1:1) | 67.40** ± 4.96 | 83.76 ± 1.4 | 489.6 ± 2.58 | +3.98 ± 0.36 | 3.51 ± 1.18 | 3.67 ± 1.26 | 4.26 | 5.61 | 35.05* ± 1.79 |
| Spray-dried – levofloxacin:L-leucine (4:1) | 79.06** ± 6.68 | 118.60 ± 4.25 | 315.0 ± 1.36 | −11.36 ± 0.61 | 4.13 ± 0.25 | 4.56 ± 0.39 | 3.21 | 3.65 | 2.24 ± 0.41 |
| Spray-dried – levofloxacin:octanoyl chitosan:L-leucine (4:1:0.25) | 72.88** ± 2.29 | 81.15 ± 4.88 | 367.2 ± 1.39 | +1.70 ± 0.19 | 3.75 ± 0.59 | 3.87 ± 0.74 | 3.11 | 3.25 | 12.52 ± 0.49 |

* Statistical difference: One-way ANOVA/Tukey versus spray-dried levofloxacin powder: $p < 0.05$.
** Statistical difference: One-way ANOVA/Tukey versus spray-dried levofloxacin powder: $p < 0.02$.

The spherical particles' surfaces are deeply corrugated and have a size in good agreement with that measured by laser diffraction (Table 3). Fig. 10K shows the effect of moisture on the morphology of the spray-dried powders containing octanoyl chitosan alone. Spray-drying often produces amorphous particles, which in a humid environment may sorb water and recrystallize. After 60 days storage at room temperature and ambient humidity there was no considerable change in morphology or evidence of a change in crystallinity of the powder, as has previously been reported for spray-dried powders of terbutaline sulphate [4]. This suggests that octanoyl chitosan formulations have reasonable physical stability in ambient conditions.

### 3.2.3. Amorphous nature of spray-dried powders

*3.2.3.1. Differential scanning calorimetry (DSC).* The thermograms of levofloxacin and spray-dried powders containing octanoyl chitosan are shown in Fig. 11. The thermogram of levofloxacin showed a sharp endothermic peak with an onset at 120 °C, indicative of melting and hence demonstrating the crystalline nature of raw material, in agreement with XRPD data (Fig. 7). The DSC thermogram of spray-dried powders containing octanoyl chitosan did not show characteristic endotherms, indicating production of an amorphous form during spray-drying. The production of an amorphous form may result in powders having a higher solubility and faster dissolution rates in lung fluids. Furthermore, exothermic transitions around 80–95 °C were observed for the spray-dried powders which were not seen in the thermograms of the raw materials. This corresponds to the heat produced by the sample on phase transition indicative of recrystallization of spray-dried powders.

*3.2.3.2. Thermogravimetric analysis (TGA).* Mass loss as a function of temperature between 20 and 130 °C (Table 3) is indicative of the

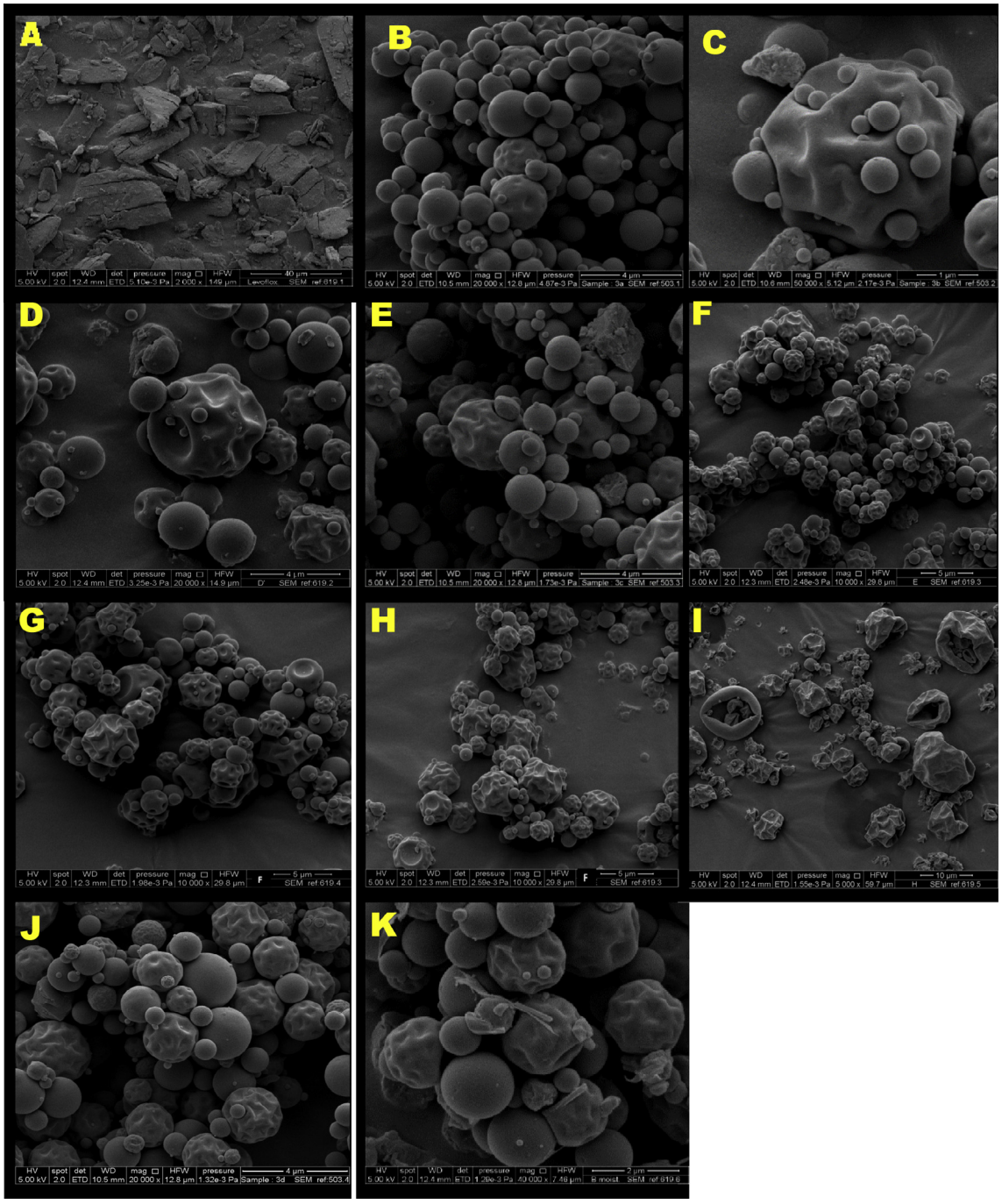


**Fig. 10.** SEM micrographs of (A) Levofloxacin raw material, (B) Spray-dried levofloxacin, (C) Spray-dried levofloxacin:octanoyl chitosan (4:1), (D) Spray-dried levofloxacin:octanoyl chitosan (2:1), (E) Spray-dried levofloxacin:octanoyl chitosan (1:1), (F) Spray-dried levofloxacin:chitosan (4:1), (G) Spray-dried levofloxacin:chitosan (2:1), (H) Spray-dried levofloxacin:chitosan (1:1), (I) Spray-dried levofloxacin:L-leucine (4:1), (J) Spray-dried levofloxacin:octanoyl chitosan:L-leucine (4:1:0.25), (K) Spray-dried levofloxacin:octanoyl chitosan (4:1) on storage at room temperature and humidity. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

moisture content of the powders. TGA thermograms showed a statistically ($p < 0.05$) greater weight loss for spray-dried powders containing chitosan than for those containing hydrophobically-modified octanoyl chitosan and L-leucine. This weight loss can be attributed to greater adsorption of water in the matrix of the hydrophilic chitosan polymer chains, preventing its loss during spray-drying and resulting in higher final water content. Spray-dried octanoyl chitosan-containing powders showed similar ($p > 0.05$) water loss as spray-dried levofloxacin powder, indicating minimal entrapment of water. Addition of octanoyl groups onto the chitosan backbone prevents hydrophilic moisture entrapment within the matrix leading to a drier final product. This may also result in a less cohesive powder with better flow properties and subsequently improved aerodynamic properties and FPF compared to spray-dried formulations with chitosan.

### 3.2.4. In-vitro aerosol properties

The in vitro aerodynamic behaviour of powder formulations delivered from a Cyclohaler into the NGI is shown in Figs. 12–14, and the derived aerodynamic parameters are presented in Table 4.

All powders were highly dispersible, with at least 71.62% of the capsule contents being emitted during aerosolization, and giving a

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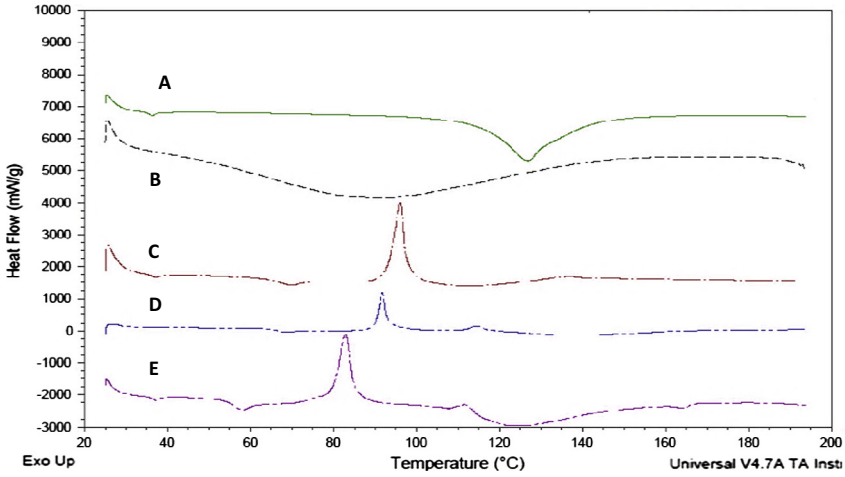


**Fig. 11.** DSC thermograms of spray-dried powders; (A) Levofloxacin raw material, (B) Octanoyl chitosan, (C) Spray-dried levofloxacin:octanoyl chitosan 4:1, (D) Spray-dried levofloxacin:octanoyl chitosan 2:1, (E) Spray-dried levofloxacin:octanoyl chitosan:L-leucine 4:1:0.25. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

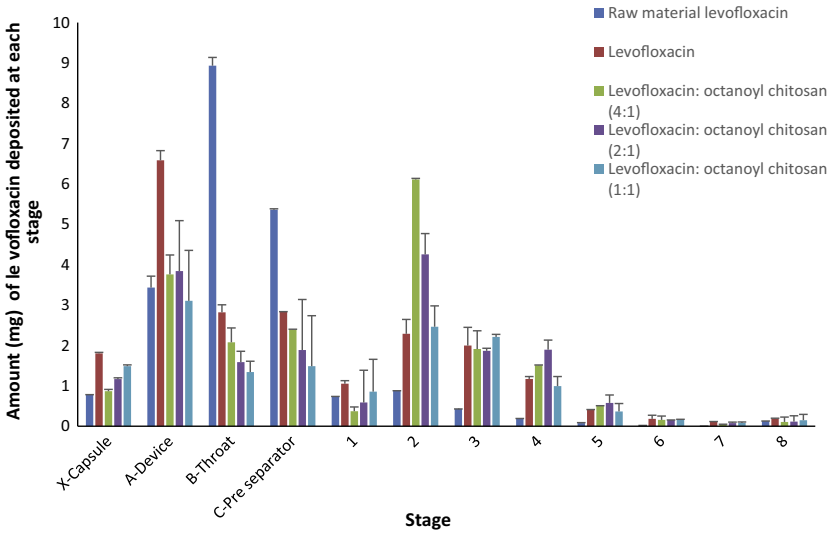


**Fig. 12.** NGI deposition profile for levofloxacin spray-dried powders containing different ratios of octanoyl chitosan. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

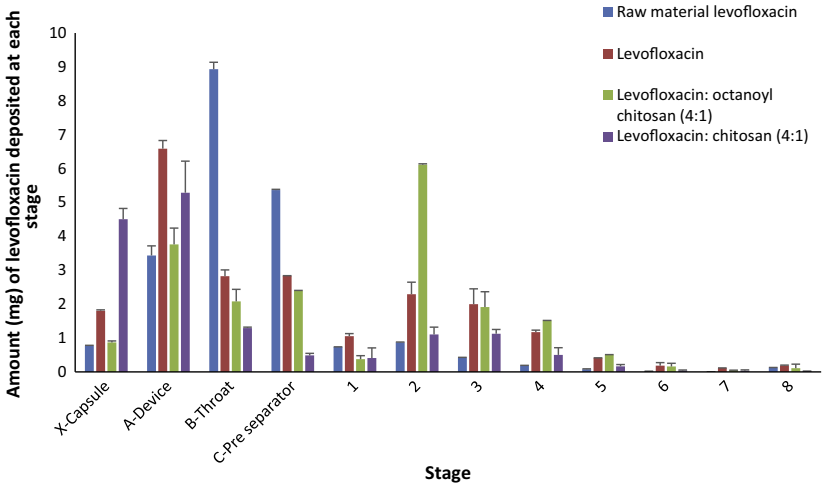


**Fig. 13.** NGI deposition profile for levofloxacin spray-dried with chitosan or octanoyl chitosan. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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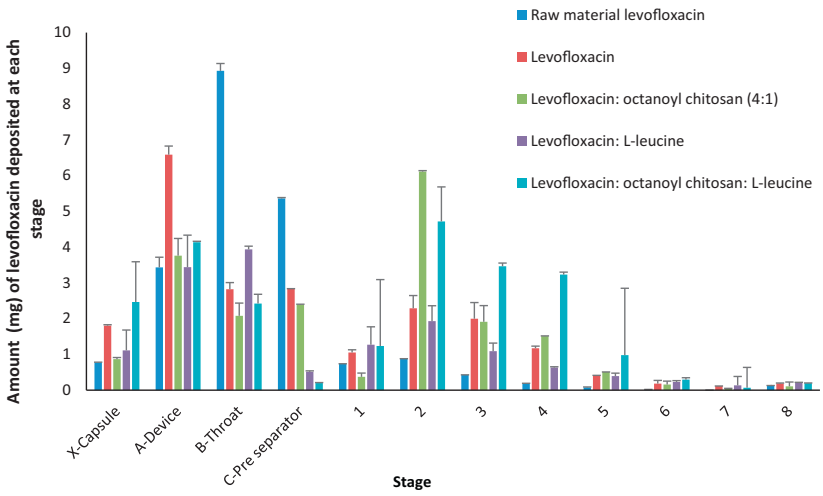


**Fig. 14.** NGI deposition profile for spray-dried octanoyl chitosan and/or L-leucine containing powders. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**Table 4**
Aerodynamic parameters for formulations delivered to the NGI.

| Formulation | Fraction recovered-FR (%) | Emitted dose-ED (%) | Fine particle fraction-FPF (%) | MMAD (μm) | GSD |
|---|---|---|---|---|---|
| Levofloxacin | 75.68 | 96.25* ± 5.70 | 8.21 ± 1.25 | 5.76 ± 0.108 | 1.89 |
| Spray-dried levofloxacin | 76.93 | 91.59 ± 3.59 | 29.65 ± 2.56 | 4.12 ± 0.13 | 1.91 |
| Spray-dried levofloxacin:octanoyl chitosan (4:1) | 82.67 | 95.62 ± 3.28 | 52.24* ± 1.89 | 4.81 ± 0.095 | 1.33 |
| Spray-dried levofloxacin:octanoyl chitosan (2:1) | 78.45 | 92.66 ± 2.21 | 49.65* ± 2.12 | 4.49 ± 0.11 | 1.46 |
| Spray-dried levofloxacin:octanoyl chitosan (1:1) | 92.63 | 89.89 ± 3.26 | 43.76* ± 1.74 | 4.19 ± 0.23 | 1.76 |
| Spray-dried levofloxacin:chitosan (4:1) | 75.71 | 71.62* ± 7.44 | 19.97 ± 4.601 | 4.16 ± 1.23 | 1.67 |
| Spray-dried levofloxacin:L-leucine (4:1) | 83.83 | 92.53 ± 6.74 | 31.08 ± 4.68 | 4.79 ± 0.64 | 1.94 |
| Spray-dried levofloxacin:octanoyl chitosan:L-leucine (4:1:0.25) | 120.24* | 89.47 ± 2.66 | 55.33* ± 2.15 | 3.84 ± 0.10 | 2.08 |
| Physical mixture levofloxacin:octanoyl chitosan (4:1) | 85.17 | 113.39 ± 2.66 | 9.77 ± 3.68 | – | – |
| Physical mixture levofloxacin:octanoyl chitosan:L-leucine (4:1:0.25) | 76.04 | 107.85 ± 3.96 | 8.14 ± 3.95 | – | – |

* Statistical difference: One-way ANOVA/Tukey versus spray-dried levofloxacin powder: $p < 0.05$ for statistically higher values.

recovery of at least 75.7% levofloxacin from the NGI; within pharmacopoeial limits of 75–125%. For spray-dried levofloxacin, a high proportion of drug is deposited in the inhaler and throat region of the NGI (Figs. 12 and 13). Spray-dried powders containing octanoyl chitosan produced the low deposition of levofloxacin in the inhaler and throat regions, with increased deposition particularly in Stages 2 and 4 of the NGI, demonstrating the increased dispersibility of this formulation. The powders generated were of suitable aerodynamic size for inhalation having a mass median aerodynamic diameter (MMAD) less than 5 μm, with formulations containing octanoyl chitosan having MMADs less than 4.5 μm.

Spray-drying levofloxacin in the presence of octanoyl chitosan at different ratios increased the FPF by approximately 76% compared to spray-dried drug alone. Comparing the FPF of formulations incorporating the commonly used dispersibility enhancer L-leucine (Fig. 14; Table 4), the FPF achieved with polymer octanoyl chitosan:L-leucine was significantly ($p < 0.05$) higher than for a comparable spray-dried levofloxacin:L-leucine formulation. In addition, the FPF of spray-dried powders containing chitosan was significantly ($p < 0.05$) lower than spray-dried powders containing octanoyl chitosan (Table 4). This can be attributed to the higher moisture entrapment in the matrix of chitosan during spray-drying, leading to cohesiveness and hence a decrease in FPF of the spray-dried powders. Moreover, the amphiphilic nature of octanoyl chitosan means that during spray-drying it is likely to migrate to the solution droplet/drying air interface, resulting in modification of the surface properties and cohesive properties of the powders.

Previous research has demonstrated that a high content of dispersibility enhancers and carriers is required to increase effectively the aerodynamics of drugs delivered as powder aerosols [4,55,56]. The present study shows that increasing polymer concentration beyond 20% did not give any significant improvement in dispersibility, and hence formulations containing 20% octanoyl chitosan would be favourable for use, incorporating the maximum amount of antibiotic for delivery of a high localized dose of levofloxacin. The dispersibility of the formulations with octanoyl chitosan was comparable with those containing L-leucine, an established dispersibility enhancer for aerosol formulation, showing that the derivatized natural polymer, octanoyl chitosan, was a highly efficient dispersibility enhancer and hence is attractive for pulmonary delivery of other drugs.

Powders comprising spray-dried levofloxacin:octanoyl chitosan and levofloxacin:octanoyl chitosan:L-leucine were compared with physical mixtures of the same composition. Both the physical mixtures showed deposition behaviour within the NGI comparable with levofloxacin raw material, with deposition high in the impactor. These formulations produced FPFs similar to raw material and significantly lower FPFs ($p < 0.05$) than their spray-dried counterparts (Table 4). Thus, simple mixing of levofloxacin with octanoyl chitosan or L-leucine did not improve the deposition profile of levofloxacin. Consequently, formulating levofloxacin within a delivery

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system, such as octanoyl chitosan via spray-drying such that components are included in individual, spherical particles is required.

## 4. Conclusion

The present investigation has shown that derivatization of chitosan with a hydrophobic octanoyl chain increased the in vitro antimicrobial activity of the polymer. Moreover, spray-dried microparticles containing octanoyl chitosan showed a greater dispersibility and higher FPF when compared to those containing non-modified chitosan, demonstrating its suitability as an alternative inhalation aerosol dispersibility enhancer. Morphological investigation showed that the engineered particles have suitable aerodynamic particle size and relatively low physical contact due to the corrugations on their surface, leading to decreased density and hence high dispersibility. Different ratios of polymer and levofloxacin were studied to explore the effect of increasing polymer concentration on aerosolization. These spray-dried microparticles would be predicted to deposit predominately in the deep lung following inhalation where infections predominate, with minimal oropharyngeal deposition, thereby maximizing the dose delivered to the region of infection, increasing the efficiency of formulation and potentially reducing the occurrence of toxicity in the oropharyngeal region or following swallowing. The dispersibility of spray-dried formulations containing octanoyl chitosan was comparable with those containing L-leucine, indicating its effectiveness as a dispersibility enhancer and suggesting its inclusion may be attractive for pulmonary delivery of other drugs. This natural polymer when hydrophobically modified would be attractive for its dispersibility enhancement property as compared to the model hydrophobic amino acid L-leucine due to its antibacterial and mucoadhesive properties. Mucoadhesion would increase the residence time of the formulations in the lungs consequently enhancing the efficiency of antibiotic for the treatment of infections. Biodegradability of this natural polymer and the non-cytotoxicity determined by MTT assay suggests the likely safety of use of this polymer as dispersibility enhancer in formulations for aerosolization.

## Conflict of interest

The authors declare there is no conflict of interest.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejpb.2014.09.005.

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