Development of drug alone and carrier-based GLP-1 dry powder inhaler formulations

Mai Babenko¹, Raid G Alany¹,², Gianpiero Calabrese¹, Waseem Kaialy³, Amr ElShaer¹,*

¹ Drug Discovery, Delivery and Patient Care (DDDPC) Theme, Department of Pharmacy, School of Life Sciences, Pharmacy and Chemistry, Kingston University London, Kingston upon Thames, KT1 2EE
² School of Pharmacy, The University of Auckland, Auckland, New Zealand
³ School of Pharmacy, Faculty of Science and Engineering, University of Wolverhampton, Wolverhampton, WV1 1LY

*Corresponding author:
Dr Amr ElShaer
²Drug Discovery, Delivery and Patient Care (DDDPC) Theme
School of Life Sciences, Pharmacy and Chemistry
Kingston University London
Penrhyn Road, Kingston upon Thames,
KT1 2EE, UK
Email: a.elshaer@kingston.ac.uk
T +44 (0)20 8417 7416 (Internal: 67416)
Abstract

The study aimed to develop two types of dry powder inhaler (DPI) formulations containing glucagon-like peptide-1(7-36) amide (GLP-1): carrier-free (drug alone, no excipients) and carrier-based DPI formulations for pulmonary delivery of GLP-1. This is the first study focusing on the development of excipient free GLP-1 DPI formulations for inhaled therapy in Type 2 diabetes. The aerosolisation performance of both DPI formulations was studied using a next generation impactor and a DPI device (Handihaler®) at flow rate of 30 L min\(^{-1}\). Carriers employed were either a 10% w/w glycine-mannitol prepared by spray freeze drying or commercial mannitol. Spray freeze dried (SFD) carrier was spherical and porous whereas commercial mannitol carrier exhibited elongated particles (non-porous). GLP-1 powder without excipients for inhalation was prepared using spray drying and characterised for morphology including size, thermal behaviour, and moisture content. Spray dried (SD) GLP-1 powders showed indented/dimpled particles in the particle size range of 1 to 5 µm (also mass median aerodynamic diameter, MMAD: <5 µm) suitable for pulmonary delivery. Across formulations investigated, carrier-free DPI formulation showed the highest fine particle fraction (FPF: 90.73% ± 1.76%, mean ± standard deviation) and the smallest MMAD (1.96 µm ± 0.07 µm), however, low GLP-1 delivered dose (32.88% ± 7.00%, total GLP-1 deposition on throat and all impactor stages). GLP-1 delivered dose was improved by the addition of SFD 10% glycine-mannitol carrier to the DPI formulation (32.88% ± 7.00% -> 45.92% ± 5.84%). The results suggest that engineered carrier-based DPI formulations could be a feasible approach to enhance the delivery efficiency of GLP-1. The feasibility of systemic pulmonary delivery of SD GLP-1 for Type 2 diabetes therapy can be further investigated in animal models.
Keywords: Dry powder inhaler formulation, Glucagon-like peptide-1(7-36) amide, D-mannitol carrier, Glycine, Spray freeze drying, Spray drying

Abbreviations
AIT, Alberta Idealised Throat; ANOVA, Analysis of Variance; BMI, body mass index; COPD, chronic obstructive pulmonary disease; CV, coefficient of variation; DPI, dry powder inhaler; DPP-4, dipeptidyl peptidase-4; DSC, Differential Scanning Calorimetry; FDA, Food and Drug Administration; FTIR, Fourier transform infrared spectroscopy; FPF, fine particle fraction; GLP-1, glucagon-like peptide-1 or glucagon-like peptide-1(7-36) amide; GLP-1RA, glucagon-like peptide-1 receptor agonist; GSD, geometric standard deviation; $^{1}$H qNMR: proton quantitative nuclear magnetic resonance; ICH, International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use; LOD, limit of detection; LOQ, limit of quantitation; MMAD, mass median aerodynamic diameter; MOC, micro orifice collector; MW, molecular weight; NGI, next generation impactor; RH, relative humidity; RP-HPLC, reversed-phase high performance liquid chromatography; RSD, relative standard deviation; SD, spray dried; SEM, Scanning Electron Microscopy; SFD, spray freeze dried; SC, subcutaneous; T2DM, Type 2 diabetes mellitus; TFA, Trifluoroacetic acid; TGA, Thermogravimetric Analysis; $t_{\text{max}}$, time reached for maximum drug concentration in plasma after administration; UK, United Kingdom.
1. Introduction

Glucagon-like peptide-1 (GLP-1) is an endogenous incretin hormone produced in intestinal L-cells and secreted in two biologically active forms, GLP-1(7-36) amide (predominant secreted form of GLP-1, Figure 1) and GLP-1(7-37) in response to elevated blood glucose levels (e.g., nutrient intake). Both bind to GLP-1 receptors present in pancreas and other tissues (e.g., gastrointestinal tract, brain, heart, and kidney) to exert antidiabetic effects advantageous for people with Type 2 diabetes mellitus (T2DM) (Pinho, et al. 2019; Vahl, et al. 2003; Yu, et al. 2018). The main effect of GLP-1 is to reduce blood glucose levels (Ismail and Csóka 2017). When glucose levels are high GLP-1 stimulates insulin secretion from pancreatic β-cells in a glucose dependent manner and suppress glucagon secretion from pancreatic α-cells therefore lower blood glucose levels (Cowart 2020; Hu and Jia 2019; Pinho, et al. 2019; Yu, et al. 2018). When glucose levels are normal the effect of insulin secretion is minimum which reduces the risk of hypoglycaemia that frequently results from insulin therapy (Yu, et al. 2018; Zheng, et al. 2011). The other physiological effects of GLP-1 are slow gastric emptying, low appetite and reduce energy/food intake promoting weight control (e.g., weight loss) (Hu and Jia 2019; Ismail and Csóka 2017; Pinho, et al. 2019; Yu, et al. 2018). However, the endogenous GLP-1 incretin hormone has a short half-life with less than 2 minutes as GLP-1 is rapidly metabolised by dipeptidyl peptidase-4 (DPP-4) in the circulation after its release limiting for its clinical applications (Ismail and Csóka 2017; Pinho, et al. 2019; Yu, et al. 2018). Therefore, GLP-1 receptor agonists (GLP-1RAs) that are resistant to DPP-4 enzymatic degradation are available as therapeutic peptides. Currently seven GLP-1RAs products are approved in the UK for the treatment of T2DM (GLP-1RAs should not be used in patients with Type 1 diabetes mellitus) (BNF 2021a; BNF 2021b; Yu, et al. 2018). These include one oral form (once daily semaglutide tablet marketed as Rybelsus®) and six injection dosage forms self-administered by subcutaneous (SC) injection (twice daily exenatide marketed as Byetta®, once daily lixisenatide marketed as Lyxumia®, once daily liraglutide marketed as Victoza®, once weekly exenatide marketed as Bydureon®, once weekly dulaglutide marketed as Trulicity®, once weekly semaglutide marketed as Ozempic®) (BNF 2021; BNF 2021; Yu, et al. 2018).

Pulmonary delivery is a non-invasive route of drug administration potential alternative to SC route, which is the most common route of administration for peptide and protein drugs
such as GLP-1RAs for the treatment of diabetes (Lin, et al. 2019; Qian, et al. 2009; Yeung, et al. 2018). The pulmonary route of administration is commonly used to deliver drugs to the lungs for the local treatment of respiratory disease (e.g., asthma, and chronic obstructive pulmonary disease (COPD)) and the lungs are also used as a route to the systemic circulation to treat systemic conditions such as diabetes (Nokhodchi and Martin 2015; Sibum, et al. 2018; Wilson, et al. 2018). The lungs offer several advantages for systemic applications such as large surface area (around 130 m²), thin (0.2 µm) epithelial cells, extensive vascularisation in the alveolar region and low hepatic first-pass metabolism allowing rapid systemic drug absorption, fast onset of pharmacological action comparable to injections and high bioavailability (Lin, et al. 2019; Nokhodchi and Martin 2015; Peng, et al. 2016). To deliver drugs to the lungs, inhaler devices such as dry powder inhalers (DPIs) are required to generate aerosols for pulmonary delivery and used for dry powder inhaler (DPI) formulations that offer better formulation stability compared to liquid-based formulations (more susceptible to physicochemical degradations facilitated by water and cold chain storage conditions required) (Emami, et al. 2018; Lin, et al. 2019; Mensink, et al. 2017; Wilson, et al. 2018). There are two main types of DPI formulations: carrier-free DPI formulations composed of drug particles in the aerodynamic diameter range of 1 to 5 µm (drug co-formulated with excipients or drug alone without excipients, no carrier) and carrier-based DPI formulations composed of drug particles (aerodynamic diameter: ≤5 µm) and carrier particles (particle size: 50-200 µm, lactose monohydrate is usually used as a carrier) prepared as adhesive mixtures (drug-carrier blends/mixtures). In carrier-based DPI formulations drug particles are adhered to the surface of the carrier via inter-particulate adhesive forces (drug-carrier adhesive forces, van der Waals, electrostatic, capillary forces) (Faulhammer, et al. 2018; Lechanteur and Evrard 2020; Yeung, et al. 2018).

Formulation approaches to develop both types of DPI formulations for peptide and protein drugs involve particle engineering such as spray drying and spray freeze drying to overcome the challenges associated with each formulation (Brunaugh and Smyth 2018; Lechanteur and Evrard 2020; Scherließ and Etschmann 2018; Yeung, et al. 2018). A common approach to develop carrier-free DPI formulations is to reduce drug-drug cohesive forces caused by the small particle size of drug particles (≤ 5 µm) by optimising the properties of drug particles (e.g., particle size, morphology) using spray drying for improved aerosolisation performance of the drug particles (e.g., powder flowability, dispersion, lung deposition of
drug particles) (Brunaugh and Smyth 2018; Yeung, et al. 2018). The high drug-drug cohesive forces lead to a high degree of drug-drug agglomeration and thus poor inhalation behaviour (Nokhodchi and Martin 2015; Yeung, et al. 2018). The development of carrier-based DPI formulations usually focuses on carrier particle engineering to address the challenge of poor drug-carrier detachment (to aid and/or reduce interaction between drug and carrier particles) (Faulhammer, et al. 2018; Scherließ and Etschmann 2018). Studies including pharmacokinetics study in Sprague Dawley rats (Peng, et al. 2017) demonstrated that engineered mannitol carriers prepared by spray drying (Peng, et al. 2017; Zhang, et al. 2018) and freeze drying (Kaialy and Nokhodchi 2013; Kaialy and Nokhodchi 2016) improved lung drug deposition (e.g., increased fine particle fraction, FPF). The results were attributed to the modified morphology (porous powders, shape irregularity) and surface roughness of mannitol carriers associated with reduced drug-carrier adhesive forces that facilitated drug-carrier detachment leading to improved lung depositions (Kaialy and Nokhodchi 2013; Peng, et al. 2017; Zhang, et al. 2018). However, freeze dried mannitol carrier exhibited variable particle size, shapes and FPF (Kaialy and Nokhodchi 2013). Freeze drying usually limits to control particle properties (e.g., particle size distribution, morphology) resulting in the formation of non-uniform particle size and shape (Ziaee, et al. 2019). Our previous impaction studies on the aerosolisation performance of saccharide carrier dry powders (mannitol, sucrose) prepared by spray drying and spray freeze drying showed that spray freeze dried (SFD) carriers with high moisture content (SFD mannitol: 5.5%, SFD sucrose: 7.5%) exhibited better powder flowability, higher delivered dose (total saccharide deposition in throat and all impactor stages, SFD mannitol: 68.99% and SFD sucrose: 66.62%) when compared to spray dried (SD) carrier powders with lower moisture content (SD mannitol: 2%, SD sucrose: 3%) exhibited lower delivered dose (SD mannitol: 49.03% and SD sucrose: 57.70%)(p< 0.05) (Babenko, et al. 2019). This was attributed to the porous powders produced by spray freeze drying (Babenko, et al. 2019).
Figure 1: A structure of glucagon-like peptide-1(7-36) amide (C₁₈₉H₂₂₆N₂₀O₄₅, Molecular weight: 3297.7 g mol⁻¹, Sequence length: 30, CAS registry number: 107444-51-9). Adapted from SciFinderⁿ Substance Results (cas.org).

Until now, no studies have been published on the development of carrier-free and carrier-based DPI formulations containing excipient free GLP-1 powder for pulmonary delivery. Previous studies with GLP-1 have focused on the preparation of GLP-1 powders in the presence of excipients to enhance GLP-1 delivery via the lungs (Qian, et al. 2009; Sanketkumar and Amit December 2015). Qian et al. (2009) used spray drying to prepare GLP-1RA dry powder (BMS-686117, 11 amino acid GLP-1RA, molecular weight (MW): 1528.7 g mol⁻¹) in the presence of mannitol (BMS-686117: mannitol=20:80 w/w) or trehalose (BMS-686117: trehalose=80:20 w/w) intended for inhalation and conducted a pharmacokinetics study in Male Sprague-Dawley rats (Qian, et al. 2009). Qian et al. (2009) selected mannitol and trehalose as excipients because these are commonly used non-reducing sugars in the formulation development for inhalation and avoid poor chemical compatibility with BMS-686117. Spray drying produced spherical particles with the particle size range of 2-10 µm and improved powder flow property in comparison to as-received BMS-686117 powder (particle size: 2-100 µm, morphology: irregular flakes associated with poor flow property) (Qian, et al. 2009). The animal study showed that SD GLP-1RA (SD BMS-686117) administered intratracheally to the lung in the rats showed faster absorption (tₘₐₓ: 0.3-0.7 hr, dose: 1 mg kg⁻¹) compared to SC administration of BMS-686117 (tₘₐₓ: 1.2 hr, dose: 0.08 mg kg⁻¹) and high bioavailability (45%) relative to SC administration (Qian, et al. 2009). Sanketkumar and Amit (2015) used spray freeze drying to develop GLP-1(7-36) amide (1% w/w) dry powder in the presence of leucine and trehalose as excipients (99% w/w, leucine: trehalose =75:25) for inhalation (Sanketkumar and Amit December 2015). Leucine and trehalose were used as cryoprotectants to reduce the risk of GLP-1 denaturation during spray freeze drying. Leucine was also used as an aerosolisation enhancer (Sanketkumar and Amit December 2015). SFD GLP-1(7-36) amide powder was highly porous and demonstrated good aerosolisation
performance (MMAD: 3.68 µm ± 0.01 µm, FPF: 60.49% ± 0.47%) suitable for pulmonary delivery (Sanketkumar and Amit December 2015). These findings show the feasibility of pulmonary delivery of GLP-1RA and GLP-1 using particle engineering and the lungs can provide rapid absorption comparable to SC injection and high bioavailability (Qian, et al. 2009; Sanketkumar and Amit December 2015). However, due to the lung safety concern, the use of excipients in DPI formulations should be minimised (Balducci, et al. 2014; Zhang, et al. 2020).

Other studies reported by Leone-Bay et al. (2009) and Marino et al. (2010) were about GLP-1 Technosphere® powders; MKC253 (GLP-1 7–36 amide) adsorbed onto Technosphere® drug carrier system composed of a novel excipient (fumaryl diketopiperazine) for pulmonary delivery of peptides and proteins through inhalation (Leone-Bay, et al. 2009; Marino, et al. 2010). Afrezza®, which is currently available in the United States as an inhaled insulin product, is based on the Technosphere® technology (Al-Tabakha 2015). The results of both studies by Leone-Bay et al. (2009) on preliminary pharmacodynamic study using female Sprague-Dawley rats and by Marino et al. (2010) on two Phase I Clinical trials (Clinical Trials Identifier number NCT00475371: healthy subjects, n=26, NCT00642538: subjects with T2DM, n=20) showed that GLP-1 inhalation powders exhibited rapid absorption (t_{max}: 2-10 mins in rats and within 5 mins in both healthy and T2DM subjects) and stimulated insulin secretion to control postprandial glucose hyperglycaemia in Type 2 diabetes rats and T2DM human subjects in a glucose dependent manner (Leone-Bay, et al. 2009; Marino, et al. 2010). Although inhaled GLP-1 might be subject to DPP-4 enzymatic activity in the lungs, both studies demonstrated the feasibility of delivering GLP-1 as inhalation powders via the pulmonary route, therefore, pulmonary route of GLP-1 administration is still prospective and attractive for T2DM treatment (Leone-Bay, et al. 2009; Marino, et al. 2010).

The aim of this paper was to develop two types of DPI formulations for pulmonary delivery of GLP-1, (i) carrier-free DPI formulations containing GLP-1 (glucagon-like peptide-1(7-36) amide) alone prepared by spray drying and (ii) carrier-based DPI formulations containing GLP-1 blended with two different carriers (sieved particle size: 90-125 µm); 10% glycine-mannitol carrier prepared by spray freeze drying (engineered carrier) or commercial mannitol carrier (non-engineered carrier). SD GLP-1 powders were prepared in the absence of excipients to minimise the lung safety concern. Both DPI formulations were studied to understand the effect of the carriers (engineered and non-engineered carriers) on the aerosolisation performance. In this study, mannitol was chosen as an alternative carrier to
Lactose is the most used carrier in DPI formulations (Nokhodchi and Martin 2015). However, lactose might not be the carrier of choice for peptides and proteins as it is a reducing sugar associated with chemical incompatibility (e.g., Maillard reaction) (Rahimpour, et al. 2014; Zhang, et al. 2020). In contrast, mannitol is non-reducing sugar and generally recognized as safe substance listed by the Food and Drug Administration (FDA) database (U.S. Food & Drug Administration 2020) that can be used in the inhalation field (Kaialy and Nokhodchi 2016) and has been used in Exubera® (former FDA approved inhaled insulin product) (Al-Tabakha 2015). Glycine was chosen as it has also been used in Exubera® (Al-Tabakha 2015; Ferrati, et al. 2018). Spray drying and spray freeze drying were used for drug and carrier particle engineering, respectively as both drying methods can optimise the properties of particles by changing process parameters (e.g., atomisation flow rate, feed flow rate) and/or chemical composition and concentration of the feedstock (Adali, et al. 2020; Shetty, et al. 2020; Wilson, et al. 2018; Ziaee, et al. 2019).

2. Materials and methods

2.1. Materials

Glucagon-like peptide-1(7-36) amide (GLP-1: C149H226N40O45, purity: 99%, MW: 3297.7 g mol⁻¹) was purchased from Henan Tianfu Chemical Co., Ltd, China. Glycine, mannitol, and sodium benzoate were all purchased from Sigma-Aldrich, UK. Acetic acid glacial, acetonitrile, 35% ammonia solution (NH₄OH), sodium hydroxide pellets (NaOH), trifluoroacetic acid (TFA) were all purchased from Fisher Scientific, UK. Deuterium oxide was purchased from Eurisotop®, UK. Sodium 3-trimethylsilyl propionate-2,2,3,3-d₄ (TSP) was purchased from Merck Sharp & Dohme Canada Limited, UK.

2.2. Quantification of GLP-1

GLP-1 in DPI formulations was analysed using a reversed-phase high performance liquid chromatography (RP-HPLC) method with an isocratic elution mode. All experiments
were performed on an Agilent Technologies 1260 Infinity II HPLC system (Agilent Technologies, UK) composed of a degasser (G7111A 1260 Quat Pump VL, Agilent Technologies, UK), vial sampler (G7129A 1260 Vialsampler, Agilent Technologies, UK), and UV detector (G7114A 1260 VWD, Agilent Technologies, UK) at ambient temperature (22°C ± 3°C). The optimal chromatography conditions used for GLP-1 quantification were as follows. The stationary phase was C8 column (4.6 mm internal diameter x 250 mm length, 5 µm particle size, 130 Å pore size, Phenomenex, UK). The mobile phase was a mixture (41:59 % v/v) of acetonitrile with trifluoroacetic acid (TFA, 0.1% v/v) and distilled water with TFA (0.1% v/v) used at a flow rate of 1.0 mL min⁻¹. The detection wavelength for GLP-1 was 215 nm. Injection volume was 20 µL. The calibration curve of GLP-1 was constructed in the concentration range of 2.0 µg mL⁻¹ to 140.0 µg mL⁻¹ (eleven GLP-1 calibration standard solutions prepared). The data acquisition and chromatograms were obtained using an Openlab ChemStation (Agilent Technologies, UK). RP-HPLC method validation was carried out based on the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines (ICH 2005). Validation characteristics, such as specificity, linearity, range, accuracy, precision, limit of detection (LOD) and limit of quantitation (LOQ), as well as robustness and system suitability (e.g., retention time, peak area, height, theoretical plates, asymmetry and tailing factor) were assessed.

2.3. Carrier dry powders preparation by spray freeze drying

Mannitol based carrier with the inclusion of 10% w/w glycine (concentration based on the total solid content of 15% w/v) was prepared using spray freeze drying. The compositions of mannitol aqueous solution (15% w/v total solid content) for spray freeze drying were glycine (1.5 g) and mannitol (13.5 g) dissolved in distilled water (100 mL). Briefly, mannitol aqueous solution was sprayed over liquid nitrogen in a round bottom flask (250 mL) and freeze dried using BenchTop Pro with Omnitronics™ freeze dryer (SP Scientific, UK) for 48 hours at 55 ± 5 µbar of pressure and condenser temperature of -59 ± 2°C. After 48 hours, the produced SFD carrier powders were sieved using an AS200 DIGIT CA sieve shaker (Retsch, Germany) with the 90 and 125 µm sieves (Fisher Brand Test Sieve, UK) for up to 10 minutes at 1.5 mm amplitude to obtain the particle size fraction of 90-125 µm. Collected SFD 10%
glycine-mannitol powders (90-125 µm) were immediately transferred into tightly closed glass vials and stored in a desiccator over silica gel at room temperature (22°C ± 3°C).

SFD 10% glycine-mannitol carrier was employed based on the results of our preliminary experiments. We assessed the effects of amino acids (glycine or leucine) at three different amino acid concentrations (5%, 10% and 15% based on mannitol content) added as excipient to SFD mannitol carrier on the aerosolisation performance of DPI formulations containing human insulin (non SD insulin and SD insulin powders). The results showed that SD insulin DPI formulation containing SFD 10% glycine-mannitol carrier demonstrated the best aerosolisation performance across formulations investigated in terms of insulin delivered dose (57.75% ± 4.24%), FPF (57.32% ± 6.81%) and MMAD (2.37 µm ± 0.34 µm) (data not shown).

2.4. GLP-1 dry powders preparation by spray drying

GLP-1 dry powders intended for inhalation were prepared by spray drying based on the method of spray drying insulin reported by Balducci et al. (2014) with minor modifications. Briefly, GLP-1 (2 mg mL⁻¹) was dissolved in acidic aqueous solution (pH 3.5) composed of acetic acid (0.1% v/v) and 1M sodium hydroxide (NaOH, 100 µL per 100 mL) or acetic acid (0.1% v/v) and 35% ammonium hydroxide (NH₄OH, 18 µL per 100 mL) at room temperature. GLP-1 aqueous solution (2 mg mL⁻¹) was spray dried using a Mini Spray Dryer B-290 (Büchi, Switzerland) under standardised processing parameters: 240 mL hr⁻¹ feeding rate, 600 L hr⁻¹ spray flow rate with compressed air, 98% aspirator speed setting, inlet temperature at 119°C and outlet temperature at 60°C ± 2°C. Collected SD GLP-1 dry powders were immediately packed into tightly closed glass vials and desiccated over silica gel at room temperature (22°C ± 3°C). Sodium hydroxide and ammonium hydroxide both were used to adjust the pH of the feed solutions to 3.5 based on the method of spray drying insulin (Balducci, et al. 2014). Acidic solution with NH₄OH was tried based on the results of insulin stability study reported by Balducci et al. (2014). Their stability study (25°C and 60% relative humidity (RH)) in terms of the degradation product contents of SD bovine insulin (i.e., A21 desamido insulin) showed that SD insulin powders prepared from acetic acid with NH₄OH (pH 3.6) kept the A21 degradant content within the required limit of 5.0% for up to six months at room temperature.
whereas SD insulin powders prepared from acetic acid with NaOH (pH 3.1) showed shorter stability of three months at room temperature (Balducci, et al. 2014).

2.5. Preparation of GLP-1 dry powder inhaler formulations

Carrier-based GLP-1 DPI formulations (100 mg total) were prepared by blending SD GLP-1 powder (SDGLP(NH₄OH), 10 mg) produced from acidic feed solution composed of acetic acid and 35% NH₄OH with SFD 10% glycine-mannitol carrier (engineered carrier, 90 mg) or commercial mannitol carrier (non-engineered carrier, 90 mg) in ratio of 1:9 (drug : carrier) in a plastic container (2 x 9 cm) using a low shear blender of Turbula® system Schatz (WAB, Switzerland) at a constant speed of 46 rpm for 30 minutes. The blends (SD GLP-1 and carrier) were stored in a desiccator over silica gel at room temperature (22°C ± 3°C) prior to the impaction study. In this study, two carrier-free DPI formulations (SDGLP(NaOH): SD GLP-1 powder produced from acetic acid with 1M NaOH and SDGLP(NH₄OH): SD GLP-1 powder produced from acetic acid with 35% NH₄OH) and two carrier-based DPI formulations (SDG10GMB: SDGLP(NH₄OH) blended with SFD 10% glycine-mannitol carrier and SDGRMB: SDGLP(NH₄OH) blended with commercial mannitol carrier) were prepared. These four DPI formulations were used for the in vitro impaction study (Section 2.8). In carrier-based DPI formulations (drug: carrier = 1:9) the drug content was limited to 2 mg in 20 mg total mass of adhesive mixtures per capsule (Section 2.8). For pulmonary delivery for the management of systemic conditions, the lungs are used as a route to the systemic circulation which require higher concentrations of drugs in DPI formulations (e.g., 5-10% of drug, mg range, Exubera®: 1 mg or 3 mg insulin per blister, Afrezza®: 0.35 mg or 0.7 mg insulin per cartridge (Al-Tabakha 2015)) compared to the common DPI formulations used for asthma and COPD (e.g., drug concentrations: 0.1-4%, µg range) (Brunaugh and Smyth 2018; Scherließ and Etschmann 2018; Sibum, et al. 2018; Yeung, et al. 2018). The total mass of drug-carrier mixtures dispersed by marketed inhaler devices is usually in the range of 10 mg to 25 mg (Sibum, et al. 2018). At higher concentration of drug, multiple layers of the drug particles on the carrier surface and drug particle agglomerates will be formed due to the limited surface area of the carrier (Scherließ and Etschmann 2018; Sibum, et al. 2018). This adversely affects the mechanical powder stability (e.g., powder handling and dosing) thus homogeneity of drug content, dose
delivery consistency (uniform drug delivery into the lungs), and dose reproducibility (Scherließ and Etschmann 2018; Sibum, et al. 2018). Consequently, the drug content should be limited to maximum 2.5 mg in 25 mg total mass of adhesive mixtures (e.g., 5-10% of drug) in the formulations that still meet content uniformity and stability (Sibum, et al. 2018).

2.6. Physicochemical characterisation

2.6.1. Scanning electron microscopy

Morphologies along with particle size of raw GLP-1, SD GLP-1 powders (SDGLP(NaOH) and SDGLP(NH₄OH)) prepared from two different acidic aqueous solutions (acetic acid with NaOH and acetic acid with NH₄OH), SFD 10% glycine-mannitol (sieved 90-125 µm) and commercial mannitol (sieved 90-125 µm) powders were characterised by Scanning Electron Microscopy (SEM, ZEISS EVO®50, UK) at an acceleration voltage of 10-25 kV. Double-sided cohesive carbon tabs were adhered to aluminium stubs and all dry powder samples were placed onto the carbon tabs. Any excess powder samples were tapped off the tabs. These samples were then coated with a palladium/gold alloy (coating thickness: around 5-8 nanometres) using a SC7640 Sputter Coater (Polaron, UK) under argon gas for 2 minutes. Multiple images of coated samples were then captured for each sample. SEM images of carrier-based DPI formulations (SDG10GMB: SDGLP(NH₄OH) blended with SFD 10% glycine-mannitol carrier and SDGRMB: SDGLP(NH₄OH) blended with commercial mannitol carrier) were also captured following the same method described above for the visual observation of the blends (Section 2.7 Blend homogeneity assessment).

2.6.2. Differential scanning calorimetry

Thermal analysis of raw GLP-1 and SD GLP-1 powders (SDGLP(NaOH) and SDGLP(NH₄OH)) was performed using a DSC822e Differential Scanning Calorimetry (DSC, Mettler Toledo, Switzerland) under nitrogen gas (50 mL min⁻¹) in the temperature range from 25°C to 400°C at a heating rate of 10°C min⁻¹. All dry powder samples were placed in aluminium crucibles (40 µL) and sealed with a pierced lid on. The dry powder samples loaded
pan and empty reference pan were placed on the DSC sample holder. The DSC curves were recorded at 22°C using STARe Software version 8.10 (Mettler Toledo, UK).

### 2.6.3. Thermogravimetric analysis

Thermogravimetric Analysis (TGA) for raw GLP-1 and SD GLP-1 powders (SDGLP(NaOH) and SDGLP(NH₄OH)) was performed to measure moisture content using a METTLER TOLEDO® TGA/DSC1 STARe System (Mettler Toledo, Switzerland) along with DSC analysis. All dry powder samples were loaded onto aluminium oxide crucibles (70 µL) and heated under nitrogen gas (50 mL min⁻¹) in the temperature range from 25°C to 400°C at a heating rate of 10°C min⁻¹. The TGA curves were recorded at 22°C using STARe Software version 8.10 (Mettler Toledo, UK).

### 2.7. Blend homogeneity assessment

After blending, the homogeneity of two carrier-based DPI formulations (SDG10GMB and SDG10MB) was assessed by quantifying the content of GLP-1 and mannitol using the developed RP-HPLC method and proton quantitative nuclear magnetic resonance (¹H qNMR) method (Babenko, et al. 2019), respectively. Blend samples (4 mg total blend; 0.4 mg of SD GLP-1 and 3.6 mg of SFD carrier) were taken from three different positions (top, middle and bottom) of each DPI formulation in the blending container and dissolved in distilled water (4 mL, theoretical GLP-1 concentration: 100 µg mL⁻¹). GLP-1 content uniformity was determined as the ratio of the calculated concentration of GLP-1 contained in the blend sample to the theoretical concentration of GLP-1 and expressed as a percentage. The coefficient of variation (%CV or relative standard deviation, RSD%) was used as a degree of GLP-1 content homogeneity. A higher %CV indicates a lower drug content homogeneity (Kaialy and Nokhodchi 2016) and drug content is considered uniform when %CV is below 6% (Kaialy and Nokhodchi 2015). Simultaneously, mannitol content uniformity was determined as the ratio of the measured concentration of mannitol contained in the blend sample to the theoretical concentration of mannitol and expressed as a percentage. All NMR data were processed using TopSpin™ software 4.1.0 (Bruker BioSpin GmbH, Germany).
2.8. Impaction study

The aerodynamic performance of four DPI formulations (SDGLP(NaOH), SDGLP(NH₄OH), SDG10GMB, and SDGRMB) was assessed in vitro using a next generation impactor (NGI, Copley Scientific, UK) with the Alberta Idealised Throat 28028 (AIT, Copley Scientific, UK). The NGI was equipped with a Critical Flow Controller (TPK 2000, Copley Scientific, UK) connected to a Vacuum pump (HPC5, Copley Scientific, UK). The flow rate was set at 30 L min⁻¹ with the test airflow duration of 3 sec using the Critical Flow Controller Model TPK 2000 (Copley Scientific, UK) and a Flow Meter Model DFM2000 (Copley Scientific, UK). The critical (sonic) flow (P₃/P₂ ratio ≤ 0.5, flow rate stability) was achieved. A leak test was performed on the NGI prior to each use. In this study, Handihaler® (Boehringer Ingelheim, Germany, a single capsule inhalation device with high resistance) was used as a DPI to deliver the content of SD GLP-1 powders filled (total fill mass per capsule: 2.0-2.3 mg for SD GLP-1 alone and 20 ± 1 mg for carrier-based DPI formulations) in the HPMC size 3 capsules (CAPSUGEL®, UK) during the impaction studies. The flow rate and test duration were adjusted because Handihaler® is a high resistance inhaler designed to generate high pressure drop and studies have shown that in vitro performance of Handihaler® was not influenced by the flow rate between at 30 L min⁻¹ and 60 L min⁻¹ with the inhalation volume of 1 L. 30 L min⁻¹ of inspiratory flow rate was sufficient for successful inhalation for Handihaler® used by patients in a broad age range from children to elderly and disease states (e.g., asthma, COPD) (Levy, et al. 2019; Lindert, et al. 2014). Patients also can produce a minimum pressure drop of around 1 kPa (∼10 cmH₂O) across DPI inhaler devices including Handihaler® (e.g., Easyhaler®, Turbohaler®/Turbuhlaer®, Diskus®) which are found to be sufficient for pulmonary deposition and patients should receive a necessary drug dose (Clark, et al. 2019). In addition, people with diabetes are associated with obesity which causes a reduction in lung volume (e.g., functional residual capacity) and lung function (e.g., forced expiratory volume in one second) due to physiological changes (increased mass of the chest wall compress the chest cavity resulting in reduced lung volume) (Lumb and Thomas 2020). This leads to a change in breathing pattern, increased work of breathing (e.g., obese subjects with body mass index, BMI 39 ± 6 kg/m², breathing frequency: 18 ± 2 breath per minute, ventilation: 12.26 ± 2.27 L min⁻¹ and control subjects with BMI 23 ± 3 kg/m², breathing frequency: 10 ± 2 breath per minute, ventilation:
7.84 ± 0.99 L min⁻¹, p<0.001 (Chlif, et al. 2009), frequent airway closure and increased risk of developing airway disease (e.g., COPD) (Chlif, et al. 2009; Lumb and Thomas 2020). The duration of time (e.g., 8 sec) to draw 4L of air (according to the European Pharmacopoeia) through Handihaler® during testing would be long for people with diabetes.

SD GLP-1 powders deposited on AIT and all NGI stages (stages 1-7 and micro orifice collector, MOC) were collected using distilled water (2 mL) and immediately quantified by the developed RP-HPLC method. SD GLP-1 powders deposited on 7 stages in the NGI were based on the aerodynamic cut-off diameters of 0.541 µm (stage 7), 0.834 µm (stage 6), 1.357 µm (stage 5), 2.299 µm (stage 4), 3.988 µm (stage 3), 6.395 µm (stage 2), and 11.719 µm (stage 1) at flow rate of 30 L min⁻¹. All NGI studies were performed at room temperature and in triplicate. The aerosolisation performance of SD GLP-1 was assessed using Microsoft® Excel and Copley Inhaler Testing Data Analysis Software (CITDAS) Version 3.10 Wibu (Copley Scientific, UK) to determine GLP-1 delivered dose, FPF, MMAD and geometric standard deviation (GSD). The delivered dose (%) was determined as the ratio of the total SD GLP-1 deposition on AIT and all the NGI stages excluding the deposition in the inhaler device and capsules to the total SD GLP-1 dose delivered from the device including the deposition in the inhaler device and capsules (i.e., the mass of the GLP-1 powders filled into the capsule). Therefore, drug loss (%) was calculated as follows: 100 - delivered dose (%). The FPF defined as the mass fraction of the delivered drug dose with less than or equal to 5.0 µm aerodynamic diameter was used to characterise the aerosolisation performance of DPI formulations.

2.9. GLP-1 stability study

Preliminary stability study on SD GLP-1 powders before and after spray drying and during storage (room temperature, 22°C ± 3°C and RH: <4% in a desiccator measured by Ebro Data logger, EBI 20-IF, Germany) for up to 7 months was performed using Fourier-transform infrared spectroscopy (FTIR) and the developed RP-HPLC method. This preliminary stability study was performed to see whether SD GLP-1 powders were stable after spray drying process and could be used for the impaction studies (also to provide information for further studies such as scale-up study). In general, room temperature stable formulations are an ideal for inhaled products (Sadrzadeh, et al. 2010). Therefore, storage conditions used for GLP-1
stability studies were room temperature (real time/real temperature conditions (ICH 1995)) and the final products will be protected against humidity. FTIR was used to study the structure integrity (e.g., secondary structure) of GLP-1 in SD GLP-1 powders (SDGLP(NaOH) and SDGLP(NH₄OH)) whether the process of spray drying using high temperatures (inlet temperature at 119°C and outlet temperature at 60 ± 2°C) had an adverse effect on the structure of GLP-1. Structural stability of SD GLP-1 powders (SDGLP(NaOH) and SDGLP(NH₄OH)) during storage was also studied using FTIR. The FTIR spectra of raw GLP-1 and SD GLP-1 powders were acquired on a Nicolet™ iS5 FTIR spectrometer (Thermo Fisher Scientific, UK) in the range of 400–4000 cm⁻¹ by accumulating 16 scans with a resolution of 4 cm⁻¹ at room temperature. FTIR spectra were obtained using OMNIC™ driver version 8.2 software (Thermo Scientific, UK). The developed RP-HPLC method was used to quantify the content of GLP-1 in SD GLP-1 powders. The stability based on the GLP-1 content in SD GLP-1 powders is the ratio of GLP-1 detected content (area) at each storage time to the detected content (area) of raw GLP-1, which is the initial powder (as-received powder) used for the GLP-1 calibration curve validated based on the ICH guideline.

2.10. Statistical analysis

Statistical analysis was performed using SPSS® statistics version 26.0 (IBM, UK) along with Microsoft® Excel at significant level of p< 0.05. One-way ANOVA (Analysis of Variance) and t-test were used to compare the mean results for data (drug content uniformity and NGI study for all DPI formulations). If the ANOVA was itself significant Post Hoc test (Tukey honestly significant difference (HSD) test) was further performed to determine which groups were different from each other (Ennos 2012).

3. Results and discussion

3.1. Quantification of GLP-1

The developed RP-HPLC method produced accurate (relative error%: 0.22-4.65%) and precise data with high repeatability (RSD%: 0.67-4.50%) in the concentration range of 2.0-
140.0 µg mL\(^{-1}\) (linear calibration curve for GLP-1, \(R^2 = 0.9999\)). The LOD was 0.79 µg mL\(^{-1}\) and LOQ was 2.39 µg mL\(^{-1}\). The RSD\% values of system suitability parameters (e.g., retention time, peak area, height, theoretical plates, asymmetry and tailing factor) were within 1.0\% (RSD\%: 0.08-1.01) and the efficiency of the column was above 2000 (3537.71). Therefore, the developed method was sufficient to use for blend homogeneity assessment (Section 3.3), impaction study (Section 3.4) and GLP-1 stability study (Section 3.5).

3.2. Physicochemical characterisation

3.2.1. Scanning electron microscopy

GLP-1 powders as received were lyophilised fluffy powders that exhibited the morphology of irregular particle shapes (e.g., flakes) with particle size ranging from 10 to 50 µm as observed under SEM (Figure 2A). Because the purchased GLP-1 powder product was not an inhalation grade (not within the suitable particle size range, ≤ 5 µm for systemic pulmonary delivery), spray drying was employed to reduce the particle size suitable for pulmonary delivery. The SEM images of SD GLP-1 powders produced from acetic acid with NaOH (Figure 2B) and acetic acid with NH\(_4\)OH (Figure 2C) both showed the particle size range of 1 to 5 µm, which is suitable for drug deposition in the lungs and similar morphologies of spherical particles with dimples on the surface. Spray drying method demonstrated to reduce the particle size of GLP-1 powders in a suitable particle size range of 1 to 5 µm for pulmonary delivery also modified the morphology of the GLP-1 powders. The formation of the indented/dimpled surfaces could be associated with the drying process (e.g., rapid evaporation of the solvent upon drying). The observed morphologies of SD GLP-1 powders tend to be common as SD peptides/proteins (e.g., insulin, glycoprotein and immunoglobin) reported in the literature (Bowey, et al. 2013; Vehring 2007). It was observed that SD GLP-1 particles were relatively agglomerated (Figure 2B,C) indicating cohesive particles. Such agglomerated particles are likely to have poor flowability (Peng, et al. 2016). Therefore, SD GLP-1 powder (SDGLP(NH\(_4\)OH)) was mixed with a carrier powder with the particle size fraction of 90-125 µm (SFD 10% glycine-mannitol or commercial mannitol) to improve the efficiency of drug aerosolisation and drug delivery.
The SEM images of SFD 10% glycine-mannitol carrier (Figure 3A) and commercial mannitol carrier (Figure 4A) both showed the suitable carrier size range (within 50-200 µm). However, different morphologies were observed: SFD 10% glycine-mannitol carrier (Figure 3A-B) showed spherical and highly porous particles whereas commercial mannitol carrier (Figure 4A-B) showed elongated particles (non-porous). The surface properties such as roughness were also different for both carriers. SFD mannitol based carrier had rough and wavy surface/uneven surface with small and shallow indentations and some open pores on the surfaces (Figure 3A-B yellow box). On the other hand, commercial mannitol carrier exhibited rather smooth surface with larger indentations (an increase in the indentation depth and length) (Figure 4A-B). This presents that spray freeze drying method demonstrated to modify the surface properties (e.g., morphology and roughness) of commercial mannitol and produced porous (and fluffy) powders compared to commercial mannitol powders.

Following the blending process, the SEM images of carrier-based DPI formulations (SDG10GMB and SDGRMB) showed that SD GLP-1 particles (SDGLP(NH₄OH)) were adhered to both carriers (Figure 3C-D and Figure 4C-D). The particles of SD GLP-1 and carriers can be clearly distinguished in the SEM images of the blends (Figure 3C-D and Figure 4C-D) as SD GLP-1 particles presented dimpled spherical particles in the small particle size range of 1 to 5 µm (Figure 2C) whereas SFD carrier was spherical and porous (Figure 3A-B) and commercial mannitol was elongated (Figure 4A-B) and both in the larger particle size range of 60 to 200 µm. As shown in Figure 3C-D, SDGLP(NH₄OH) particles were less agglomerated and relatively evenly distributed on the surface of SFD 10% glycine-mannitol carrier. This could be attributed to the rough and wavy surface of the porous and spherical SFD carrier with small and shallow indentations. Such indentations were not deep enough to hold large amounts of small drug particles on the SFD carrier, therefore more irregularities associated with larger surface areas (Kaialy 2016; Rudén, et al. 2019). This would have kept the GLP-1 particles relatively separated by providing more contact areas available for drug-carrier adhesion. This suggests that the inclusion of SFD 10% glycine-mannitol carrier in the formulation demonstrated to break up SD GLP-1 cohesive particles/agglomerates (Figure 2C) followed by adhering the drug particles to the small indentations on the surface of the SFD carrier as a single adhesion layer of drug particles during blending. However, some SD GLP-1 particles were seen between the small indentations on the surface of SFD 10% glycine-mannitol carrier (Figure 3D yellow circles) where the drug particles could fit into the void spaces (macroscale roughness, carrier surfaces
with large scale asperities that are larger than the drug particle size (Nokhodchi and Martin 2015; Shalash, et al. 2015). The small indentations yet slightly larger than the drug particle size could shield the drug particles from forces (e.g., drag and lift forces) during aerosolisation (Nokhodchi and Martin 2015; Shalash, et al. 2015). This could possibly lead to poor drug detachment from the SFD carrier and affect the aerosolisation performance of the formulations. On the other hand, the carrier surface of commercial mannitol particles was overloaded with SDGLP(NH$_4$OH) particles (Figure 4C-D) in comparison to the surface of SFD mannitol-based carrier particles (Figure 3C-D). It can be seen from Figure 4D that SDGLP(NH$_4$OH) particles were present as agglomerates between the large indentations on the surface of the commercial mannitol carrier. This could be attributed to fewer overall contact areas available on the surface of the non-engineered commercial mannitol carrier for drug-carrier adhesion. Commercial mannitol particles with large indentations therefore less irregularities would be associated with small surface areas providing a small contact area for drug-carrier adhesion. This suggests that only some GLP-1 particles could adhere to indentations/irregularities on the surface of commercial mannitol carrier and start to form multi adhesion layers of drug particles (SD GLP-1 particles aggregates) (Rudén, et al. 2019). Consequently, these differences in particle surface properties (e.g., morphology and surface roughness) affected the GLP-1 content uniformity (Section 3.3) and aerosolisation performance of DPI formulations (Section 3.4).
Figure 2: Scanning electron microscopy (SEM) images of (A) raw GLP-1 powder as received, (B) SDGLP(NaOH): spray dried (SD) GLP-1 powder produced from acetic acid with NaOH, and (C) SDGLP(NH₄OH): SD GLP-1 powder produced from acetic acid with NH₄OH.
Figure 3: SEM images of spray freeze dried (SFD) 10% glycine-mannitol carrier powder (sieved 90-125 µm) (A,B) and spray dried (SD) GLP-1 powder (produced from acetic acid with NH₄OH) blended with SFD 10% glycine-mannitol carrier powder (C,D). Yellow boxes indicate SEM images with 2 µm scale bar. Yellow circles indicate SD GLP-1 particles fitting into the void spaces between indentations.
3.2.2. Differential scanning calorimetry

The results of DSC analysis for raw GLP-1 and two SD GLP-1 powders (SDGLP(NaOH) and SDGLP(NH₄OH)) showed no significant sharp peaks as all the peaks observed were small and broad indicating amorphous nature of GLP-1 powders (Figure S1). This suggests that spray drying maintained GLP-1 in an amorphous state. The DSC curves for all GLP-1 powders showed three endothermic peaks; a broad endothermic peak between 40°C and 100°C, a small endothermic peak between 140°C and 160°C, and a broad endothermic peak started around 180°C (Figure S1). SDGLP(NaOH) powder showed an extra small endothermic peak around 235°C followed by another broad endothermic peak at 257°C (Figure S1B), whereas SDGLP(NH₄OH) powder showed a small endothermic peak around 250°C (Figure S1C). The first broad endothermic peaks seen below 100°C were attributed to moisture loss. This
indicates that spray drying did not remove all water resulting in the production of moisture-contained SD GLP-1 powders or SD GLP-1 powders may be considered susceptible to moisture if exposed to atmosphere (i.e., during sample preparation for DSC). SD GLP-1 powders were found to be amorphous therefore easier to absorb water vapor from air relative to crystalline materials (Weers and Miller 2015). This may affect the long-term stability of the DPI formulations (Banga 2015). The second small endothermic peak seen around 150°C would be ascribed to the onset of GLP-1 degradation. The small and broad endothermic peaks observed between 180°C and 260°C could be attributed to its decompositions induced by increasing temperature. This could indicate that there would be a series of decomposition processes involved in the thermal breakdown of GLP-1 during the temperature change. Therefore, this could be attributed to the degradation of GLP-1 occurred via the chemical or physical process influenced by the temperature conditions (Ansari, et al. 2016). Peptides and proteins undergo degradation that can result in a change or loss of biological activity and main mechanism of degradation can be either physical degradation such as denaturation and aggregation or chemical degradation involving oxidation, hydrolysis and deamidation (Depreter, et al. 2013; Mensink, et al. 2017).

3.2.3. Thermogravimetric analysis

The water content for raw GLP-1 and two SD GLP-1 powders (SDGLP(NaOH) and SDGLP(NH₄OH)) was measured by TGA. This study was performed because peptides and proteins are generally not stable for long, characteristics of formulations/products can change over time and chemical degradation can occur when exposed to stresses (e.g., moisture, high/cold temperature, etc.) during manufacturing process and storage therefore affecting stability, shelf-life, safety and efficacy (Depreter, et al. 2013; Mensink, et al. 2017). In addition, for DPI formulations containing amorphous drugs, they are generally hygroscopic therefore powders absorbed moisture during storage or the presence of moisture in dry powders can increase powder/particle stickiness that affect particle size and aerosolisation performance (Weers and Miller 2015).

The TGA curve of all GLP-1 powders showed apparent weight gains (4-8%, the mass was higher than the initial mass) below 40°C (Figure S2). This could be due to the drastic change of the gas density in the furnace atmosphere on heating in the beginning of the
measurement that influenced the mass change on the TGA curve (Craig and Reading 2006; Mettler-Toledo International Inc 2021). There are generally some delays in heat transfer from the furnace to the sample due to the thermal conductivity of the samples and the delay is generally great if samples are poorly conducting materials (Craig and Reading 2006). This could be attributed to the amorphous nature of the GLP-1 powders. Amorphous solids, which are not structurally ordered (non-crystalline lattice), are associated with low thermal conductivity compared to crystalline materials as heat energy is transferred through lattice vibrations (Zhou, et al. 2020). Therefore, all GLP-1 powders can be considered poorly conductive materials or non-conductive materials.

Continuous weight gains were also observed for SDGLP(NaOH) and SDGLP(NH₄OH) until around 200°C (Figure S2B,C). This would have resulted from interactions with a trace of oxygen in the nitrogen purge gas or volatilised products generated in the furnace atmosphere (Craig and Reading 2006). The TGA curve of raw GLP-1 showed few steps of mass loss (Figure S2A). The first step (about 4% w/w) observed in the temperature range of 50°C to 100°C is associated with moisture content where water evaporation would have taken place. This supports the DSC curve for raw GLP-1 where the endothermic peak observed below 100°C (Figure S1A) was attributed to moisture loss. The mass losses observed around 150°C (about 2% w/w), 200°C (about 12% w/w), 240°C (3% w/w) and 260°C (34% w/w) (Figure S2A) could be attributed to the multi-step thermal decompositions caused by the thermal breakdown of GLP-1 on heat stress. This also supports the DSC curve of raw GLP-1 where the small endothermic peaks observed above 140°C (Figure S1A) were attributed to its thermal decompositions. There were no steps observed below 200°C for both SD GLP-1 powders (SDGLP(NaOH) and SDGLP(NH₄OH)) due to the continuous weight gains observed (Figure S2B,C). Steps associated with moisture content below 100°C could be hidden as the DSC curves for both SD GLP-1 powders showed broad endothermic peaks below 100°C (Figure S1B,C) attributed to moisture loss. However, above 200°C, SDGLP(NaOH) powder showed three steps of mass loss starting at around 200°C (12% w/w), 240°C (4% w/w) and 260°C (24% w/w) in the TGA curves (Figure S2B). These mass losses can be associated with its decomposition therefore support the DSC curve where the broad endothermic peaks observed around 180°C, 240°C and 260°C (Figure 1SB) were attributed to its decomposition.

It was observed that raw GLP-1 and SDGLP(NaOH) showed a similar trend of the mass change above 240°C in the TGA curves (Figure S2A,B) whereas SDGLP(NH₄OH) showed a small
change in mass (about 6% w/w) in the temperature range of 220°C and 340°C (Figure S2C). This could indicate that SDGLP(NH₄OH) would be more thermostatically stable (e.g., less susceptible to change in temperature) than SDGLP(NaOH). Overall, raw GLP-1 powder had the highest loss of moisture content (51% w/w) at above 200°C followed by SDGLP(NaOH) powder (40% w/w) and SDGLP(NH₄OH) powder exhibited the lowest mass change (6% w/w). Although SD GLP-1 powders were stored in a desiccator over silica gel, SD GLP-1 powders may be considered susceptible to moisture when exposed to atmosphere (i.e., during sample preparation for TGA) due to the amorphous powders. SDGLP(NH₄OH) powder with less moisture content compared to SDGLP(NaOH) powder could be due to the use of volatile ammonia solution associated with rapid evaporation during spray drying.

3.3. Blend homogeneity assessment

Both carrier-based DPI formulations exhibited a high degree of mannitol carrier content uniformity (SDG10GMB: 99.67 ± 0.06% and SDGRMB: 104.16 ± 0.12%) with low %CV (SDG10GMB: 0.06 and SDGRMB: 0.12). The intensity of all mannitol peaks observed by NMR showed comparable for both formulations presenting similar mannitol concentrations were determined (Figure 5). However, it was found that they exhibited significantly different mannitol uniformity from each other (t-test: p<0.05). DPI formulation with commercial mannitol carrier (SDGRMB) showed higher mannitol content present in the formulation (104.16%, %CV: 0.12) than DPI formulation with SFD 10% glycine-mannitol carrier (SDG10GMB, 99.67%, %CV: 0.06) (t-test: p<0.05). Similarly, GLP-1 uniformity was significantly different between SDG10GMB and SDGRMB (t-test: p<0.05). These findings could imply that drug (GLP-1) content influences carrier (mannitol) content or vice versa consequently affecting the overall homogeneity of the formulation blends (One-way ANOVA: p<0.05).

SDGRMB demonstrated better drug content uniformity (95.42 ± 1.66%) with a low CV (1.81%) when compared to SDG10GMB that resulted in lower drug content uniformity (79.75 ± 2.00%) with a higher CV (2.55%). This presents that SDGRMB exhibited a better homogeneous formulation blend (GLP-1: 95.42%, and mannitol: 104.2%) with good drug content uniformity (%CV: 1.81). The drug content uniformity for SDG10GMB (79.75%) was outside the acceptable range of 85-115% of the nominal dose (British Pharmacopoeia Volume
This could be related to the width of the particle size distribution (i.e., span value) that SFD 10% glycine-mannitol carrier exhibited the wider size distribution, thus higher span value (span: 5.82 determined by laser diffraction from our previous study, data not shown) than commercial mannitol carrier (span: 2.25 (Babenko, et al. 2019)). Low span of particle size distribution is associated with low variation in drug content (e.g., low %CV) which is related to the uniformity of the dose (Kaialy and Nokhodchi 2015). Carrier particles with higher span value could contain different amounts of drug particles per unit mass (Kaialy and Nokhodchi 2015). Also, this could be attributed to different morphologies observed between SFD 10% glycine-mannitol carrier (Figure 3A-B) and commercial mannitol carrier (Figure 4A-B). SFD 10% glycine-mannitol carrier (Figure 3A-B) showed highly porous particles that would have been associated with lower density in comparison to non-porous commercial mannitol carrier (heavier) (Figure 4A-B). SFD carrier would have had lower shear forces (e.g., press-on forces) in the low shear blender used (Turbula®) resulting in poor drug-carrier adhesion forces therefore poor distribution of SD GP-1 powders on the surface of SFD carrier particles (Hertel, et al. 2020). This indicates that the fraction of the drug particles available in SDG10GMB for systemic pulmonary delivery would be lower than SDGRMB as SFD carrier retained less GLP-1 particles.
Figure 5: $^1$H NMR spectra of mannitol from three different positions (top, middle and bottom) of carrier-based DPI formulations in the blending container. (A) SDG10GMB: Spray dried GLP-1 (SDGLP(NH$_4$OH)) blended with spray freeze dried 10% glycine-mannitol carrier. (B) SDGRMB: SDGLP(NH$_4$OH) blended with commercial mannitol carrier. Dashed boxes indicate peaks of internal standard (sodium benzoate), mannitol, chemical shift reference (sodium 3-trimethylsilyl propionate-2,2,3,3-$d_4$, TSP).

3.4. Impaction study

SD GLP-1 powder (SDGLP(NH$_4$OH)) prepared from acidic aqueous solution (pH 3.5) composed of acetic acid and 35% NH$_4$OH was selected over SD GLP-1 powder (SDGLP(NaOH)) prepared from acetic acid with 1M NaOH acidic aqueous solution (pH 3.5) in order to prepare carrier-based DPI formulations (SDG10GMB and SDGRMB). SDGLP(NH$_4$OH) presented better aerosolisation performance in terms of FPF and MMAD (Table 1). Carrier-free DPI formulations, SDGLP(NaOH) and SDGLP(NH$_4$OH) both demonstrated similar GLP-1 delivered dose (SDGLP(NaOH): 33.31 ± 12.52% and SDGLP(NH$_4$OH): 32.88 ± 7.00%) (Table 1) as the total GLP-1 depositions between AIT and all NGI stages (stage 1-7 and MOC) were not significantly different from each other (t-test: p>0.05). This presents that using two different acidic aqueous solutions to produce SD GLP-1 powders did not affect the overall GLP-1 delivered dose. However, SDGLP(NH$_4$OH) showed significantly low GLP-1 deposition between AIT and stage 2 (AIT and stage 1 generally represent the oropharynx region) and significantly high GLP-1 deposition in the lower NGI stages (i.e., stage 5-6, the lower NGI stages represent the desired deep lung regions for systemic pulmonary delivery) in comparison to SDGLP(NaOH) (t-test: p<0.05) (Figure 6). Therefore, SDGLP(NH$_4$OH) generated higher FPF (90.73 ± 1.76%) and smaller MMAD (1.96 ± 0.07 µm) with lower GSD (1.71 ± 0.07) than SDGLP(NaOH) (FPF:
52.21 ± 7.30%, MMAD: 3.49 ± 0.61 µm and GSD: 2.64 ± 0.31 (Table 1). This implies that using feed solution composed of acetic acid and NH₄OH to spray dry GLP-1 resulted in significantly higher FPF by decreasing drug deposition in high NGI stages (AIT and stage 1-2) in comparison to using feed solution composed of acetic acid and NaOH (t-test: p<0.05). This trend is in agreement with the results of aerodynamic assessment for SD insulin powders (device used: Turbospin®, flow rate: 60 L min⁻¹) reported by Balducci et al. (2014). Balducci et al. (2014) prepared SD insulin powders from acetic acid (0.4N) with NH₄OH (10% v/v, pH 3.6) that showed higher FPF (83.6 ± 4.7) and smaller MMAD (1.79 ± 0.18 µm) in comparison to SD insulin powders prepared from acetic acid (0.4N) with NaOH (1N, pH 3.1) had low FPF (65.5 ± 3.0%) and larger MMAD (3.21 ± 0.11 µm) (Balducci, et al. 2014). Their results were attributed to the different particle shapes of SD insulin powders produced from their two different acidic feed solutions; more volatile ammonium acetate was present in the solution than sodium acetate in the solution. This resulted in the formation of more deeply shrivelled particles of SD particles with NH₄OH and smaller particles due to the evaporation rate of solvent relative to diffusion of insulin molecule during spray drying compared to SD particles with NaOH which was wrinkled raisin-like shapes (Balducci, et al. 2014). In the present study, SDGLP(NaOH) and SDGLP(NH₄OH) produced from two different feed solutions with the same drug concentration (2mg mL⁻¹), pH (3.5) and processing parameters of spray drying showed no apparent difference in the morphologies of SD GLP-1 powders in the same particle size range of 1-5 µm (Figure 2B,C). Therefore, further characterisation studies (e.g., particle size distribution, density, fluidity, powder flowability, electrostatic forces) are needed to identify the specific factors contributed to the difference in the FPF values.

All four DPI formulations showed significantly different GLP-1 depositions in the high NGI stages (i.e., AIT and stage 1-2) and lower NGI stages (i.e., stage 3-6) (One-way ANOVA: p<0.05). This means that GLP-1 deposition patterns in high and lower stages were dependent on the formulations; therefore, affected the aerosolisation performance of SD GLP-1 powders. Carrier-free DPI formulation of SDGLP(NH₄OH) produced the highest FPF (90.73 ± 1.76%) with the smallest MMAD (1.96 ± 0.07 µm) whereas carrier-based DPI formulation of SDG10GMB exhibited the lowest FPF (29.20 ± 5.62%) with the largest MMAD (5.85 ± 1.25 µm) (One-way ANOVA: p<0.05)(Table 1). The highest FPF obtained for SDGLP(NH₄OH) could indicate that higher amount of GLP-1 (aerodynamic diameter: ≤ 5 µm) would be expected to reach the deep lung regions for systemic pulmonary application compared to the lowest FPF
value obtained for SDG10GMB (29.20 ± 5.62%). However, despite that SDGLP(NH₄OH) carrier-free DPI formulation had the highest FPF, GLP-1 delivered dose was just over 30% (32.88 ± 7.00%) (Table 1) representing a high amount of drug (GLP-1) loss (about 70% of drug loss). In contrast, DPI formulation using SFD 10% glycine-mannitol carrier (SDG10GMB) demonstrated the highest GLP-1 delivered dose (45.92 ± 5.84%) therefore reduced the drug loss by about 15% (70% - 55% drug loss). This indicates that the addition of SFD 10% glycine-mannitol carrier to the DPI formulation enhanced the powder flow and powder release from the DPI, therefore facilitated the delivery of SD GLP-1 powder (1.4 fold increase in GLP-1 delivery dose against SD GLP-1 alone, 32.88 ± 7.00% - 45.92 ± 5.84%) (Table 1). This could be due to the porous and fluffy particles of 10% glycine-mannitol carrier produced by spray freeze drying that resulted in better fluidisation and powder emission/release from the inhaler device. The engineered SFD carrier provided a means to enhance the flowability and improved the drug delivery via the DPI. Many studies on the inhalation performance of carrier-based DPI formulations also have shown the advantages (e.g., increase FPF) of the use of carriers/engineered carriers (Kaialy and Nokhodchi 2013; Kaialy and Nokhodchi 2016; Rashid, et al. 2019). However, the addition of commercial mannitol carrier to the DPI formulation (SDGRMB) resulted in the lowest GLP-1 delivered dose (25.74 ± 8.50%) (Table 1) therefore the highest drug (GLP-1) loss (>74%). Most of the drug remained on the wall of the capsule covered in a layer of white powders containing GLP-1 (i.e., the capsule retained a high fraction of drug particles). This suggests that this formulation did not fluidise efficiently with Handihaler® and commercial mannitol carrier is not a good carrier candidate to enhance the powder flow and facilitate the drug delivery. This could be explained by the morphology of commercial mannitol carrier powder that is not porous (Figure 4A-B) resulting in poor flowability and low drug delivered dose. Further, SDG10GMB and SDGRMB both showed significantly different GLP-1 depositions between AIT and stage 2 (t-test: p<0.05) (Figure 6). SDGRMB exhibited lower SD GLP-1 particles deposition in AIT and stage 1-2 therefore more SD GLP-1 particles were available for deposition in the lower NGI stages (e.g., stage 3-5) resulting in higher FPF (FPF: 60.41 ± 11.12%). On the other hand, SDG10GMB demonstrated significantly higher GLP-1 depositions between AIT and stage 2, consequently, only a small amount of the drug remained to reach the lower NGI stages resulting in the lowest FPF (29.20 ± 5.62 %, t-test: p<0.05) with the highest MMAD (5.85 ± 1.25 µm) (Table 1). This suggests that SD GLP-1 particles adhered to the surface of commercial mannitol carrier detached more
easily than SD GLP-1 particles attached to SFD 10% glycine-mannitol carrier. This could be attributed to the agglomerates of SD GLP-1 particles (multi layers of drug particles) seen on the surface of commercial mannitol particles (Figure 4C-D). These particles were associated with better dispersion as some drug particles were not attached to the commercial mannitol carrier surface and freely available for aerosolisation that resulted in higher FPF values (Leung, et al. 2016). However, this adversely can affect the mechanical powder stability (e.g., powder handling and dosing) and dose delivery consistency (uniform drug delivery into the lungs) as drug-carrier adhesive forces are to stabilise the drug-carrier mixtures (Kaialy 2016; Scherließ and Etschmann 2018; Sibum, et al. 2018). It could be speculated that the process of drug-carrier detachment for SDGRMB would have been associated with more like drug-drug particle de-agglomeration rather than drug-carrier detachment. As shown in Figure 6 both SDGRMB and SDGLP(NH₄OH) showed similar drug deposition profiles in stage 3-6 and statistically GLP-1 depositions in stage 3-6 for SDGRMB and SDGLP(NH₄OH) were not significantly different from each other (t-test: p>0.05). SDG10GMB with the lowest FPF could be due to the small indentations on the surface of SFD 10% glycine-mannitol carrier where some SD GLP-1 particles fitted into the void spaces between the small indentations (Figure 3D yellow circles). The small indentations yet slightly larger than the drug particle size observed by SEM (Figure 3D) would have shielded the drug particles from forces (e.g., drag and lift forces) during aerosolisation affecting drug dispersion/the drug-carrier detachment therefore the aerosolisation performance of the formulation. GLP-1 depositions in stage 3-6 for SDG10GMB was significantly lower than carrier-free SDGLP(NH₄OH) (t-test: p<0.05). This could be interpreted that Handihaler® with high resistance can fluidise SDG10GMB formulation relatively well as the highest GLP-1 delivered dose (45.92 ± 5.84%) was achieved because of the porous carrier powder used. However, this formulation did not detach the drug particles from the SFD carrier efficiently via the air stream generated by Handihaler® as FPF obtained was the lowest indicating the poor drug-carrier detachment. The device might not be designed to separate drug-SFD carrier mixtures effectively. This can lead to insufficient therapeutic GLP-1 deposition in the desired deep lung regions for systemic pulmonary delivery. Handihaler® is designed for patients with COPD who have difficulty in generating sufficient inspiratory flow through DPIs therefore less dependent on patient inspiration flow rate (inhalation effort required by the patients is low to fluidise the powders) (Altman, et al. 2018). Airflow generated by high resistance DPIs (using low flow rate) might not be sufficient
to disperse the drug-carrier powders efficiently when SFD carrier-based DPI formulations (drug: carrier = 1:9) were used (more inspiratory flow would be required for drug-carrier detachment).

![Figure 6: Next Generation Impactor (NGI) deposition profiles of spray dried (SD) GLP-1 in four different dry powder inhaler formulations aerosolised from Handihaler® at flow rate of 30 L min⁻¹. GLP-1 deposition is expressed as delivered dose (%) per NGI stage (Data presented as mean ± standard deviation, n=3). AIT: Alberta idealised throat, MOC: Micro orifice collector. SDGLP(NaOH): SD GLP-1 prepared from acetic acid with NaOH, SDGLP(NH₄OH): SD GLP-1 prepared from acetic acid with NH₄OH, SDG10GMB: SDGLP(NH₄OH) blended with SFD 10% glycine-mannitol carrier and SDGRMB: SDGLP(NH₄OH) blended with commercial mannitol carrier.]

Table 1: Results of GLP-1 delivered dose (%), fine particle fraction (FPF% ≤5.0 µm), mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) assessed by Next Generation Impactor with Handihaler® at flow rate of 30 L min⁻¹ for dry powder inhaler (DPI) formulations (Data presented as mean ± standard deviation, n=3). SDGLP(NaOH): spray dried (SD) GLP-1 prepared from acetic acid with NaOH, SDGLP(NH₄OH): SD GLP-1 prepared from acetic acid with NH₄OH, SDG10GMB: SDGLP(NH₄OH) blended with SFD 10% glycine-mannitol carrier and SDGRMB: SDGLP(NH₄OH) blended with commercial mannitol carrier.

<table>
<thead>
<tr>
<th>DPI Formulation</th>
<th>Dose</th>
<th>Size Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GLP-1 delivered dose (%)</td>
<td>FPF (%)*</td>
</tr>
<tr>
<td>SDGLP(NaOH)</td>
<td>33.31 ± 12.52</td>
<td>52.21 ± 7.30a</td>
</tr>
<tr>
<td>SDGLP(NH$_4$OH)</td>
<td>32.88 ± 7.00</td>
<td>90.73 ± 1.76$^a$</td>
</tr>
<tr>
<td>----------------</td>
<td>--------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>SDG10GMB</td>
<td>45.92 ± 5.84</td>
<td>29.20 ± 5.62$^b$</td>
</tr>
<tr>
<td>SDGRMB</td>
<td>25.74 ± 8.50</td>
<td>60.41 ± 11.12$^b$</td>
</tr>
</tbody>
</table>

Asterisks (*) denote statistically significant differences between DPI formulations (One-way ANOVA, p<0.05). Groups denoted by the same letter (a,b,c,d,e) are significantly different from each other (t-test, p<0.05).

### 3.5. GLP-1 stability study

The FTIR spectra between raw GLP-1 as received (Figure S3A) and two SD GLP-1 powders (SDGLP(NaOH) in Figure S3B and SDGLP(NH$_4$OH) in Figure S3C) produced from two different acidic solutions showed similarities as no major shift of the characteristic band positions were observed: 3292-3290 cm$^{-1}$ for N-H stretching vibration, 3065-2960 cm$^{-1}$ for C-H stretching, 1655-1651 cm$^{-1}$ for C=O stretching vibration of the amide groups (Amide I), 1542-1540 cm$^{-1}$ for C-N stretching vibration of Amide II, 1456-1453 cm$^{-1}$ for C-H bending, 1202-1201 cm$^{-1}$ for C-C=O stretching vibration, and 1137-1135 cm$^{-1}$ for C-C stretching vibration. The common proteins/peptides characteristic absorption bands such as Amide I (1700-1600 cm$^{-1}$) and Amide II bands (1575-1480 cm$^{-1}$) were observed (Sarmento, et al. 2006; Tiernan, et al. 2020). These characteristic band positions observed for GLP-1 were similar to the FTIR result of exenatide (the first approved GLP-1RA, synthetic form of 39-amino acid peptide incretin for the treatment of T2DM) reported by Zhu et al. (2015). The similarity of the FTIR spectra between raw GLP-1 (before spray drying) and SD GLP-1 powders (after spray drying) indicates that the integrity of GLP-1 structure (e.g., secondary structure) was maintained after the process of spray drying despite the use of relatively high temperatures. Therefore, the employed spray drying parameters did not have an influence of thermal stress on the GLP-1 structure. In addition, dissolving GLP-1 in two different acidic aqueous solutions for spray drying did not influence the secondary structure of GLP-1. The FTIR spectra of SD GLP-1 powders (SDGLP(NaOH) and SDGLP(NH$_4$OH)) stored at room temperature for up to 7 months also showed no major changes in the characteristic band positions (e.g., Amide I and Amide II) between raw GLP-1 and two SD GLP-1 powders (FTIR spectra of SD GLP-1 powders stored at room temperature for 7 months are available on request). This suggests that the integrity of secondary structure of GLP-1 in SD powders stored at room temperature was retained for up 7 months.
The stability of GLP-1 was also assessed by RP-HPLC. No structural changes or degradation of GLP-1 molecule would have occurred during the process of spray drying as GLP-1 content determined by RP-HPLC in both SDGLP(NaOH) and SDGLP(NH₄OH) within 24-48 hours of SD powders production were 100% ± 1% (Figure 7A and Figure 7B, respectively). SDGLP(NaOH) maintained their stability for up to 27 days at room temperature after the process of spray drying then dropped by over 10% within 15 days (87% at 41 days, Figure 7A) and reached a plateau for about 6 months (84-86% at 62-209 days, Figure 7A). In comparison, SDGLP(NH₄OH) maintained their stability for up to 7 months (GLP-1 content: 100% ± 3%) after the process of spray drying (Figure 7B). The different stability profiles of two SD GLP-1 powders could be attributed to the presence of different moisture content in SD GLP-1 powders observed by TGA (Section 3.2.3) where SDGLP(NH₄OH) showed less mass change overall compared to SDGLP(NaOH). This could suggest that SDGLP(NH₄OH) powder might exhibit slower degradation rate (e.g., decompose slower by hydrolysis), therefore, greater kinetic stability compared to SDGLP(NaOH) powder when SD GLP-1 powders were dissolved in distilled water for HPLC analysis.

Figure 7: GLP-1 content determined by RP-HPLC. (A) SDGLP(NaOH): Spray dried (SD) GLP-1 powder produced from acetic acid with 1M NaOH stored at room temperature for up to 209 days (7 months). (B) SDGLP(NH₄OH): SD GLP-1 powder produced from acetic acid with 35% NH₄OH stored at room temperature for up to 203 days (7 months). (Data presented as mean ± standard deviation, n=4).
4. Conclusion

Both carrier-free and carrier-based DPI formulations exhibited significantly different aerosolisation performance. Handihaler® aerosolised carrier-based DPI formulation better when the porous engineered SFD carrier was used compared to non-engineered carrier. In addition, low GLP-1 delivered dose obtained from carrier-free DPI formulation of SDGLP(NH\textsubscript{4}OH) was improved by the use of SFD 10% glycine-mannitol carrier because of the porous carrier produced by spray freeze drying. This study demonstrated the advantages of using the engineered carrier-based DPI formulation and without the addition of the engineered SFD carrier to the formulation, SD drug particles showed poor flowability. The airflow generated from Handihaler® might be too low for drug-carrier detachment (when high concentration of drug is employed in DPI formulations, drug: carrier = 1:9), therefore, higher inspiratory flow would be required for drug-carrier detachment. The process of drug-carrier detachment in carrier-based DPI formulations is crucial for drug delivery to the lung. Inhaler devices with low resistance which use higher flow rate might be more applicable for carrier-based DPI formulations.

Overall, both types of DPI formulations have shown advantages with different challenges for pulmonary administration of GLP-1. The optimised DPI formulations can be achieved by developing DPI formulations together with developing new devices or using particle engineering to prepare engineered particles based on the type of DPI formulations (carrier-free or carrier-based) and the optimised formulations adapt to inhaler devices suited for the intended formulations instead of formulations adapting to the already available inhalers (e.g., inhalers could be designed based on the formulations). When selecting DPIs for the development of DPI formulations type of DPI formulations should be taken into consideration. The successful inhaled GLP-1 product will provide an alternative treatment option for patients with T2DM. Therefore, pharmacokinetics and pharmacodynamics profiles of inhaled SD GLP-1 powders can be further investigated in animal models for the feasibility of systemic pulmonary delivery of GLP-1 for T2DM therapy.


BNF, 2021a. SEMAGLUTIDE. BNF: British National Formulary - NICE.

BNF, 2021b. Type 2 diabetes. BNF: British National Formulary - NICE.


