REVIEW ARTICLE

Challenges and Strategies to Enhance the Systemic Absorption of Inhaled Peptides and Proteins

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Abstract

Proteins and peptides-based therapeutics are making substantial access to the market due to their obvious advantages of strong potency, high specificity and desirable safety profile. However, most clinical products are mainly delivered via parenteral route with inferior convenience. Lung is an attractive non-invasive alternative passage for systemic administration of biologics with numerous outstanding features, as examples of large absorptive surface area, extensive vascularization and mild local microenvironment. Even so, mucociliary clearance, alveolar macrophage phagocytosis, enzymatic metabolism, pulmonary surfactant adsorption and limited epithelium permeability constitute major obstacles affecting the systemic absorption of inhaled proteins and peptides. This article begins by giving a brief overview of challenges for the systemic absorption of inhaled proteins and peptides, and then goes on to a comprehensive review of possible strategies for enhanced pulmonary absorption, including chemical modification, addition of protease inhibitors, incorporation of absorption enhancers, modification with fusion proteins and development of particulate-based drug delivery systems. These strategies can provide enhanced transmembrane absorption capacity while avoiding pulmonary clearance, offering a valuable reference for designing pulmonary delivery systems of protein and peptide drugs.

Keywords absorption enhancers · clearance mechanisms · formulation strategies · proteins and peptides · pulmonary absorption

Introduction

Peptides and proteins have a great potential as therapeutic agents owing to their strong specificity, high potency, targeting abilities and lower toxicity relative to traditional chemical drugs [1]. The market of peptide and protein drugs has developed rapidly in recent years, with an annual growth rate of 20%, far outpacing the overall pharmaceutical market's annual growth rate of 9%, which is expected to reach $388 billion by 2024 [2]. According to the general pharmaceutical classification, proteins are macromolecules made up of multiple peptide subunits and contain 100 or more amino acids, while the biologics with less than 100 amino acid molecules belong to peptide drugs. Most proteins naturally fold into a complicated three-dimensional structure called the native conformation with known amino acid sequence

and higher order structure. Any alteration in their conformational structure mediated by various stresses, such as exposure to extreme temperature or pH, light, shear force, high osmolality and interfacial adsorption, renders them not only inactive but also immunogenic [3, 4]. Other areas of concern for proteins and peptides are their large molecular size and high polarity, resulting in limited mucosal permeability across the epithelium, and thus, injection has become the main delivery route of biological agents in clinical practice [5]. However, parenteral therapy has its own drawbacks such as invasiveness, frequent dosing and short plasma half-life, with low patient compliance especially for the treatment of chronic diseases [6]. Hence, developing a non-invasive administration route is highly desirable and essential for the better delivery of biologics. Despite the considerable efforts and almost a century of research for oral delivery, oral bioavailability of peptide and protein drugs is extremely low (typically less than 1%) mainly because of the poor gastrointestinal stability and limited permeability [7, 8].

Pulmonary route has been considered as an accessible alternative to parenteral delivery for treating respiratory

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disorders and systemic diseases, with attractive advantages such as the large absorptive surface area [100 m2], thin alveolar epithelial layer (0.2–0.7 µm), rich blood supply (5 L/min), extensive vascularization, mild environment, low intrinsic enzymatic activity, avoidance of first pass metabolism and fast onset of drug action [9, 10]. Moreover, pulmonary administration can offer a comparatively wide design space as several forms of inhaled products have been developed, including solutions, suspensions and dry powders [11]. The clinical application of Affreza® (MannKind) insulin inhalation system has also provided inspiring evidence for exploitation of inhaled proteins and peptides for systemic action [12]. Despite of these potential superiorities, it is still challenging to develop inhalable biologics for efficient lung delivery and enhanced systemic absorption. There are a few anatomical, physiological and immunological obstacles that influence the *in vivo* fate of inhaled biopharmaceuticals, including highly branched structure of the airways, mucociliary clearance, alveolar macrophage phagocytosis and enzymatic metabolism [13]. Meanwhile, the respiratory mucus, alveolar lining layer, airway epithelium, basement membrane, interstitium and capillary endothelium could pose a critical barrier for systemic absorption of large proteins with numerous hydrophilic domains. To overcome these limitations, tremendous efforts have been focused on the formulation strategies and particle engineering technologies with the goal to enhance pulmonary deposition and further absorption of proteins and peptides [14]. Apart from these biological barriers, physical degradation (e.g. aggregation, unfolding, adsorption

and precipitation) and chemical degradation (e.g. hydrolysis, oxidation, deamidation, racemization and disulfide bond shuffling) mediated by conformational instability can usually occur during production, delivery and storage of proteins, and have been extensively discussed elsewhere [4].

In this review, first of all, the main challenges affecting the systemic absorption of inhaled proteins and peptides in the respiratory airways were elucidated. Thereafter, possible strategies for enhancing the systemic absorption of inhaled peptides and proteins were addressed, including a) chemical modification to evade pulmonary surfactant adsorption and macrophage phagocytosis; b) addition of protease inhibitors to prevent enzymatic degradation; c) incorporation of absorption enhancers to improve transmembrane transport; d) modification with fusion proteins to enhance transcytosis and e) development of polymeric or lipid-based micro/nanoparticles delivery systems to prolong lung retention and reduce dosing frequency.

Challenges for the Systemic Absorption of Inhaled Proteins and Peptides

Protease and Peptidase Metabolization

Diverse metabolic enzymes as examples of serine proteases, cysteine proteases, aminopeptidases, metalloproteases, trypsin and cathepsin, which are either membrane-bound or secreted by pulmonary epitheliums and macrophages, are mainly located in the peripheral respiratory zone for protease
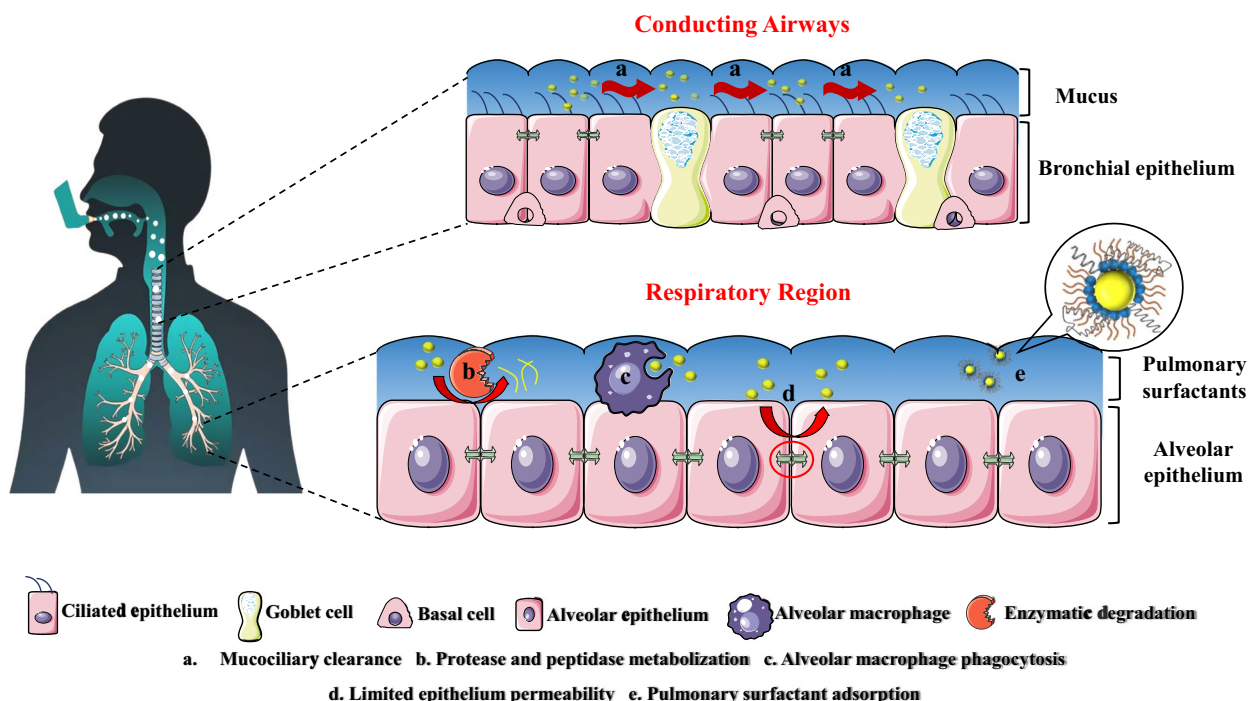

Fig. 1 Schematic representation of challenges for the systemic absorption of inhaled proteins and peptides in the respiratory airways.

and peptidase metabolism, despite with less enzymatic activity compared to the ones in the gastrointestinal tract [15, 16] (Fig. 1b). The proteomic analysis of human airway lavage fluids derived from healthy volunteers further identified 14 proteolytic species in lung lining fluid, of which the four most abundant confirmed proteases are cathepsin D, cathepsin H, angiotensin-converting enzyme (ACE) and dipeptidyl peptidase (DPP) IV, respectively [17]. Degradation of inhaled proteins and peptides could occur upon deposited in the respiratory tract or transported through the lung epithelium. The degree of enzymatic hydrolysis is drug molecular weight-dependent, presenting a higher metabolization for small natural peptides with less than 30 amino acids, compared with the proteins containing greater tertiary and quaternary structure [18]. In general, proteins with molecular weight ranging from 6 and 50 kDa are relatively resistant to ubiquitous peptidases with good bioavailability following inhalation, partially because the ends of their amino acid chains are frequently tucked inside the globular structure of proteins and are not accessible for hydrolysis. In most cases, the proteins degradation occurs inside cells in specialized organelles (endosomes, lysosomes) [19]. In addition, alveolar epithelial cells could exhibit a dominating enzymatic barrier for systemic delivery of peptide or protein drugs, in which alveolar type I cells have lower proteolytic activities relative to type II cells due to their late state of differentiation. *Yang et al.* demonstrated that alveolar type II epithelial cells possess significant metabolic activities against luteinizing hormone releasing hormone than type I cells with a higher ACE expression [20].

Mucociliary Clearance

In the conducting airways, the mucociliary clearance is an efficient self-respiratory defense mechanism, with a half-life of approximately 1.5 h [21] (Fig. 1a). The conducting airways are primarily lined by two types of airway epithelial cells: ciliated columnar cells (80%) and mucus-producing goblet cells (20%), which are collectively referred to as a mucociliary escalator [22]. The ciliated epithelium is covered with the lung lining fluid, composed of a double-layered mucus blanket: the watery periciliary sol layer in which the cilia can rhythmically swing without too high resistance, and the gel layer overlying the sol layer that contains mucins and other glycoproteins, with a thickness of 10–30 µm in the trachea and 2–5 µm in the bronchi [23, 24]. The most relevant constituents of mucus, known as mucins, consist of a protein backbone with a high number of tandem repeats of the amino acids proline-serine-threonine (PTS sequences), flanked by cysteine-rich regions that serve to establish inter-mucin disulfide bonds, creating a three-dimensional and covalently-linked mesh-like network [25]. On the one hand, mucus acts as a physical filtering barrier for larger proteins with intricate spatial conformations

(e.g. immunoglobulin) owing to the limited mesh-spacing of mucin network, with an average pore size of 340 ± 70 nm for human respiratory mucus [26], leading to the entrapment of inhaled proteins in mucus blanket prior to moving to the lower respiratory regions, and then being propelled and transported together with mucus towards the proximal direction and eventually either expectorated or swallowed [27]. On the other hand, mucus serves as a physicochemical interaction barrier due to its complex chemical composition. Of which, high sialic acid and sulfate content in most mucin glycoproteins confer a strongly net-negative surface charge that induces the interactions with cationic proteins. Meanwhile, mucus contains various functional groups which can participate in hydrogen bonding, including hydroxyl, carboxylic and sulfate groups from glycans and amide groups from the peptide backbone, impeding the diffusion of hydrophilic proteins and peptides, and thus limiting drug absorption [28]. One feasible strategy for overcoming rapid mucociliary clearance is the development of mucus-penetrating particles (MPP), through rendering the particle surface non-mucoadhesive via lessons learnt from viruses characterized by small size and net neutral hydrophilic surface [29, 30].

Alveolar Macrophage Phagocytosis

In the respiratory region, the clearance of inhaled peptides and proteins are predominantly performed by alveolar macrophages (Fig. 1c). The airspaces of each of the estimated 500 million alveoli in a healthy individual are typically "patrolled" by 12–14 alveolar macrophages to keep it free from any inhaled/foreign particles deposited in the alveolar zone [31]. After phagocytosis, alveolar macrophages travel along the alveolar surface to the mucociliary escalator or translocate into tracheobronchial lymph for clearance. In general, the molecular weight (MW) and size are the factors of great importance for regulating the drug-macrophage interactions and subsequent uptake. Macromolecules with MW ≤ 25 kDa are rapidly cleared, while the ones with MW ≥ 40 kDa are slowly endocytosed by the macrophages [32]. Meanwhile, phagocytosis appears optimal for particles of 1.5–3 µm in size. Apart from the size, the shape, density, charge and hydrophilic/lipophilic characteristics of particulate carriers also play an indispensable role in alveolar macrophage clearance and their effects have been specifically reviewed in another literature [33]. Furthermore, the macrophages could also affect the *in vivo* fate of therapeutic biologics owing to their immunogenicity. The inhaled proteins or peptides processed by alveolar macrophages may be transferred back to regional lymph nodes and subsequently activate naïve T cells and B lymphocytes, resulting in the unwanted immune responses, which could reduce the therapeutic efficacy of these macromolecules [23, 34]. *Lombry et al.* demonstrated that depletion of alveolar macrophages

by liposome-encapsulated dichloromethylene diphosphonate could cause 2- and 13-fold enhancement in systemic bio-availability of IgG and human chorionic gonadotropin (hCG) respectively after intratracheal instillation in rats [35].

Pulmonary Surfactant Adsorption in the Alveolar Lining Layer

Pulmonary surfactant is a complex mixture of lipids and proteins, covering the surface of epithelial cells in the entire respiratory zone (Fig. 1e). Phospholipids are the main component of pulmonary surfactant (around 90%), with the fully saturated dipalmitoyl phosphatidylcholine (DPPC) constituting approximately 41% of the mass, along with other unsaturated phosphatidylcholines (PC, 25%), phosphatidylglycerol (PG, 9%), phosphatidylethanolamine (PE, 5%), phosphatidylinositol (PI, 3%) and cholesterol (8%). In addition to lipids, surfactant proteins constitute the remaining 8–10% of surfactants by mass, including hydrophilic proteins (6% SP-A and 0.5% SP-D) and hydrophobic proteins (1% SP-B and 1% SP-C) [36]. Deep within the lung, pulmonary surfactant is considered as the first barrier of respiratory surface to come into contact with external elements. When deposited in the respiratory zone, proteins and peptides with diverse physicochemical characteristics (size, surface charge and hydrophobicity/hydrophilicity) could dynamically adsorb various surfactant components onto their surface, which may subsequently alter their clearance and translocation [37]. As for inhaled proteins with charged hydrophilic traits, on the one hand, they will interact with these surfactant components via electrostatic interaction, such as negatively charged phospholipids (PG, PI) and positively charged SP-B and SP-C. On the other hand, the hydrophilic proteins (SP-A and SP-D) and hydrophilic head group of phospholipids preferentially adsorb to the hydrophilic domains of inhaled proteins, forming the larger aggregates. The surfactant lipids/proteins-triggered agglomeration of the biologics in the lung could be eventually degraded by alveolar macrophages, resulting in reduced systemic absorption of therapeutic proteins and peptides.

Limited Epithelium Permeability

The optimal absorption characteristics of inhalable biologics depend on the site of drug action. For locally-acting drugs, the aerosolized proteins and peptides need to be absorbed and terminated in lung tissues, whilst for systemically-acting biological drugs, these macromolecules must diffuse or actively transport through the surfactant layer/mucus layer, epithelium, basement membrane and interstitium and capillary endothelium to access the blood circulation [38]. Whereas, the pulmonary epithelium serves as the main barrier for the transportation of peptide and

protein drugs to the bloodstream due to their high MW and hydrophilic features (Fig. 1d), whether via paracellular transport or transcytosis [39, 40]. In the paracellular pathway, the tight junctions (integral proteins of claudins and occludins) of respiratory epithelium with a cut-off of 0.5–0.9 nm are considered the critical obstacle affecting the absorption of proteins and peptides into the systemic and/or lymphatic circulation [41]. The absorption rate of these macromolecules from the airway lumen into the systemic circulation is inversely related to their molecular weight. The peptides and smaller proteins with MW below 40 kDa are rapidly detected in the bloodstream within a few minutes following inhalation, whereas those with MW above 40 kDa are slowly absorbed in the lung over hours to days [42]. In addition, some of larger proteins are also able to be absorbed into the bloodstream via the transcellular pathway (non-specific pinocytosis and receptor-mediated endocytosis), but only to a limited extent. Studies have revealed the presence of membrane vesicles recognized as caveolae, within both the epithelial type I cells and alveolar endothelial cells, which are assumed to mediate transport of peptides and proteins [43]. In parallel, transporter molecules expressed on the apical membrane of epithelial cells in bronchi and alveoli, such as the solute carrier (SLC) and ATP binding cassette (ABC) transporters could also mediate the transmembrane absorption of proteins and peptides [44]. As an example, the proton-coupled peptide transporters (PEPT) 2 with a high expression level are involved in the transmembrane transport of di- and tripeptides, as well as some peptidomimetic drugs. Moreover, the translocation of antibodies and plasma proteins, like albumin, transferrin and immunoglobulin G from the apical to the basolateral side could occur in alveolar monolayers through the receptor-mediated endocytosis [45, 46]. Given the role of paracellular /transcellular pathways in transepithelial trafficking of inhaled proteins and peptides, modulation of epithelial tight junctions and identification of specific transporters/receptors involved in the cellular uptake are important for the development of efficient pulmonary delivery strategies for biological drugs.

Strategies to Enhance the Systemic Absorption of Inhaled Proteins and Peptides

Chemical Modification

Covalent modification of polymers onto sulfhydryl, amino and carboxyl groups on the side chain of protein molecules could be deemed as a viable strategy to reduce the rapid lung clearance of inhaled biologics, thus improving the drug retention within the lungs and further systemic

absorption. In addition, the polymer-protein conjugates could also effectively ameliorate the stability of therapeutic proteins, which is beneficial to the industrial production of protein drugs [47]. Of these polymer conjugates, the most developed and in-depth study focuses on PEGylated proteins. Zwitterionic polymers have recently showed exceptional potential for enhanced systemic absorption of inhaled proteins and peptides [48].

PEGylated Proteins

PEG, which is made up of repeated ethylene glycol units, is a biocompatible, neutral and non-immunogenic polyether available in a wide range of molecular weights. Its flexibility in terms of structure and hydration can create steric hindrance that prevent access of proteases, antibodies and opsonin [49]. PEGylation is a desirable pharmaceutical technology for sustaining the residence time of peptides and proteins in the lungs on account of the increased molecular size, enhanced proteolytic resistance and lessened macrophage phagocytosis [50]. Moreover, pulmonary administration of PEGylated proteins is also attractive as a non-invasive route for systemic absorption with extended plasma half-life, and the altered pharmacokinetic behavior (local or systemic action) is strongly correlated with size of the PEG substituent [51]. *Youn et al.* demonstrated that PEGylated salmon calcitonin derivatives administered intratracheally could enhance the *in vivo* systemic efficacy due to their increased proteolytic stability and extended circulating half-life, both of which showed the dependences on PEG size (1–5 kDa). Conjugation of salmon calcitonin to 1 kDa, 2 kDa or 5 kDa PEGs exhibited 2.5-, 4.3- and 7.0-fold increase in the area under plasma concentration–time curves (AUC), and prolonged the plasma half-life ($t_{1/2}$) from 35 to 54 min, 101 min and 119 min in comparison with native salmon calcitonin, respectively (Fig. 2A) [52, 53]. In contrast, *Patil, Mahri and Mcleod et al.* observed declined systemic exposure and increased lung residence for larger PEGs (20–40 kDa)-protein conjugates after intratracheal administration, such as

linear 20 kDa PEG/two-armed 40 kDa PEG-anti-interleukin (IL) antibody fragments, 40 kDa PEG-interferon α2 and 20 kDa/ 30 kDa /40 kDa PEG-recombinant human deoxyribonuclease I (rhDNase) [545556]. A particular note is that protein conjugation to PEG molecules could cause an interference with their original biological activities, and thus, it is necessary to take site-specific PEGylation into consideration rather than random-site attachment of PEG chains [50]. Furthermore, repeated administration of PEGylated proteins, liposomes or nanoparticles could trigger an accelerated blood clearance (ABC) phenomenon mediated by anti-PEG antibodies, potentially affecting therapeutic value of biologics in chronic diseases [57].

Zwitterionic Polymer Conjugated Proteins

Zwitterionic polymers, which are primarily composed of three types of compounds, including phosphorus-ammonium (phosphorylcholine), sulfo-ammonium (sulfobetaine) and carboxy-ammonium (carboxybetaine), have emerged as a significant class of superhydrophilic biomaterials with exceptionally high hydration and ultra-low protein adsorption [58]. Among these materials, zwitterionic poly(carboxybetaine) (PCB) presents the great aptitude of avoiding non-specific interactions with lung environment and enhancing the protein absorption into the bloodstream with negligible immunogenicity/antigenicity due to its superior hydration capability [59, 60]. Meanwhile, PCB conjugation also enhances protein stability without sacrificing the bioactivity owing to its structural resemblance to the naturally occurring protein stabilizer glycine betaine, and it is viewed as a feasible alternative to PEG substitution [61]. *Tsao et al.* demonstrated that organophosphate hydrolase (OPH) conjugated with PCB (5, 10 and 20 kDa) administered via intratracheal instillation into the lung exhibited improved pharmacokinetic properties and prophylactic efficacy against organophosphate poisoning, with OPH-5 kDa PCB systemic absorption being the most and OPH-20 kDa PCB being the least due to the natural size-dependent

Fig. 2 Pharmacokinetic profiles of intratracheally administered (**A**) salmon calcitonin (sCT) and PEG (1 k, 2 k and 5 k)-sCT derivatives, and (**B**) organophosphorus hydrolase (OPH) and zwitterionic poly(carboxybetaine) (pCB, 5 k, 10 k and 20 k)-OPH derivatives. Adapted with permission from [52, 62].
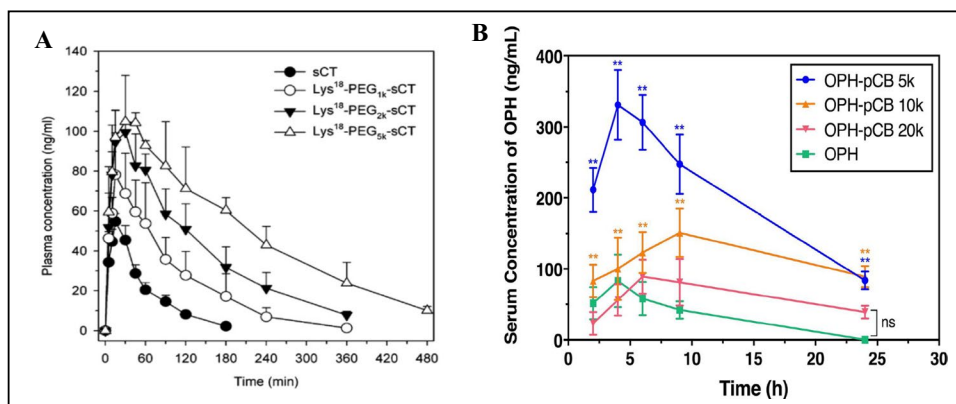
limitation of mucus and lung epithelium, and the bioavailability of OPH significantly increased from 5.1% to 53.4% following 5 kDa PCB conjugation (Fig. 2B) [62].

Adding Protease Inhibitors

Considering the incomplete absorption of proteins and peptides mediated by enzymatic degradation, protease inhibitors as one of the promising approaches, can reduce the breakdown of various peptides and proteins at the absorption sites and in turn enhance the systemic absorption. The degree of absorption enhancement provided by these protease inhibitors typically relies on what enzymes the protease inhibitor utilized restrains and how susceptible the delivered protein is to degradation [11]. Several compounds have been demonstrated as protease inhibitors for enhancing the stability and subsequent pulmonary absorption of inhaled peptides and proteins, such as aprotinin (a serine protease inhibitor), bacitracin (an aminopeptidase inhibitor), bestatin, leupeptin, chymostatin, soybean trypsin inhibitors and sodium glycocholate [63]. *Amancha et al.* evaluated the effect of protease inhibitors (aprotinin/bestatin) on the pulmonary absorption of therapeutic peptides and proteins with different molecular weights, including leuprolide (1.2 kDa), calcitonin (3.4 kDa), insulin (5.8 kDa), leptin (16.0 kDa) and human chorionic gonadotropin (36.5 kDa). The authors discovered that the *in vitro* stability in rat lung homogenate and *in vivo* systemic bioavailability were enhanced to varying degrees with the specificity of protein species. Among them, the bioavailability of calcitonin improved the most significantly with a twofold increase in the presence of aprotinin/bestatin [64]. *Fukuda and Park et al.* revealed the effect of various protease inhibitors on the lung absorption and metabolism of insulin after intratracheal instillation, with the effectiveness in the order of bacitracin > aprotinin > soybean trypsin inhibitor > sodium glycocholate [65, 66]. Moreover, the safety issues should be noted that addition of exogenous protease inhibitors could increase local protein concentration in the lung parenchyma and therefore break airway surface liquid homeostasis [67].

Adding Absorption Enhancers

A fundamental challenge in systemic pulmonary drug delivery is improving the transportation of peptides and proteins across the airway epithelium. Absorption enhancers are one of the most straightforward and well-proven methods for increasing the transmucosal permeability of co-administered payloads via paracellular or transcellular pathways, with a dose-dependent efficacy and potential toxicity [68]. The three major classes of absorption enhancers for pulmonary delivery include surfactants, cationic polymers and cyclodextrins (Table I).

Surfactants

Phospholipids as biocompatible, biodegradable and endogenous surfactants, have been proved to enhance the systemic absorption of proteins and peptides after intratracheal delivery in several studies. *Codrons et al.* demonstrated that the dry powders containing DPPC could promote the transport of parathyroid hormone 1–34 (PTH) from the airspaces into the bloodstream with 1.8-fold increase in PTH bioavailability [69]. *Gao et al.* verified the potential of DSPE-PEG polymers on improving systemic absorption of calcitonin via intrapulmonary administration with no damage to local pulmonary membranes, exhibiting threefold enhancement for hypocalcemic effect [70]. Moreover, the phospholipids with different types of hydrophobic fatty acid chains could result in distinct systemic bioavailability of inhaled proteins. Several research groups have reported that lipids including the palmitoyl group, such as DPPC and egg yolk phosphatidylcholine (EPC), enhanced the pulmonary absorption of insulin with 6.7- and 3.4-fold increase, respectively. In contrast, dilauroyl phosphatidylcholine (DLPC), distearoyl phosphatidylcholine (DSPC) or dimyristoyl phosphatidylcholine (DMPC) devoid of the palmitoyl group might display no delivery-enhancing effects [71, 72].

Bile salts and their derivatives have received considerable interest in pulmonary delivery of biologics owing to their ability of enhancing systemic bioavailability. Bile salts are ionic amphiphilic compounds with a steroid skeleton, and the commonly used bile salts as absorption enhancers include the sodium salts of cholate, deoxycholate, taurocholate, taurodeoxycholate, glycocholate and glycodeoxycholate [38]. Among them, sodium taurocholate is one of the most widely used bile salts to enhance the systemic absorption of aerosolized proteins. *Johansson et al.* observed that the absolute bioavailability of inhaled insulin combined with taurocholate sodium (32 mM) was about nine times higher relative to pure insulin [73]. The enhancement for insulin absorption by bile salts have been ordered as sodium deoxycholate > sodium cholate > sodium glycocholate > sodium glycodeoxycholate > sodium taurodeoxycholate. However, bile salts have limited clinical use on account of their irreversible damage to the mucosa and ciliotoxicity. *Sorli et al.* evaluated the potential toxicity of bile salts on lung tissues, and the bile salts were ranked as sodium deoxycholate > sodium glycocholate > sodium taurodeoxycholate > sodium taurocholate according to potency *in vitro* for surfactant function disruption and *in vivo* for induction of pulmonary irritation [74]. *Moghimipour et al.* also demonstrated the intense ciliotoxicity of sodium deoxycholate, with ciliary arrest occurring within 1 min at a concentration of 5 mM, whereas sodium taurocholate caused less mucosa damage and ciliary arrest does not occur until 30 min, even

Table I Commonly Used Absorption Enhancers for Pulmonary Delivery of Proteins and Peptides

Class	Enhancers	Examples	Concentration	Biologics	Enhancement	Refs
Surfactants	Phospholipids	DPPC	19.4 mmol/L	Insulin	6.7-fold increase for pulmonary absorption (AUC) of insulin	[71]
		DSPE-PEG	1% (w/v)	Calcitonin	threefold enhancement for hypocalcemic effect of calcitonin	[70]
	Bile salts	Sodium taurocholate	32 mM	Insulin	ninefold improvement for absolute bioavailability of inhaled insulin	[73]
	Fatty acids	Arachidonic acid	25 mM	IFN-α	2.7- and 2.5-fold increase for C_{max} and AUC of IFN-α, respectively	[84]
Cationic polymers	Chitosan and its derivatives	N-trimethylated chitosan	1.5% (w/v)	Octreotide acetate	2.4-, 2.5- and 3.9-fold enhancement for *in vivo* bioavailability of octreotide with chitosan, 20% and 60% N-trimethylated chitosan	[91]
		Chitosan oligomers (hexamer)	0.5% (w/v)	Calcitonin	1.9-fold increase for pharmacological availability of calcitonin	[92]
	PAMAM	Generation 3 (G3) PAMAM dendrimer	0.1–1% (w/v)	FD4	2.0-, 2.7- and 2.9-fold enhancement for pulmonary absorption (AUC) of FD4 at concentrations of 0.1%, 0.5% and 1% (w/v)	[93]
	Cationic dextran	Sperminated dextran (SD)	0.1% (w/v)	Insulin	1.2 times more pharmacological active than inhaled insulin alone	[94]
Cyclodextrins (CD)	Dimethyl-β-CD		0.25% (w/v)	Insulin	2.3- and 6.5-fold increase for AUC and C_{max} of inhaled insulin, respectively	[97]
	Hydroxypropyl-β-CD-PEI$_{1800}$		5% (w/v)	Insulin, Calcitonin	2.8- and 3.2-fold increase for pharmacological availability of insulin and calcitonin, respectively	[98]

at concentration of 30 mM [75]. The mechanisms of absorption enhancement by bile salts may involve the transcellular transport via extraction of membrane phospholipids and proteins relying on formation of micelles, paracellular transport by destroying the hemidesmosomes or binding to calcium in the regions of tight junctions, or some combination of both [76]. Additionally, some studies have reported that bile salts can reduce the viscosity and elasticity of the mucus layer and block the mucosal membrane peptidases and protease, conducive to the pulmonary absorption of biologics [77].

Fatty acids and their derivatives with different structures have also been demonstrated for enhancing pulmonary absorption of peptides and proteins. Fatty acids are classified based on the level of chain length or unsaturation, including short-, medium- and long-chain fatty acids, or saturated and unsaturated fatty acids [78]. Of these fatty acids, medium-chain fatty acids such as capric acid (C10) and lauric acid (C12) have been extensively researched as absorption enhancers, and the potential mechanisms of action might be related to the phospholipase C-mediated elevation of intracellular calcium levels, leading to contraction of calmodulin-dependent actin microfilaments and dilation of tight junctions, or the detergent-induced plasma membrane fluidization [798081]. *Ghadiri et al.* revealed that sodium decanoate could enhance the bronchial epithelial transport of poorly permeable biologics with twofold increase in apparent permeability coefficient (P_{app}) [82]. Moreover, polyunsaturated fatty acids (PUFAs) have exhibited an essential involvement in airway epithelial permeability by altering the local lipid environment. *Traini et al.* indicated that PUFAs (docosahexaenoic acid, eicosapentaenoic acid, linoleic acid, palmitoleic acid, arachidonic acid) could enhance the systemic absorption of fluorescein sodium (Na-flu) delivered via inhalation with decreased transepithelial resistance and depressed mucus production of calu-3 cells. Of which, the increase was prominently higher for palmitoleic acid treatment with the P_{app} increasing 1.5 times over control group. [83]. *Miyake et al.* demonstrated that arachidonic acid (25 mM) may improve the pulmonary absorption of interferon-α (IFN-α) after intratracheal administration in rats through opening the tight junction domains with 2.7- and 2.5-fold increase in C_{max} and AUC of IFN-α, respectively [84]. However, despite the favorable outcomes achieved with these sorts of absorption enhancers, there remain worries about their propensity to harm essential mucous membrane, especially regarding application in the treatment of chronic diseases, like diabetes.

Cationic Polymers

Cationic polymers characterized by the inherently positive charges in the polymer side chains and/or its backbone, have shown the potential to enhance absorption of macromolecules across mucosal surfaces. Natural cationic polymers such as cationic gelatin, dextran, chitosan, and synthetic cationic polymers like poly (amido amine) (PAMAM), poly (L-lysine) (PLL), poly (ethylenimine) (PEI) are some examples of these polymers [85, 86]. Among them, chitosan and its derivatives have been employed commonly as absorption enhancers duo to their good biocompatibility and biodegradability. Chitosan, composed of 2-amino-2-deoxy-D-glucopyranose and 2-acetamido-2-deoxy-D-glucopyranose units randomly linked together via β-(1,4) glycosidic bonds, is a linear polysaccharide with varied molecular chain length, sequence and composition [87]. The mechanism by which chitosan enhances mucosal absorption could be attributed to a combination of mucoadhesion and tight junctions' regulation [88, 89]. On the one hand, protonated amino groups of chitosan can interact electrostatically with the negatively charged sialic acid residues on mucin. On the other hand, chitosan can transiently open the epithelial tight junctions embodied by a reduction in transepithelial electric resistance (TEER), and the spatial distribution of tight junction associated proteins, as an example of zonula occludens-1 (ZO-1), along with cytoskeletal F-actin could be altered in the presence of chitosan, thus affecting paracellular transport [90]. Additionally, several analogues have been developed to address low solubility of chitosan under pulmonary physiological environment (pH 7.4), and the most comprehensively tested chitosan derivative is *N*-trimethyl chitosan (TMC), which has superior aqueous solubility and remains charged over a broad pH range (pH 1–9). *Florea et al.* validated that chitosan, 20% and 60% *N*-trimethylated chitosan derivatives (TMC20 and TMC60) could decrease TEER of bronchial epithelium significantly and enhance octreotide acetate permeation *in vitro* by 21-, 16- and 30-fold, as well as bioavailability *in vivo* by 2.4-, 2.5- and 3.9-fold, respectively [91]. Furthermore, chitosan oligomers including chitosan dimer, tetramer and hexamer have been well-researched for promoting the pulmonary absorption of therapeutic peptides and proteins owing to their high solubility in water and low molecular weight compared with conventional chitosan. *Zhang et al.* demonstrated that chitosan oligomers, especially chitosan hexamer with the optimal concentration of 0.5% (*w/v*), were effective to increase absorption of salmon calcitonin from pulmonary routes, with 1.9-fold enhancement in pharmacological availability [92]. Except for chitosan and its derivatives, the PAMAM dendrimers constructed by the successive grafting of amino acrylate units (generation) with terminal amino groups (pKa of 8–9) have also proven as an efficient absorption promoter for pulmonary delivery of proteins and peptides. *Lu et al.* elucidated that the absorption-enhancing effects of PAMAM dendrimers on the pulmonary absorption of fluorescein isothiocyanate-labeled dextrans (FDs) were in a generation-dependent manner, generation 3 (G3)
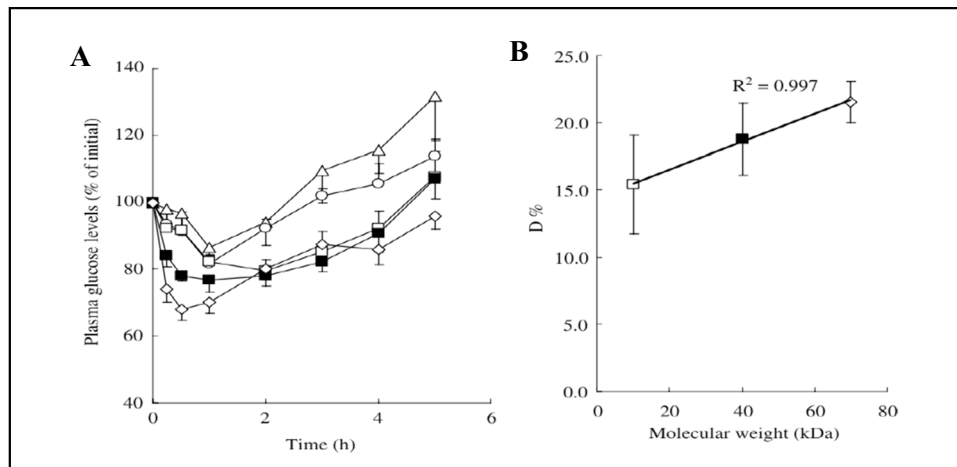
Fig. 3 Effects of different molecular weights of sperminated dextran (SD, 10 kDa, 40 kDa and 70 kDa) on the absorption of insulin in rats. (A) Plasma glucose levels after pulmonary administration of insulin (10 IU/kg). (b) Relationships between the molecular weights of SD and the D% as indexes of pharmacological effects after pulmonary administration of insulin (10 IU/kg). △ PBS, ○ insulin without enhance, □ insulin with SD-10, ■ insulin with SD-40, ◇ insulin with SD-70. Reprinted with permission from [94].

PAMAM dendrimer presenting the highest effectiveness and negligible toxicity, with 2.9-fold increase in AUC, and the absorption mechanism might be related to the up-regulation of organic cation transporters (OCTs) secretion, for instance, OCT1, OCT2 and OCT3 [93]. *Morimoto et al.* noticed that sperminated dextran, a cationic polymer, enhanced the systemic absorption of insulin in rats following inhalation, presenting a positive correlation with the molecular weights of sperminated dextran over the range 10–70 kDa (Fig. 3) [94]. Of note, some of these cationic polymers could induce a significant dose-dependent pulmonary inflammatory response, and thus, a balance between efficacy and adverse effects towards the respiratory epithelium is crucial to their acceptance.

Cyclodextrins

Cyclodextrins (CDs) are a family of cyclic oligosaccharides with hydrophobic cavity and hydrophilic exterior, which is mainly sub-divided into α, β and γ—CDs containing six, seven and eight glucopyranose units, respectively [95]. CDs in combination with therapeutic proteins have been proposed as candidates that display enhanced mucosal absorption capacity. Nevertheless, the specific mechanism of penetrating enhancer is still unclear, possibly involving the removal of membrane proteins, complexation with lipophilic penetrants or inhibition of proteolytic enzyme activity. The effectiveness of these compounds in enhancing transmucosal protein transport was observed to be in the sequence of dimethyl-β-CD > α-CD > β-CD > γ-CD > hydroxypropyl-β-CD [66]. *Jalalipour et al.* showed the absolute bioavailability of 25.38%, 76.52% and 63.97% after intratracheal insufflation of dry powders containing dimethyl-β-CD and recombinant human growth hormone (rhGH) at the molar

ratios of 10, 100 and 1000, respectively, presumably due to the increased powder dispersion and good absorption-promoting behavior of dimethyl-β-CD [96]. *Hussain et al.* revealed that dimethyl-β-CD (0.25%, *w/v*) was efficacious to enhance insulin absorption via pulmonary administration with 2.3-fold increase in AUC and less cellular toxicity as well [97]. *Zhang et al.* synthesized the hydroxypropyl-β-cyclodextrin grafted polyethyleneimine (HP-β-CD-PEI$_{1800}$) conjugate, and validated its potential as a safe absorption enhancer for improving the pulmonary absorption of proteins, with 2.8- and 3.2-fold increase for pharmacological availability of insulin and calcitonin, respectively [98].

Using Fusion Proteins to Enhance Transcytosis

Aiming at lung epithelium barrier, enhanced transcytosis could be regarded as another potential strategy to improve the systemic absorption of inhaled proteins and peptides. Enhanced transcytosis via the lungs have been achieved by guiding the delivery through a particular active uptake pathway by merging the protein of interest with another protein that selectively interacts to a receptor on the lung epithelium [99]. The neonatal constant region fragment (Fc) receptor (FcRn) acts as a transporter from the lumen of the upper and central airways to the blood allowing pulmonary delivery of Fc-containing proteins [100]. As a consequence, incorporation of the Fc domain of immunoglobulin G (IgG) into protein molecules is deemed to be an effective approach for improving the transportation of biologics across the respiratory epithelial barrier [1]. Additionally, these hybrid biomacromolecules have also extended plasma half-life on account of FcRn-mediated receptor binding and subsequent salvaging of IgG from intracellular lysosomal degradation pathways [101, 102]. Several researchers have reported that

the absorptive transcytosis of Fc-fusion proteins is pH and temperature sensitive together with concentration dependence, showing stronger association with FcRn at slightly acidic pH (at or below 6.5) and comparatively higher temperature (37 ℃) [103]. Meanwhile, IgG1 is recognized to display a higher affinity binding interaction among these IgG subclasses. *Bitonti et al.* developed a conjugation of erythropoietin and Fc domain of IgG1 and demonstrated a 2.3-fold increase in lung absorption through FcRn-mediated transport channel relative to erythropoietin delivered alone, with the effects being more pronounced following deposition in the upper airways as opposed to the peripheral lung [104]. *Vallee et al.* revealed that the monomeric Fc fusion protein of interferon beta (IFNβ) was absorbed via an immunoglobulin transport system upon inhalation, with 2.2-fold enhancement in systemic absorption and 3 times longer circulating half-life [105]. Despite the limited available data, pulmonary delivery of such therapeutic Fc fusion proteins, especially for large proteins exceeding 10 kDa in MW, showed a potentially important alternative to the parenteral dosage forms.

Polymeric Particulate Delivery Systems

Polymeric micro/nanoparticles as controlled pulmonary drug delivery systems, could overcome the drawbacks of inhaled proteins and peptides, such as poor absorption, rapid metabolism and elimination, and enhance the therapeutic index of biologics through one or more of the following features: a) prolonging the lung residence time by avoiding clearance mechanisms, together with sustained drug release behavior; b) improving the storage and *in vivo* stability; c) increasing the proportion of biologics at the site of action (systemic or local, intracellular or extracellular domain); d) evading the immunogenicity of biologics; e) decreasing the irritation and toxicity due to high initial doses [106, 107]. Several types of carriers have been utilized for formulating polymeric particulate delivery systems, for instance, natural polymers [chitosan (CS), hyaluronic acid (HA), alginate, gelatin, dextran, carrageenan, albumin, cyclodextrin] and synthetic polymers [poly (lactic acid) (PLA), poly (lactic-co-glycolide acid) (PLGA), polyethylene glycol (PEG), poly (vinyl alcohol) (PVA), poly(butylcyanoacrylate) (PBCA)] [108, 109]. The representative particle engineering technologies for pulmonary administration of proteins and peptides are summarized in Table II.

Polymeric Nanoparticles

Polymeric nanoparticles are particularly interesting carriers for pulmonary delivery of proteins and peptides by virtue of their unique advantages over other carrier systems. Smaller size relative to microspheres renders nanoparticles a stronger

translocation efficiency across airway epithelium. Meanwhile, polymeric nanoparticles also exhibit higher loading capacity and good stability in lung lining fluids compared to liposomes and lipid nanoparticles [110]. Moreover, the extended drug release, together with circumvention of macrophages phagocytosis and enzymatic degradation prolong the lung residence of biologics and exert a longer pharmacological activity of payloads for weeks [111]. *Markwalter et al.* developed a solubility-driven sequential assembly process called inverse Flash NanoPrecipitation (iFNP) that produces highly loaded polymeric nanoparticles with 50–200 nm in diameter. The polymyxin B-loaded nanoparticles prepared using poly (aspartic acid)-*b*-poly (lactic acid) copolymer and iFNP technique exhibited therapeutic loadings as high as 27 wt% and 2.7-fold reduction in lung bacterial burden below that of unencapsulated one [112]. *Makhlof and Varshosaz et al.* demonstrated that calcitonin-loaded thiolated glycol chitosan nanoparticles and poly (methyl vinyl ether maleic acid) bioadhesive nanoparticles obtained by ionic gelation method could cause a pronounced hypocalcemic effect for at least 24 h after pulmonary administration, and a corresponding pharmacological availability of 40 and 65%, respectively [113, 114]. *Huang and Zhao et al.* revealed that insulin-loaded nanoparticles made from low-molecular-weight chitosan and poloxamer 188 modified gelatin promoted insulin pulmonary absorption effectively, and showed good relative pharmacological bioavailability of 73.7% and 76.4% in comparison with subcutaneously administered insulin solution, respectively [115, 116]. Notable, too, is the toxicity issues of polymer accumulation within the lungs [18]. Nanoparticles, unlike micron fragments, are less accessible to alveolar macrophages and are usually retained in the alveoli for a long time upon inhalation, which could result in the potential hazard to lung epithelium. Particularly for PLGA nanoparticles, the bulk degradation of PLGA matrix produces an acidic zone that can cause a damage to pulmonary mucosa and pH sensitive proteins and peptides.

Polymeric Microparticles

Polymeric microparticles are a frequently used strategy for controlled pulmonary drug delivery. Compared with nanoparticulate system, microparticles are usually fabricated in the form of dry powders with a higher storage stability of proteins and peptides, and the larger particle sizes can avoid the low inertia properties, making it much more available in practical use. Conventional inhaled microparticles with geometric sizes between 1 and 5 μm and mass density near 1 g/cm^3 are prone to particle aggregation and poor dispersibility during inhalation, resulting in decreased aerosolization efficiency [117]. In recent years, sophisticated microparticulate delivery systems with versatile structures have been developed to alleviate these limitations, such as large

Table II Representative Particle Engineering Technologies for Pulmonary Administration of Proteins and Peptides

Delivery systems	Biologics	Carriers	Key Findings	Refs
Polymeric nanoparticles	Polymyxin B	PAsp-b-PLA/ PLA-b-PEG	2.7-fold reduction in bacterial burden below that of unencapsulated one	[112]
	Salmon calcitonin	Thiolated glycol chitosan	Enhanced mucoadhesion and hypocalcemic effect for at least 24 h, with a pharmacological availability of 40%	[113]
	Salmon calcitonin	P(MVEMA)	2.8-fold enhancement for normalized reduction of plasma calcium levels compared to free solution, with a pharmacological activity of 65%	[114]
	Insulin	Gelatin	Improved relative pharmacological bioavailability than inhaled insulin alone with 1.2-fold enhancement	[116]
	Salmon calcitonin	PLGA	Superior hypocalcemic action over calcitonin solution with 1.1-fold increase	[148]
	Insulin	PBCA	Prolonged hypoglycemic effect for 12 h and a relative pharmacological bioavailability of 57.2%	[149]
	Synthetic LL37 peptide	Albumin	Sustained release and extended antimicrobial effects over 48 h with alleviated lung damage	[150]
Large porous particles	Exendin-4	PLGA	Sustained release profiles and longer *in vivo* pulmonary hypoglycemic duration over 5 d	[119]
	Insulin	PLGA	Desired aerodynamic properties with a MMAD < 5 μm, and fivefold increase in pharmacological availability as compared to inhaled insulin solution	[121]
	Insulin	PLA	Good aerosolization characteristics with a MMAD of 4.31 μm and a FPF of 65.57%	[122]
Porous nanoparticle- aggregate particles	Bovine serum albumin	PGA-co-PDL	A high FPF of 78.57% and low mucosal toxicity with above 85% cell viability	[124]
	Insulin	Chitosan/ Mannitol	An D_a of 2–3 μm and 1.7-fold increase in systemic absorption compared with insulin alone	[126]
Technosphere® microparticles	Insulin	FDKP	A respirable MMAD of 2–2.5 μm and a rapid systemic absorption within 30 min, along with twofold increase in AUC compared with insulin alone	[151]
Liposomes	Insulin	DPPC/EPC	6.7- and 3.4-fold increase in AUC for DPPC- and EPC-based liposomes compared with insulin solution, respectively	[71]
	Insulin	SPC	Long-lasting period with low blood glucose level for 10 h, and a relative pharmacological bioavailability of 38.4%	[135]
	Elcatonin	DSPC	twofold enhancement in systemic absorption for CS modified liposomes	[136]

Table II (continued)

Delivery systems	Biologics	Carriers	Key Findings	Refs
Solid lipid nanoparticles	Insulin	SPC	A higher nebulization efficiency of 63.3%, good stability in vitro and a relative bioavailability of 22.3% for inhalation	[141]
	Papain	Glyceryl dibehenate / Glyceryl tristearate	Good protein stability and high enzymatic activity above 90%	[152]
Nanostructured lipid carriers	Octreotide acetate	SPC/Glycol tristearate/Glyceryl monostearate/ Oleic acid	A preferred encapsulation efficiency of 95% and a MMAD of 3 μm, as well as 2 times higher AUC in comparison to octreotide acetate solution	[143]
Pulmosphere™ microparticles	IgG	DSPC/DPPC	Rapid drug release profiles for 80% release within 6 h, and a systemic bioavailability of 26%	[147]

PLGA [poly(lactide-co-glycolide)]; PLA (poly-l-lactide); PAsp [poly(aspartic acid)]; PEG [poly(ethylene glycol)]; P(MVEMA) [poly(methyl vinyl ether maleic acid)]; PBCA (polybutylcyanoacrylate); PGA-co-PDL [poly(glycerol adipate-co-pentadecalactone)]; FDKP (fumaryl diketopiperazine); IgG (immunoglobulin); SPC (soybean phosphatidylcholine); EPC (egg phosphatidylcholine); DPPC (dipalmitoyl phosphatidylcholine); DSPC (disaturated phosphatidylcholine)

porous particles (LPPs) and porous nanoparticle aggregate particles (PNAPs).

LPPs characterized by large geometric diameters (> 5 μm, up to 30 μm) and low bulk densities (typically < 0.4 g/cm³), present numerous advantages over their nonporous counterparts, including improved dispersibility, efficient deep lung deposition and avoidance of phagocytic clearance [118]. These characteristics are capable of enhancing the aerosolization effectiveness of therapeutic biologics to the lung periphery, facilitating transportation into systemic circulation. In general, such porous polymeric microparticles are prepared using several unique pore-forming agents, as examples of osmogens (cyclodextrins etc.), extractable porogens (Pluronics and polyvinylpyrrolidone etc.) and effervescent agents (ammonium bicarbonate etc.) [119, 120]. *Ungaro et al.* developed insulin-loaded PLGA-based LPPs fabricated by a double emulsion method by aid of hydroxypropyl-β-cyclodextrin (HPβCD). The PLGA/HPβCD/insulin LPPs showed the desired aerodynamic properties with a fine particle fraction (FPF) of 27–90% at the airflow rates tested of 30–90 L/min, and the inhalable LPPs exerted a very significant and longer hypoglycaemic effect even at insulin doses as low as 0.5 IU/kg as compared to aerosolized insulin solution, with pharmacodynamic availability of 10% and 94% for insulin solution and LPPs groups, respectively [121]. *Kim et al.* devised a PLGA-based LPP adsorbed with palmitylacylated exendin-4 using extractable Pluronic F68/F127 as porogen through a single o/w emulsification-solvent evaporation method. The porous microspheres displayed sustained *in vitro* release profiles and longer *in vivo* pulmonary hypoglycemic duration over 5 days [119]. *Chen et al.* also encapsulated insulin into large porous PLA particles manufactured in supercritical CO_2 using ammonium bicarbonate as a porogen, exhibiting good aerosolization characteristics with an D_a of 4.31 μm and a FPF of 65.57%, which have considerable pharmaceutical potential as a type 2 anti-diabetic inhalation treatment [122].

PNAPs, also known as Trojan microspheres, have recently received more attention for pulmonary delivery of proteins and peptides. These particulates are composed of drug-loaded nanoparticles assembled into microstructures [123]. Once aerosolized, the PNAPs deposit homogeneously on alveolar epithelial cells surface owing to their optimal aerodynamic particle size, and subsequently, the nanoparticles are released into the lung lining fluids and are retained for a longer period due to their resistance to immune cells. The Trojan particles combine the advantages of polymeric nanoparticles and porous microparticles such as high drug loading, superior mucus penetration and lung retention capacity, as well as favorable aerodynamic characteristics [18]. *Alfagih et al.* developed bovine serum albumin (BSA)-loaded nanoparticles using a biodegradable polymer poly

(glycerol adipate-co-pentadecalactone) (PGA-co-PDL) by the double emulsion evaporation process followed by spray-drying with leucine to form nanocomposite microparticles. The formed PNAPs are suitable for pulmonary delivery via dry powder inhalers (DPIs), with an D_a of 1.46 μm and a FPF of 78.57%, as well as low mucosal toxicity with > 85% cell viability even at high concentrations of 5 mg/mL [124]. *Sinsuebpol and Al-Qadi et al.* reported similar results with insulin-loaded PNAPs. These researches constructed a new dry powder system consisting of microencapsulated insulin-loaded chitosan nanoparticles, exhibiting adequate aerodynamic properties for deposition in deep lungs with an D_a of 2–3 μm and a more pronounced hypoglycemic effect following intratracheal administration compared to insulin alone with 1.5 ~ twofold increase in systemic absorption [125126127128].

Although numerous researchers are currently working on formulating inhaled therapies for proteins and peptides, only two inhalable formulations of delivering biologics with DPIs have been approved by FDA, they are the Exubera® (approved by FDA and European agency in January, 2006) and Affreza® (approved by FDA in June, 2014) [12]. Exubera® (Pfizer) insulin inhalation system contains amorphous insulin, mannitol, glycine, sodium citrate dihydrate and sodium hydroxide. The spray drying of insulin/excipients combination produces the microparticles with desirable aerodynamic particle diameter of approximately 3 μm and excellent room-temperature stability for 2 years [129]. However, Exubera® was withdrawn from the market in 2007, after only 1 year of commercialization, owing to unexpectedly low sales. On the one hand, the cumbersome inhalation device and the requirement for pulmonary function testing could discourage patients' acceptability. On the other hand, the safety concerns about antibody production, lung function, cough episodes, and in particular, risk of bronchial carcinoma may compromise clinical therapeutic benefits and potentially cause product delisting [130]. Affreza® (Technosphere insulin, MannKind) is composed of a dry powder formulation of recombinant human insulin and biologically inert excipient fumaryl diketopiperazine (FDKP) (1:9, *w/w*). Technosphere insulin microparticles are formed via insulin adsorption (electrostatically) into the acid-induced intermolecular self-assembly (hydrogen bonds) of FDKP molecules, followed by freeze-drying to produce attractive respirable microparticles with an MMAD around 2–2.5 μm [131]. Once deposited into the lung, FDKP microparticles can rapidly dissolve at neutral pH and then release insulin into the systemic circulation [132]. Higher bioavailability and faster absorption were observed for Afrezza® in comparison to those of Exubera®, with a bioavailability of 26% *vs.* 10% relative to subcutaneous (s.c.) regular insulin

and a T_{max} of 13 min *vs.* 45 min, respectively [40]. The marketed insulin inhalation system could be recognized as a good exemplification for development of inhaled biologics intended for systemic action.

Lipid-Based Particulate Delivery Systems

Liposomes

Liposomes are phospholipids assembled into one or more multilamellar vesicles with a size range within the nano to micrometer scale and are the only sustained-release strategy for pulmonary administration that has achieved clinical development [133]. The main drive for liposomes research in the field of biologics derives from their advantages with the structural similarity to cellular membrane and chemical similarity to lung surfactant as well as excellent biocompatibility, facilitating protein absorption across the airway epithelium intact. Moreover, the organized structure of liposomes permits the association of payloads to both aqueous and lipid phase and provides a reservoir for controlled release by adjusting the bilayer's number and composition, prolonging local and systemic therapeutic levels [134]. *Chono et al.* evaluated the enhancing effect on insulin delivery by monitoring plasma glucose levels after aerosolized liposomes containing insulin were administered into rat lungs. This finding demonstrated that DPPC- and EPC-based liposomes enhanced pulmonary insulin absorption by opening the epithelial cell space in the pulmonary mucosa without mucosal cell damage, with 6.7- and 3.4-fold increase in AUC compared with insulin solution, respectively [71]. *Bi et al.* developed a dry powder inhalation of insulin-loaded soya lecithin-based liposomes prepared via combining reverse-phase evaporation technique and spray freeze drying method for enhanced pulmonary delivery and long-term stability. The *in vivo* research of intratracheal instillation of insulin-loaded liposomes to diabetic rats revealed a successful hypoglycemic effect with a relative pharmacological bioavailability as high as 38.38% in the group of 8 IU/kg dosage [135]. *Murata et al.* investigate the feasibility of surface-modified liposomes for pulmonary delivery of peptides using chitosan oligosaccharide (oligoCS) and polyvinyl alcohol with a hydrophobic anchor (PVA-R) as surface modifiers. The elcatonin-loaded DSPC-assembled liposomes slightly enhanced pharmacological efficacy relative to elcatonin solution alone with no statistical difference, and further surface modification of liposomes markedly promoted the peptide absorption into systemic circulation with a near twofold enhancement, possibly attributable to tight junctions' regulation of oligo CS and steric hindrance of PVA-R layer [136].

Lipid Nanoparticles

Lipid nanoparticles, including solid lipid nanoparticles (SLNs) and nanostructured lipid carriers (NLCs), are nanosized colloidal systems in which solid particles consisting of a lipid matrix are stabilized by surfactants in an aqueous phase [137]. The composition and structure of the lipid matrix serve as a defining characteristic between the two types of lipid nanoparticles. The SLNs matrix is composed of a solid lipid or a combination of various solid lipids which construct almost a perfect crystalline structure. In contrast, the NLCs matrix consists of binary mixtures of solid and liquid lipids forming a crystal structure with more imperfections, attaching more flexibility and stability to the system [138, 139]. Both lipid nanoparticles confer improved protein stability, depressed proteolytic degradation and sustained release of incorporated molecules [140]. *Liu et al.* developed nebulizer-compatible SPC-based SLNs by reverse micelle-double emulsion method for pulmonary drug delivery of insulin. The optimized SLNs exhibited a higher nebulization efficiency of 63.28% and an enhanced pharmacological bioavailability of 24.33% following aerosol cloud of insulin-loaded SLNs (20 IU/kg) into rat lungs [141]. *Yang et al.* prepared biodegradable insulin-loaded SLNs flocculates through self-assembly of cationic and anionic SLNs with a prolonged hypoglycemic effect for 12 h at least and a relative pharmacological bioavailability of 35.62% after intratracheal instillation to diabetic rats at the dose of 8 IU/kg dosage [142]. *Gong et al.* constructed the octreotide/sodium deoxycholate complex-loaded NLCs and PNAPs with an excellent entrapment efficiency (> 95%) and proper aerodynamic size (~3 µm), and the PNAPs intrapulmonary delivery showed enhanced systemic absorption with 2 times higher AUC values in comparison to octreotide acetate solution [143].

## Lipid-Based Hollow-Porous Microparticles

Pulmosphere™ and Aerosphere™ particles composed mainly of DSPC are manufactured by an emulsion-based spray-drying process with a sponge-like morphology, geometric diameters < 5 µm and low tap densities of approximately 0.1 g·cm^{-3}, and specifically engineered for pulmonary delivery via dry powder inhaler (DPI) and pressurized metered dose inhaler (pMDI), respectively [144]. Active pharmaceutical ingredients (APIs) incorporated or absorbed into the hollow-porous phospholipid microparticles can achieve a constant release and improved dose consistency as well as peripheral lung deposition independent of their physicochemical properties and lung dose [145]. Meanwhile, the endogenous phospholipids as pulmonary surfactants, play a stabilizing role in lung tissues and participate in the anti-infection barrier following inhalation, such as the prevention of coronavirus disease 2019 (COVID-19) [146]. *Bot et al.* developed human immunoglobulin (hIgG)-loaded lipid-based porous microparticles for administration to the lower respiratory tract utilizing Pulmosphere™ technology, which presented rapid drug release profiles, with 80% IgG release in 6 h and 26% systemic bioavailability [147].

Commonly used strategies and related mechanism to enhance the systemic absorption of inhaled proteins and peptides are schematically presented in Fig. 4.

Concluding Remarks and Future Perspectives

Inhalation is a feasible non-invasive option for systemic administration of proteins and peptides. However, mucociliary clearance, alveolar macrophage phagocytosis, enzymatic metabolism, pulmonary surfactant adsorption

Fig. 4 Schematic representation of strategies to enhance the systemic absorption of inhaled proteins and peptides.
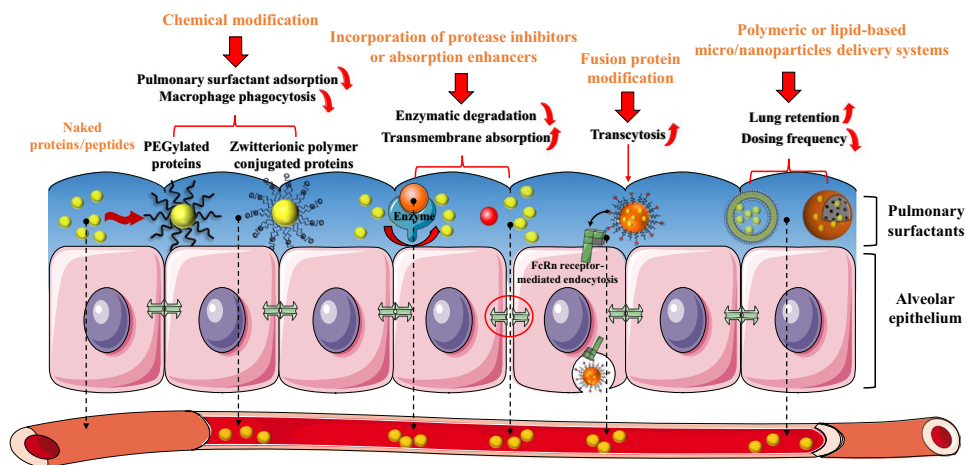
and poor epithelium permeability limited the pulmonary absorption of inhaled biologics. Given these delivery challenges, several strategies have been attempted to enhance systemic absorption of proteins and peptides following pulmonary delivery. Chemical modification and adding protease inhibitors could evade pulmonary clearance mechanisms with improved drug lung retention ability. Incorporation of absorption enhancers and Fc-fusion proteins could promote transmembrane transport of inhaled proteins and peptides. Particulate-based delivery systems could prolong lung residence and absorption of these biologics with decreased dosing frequency. Even so, the product development in this field is still slow. In particular, polymeric or lipid-based micro/nanoparticles delivery systems have barely achieved clinical transformation, although they have been successfully proved to be effective for pulmonary delivery (seen in 3.5 and 3.6 sections). The main obstacle for particle engineering and formulation development of proteins/peptides could be attributed to the limited number of approved excipients for safe delivery to the lung. Despite some researchers have validated the short-term safety of numerous excipients for lung delivery, long-term safety remains a knowledge gap to be filled. Another crucial factor limiting clinical application of inhaled proteins and peptides is the adaptation of inhaler devices based on the formulation type and patient condition. Even though the fact that there are many well-established market products for nebulizers, DPI and pMDI, advancements are still required to employ these devices for optimal delivery of macromolecules with good aerosolization efficiency, excellent stability and easy operation. Moreover, lessons learnt from Exubera® evidently demonstrate that patient acceptance, cost and market positioning of delivery devices are also indispensable for successful commercial development of inhaled biologics, with an ideal inhalation device being small, portable, convenient and effective.

Up to now, only 4 inhaled biologics have already been approved on the market, including Beractant®/Survanta® in 1991, Pulmozyme® in 1993, Afrezza® in 2014 and Exubera® in 2006 (withdrawn in 2007). 2 of these products are engineered particles DPIs (Afrezza® and Exubera®, both delivering insulin), 1 is a nebulizer (Pulmozyme®) and Survanta® is a one-off intratracheal dosing with a syringe (no inhaler device is used with Survanta®). A scan of the scientific literature from 2020 and 2021 revealed that out of 40 companies developing inhaled biomolecule therapies, the majority of them use nebulizers in Phase 2 trials. Nebulizers are ideal in a clinical setting and critical care, whereas better options for chronic use and home use of the biomolecules are SMIs and DPIs. Moreover, Technosphere platform and Pulmosphere/Aerosphere technology are also worth learning from Mannkind and Novartis for the development of inhaled biologic products owing to their accepted clinical practice.

Overall, developing inhaled protein and peptide drugs is a systematic process and requires to ensure therapeutic effectiveness, clinical safety and patient/market acceptance. A quick count on a popular pharmaceutical market database has displayed that 717 companies are developing or already producing inhaled biologics products. Meanwhile, the scientific literature is equally rich in reviews and research articles commenting on inhaled biologics, which is a good sign of a market in expansion. In the future, more available safe excipients and sophisticated devices need to be further explored for efficient and precise pulmonary delivery of inhaled proteins/peptides.

Declarations

Competing Interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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