

Innovative nebulisation delivery of GalNAc conjugated siRNA - a potential route for systemic drug delivery through the lungs

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Summary

Small interfering RNA (siRNA) therapeutics enable gene-specific inhibition by selectively degrading disease-related mRNAs, offering strong potential for treating genetic and inflammatory disorder. However, systemic delivery remains a key limitation due to siRNA's inherent instability *in vivo*. Conjugating siRNA to ligands or polymers significantly enhances both its stability and functionality, making it a more viable therapeutic agent for gene therapy. Among these, inhalable siRNA formulations can deliver drugs directly to the lungs, showing great potential for treating respiratory diseases. In this study, we evaluated the nebulisation of GalNAc-conjugated siRNA using the Nebu~Flow acoustic-based nebuliser. Our results demonstrate that GalNAc-conjugated siRNA can be nebulised without degradation. The aerosolised siRNA maintained structural integrity and retained its gene-silencing activity in primary mouse hepatocytes, demonstrating comparable knockdown efficiency to un-nebulised samples across both free uptake and transfection conditions. These findings demonstrate the capability of efficiently nebulised GalNAc-conjugated siRNAs without losing the biological activity. This has the potential for stable, effective, and non-invasive respiratory delivery for pulmonary and systemic gene therapy applications, which requires further validation in next studies.

Key Message

The acoustic nebuliser technology leverages high-frequency acoustic waves to generate a low-shear aerosol, enabling efficient nebulisation of GalNAc-conjugated siRNA formulations while preserving the structural integrity and biological activity of siRNA and GalNAc moieties.

Introduction

Small interfering RNA (siRNA) therapeutics offer a gene-specific approach to treating genetic and inflammatory diseases by selectively degrading disease-driving mRNAs. Despite their potential, siRNA-based drugs face critical challenges related to stability and delivery. Unmodified siRNAs are rapidly degraded and cleared *in vivo*, limiting their clinical potential [1]. To address this, chemical modifications and ligand conjugation strategies—particularly trivalent N-acetylgalactosamine (GalNAc) conjugation—have been developed to enhance siRNA stability and pharmacokinetics. These approaches also support sustained gene-silencing activity through targeted uptake and silencing via receptor-mediated endocytosis. Notably, the therapeutic effects have been observed to persist for several months in both preclinical models and humans [2].

Pulmonary delivery has gained increasing attention as a non-invasive and efficient route for siRNA administration. The lung's large surface area, extensive vasculature, and relatively low enzymatic activity support both local and systemic drug delivery via the alveolar–capillary interface [3]. It is important to note that while GalNAc-conjugated siRNA is specifically designed to target liver hepatocytes via injection, the pulmonary delivery of such conjugates has not yet been explored. Although GalNAc targets hepatocytes via the asialoglycoprotein receptor, with proper particle size optimisation, nebulisation can facilitate systemic uptake, offering a promising vector-free platform for inhaled siRNA therapeutics. However, conventional nebulisers often expose siRNAs to mechanical shear stress, leading to degradation and reduced bioactivity [4].

In this study, we investigated the feasibility of nebulising GalNAc-conjugated siRNA using an acoustic-based nebuliser. This technology employs interdigitated transducers to generate high-frequency acoustic waves and disperse liquid on its propagation path, in the form of a fine-mist, as presented in the previous publications [5,6].

Experimental method

GalNAc-conjugated siRNA targeting mouse transthyretin, designated TEGSIDI, was synthesized with full chemical modifications and a triantennary GalNAc moiety conjugated to the 3' end of the sense strand. The GalNAc-conjugated siRNA was kindly supplied by Argonaute RNA.

In this study, nebulisation experiments (n=3) were performed using Nebu~Flow's acoustic-based nebuliser, as described in previous studies [5,7,8]. A total of 500 μ L of GalNAc-conjugated siRNA (100 μ g/mL in RNase-free phosphate-buffered saline) was nebulised at a flow rate of 200 μ L/min. The aerosolised output was delivered to a Next Generation Impactor (NGI; Copley Scientific, UK) operating at 15 L/min. Samples deposited across all NGI stages were pooled together and collected from parafilm-lined NGI cups. The aerosol characterisation studies were conducted in earlier study [9] and in this work, we solely consider the effect of nebulisation on siRNA's structure and biological activity. A positive control was included to confirm the absence of non-specific siRNA binding to parafilm [10]. The collected samples were subsequently used for structural stability and biological activity testing of GalNAc-conjugated siRNA post-nebulisation.

To assess the structural stability of the GalNAc-conjugated siRNA a non-denaturing polyacrylamide gel electrophoresis (PAGE) was carried out. A siRNA (10ng per well) was run on a 20% TBE gel at 200 V for 75 minutes. The gel was subsequently stained with SYBR Safe and visualised for evidence of degradation or shearing.

Furthermore, mouse transthyretin gene (mTTR) silencing efficacy of the nebulised GalNAc-conjugated siRNA was assessed in primary mouse hepatocytes. Cells were treated with nebulised and un-nebulised (Control) GalNAc-conjugated siRNA at 1nM and 4nM under free uptake conditions, with 4nM selected to achieve maximal gene silencing and 1 nM representing the lowest effective dose prior to a decline in knockdown efficiency; 10 nM was used under lipofectamine-mediated transfection as a standard concentration. After 48 hours of incubation, cells were lysed using Cells-to-Ct lysis buffer, and mTTR mRNA expression levels were quantified by qPCR.

Results

Both the physical characteristics and biological activities of the siRNA were evaluated to assess the effect of the nebulisation process on the stability of siRNA and GalNAc conjugate. These included determinations of structural integrity and gene-silencing efficacy under free uptake and transfection conditions.

Physical stability of GalNAc-conjugated siRNA post nebulisation.

The structural integrity of the moiety was studied using a non-denaturing polyacrylamide gel electrophoresis (PAGE). As shown in Figure 1, the gel results showed an increase in the molecular size of the GalNAc-conjugated siRNA (Lane 2), compared to un-conjugated siRNA (Lane 1), confirming the successful conjugation of GalNAc to siRNA. A comparison between the un-nebulised (Lane 2) and nebulised samples (Lane 3 to 5) shows no change in molecular size in collected sample. This confirms that the conjugated siRNA maintained its hybridised, double-stranded structure post-nebulisation. No evidence of degradation or shearing was observed, and the GalNAc moiety remained intact, indicating molecular stability throughout the process.

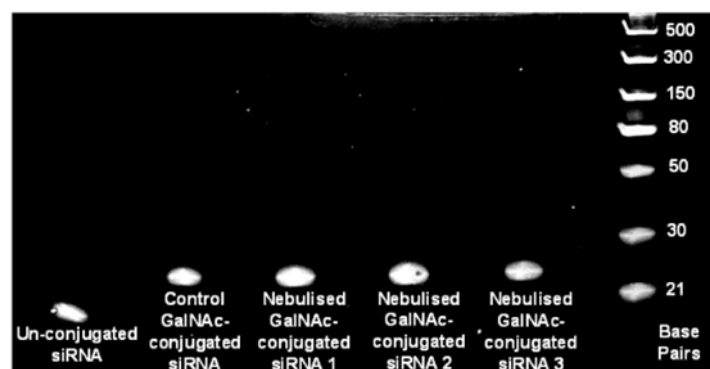


Figure 1 – Integrity of nebulized GalNAc-conjugated siRNA assessed via non-denaturing polyacrylamide gel electrophoresis (n=3). Lane 1: the naked, un-nebulized siRNA. Lane 2: Un-nebulized GalNAc-conjugated siRNA (control). Lanes annotated 3-5 correspond to nebulized GalNAc-conjugated siRNA

recovered from NGI stages. Lane 6: dsRNA ladder (New England Biolabs, N0363S) with a 21 bp reference band at the base.

Biological activity of GalNAc-conjugated siRNA post nebulisation.

The biological activity was evaluated by measuring the gene-silencing efficacy of nebulised siRNA, targeting mTTR mRNA. mTTR was chosen because TTR encodes transthyretin - a liver-delivered protein primarily synthesized by hepatocytes - making it an ideal target for GalNAc-conjugated siRNA. Gene silencing was assessed under both free uptake and lipofectamine mediated transfection. Under free uptake conditions, siRNA was administered at two concentrations: 1nM and 4nM. Quantitative PCR analysis revealed potent gene knockdown, with 90–95% reduction in mTTR mRNA levels at 1 nM and 95% knockdown at 4 nM, relative to non-treated controls. These are consistent with the knockdown effect from un-nebulised sample (Figure 2a, b). Additionally, under transfection conditions using nebulised siRNA at 10 nM, a 95% knockdown was also achieved, confirming that the functional activity of the siRNA was retained after nebulisation and comparable to pre-nebulised sample (Figure 2c).

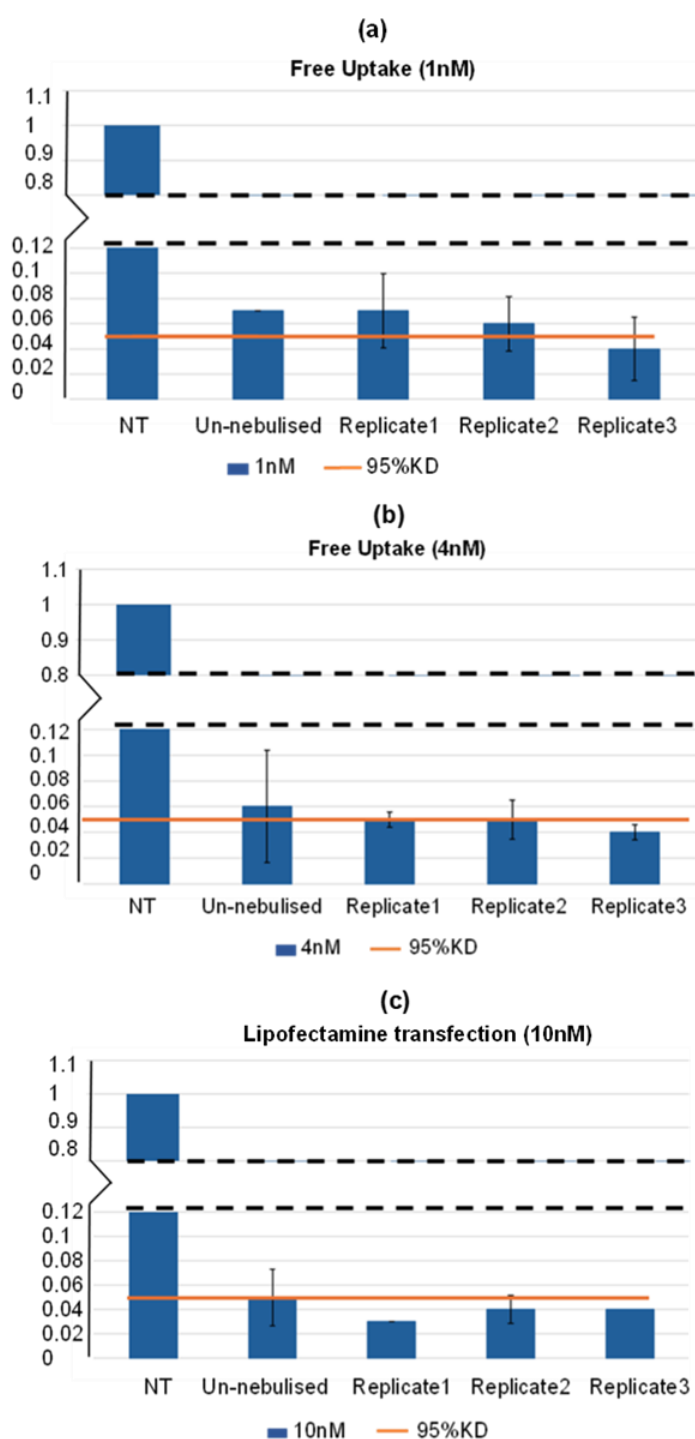


Figure 2 – Knockdown efficacy (KD) of nebulized GalNAc-conjugated siRNA in primary mouse hepatocytes with 3 replicates. (a), (b) Free uptake: Knockdown was evaluated at siRNA concentrations of 1 nM and 4 nM. (c) Lipofectamine-mediated transfection: Knockdown was assessed at 10 nM siRNA concentration. Controls included un-nebulised GalNAc-conjugated siRNA tested at 1nM and 4nM (Free uptake) and 10nM (lipofectamine transfection), as well as non-treated cells (NT). mTTR mRNA levels in mouse hepatocytes were quantified post-treatment via qPCR. Data are presented as mean $\pm$ standard deviation.

Discussion and conclusions

Pulmonary drug delivery offers a non-invasive and efficient route for the lung and systemic drug administration via the alveolar-capillary interface; however, efficient delivery of biologics remains a significant challenge due to their vulnerability to physical stresses encountered during nebulisation [11].

Our findings demonstrate that acoustic nebuliser technology can effectively nebulise GalNAc-conjugated siRNA without compromising siRNA integrity and bioactivity. Structural assessment using polyacrylamide gel electrophoresis confirmed the siRNA remained intact post-nebulisation, maintaining its double-stranded form with no evidence of degradation. Furthermore, functional assays using primary mouse hepatocytes validated the biological activity of the nebulised siRNA, indicating successful gene silencing consistent with untreated controls. Although these data support the stability and bioactivity of the GalNAc-conjugated siRNA, further investigation is required to study both aerosol characterisation and in vivo animal models to demonstrate the efficacy of nebulised samples.

These results highlight the potential of Nebu~Flow's acoustic-based nebuliser for delivering fragile RNA therapeutics via the pulmonary route. The ability to maintain siRNA integrity and activity during aerosolisation represents a significant step toward expanding non-invasive delivery options for RNA-based drugs, particularly for applications beyond the lungs.

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