

Potential mucus rheological consequences of off target siRNA loaded nanoparticle deposition in the lungs

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ABSTRACT

The gas exchange surfaces of the lungs is an attractive target for local drug delivery because this area represents the pathological site for a number of lung diseases, and for systemic drug delivery as it is a non-invasive route avoiding the enzyme rich GI tract. Nucleic acid-based drugs, *e.g.* RNA interference (RNAi) therapeutics like small interfering RNA (siRNA), are promising next-generation biopharmaceuticals due to their unprecedented specificity and potency. For non-invasive administration routes such as the intrapulmonary route, drugs in this class may be loaded into delivery systems to enhance intracellular delivery and to protect the cargo against enzymatic degradation. The deposition target for these formulations may be the deep lung, but deposition in the conducting airways must also be considered during development of inhalable dosage forms. Potential consequences of deposition in the upper airways is that the nanoformulations are cleared before absorption and/or may interfere with the rheological properties of mucus, eventually resulting in altered physiological mucociliary clearance parameters.

The purpose of this study was to investigate whether different nanoformulation had different propensities

to alter mucus rheology. We compared the effect of three different nanoformulations loaded with the same siRNA cargo on the rheological properties of a model mucus matrix. We found that the presence of nanoformulations or naked siRNA increased the elastic modulus of the mucus matrix, but the phase angle, which determines the functional properties of mucus, remained essentially unchanged. There were no statistically significant difference between the nanocarriers tested in this study. This suggests that potential mucus rheological consequences of off target deposition need not be a major consideration when selecting siRNA carrying nanoformulations.

INTRODUCTION

Biopharmaceuticals are inherently large and biologically unstable and pose challenges for non-invasive drug delivery. Both the labile nature and unfavourable physicochemical properties (size, hydrophilicity) of biopharmaceuticals, *e.g.*, RNAi-based therapeutics, have driven the development of nanoformulations to protect the drug cargo and improve intracellular delivery to the drug's biological target¹. The gas exchange surfaces of the lungs are an attractive target for both local and systemic delivery of such drugs, because of their

relative accessibility, rich blood supply, large surface area and relatively low levels of enzymatic activity². Nevertheless, formulations of biopharmaceuticals have to pass through the conducting airways, if the target is located in the alveoli.

The conducting airways (trachea, bronchi and bronchioles) are cilia lined and have a continuous mucus secretion. Inhaled particles are trapped in the sticky mucus and, along with the mucus, are removed from the airways by the beating cilia in a process known as mucociliary clearance, which plays a central role in keeping the delicate gas exchange surface of the alveoli free of particulate matter. Turbulent airflow through the airways increases the chances of inhaled particulate matter colliding with the mucus surface of the airway walls and thus being removed from the lungs. The rheological properties of the mucus are important for effective mucociliary clearance³. Although the mucociliary clearance system has evolved to entrap and remove inhaled particles, it has also been shown that nano-sized particles can affect the rheology of mucus secretions^{4,5}, which suggests that inhaled nanoparticles may potentially alter the effectiveness of the mucociliary clearance system.

The aerodynamic size of inhaled particles is a major determinant for their deposition pattern in the respiratory system. The majority of nanoparticles will be exhaled without deposition. To achieve alveolar deposition, nanoformulations of biopharmaceuticals must be embedded within microscale particles, either as nebulised aqueous micro-droplets, or as a solid micron-sized formulation⁶.

In this study we considered a potential nebulised delivery of an siRNA drug to the lung and investigated whether the nature of the nanoformulation is important for determining the degree of rheological changes induced in mucus upon deposition in the airways. Two different architectures of poly(lactide-co-glycolide) acid (PLGA)

nanoparticle formulations loaded with an siRNA directed against TNF α were investigated. The first was composed of a PLGA core with a positively charged chitosan coating⁷, and the second was lipidoid-PLGA hybrid nanoparticles (LPNs)⁸. For reference purposes, positively charged cell-penetrating peptides (the so-called Pepfects)⁹ were tested. The first two carry siRNA on their surface, whereas the Pepfects form nanoscale complexes upon mixing with the negatively charged siRNA. The sequence of the siRNA used was as follows: Sense strand 5'-pGUC UCA GCC UCU UCU CAU UCC UGct-3', antisense strand 5'-AGC AGG AAU GAG AAG AGG CUG AGA CAU-3', where lower case letters represent deoxyribonucleotides, underlined capital letters represent 2'-O-methylribonucleotides and p represents a phosphate residue.

We assumed that the nanoformulations will be administered as nebulised aqueous micro-droplets with a final mucus:droplet ratio of 7.5:1 (w:w), which is high but not unachievable. The amount of nanoformulation within the aqueous suspension was normalised to the amount of loaded therapeutic siRNA to allow a relevant comparison between the formulations.

METHODS

Sample preparation

A large batch of mucus substrate was prepared from gastric mucus obtained from pigs at slaughter. In contrast to lung mucus, this type of mucus is available in large quantities. In addition, it has both biochemical and rheological similarities to tracheobronchial mucus and most importantly allows the study to be carried out on a mucus substrate with a normal, intact mucin polymeric matrix. *Ex vivo* pig gastric mucus was suspended in 10x volume of 0.9% saline with vigorous magnetic stirring for 2 hours at 4°C, followed by

centrifugation at 8000 g for 1 hour. Particulate matter from the base of the pellet and the supernatant were discarded and the procedure repeated. The resultant mucus material was relatively free from particulate debris and homogenous allowing direct rheological comparisons between samples. Mucus was stored frozen in sealed aliquots of 1.5g.

The day before rheological experiments were to be carried out, an aliquot of mucus was thawed, 200 μ l of either 0.9% saline (control), 8 μ g siRNA in 0.9% saline or a suspension of nanocarrier (see introduction) loaded with 8 μ g (0.4nmol) siRNA in 0.9% saline, was added. The mucus was stirred thoroughly, and the treated mucus was stored in an airtight container overnight at 4°C. Mucus aliquots from the same batch were used for all experiments.

Rheological testing

For each treated mucus sample, rheological studies were performed in triplicate. Each run required 0.3 g mucus, and all samples were taken from the same aliquot so variations in nanoparticle distribution within the mucus should be reflected within the variability of the results.

A Malvern Kinexus rheometer fitted with a CP 40-1 cone plate geometry was used in all experiments. An amount of 0.3 g mucus was loaded on to the rheometer and subjected to the following test protocols:-

- Isothermal temperature (25°C, 10 min);
- single frequency oscillation (1Hz, 1% strain, 100 samples, interval: 5 s);
- isothermal temperature (25°C, 10 min); frequency sweep (0.01 Hz to 10 Hz, 1% Strain, 10 samples per decade, logarithmic sampling);
- isothermal temperature (25°C, 10 min);
- relaxation (target strain: 5%, rise time: 0.001 s, 10 samples per decade, maximum time: 30 min);
- isothermal temperature (25°C, 10 min);

- strain sweep (1Hz, 0.01-100% Strain, 10 samples pr decade, logarithmic sampling).

RESULTS

During the single frequency oscillation, all samples showed apparent equilibrium behaviour (not shown). All treated mucus samples showed a rheological behaviour profile typical of mucus with the frequency sweep showing $G' > G''$ across the frequency range and a slight frequency dependence of the moduli (not shown). Relaxation measurements were unreliable due to apparent slip effects, and they were therefore not considered further. The strain tolerance of the treated mucus samples was similar to the control mucus, both in terms of linear viscoelastic region and the stress strain relationship, and all samples maintained their elastic dominant properties at 100% strain (not shown).

Compared to the mucus control with only saline added, siRNA alone and all siRNA-loaded nanoparticle formulations increased G' and G'' across the frequency range to between 110 and 140% of the control value (figs. 1 and 2). There were no significant differences in G' with the addition of siRNA-loaded nanoparticles when compared to the addition of naked siRNA (ANOVA with Dunnett's multiple comparisons test, GraphPad Prism).

Whilst the moduli values were increased by the presence of siRNA or siRNA-loaded nanoformulations, the phase angle remained relatively constant in all cases (figs. 3 and 4) with no significant difference to the control or between siRNA without and with nanoformulations by ANOVA with Dunnett's multiple comparisons test (GraphPad Prism).

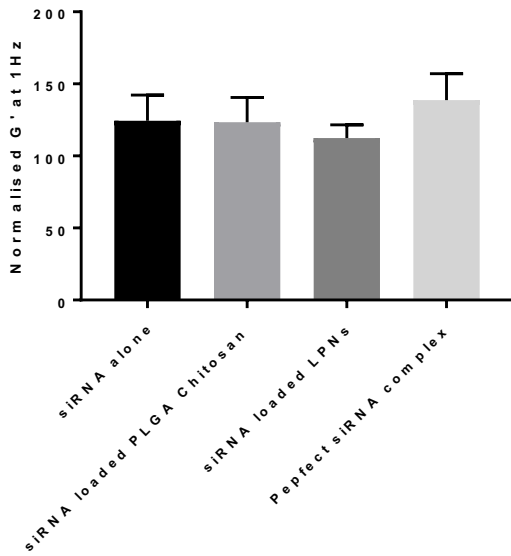


Figure 1A. G' at 1Hz for treated mucus gels as % of the G' of control mucus.

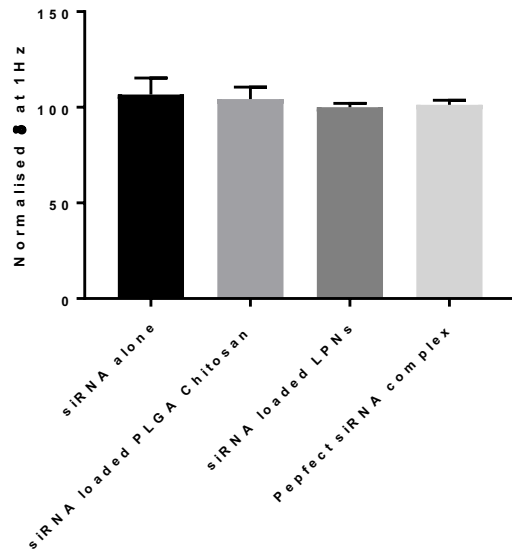


Figure 3. Phase angle δ at 1Hz for treated mucus gels as % of δ of control mucus.

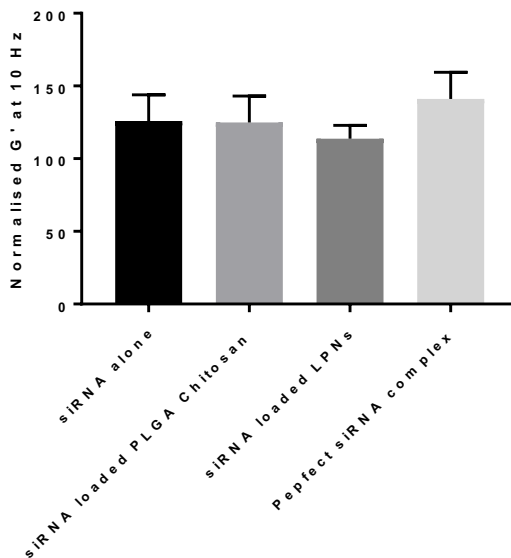


Figure 2. G' at 10Hz for treated mucus gels as % of the G' of control mucus.

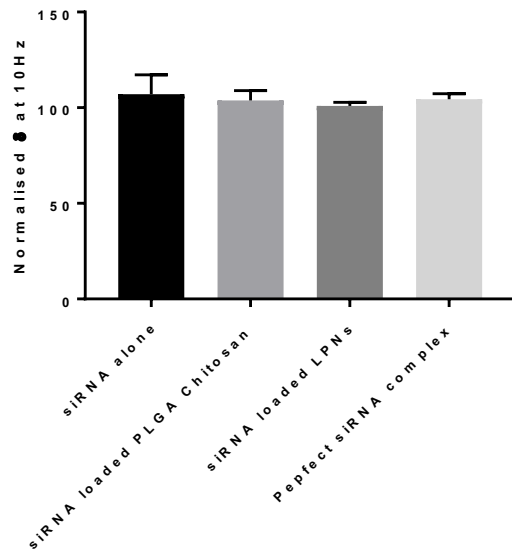


Figure 4. Phase angle δ at 10Hz for treated mucus gels as % of δ of control mucus.

DISCUSSION

The phase angle of the mucus substrate, which is a major determinant of mucus physiological function¹⁰, remained

essentially unaltered by the addition of siRNA with or without nanoformulations. This suggests that the alterations in mucus rheological properties resulting from the deposition of biopharmaceuticals may have only minor physiological consequences. Nevertheless, addition of the siRNA drug brings about a clear increase in the moduli values, albeit independent of the formulation. Whether or not these rheological changes will be able to influence physiological function will depend on several additional parameters. Firstly, the distribution of nanoparticles in mucus should be considered. Nanoparticles (or drugs) with good mucomobility would be expected to diffuse after deposition both throughout the depth of the mucus layer and laterally from the deposition point, whereas nanoparticles (or drugs) with poor mucomobility will remain in higher concentrations at their point of deposition and exert minor influence on the surrounding mucus. Equally, the frequency of administration and the rate of mucus clearance will influence the degree of mucus accumulation of the nanoparticles (or drug). These factors combined will control the concentration of nanoparticles (or drug) within the mucus, and further studies to investigate the effects of nanoparticle concentration on the observed alterations in mucus rheological properties would be beneficial to understand in more details the physiological consequences of mucus deposition. Finally, it should be remembered that the primary function of the mucociliary transport system is to entrap and remove inhaled particulate matter so it can be assumed that the system is able to handle local variations in mucus rheological properties without the occurrence of local mucus stasis and clearance failure.

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