Carrier-based Strategies for Targeting Protein and Peptide Drugs to the Lungs

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ABSTRACT

With greater interest in delivery of protein and peptide-based drugs to the lungs for topical and systemic activity, a range of new devices and formulations are being investigated. While a great deal of recent research has focused on the development of novel devices, attention must now be paid to the formulation of these macromolecular drugs. The emphasis in this review will be on targeting of protein/peptide drugs by inhalation using carriers and ligands.

KEYWORDS: Protein, peptide, inhalation, liposomes, microspheres, targeting

INTRODUCTION

The Barriers to Effective Protein/Peptide Delivery

Issues that must be addressed when preparing proteins/peptides for delivery include the large size, hydrophilicity, and physical and chemical lability of the drug molecule. These factors impact on both the pharmacokinetics and pharmacodynamics of the drug in vivo and must be considered when selecting a suitable formulation, storage, and delivery method. Technology currently in use for aerosol delivery was originally developed for small molecule drugs and not for the delivery of proteins/peptides,[1] necessitating the reengineering of inhalers for macromolecular delivery. The focus of this review, however, is on the carriers used in the formulation of protein/peptide-based drugs, so only a brief overview of devices will be provided.

The Advantages of Delivery to the Lungs

There are several advantages in delivering protein/peptide-based drugs to the lungs including a noninvasive method of delivery (locally targeted delivery of drugs acting in the lungs can improve efficacy and decrease unwanted systemic side effects); a large surface area for absorption (~75m^2); thin (0.1 to 0.5 μm) alveolar epithelium, permitting rapid absorption; absence of first-pass metabolism; rapid onset of action; and high bioavailability.

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Devices for Delivery

The delivery device plays a major role in the efficiency of pulmonary delivery, and great strides have been made in the development of new devices in recent years. The devices most commonly used for respiratory delivery, including nebulizers, metered-dose inhalers (MDIs), and dry powder inhalers (DPIs), can all be adapted for use with protein/peptide drugs. The choice of device will depend on the drug, the formulation, the site of action, and the pathophysiology of the lungs. For example, liposomes do not form in conventional MDIs and would therefore be better suited for nebulization or drying to form a DPI.

Drugs for inhalation can be dissolved/suspended in aqueous-based formulations for nebulization.[2] DNase, the only protein-based pharmaceutical licensed for inhalation, is delivered using a jet nebulizer. The stability of proteins and peptides on nebulization is a potential limitation. Many biopharmaceuticals are unstable in aqueous solutions, and penetration can occur due to the thermal[3] and surface effects during nebulization.[4] These drawbacks have led to the development of newer devices such as the AERx (Aradigm, Hayward, CA) (Figure 1A)[5] and Respimat (Boehringer, Germany) (Figure 1B)[6] that generate an aerosol mechanically and vibrating mesh technologies such as AeroDose (Aerogen Inc, Mountain View, CA) (Figure 1C)[7] that have been used successfully to deliver proteins to the lungs[8-11] and are currently being used in the clinical trials of protein and peptide-based pharmaceuticals.

MDIs are not generally the delivery method of choice for proteins/peptides owing to their susceptibility to penetration when they come into contact with the propellants or with the large air-liquid interface generated.[12,13] It is a feasible delivery system, however, when stability is not an issue. There are examples of both solution and suspension-based formulations of protein/peptide drugs in MDIs. The peptide leuprolide acetate was delivered systemically in humans as a suspension formulation in an MDI,[14] and a solution formulation of this peptide has also recently been reported in an HFA134a propellant system using ethanol and water as cosolvents.[15] Larger proteins have also been incorporated into MDIs including antigenic proteins, enzymes, and antibodies.[16,17] A solution of cyclopeptide FK224 provided a much greater bioavailability than the suspension formulation when delivered to rat lungs using an MDI.[18]

Figure 1. New generation of nebulizers: (A) AERx (Aradigm), (B) Respimat (Boehringer), (C) AeroDose (AeroGen Inc).

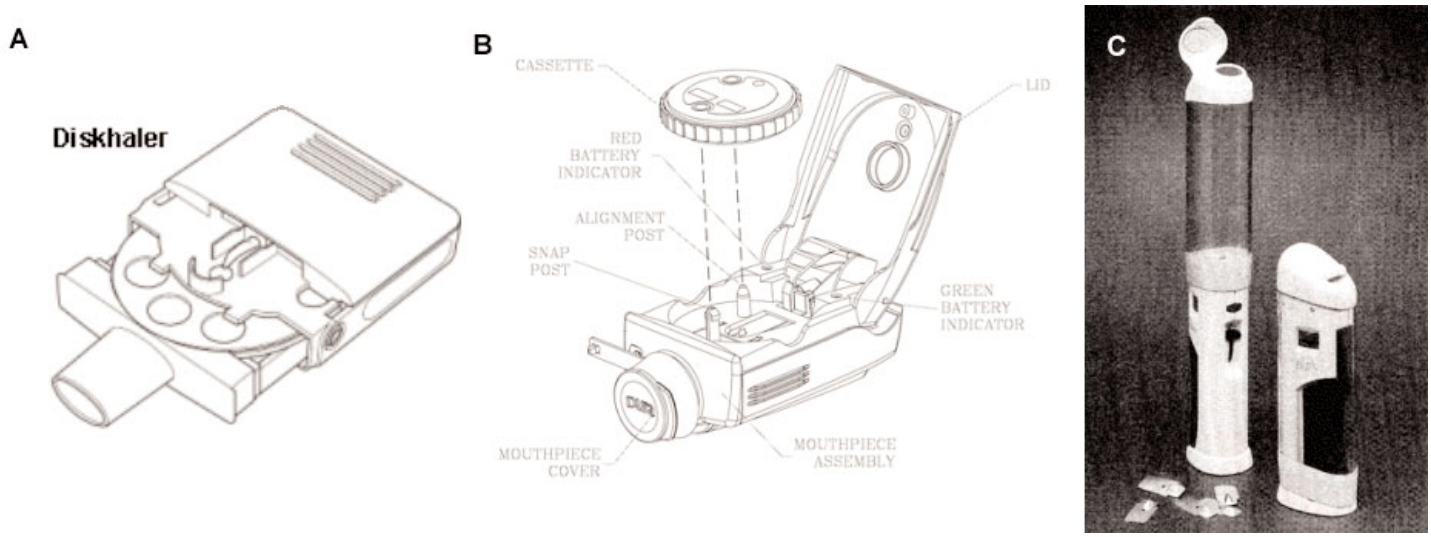

Figure 2. Dry powder inhalers for protein delivery: (A) Diskhaler (GSK), (B) Dura dry powder inhaler (Spiros), (C) Nektar dry powder inhaler (Nektar).

Dry powder inhalers are one of the most popular methods of protein delivery to the lungs. An array of dry powder devices is available including multidose and unit dose, patient-driven, and powered systems. For stability reasons, the unit-dose devices are the most suitable for protein delivery. These devices range from the original capsule unit doses of the Spinhaler (Fisons Pharmaceuticals, Rochester, NY) and Rotahaler (GSK, RTP, NC) to the more elegant and stable foil blister pack in either single- or multidose of disks/tape (see Figure 2A, Diskhaler (GSK, RTP, NC). In general, patient-driven devices exhibit variable, often poor, delivery efficiency that is flow-rate dependent. This inefficiency has driven interest toward the use of powered inhaler systems (eg, Spiros [Dura Pharmaceuticals, San Diego, CA] and Nektar [Nektar Pharmaceuticals, San Carlos, CA]) that produce inhalable aerosols independent of the patient's inspiratory flow rate and volume (see Figure 2B and C). This does not eliminate the need to control inspiratory flow rate, which can still influence the location and degree of deposition of the protein powder. All these devices require a dry powder formulation of the active ingredient with good flow, dispersability, and stability. Many of the carrier systems discussed in this review have been developed specifically for use in dry powder devices. While more complex formulation issues arise in developing such formulations, the prolonged shelf life of amorphous, protein powders compared with aqueous systems means that the investment would be returned in the form of a commercially viable, patient-friendly product.

The selection of device for delivery of proteins to the lungs is an important factor in the formulation design. This relates to fundamental choices of the state of the protein (ie, solution or dry powder) to be used, the method and state of storage, the choice of excipients, and the interactions between the formulation and the device (eg, adsorption). If the drug is being targeted to a specific region of the lungs, then a device capable of generating and delivering droplets/particles with the requisite aerodynamic diameter will be required. Efficient dose delivery to specific sites of action is of paramount importance if proteins/peptides for inhalation are to become a commonly used clinical format. Inhalers/nebulizers that deliver only a small percentage of the dose would make the cost of therapy in many cases prohibitive.

Table 1. Examples of Proteins/Peptides for Inhalation*

Disease State	Peptide/Protein
Local	
Adult Respiratory Distress Syndrome	Surfactant Proteins (approved)
Cystic fibrosis (CF)	DNase (approved)
Emphysema/CF	Alpha-1-antitrypsin
	Secretory leukoprotease inhibitor
Lung transplant	Cyclosporin A
Cancer/Pneumocystis carnii	Interferon-γ
	Interleukin-2
Alpha-1-antitrypsin deficiency	$Alpha_1$ proteinase inhibitor
Asthma	IL-1R
	Anti-IgE Mab
Anti-TB vaccine	Muramyl dipeptide
Oxidative stress	Catalase
	Superoxide dismutase
Systemic	
Osteoporosis	Calcitonin
	Parathyroid hormone
Growth deficiency	Human growth hormone
Multiple sclerosis	Interferon-β
Diabetes	Insulin
Cancer	LH-RH analogs
Viral infections	Ribavirin
	Interferon-α
Neutropenia	rhG-CSF
Anemia	Erythropoetin
Anticoagulation	Heparin
Diabetes insipidus	dDAVP (1-deaminocysteine-8-D-arginine vasopressin)

*Interleukin (IL); Luteinising Hormone-Releasing Hormone (LH-RH); and Colony Stimulating Factor (CSF).

The General Formulation Strategies for Proteins/Peptides

The vast majority of protein-based pharmaceuticals are given parenterally (including intravenous, intramuscular, subcutaneous, and intraperitoneal injections). Many are delivered as solutions with the exception of recombinant vaccines and insulin. Excipients, commonly used in the parenteral delivery of small drug molecules, including solubility enhancers, osmotic agents, buffers, and preservatives, are often included in protein formulations. The increased stability concerns when formulating and delivering proteins has led to the inclusion of antiaggregation and antiadsorption agents, such as surfactants and albumin. These agents decrease the risk of the active protein interacting with an interface, which can lead to unfolding, aggregation, and even precipitation.[19]

Proteins in solution can be unstable with limited shelf life. One of the most common approaches for improving stability is the use of freeze-drying, but lyophilized proteins are unsuitable for inhalation without further processing.[13]

The Range of Protein/Peptide-Based Drugs Currently Being Investigated for Respiratory Delivery

Parenteral delivery of proteins has several drawbacks, including an invasive delivery method (often requiring medical professional administration), a sterile dosage form, systemic side effects, and rapid clearance.

Pulmonary protein delivery offers both local targeting for the treatment of respiratory diseases and increasingly appears to be a viable option for the delivery of proteins systemically.[20] The lung is easy to access, has decreased proteolytic activity compared with the gut, and allows rapid absorption and avoidance of first-pass metabolism for systemically delivered drugs.[21]

Hundreds of proteins and peptides are undergoing clinical investigation for a range of clinical conditions. These include growth factors, hormones, monoclonal antibodies, cytokines, and anti-infective agents. For those being investigated for delivery via inhalation, the ultimate site of action may be the airway surface (eg, DNase), the airway cells (eg, cyclosporin), or the systemic circulation (eg, insulin). It is important to note that despite a huge body of work over recent years in this area, to date relatively few treatments have made it through regulatory procedures to full license. Careful choice of carrier and device can facilitate delivery to a specific area of the lungs. Once delivered, a carrier can further influence the distribution and rate of clearance from the site of action.

The first investigation of insulin delivery via the lung took place in the 1920s, and the interest in this route has increased in recent years with the advances made in recombinant technology. The only protein for inhalation currently available on the market is DNase, but a growing number of proteins/peptides are in various phases of clinical trials. Systemic inhaled insulin is in late phase 3 trials. Other proteins/peptides in phase 3 trials include leuprolide and gamma-interferon.[22] Examples of some the proteins/peptides being considered for delivery to/via the lungs are shown in Table 1.

The Advantages of Carrier-based Systems for Sustained and Targeted Delivery

In this context a broad definition of carrier is used and will include molecules conjugated to, mixed with, or used for encapsulating protein/peptide drugs.

Carrier-based systems for protein and peptide delivery can play a role in improving the therapeutic index of a drug by one or more of the following:

- Increasing the proportion of protein that reaches its site of action (be it intracellular or extracellular)
- Improving the transport of the drug to its site of action

- Allowing colocalized deposition of protein with other proteins or excipients (eg, protein and protease inhibitor)
- Improving the stability of the drug in vivo
- Prolonging the residence time of the drug at its site of action by reducing clearance
- Decreasing the nonspecific delivery of the drug to nontarget tissues
- Decreasing irritation caused by the drug
- Decreasing toxicity due to high initial doses of the drug
- Altering the immunogenicity of the protein
- Improving taste of the product
- Improving shelf life of the product

There is now a greater understanding of the molecular and biochemical composition of the lung, the molecular basis of disease, and the barriers to drug delivery. This knowledge, with recent improvements in delivery devices, means that advanced, targeted drug delivery systems can be developed.

Carriers can be employed to provide passive and/or active targeting. Delivery topically to the airways is itself a targeting strategy for diseases of the lungs. A carrier might be used to alter the nature (ie, size, shape, charge, hydrophobicity, or density) of the aerosol droplet or particle in order that deposition might be altered. Active targeting refers more specifically to the use of a homing device (eg, antibody that when attached to the protein or protein carrier system can target specific tissues, cells, or organelles).

Carriers

It should be noted that most of the carrier systems discussed throughout this review are not yet licensed for use in humans, and many are only in the early stages of development. The choice of carrier depends on several factors, including the nature of the protein to be delivered, the device for delivery, the site of action, the disease state, and the nature and safety of the carrier.

Safety Aspects of Particulate Carriers

Peptides/Proteins

The major safety concern in delivering therapeutic peptides and proteins to the lungs is the possibility of immunologic reactions. The body may recognize the native protein or the denatured protein as an antigen, which would trigger an immune response. Work to date has suggested that pulmonary delivery of most peptides and proteins via the pulmonary route is safe,[23] at least in the short term. Longer-term studies with insulin have shown that it is safe over a 2-year period.[24]

Carriers

When carriers are used for delivery of proteins and peptides either locally or systemically, the safety of the adjuvant itself must be determined. Microparticulate carriers and targeting moieties composed of natural or synthetic materials may be incompatible with lung tissue. While the safety of some carriers has been examined (eg, conventional liposomes),[25] many others have not. Cationic liposomes, for example, that have gained popularity for gene delivery have been found to induce oxygen radical-mediated pulmonary toxicity.[26] For carriers that are used to prolong release (eg, polymeric microspheres), there is a danger that with long-term use the carrier material may accumulate in the lung, especially in the lung periphery, which is not served by mucociliary clearance. Long-term inhalation of carrier particles has been shown to induce depletion of surfactant with subsequent recruitment of phagocytic cells.[27]

Residual solvents remaining after formulation processes for microencapsulation or liposome preparation may also cause toxicity. Processing and/or excipients that denature the protein/peptide may lead to increased immunogenicity; therefore processing techniques and formulation components must be considered carefully. Excipients used in dry powder formulations to promote the stability of proteins, such as salts and sugars, can cause bronchoconstriction in hyperresponsive patients.[13,28] Issues regarding local irritancy and toxicity, long-term accumulation, and immunogenicity will all have to be addressed using suitable models.[29]

Concerns have also been raised about the use of other excipients such as absorption enhancers and enzyme inhibitors.[30,31] Increased transepithelial transport of the native or denatured protein may lead to increased interactions with the systemic immune system, and increased permeability may also allow transport of other toxins and antigens across the epithelial barrier.

It is important to note that lecithin (phosphatidylcholine) is the only excipient currently approved by the FDA for lung delivery, so there is a long regulatory road ahead before some of the more sophisticated polymeric and targeted carriers are used in clinical practice. This is an important point to note for all the carriers discussed below including liposomes (containing lipids other than phosphatidylcholine), microspheres, carbohydrates, and the more specific targeting ligands. Lactose is an approved carrier in dry powder products, but it is not intended to enter the lungs. Its particle size limits deposition to the oropharynx.

Lipids and Liposomes

Background on Lipids and Liposomes

Liposomes have been used in drug delivery for many years. Liposomal aerosols have several advantages, including sustained release, prevention of local irritation, reduced toxicity,

Table 2. Examples of Liposomal Formulations of Proteins/Peptides for Respiratory Delivery

Drug	Effect	Reference
Cyclosporin	The lung rapidly and preferentially absorbed the liposomal cyclosporine; the drug was retained for 120 minutes in a dog model.	61
Insulin	Liposomal formulation facilitated pulmonary absorption and enhanced the hypoglycemic effect.	184
Catalase	Liposome formulations conferred resistance to pulmonary oxygen toxicity.	63
Superoxide dismutase	Intratracheal administration of liposomal SOD minimized toxicity to subsequent hyperoxia and improved survival.	63
Interleukin-2	Local delivery of liposomal IL-2 to the lungs facilitated bioactivity and reduced toxicity.	188
Ricin vaccine	Improved safety profile for intrapulmonary vaccination using liposomes.	189

improved stability in the large aqueous core, and the possibility to manipulate release and targeting by altering the bilayer constituents and changing the preparation technique.[32] Several injectable liposome-based products are now on the market including Ambisome, Fungisome, and Myocet.

The drug carrying capacity, release rate, and deposition of liposomes in the lungs is dependent on the lipid composition, size, charge, drug/lipid ratio, and method of delivery.[33-35] Conventional liposomes are composed of neutral or anionic lipids (natural or synthetic). The most commonly used are the lecithins (phosphatidylcholines), phosphatidylethanolamines (PE), sphingomyelins, phosphatidylserines, phosphatidylglycerols (PG), and phosphatidylinositols (PI).[36] The recent attention to the use of liposomes for delivery of DNA to the lungs means that a greater understanding of their use in macromolecular delivery via inhalation is now emerging.[37-42] Much of this new knowledge, including new lipids and analytical techniques, can be used in the development of liposome-based protein formulations.

Liposomes may be prepared for inhalation in liquid[43] or dry powder form.[44] Drug release can occur during nebulization, but manipulation of lipid composition,[45,46] size,[47] and operating conditions[48] can minimize this loss. Dry powder liposomes have been produced by lyophilization followed by milling,[44,49] or by spray-drying.[50-52]

Several proteins for inhalation have been formulated in liposomes (Table 2). This includes both proteins with local and systemic activity.

It appears that by manipulating the liposome composition, the pharmacokinetics of protein and peptide drugs in the lungs can be altered.[53] Liposome composition can be altered to enhance transport across the epithelium for systemic delivery, to improve drug retention within the lung, or to delay release. Three major factors must be taken into consideration: (1) the interaction of the protein with the lipids, (2) the interaction of the formulation with the lungs, and (3) the efficiency of delivery from a given device.

Encapsulation Efficiency and Release

The interaction of proteins with lipids and the subsequent encapsulation efficiency and stability of the formulation is dependent on both the choice of lipids and the method of production. The diversity in structure of proteins/peptides requires careful consideration. Electrostatic and hydrophobic interactions have a role in their behavior. These interactions may be monitored using differential scanning calorimetry (DSC).[54] Recent studies at Advanced Drug Delivery Research Centre, RCSI, looking at the encapsulation of the cationic protein Secretory Leukocyte Protease Inhibitor in liposomes clearly indicated that inclusion of a negatively charged lipid such as phosphatidylserine (PS) in the liposome could improve encapsulation efficiency. Rehydration of a lipid film with the protein solution followed by freeze-thawing and extrusion is the most commonly used technique.[55] However, it has been shown that this may not be the best method for all proteins. For example, the encapsulation efficiency of superoxide dismutase (SOD) was much greater using a proliposome technique compared with simple rehydration.[56] Newer methods for encapsulation of proteins and peptides, involving injection techniques, allow pilot scale batches to be produced efficiently.[57] Care must be taken when using size reduction techniques (eg, sonication, homogenization, extrusion), so that the stability of the protein/peptide is not compromised. The desired release rate can also be achieved by careful choice and preparation of liposomes. Suarez et al[34] showed that larger liposomes tend to slow the release of encapsulated water-soluble solutes.

Examples

Liposomal encapsulation frequently increases residence time and/or decreases toxic side effects of the drugs delivered to the lungs. The residence time of any liposomal preparation will depend on the area of deposition in the lungs. If the liposomal drugs are deposited in the tracheobronchial tree, then they are more likely to be removed rapidly by the mucociliary escalator. The fate of liposomes in the lungs has been studied. Intratracheal administration of radiolabeled dipalmi-

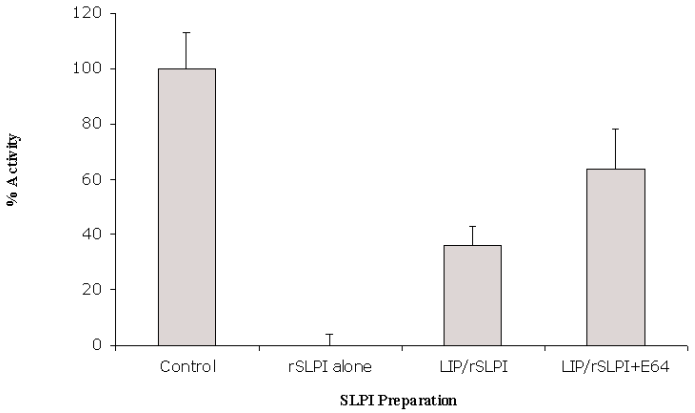

Figure 3. Effect of liposomes: effect of encapsulation (and co-encapsulation with protease inhibitor E64) in DOPC:Chol (2:1) liposomes (LIP) on the neutrophil elastase (NE) inhibitory activity of rSLPI. Free (rSLPI), encapsulated rSLPI (LIP/rSLPI), and co-encapsulated rSLPI and cathepsin inhibitor E64 (LIP/rSLPI+E64) were treated with Cathepsin L (1:400) for 2 hours and were then assayed for the NE inhibitory activity remaining. The control represents inhibition by undigested rSLPI (Cryan et al, unpublished data, Nov 2003).

toyl-phosphatidylcholine (DPPC):cholesterol (7:2) led to 90% of the liposomes being taken up by the lungs and 50% being retained longer than 24 hours.[58] Clearance rates of liposomes are also affected by the degree of ventilation of the lung[59] and the inclusion of fusogenic lipids, such as PG.[60]

An example of local therapy is cyclosporin, the drug of choice for the treatment of heart-lung transplant rejection. Systemic treatment is often associated with serious side effects, such as nephrotoxicity and hepatotoxicity. Letsou et al[61] reported that liposomal formulations of the immunosuppressant selectively deposited and concentrated the drug in the lungs of dogs following pulmonary delivery. This finding is particularly important for a drug that has a narrow therapeutic window, such as cyclosporine. Its poor water solubility meant that previous attempts to aerosolize the drug were done using non-aqueous formulations. Liposomal delivery allowed an aqueous-based formulation to be developed and, when tested in mice, also increased lung retention times significantly from 17 minutes for free drug to 4.8 hours in normal lungs.[62]

The antioxidant enzymes, catalase and SOD, have also been entrapped in liposomes for local delivery to the airways.[63] Intravenous and intraperitoneal dosing requires high doses to be used in order to augment the antioxidant protection of the lungs. Liposomal formulations were found to increase intracellular delivery of the enzymes, thereby increasing access to the mitochondria and endoplasmic reticulum, which are production sources of O_2 and H_2O_2. Intratracheal instillation of liposome-encapsulated antioxidant enzymes to oxidant-sensitive alveolar epithelium renders it resistant to pulmonary O_2 toxicity.[63] If intracellular targeting is required, then the choice of lipids for the formulation is particularly important and will be discussed in more detail in a later section (see Intracellular Targeting). The phospholipid composition including acyl chain length, charge, and concentration significantly affects the systemic absorption and subsequent hypoglycemic effect of liposome-encapsulated insulin.[64]

Liposome encapsulation can prolong residence time by decreasing degradation of protein/peptide drugs by proteases in the lungs. For example, studies have shown that rSLPI is proteolytically susceptible to cleavage and inactivation by Cathepsin L in the diseased emphysematous lungs, and this may be limiting its therapeutic effect after aerosol administration. We have shown that encapsulation in liposomes can significantly improve the stability of this protein after in vitro challenge with Cathepsin L (Figure 3).

Targeted Liposomes

Different strategies have been employed to control the interaction of liposomes with the environment (in this case, the lungs) including the development of targeted and reactive liposomes. Targeted liposomes have targeting ligands, such as monoclonal antibodies or lectins, attached to their surface, and this allows them to interact with specific receptors and/or cell types. Reactive or polymorphic liposomes include a wide range of liposomes, the common property of which is their tendency to change their phase and structure upon a particular interaction (eg, pH-sensitive liposomes).[36]

Lipid-based Microparticles

Lipid-based microparticles have also been examined as carriers for lung delivery and are one of a range of materials used to produce these carriers (see next section). Pulmospheres, lipid-based hollow-porous microparticles, were loaded with human immunoglobulin (IgG) and instilled into the upper and lower respiratory tract of mice, which triggered local and systemic immune responses.[65]

Microparticles

Background on Microparticles

The second of the major carrier types are microspheres. Microspheres are produced using naturally occurring or synthetic polymers to produce particulate systems in the size range of 0.1 to 500 μm. The fate of microspheres in the lungs is dependent on the polymeric material chosen, the preparation technique, and the delivery device. Microspheres are physically and chemically more stable than liposomes and allow for higher drug loading. They are, therefore, a useful carrier system for proteins and peptides.[66,67] Examples of microsphere technologies to deliver drugs to the airways are shown in Table 3.

Table 3. Examples of Microparticle Formulations of Proteins/Peptides for Delivery to the Lungs*

Drug	Polymer Material	Effect	Ref
Peroxidase	PLGA coated with DPPC	DPPC coating reduced macrophage uptake from 70% to 25%	75
Calcitonin	Gelatin	Positively charged gelatin microspheres produced a higher pharmacological response after IT instillation in rats.	190
Leuprolide	Albumin	Leuprolide delivered efficiently to the systemic circulation.	191
TB Vaccine	DL-PLG	Sustained protection and greater clearance of the bacterial load after challenge with viable bacilli.	74
IgG	SDLMs: DPPC and DSPC (pulmospheres)	Enhanced local and systemic immune responses associated with receptor-mediated loading of alveolar macrophages.	65
Insulin	PLGA	Blood glucose level reduced significantly; hypoglycemia prolonged over 48 hours, compared with the nebulized aqueous solution of insulin (6 hours).	69
	large porous particles (PLGA) (AIR)	High levels of insulin achieved systemically within 1 hour after aerosolization; remained high for 96 hours.	83
	PEG (ProMaxx)	Microspheres of almost 100% protein formed: rapid glucose depression in nondiabetic dogs.	80
	Sodium hyaluronate	Altered pharmacokinetic profile with increased MRT (9-fold), increased AUC/dose (2.5-fold), increased Tmax (3-fold)	70
	Diketopiperazine derivatives (Technosphere)	Rapid onset of action and greater metabolic effect than SC injection over 3 hours.	192, 82
	Calcium phosphate-PEG particles	Longer $t_{1/2}$, longer MRT, slower elimination than insulin solution; increase bioavailability (1.8-fold) compared with SC injection.	71
	DPPC-coated insulin microspheres	The biological effect was extended in proportion to the amount of lipid present.	68
	Oligosaccharide derivative DPPG (Solidose)	Improved pharmacokinetic profile and a prolonged duration of action (8 hours)	72

*PLGA indicates polylactic-co-glycolic acid; DPPC, dipalmitylphophatidylcholine; Intratracheal (IT), DL-PLG, DL-lactide-co-glycolide; SDLMs, spray-dried lipid-based microparticles; DSPC, distearylphosphatidylcholine; AIR (large porous PLGA particles); PEG, polyethylene glycol; MRT, mean residence time; AUC, area under the curve; SC, subcutaneous; and DPPG, dipalmitylphosphatidylglycerol.

The synthetic polymers, polylactic acid (PLA) and polylactic-co-glycolic acid (PLGA), are the 2 most commonly used. Other options include natural polymers such as albumin, gelatin, chitosan, and dextran. As mentioned previously, lipid-based microparticulate systems are also gaining popularity.[65,68] A range of polymeric systems including PLGA,[69] sodium hyaluronate,[70] calcium phosphate-polyethylene glycol (PEG) particles[71] and oligosaccharide derivatives[72] have been used to prepare protein/peptide microspheres for inhalation. The use of novel polymers in microsphere technology, including both the oligosaccharide-lipid mix of Solidose and the lipid-based Pulmosphere, provides even greater formulation options.

Microparticles can be manufactured using several different techniques. When encapsulating proteins/peptides, the effect of solvents, heat, moisture, pH, oxygen, and mechanical stresses must be assessed. Preparation of microparticles for aerosol delivery can be performed using supercritical fluid technology,[73] emulsion-solvent evaporation,[69,74-76] spray-drying,[65,68,77,78] emulsion-solvent diffusion, and phase separation.[79] New techniques that produce microspheres of pure protein are also being developed.[80]

Release Rate

Release rate from microparticles is dependent on both dissolution and diffusion of the drug. The protein/peptide release rate will depend on many factors including the concentration, solubility, size, and nature of the macromolecular drug, and the nature, molecular weight, porosity, tortuosity, size, and uniformity of the polymer. Manipulation of these parameters allows controlled delivery of proteins to the lungs. This control may be used to prolong exposure, improve aerosol particle characteristics, and/or improve stability of the preparation.

Coating of microparticles can be used to alter the properties in vivo. For example, coating of PLGA microspheres with the lipid DPPC decreased uptake of the cargo protein peroxidase into macrophages.[75] Coating of particles with mucoadhesive polymers such as chitosan and hydroxypropylcellulose increased residence time of peptide drug carriers in the lungs.[79] Coating of carriers with active targeting ligands such as antibodies will be discussed later.

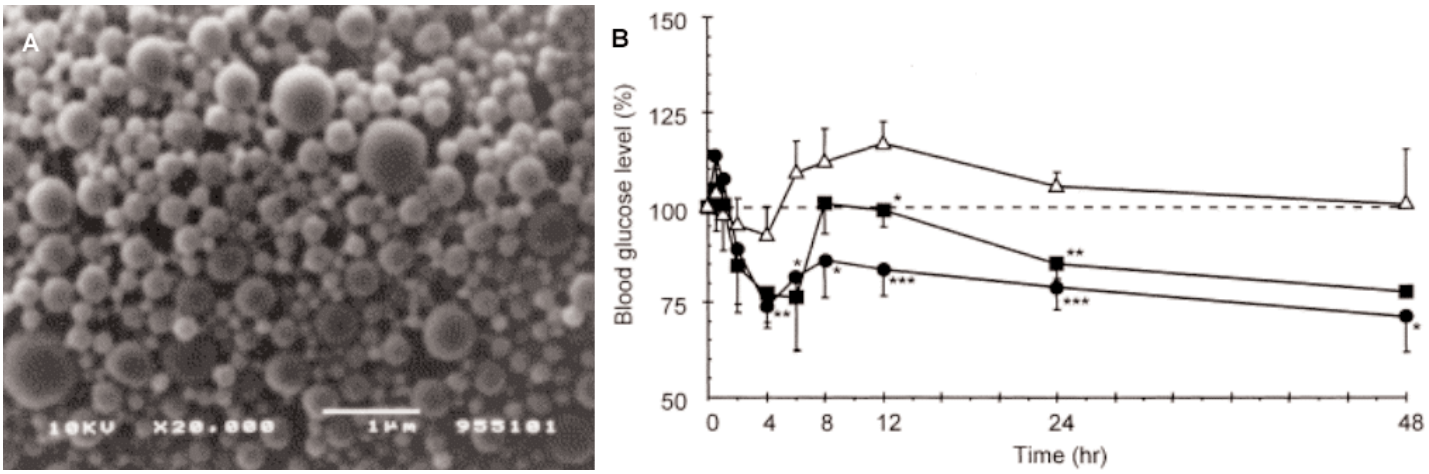

Figure 4. (A) SEMs of PLGA nanospheres containing insulin[69]; (B) Profiles of blood glucose level after pulmonary administration of insulin nanosphere suspension. Data are presented as means ± SD (n = 5), ***: $P < .001$, **: $P < .01$, *: $P < .05$ (n = 5). (△): control (blank NS), (■): insulin solution, (•): insulin-loaded nanosphere suspension (reprinted with kind permission from Ref 69).

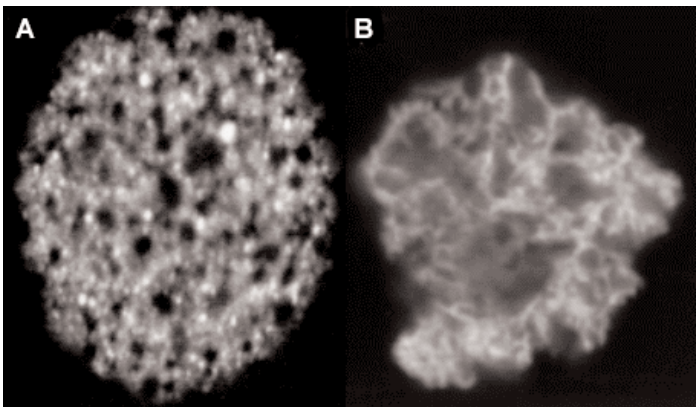

Figure 5. Confocal microscopy images of (A) porous PLGA and (B) porous PLAL-Lys particles. Fluorescein isothiocyanate-dextran was encapsulated in the PLGA particle to render the pore spaces of the particle visible in the fluorescent confocal image. The PLAL-Lys particles were fluorescently labeled through the reaction of rhodamine isothiocyanate with lysine amine groups on the surface of the particles. The PLGA and PLAL-Lys particles are highly porous, as evidenced by the appearance of fluorescence throughout the particle structure (reprinted with kind permission from Ref 69).

Examples of Local Delivery

Studies on the use of microspheres for locally acting protein/peptide drugs have focused on vaccination. Encapsulating antigenic proteins/peptides has many potential advantages when modulating the immune response. It can improve the stability of often labile antigens, prolong exposure, allow coformulation with adjuvants, and target antigen-presenting cells (APCS) by promoting phagocytosis. Evidence of improved targeting was seen when IgG and an inactivated flu virus vaccine were coformulated into spray-dried lipid particles and delivered to the lungs of rodents. This formulation led to improved targeting of alveolar macrophages (AM) by enhancing receptor-mediated uptake and triggered enhanced local and systemic immune responses.[81]

Examples of Improved Systemic Delivery

The most widely studied peptide cargo for delivery to the lungs is insulin, which has been formulated with a range of polymeric systems including PLGA,[69] sodium hyaluronate,[70] PEG,[80] calcium phosphate-PEG particles,[71] and oligosaccharide derivatives.[72] Different effects have been observed depending on the particle technology used. For example, insulin incorporated into the diketopiperazine-based technosphere particles improved bioavailability from the lungs of healthy, nonsmoking volunteers compared with nonencapsulated insulin with a rapid onset but short duration of action, similar to insulin delivered parenterally.[82] This formulation, therefore, improves systemic delivery but does not significantly prolong action. Microparticles of PLGA (mean diameter, 400 nm), however, extended the duration of action of an inhaled dose of insulin significantly from 6 hours (for a nebulized aqueous solution of insulin) up to 48 hours in guinea pigs[69] (Figure 4). Most of the microparticles appear to enhance systemic delivery and, therefore, bioavailability of insulin.[71,72,83]

Large Porous Particles

Until recently, particles of 1 to 3 μm geometric diameter and unit density were thought to be most suitable for lung delivery. This size range minimized losses from oropharyngeal impaction (large particles) and exhalation (small particles). Unfortunately, particles of this size tended to aggregate[84] and were cleared rapidly by AMs.[85] The development of large porous particles has revolutionized the thinking in this area. These particles have geometric diameters >5 μm but have aerodynamic diameters <5 μm owing to their low density (generally <0.1 mg/mL).[83] These particles have good flow and aerosolization properties and can evade alveolar phagocytosis because of their large size (Figure 5). Once aerosolized, large porous particles (LPPs) deposit homogeneously and reproducibly on the cell surface and appear relatively nontoxic to airway cells from microscopy studies performed in culture.[76]

Even more recent developments have led to "Trojan" particles, so-called because of their ability to escape both phagocytic and mucociliary clearance within the airways. Trojan particles are prepared from nanoparticles made from different materials (eg, polystyrene), which upon spray-drying, assemble into a microparticle with low unit density (<0.1 mg/mL).[86] These particles have yet to be assessed with a drug-load, but in preliminary studies they aerosolize easily from a dry powder inhaler and redisperse into nanoparticles once in solution. For protein/peptide delivery, Trojan particles offers a method of producing a DPI with good flow and dispersability properties, which, once delivered to the peripheral airways, will liberate nanoparticles that should avoid clearance mechanisms and provide sustained drug release. Previous attempts to use nanoparticle technology in the lungs failed to address the difficulties involved in effectively delivering very small particles to the lungs.[69]

Carbohydrates

Background on Carbohydrates

Powder carriers, generally lactose, have been a mainstay of DPI formulation for some time. In fact, lactose is the only excipient approved for general use in the United States. It can be difficult to produce a micronized powder of a drug molecule that has the properties required for effective lung delivery (ie, size, shape, density, flowability, and dispersability), especially when using DPI devices that are patient-driven. Thus, carriers are often used to aid in handling and to impart aerodynamic benefits to the formulation. Given the increasing interest in the use of DPIs for protein/peptide delivery, the role of carbohydrates in protein powder production/dispersion[87] and/or encapsulation[72] is growing.

Sugars and Polyols

Sugars (eg, lactose) and polyols (eg, mannitol) can play several roles in dry powder formulations. As well as aiding flow and dispersability, they can also serve as stability enhancers during processing. For example, the stability of interferon β to jet milling, required to produce a respirable powder, was found to be dependent on the presence of sorbitol in the formulation.[88,89] To date, most developmental work has concentrated on the aerodynamic effects of these carriers, but reducing sugars such as lactose can influence the stability of proteins and peptides.[90,91] In fact, the use of lactose with protein powders may lead to a reaction with lysine residues present in the protein, producing lactosylated protein molecules.[92]

In an interesting corollary to this finding, a group investigating methods of improving intracellular delivery to airway epithelium found that lactose enhanced the uptake of polylysine into airway cells.[93,94] This interaction may therefore (in cases where intracellular delivery is desired and stability is not compromised by lactosylation) be a method of improving intracellular localization of therapeutic proteins/peptides. Thus, sugars can be active carriers manipulated for their targeting potential.

Nonreducing sugars (eg, trehalose) have been assessed for their suitability in protein DPIs.[95] A recent study examined alternative carriers including mannitol, glucose, sorbitol, malitol, and xylitol for their potential use in DPIs.[96] After physicochemical properties and aerosolization behavior of the powders were monitored, the study concluded that mannitol appeared to be the best candidate for DPI formulations as the more hydroscopic sugars showed poor dispersability.

The methods used for preparation of protein powders can be limited by the protein's sensitivity to the processes used. Several different technologies can be used to produce respirable protein/peptide powders including freeze-drying followed by milling, or spray-drying. Respirable particles of salmon calcitonin were produced by lyophilizing a solution of the protein with lactose prior to jet-milling.[97] Spray-drying is the most popular method and is a particularly interesting option for the formulation of proteins and peptides as it is a single-unit process and avoids some of the technical difficulties associated with freeze-drying and crystallization. Spray-dried lactose, sucrose, mannitol, and trehalose have been assessed as potential excipients for DPIs.[98] Supercritical fluid technology[99,100] and spray-freeze drying[101,102] are gaining popularity. Maa et al[102] assessed spray-freeze-dried particles of anti-IgE monoclonal antibody and recombinant human deoxyribonuclease (rhDNase) prepared with a range of carbohydrate excipients including mannitol, trehalose, and sucrose. The fine particle fraction of these formulations was found in most cases to be highly dependent on the method of production, with spray-freeze drying producing larger, more aerodynamic, particles. Exceptions to this rule appeared to be linked with crystallization or coalescence of the excipients during spray-freeze drying. Thus, when choosing a process for powder preparation it is important to monitor the protein and excipient stability.

Cyclodextrins

Cyclodextrins (CDs) are cyclic oligosaccharides with many properties that have made them useful as excipients in respiratory delivery of small molecules.[103] To date, their use in the delivery of proteins/peptides to the lungs has been limited to penetration enhancement. Kobayashi et al[97] used dimethyl-β-CD to enhance the systemic absorption of salmon calcitonin after intratracheal administration of the protein in rats, and recent studies have shown that it is also capable of enhancing the pulmonary absorption of insulin in rats.[104] It appeared that the greatest effect was seen when the enhancer was

delivered in a dry powder form versus a solution. β-CD was also found to improve the systemic delivery of the cyclopeptide, FK224.[18] The bioavailability increased as the concentration of CD increased. Of interest, a metered-dose approach was assessed in this study for delivery of the peptide formulation. CDs are potentially useful excipients, rather than carriers, in the respiratory delivery of proteins.

These carriers-liposomes, microspheres, and carbohydrates-are therefore useful tools for delivery. But how can these carriers be manipulated in order to control drug targeting?

Passive Targeting

The delivery of a drug to its site of action in the lungs is dependent on several factors including the physicochemical properties of the aerosol droplet/powder particle. Carriers such as liposomes and polymeric microparticles offer the possibility of altering the aerosol characteristics to allow passive targeting. Certain protein and peptide drugs may be active in the tracheobronchial tree, whereas others will need to reach the periphery of the lungs. For those being delivered primarily for systemic absorption, transport is generally most efficient across the alveolar epithelial cells in the peripheral airways.

The physical factors affecting deposition include size, shape, density, and hygroscopicity. Many years of research and development have been dedicated to the characterization of aerosols for delivery of small drug molecules, and this know-how can be applied to macromolecular delivery. The first principle of delivery to the airways is avoidance of impaction in the oropharyngeal region. Given the expense of many protein- and peptide-based therapeutics, it is not only clinically relevant but also economically important to diminish losses and deliver the maximum dose possible to its site of action. Drug-carrying particles 1 to 3 μm in aerodynamic diameter generally avoid oropharyngeal deposition. Micron-sized particles often have poor flow properties, so carrier material such as lactose, which is deposited in the mouth, is added. The size distribution of aerosols also affects deposition.[105] Many studies focus on the primary particle size characteristics, but it is the aerosol (powder or droplet) size that determines whether it eaches its site of action. In many cases the aerosol size is a function of the aggregate state of the primary particles.

Particle shape is also important. Generally, spherical particles are preferred because of their good flow properties, but long, thin particles have also been shown to deposit in the alveoli and may provide a method for selective delivery if the dissolution rate is slow.[105] Recent studies have shown that large, porous particles can be deposited in the peripheral airways owing to their low density (meaning their aerodynamic diameter is far less than their geometric diameter).[83] These particles also appear resistant to AM phagocytosis and could therefore prolong the duration of action and target delivery to the alveolar epithelial cells. Controlling the hydroscopic growth of a drug particle in the humid environment of the lungs can also alter deposition, and hydrophobic film coatings have been examined for this purpose.[106]

Clearance Mechanisms Within the Lung and Their Effect on Delivery

Carrier delivery systems have the ability to control drug delivery and release. There are several factors that determine the rate of drug delivery including the physicochemical nature of the drug itself (eg, solid or liquid state, molecular weight, charge, partition coefficient), the nature of the carrier (eg, choice of polymer, size, surface characteristics, bioadhesive properties, biodegradability), and the rate of clearance from the lungs. The mechanism of clearance of aerosol particles depends on their site of deposition within the lungs. If particles are deposited in the tracheobronchial tree, then they will be rapidly removed by the mucociliary escalator.[107] Particles deposited in the lower, alveolar regions of the lungs are more likely to be scavenged by AMs (phagocytosis) and/or transported across the epithelium. While the lungs are a far less hostile metabolic environment than the gastrointestinal tract, enzymes are still present (though in smaller amounts).[108] Particles may be enzymatically degraded intracellularly (within macrophages) and/or extracellularly by membrane-associated proteases and peptidases (epithelial and endothelial). The degree of alveolar phagocytosis and transport across the epithelial barrier may be controlled to some extent by altering the nature of the drug and carrier (as described previously).

The deposition, degradation, and retention of carrier delivery systems also depends on the lung pathophysiology.[109] For example, the mucus and surfactant layer in the healthy lung contains high concentrations of protease inhibitors, which could improve the stability of protein drugs, but this antiprotease protection may be diminished in certain disease states (eg, emphysema). Another example is the highly viscous mucus present in the cystic fibrotic (CF) lungs. This highly dehydrated mucous can pose a diffusional and/or electrostatic barrier to particulate carriers and proteins.[110,111]

Careful choice of excipients and manufacturing technology can therefore provide a means of targeting delivery and controlling clearance without the use of ligands. It must be remembered that controlling the physical properties of the particles is only one of the factors affecting deposition; the generation system and patient inhalation technique also influence the site of deposition.

Pegylation

The conjugation of polyethylene glycol (PEG) to proteins serves as a mechanism of prolonging the residence time by

decreasing degradation and prolonging half-life in the lungs while reducing side effects by limiting systemic absorption.[112] It appears to be a safe carrier[113] and prolonged the effect of rhG-CSF[114] upon delivery to the airways of male rats. PEGylated SOD delivered to the lungs has been found to be effective in protecting rats from oxygen toxicity compared with SOD alone, which offered no protection.[115] Nektar Pharmaceuticals are in the very early stages of developing a long-acting inhaled insulin using PEGylation. The PEGylation of proteins can have many other benefits including improved solubility, reduced immunogenicity, and increased storage stability.

Once the protein/peptide has reached the lungs, its site of action may be on the cell surface, intracellularly in airway cells, or in the systemic circulation. A carrier/drug can be modified in several ways to improve distribution into the desired target cell/tissue.

Active Targeting

Targeting proteins/peptides or protein-loaded liposomes/microparticles using ligands offers the opportunity to improve delivery to target cells while decreasing unwanted side effects. Table 4 gives examples of specific targeting ligands that have been assessed either in vitro or in inhalation studies for targeting airway cells. Many ligands can be used either directly conjugated to the protein/peptide or attached to the carrier (eg, microsphere or liposome). Ligands can aid bioadhesion, cell uptake, and/or transcytosis. The examples are all macromolecular cargoes or carriers. DNA delivery is also included as much of the recent research into intracellular and cell-type specific ligands have been based on the development of gene-delivery vectors. Gene therapy is another means of indirectly delivering proteins to the lungs. The technologies developed for DNA delivery may be adapted for delivery of recombinant proteins and peptides. In many cases the gene delivery vectors are themselves peptide-based systems (eg, mannosylated poly-L-lysine).

Developments in molecular biology and proteomics have provided more thorough processes for screening likely ligands. Several groups have used phage display in order to identify likely binding moieties on the epithelial cell surface. Jost et al[116] used a phage display library to screen peptides for binding affinity to apically located receptors on airway cells. This screening process identified the Thr-His-Ala-Leu-Trp-His-Thr (7-mer THALWHT) peptide as having a highly specific binding affinity for airway cells. A more recent screen of 3 peptide libraries identified lung epithelium binding peptide 1 LEBP-1 (15-mer QPFMQCLCLIYDASC), LEBP-2 (15-mer RNVPPIFNDVYWIAF), and LEBP-3 (14-mer VFRVPWYQSTSQS).[117] These peptides selectively bound to alveolar epithelial cells compared with Hep2 cells. PTD-4 and PTD-5, two 12-mer peptides of the M13 phage library were found to improve protein translocation 600-fold.[118] Similar studies have been performed to identify ligands that target the lung vasculature.[119]

Bioadhesives

Bioadhesives can be used to prolong tissue exposure to the therapeutic entity. Bioadhesion in this context refers to interactions that involve multiligand binding (generally noncovalent) of the surface material of the carrier to cell surface determinants on airway cells, which can induce rapid envelopment of the carrier by either transcytosis of migrational overgrowth mechanisms.

Multivalent binding agents such as lectins, peptides, and antibodies may be included in carrier-based systems, and microaggregates such as albumin microspheres can have inherent mucoadhesive properties.

Lectins are naturally occurring glycoproteins that have the ability to recognize and bind to carbohydrate residues on the surface of epithelial cells. Several lectins when bound to the apical surface are actively taken up into the cells.[120] Recent studies have shown that lectins are capable of enhancing the binding and uptake of liposomes into airway cells.[121,122]

Other bioadhesive moieties include heparin and heparin sulphates.[123] Their interaction with cell-surface proteoglycans can improve adhesion. Other anionic sites on the alveolar epithelium bind cationic ferritin.[124] The abundance of anionic sites on the cell membrane and in the glycocalyx means that cationic moieties have a tendency to "stick." This has been manipulated with cationic liposomes and cationic polymers. Small, cationic peptides are a less cumbersome and safer method of conferring cationic charge on a protein/carrier. Our recent findings suggest that octa-arginine and other cell-penetrating peptides are capable of improving the cell adhesion of liposomes to airway epithelial cells[125] (see Figure 6).

These generalized interactions with cell membranes are well suited to bioadhesion. When cell/tissue specificity or efficient intracellular delivery is required, more specific receptor-ligand interactions can be harnessed. A good example is the use of antibodies. Antibodies will bind to specific cell surface antigens targeting the drug/carrier to a specific tissue/cell type and often triggering a more efficient receptor-mediated uptake process than that of adsorptive endocytosis.

Cell-type Specific Targeting

The ability to target specific cell types within the lungs could prove extremely useful for both disease treatment and for prevention and/or diagnosis. With increasing interest in and understanding of the role of lung cell types in disease pathology, it is likely that targeting to mast cells (eg, for asthma) and mucus cells (eg, for CF) will be required. Gene therapy

Table 4. Examples of Ligands Capable of Modulating the Delivery of Drugs to the Lungs*

Ligand	Receptor	Examples	Effect	Ref
Lectin	Lectin receptor	Plant lectins, GS-I lectin, (WGA)	Improved uptake of drugs and lipsomes into airway cell cultures	121,122,146
Sugars	Lectin receptors	Glucose, mannose, lactose	More efficient uptake by airway epithelial cells in culture	147,148,193,194
	Alveolar macrophage receptors	Mannose	Specific, enhanced endocytic uptake into alveolar macrophages	131, 133, 134
Immuno-globulins	IgG	Fc receptor		65,135,195
Lipo-proteins	SP-A	SP-A receptor	Increased drug and liposome delivery to airway cells	145,196,197
EGF	rEGF	EGF receptor	Efficient DNA uptake into cancer cells	138,139,150
Mono-clonal Abs	ICO-25 Mab	Mucin-like human epithelial membrane antigen	Specific delivery to tumor cells of epithelial origin	198
	Anti-ICAM-1 antibodies (mAb F10.2)	ICAM-1	IFN-gamma activated human bronchial epithelial cells (BEAS-2B)	151
Peptides	THALWHT		Specific binding and uptake into human airway epithelial cells in vitro	116
	[D-R(6),D-Trp(7,9)-N(me)Phe(8)]-substance P(6-11)	Growth factor antagonist	Improved binding and uptake into SCLC cells in culture	140
	linear/cyclic PLAEIDGIEL	$\alpha 9\beta 1$-integrin	Efficient DNA delivery to airway cells in culture	199
	RGD	α-integrin receptor	Increased gene transfer efficiency	200
	HIV-TAT	Proteoglycans	Improved intracellular localization of liposomes	201
	LEBP-1 LEBP-2	Postulated to bind to IRF-7	Targeted binding to alveolar cells	117
Receptor agonists/ Antagonists	UTP	G-protein-coupled P2Y2 receptor	Efficient gene transfer in human airway cells	149
	Folate	Folate receptor upregulated in cancer cells	Efficient internalization into FR-expressing murine lung carcinoma cell line	137,202
	Transferrin	Transferrin receptor	Improved uptake into alveolar epithelial cells	150,203,204

*GS-I, indicates griffonia simplicifolia-I lectin; WGA, Wheat germ agglutinin; IGg, immunoglobulin; IRF-7, interferon regulatory factor 7; rEGF, recombinant epidermal growth factor; SP-A, surfactant protein A; ICAM-1, intercellular adhesion molecule; Interferon (IFN); PLAEIGIEL (peptide sequence); RGD (peptide sequence), Human Immunodeficiency virus-TAT (HIV-TAT); lung epithelial binding protein (LEBP); Uridine 5' Triphosphate (UTP).

studies have already started using cell-selective gene promoters to target genes to specific lung cell types.[126] Much of the work to date has focused on targeting AMs and cancer cells.

Alveolar Macrophage

The ability to specifically target AMs is desirable for vaccination, modulation of the inflammatory response, and cancer and parasite treatment. Lymphatic targeting is also possible, as a proportion of AMs are transported to this system.

Liposomes and microspheres are generally engulfed by AMs owing to their particulate nature, and understanding this interaction can aid the formulator in developing preparations to enhance or avoid uptake. When the site of action for a protein/peptide-based drug is not the AM, then phagocytosis can lead to rapid clearance and degradation thereby limiting therapeutic efficacy; therefore strategies have been developed that appear to limit uptake. These strategies include large porous particles,[83] coating of microspheres with DPPC, phospholipids, decreasing macrophage uptake[75,127,128]

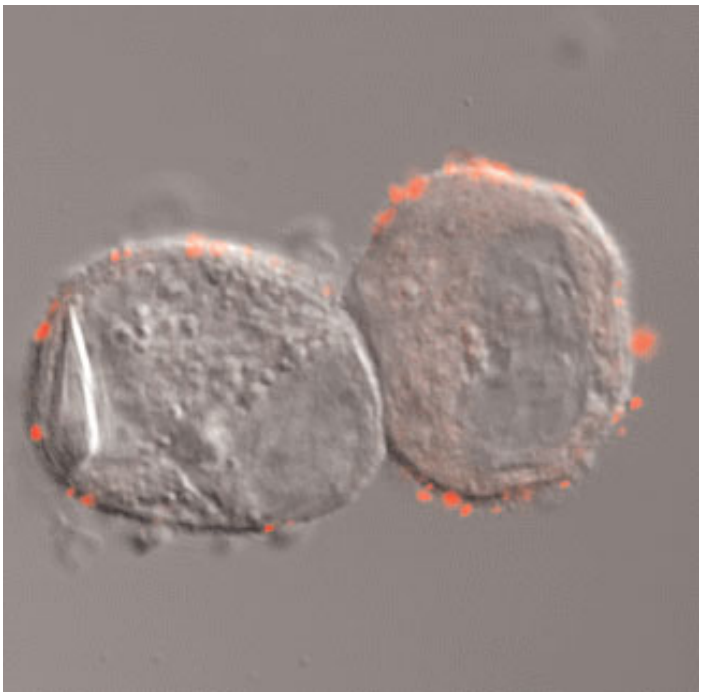

Figure 6. Increased bioadhesion of DOPC:Chol liposomes (rhodamine labeled) to airway cells when conjugated with octa-arginine.

(Figure 7), and precoating particles with bovine serum albumin (BSA) and nonproteinaceous macromolecules. In contrast, precoating particles with bovine gamma-globulin, human fibronectin, and gelatin enhances phagocytosis,[85] and dense particles of <5 μm tend to be engulfed by AMs.

Liposomes tend to be unstable after phagocytosis, leading to a rapid discharge of their contents. The rate of microsphere degradation and subsequent protein release in AMs can be controlled by changing the molecular weight and the monomer composition of the copolymers comprising the microspheres.

Receptors for transferrin, lactoferrin, immunoglobulins, interleukin-2, and granulocyte-macrophage colony-stimulating factors are present on AM cell membranes,[129,130] and mammalian macrophages can internalize glycoproteins with exposed mannose residues[131,132]; several studies have used this mechanism for targeting proteins/peptides to AMs.[131,133,134] Others have harnessed immunoglobulins to target the AM Fc receptor.[65,81,135]

Cancer Cells

Specific targeting of cancer cells is always desirable given the toxic side effects of many anticancer agents. Inhalation therapy versus parenteral delivery aims to limit unwanted systemic side effects, but deleterious effects on the normal airway cells will result.

Several receptors are overexpressed in cancer cells including folic acid and epidermal growth factor (EGF), and this phenomenon has been harnessed to target lung cancer cells.[136-140] Another moiety that has been used to target cancer cells is low-density lipoprotein (LDL). Receptor-mediated assimilation of LDL by many cancer cells is much higher than that of normal cells, and this property can be harnessed for targeting.[141]

Intracellular Targeting

So far, discussion has focused on delivering the drug to the desired area of the lungs or the desired cell type, but it is becoming increasingly clear that drugs may need to be targeted to specific intracellular sites of action.

The focus of the majority of intracellular targeting work has been in the area of gene therapy but intracellular delivery is also essential for a range of protein and peptide drugs. Examples include antimicrobial peptides, anti-inflammatory proteins, and antioxidants such as catalase and dismutase. Some of the fundamental obstacles to intracellular macromolecule delivery to the lungs have been inefficient vectors and the absence of appropriate receptors on the apical surface of airway epithelial cells.[94]

To date, attention has focused on the alteration of charge to produce positively charged complexes that can interact effectively with cell membranes. More recent advances have led to the use of a range of complexing and condensing agents, involving a wide range of chemical moieties including polycations, peptides, proteins, lipids and liposomes, and polysaccharides, as well as other condensing and noncondensing polymers.[142] Many of these "standard" transfection reagents do not facilitate efficient intracellular delivery of proteins. Two exceptions are the BioPORTER (a cationic lipid-based carrier)[143] and TransIT[144](a histone-based polyamine), which have both been used to deliver proteins intracellularly.

Briscoe et al[145] used pH-sensitive liposomes to improve the intracellular delivery of SOD to cultured fetal rat lung distal epithelial (FRLE) cells. Upon interaction with an acidic environment, pH-sensitive liposomes have a tendency to change their phase and structure.[36] Briscoe et al[145] also examined the effect of including surfactant protein A (SP-A) in the liposome and found that it improved intracellular delivery by 6.2-fold. Many ligands (see Table 4), once bound to the cell membrane, can trigger cell uptake including lectins,[146] sugars,[147,148] Uridine 5'-triphosphate (UTP),[149] transferrin,[150] and EGF.[138,139] An interesting example of ligand-based intracellular delivery is based on anti-intercellular adhesion molecule-1 (ICAM-1) antibody (mAb F10.2). The intracellular delivery of anti-inflammatory drugs to sites of inflammation characterized by an increased expression of ICAM-1 has been improved by using ICAM-1-targeted immunoliposomes.[151]

It should be noted that some peptides and proteins are naturally internalized by cells. Several small regions of proteins called protein transduction domains (PTD) have been identi-

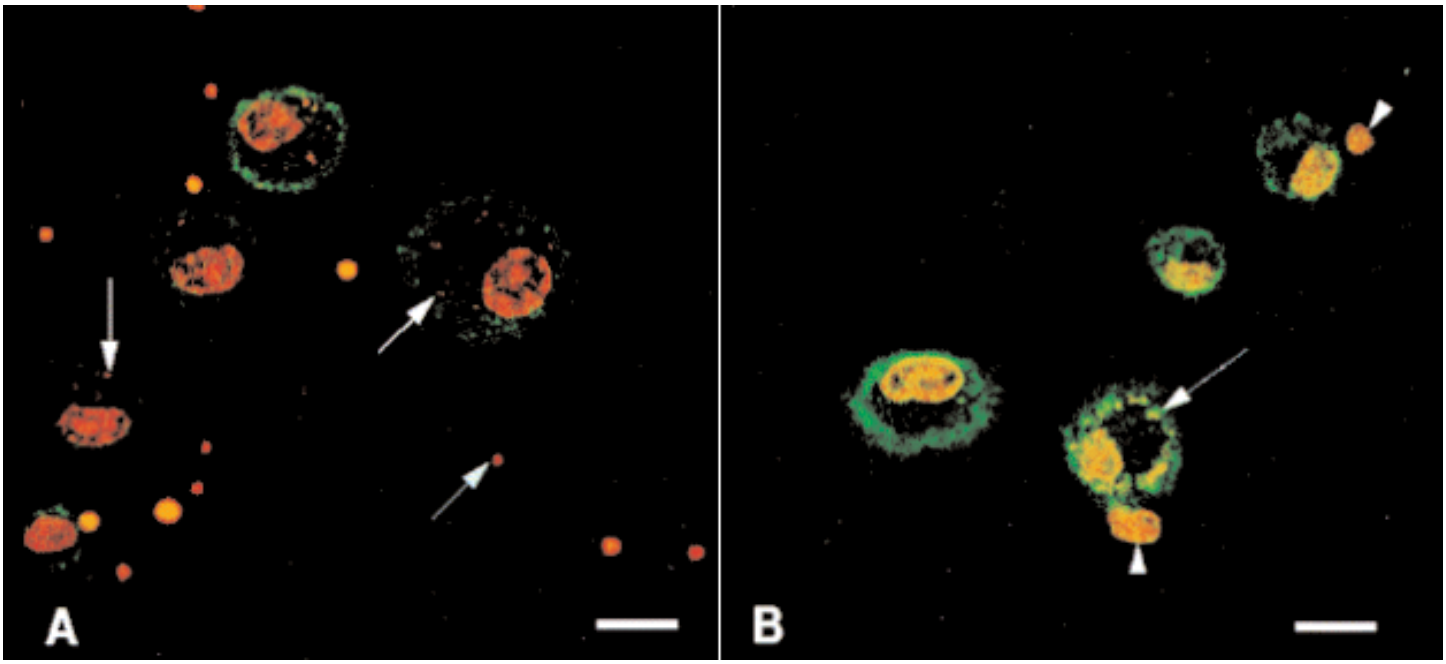

Figure 7. (A) Fluorescent confocal micrographs of rat alveolar macrophages (green) stained with FITC-phalloidin after a 1-hour exposure to peroxidase-containing PLGA particles (arrows). (B) Rat alveolar macrophages exposed to peroxidase-containing PLGA particles (arrow) prepared with DPPC. Large particles are occasionally seen bound to cell surfaces. Bar represents 10 microns. (reprinted with kind permission from Ref 75)

fied, which are rapidly and efficiently internalized by cells.[152] These peptides appear to represent a highly evolved macromolecular delivery system and include the *Drosophilia* homeotic transcription protein antennapedia (Antp),[153] the human immunodeficiency virus I transcriptional activator (HIV-TAT),[154] and the herpes-virus derived VP22.[155] It has already been shown that these peptides can be used as vectors for delivery of macromolecules, including proteins.[156] Other protein delivery strategies have employed transduction domains of bacterial or plant toxins[157] and hydrophobic cell penetrating peptides based on signal sequences.[158] Protein delivery is accomplished by conjugating the delivery peptide to the cargo protein or by forming chimeric proteins containing the therapeutic protein and the delivery protein.

Intracellular Trafficking

The mechanism of uptake and the subsequent intracellular pathway can determine whether the drug is available at the site of action. It is becoming increasingly evident that an understanding of the mechanism of cell-carrier interaction is imperative in formulation development. Many proteins and carriers are candidates for cellular uptake by constitutive and ligand-stimulated pathways. These pathways can lead to recycling out of the cell, entrainment in the endolysosomal system, delivery to organelles (eg, the Golgi apparatus) or transcytosis. Ligands can facilitate uptake via several different pathways (eg, clathrin-coated pits or caveolae) and could therefore alter the intracellular fate of protein/peptide cargo.

After binding to a cell-surface receptor, the transport of ligands (and their cargo) to specific intracellular sites is facilitated by membrane-trafficking proteins. Several cytosolic proteins, including serpins, nexins, and Grb2, regulate downstream trafficking of receptors (and their ligand complexes).[159] Cytoskeletal elements are responsible for most trafficking between organelles. Trafficking of endosomal vesicles and their cargo is driven by molecular motors along microtubules and microfilaments. The trafficking of some carriers is also linked to these motor proteins. For example, intracellular trafficking of liposomes has been found to involve microtubules.[160] Thus, a protein within a liposome carrier may exhibit altered trafficking, compared with the free protein, within the cell. There is a growing need to understand how airway cells regulate the trafficking of carriers and ligands after interaction with the cell membrane.

Endosomal Release

Many of the ligands and carriers described trigger adsorptive endocytic internalization by cells. Inefficient cytoplasmic delivery is a fundamental and well-recognized problem with many delivery vectors including liposomes, polymers, and peptides. Prolonged retention in the endolysosomal system can lead to rapid degradation of proteins and peptides.

Several strategies have been employed to overcome this issue. Lysomotrophic agents include reagents such as ammonium chloride and chloroquine, whose effects are exerted by preventing endosomal acidification and hence routing of endocytosed complexes to the lysosome for degradation. The

use of chloroquine is restricted to in vitro studies since the concentrations required for a response in vivo are likely to result in unacceptable toxicity.

pH-sensitive liposomes have demonstrated enhanced cytoplasmic delivery of macromolecules owing to the destabilization of the liposome bilayer at acidic pH within the endosome.[161] Inclusion of colipids such as dioleoylphosphatidylethanolamine (DOPE) in liposomal formulations is thought to improve cytoplasmic delivery of liposomal contents due to its tendency to undergo a transition from a bilayer to a hexagonal configuration under acidic pH leading to fusion with or destabilization of target membranes, especially endosomal membranes.[162,163]

Certain enveloped viruses use fusogenic peptides to infect cells, and several studies using both polymeric delivery systems have looked at incorporating fusogenic peptides into the formulation in order to destabilize the endosomal membrane in a virus-like fashion.[164,165] By using only the fusogenic peptide sequences, the dangers associated with viruses are eliminated. The best characterized fusogenic system is present within the influenza virus hemaglutinnin (HA), whose fusion domain is located at the N terminus of subunit HA-2 and has been shown to significantly enhance intracellular macromolecule delivery.[165,166] Another pH-sensitive fusion peptide is GALA, which is a water-soluble, 30 amino acid containing amphipathic peptide that undergoes a conformational change from random coil at pH 7.5 to an amphipathic helix at pH 5. JTS-1 is a second synthetic pH-sensitive fusion peptide designed by molecular modeling.[167]

Another approach involves the use of polymers, with a substantial buffering capacity below physiological pH, which is potentially capable of producing membrane disruption. Examples of these include the polyamidoamine cascade polymers,[168] interpolyelectrolyte complexes (IPECs),[169] the organic macromolecular polyethyleneimine (PEI),[170] and pH-sensitive polymeric carriers based on a poly(propylacrylic acid).[171,172]

Many ligands interact with cell membranes and stimulate endocytic internalization of the complex. Ligands that evade endocytic uptake are being sought avidly. Early indications were that the HIV-TAT and Antp peptides evaded endocytic uptake, but this has since been refuted.[141]

Once free in the cytoplasm, protein/peptides may need to interact with specific sites in given organelles, including in some cases the nucleus.

Nuclear Localization

Many of the strategies employed in nuclear targeting have their origin in the natural nuclear localizing ability of proteins/peptides. Transport across the nuclear envelope is mediated by large supramolecular structures called the nuclear pore complexes (NPC), which span the membrane. Small ions and metabolites diffuse freely through the NPC, while most macromolecules are transported through gated channels via signal- and energy-dependent mechanisms. Certain proteins, histones, transcription factors, viral proteins, and the like bear Nuclear Localization Signal (NLS) proteins, which allow them to be transported into the nucleus via a receptor-mediated process.[173] Typically, an NLS will contain a cluster of 4 or more cationic residues that are often flanked by proline of glycine. Some mammalian nucleoproteins have "bipartite" sequences of cationic residues separated by a spacer (10-12 residues).

The best-known example of an NLS is the SV40 motif, -PKKKRKV-. Coupling of a single copy of this sequence to proteins failed to produce rapid nuclear uptake.[174,175] Multiple copies of NLS appear to be more efficient. Peptides, composed of a short stretch of 5 to 10 basic amino acids based on the SV40 core NLS, have been conjugated to polycations and associated with liposomes in order to enhance nuclear localization.[176] Where required, these sequences, either conjugated directly to the drug or to a carrier, could prove useful for targeting protein/peptides to the nucleus.

Systemic Delivery: Transport Mechanisms Across the Airway Cells

Early work using several different peptides indicated that the pulmonary route offered a feasible alternative for systemic delivery of large molecules.[1] A wide range of proteins and peptides for systemic delivery via the lungs are being investigated, including insulin, calcitonin, growth hormone, immunoglobulins, and granulocyte colony-stimulating factor (G-CSF) to name a few.

The alveolar epithelium is the major site of absorption of peptides and proteins in the lungs because of the large surface area and the low permeability of the upper airways to proteins. The alveolar epithelial cells and not the underlying endothelial cells are the major barrier to transport.[177] The lung epithelium is composed of polarized epithelial cells with tight junctions between the cells. Though the alveolar epithelium is highly permeable to water, gases, and lipophilic substances, the permeability of large, hydrophilic substances, such as proteins is limited.[178] The routes of absorption across the airways' epithelium include passive and active transport mechanisms involving paracellular and transcellular transport, pore formation, vesicular transport, and drainage into the lymphatics.

The apical surfaces of these cells contain a high level of actin, which strengthen them and inhibits endocytic activity, making uptake of macromolecules more difficult. Rapid clearance and degradation of drug in the lungs will limit the amount available for transport. While mucociliary clearance

does not play a major role in the alveoli, the epithelial surface is covered with a complex surfactant layer (a mixture of lipids and proteins), 0.1 to 0.2 μm thick, which can limit absorption. The delivery of protein/peptide drugs systemically may also be hampered by protease and peptidase degradation and/or alveolar phagocytosis.[179,180]

Various approaches such as the use of penetration enhancers (eg, surfactants, bile salts, cyclodextrins), enzyme inhibitors (eg, chymostatin, leupeptin, bacitracin), and carriers have been used to overcome these barriers. The pulmonary absorption of salmon calcitonin and insulin is more efficient with absorption enhancers and enzyme inhibitors.[31,97,181-183] The use of enzyme inhibitors is not limited to systemically delivered proteins. The efficacy of locally acting proteins/peptides may also be improved by decreased degradation. Formulation of absorption enhancers and enzyme inhibitors with the drugs in carriers, such as liposomes and microspheres, would provide not only effective co-administration but also colocalization within the lungs.

Microparticles themselves can aid the absorption process. Edwards et al[83] enhanced insulin absorption using large, porous PLGA particles and prolonged action for up to 96 hours. Both microspheres[69,83] and liposomes[64,184] have been used to enhance the systemic effect of insulin after inhalation. The effects of microspheres were attributed to the sustained release of the insulin from the polymeric carriers.[185] The effects of liposomes on systemic absorption depend on the concentration, charge, and acyl chain length of the phospholipid components.[64]

The attachment of bioadhesive and targeting ligands to the carriers could also be used as a strategy to alter the systemic absorption of proteins/peptides. Ligands that specifically trigger transcytosis, for example, by harnessing caveolae-mediated transport,[186] of the conjugated/encapsulated proteins may be preferable from a safety perspective to generalized permeation enhancement that allows the nonspecific passage of other particles across the airways.

Limitations

Effective formulation requires the use of relevant models. Reproducible and well-characterized models of the airways have been difficult to develop. In order to effectively deliver macromolecules to the desired site, knowledge of the mechanisms of cell adhesion and cell transport in the lung is vital. Impingers, impactors, and dissolution studies provide some important data on aerosol characteristics but do not advance the understanding of the interaction (including release, uptake, transport) of the formulation with the airway cells. Despite the interest in this field, many important mechanisms have yet to be elucidated. Advances in recent years mean that primary human and animal cell culture models as well as cell lines such as A549, Calu-3s, and HBE16σs are available. Their relevance to in vivo conditions, however, needs to be carefully assessed when using the data as a basis for further testing. One major issue is the expression of peptidases in cell culture models. Peptidases represent a major barrier to both local and systemic delivery of stable proteins and peptides. Forbes et al[187] assessed several alveolar epithelial cell models for their ability to produce ectopeptidases and found that peptidase production was time dependent. Thus, culture conditions and duration can affect the relevance of the results of such studies. Several groups have developed impactor/impinger models that incorporate airway cell culture monolayers with the aim of simulating the lungs more effectively.[76]

A point that must be noted when discussing the development of protein/peptide-based drugs is cost. Many recombinant proteins and peptides are expensive to produce. Thorough formulation and bioavailability studies require large quantities of protein, the cost of which can be prohibitive.

CONCLUSION

With increasing knowledge of molecular targets for drugs and drug targeting and the sophisticated techniques now available to visualize trafficking at the cellular level, the temptation to forget the bigger picture grows. Knowledge of the dosage form, whole organism, cell, and molecular biology is crucial to the success of inhaled protein/peptide drug delivery. In short, the role of the pharmaceutical scientist in the development of proteins for inhalation grows ever more complex and exciting.

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