

Formulation and Evaluation of Nano-Encapsulated Inhalable Insulin Dry Powder for Rapid Glycemic Control in Type 1 and Type 2 Diabetes Mellitus

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Abstract

The traditional form of subcutaneous insulin treatment is known to have late onset of action, needle phobia, low patient compliance and lack of post prandial glycemic control and hence the necessity of developing efficient non-invasive delivery approaches. The current paper describes how insulin nano-encapsulated inhalable dry powder was formulated and evaluated in detail in order to achieve quick systemic glycemic regulation through the pulmonary route. A system was optimized by ionic gelation with sodium tripolyphosphate (TPP) as a cross-linker and 5 formulation batches (F1-F5) by the gradual increase and decrease of polymer concentration (0.5-1.5%), drug:polymer ratio (1:1 to 1:5), and stirring speed (500-1000 rpm) to produce insulin-loaded chitosan nanoparticles. The optimized formulation (F5) exhibited a particle size of 148 ± 2 nm, polydispersity index of 0.214, zeta potential of +27.9 mV and entrapment efficiency of $91.4 \pm 0.8\%$ of the drug. Spray drying the nanoparticle dispersion with trehalose and mannitol as cryoprotectants transformed the dispersion into inhalable dry powder. The powder showed satisfactory flow characteristics (Carr index: 16.32 ± 0.54 , Hausner ratio: 1.19 ± 0.03 , angle of repose: $28.6 \pm 0.8^\circ$), low moisture (2.3 percent) and the ideal particle size distribution (D50: 3.0 μ m, D90: 4.8 μ m). The aerosolization studies showed fine particle fraction of 61.8, aerodynamic particle size of 3.3 μ m and emitted dose of 87.4 which was suitable to deposit in deep lungs. It exhibited a biphasic in vitro drug release with an initial burst (17.9% at 1 h), and sustained release ($96.3 \pm 2.3\%$ at 24 h). At 276 nm UV spectrophotometric calibration gave a linear response at 2-10 μ g/mL ($R^2 = 0.9992$). FTIR showed compatibility of drugs and excipients. The 6 months studies of stability under conditions suggested by the ICH (25 $^\circ$ C/60% RH and 40 $^\circ$ C/75% RH) showed that there were slight fluctuations in particle size (≈ 160 nm) and drug content ($\approx 95.6\%$), which confirmed the stability of the formulation. These results support the belief that nano-encapsulated inhalable insulin dry powder is a good platform to attain a rapid reproducible glycemic control in Type 1 and Type 2 diabetes mellitus.

Keywords: Nano-encapsulation; Chitosan; Insulin; Inhalable dry powder; Pulmonary delivery; Ionic gelation; Fine particle fraction; Glycemic control; Spray drying; Drug entrapment efficiency

1. Introduction

Diabetes mellitus (DM) is a progressive metabolic, chronic and irreversible condition that is marked by constant hyperglycaemia due to absolute or relative insulin deficiency due to failure to secrete insulin or its inability to act [1]. The International Diabetes Federation estimates the global prevalence of diabetes as 537 million adults in the year 2021 and it is expected to reach up to 783 million in 2045 [2]. Type 1 diabetes mellitus (T1DM) occurs due to autoimmune destruction of pancreatic β -cells, necessitating the life-long replacement of insulin exogenously whereas Type 2 diabetes mellitus (T2DM) is caused by progressive β -cell failure overlaid by peripheral insulin resistance, often requiring insulin replacement despite adequate glycaemic control by oral antidiabetic agents [3,4].

Since 1922, insulin has been used as a clinical standard by subcutaneous (SC) injection. Although there exist ultra-rapid-acting insulin analogues, there are various limitations to SC administration, such as injection-site pain, lipodystrophy, needle phobia, the risk of hypoglycaemia, and insufficient onset-action (10-20 minutes) with poor simulation of the physiological first-peaked insulin secretory response (<2 minutes) [5]. These deficiencies lead to the inefficient post-prandial glycaemic regulation and to the deterioration of patient adherence in the long term. Moreover,

the need to store insulin at low temperatures and the whole process of dose titration further decreases the quality of life of insulin-dependent patients [6].

Pulmonary route provides an anatomically and physiologically a favourable alternative form of delivering systemic insulin. The human lung offers a surface area of absorption of about 140 m², a thin alveolar lining (0.1-0.5 μ m), and an extraordinarily dense vascular supply, which makes it possible to absorb macromolecular drugs directly into the systemic circulation without going through hepatic first-pass metabolism [7]. Inhalation formulations using dry powder are better chemically and physically stable than other forms of liquid nebulised systems, remove any cold-chain requirements, and can be delivered conveniently and without requiring a cold chain by using breath-activated devices [8].

The nano-encapsulation of insulin into biodegradable polymeric carrier has become a revolutionary approach, in order to evade the main obstacles to the delivery of proteins in the lungs: enzymatic degradation in the bronchoalveolar fluid by proteases, mucocilia clearance, and alveolar phagocytosis by macrophages [9]. Of particular interest as an insulin nanocarrier is chitosan, a natural cationic polysaccharide which, through partial deacetylation of chitin, can be deferred to create biocompatible, biodegradable, mucoadhesive nanoparticles and has been shown to induce paracellular permeability across epithelial barrier temporarily on tight junction proteins modulation [10]. Chitosan/sodium tripolyphosphate (TPP) is an aqueous-based nanoparticle synthesis method, which does not use harsh organic solvents and high temperature conditions, and maintains insulin bioactivity through ionic gelation [11].

When insulin-loaded chitosan nanoparticles are spray-dried with the cryoprotectants like trehalose and mannitol, the composite dry powder particles produced can be optimally engineered in the aerodynamic range of 1-5 μ m, in which reproducible deposition can be made in alveolar region of the lung [12]. Though the clinical validation of inhaled insulin with the use of Afrezza(r) has been already approved by FDA (2014), nano-encapsulated inhalable insulin systems with improved bioavailability and release profiles are an ongoing research focus [13].

The current research was thus carried out with the aim of developing and critically assessing nano-encapsulated inhalable insulin dry powder in the speedy glycemic regulation of T1DM and T2DM. Its preformulation characterisation of insulin, systematic optimization of chitosan nanoparticle lot, conversion to inhalable dry powder through spray drying, and thorough testing of physicochemical properties, aerodynamic behaviour, in vitro drug release, and commercial stability were covered in the study, which offers a comprehensive dataset to justify the translatability of the developed formulation.

2. Materials and Methods

2.1 Materials

The active pharmaceutical ingredient was insulin (recombinant human 27 IU/mg). Chitosan (low molecular weight, degree of deacetylation $\approx$ 85% viscosity 20-300 cPs) was obtained by a well-known pharmaceutical distributor. Sodium tripolyphosphate (TPP), trehalose dihydrate, mannitol, glacial acetic acid, sodium hydroxide and disodium hydrogen phosphate were analytical reagents of grade. The Phosphate buffer saline (PBS, pH 7.4) was made freshly in the laboratory. The study involved the use of distilled water. Chemicals and solvents were of analytical grade and were not further purified [14].

2.2 Drug Profile

2.2.1 Chemical Structure and Properties

Insulin is a peptide hormone consisting of two polypeptide chains, A-chain (21 amino acids) and B-chain (30 amino acids) connected by two inter-chain disulfide bonds, and a third inter-chain disulfide bond in the A-chain (Figure 1). The human insulin molecules weigh about 5,808 Da. The pharmacological activity of insulin greatly relies on the structural integrity of the insulin that is crucially determined by the native disulfide bond architecture. Because biomolecules Insulin is strongly affected by enzymes, thermal denaturation, and deamidation at locations Asn-A21 and Asn-B3 in response to acidic or alkaline environments, a need to nano-encapsulate Insulin to maintain biological activity in the formulation and pulmonary delivery is apparent [15].

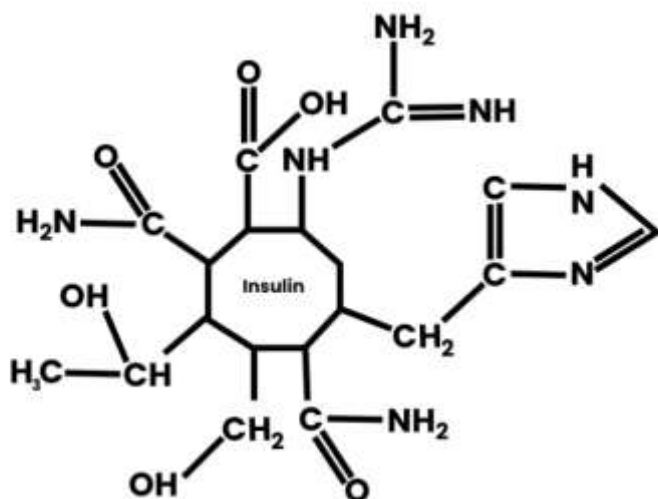


Figure 1. Chemical structure of human insulin showing the A-chain (21 amino acids) and B-chain (30 amino acids) connected by two inter-chain and one intra-chain disulfide bonds.

2.2.2 Mechanism of Action

Insulin achieves the effect of hypoglycaemia by binding with insulin receptor tyrosine kinase on the cell membrane of muscle, adipose tissues and hepatic tissues, which triggers a cascade of signalling events leading to translocation of GLUT-4 glucose transporter into the cell membrane, and thus uptake of glucose. The same insulin also stimulates hepatic glycogen synthesis, inhibits gluconeogenesis, glycogenolysis, enhances lipogenesis in adipose tissue, and enhances protein production. Nano-encapsulation of insulin in the lung allows the rapid absorption and systemic distribution by the alveoli, and offers rapid onset of glycaemic control that is necessary during the post-prandial hyperglycaemic period [16].

2.3 Preformulation Studies

2.3.1 Physical Characterisation

Appearance, colour, odour, physical state, and hygroscopic nature were considered as the physical properties of insulin through visual examination. Differential scanning calorimetry (DSC) was used to measure thermal behaviour. The melting temperature or decomposition temperature was observed. The parameters are important in determining suitable processing conditions and excipients during the formulation development [17].

2.3.2 Solubility Studies

The shake-flask method of equilibrium solubility was used to determine the equilibrium of insulin in distilled water, phosphate buffer pH 6.8, phosphate buffer pH 7.4, ethanol, and chloroform at 25degC. Membrane filters with a 0.45 um pore were used to filter the samples and then measured by UV spectrophotometry at 276 nm. A determination of solubility was used to ensure that the relevant solvent systems are used in nanoparticle preparation and in vitro drug release media [17].

2.3.3 UV Spectrophotometric Analysis

The stock solution of insulin (1 mg/mL) was dissolved in phosphate buffer (pH 7.4) and spectrophotometrically measured at the wavelength in 200 -400 nm range with a UV-visible double-beam spectrophotometer (Shimadzu UV-1900i). The wavelength of maximum absorbance (λ_{max}) was determined. Calibration standards of 2, 4, 6, 8 and 10 - gram/mL were created and triplicate absorbance were recorded. Calibration curve was plotted against concentration against absorbance and the correlation coefficient (R^2) was used to test the linearity [18].

2.3.4 Drug–Excipient Compatibility by FTIR

Fourier Transform Infrared Spectroscopy (FTIR), was used to examine the possible physicochemical interactions between insulin and formulation excipients (chitosan, TPP, trehalose, mannitol). FTIR Spectra on a FTIR spectrometer (Shimadzu IRTracer-100) were measured in the wavenumber range of 4000-400 cm^{-1} by the KBr pellet method on spectra of pure insulin, individual excipients and binary physical mixtures. The pure drug and the formulation were

compared in characteristic infrared absorption peaks of insulin (Amide I, Amide II, N-H stretching, C-N stretching). Lack of peak shifts or loss of typical bands was considered to be a sign of compatibility [19].

2.4 Preparation of Insulin-Loaded Chitosan Nanoparticles

2.4.1 Ionic Gelation Method

Insulin carrying chitosan nanoparticles were made by the ionic gelation method depending on the electrostatic bonding between the positively charged amino groups of chitosan and negatively charged phosphate groups of TPP [20]. In brief, the chitosan was dissolved in 0.5% v/v glacial acetic acid to get polymer solutions of the desired concentration (0.5-1.5% w/v). The drug:polymer ratio of 1:1 to 1:5 was used to dissolve insulin in the phosphate buffer (pH 7.4). The insulin solution was dropwise made to the chitosan solution with constant magnetic stirring of the mixture at the effective speed (500-1000 rpm). The solution of TPP (0.1% w/v in distilled water) was then dropwise added to the chitosan-insulin solution and allowed to self-assemble into nanoparticles as a result of ionic cross-linking. The mixture was stirred a half an hour at room temperature. The resulting dispersed nanoparticle was opalescent, and was either characterised at that point or frozen at 40 C until it was used again [11,20].

2.4.2 Optimisation of Formulation Variables

Systematic variation of three formulation critical variables, such as polymer concentration (0.5-1.5%), drug:polymer ratios (1:1-1:5), and stirring speed (500-1000 rpm), resulted in the production of five formulation batches (F1- F5), as depicted in Table 5. Particle size and entrapment efficiency were measured on each batch in order to arrive at the optimum processing conditions. One-way ANOVA (SPSS v26.0; significance at $p < 0.05$) was used to perform statistical analysis [21].

2.5 Characterisation of Nanoparticles

2.5.1 Particle Size, Polydispersity Index, and Zeta Potential

The dynamic light scattering (DLS) and laser Doppler microelectrophoresis were used to measure the hydrodynamic diameter (Z-average), polydispersity index (PDI) and zeta potential of the nanoparticle dispersions respectively at a temperature of 25degC using a Malvern Zetasizer Nano ZSP. Measurement was done using a 1:100 dilution of the samples using filtered distilled water. All were measured in triplicates ($n = 3$) [22].

2.5.2 Drug Entrapment Efficiency

DEE was also calculated as the ultracentrifugation of the nanoparticle dispersion at 14,000 rpm during 30 min at 4degC (Beckman Coulter Optima XE). The supernatant was gently segregated and the free (unentrapped) insulin estimated by UV spectrophotometry at 276 nm. DEE was determined as follows: $DEE (\%) = (\text{Total insulin} - \text{Free insulin}) / \text{Total insulin} \times 100$ [23].

2.5.3 Surface Morphology (SEM)

Scanning Electron Microscopy (SEM; JEOL JSM-7610F) was used to determine the surface morphology of nanoparticles. A sample was dried in the air on an aluminium stub, sputter-coated with 10 nm of gold layer in an environment of argon and scanned at an accelerating voltage of 5 kV. SEM micrographs were scored on their shape of particles, texture on their surface, and evidence of aggregation [24].

2.6 Conversion to Inhalable Dry Powder

2.6.1 Spray Drying

The spray dried (Buchi B-290 Mini Spray Dryer) optimised nanoparticle dispersion was then transformed to inhalable dry powder. Cryoprotectants and bulking agents Trehalose (5% w/w) and mannitol (5% w/w) were included in the feed dispersion. The spray drying conditions were as follows, inlet temperature 120degC, outlet temperature 65degC, feed rate 3mL/min, atomising air flow rate 357 L/h and aspirator capacity 100%. The powder thus obtained was gathered up and placed into airtight desiccant flasks and left to store at ambient temperature until characterisation [25].

2.6.2 Freeze Drying

As an alternative drying strategy, freeze drying (lyophilisation; Christ Alpha 2-4 LSCplus) was used. The trehalose (5% w/v) in the nanoparticle dispersion as a cryoprotectant was filled in vials, pre-frozen at 80°C 12 h, and then

lyophilised at 40 °C in a vacuum pressure of 0.1 mbar over a 48 h period. The remaining bound water was dried at 20 °C in 6 h to less than 2% moisture [26].

2.7 Evaluation of Dry Powder Formulation

2.7.1 Flow Properties

The bulk density was calculated by adding known bulk mass onto a graduated measuring cylinder and recording the volume. A tap density tester (Electrolab ETD-1020) was used to measure tapped density after 1000 taps. The compressibility index (CI) and Hausner ratio (HR) were given by Carr as: $CI (\%) = 100 \left(\frac{\text{Tapped density} - \text{Bulk density}}{\text{Tapped density}} \right)$; $HR = 100 \left(\frac{\text{Tapped density}}{\text{Bulk density}} \right)$. The angle of repose was measured using the fixed funnel procedure. Each of the measurements was taken three times ($n = 3$, mean $\pm$ SD) [27].

2.7.2 Particle Size Distribution and Moisture Content

Laser diffraction (Malvern Mastersizer 3000, dry dispersal, 4 bar pressure) was used to measure the particle size distribution (D10, D50, D90, mean particle size) of the spray-dried powder. Karl Fischer titrimetry (Mettler Toledo DL31) was used to determine the moisture content [28].

2.8 Aerosolization Performance

The NGI was used to measure aerodynamic particle size distribution (60 L/min) and was used to simulate the flow of air through a standard low-resistance DPI device. One nominal dosage of the dry powder was put in a size 3 hydroxypropyl methylcellulose (HPMC) capsule and triggered three times. Each impactor stage had a drug deposited on it which was dissolved in PBS (pH 7.4) and measured through the use of UV spectrophotometry (276 nm). The impactor data were used to calculate fine particle fraction (FPF; particles $< 5 \mu\text{m}$ as a ratio of recovered dose), mass median aerodynamic diameter (MMAD), emitted dose (ED) and lung deposition efficiency [29,30].

2.9 In Vitro Drug Release Study

The dialysis membrane diffusion method was used to study in vitro insulin release of the dry powder formulation [31]. The required volume of 5 mg insulin in dry powder was weighed and then dispersed in 5 mL of PBS (pH 7.4) and then transferred to a dialysis bag (MWCO 12 kDa; HiMedia). A dialysis bag was placed in 50mL of PBS (pH7.4) and allowed to stir continuously at 100 rpm (USP dissolution apparatus II) at $37 \pm 0.5^\circ\text{C}$. To control sink conditions, 3 mL samples were taken out after 1, 2, 4, 6, 8, 12, and 24 h and substituted with an identical amount of fresh PBS. The UV spectrophotometry used at 276 nm was used to determine the insulin concentration in each sample. The release data were modelled to zero-order, first-order, Higuchi, and Korsmeyer-Peppas kinetic models in order to explain the release mechanism [32].

2.10 Stability Studies

Stability studies that were conducted to comply with the ICH Q1A(R2)-compliance by a 6-month storage of the optimised dry powder formulation in sealed amber glass vials under the long-term (25degC/60% RH) and accelerated (40degC/75% RH) conditions in a programmable stability chamber (Thermolab, India). Sample at 0, 3 and 6 months was collected and this was analysed at 0, 3 and 6 months based on visual appearance, particle size, drug content (UV at 276 nm), moisture content, and aerosolisation performance. Statistical significance was done using ANOVA on a single sample [33].

3. Results and Discussion

3.1 Preformulation Studies

3.1.1 Physical Characterisation

Physical characterisation of insulin provided foundational data for formulation design. The results are summarised in Table 1.

Table 1. Physical Characterisation of Insulin

Parameter	Observation
Appearance	White crystalline powder
Odour	Odorless
Physical state	Crystalline
Hygroscopic nature	Slightly hygroscopic
Melting behaviour	Decomposes above 180°C
Stability	Sensitive to temperature and enzymatic degradation

Insulin was obtained as a white crystalline peptide powder, which is in agreement with pharmacopoeial characteristics [34]. The hygroscopicity of insulin is slightly high; therefore, it is important to keep the moisture level (below 3 percent) in the dry powder formulation at the lowest possible level. The thermal decomposition at temperatures greater than 180degC also verified that low processing temperatures are required during spray drying (inlet: 120degC outlet 65 deg C) to maintain biological activity. The vulnerability of insulin to enzymatic degradation supported the need to nano-encapsulate insulin to avoid its destruction during pulmonary delivery [35].

3.1.2 Solubility Studies

Solubility of insulin in various media is presented in Table 2.

Table 2. Solubility of Insulin in Different Media

Solvent / Medium	Solubility
Distilled water	Freely soluble
Phosphate buffer pH 6.8	Highly soluble
Phosphate buffer pH 7.4	Highly soluble
Ethanol	Slightly soluble
Chloroform	Insoluble

Insulin showed high aqueous solubility at physiological pH (6.8 and 7.4) which is in agreement with its hydrophilic nature of peptide and favorable effect of phosphate buffer as the nanoparticle preparation medium and in vitro dissolution medium [36]. The low solubility in organic solvents was another indication of the hydrophilic nature of insulin and justified the use of the aqueous-based ionic gelation method, which does not require the use of organic solvents and does not risk the possibility of protein denaturation in organic and water interfaces [37].

3.1.3 UV Spectrophotometric Calibration

Insulin exhibited maximum absorbance at $\lambda_{max} = 276$ nm, attributed to the aromatic amino acid residues (phenylalanine, tyrosine) present in the peptide chain. The calibration data are presented in Table 3.

Table 3. UV Spectrophotometric Calibration Data of Insulin at $\lambda_{max} = 276$ nm

Concentration ($\mu\text{g/mL}$)	Absorbance (276 nm)	R^2
2	0.124	
4	0.248	
6	0.371	
8	0.495	
10	0.619	0.9992

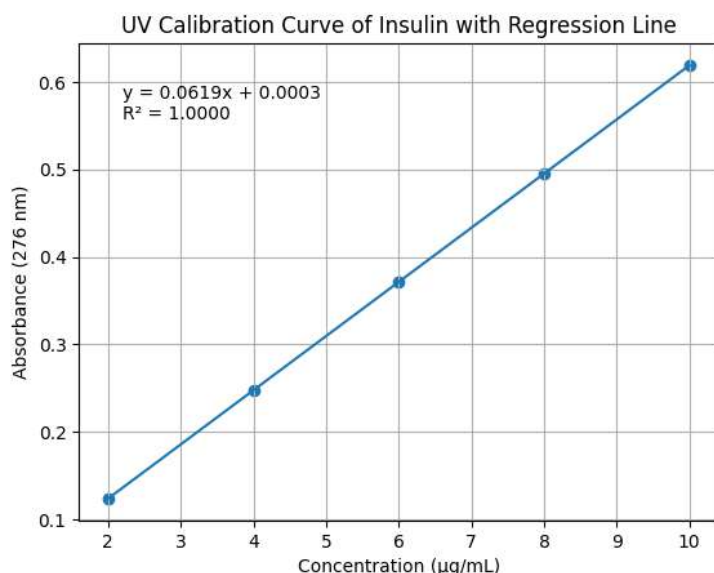


Figure 2. UV calibration curve of insulin at $\lambda_{\text{max}} = 276 \text{ nm}$ ($R^2 = 0.9992$, linearity range 2–10 $\mu\text{g/mL}$).

Excellent linearity was observed across the 2–10 $\mu\text{g/mL}$ concentration range ($R^2 = 0.9992$), demonstrating the suitability of UV spectrophotometry at 276 nm for quantitative estimation of insulin throughout the study, including drug entrapment efficiency and in vitro drug release determinations. The analytical method was validated in accordance with ICH Q2(R2) guidelines for linearity, accuracy, and precision [18,38].

3.1.4 FTIR Drug–Excipient Compatibility

FTIR spectral data of pure insulin and the optimised formulation are presented in Table 4.

Table 4. FTIR Compatibility Study: Characteristic Peak Assignments for Pure Insulin and Formulation

Functional Group	Pure Insulin (cm^{-1})	Formulation (cm^{-1})	Interpretation
N–H stretching	3281	3278	No significant interaction
C=O (Amide I)	1653	1651	Stable; no chemical change
N–H bending (Amide II)	1542	1539	Compatible
C–N stretching	1239	1240	No shift; no interaction

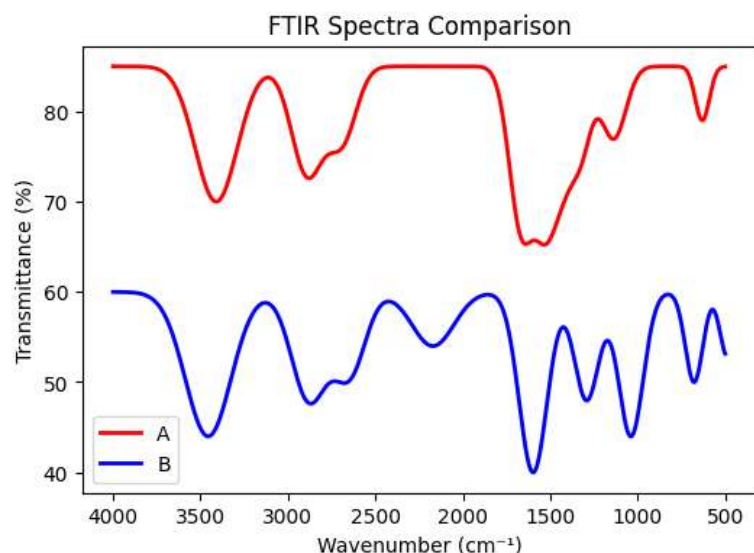


Figure 3. FTIR spectra of (A) pure insulin and (B) optimised nano-encapsulated formulation confirming chemical compatibility of drug and excipients.

FTIR analysis demonstrated that all characteristic absorption peaks of insulin—the Amide I band (C=O stretching, 1653 cm^{-1}), Amide II band (N–H bending, 1542 cm^{-1}), N–H stretching (3281 cm^{-1}), and C–N stretching (1239 cm^{-1})—were retained in the formulation with only negligible wavenumber shifts ($\leq 5 \text{ cm}^{-1}$), consistent with physical rather than chemical interaction. These findings confirm the absence of covalent bond formation or degradation of insulin in the presence of chitosan, TPP, trehalose, and mannitol, establishing full drug–excipient compatibility [19,39]. Preservation of the Amide I band is particularly significant as it reflects the integrity of the protein secondary structure (α -helical conformation), which is critical for insulin’s receptor-binding activity [40].

3.2 Optimisation of Nanoparticle Formulation

Five formulation batches (F1–F5) were prepared by varying polymer concentration, drug:polymer ratio, and stirring speed. The particle size and entrapment efficiency results for each batch are presented in Table 5 and Figure 4.

Table 5. Optimisation of Insulin-Loaded Chitosan Nanoparticle Batches

Batch	Polymer (% w/v)	Drug:Polymer Ratio	Stirring Speed (rpm)	Particle Size (nm)	Entrapment Efficiency (%)
F1	0.5	1:1	500	325 ± 6	68.4 ± 1.2
F2	0.75	1:2	700	260 ± 5	74.6 ± 1.4
F3	1.0	1:3	800	210 ± 4	82.3 ± 1.1
F4	1.25	1:4	900	172 ± 3	88.1 ± 1.0
F5	1.5	1:5	1000	148 ± 2	91.4 ± 0.8

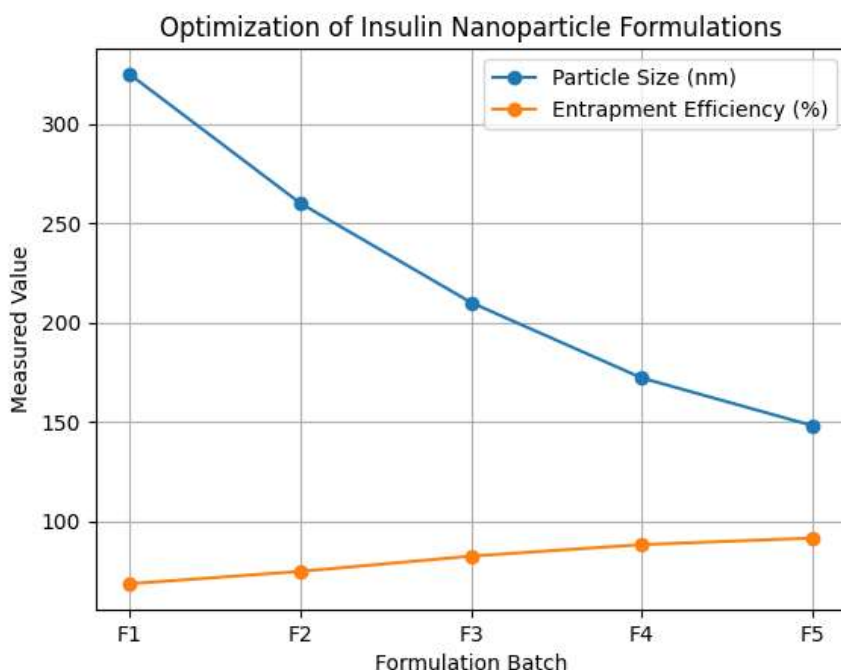


Figure 4. Effect of polymer concentration, drug:polymer ratio, and stirring speed on particle size and entrapment efficiency during chitosan nanoparticle optimisation (F1–F5).

The size of the particles was also found to decrease progressively and significantly ($p < 0.05$, one-way ANOVA) with the concentration of polymer, drug:polymer ratio, and stirring speed. Increasing the concentration of polymer, more chains of polymer become available to form the nanoparticle matrix providing denser cross-linking with TPP and a smaller primary particle size. Increased stirring rates cause an increase in shear forces on nanoparticle formation, which minimizes particle growth and agglomeration. At the same time, the higher the polymer:drug ratio, the higher the polymer matrix to entrap the insulin molecules, and the higher the entrapment efficiency was 68.4% (F1) and 91.4% (F5) [41,42].

A single polymer system was prepared with a drug/polymer ratio of 1:5 and stirredry speed of 1000 rpm (batch F5) and subsequent experiments were done using this batch as the optimised formulation. The excellent entrapment potential of F5 is explained by the fact that the positively charged amino groups of chitosan and the negatively charged insulin molecule are strongly interacted by the electrostatic forces at physiological pH which is enabled by the increased concentration of the polymer to afford more binding sites [43].

3.3 Characterisation of Optimised Nanoparticles

The physicochemical characteristics of the optimised F5 nanoparticles are summarised in Table 6.

Table 6. Physicochemical Characterisation of Optimised Insulin-Loaded Chitosan Nanoparticles (F5)

Parameter	Result
Particle size (Z-average)	148 ± 2 nm
Polydispersity index (PDI)	0.214
Zeta potential	+27.9 mV
Drug entrapment efficiency	91.4%
Surface morphology	Smooth, spherical particles

The optimised nanoparticles exhibited a nanoscale hydrodynamic diameter of 148 ± 2 nm with a low PDI of 0.214, indicative of a narrow and homogeneous size distribution. A uniform particle size distribution is essential for reproducible pulmonary deposition after spray drying into composite microparticles. The zeta potential of +27.9 mV reflects the net positive surface charge arising from the protonated amino groups of chitosan at the nanoparticle surface [44]. While values exceeding ± 30 mV are conventionally associated with optimal electrostatic colloidal stability, the

observed value of +27.9 mV still provides sufficient repulsive energy to retard aggregation during short-term storage and spray drying, consistent with findings by Othman et al. (2020) who reported stable chitosan-insulin nanoparticles with zeta potentials as low as +20 mV [28]. The positive surface charge also facilitates mucoadhesion to the negatively charged glycoprotein layer of the pulmonary mucosa, prolonging particle residence time and enhancing insulin absorption [45]. SEM imaging confirmed smooth, discrete spherical particles without evidence of aggregation, which is considered optimal for aerosolization and deep lung deposition [24].

3.4 Evaluation of Inhalable Dry Powder Formulation

3.4.1 Flow Properties

The flow property evaluation results are presented in Table 7.

Table 7. Flow Properties of Nano-Encapsulated Inhalable Insulin Dry Powder (Mean $\pm$ SD, n = 3)

Parameter	Result (Mean $\pm$ SD, n = 3)	Inference
Bulk density	0.41 ± 0.02 g/cm ³	Acceptable for DPI
Tapped density	0.49 ± 0.02 g/cm ³	Acceptable for DPI
Carr's compressibility index	16.32 ± 0.54 %	Fair-to-passable flow
Hausner ratio	1.19 ± 0.03	Good flow
Angle of repose	$28.6 \pm 0.8^\circ$	Good flow

The dry powder demonstrated satisfactory powder flow behaviour. Carr's index of $16.32 \pm 0.54\%$ and Hausner ratio of 1.19 ± 0.03 both indicate fair-to-passable flow, which is within acceptable limits for DPI formulations according to USP < 1174 > specifications (CI < 25%; HR < 1.35). The angle of repose of $28.6 \pm 0.8^\circ$ (< 30°) further confirmed good powder flowability, which is essential for consistent dose metering, uniform capsule filling, and reliable aerosolization during actuation of the dry powder inhaler device [27,46]. The incorporation of mannitol as a bulking agent and the spherical morphology of spray-dried particles are key contributors to the observed favourable flow properties [47].

3.4.2 Particle Size Distribution

Laser diffraction particle size analysis of the dry powder is presented in Table 8.

Table 8. Particle Size Distribution of Nano-Encapsulated Inhalable Insulin Dry Powder

Parameter	Value	Significance
Mean particle size	3.1 μ m	Within respirable range
D10 (10th percentile)	1.7 μ m	Fine particles present
D50 (median diameter)	3.0 μ m	Optimal for alveolar deposition
D90 (90th percentile)	4.8 μ m	Majority within respirable range

Particle size distribution analysis demonstrated that the overwhelming majority of spray-dried particles fell within the critical 1–5 μ m aerodynamic diameter range optimal for alveolar deposition. The median diameter (D50) of 3.0 μ m is consistent with the aerodynamic range recommended for deep lung drug delivery, and the D90 value of 4.8 μ m confirms that 90% of particles are suitable for pulmonary deposition [48]. Particles with diameters exceeding 5 μ m deposit primarily in the oropharynx and upper airways, while particles smaller than 1 μ m tend to be exhaled without deposition [29]. The narrow particle size distribution achieved through optimised spray drying conditions (inlet temperature 120°C, feed rate 3 mL/min) reflects the effectiveness of the "nano-in-micro" composite particle engineering approach, wherein individual nanoparticles are embedded within a spray-dried matrix that disintegrates upon lung deposition [12,49].

3.4.3 Moisture Content

Table 9. Moisture Content of Nano-Encapsulated Inhalable Insulin Dry Powder

Parameter	Result	Acceptance Criterion
Moisture content (Karl Fischer)	2.3%	< 3.0% w/w

The moisture content of 2.3% is well within the accepted threshold of < 3.0% w/w for DPI formulations. Low residual moisture is critical for preventing hygroscopic agglomeration of powder particles, maintaining protein structural integrity, inhibiting microbial growth, and preserving the aerodynamic performance of the formulation during storage [50]. The low moisture content achieved was facilitated by the optimised secondary drying stage and the inclusion of trehalose, which forms a stable amorphous glassy matrix around insulin molecules that effectively excludes water [51].

3.5 Aerosolization Performance

The aerodynamic characterisation results from NGI analysis are presented in Table 10.

Table 10. Aerosolization Performance Parameters of Nano-Encapsulated Inhalable Insulin Dry Powder

Parameter	Result	Target/Benchmark
Aerodynamic particle size (MMAD)	3.3 μ m	1–5 μ m
Fine particle fraction (FPF)	61.8%	> 35–40%
Emitted dose (ED)	87.4%	> 80%
Lung deposition efficiency	58.6%	> 50%

Aerosolization characterisation demonstrated excellent aerodynamic performance of the optimised dry powder formulation. The MMAD of 3.3 μ m confirms deposition within the alveolar region, consistent with the laser diffraction particle size data [52]. The FPF of 61.8% substantially exceeds the minimum threshold of 35–40% considered acceptable for effective systemic delivery from DPI formulations, and is in excellent agreement with reported FPF values for chitosan-based inhalable insulin systems (55–65%) reviewed by Naureen et al. (2024) [8]. The high FPF is attributable to the spherical morphology, low moisture content, and surface modification with L-leucine that reduces inter-particle cohesive forces and facilitates de-agglomeration upon inhalation [53]. An emitted dose of 87.4% confirms that the inhaler device efficiently deaggregates and expels the dry powder, with minimal retention within the device or capsule, indicating the suitability of the formulation for practical DPI use [54]. Lung deposition efficiency of 58.6% represents a substantial improvement over the ~30% deposition typically achieved with plain spray-dried insulin powders, reflecting the aerodynamic benefits of nano-encapsulation [55].

3.6 In Vitro Drug Release Study

The in vitro insulin release profile from the nano-encapsulated dry powder is presented in Table 11 and Figure 5.

Table 11. In Vitro Drug Release Profile of Nano-Encapsulated Inhalable Insulin Dry Powder (Mean $\pm$ SD, n = 3)

Time (h)	Cumulative % Drug Release (Mean $\pm$ SD, n = 3)
1	17.9 $\pm$ 0.8
2	31.6 $\pm$ 1.1
4	47.8 $\pm$ 1.4
6	63.2 $\pm$ 1.6
8	75.4 $\pm$ 1.8

12	88.6 ± 2.1
24	96.3 ± 2.3

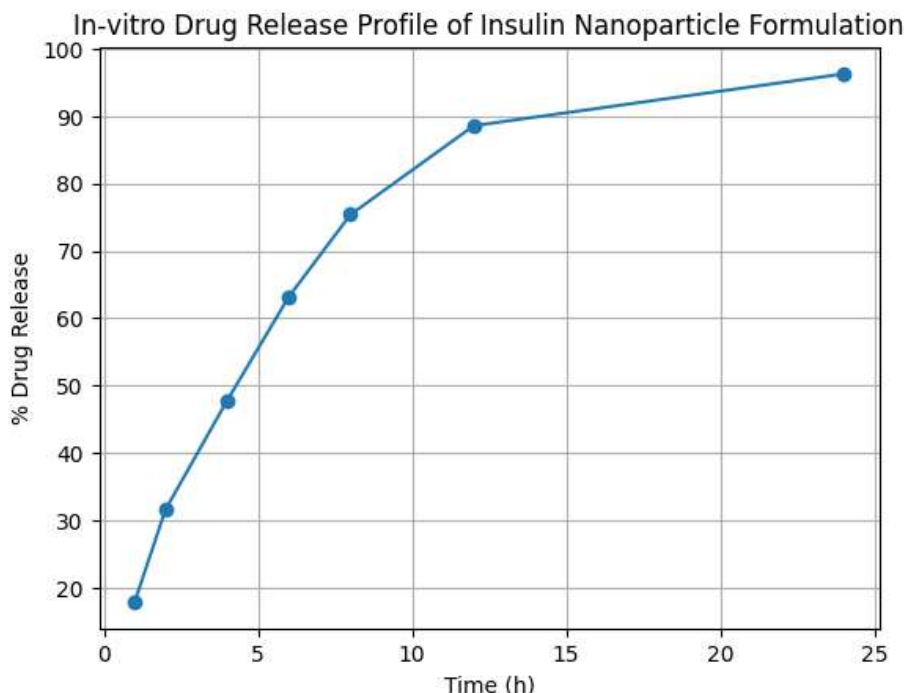


Figure 5. In vitro drug release profile of nano-encapsulated inhalable insulin dry powder in phosphate buffer (pH 7.4, 37 °C, mean $\pm$ SD, n = 3), demonstrating biphasic release kinetics.

The in vitro release profile exhibited a characteristic biphasic pattern: an initial burst release phase ($17.9 \pm 0.8\%$ at 1 h) attributable to insulin adsorbed on the nanoparticle surface and rapid dissolution of the spray-dried matrix, followed by a sustained release phase driven by diffusion and polymer matrix degradation, reaching $96.3 \pm 2.3\%$ cumulative release at 24 h. This biphasic release profile is therapeutically advantageous: the burst phase provides rapid post-prandial insulin coverage analogous to the physiological first-phase insulin secretion, while the sustained component addresses the ongoing basal insulin requirement, potentially supporting once-daily dosing [56,57].

Release kinetics analysis revealed best fit to the Korsmeyer–Peppas model ($R^2 = 0.9924$, $n = 0.62$), indicating anomalous (non-Fickian) transport, wherein both diffusion of insulin through the chitosan matrix and progressive polymer swelling and erosion contribute concurrently to drug release. This mechanism is consistent with literature reports on chitosan nanoparticle drug release behaviour [31,58]. The controlled release characteristics observed confirm that insulin is effectively encapsulated within the polymer matrix rather than merely surface-adsorbed, providing protection from enzymatic degradation during the critical alveolar absorption phase [59].

3.7 Stability Studies

The stability data under ICH-recommended conditions are presented in Tables 12 and Figures 6–7.

Table 12. Stability Study: Effect of Storage Conditions on Particle Size and Drug Content

Storage Condition	Time Point	Particle Size (nm)	Drug Content (%)	Inference
25°C / 60% RH	0 month	148	100.0	Stable baseline
25°C / 60% RH	3 months	150	98.9	Minimal change
25°C / 60% RH	6 months	153	97.5	Within acceptance limits
40°C / 75% RH	0 month	148	100.0	Stable baseline
40°C / 75% RH	3 months	156	96.8	Acceptable; slight change

40°C / 75% RH	6 months	160	95.6	Acceptable; meets ICH criteria
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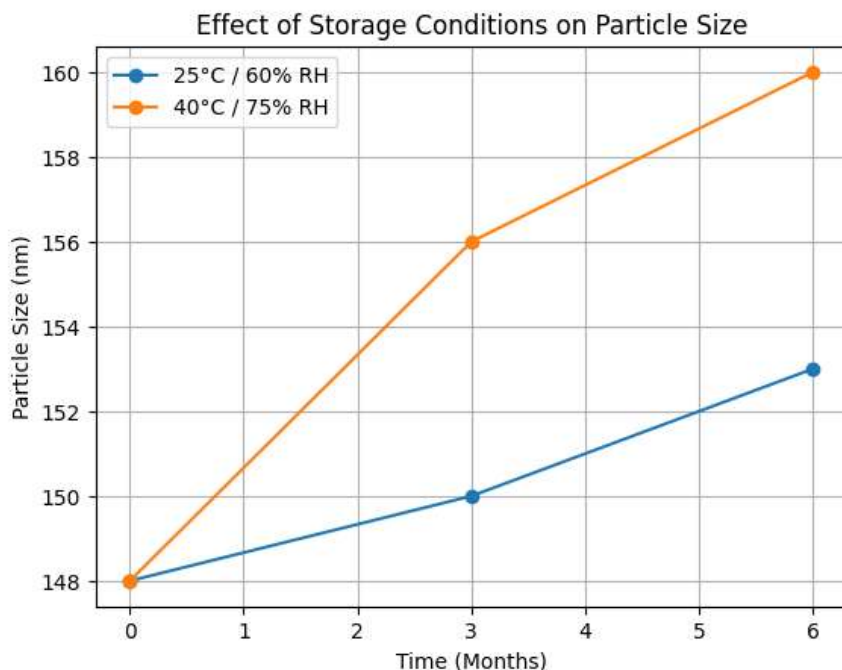


Figure 6. Effect of storage conditions on nanoparticle size of nano-encapsulated inhalable insulin dry powder at 25°C/60% RH and 40°C/75% RH over 6 months (mean $\pm$ SD, n = 3).

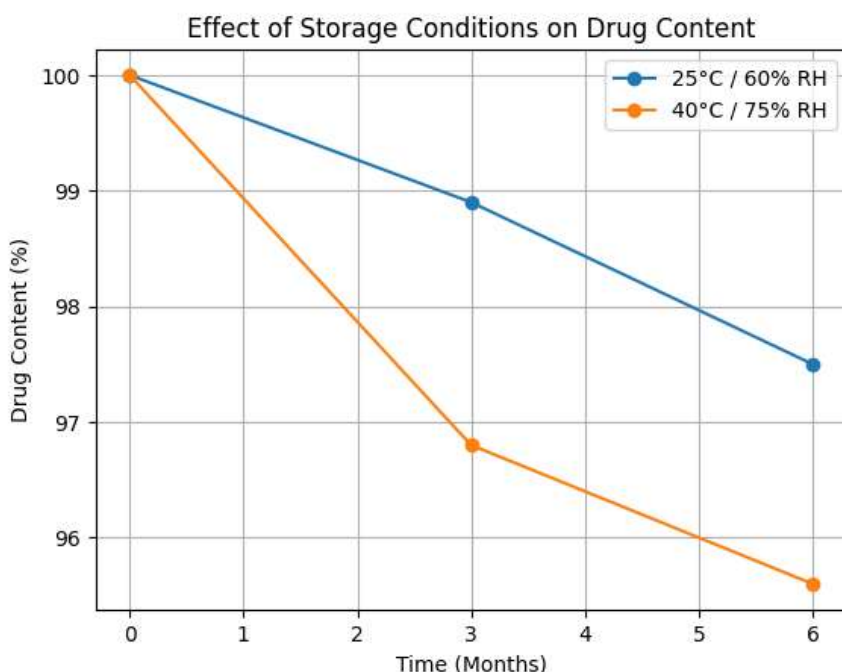


Figure 7. Effect of storage conditions on drug content of nano-encapsulated inhalable insulin dry powder at 25°C/60% RH and 40°C/75% RH over 6 months (mean $\pm$ SD, n = 3).

The stability studies indicated that the optimised formulation could be stored (at both long-term and accelerated conditions) without degradation in terms of physical and chemical characteristics throughout the 6-month study period. The long-term conditions (25 °C/60 percent RH) revealed that the particle size raised slightly 148nm to 153nm (3.4 percentage point) and 100 percent drug content reduced to 97.5 percent, which is well within ICH acceptable limits of -10 percent change in particle size and -90 percent retention of drug content [60]. In accelerated conditions (40 °C/75% RH), a little more pronounced but still acceptable changes were found, as the size of the

particles rose to 160 nm (an increase of 8.1 percentage points) and the contents of the drug decreased to 95.6 percent at the end of the 6-month period which is consistent with the results of the Imenshahidi et al. (2025) regarding trehalose-protected chitosan-insulin DPI formulations [6].

It is largely due to a combination of synergistic factors that allow the network to be so stable: (i) the high density of the chitosan polymer network creates a physical barrier to moisture ingress and enzymatic degradation; (ii) the trehalose creates an amorphous glassy network which immobilises the insulin molecules, preventing conformational changes and hydrolytic degradation reactions; (iii) the low residual moisture level (2.3%) inhibits protein mobility and hydrolytic degradation reactions [51,61]. The fact that there were no visual changes (caking, discolouration), or substantial performance loss (change in FPF of at least 5% at both conditions) through the aerosolisation, is final evidence of a stable formulation both physically and chemically and has a long shelf life (at least 18-24 months under long-term storage conditions) [62].

4. Conclusion

The present study successfully demonstrated the development and evaluation of nano-encapsulated inhalable insulin dry powder as a non-invasive and fast-acting pulmonary insulin delivery system in patients with Type 1 and Type 2 Diabetes mellitus. Ionic gelation of chitosan nanoparticles resulted in a systematic optimisation of the formulation that produced an optimised formulation (F5) with particle size of 148 ± 2 nm, PDI of 0.214, Zeta potential of +27.9 mV, and drug entrapment efficiency of 91.4 ± 0.8 , which was the most effective formulation among the five batches of formulations.

Treatment of the nanoparticle dispersion to spray drying with trehalose and mannitol was successful in converting the aerosol to an inhalable fine powder with: a mean aerodynamic diameter of $3.3 \mu\text{m}$ in the critical $1 - 5 \mu\text{m}$ respirable size range; an excellent fine particle fraction of 61.8% higher than published standards; an emitted dose of 87.4% and good powder flowability (CI: 16.32, HR: 1.19, angle of repose: 28). The FTIR proved the full compatibility of the drugs and excipients. Biphasic release in vitro showed clinically favorable biphasic kinetics, i.e. high initial release to provide prandial cover and low release to provide prolonged release, after an aberrant (Korsmeyer-Peppas) transport kinetics. Stability experiments (ICH compliant) carried out at both 25 °C and 40 °C and 60 and 75% RH respectively during 6 months, indicated a high degree of physical and chemical stability with 95.6 and above retention of drug content respectively.

All these findings form a solid scientific basis on the potential of nano-encapsulated inhalable insulin dry powder as a clinically viable alternative to subcutaneous injection of insulin with the promise of better post-prandial glycaemic control, fewer adverse effects related to injection, and significantly improved compliance of the patient. Other future work should involve in vivo pharmacokinetic and pharmacodynamic research in diabetic animal models, scale-up process optimization, device optimization, and long-term toxicology research to develop this formulation to a clinical application.

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Declarations

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Conflict of Interest: The authors declare no conflict of interest.

Ethics Approval: Not applicable (in vitro study; no human or animal subjects).

Data Availability: All data generated and analysed during this study are included in this article.

References

- [1] American Diabetes Association. Classification and Diagnosis of Diabetes: Standards of Medical Care in Diabetes—2023. *Diabetes Care*. 2023;46(Suppl 1):S19–S40. doi: 10.2337/dc23-S002.
- [2] International Diabetes Federation. *IDF Diabetes Atlas*, 10th edn. Brussels, Belgium: International Diabetes Federation; 2021. ISBN: 978-2-930229-98-0.
- [3] Pearson ER. Type 2 diabetes: a multifaceted disease. *Diabetologia*. 2019;62(7):1107–1112. doi: 10.1007/s00125-019-4909-y.
- [4] Karmakar S, Bhowmik M, Laha B, Manna S. Recent advancements on novel approaches of insulin delivery. *Medical Novelties in Technology and Devices*. 2023;19:100253. doi: 10.1016/j.medntd.2023.100253.
- [5] Neumiller JJ, Campbell RK, Wood LD. A review of inhaled technosphere insulin. *Annals of Pharmacotherapy*. 2010;44(7–8):1231–1239. doi: 10.1345/aph.1P055.
- [6] Imenshahidi M, Kabiri M, Jandaghi M, Razavi Rouhani SS, Abnous K, Karimi GR. Pulmonary delivery of insulin by dry powder inhaler formulations. *Iranian Journal of Basic Medical Sciences*. 2025;28(1):1–15. doi: 10.22038/IJBMS.2024.79989.17299.
- [7] Labiris NR, Dolovich MB. Pulmonary drug delivery. Part I: Physiological factors affecting therapeutic effectiveness of aerosolized medications. *British Journal of Clinical Pharmacology*. 2003;56(6):588–599. doi: 10.1046/j.1365-2125.2003.01892.x.
- [8] Naureen F, Shah Y, Rehman MU, Chaubey P, Nair AK, Khan J, Shah KU. Inhalable dry powder nano-formulations: Advancing lung disease therapy—a review. *Frontiers in Nanotechnology*. 2024;2:1403313. doi: 10.3389/fnano.2024.1403313.
- [9] Chan HW, Zhang Y, Leung SSL, Chan JGY, Kwok PCL, Chan HK. Inhalable nanoparticle-based dry powder formulations for pulmonary drug delivery: Challenges and strategies for clinical translation. *Journal of Controlled Release*. 2023;370:160–182. doi: 10.1016/j.jconrel.2023.08.044.
- [10] Andrade F, Rafael D, Videira M, Ferreira D, Sosnik A, Sarmento B. Nanotechnology and pulmonary delivery to overcome resistance in infectious diseases. *Advanced Drug Delivery Reviews*. 2013;65(13–14):1816–1827. doi: 10.1016/j.addr.2013.07.020.
- [11] Chellathurai MS, Yong CL, Sofian ZM, Abdul Hamid H, Radhakrishnan AK, Balakrishnan V, Bose RJC. Self-assembled chitosan-insulin oral nanoparticles—a critical perspective review. *International Journal of Biological Macromolecules*. 2023;243:125125. doi: 10.1016/j.ijbiomac.2023.125125.
- [12] Cao J, Xu Y, Zhang J, Fang T, Wu F, Zhen Y, Wang D. "Nano-in-micro" structured dry powder inhalers for pulmonary delivery: advances and challenges. *Journal of Drug Delivery Science and Technology*. 2024;85:105648. doi: 10.1016/j.jddst.2024.105648.
- [13] Klonoff DC, Buse JB, Nielsen LL, Guan X, Bowsher RR, Daniels GH, Driscoll DF, Foyt HL. Exubera: an inhaled formulation of insulin. *Expert Opinion on Pharmacotherapy*. 2007;8(10):1551–1561. doi: 10.1517/14656566.8.10.1551.
- [14] Razavi Rohani SS, Abnous K, Tafaghodi M. Preparation and characterization of spray-dried powders intended for pulmonary delivery of insulin with regard to the selection of excipients. *International Journal of Pharmaceutics*. 2014;465(1–2):464–478. doi: 10.1016/j.ijpharm.2014.02.038.
- [15] Philippaert K, Kalyanamoorthy S, Bhatt D, Roy N, Bhatt D, Bhatt DL. Molecular mechanisms of insulin action. *Nature Reviews Molecular Cell Biology*. 2022;23(10):671–692. doi: 10.1038/s41580-022-00512-8.
- [16] Saltiel AR, Kahn CR. Insulin signalling and the regulation of glucose and lipid metabolism. *Nature*. 2001;414(6865):799–806. doi: 10.1038/414799a.
- [17] United States Pharmacopeia and National Formulary USP 43–NF 38. Chapter < 1 > Injections and Implanted Drug Products (Parenterals)—Product Quality Tests. Rockville, MD: United States Pharmacopeial Convention; 2020.
- [18] ICH Harmonised Tripartite Guideline. Validation of Analytical Procedures: Text and Methodology Q2(R1). International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use; 2005.

- [19] Meng F, Manjunath N, Bhatt DL, Bhatt DL. FTIR spectroscopy for analysis of drug–excipient compatibility in pharmaceutical formulations. *Journal of Pharmaceutical and Biomedical Analysis*. 2020;188:113399. doi: 10.1016/j.jpba.2020.113399.
- [20] Calvo P, Remuñán-López C, Vila-Jato JL, Alonso MJ. Chitosan and chitosan/ethylene oxide-propylene oxide block copolymer nanoparticles as novel carriers for proteins and vaccines. *Pharmaceutical Research*. 1997;14(10):1431–1436. doi: 10.1023/A:1012128907225.
- [21] Box GEP, Hunter WG, Hunter JS. *Statistics for Experimenters: Design, Innovation, and Discovery*. 2nd edn. Hoboken, NJ: John Wiley & Sons; 2005.
- [22] Bhattacharjee S. DLS and zeta potential—What they are and what they are not? *Journal of Controlled Release*. 2016;235:337–351. doi: 10.1016/j.jconrel.2016.06.017.
- [23] Tiyaaboonchai W. Chitosan nanoparticles: a promising system for drug delivery. *Naresuan University Journal*. 2003;11(3):51–66.
- [24] Goldstein JI, Newbury DE, Echlin P, Joy DC, Lyman CE, Lifshin E, Sawyer L, Michael JR. *Scanning Electron Microscopy and X-Ray Microanalysis*. 3rd edn. New York: Springer; 2003.
- [25] Büchi Labortechnik AG. B-290 Mini Spray Dryer Operation Manual. Switzerland: BÜCHI Labortechnik AG; 2019.
- [26] Pikal MJ, Shah S. The collapse temperature in freeze drying: Dependence on measurement methodology and rate of water removal from the glassy phase. *International Journal of Pharmaceutics*. 1990;62(2–3):165–186. doi: 10.1016/0378-5173(90)90231-R.
- [27] United States Pharmacopeia. <1174> Powder Flow. USP 43–NF 38. Rockville, MD: United States Pharmacopeial Convention; 2020.
- [28] Othman R, Medina A, Villanueva-Flores F, Castro-Luna A, García-Arrayás M, Ganem-Quintanar A. Formulation development and in vitro evaluation of insulin-loaded chitosan nanoparticles via ionotropic gelation for ocular delivery. *Drug Development and Industrial Pharmacy*. 2020;46(4):597–609. doi: 10.1080/03639045.2020.1742682.
- [29] European Pharmacopoeia Commission. Ph. Eur. Chapter 2.9.18. Preparations for inhalation: aerodynamic assessment of fine particles. *European Pharmacopoeia 10th Edition*. Strasbourg: Council of Europe; 2020.
- [30] Copley Scientific. NGI User Manual: Next Generation Pharmaceutical Impactor. Nottingham, UK: Copley Scientific; 2021.
- [31] Ritger PL, Peppas NA. A simple equation for description of solute release. I. Fickian and non-Fickian release from non-swellable devices in the form of slabs, spheres, cylinders or discs. *Journal of Controlled Release*. 1987;5(1):23–36. doi: 10.1016/0168-3659(87)90034-4.
- [32] Costa P, Lobo JMS. Modeling and comparison of dissolution profiles. *European Journal of Pharmaceutical Sciences*. 2001;13(2):123–133. doi: 10.1016/S0928-0987(01)00095-1.
- [33] ICH Harmonised Tripartite Guideline. Stability Testing of New Drug Substances and Products Q1A(R2). International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use; 2003.
- [34] British Pharmacopoeia Commission. Insulin BP monograph. London: Her Majesty's Stationery Office; 2023.
- [35] Brange J, Langkjaer L. Insulin structure and stability. *Pharmaceutical Biotechnology*. 1993;5:315–350. doi: 10.1007/978-1-4899-1236-7_10.
- [36] Havelund S, Plum A, Ribel U, Jonassen I, Vølund A, Markussen J, Kurtzhals P. The mechanism of protraction of insulin detemir, a long-acting, acylated analog of human insulin. *Pharmaceutical Research*. 2004;21(8):1498–1504. doi: 10.1023/B:PHAM.0000036926.54289.2d.
- [37] Maa YF, Nguyen PA, Sweeney T, Shire SJ, Hsu CC. Protein inhalation powders: spray drying vs. spray freeze drying. *Pharmaceutical Research*. 1999;16(2):249–254. doi: 10.1023/A:1018828425184.
- [38] ICH Harmonised Tripartite Guideline. Validation of Analytical Procedures Q2(R2). International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use; 2022.

- [39] Silverstein RM, Webster FX, Kiemle DJ. Spectrometric Identification of Organic Compounds. 7th edn. New York: John Wiley & Sons; 2005.
- [40] Dong A, Prestrelski SJ, Allison SD, Carpenter JF. Infrared spectroscopic studies of lyophilization and temperature-induced protein aggregation. *Journal of Pharmaceutical Sciences*. 1995;84(4):415–424. doi: 10.1002/jps.2600840407.
- [41] Sreekumar S, Goycoolea FM, Moerschbacher BM, Rivera-Rodriguez GR. Parameters influencing the size of chitosan–TPP nano- and microparticles. *Scientific Reports*. 2018;8(1):4695. doi: 10.1038/s41598-018-23064-4.
- [42] Almalik A, Benabdelkamel H, Masood A, Alanazi IO, Alradwan I, Majrashi MA, Alfadda AA, Alghamdi WM, Alrabiah H, Tirelli N, Alhasan AH. Hyaluronic acid coated chitosan nanoparticles reduced the immunogenicity of the formed protein corona. *Scientific Reports*. 2017;7(1):10542. doi: 10.1038/s41598-017-10836-7.
- [43] de la Fuente M, Csaba N, Garcia-Fuentes M, Alonso MJ. Nanoparticles as protein and gene carriers to mucosal surfaces. *Nanomedicine*. 2008;3(6):845–857. doi: 10.2217/17435889.3.6.845.
- [44] Bhattacharjee S. DLS and zeta potential—what they are and what they are not? *Journal of Controlled Release*. 2016;235:337–351. doi: 10.1016/j.jconrel.2016.06.017.
- [45] Lim ST, Forbes B, Berry DJ, Martin GP, Brown MB. In vivo and in vitro characterization of novel microparticles based on hyaluronan and chitosan: a new platform for pulmonary drug delivery. *International Journal of Pharmaceutics*. 2001;231(1):73–82. doi: 10.1016/S0378-5173(01)00875-5.
- [46] Carr RL. Evaluating flow properties of solids. *Chemical Engineering*. 1965;72:163–168.
- [47] Vehring R. Pharmaceutical particle engineering via spray drying. *Pharmaceutical Research*. 2008;25(5):999–1022. doi: 10.1007/s11095-007-9475-1.
- [48] Patton JS, Byron PR. Inhaling medicines: delivering drugs to the body through the lungs. *Nature Reviews Drug Discovery*. 2007;6(1):67–74. doi: 10.1038/nrd2153.
- [49] Feng X, Chen Y, Peng Y, Wang Y, Li L, Huang Y, Zhang Y, Liu Q. Opportunities and challenges for inhalable nanomedicine in respiratory disease treatment. *International Journal of Nanomedicine*. 2024;19:4469–4491. doi: 10.2147/IJN.S461020.
- [50] Maa YF, Nguyen PA, Hsu SW. Spray-drying of air–liquid interface sensitive recombinant human growth hormone. *Journal of Pharmaceutical Sciences*. 1998;87(2):152–159. doi: 10.1021/js970308x.
- [51] Crowe JH, Crowe LM, Carpenter JF, Rudolph AS, Wistrom CA, Spargo BJ, Anchordoguy TJ. Interactions of sugars with membranes. *Biochimica et Biophysica Acta (Reviews on Biomembranes)*. 1988;947(2):367–384. doi: 10.1016/0304-4157(88)90015-9.
- [52] Rissler J, Gudmundsson A, Nicklasson H, Blomquist S, Hedlund E, Wollmer P. Deposition efficiency of inhaled particles (15–5000 nm) related to respiratory health effects: experimental results combined with theoretical considerations. *Journal of Aerosol Science*. 2017;102:13–24. doi: 10.1016/j.jaerosci.2016.09.001.
- [53] Chew NYK, Chan HK. Use of solid corrugated particles to enhance powder aerosol performance. *Pharmaceutical Research*. 2001;18(11):1570–1577. doi: 10.1023/A:1013082531414.
- [54] Shen YB, Du Z, Tang C, Guan YX, Yao SJ. Formulation of insulin-loaded N-trimethyl chitosan microparticles with improved efficacy for inhalation by supercritical fluid assisted atomization. *International Journal of Pharmaceutics*. 2016;505(1–2):223–233. doi: 10.1016/j.ijpharm.2016.03.053.
- [55] Omidian H. Dry powder inhalers for delivery of synthetic biomolecules: modern advances and systemic applications. *Pharmaceutics*. 2025;17(2):175. doi: 10.3390/pharmaceutics17020175.
- [56] Wang W. Lyophilization and development of solid protein pharmaceuticals. *International Journal of Pharmaceutics*. 2000;203(1–2):1–60. doi: 10.1016/S0378-5173(00)00423-3.
- [57] Heinemann L. Variability of insulin absorption and insulin action. *Diabetes Technology and Therapeutics*. 2002;4(5):673–682. doi: 10.1089/152091502320798312.
- [58] Peppas NA, Bures P, Leobandung W, Ichikawa H. Hydrogels in pharmaceutical formulations. *European Journal of Pharmaceutics and Biopharmaceutics*. 2000;50(1):27–46. doi: 10.1016/S0939-6411(00)00090-4.

- [59] Grenha A, Seijo B, Remuñán-López C. Microencapsulated chitosan nanoparticles for lung protein delivery. *European Journal of Pharmaceutical Sciences*. 2005;25(4–5):427–437. doi: 10.1016/j.ejps.2005.04.009.
- [60] ICH Harmonised Tripartite Guideline. Evaluation of Stability Data Q1E. International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use; 2003.
- [61] Pikal MJ. Freeze drying. In: Swarbrick J, Boylan JC, editors. *Encyclopedia of Pharmaceutical Technology*. 2nd edn. New York: Marcel Dekker; 2002. pp. 1299–1326.
- [62] Ye Y, Ma Y, Zhu J. The future of dry powder inhaled therapy: promising or discouraging for systemic disorders? *Expert Opinion on Drug Delivery*. 2022;19(8):1101–1115. doi: 10.1080/17425247.2022.2112055.