

# Chapter 5

## Mucosal Delivery of RNAi Therapeutics

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**Abstract** The effectiveness of RNA interference-based drugs is dependent on accumulation at the target site in therapeutically relevant amounts. Local administration to the mucosal surfaces lining the respiratory, gastrointestinal and genitourinary tracts allows access into diseased areas without the necessity to overcome serum nuclease degradation, rapid renal and hepatic clearance and non-specific tissue accumulation associated with systemic delivery. This work describes RNAi therapeutics focused on pulmonary, oral, rectal and intravaginal routes of administration. Mucosal barrier components including site variations and delivery considerations are addressed in order to design an effective mucosal delivery strategy.

### 5.1 Introduction

Regulation of cellular gene expression by harnessing the natural process of RNA interference (RNAi) offers an exciting gene medicine approach [1, 2]. Post-transcriptional silencing occurs by mRNA engagement with small interfering RNA (siRNA) or microRNA (miRNA) facilitated by complementary base pairing [3]. Gene specificity coupled with the capability for externally introduced synthetic siRNA and miRNA to be recruited into the cellular RNAi pathway provides the rationale for RNAi drug development. A greater understanding of the molecular mechanism of RNAi has resulted in a wide repertoire of potential RNAi drugs involved in the RNAi pathway cascade that offers diverse therapeutic options.

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The clinical potential of nucleic acid-based drugs is restricted by the susceptibility to serum nuclease degradation, rapid renal clearance and non-specific tissue accumulation [4]. Furthermore, the macromolecular and polyanionic nature reduces interaction and uptake across the cellular membrane required for recruitment into the intracellular RNAi machinery. Improvements in both extracellular and intracellular delivery are key to the therapeutic success of RNAi therapeutics. Chemical modification [5], conjugation [6] and incorporation into nanoparticle-based delivery systems [7, 8] are common strategies that have been employed to maximise delivery [9, 10].

The route of administration is an important determinant for successful RNA-based silencing therapeutics. The administration route dictates both migratory pathway and biological barriers the drug must undertake in order to reach its target. Local administration to the mucosal surfaces lining the respiratory, gastrointestinal and genitourinary tract is an attractive alternative to the intravenous route [11–13]. It is a non-invasive method that avoids hepatic and renal clearance associated with the systemic route and allows direct access to regions that are the main portal of entry and pathogenesis for many pathogens, inflammation and cancer.

Recent Phase II clinical trials with RNAi therapeutics delivered directly to the lung [14] highlight the potential and support the use of the mucosal route.

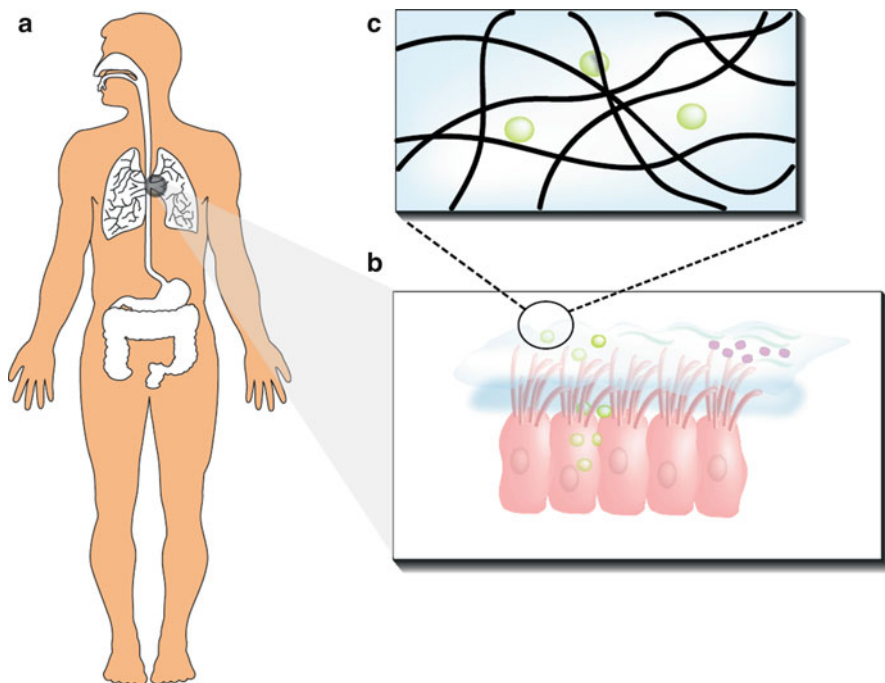
This work describes pulmonary, oral, rectal and intravaginal delivery of RNAi therapeutics focused on nanoparticle-based delivery of synthetic siRNA. Attention will be given to the biological and physical barriers occurring at the mucosal surfaces that restrict uptake of luminal material. Strategies to improve mucosal penetration will be discussed with a view to better design of mucosal delivery systems.

## 5.2 Mucosal Barriers

RNAi-based therapeutics must overcome the physical barrier of the mucus gel layer and tightly packed epithelial cells combined with mucus capture and consequent active clearance mechanism. Understanding these barriers and their evolutionary differences can provide guidelines for siRNA-based therapy targeted at specific mucosal sites. This section focuses on mucus and epithelial components relevant to naked siRNA and nanoparticle-based siRNA delivery.

### 5.2.1 Mucus

Mucus is a hydrated protein gel which overlays the luminal surface at mucosal sites and serves as a barrier between the external environment and the underlying tissue. It lines the respiratory, gastrointestinal and genitourinary tracts, and eyes (Fig. 5.1). Its role is to serve as a first line of defence against various pathogens [15] and toxins



**Fig. 5.1** Schematic representation of nanoparticle uptake across mucosal epithelium. **(a)** The mucosal surfaces lining the respiratory, gastrointestinal and genitourinary tracts and eyes. **(b)** The mucosal epithelial border restricts penetration of luminal particulates (*right*) through a combination of overlying mucus composed of a lower steady layer (dark) and an upper layer (light), tight cellular junctions and ciliary clearance. Exploitation of mucoadhesive and mucopermeable particles allows cellular uptake across mucosal surfaces (*left*). **(c)** Particles diffusing through the network of mucin fibres

[16] and to facilitate continuous exchange of nutrient, water and gases. Mucus has macroscopic properties of a gel and exhibits non-Newtonian rheological behaviour [17, 18]. It is composed of ions, glycoproteins (termed mucins), proteins, lipids, DNA and cellular debris [18]. The mucins are extended 0.5–40-MDa molecules that are produced and secreted by goblet cells. It has a two-layer composition composed of a lower steady-state layer in contact with the epithelium and a mobile outer layer. Mucus site variations have evolved to suit the role performed at particular sites.

### 5.2.1.1 Site Variations

Mucus thickness, rate of renewal and pH are properties that can vary between tissues. The mucus layer thickness determines the accessibility to the underlying epithelium and depends on both luminal conditions and functional requirements of the underlying tissue. The layer thickness varies along the human gastrointestinal tract with the thickest layer in the stomach (~50–450  $\mu\text{m}$ ) [19] and in the colon

(~110–160  $\mu\text{m}$ ) [20]. In the eyes, the thickness of the mucus layer has been reported to be 30–40  $\mu\text{m}$  [21, 22], in the airway 7–30  $\mu\text{m}$  [23–25] and in the bronchiole ~55  $\mu\text{m}$  [26]. The most accessible mucosal surfaces are found in the nasal region [27] due to a very thin layer of mucus, and in the deep lung where the epithelial lining is devoid of mucus and instead contains surfactants which reduces the surface tension and potentiates gas transfer in the alveoli [28]. Relevant to mucosal therapeutic delivery is the thickness and integrity of the mucus layer may be compromised under various pathological conditions. For example increase in mucus thickness has been observed in asthma [29], cystic fibrosis [26] and chronic obstructive pulmonary disease [30], whilst a decrease in thickness has been observed in ulcerative colitis [20]. This variation in mucus thickness can lead to a predisposition for disease or be induced by pathological consequences of the disease such as loss of epithelial integrity as seen in ulcerative colitis.

Despite the existence of a steady layer just above the epithelial cells, mucus is a dynamic substance which undergoes continuous renewal by secreting goblet cells dispersed throughout the epithelial layer. This produces an outward moving barrier to any entity that aims to reach the epithelial layer and determines the timeframe allowed for particles to penetrate the epithelium before clearance. Renewal rates are tissue dependent which has implications for designing therapeutic strategies. Mucus in the nasal cavity is replaced approximately every 20 min [31] and between 10 and 20 min in the respiratory tract [32] compared to a clearance rate of 4–6 h in the gastrointestinal tract [33] as found in rats but values have not been fully determined in humans.

In addition to thickness and rate of renewal, the pH value varies at different sites, with the lung and nasal mucus being nearly neutral [34, 35], the eye possessing a weak basic pH [21] and the mucus of the stomach having a pH gradient from pH 1–2 at the luminal side to approximately pH 7 at the surface of the epithelial cells underlying the mucus [36, 37]. These pH variations could be utilised for pH-responsive delivery systems which release their cargo at specific mucosal sites.

### 5.2.1.2 Mucus Penetration

The protective properties of mucus pose a barrier to RNAi-based therapeutics both in naked or nanoparticle form. Mucus constituents such as the glycoproteins (mucins), cellular debris and lipids form a heterogeneous environment through which drugs and/or drug carriers need to diffuse to reach their target [38]. Glycosylated domains of the mucin fibres possess a negative charge under physiological conditions, and hence mucus selectively controls the diffusion of particles not only through particle physical parameters such as size, but also by their chemical surface properties. Based on the net negative charge of mucins, one could speculate that naked siRNA might be repelled by the mucin fibres. Multivalent interactions between particles and the mucus network are main determinants of particle diffusion. Both electrostatic and hydrophobic interactions occur, and the possibility of making large numbers of non-specific hydrophobic interactions together with the more thermal stable electrostatic interactions enables mucus to trap particles. Several studies have demonstrated the efficacy of hydrophobic interactions to immobilise particles

within mucus [38–41] and Ribbeck and co-workers have shown that particle surface charge, density and pH of mucin hydrogels can alter particle diffusion [42]. Particles with a PEGylated surface possessing a neutral charge had a greater diffusion rate compared to its negative or positive charged counterpart [42]. Two approaches govern the design of particles for mucosal delivery (1) the mucoadhesive and (2) mucus penetrative approach.

Regarding the mucoadhesive strategy, much attention has been given to the design of particles which associate with the mucus barrier and hence lower the clearance rate. The essence of this strategy lies with the previously mentioned bilayered structure of mucus. Association of particles with the lower undisturbed layer will avoid clearance and enhance the bioavailability of the bioactive entity. Another positive effect from mucus association is increased viscosity due to greater cross-linking of mucus fibres, which in turn may lower the clearance rate. Materials of choice have been the so-called mucoadhesive materials. A common characteristic of these materials is their adherence to mucus through various forces. A widely used mucoadhesive polymer is chitosan [43], which have been utilised to form particles with siRNA and exhibit mucosal silencing [7]. Thiolation of polymers have been shown to enhance mucus interaction through formation of disulfide linkages with mucins [44] and various thiolated chitosan's have been synthesised [45, 46]. Not only does the mucoadhesiveness prolong the bioavailability of particles at mucosal surfaces, but it can also alter the structure of mucus so that it becomes more permeable to siRNA-loaded particles [47]. As an alternative, mucus-penetrating particle with limited interaction with mucus and increased diffusion rates is an exciting approach. Coating with PEG has been demonstrated to mediate such surface properties on latex particles (200–500 nm) [48] and nanoparticles composed of a biodegradable di-block copolymer of poly (sebacic acid) and PEG [49]. It has been further shown that both molecular weight and degree of surface coverage determined the mobility of the coated particles [50]. Reports, however, have previously classified PEG as a mucoadhesive polymer [51–53]. As speculated by Lai et al. the contradicting reports might be attributable to variations in type of PEGylation used, but the use of PEG-coated particles have yet to demonstrate intracellular delivery of nucleic acids to the respiratory or gastrointestinal tract. Interestingly, the design of mucus-penetrating particles builds on lessons learned from nature where viruses with an equal surface density of positive and negative charges readily penetrate mucus barriers [54]. Thus, surface chemistry together with size appear to be determining factors for mucus penetration and evidence points towards neutral surfaces for effective diffusion.

### 5.2.2 *Epithelial Cell Barrier*

An ordered array of closely packed epithelial cells overlaying a basement membrane constitutes the mucosal epithelium. Cell type, morphology and arrangement differ dependent on site and function. For example the small intestine comprises of single-layered enterocytes assembled into structured villi that increase the

adsorptive surface area. Whilst in the upper respiratory tract, movement of apical cilia on pseudostratified epithelium restricts interactions at the luminal surface. Common to all mucosal epithelium is the close packing of adjacent cells separated by tight junctions.

Material uptake across the epithelium can occur by transcellular or paracellular pathways that are determined by the physicochemical characteristics of the material. The main transcellular mechanism for nanoparticle transport across the epithelium is adsorptive or cell-mediated endocytosis. Modification of material properties or targeting specific sites can be used to maximise delivery across the mucosa. It is generally accepted that the tight junctions restrict paracellular transport of micro-nanoparticles; however, mucopenetration enhancers have been used to facilitate transient opening of the junctions and mediate paracellular movement of small molecules.

The migration of particles from local to systemic tissue relies on translocation through the lymphatic or vasculature system and is dependent on the physicochemical properties of the material and the site. Disease pathogenesis dictates whether there is a necessity for local and/or systemic delivery and should determine the therapeutic strategy adopted.

### 5.3 Pulmonary Delivery

Direct access to a vast array of lung-associated diseases makes the lung an ideal target for RNAi-based therapies. With a total surface area of 140 m<sup>2</sup> [54], the pulmonary route offers an attractive alternative to the invasive nature of intravenous injections. The future clinical potential for pulmonary RNAi therapeutics holds promise based on the large number of current inhalable traditional drugs and established pulmonary delivery technologies provided by pressurised metered dose inhalers (pMDIs) or dry powder inhalers (DPIs) [55]. The respiratory system also provides an opportunity for drugs to reach the systemic circulation by uptake across the thin epithelium of the alveoli.

Lung-associated diseases such as influenza and respiratory syncytial virus (RSV) infection are prime candidates for siRNA therapy. The transient nature of gene silencing is sufficient for acute viral disease treatment. Moreover, silencing of host factors or conserved genes involved in viral replication could overcome the necessity for seasonal drugs directed towards surface proteins that are susceptible to mutational changes. Several host factors critical for viral replication [56] have now been identified in influenza which provides a selection of novel targets for RNAi-based therapies.

#### 5.3.1 Considerations for Pulmonary Delivery of siRNA

The anatomy, physiology and immunology of the lung present a challenge to delivery of nanoparticle-based or naked siRNA. The lung is composed of the conducting

and respiratory regions in which site variations in both structure and cell composition pose specific regional challenges to siRNA delivery.

The main role of the upper respiratory tract is to filter and conduct air to the lower respiratory segment. As a consequence, its anatomical and cellular features restrict material adsorption that includes naked siRNA or nanoparticle-based delivery systems. The trachea divides into the two primary bronchi at the carina, after which, heavy branching from the lobular bronchi occurs and then onto continuously narrowing tubes to the respiratory segment beginning with the respiratory bronchioles and the alveolar ducts and sacs. Delivery of siRNA to the lungs, whether naked or incorporated in a particle, needs to address the branching of airways and the mucus layer covering the conducting segments. Ciliated cells are abundant in the nasal cavity and trachea, with apical cilia working in coordinated sweeps to transport mucus along with trapped material towards the oesophagus. The constant removal of mucus by the ciliated cells, termed the “mucociliary escalator” plays a critical role in preventing inhaled particulates and pathogens from residing within the trachea and the upper bronchiolar tree. The respiratory mucus consists of an outer luminal layer and an inner layer (termed periciliary liquid) in direct contact with the cilia. Under normal physiological conditions, the luminal mucus layer is refreshed every 10–20 min, whereas renewal of the underlying layer is cleared much slower [38]. The mucus layer is swept away and replenished continually requiring trapped material or nanoparticles to diffuse across a current gradient in order to reach the epithelial surface [17].

Deeper into the lung, the mucus layer diminishes, but the passageways narrow. This restricts the transit of particle-based delivery systems into the alveoli. If administered as an aerosol, inertia determines whether or not particles will impact on the epithelium walls, in which case they will be cleared by the mucociliary escalator. Furthermore, surfactant that covers the deeper regions to prevent collapse of the respiratory sections during exhalation may interfere with particle integrity [57], leaving the siRNA exposed to enzymatic degradation. Alveoli macrophages that compensate for the lack of mucus protection are able to scavenge foreign material by extending processes into the lumen of the alveolus. This could limit the effectiveness of nanoparticle-based RNAi therapeutics; however, subsequent macrophage migration may offer a mechanism for systemic delivery of the nanoparticles.

### 5.3.2 *Naked siRNA Delivery*

There is an ongoing discussion on whether non-formulated naked siRNA is sufficient or a particle formulation is needed for effective pulmonary siRNA delivery. Both approaches have been used (Table 5.1).

Non-formulated siRNA administered by intranasal or intratracheal instillation have been able to mediate a reduction in target gene expression [58–62, 74] or viral titres [63, 65, 76] in mice and non-human primates [64]. In an interesting study from 2005, Bitko et al. demonstrated that naked phosphoprotein-specific anti-RSV siRNA (70 µg single dose) performed near equally as siRNA complexed with the commercial

**Table 5.1** Pulmonary delivery of siRNA

Formulation	Route/animal	Molecular target/model	Effect (dosage)	Ref./year
<i>Naked siRNA</i>				
Naked	Intranasal C57BL/6 mice	PAI-1/bleomycin induced pulmonary fibrosis model	Suppression of PAI-1 resulted in prevention of fibrosis (multiple doses, 2 $\mu$ M in 50 $\mu$ l)	[58]/2010
Naked	Intranasal C57BL/6J mice	HO-1/ischaemia reperfusion injury	Administration of HO-1 siRNA increased apoptosis in lung (1 $\times$ 2 mg/kg)	[59]/2004
Naked	Intratracheal C3H/HeN mice	KC and MIP-2/acute lung injury	~40% reduction of KC and MIP-2 mRNA (1 $\times$ 75 $\mu$ g)	[60]/2005
Naked	Intratracheal C57BL/6 mice	XCL-1/ <i>M. tuberculosis</i>	50% reduction in xcl1 mRNA levels and 40% reduction in protein levels (1 $\times$ 5–15 $\mu$ g)	[61]/2009
Naked	Intratracheal C3H/HeN mice	Fas and caspase-8/acute lung injury	Reduction of Fas and caspase-8 mRNA (1 $\times$ 75 $\mu$ g)	[62]/2005
Naked/Mirus TKO	Intranasal BALB/c mice	Phosphoprotein/RSV	Several log reduction of viral titres (1 $\times$ 70 $\mu$ g)	[63]/2005
Naked	Intranasal BALB/c mice	Nucleocapsid mRNA/RSV	2.5–3 log reduction in RSV lung concentration (single or multiple doses, 40–120 $\mu$ g)	[76]/2009
Naked/Lipofectamine	Intranasal BALB/c mice	Ori and glycoprotein B/EHV-1	Antiviral effect observed (1 $\times$ 62.5 pmol)	[65]/2009
Naked	Intranasal rhesus macaque	SARS Corona virus/replicase, transcriptase and structural proteins	Anti-SARS effect by prophylactic or therapeutic regimens (1 $\times$ 30 $\mu$ g)	[64]/2005
Naked	Nasal spray human	Nucleocapsid mRNA/RSV	38% reduction in experimentally infected patients (150 mg/day for 4 days)	[14]/2010
Naked LNA modified	I.V. C57BL/6-Yg mice	EGFP/EGFP transgenic mice	55% reduction of EGFP (5 $\times$ 50 $\mu$ g)	[66]/2009
<i>Polymers</i>				
Chitosan	Intranasal C57BL/6-Yg mice	EGFP/EGFP transgenic mice	~40% reduction in EGFP expressing bronchoepithelium cells (5 $\times$ 30 $\mu$ g)	[7]/2006



Chitosan	Intratracheal B6;129P2-RAGE tm1.1 mice	EGFP/EGFP transgenic mice	68% silencing of EGFP (3 × 0.26 µg)	[13]/2010
Chitosan/imidazole-PEG modified chitosan	I.V./intranasal BALB/c-C57BL/6 mice	GAPDH	40–50% silencing of GAPDH after I.V. or intranasal administration (1–3 × 0.5–1 mg)	[67]/2010
PEI	Retroorbital injection—C57BL/J mice	Nucleocapsid protein influenza A	10- to 1,000-fold reduction in viral titres (1 × 120 µg)	[68]/2004
Fully deacetylated PEI	Retroorbital injection—C57BL/J mice	Nucleocapsid protein influenza A	94% drop in viral titre (1 × 120 µg)	[69]/2005
PEG-PEI	Intratracheal C57BL/6-Tg (CAG-EGFP) I Osb/J mice	EGFP DsiRNA	42% knockdown of EGFP (1 × 50 µg)	[57]/2009
Fatty acid modified PEG-PEI	Intratracheal C57BL/6J-Tg mice	EGFP	69% knockdown of EGFP (1 × 35 µg)	[70]/2011
<i>Lipid-based vectors</i>				
Oligoectamine/naked siRNA	Intranasal /hydrodynamic injection BALB/cAnNCR mice	Nucleoprotein, acidic polymerase/influenza A	63-fold reduction of viral titres (1 × 50 µg + 20 µg)	[71]/2004
DharmaFECT	Intratracheal C57BL/6 mice	SPARC/bleomycin induced lung fibrosis	58% reduced collagen content in lung (3 × 3 µg)	[72]/2010
GL67	Intranasal BALB/c mice	lacZ/β-galactosidase	33% lower β-galactosidase mRNA levels (1 × 40 µg)	
Cholesterol/cell penetrating peptides	Intratracheal BALB/c mice	p38 MAP kinase	45% knockdown of p38 MAP kinase mRNA, no change in protein levels (1 × 10 nmol)	[73]/2007
AtuPLEX	I.V. C57BL/6N mice	E-cadherin	~50% reduction of VE-cadherin mRNA (4 × 50 µg)	[74]/2010
Infasurf	Intranasal C57BL/6J mice	GAPDH	50–67% lowered lung concentration of GAPDH protein at 24 h and 7 days (1 × 10 µg)	[75]/2004

transfection agent Mirus TKO in a RSV mouse model. In this study, viral titres were reduced several logs after intranasal administration with no adverse or immunostimulatory effects observed [63]. Alvarez et al. likewise, was able to reduce RSV titres (2.5–3 log reduction, 100 µg single dose) using the naked Alnylam siRNA against RSV nucleocapsid gene (ALN-RSV01) after intranasal delivery in mice. By RACE analysis of the ALN-RSV01 cleavage product, it was also confirmed that the reduction of the viral titre was in fact an RNAi-mediated effect [76]. Recently, the results from an Alnylam phase II clinical trial was published showing a 38% reduction of experimentally RSV infected test subjects receiving ALN-RSV01 (150 mg) compared to subjects receiving a placebo [14]. A nasal spray was used to deliver the PBS/ALN-RSV01 solution. As mentioned by the authors of the report, the induced RSV infection in the study resulted in a mild to moderate upper respiratory tract illness in the region the nasal spray is likely to reach. Studies are currently underway to evaluate the effect of ALN-RSV01 in naturally infected patients, and these are likely to use aerosolised delivery methods in order to reach both the upper and lower respiratory segments simultaneously. The success achieved in studies using non-formulated siRNA is unexpected when one considers the polyionic nature of the siRNA molecule that restricts cellular uptake. A possible explanation could be loss of epithelial integrity due to infection that might allow entry of naked siRNA. Nonetheless, at the time of writing, the Alnylam RSV programme is one of the most advanced RNAi clinical trial programmes and the simplistic naked siRNA approach could fulfil the clinical requirement of cost-effectiveness.

Whilst direct administration of naked siRNA to the mucosa has been extensively used, the susceptibility of the duplex to serum nucleases makes intravenous (i.v.) delivery less attractive. Modification of the siRNA backbone, however, is now standard to reduce serum degradation [77]. In a recent study, serum stability and silencing of enhanced green fluorescent protein (EGFP) in the bronchoepithelium of mice have been demonstrated after i.v. administration of naked LNA modified siRNAs [66]. Intravenous injections of naked LNA modified siRNA (five doses of 50 µg siRNA) resulted in comparable reduction of EGFP (55% reduction) in the bronchoepithelium as animals dosed intranasally with chitosan/siRNA particles (single 30 µg dose). Naked modified siRNA was less effective after intranasal dosing. The authors suggest that the success of the naked modified siRNA to reach the lung epithelium after i.v. injection might result from increased serum stability, allowing for longer circulation time compared to unmodified siRNAs.

### 5.3.3 Nanoparticle Delivery

It is generally accepted that nanoparticle-based systems are needed to improve the therapeutic potential of the siRNA despite the success of naked siRNA. The ability to package high levels of siRNA into nanoscale carriers with a predisposition to enter cells has promoted their use. Two prominent classes are polyplexes and lipoplexes formed by self-assembly of polycations or cationic lipids with siRNA

resulting from ionic interaction between cationic amines and siRNA-bearing anionic phosphates [78]. The net positive charge facilitates cellular uptake, and incorporation of mucopenetrative components into the design promotes use for mucosal siRNA delivery applications.

### 5.3.3.1 Polymer-Based Systems

#### Chitosan-Based Systems

The polysaccharide chitosan has been used extensively for the mucosal delivery of drugs. It is a deacetylated derivative of the natural polymer chitin and is composed of randomly distributed repeating units of  $\beta$  (1,4)-*N*-acetyl-D-glucosamine and  $\beta$  (1,4)-D-glucosamine and is non-toxic, biocompatible and biodegradable [79]. The cationic glucosamine component facilitates mucoadhesion, mucopermeation and polyplex formation. It is involved in transient opening of epithelial tight junctions improving paracellular drug transport [80, 81]. Moreover, it adheres to the mucus layer by interaction with sialic acid in mucus glycoproteins that increases viscosity, leading to decreased mucociliary clearance and prolonged residence time [43, 82]. The cationic amine has been utilised for entropy-driven formation of sub-micron particles with polyanionic DNA [83] and siRNA [84]. Chitosan has demonstrated excellent transfection abilities and several *in vivo* studies have revealed the ability of chitosan to enhance respiratory delivery of siRNAs and DNA. A study by Köping-Höggård et al. achieved expression of  $\beta$ -galactosidase after intratracheal delivery of chitosan/DNA polyplexes [85] and another study managed to partly immunise mice from RSV by intranasal application of chitosan/DNA particles coding for RSV epitopes [86].

Chitosan-based nanoparticle delivery of siRNA was first introduced by Howard and co-workers [7]. Parameters such as high molecular weight (~100 kDa) and highly deacetylated (>80%) chitosan at N:P (amine:phosphate ratio) >30 showed improved formation, stability and knockdown *in vitro* [84]. It is proposed that excess chitosan at high N:P ratio may improve mucosal properties. Silencing (~40%) of enhanced green fluorescent protein (EGFP) was observed in the bronchiolar epithelium in transgenic mice after intranasal administration (30  $\mu$ g of siRNA per dose) over 5 consecutive days of the chitosan/siRNA polyplexes [7]. Intranasal administration suffers from particle adsorption in the mucus layers of the nasal cavity and the amount of drug reaching the lung can, therefore, only be estimated.

In a recent study by the same group, the airway deposition of the chitosan/siRNA particle system was improved with an aerosolised formulation using a nebulising catheter (AeroProbe™, Trudell Medical Instruments) inserted directly into the trachea of the mouse. Silencing of the target gene (EGFP) was accomplished with a very low dose of siRNA (three doses of 0.26  $\mu$ g) [13]. The low dose is a significant step towards reduction of potential off-target and immunological side effects [87].

Chitosan has predominately been used for local delivery; however, a recent report showing chitosan/siRNA particles accumulated in the kidneys after *i.v.*

administration [4] suggests circulatory properties. Furthermore, modification of chitosan with an imidazole group and PEG has been used for intravenous delivery of siRNA in mice resulting in a 49% reduction of mRNA levels of GAPDH in the lungs, suggesting that chitosan/copolymers might be useful as an intravenous delivery vector [67].

### PEI-Based Systems

Since the introduction of polyethylenimine (PEI) as a gene transfer reagent in 1995 [99], this cationic polymer has been studied extensively for both DNA and siRNA delivery [100]. Effective polyplex formation, protection from nucleases and endosomolytic properties attributed to its high charge amino density have promoted its use. PEI has been used for systemic delivery of siRNA to a number of tissues including the lungs in mice [68, 69]. Ge et al. and Thomas et al. used PEI/siRNA polyplexes (N:P 5) against the influenza nucleocapsid protein (120 µg single dose) administered by retroorbital injection. The study by Thomas et al. expanded on the previous study by Ge et al., by evaluating the ability of various high molecular weight PEI polymers to enhance the delivery of siRNA and in both studies, a significant reduction of viral titres was observed (10- to 1,000-fold reduction).

The mechanism of antiviral effects from the studies by Thomas and Ge, however, has been brought to question in a landmark paper from Robbins et al. [101]. In this work, several published siRNA sequences, including the nucleocapsid sequence used by Ge et al. and Thomas et al., were tested for an ability to stimulate the innate immune system ascribed to intracellular Toll-like receptor activation. Remarkably, it was shown that the control GFP sequence used in several in vivo studies [60, 68, 69, 71] did not elicit an immune response, whereas the nucleocapsid sequence (among others) stimulated the production of interferon  $\alpha$  suggested to be largely responsible for the observed antiviral effect.

The ability of systemic PEI-based siRNA systems to reach the lungs [102, 103] could result from serum-induced aggregation and its consequent entrapment within the lung capillary beds. This, however, could result in lung embolisms and restricts the likelihood for clinical translation.

PEI, unlike chitosan, is not generally thought to be a mucoadhesive polymer, but it is reasonable to speculate that some amino-mediated interaction with mucins can occur if delivered locally. *Hitherto*, PEI interactions and effects on mucus have not been studied in detail. Two recent studies have demonstrated pulmonary EGFP silencing in transgenic mice. Using intratracheal administration of PEG-PEI/siRNA polyplexes, Merkel et al. showed a 42% reduction of EGFP expression compared to luciferase siRNA control (single 50 µg siRNA dose) [57]. Moderate inflammation was seen by analysis of cytokine levels, but no histological abnormality was observed. Beyerle et al. used a fatty acid modified PEG-PEI/siRNA polyplex to achieve a 69% reduction of EGFP expression compared to untreated controls (35 µg single dose) [70]. As in the previous study by Merkel et al., PEGylation increased inflammation, whilst at the same time also decreased cytotoxicity. These findings seem to contradict the usual perception that PEG limits interaction with the immune system.

Whilst PEGylated PEG-PEI polymers appear less cytotoxic than non-modified PEI [104], they and the fatty acid modified PEI may have a higher proinflammatory potential that is of concern in a clinical setting. Although PEI has been used extensively for several years in animal studies, safety concerns still restrict its use in the clinic.

### 5.3.3.2 Lipid-Based Systems

Lipid vectors have been widely used for *in vitro* and *in vivo* delivery of siRNA [105], most based on cationic lipids that form lipoplexes with siRNA. Mirus TKO, a cationic lipid/polymer formulation, has been used by Bitko et al. to deliver siRNA against RSV [63]. 70 µg siRNA delivered intranasally with Mirus TKO was able to reduce viral titres in mice without inducing an interferon response, an effect shown to be a 20–30% improvement on naked siRNA. In a mouse influenza model, the animals received hydrodynamic injections (3.78 nmol) of naked siRNA followed 16–24 h later by intranasal delivered oligofectamine/siRNA (1.51 nmol) complexes against the viral nucleoprotein and acidic polymerase to reduce viral titres in the lung (63-fold compared to EGFP siRNA) [71]. Whilst interferon levels were investigated and found not to be upregulated, concerns remain for the use of the EGFP sequence as a negative control due to its non-stimulatory uniqueness [101]. A third commercial lipid-based transfection reagent, DharmaFECT, has been used as a pulmonary delivery vector in a bleomycin-induced lung fibrosis mouse model [72]. siRNA against SPARC, a matricellular protein overexpressed in fibrotic diseases, markedly reduced collagen content in the lung (58%) compared to the bleomycin-only group after intratracheal dosing (3 × 3 µg siRNA).

The cationic lipid from Genzyme, GL67, has been used in k18-lacZ mice which express β-galactosidase in airway epithelial cells [106]. A 33% reduction of mRNA level (but no change in protein levels) was observed after intranasal administration of lacZ siRNA (40 µg siRNA). Histological analysis showed that the GL67/siRNA lipoplexes were mainly associated with pulmonary macrophages which could explain the lack of change in protein levels.

Direct conjugation of siRNA to cholesterol has been explored by Moschos et al. [73]. A single intratracheal administration of siRNA–cholesterol conjugates (10 nmol) facilitated a 45% knockdown of p38 MAP kinase mRNA in mouse lungs after 12 h compared to vehicle-only controls. The effect appeared to be transient as the detected mRNA levels were almost back to normal after 24 h which was attributed to poor stability of the siRNA. It was suggested that chemical modification of the backbone might increase the silencing effect.

The respiratory vasculature in mice can be targeted by systemic delivery of cationic lipoplexes (AtuPLEX) [74]. A ~50% reduction of the endothelial cell-specific protein VE-cadherin was achieved in the lungs after intravenous injection of 50 µg of siRNA on 4 consecutive days compared to a luciferase-specific siRNA. The lipoplexes were also administered intratracheally, but only a 21% reduction of epithelial

E-cadherin was observed compared to a luciferase control, suggesting better suitability for systemic delivery. Capture within the lung microvasculature and subsequent endothelial uptake were proposed by the authors as the mechanism of delivery.

### **5.3.4 Aerosolised Formulations**

It is anticipated that clinical translation will require inhalation technology based on aerosols of dry powder formulation or solutions. Aerosols are by definition a gaseous suspension of fine solid particles or liquid droplets. The size and weight of these particles or droplets determine their ability to follow the flow of inhaled air through the airways.

The main parameter for linking particle or droplet size and weight in regard to lung deposition is the aerodynamic diameter. This parameter takes into account shape, roughness and porosity of the particles or droplets in an aerosol. The aerodynamic diameter is the diameter of a unit density ( $1 \text{ g/cm}^3$ ) sphere having the same gravitational settling velocity as the particle being investigated. The mass median aerodynamic diameter (MMAD) is the diameter at which 50% of the particle/droplet distribution by mass will have a larger or smaller diameter. In other words, if deposition at a specific airway depth is required and is achieved at a given aerodynamic diameter (e.g.  $5 \text{ }\mu\text{m}$ ), then if the MMAD of an aerosol is  $5 \text{ }\mu\text{m}$ , then 50% of the total aerosol mass will in principle deposit above the selected depth and 50% will deposit below. This restricts nanoparticle diameter to a narrow size distribution if deposition at a certain depth is required.

Investigation of MMADs of aerosols is typically carried out on cascade impactors mimicking different airway depths. Particles with an aerodynamic diameter between 1 and  $5 \text{ }\mu\text{m}$  are likely to reach the pulmonary regions, whereas larger particles will be deposited in the upper airways [55]. However, if particles become too small, they are prone to being exhaled before depositing. This means solid nanoparticles, per definition, are in principle too small to be effectively deposited in the lungs, and a large portion of these particles may end up leaving the lung again after inhalation. There are two solutions to this problem. Either the particles are kept in solution or they are attached to a carrier formulation which will facilitate deposition at the required depth. Nanoparticles such as those consisting of polymers and siRNAs are formed in solution, but subsequent drying by either spray drying [107] or freeze drying [108] can produce particles retaining their silencing ability which in terms of storage and stability of a therapeutic agent might be preferable compared to a solution-based formulation.

Intratracheal administration in animal models has provided preclinical evaluation of aerosolised siRNA formulations and is more cost-effective than inhalation chambers. Nebulisers developed specifically for delivery of aerosols to animals such as the “AeroProbe” from Trudell Medical and the “Microsprayer” from PennCentury are examples of devices used for particle delivery directly to the

mucosal surfaces of the respiratory tract. These devices overcome the difficult nature of the mouse breathing pattern and anatomy [109] and allow dose-response studies to be conducted.

Substantial clinical evaluation of dry powder-based siRNA formulations is lacking, although Alnylam (<http://www.alnylam.com>, 2011) has used a handheld battery-driven nebulising system (<http://www.paripharma.com>, 2010) in their current phase II RSV clinical trial with naked siRNAs. The promise of nanotechnology and the advances with surface and particle engineering combined with recent advances in inhaler technology hold promise for future inhalable siRNA-based particles.

## 5.4 Oral Delivery

Oral administration of therapeutics is considered the most favourable in terms of cost-effectiveness, ease of administration and patient compliance. This route potentially provides rapid systemic distribution of the drug [110] due to the enormous adsorptive surface area (~200 m<sup>2</sup>). Utilisation of this route depends on overcoming the challenges of enzymatic degradation, mucus and epithelial penetration. Oral administration of RNAi-based drugs offers great potential for both the treatment of diseases occurring locally within the gastrointestinal (GI) tract, such as inflammatory bowel disease (IBD), and to combat systemic pathologic conditions.

### 5.4.1 Considerations for Oral Delivery of siRNA

The GI tract possesses a specialised epithelium involved in the degradation of macromolecules and assimilation of the obtained products while restricting the transport of pathogens. Unfortunately, these processes often compromise the integrity and absorption of therapeutics. In this respect, exposure to a highly active enzymatic environment, extreme pH conditions and the existence of a selective-permeability epithelial barrier are the main challenges for oral delivery of RNAi therapeutics. Nucleases, highly abundant in pancreatic secretions, constitute the main enzymatic barrier to nucleic acids. Moreover, the delivery system itself may be susceptible to degradation by other enzymes present in the lumen (e.g. lipases, glycosidases or proteases) or the microvillus (e.g. P450).

pH extremes along the GI tract ranging from 1 to 2 in the stomach to >7 at the terminal part of the small intestine and colon may affect acid- or base-labile components of the delivery system, although increased stability of nucleic acids under these pH conditions can be achieved by chemical modifications [111, 112] or incorporation into delivery systems. Exploitation of the localised pH conditions could offer an exciting strategy for site-specific release of siRNA using pH-sensitive carriers. Luminal pH determines the drug's ionisation degree that affects transcellular passive diffusion and/or the interactions between the formulation components [84].



Since the GI tract epithelium is covered by mucus, drugs must diffuse through this lubricant and protective layer in order to reach the absorptive surface. Therefore, the uptake of a therapeutic compound will depend upon the interactions of the drugs with the mucus components as well as the thickness of this layer, which in experimental animal models has been shown to vary along the gastrointestinal tract [113, 114].

Whilst paracellular transport across the GI epithelium is limited to ions and small hydrophilic molecules that can diffuse across tight junctions, the hydrophobic nature of the cell membranes impede the diffusion of most polar and charged molecules [110, 115]. Consequently, macromolecular siRNA absorption across the epithelia is restricted, although binding of specific ligands may facilitate uptake as demonstrated in different cell types [6, 116–118]. The capability to attach different chemical components by simple nucleic acid chemistry could promote this approach.

Nanoparticle-based carriers are taken up by adsorptive or receptor-mediated endocytosis across enterocytes dependent on surface moieties. The level of uptake is thought to be low, although penetration enhancers may potentiate paracellular delivery. An important consideration is delivery and breakdown in the liver due to the first-pass effect commonly encountered by absorbed drugs. An alternative route through the gut-associated lymphoid tissue (GALT) has been exploited for the delivery of micro and nanoparticles [119–122]. The overlying follicle-associated epithelium (FAE) contains specialised cells termed M-cells [115] that are anatomically designed to sample luminal particles as part of the mucosal immune response. The lymphoid follicle domes are highly populated with macrophages, which have been shown to capture material. Systemic dissemination of these macrophages has been proposed as a mechanism of transport to peripheral tissue. Although particle transport through M-cells may be augmented by increasing particle-surface hydrophobicity or attachment of specific targeting ligands [123, 124], it is important to bear in mind when utilising this route for intestinal absorption that GALT only constitutes a small fraction of the GI tract, with the numbers decreasing with age. Recent attention has focused on transport across the epithelial barrier directly mediated by dendritic cells [125]. These phagocytic cells, widespread throughout the epithelia, have been shown to disrupt tight junction and sample luminal content through the projection of dendrites, providing an exciting opportunity for the design of oral vaccines.

Inter-species differences exist between humans and the animal models commonly used for the *in vivo* evaluation of oral drug administration. For example in contrast to humans, mice and rats exhibit a less acidic stomach pH (~4 vs. ~1.7) and lower mean intestinal pH [126]. These are important considerations when assessing clinical translation.

### 5.4.2 Oral Studies

A number of studies have used the oral route for siRNA delivery (Table 5.2). A high profile study was reported in 2009 by Aouadi et al. [88]. In this study, porous  $\beta$ -1, 3-D-glucan shells were loaded with siRNA targeting expression of tumour necrosis factor-alpha (TNF- $\alpha$ ) or mitogen-activated protein kinase 4 (Map4k4) in mice. The



**Table 5.2** Oral, rectal and intravaginal delivery of siRNA

Formulation	Route/animal	Molecular target/model	Effect (dosage)	Ref./year
$\beta$ -1, 3-D-glucan shells	Oral C57BL/6J mice	TNF- $\alpha$ , Map4k4 Untreated animals and LPS lethality test	Knock-down of target genes. Protection from LPS-induced lethality (~0.4 $\mu$ g/dose for 8 consecutive days)	[88]/2009
Thioketal nanoparticles	Oral C57BL/6 mice	TNF- $\alpha$ DSS-induced colitis	TNF- $\alpha$ Knock-down. Protection from colitis (~46 $\mu$ g/dose or 4.6 $\mu$ g/dose for 5 consecutive days)	[89]/2010
NIMOS	Oral Balb/c mice	TNF- $\alpha$ DSS-induced colitis	TNF- $\alpha$ knock-down. Milder colitis symptoms (~24 $\mu$ g/dose for 3 alternated days)	[90]/2011
Stabilised unilamellar vesicles $\beta$ 7 integrin-targeted	Intravenous C57BL/6 mice	CyD1 DSS-induced colitis	CyD1 knock-down. Alleviated colitis symptoms (~50 $\mu$ g/dose for 4 alternated days)	[91]/2008
Lipoplex (Lipofectamine)	Rectal C57BL/6 mice	TNF- $\alpha$ DSS-induced colitis	TNF- $\alpha$ knock-down in descending colon. Mild or moderate inflammation (~53 $\mu$ g/dose for 2 alternated days)	[92]/2006
Lipoplex (DOTAP) chemical modified siRNA	Rectal Swiss-Webster mice	None detection of fluorescent-labelled siRNA	Uptake in spleen, colon and bone marrow (single dose of ~20 $\mu$ g)	[93]/2007
Lipoplex (Oligofectamine)	Intravaginal Balb/c mice	UL27 & UL29 viral genes HSV-2 lethal challenge	Protection from HSV-2 lethal infection (two doses of ~7 $\mu$ g)	[94]/2006
Naked (cholesterol conjugated chemical stabilised siRNA)	Intravaginal Balb/c mice	Viral (UL29) & host (nectin-1) genes HSV-2 lethal challenge	Protection from HSV-2 lethal infection (~27 $\mu$ g/dose for 2 consecutive days)	[95]/2009
PLGA nanoparticles	Intravaginal FVB Cg-Tg (GFPU)5 Nagy mice	GFP transgenic GFP mice	GFP gene silencing throughout reproductive tract (single dose of ~7 $\mu$ g)	[96]/2009
Lipoplex (Lipofectamine)	Intravaginal C57BL/6 mice	Lamin A/C, CCR5	Knock-down of target genes (single dose of ~53 $\mu$ g)	[92]/2006
PEGylated Lipoplexes entrapped in alginate scaffold	Intravaginal C57BL/6 and K14E7 mice	Lamin A/C	Lamin A/C knock-down (two doses of ~8 $\mu$ g)	[97]/2011
Naked (CD4 aptamer-siRNA chimera)	Intravaginal NOD/SCID-BLT & NSG-BLT mice	Viral ( <i>gag, vif</i> ) and host ( <i>CCR5</i> ) genes Humanised mice	Protection against HIV vaginal transmission (~4 $\mu$ g in complex dosing regimen)	[98]/2011

internal element of the 2–4  $\mu\text{m}$  particles contained a tRNA-core coated with consecutive layers of PEI and siRNA. Daily particle administration ( $\sim 0.4 \mu\text{g}$  siRNA/dose) by oral gavage over an 8-day period resulted in reduced mRNA levels of Map4k4 ( $\sim 60\text{--}70\%$ ) or TNF- $\alpha$  ( $\sim 40\text{--}60\%$ ) in peritoneal exudate cells (PECs) compared to animals receiving scrambled-siRNA containing particles. Map4k4 down-regulation elicited a concomitant reduction in TNF- $\alpha$  expression that suggests a role for Map4k4-mediated control of TNF- $\alpha$ . In addition extended knockdown duration of  $\sim 8$  days was observed after the final dose. Interestingly, the authors proposed that siRNA release from the glucan shell was triggered by the acidic environment in phagosomes; this, however, could compromise particle integrity at low pH within the GI tract. Notably, no unspecific interferon- $\gamma$  response was detected even though non-modified siRNA was used. Map4k4 and TNF- $\alpha$  silencing in macrophage-enriched cells isolated from spleen, liver and lung tissues was observed ascribed to particle uptake across GALT and subsequent dissemination in migrating macrophages. No direct evidence for M-cell uptake or adsorption levels was provided; however, this study does suggest the possibility for systemic silencing via the oral route.

In certain pathologies, such as IBD, a localised rather than systemic effect is more desirable. IBD encompass a group of complex autoimmune diseases, which are broadly categorised as Crohn's disease or ulcerative colitis found in the small intestine and colon respectively. An attractive pathological condition that could be exploited for improved siRNA-based therapeutic delivery is mucosa integrity loss in IBD. Despite the numerous targets investigated that includes IL-12, IL-23, IL-17 and IL6 [127], the current biologic treatment of IBD is based on anti-TNF- $\alpha$  molecules [128]. TNF- $\alpha$  has been the preferred target for oral-based siRNA therapies in two recent studies employing orally delivered siRNA for the prevention/treatment of dextran sodium sulphate (DSS)-induced ulcerative colitis in mice. In the first of these studies, Wilson et al. [89] used thioketal (poly-1, 4-phenyleneacetone dimethylene thioketal) nanoparticles (TKNs) designed for triggered anti-TNF- $\alpha$  siRNA release in response to raised levels of reactive oxygen species (ROS) common to inflamed regions. A tenfold specific decrease in colonic mRNA levels of TNF- $\alpha$  and other proinflammatory cytokines (IFN- $\gamma$ , IL-6 and IL-1) was detected after oral administration of anti-TNF- $\alpha$  TKNs ( $\sim 46 \mu\text{g}$  siRNA/dose) over 5 consecutive days during colitis induction. Furthermore, the authors also demonstrated by histological and weight analysis that a ten times lower siRNA dose ( $\sim 4.6 \mu\text{g}$  siRNA/dose) was sufficient to protect mice from DSS-induced colitis.

An alternative approach for site-specific delivery was reported by Kriegel et al. [90]. The nanoparticles-in-microsphere oral system (NiMOS) is based on lipase-mediated intestinal degradation of poly epsilon-caprolactone microspheres for triggered release of gelatin nanoparticles containing TNF- $\alpha$ -specific siRNA. Administration by oral gavage at days 2, 4 and 6 after DSS treatment of anti-TNF- $\alpha$  siRNA-loaded NiMOS ( $\sim 24 \mu\text{g}$  siRNA/dose) resulted in reduced intestinal mRNA and protein levels compared with controls. In addition, ELISA showed decreased levels of several proinflammatory cytokines (IFN- $\gamma$ , IL-1b, IL-2, IL-5, IL-6 and

IL-12p70); however, some non-specific silencing intrinsic to the formulation was observed. Colitis protection (moderate intestinal inflammation and healthy colon morphology) was only evident in the anti-TNF- $\alpha$  siRNA-treated mice.

An alternative strategy based on a systemic RNAi-based IBD treatment was revealed by the elegant study by Peer et al. [91]. In this study, i.v. administration of particles targeting a specific leucocyte subset ( $\beta$ 7 integrin expressing gut mononuclear leucocytes) was utilised for the treatment of DSS-induced colitis. The design of the system, termed  $\beta$ 7-I-tsNPs, has a protamine/siRNA core complex coated within a unilamellar vesicle decorated with an anti-integrin  $\beta$ -7 antibody. Administration of CyD1-specific siRNA (~50  $\mu$ g/dose)  $\beta$ 7-I-tsNPs at days 0, 2, 4 and 6 resulted in reduced intestinal mRNA levels of this cell cycle regulatory molecule and simultaneous mRNA reduction of the proinflammatory cytokines TNF- $\alpha$  and IL-12. This resulted in significantly less severe lesions at the intestinal tissue and the reversal of clinical and pathological characteristics associated with the onset of the DSS-induced colitis. The observed local effect may be attributed to the CyD1 silencing of peripheral blood and spleen leucocytes prior their recruitment to the inflamed gut.

We are currently evaluating the potential of siRNA nanoparticles formulated with the non-toxic, biodegradable and mucoadhesive polymer chitosan for the reduction of proinflammatory cytokines after oral administration. Encouraging results have been recently obtained in animal experiments, suggesting strong nuclease-protection and high gastrointestinal siRNA deposition provided by this system (unpublished results).

## 5.5 Rectal Delivery

Rectal administration is an attractive route for siRNA delivery as it circumvents the low stomach pH, is an established route for traditional drugs and the colon presents a low enzymatic milieu. In addition, direct access to the site of several diseases such as colorectal carcinoma or ulcerative colitis further promotes this route.

Zhang et al. [92] demonstrated that rectal administration of lipoplexes containing anti-TNF- $\alpha$  siRNA (two doses of ~53  $\mu$ g siRNA) significantly reduced the upregulation of TNF- $\alpha$  mRNA in a DSS-induced ulcerative colitis mouse model. Reduced perirectal TNF- $\alpha$  mRNA levels were associated with mild or moderated inflammation at the mucosa of the descending colon compared to the severe inflammation observed in the controls. Interestingly, despite toxicity previously reported with similar liposomal formulations, no increase in proinflammatory cytokines (IL-1, IL-10, TNF- $\alpha$ ) or interferon responses was found. In a later study [93], fluorescent and chemically modified siRNA contained within DOTAP liposomes was detected in the spleen, bone marrow, colon and liver after rectal administration in mice. This supports the capability for nanoparticles to migrate into the systemic circulation that could be exploited for both local and systemic gene silencing.

## 5.6 Intravaginal Delivery

Advantages such as an established therapeutic route, marketed products for sustained drug release, low enzymatic activity and possible avoidance of the first-pass effect [129] promote vaginal administration of siRNA. Poor systemic absorption of polar high molecular weight molecules across the epithelium, however, seemingly restricts siRNA-based therapies to local vaginal treatments.

### 5.6.1 Vaginal Studies

A number of studies have utilised acute infection of mice with herpes simplex virus 2 (HSV-2) as a model for the development of antiviral siRNA-based therapeutics. In this setting, the capacity of the treatment to inhibit viral spread across the genital mucosa after HSV-2 challenge is evaluated. In 2006, a study by Palliser et al. [94] assessed the protection provided by lipid-complexed siRNA (~7 µg/dose) targeting essential HSV-2 viral genes. Two siRNA (UL27.2 and UL29.2) conferred significant protection with considerable reduction in the lethality and severity of the lesions, when administrated in a double regime 2 h prior and 4 h after an otherwise lethal HSV-2 vaginal challenge. The protection was, however, transient and a post-exposure treatment (3 and 6 h after the viral challenge) was only effective when both siRNA were administrated in combination but not individually. No inflammatory response or interferon induction was detected by histological and expression analysis respectively in this study. More exhaustive follow-up studies have revealed, however, several undesirable features and toxic side effects related to lipid formulations [95, 96].

A chemically modified siRNA approach has been also used for the treatment of HSV-2 [95]. The cholesterol (Chol)-siRNA conjugate, stabilised with phosphorothioate residues, was used to knockdown viral and host gene expression. Consistent with previous results [94], targeting an essential viral gene (UL29) exclusively conferred protection when the siRNA was administrated within a few hours of the viral challenge. Interestingly, this protection could not be replicated if a too high siRNA dose (~135 µg) was employed, a matter that requires further investigation. In contrast, targeting of nectin-1, the receptor used by HSV-2 to penetrate in the cells, conferred protection only when administrated 1–7 days prior, but not immediately before or after the HSV-2 challenge. Treatment of the mice with two doses (~27 µg/dose) of Chol-siRNA combining the targeting of nectin-1 and viral genes provided significant protection for 1 week irrespective of the time of challenge.

Woodrow et al. [96] developed a delivery system based on a siRNA/polyamine (spermidine) core encapsulated into PLGA nanoparticles. A single dose (~7 µg) of these particles induced sustained GFP mRNA silencing throughout the female

reproductive tract for at least 14 days in a transgenic GFP mouse model. Reduction of fluorescence was maximal at day 10 in the vaginal tract, with only 30–40% of the siRNA dose (~2.8 µg) released due to the slow degradation rate of the nanoparticles.

In the aforementioned studies, thorough cleaning of the vaginal tract and/or progesterone treatment of the animals prior to particle administration was performed [94–96]. Whilst mucus removal eliminates one of the main barriers for vaginal epithelial transfection, the hormone treatment arrests the oestrous cycle in the dioestrus phase in which the epithelium is thin and porous that most probably contributed to higher drug absorption [130]. In addition this treatment has been associated with a reduced immune response in the vagina, which may mask potential undesirable side effects of the evaluated drugs.

In a model more closely resembling the normal physiological conditions, Zhang et al. [92] reported liposome-mediated transfection of the squamous epithelia layer and submucosa. A single dose of siRNA (~53 µg siRNA) was sufficient to induce a significant and consistent knockdown of the targeted gene (lamin A/C or CCR5) over a 7-day period. Analysis of proinflammatory cytokines (IL-1, IL-10, TNF-α) and interferon-related genes did not detect any significant changes in the treated animals compared to controls.

In contrast, Wu et al. [97] suggested that vaginal epithelium transfection in physiological conditions with conventional lipoplexes was unlikely, most probably due to the combination of poor drug retention at the vaginal cavity and an inefficient transport across the mucus layer. In order to overcome these limitations and achieve sustained release of the entrapped therapeutic, the authors developed and characterised a system based on a biodegradable alginate scaffold. Upon exposure to sodium ions, a common element of cells and body fluids, scaffold degradation occurs, resulting in the slow release of incorporated PEGylated lipoplexes. PEGylated, but not conventional, liposomes were capable of mucosal diffusion and induce siRNA-mediated gene knockdown at the vaginal epithelium. Intra-vaginal administration of the scaffold over 2 consecutive days (daily dose of 8 µg/animal) resulted in an 85% knockdown of lamin A/C mRNA. In this report, evaluation of proinflammatory cytokine levels and unspecific interferon activation was not reported.

Encouraging results have been recently reported by Wheeler et al. [98], who by targeting viral (*gag* and *vif*) and host genes (*CCR5*) could inhibit HIV vaginal transmission in a humanised mouse model. Macrophage and CD4+ T-cell-specific targeting was achieved by the fusion of the siRNAs to a CD4 receptor-specific aptamer. The observed protection is probably due to the combination of selective gene knockdown by the siRNAs (*CCR5*, *gag* and *vif*) and a viral–aptamer competition for CD4 receptor binding. Despite the apparent absence of cellular toxicity or lymphocyte activation, caution should be taken with molecules interacting with the CD4 receptor due to its role in the host immune response and susceptibility for HIV infections in activated T lymphocytes.

## 5.7 Future Perspectives

Mucosal delivery of RNAi therapeutics is an exciting approach that is set to progress rapidly building on encouraging clinical studies. The relative ease of access to surfaces common to pathogen, cancer and inflammatory disease promotes their use. Local delivery avoids the necessity to install “stealth” characteristics required for systemic delivery that reduces the complexity of design that has manufacturing, cost and clinical approval benefits. The restricted entry, however, encountered by macromolecules across mucosal barriers still requires delivery strategies to improve penetration. In this context, nanoparticles rather than naked forms seem the most promising. Research to identify surface characteristics that promote mucus penetration including hydrophilic coats is set to continue, whilst coatings that mimic pathogens evolved to penetrate the mucosa is an interesting approach. Detailed studies of nanoparticle penetration in mucus and changes in the mucus morphology in response to mucopenetrative materials are a future trend. Variations in mucus characteristics at different sites and disease states are an important consideration in the design of the delivery strategy. The polyplex systems composed of siRNA and cationic polymers such as chitosan could proceed rapidly into clinical trials due to their simplistic design and mucoadhesive and mucopermeable properties. A current trend is to identify new biopolymers to improve mucosal delivery and expand the selection of available materials.

Recent attention has been directed towards oral formulations focused on treatment of inflammatory diseases of the gastrointestinal tract. Anti-inflammatory effects in IBD preclinical models using particle formulations suggest that IBD will be a primary candidate for clinical translation. The development of bioresponsive particles or coatings composed of pH-sensitive materials, employed for other drug types, will offer the possibility for localised site-specific delivery utilising the differing pH found throughout the GI tract. The necessity for particle disassembly needed for siRNA incorporation into the cellular RNAi machinery calls for intracellular release mechanisms [131, 132]. Reducible disulphide links that are cleaved in the cytoplasm is a strategy, but cost may preclude clinical translation. In contrast to the necessity for stable particles in the circulatory environment, mucosal delivery allows the use of less stable systems that could facilitate siRNA release.

The ability of nanoparticles to translocate the mucosa and enter systemic circulation is set to be exploited to elicit local and systemic silencing effects often needed to match pathogenesis. This, however, will require further modifications to avoid serum-induced aggregation and hepatic clearance. The success of this approach will depend on the technologies currently pursued for systemic nanoparticle delivery. To this end, improving nanoparticle delivery across lymphoid tissue as a route for systemic delivery is set to continue with identification of new targeting approaches running in parallel.

In addition to improved delivery systems, siRNA design is an important consideration relevant to all routes of administration. Some of the initial siRNA-mediated antiviral effects were seemingly attributed to non-specific induction of innate

immune responses due to Toll-like receptor (TLR) engagement. Proinflammatory responses are highly detrimental, particularly in inflammatory disorders. The endocytic pathway undertaken by particles can increase delivery into a TLR-rich environment that can inadvertently potentiate the response. Fortunately, these TLR-dependent (through TLR-3, -7 and -8 signalling) or independent (through RIG-1 and PKR activation) adverse side effects can be avoided by siRNA structure and sequence modification such as 2'-*O*-methyl substitutions. Induction of non-specific immune responses is particularly pertinent in the mucosal immune system rich in immunocompetent sites evolved to recognise and protect against foreign luminal material. Evaluation of immune responses to both siRNA and carrier needs to be adequately addressed going forward.

In the foreseeable future, clinical trials are set to seemingly follow the lead towards treatment of pulmonary diseases such as RSV infection. Established pulmonary delivery technologies used for traditional inhalable drugs should allow rapid clinical translation. Identification of novel targets will push the field forward. An interesting approach is targeting host factors required for viral replication such as influenza rather than viral-specific targets [56].

There is a general shift in the RNAi field from conventional siRNA to miRNA-based agents that is set to follow for mucosal RNAi therapeutics. Deep sequencing technologies are set to be used for rapid identification of mucosal miRNA targets.

Mucosal delivery holds many advantages over the systemic approach and is now showing promise for delivery of siRNA that could lead to rapid clinical translation of mucosal-based RNAi therapeutics.

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