Efficient SiRNA Delivery by Secondary and Tertiary Amine Modified Polysaccharides

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ABSTRACT

As siRNA mediated therapy develops the need for a biocompatible, effective delivery vector is apparent. We report a novel nanocarrier for siRNA delivery via targeted chemical modification of chitosan. Despite its wide use in mucosal drug delivery applications, chitosan is limited by minimal solubility and buffering capacity at physiological pH resulting in poor cellular transfection efficacy. We hypothesized that introduction of secondary and tertiary amines to chitosan would enhance solubility, endosomal escape and cytoplasmic siRNA dissociation, thus resulting in highly efficient gene silencing. We have successfully introduced imidazole acetic acid to chitosan via carbodiimide chemistry, and demonstrated enhanced buffering capacity and solubility even at high pH. As predicted, *in vitro* gene knockdown was greatly enhanced, nearly doubling in efficacy, while preliminary *in vivo* studies showed effective gene knockdown in mice following intranasal administration of polymer-siRNA nanocomplexes.

Keywords: siRNA, chitosan, *in vivo*, delivery, non-viral

1 INTRODUCITON

With increased development of nucleic acids such plasmid DNA and siRNA, the need for highly efficient, biocompatible nanocarriers to overcome both tissue and cellular barriers of nucleic acid delivery has grown critical. Such nanocarriers are necessary in addressing the limitations in tissue and cellular uptake, nucleic acid stability, intracellular transport, endosomal escape, and cell targeting [1,2]. Numerous past studies have focused on the use of both viral and non-viral nucleic acid delivery systems. Despite their extreme efficiency *in vivo and in vitro*, viral vectors pose considerable risk of immunogenic, cytotoxic, and oncogenic responses [3,4]. Alternatively, non-viral vectors provide a safer alternative to viral vectors, but suffer from much lower transfection efficiencies as well as toxicity when used at high doses. More recent studies have focused on cationic molecules for delivery due to the intrinsic capability to form nano- and microparticles by electrostatic interaction of the positively charged molecules with negatively charged nucleic acids [5]. Such vectors include cationic polymers and lipids such as polyethyleneimine (PEI), dendrimers, and polylysine. Despite reasonable transfection efficiencies that are significantly lower than viral carriers, both *in vitro* and *in vivo*, most of these materials are limited for use in humans as a result of toxicity at high doses. Limitations with such carriers provide a significant challenge when route specific delivery is desired, including intranasal and mucosal delivery. Therefore, the necessity for development carriers not only requires biocompatibility and enhanced transfection efficacy, but also the capability for effective route-specific delivery.

The natural polymer chitosan is a cationic polysaccharide noted for its biocompatibility that is limited in transfection capabilities. Recent research has focused on many modifications in order to enhance the transfection capabilities of chitosan. Such modifications include conjugation of polyethylene glycol (PEG), trimethylation, thiolation, galactosylation, and conjugation of deoxcholic acid [6-10]. PEG conjugation to chitosan provides enhanced particle stability and minimized aggregation without reduction in transfection efficiency [6]. Further conjugations of galactose to chitosan-PEG conjugates have demonstrated enhance targeting to the liver [10]. Trimethylation of chitosan has been shown to enhance polysaccharide solubility at physiological pH [7], while conjugation of deoxycholic acid provides greater formation of self-aggregated plasmid DNA nanoparticles formed with chitosan [8]. Thiolated polysaccharides have been demonstrated enhanced mucoadhesive characteristic, providing potential for chitosan as a carrier via multiple routes of administration [9]. Grafting of PEI to chitosan was recently demonstrated to enhance the transfection efficiency of chitosan; however cytotoxicity remains a concern [11]. Despite efforts, these modifications were unable to address the specific barriers present in delivery of nucleic acid using chitosan while also preserving polymer biocompatibility.

One approach for enhanced nucleic acid delivery with chitosan is to introduce secondary and tertiary amines to the polymer allowing for further exploitation of the "proton sponge" mechanism. The introduction of chemical groups containing amines, such as imidazole acetic acid (IAA), can provide improved solubility, buffering capacity, and release kinetics at endosomal and physiological pH levels. The covalent conjugation of IAA to cationic polymers has been previously demonstrated to enhance transfection efficiency for pDNA [12,13]. In this study, we report the development and characterization of chitosan conjugated with IAA as an enhanced nucleic acid delivery vector both *in vitro* and *in vivo*.

2 MATERIALS AND METHODS

2.1 Materials

Imidazole-4-acetic acid monohydrochloride was purchased from Acros Organics, Beel, Germany. Chitosan (Protasan UP CL113, MW = 130,000 Da, Degree of Deacetylation = 86%) was purchased from Novamatrix, Norway. EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) and Snakeskin pleated dialysis tubing (10,000 MW cut-off) were both purchased from Pierce Biotechnology, Inc., Rockford, IL. Silencer GAPDH siRNA and SiPORT Amine were obtained from Ambion, Inc., Austin, TX. HEK293T cells were obtained from the American Type Culture Collection, Manassas, VA. Gibco modified DMEM used for cell culture medium and all remaining cell culture reagents were purchased from Invitrogen, Carlsbad, CA. C57BL/6 mice for in vivo studies were purchased from The Jackson Laboratory, Bar Harbor, ME.

2.2 Synthesis of Modified Chitosan

Conjugation of Imidazole-4-acetic acid monohydrochloride to the primary amines of chitosan was performed via carbodiimide chemistry. The ratio of IAA to the number of primary amines in chitosan was varied to provide variable degrees of modification. The following is the general synthesis: A 0.5% w/v solution of chitosan was prepared in 0.1 M MES buffer at pH 5. A 2.0% w/v solution of imidazole acetic acid (IAA) was also prepared in the same reaction buffer. The solutions were held on ice prior to mixing. IAA solution was added to the chitosan solution at variable amounts to provide different degrees of modification. The mixed solution of chitosan-IAA was then added to a 20 M excess (in relation to IAA) of EDC and immediately vortexed for 60 seconds to catalyze conjugation of IAA along the chitosan backbone. The final solution was left to react overnight while on a rotator. Solutions were then dialyzed using Snakeskin pleated dialysis tubing for 24 hours against 3mM HCl for 2 hours (3 times), then against deionized water for 6 hours (3 times). Samples were then lyophilized overnight.

2.3 Modified Chitosan Characterization

Characterization of IAA-modified chitosans is described in our previous publication [13]. This includes degree of modification as well as characterization of buffering capacity and solubility.

2.4 Preparation of Chitosan/siRNA Nanoparticles

Nanoparticle formulations with siRNA (Silencer GAPDH siRNA) were prepared as previously described at a N/P ratio of 50 with chitosan and various chitosan-IAA. In brief, the general preparation was as follows: siRNA nanoparticles, solutions of chitosan and chitosan-IAA (0.01–0.06% in RNase-free 200 mM sodium acetate buffer, pH 4.5) and siRNA solutions (20 µg/mL in RNase-free water) were preheated to 50–55ºC. An equal volume of chitosan solution was added to siRNA solution (drop-wise) and vortexed for 20–30 seconds resulting in a final volume of 200 µL followed by incubation at room temperature for 30 minutes. Nanoparticle solutions were later used for transfection. Nanoparticles were characterized for size and zeta potential as previously described [13].

2.5 *In Vitro* Transfection of HEK293T Cells

24 hours prior to transfection, 96-well plates were seeded with HEK293T cells were seeded at a density of $1x10^4$ cells per well in 0.2 mL of complete medium (DMEM containing 10% FBS, supplemented with 1% penicillin and streptomycin). Polymer/siRNA formulations were studied for various N/P ratios. Nanoparticles carrying 50 nM of siRNA were added to each well followed by incubation for 4 hr in 0.1 mL of serum-free medium. After the 4 hour incubation 0.1 mL of balancing DMEM medium (containing 20% FBS and 2% penicillin and streptomycin) was added to each well to cells, which were then incubated for 44 hr at 37ºC. As a positive control, transfection of the siRNA was performed using siPORT Amine. All transfection experiments were performed in triplicate. Following incubation, GAPDH knockdown was determined using the KDAlert GAPDH assay kit purchased from Ambion, Inc.

2.6 *In Vivo* GAPDH Knockdown in Mice

In vivo gene knockdown was studied on naïve C57BL/6 mice. Mice (N=5) were injected with siRNA nanoparticles complexed with either chitosan, IAA-modified chitosan, or siPORT Amine (positive control, Ambion, Inc.). Mice were each intransally administered particles containing 10 µg for each administration. Mice were either given a single dose or multiple doses over repetitive (three) days. Miice were sacrificed and lung tissues collected three days following the final administration. Lung tissue were immediately homogenized and analyzed to determine GAPDH knockdown using the KDAlert GAPDH activity assay. Protein content of samples was also analyzed for normalization using a Micro BCA protein assay kit purchased from Pierce Biotechnologies, Rockford, IL.

3 RESULTS

3.1 Introduction of Imidazole Acetic Acid to Chitosan and Characterization

Through the use of carbodiimide chemistry (schematic presented in Figure 1), we have successfully covalently conjugation IAA to chitosan at various degrees of

modification. Modification efficiency was demonstrated at greater than 60% with degrees of modification achieved as high as 90%. Degrees of modification were assessed by two separate methods using a ninhydrin colorimetric assay or by ^{1}H NMR as previously described [13]. Further characterization of the IAA-modified chitosans has shown an increase in polymer solubility and enhanced buffering capacity at endosomal and physiological pH as well as up to greater than pH 9 [13]. Solubility and buffering capacity within this pH range has previously been linked to efficacy of the proton sponge mechanism [14], thus enhancing these characteristics should provide improved transfection efficiency. Also key for the polymers is retention of biocompatibility, which was demonstrated with an MTT assay as previously presented [13].

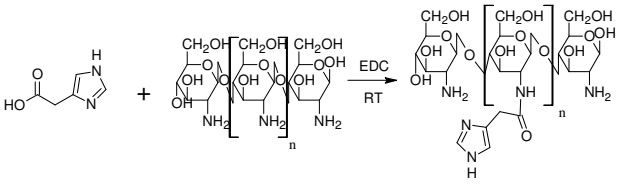

Figure 1. Schematic of IAA modification of chitosan.

3.2 SiRNA Nanoparticle Formations and Characterization

For further studies concerning formation of nanoparticles with siRNA, Chi-IAA 20 (20% IAA-modified chitosan) and Chi-IAA 30 (30% IAA-modified chitosan) were used. Nanoparticles were prepared as described in section 2.4, and then analyzed for zeta potential and particle size. Particles were prepared at N/P ratios of 5, 10, 25, and 50. Dynamic Light scattering analysis demonstrated particle sizes ranging from 75 nm (N/P=50) to 300 nm (N/P=5), well within the range accepted for effective particle-mediated delivery. Size varied based on N/P ratio and degree of modification. Zeta potential for the particles was determined to range from +15 mV (N/P=5) to 38mV (N/P =50), increasing as N/P ratio rose. Particle size was also dependent upon degree of modification with larger sized particles for IAA-modified polymers; however zeta potential values were also higher for these polymers when compared to non-modified chitosan.

3.3 Cellular Transfection of GAPDH siRNA/IAA-modified Chitosan Nanoparticles

*In vitro c*ellular studies were performed to in HEK293T cells to demonstrate the enhanced transfection capabilities of modified chitosan. Chitosan, Chi-IAA 20, and Chi-IAA 30 were used to produce nanoparticles with siRNA for these studies at a N/P ratio of 50. As seen in figure 2 below, siRNA nanoparticles at a concentration of 50nM/well provided increased knockdown with increased degree of IAA modification. Here, 30% IAA-modified chitosan nanoparticles produced a 90% gene knockdown which matched the positive control (siPORT Amine), while nearly doubling the knockdown effects of non-modified chitosan and Chi-IAA 20. As predicted, the presence of the secondary and tertiary amines has provided enhanced siRNA transfection *in vitro*.

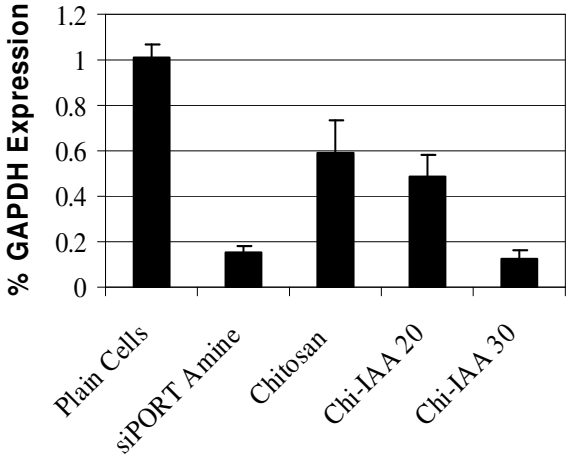

Figure 2. *In vitro* transfection of siRNA nanoparticles with chitosan, chitosan-IAA, and siPORT Amine at N/P=50.

3.4 Particle-Mediated GAPDH Knockdown in Murine Model

Murine studies were performed to study the effects of IAA modification on the chitosan-mediated delivery of siRNA via intranasal delivery. Nanoparticles were prepared as previously described in section 2.4 with the following changes: Several particle solutions were combined together and concentrated using Amicon-15 centrifugal filters (Millipore, Billerica, MA) to provide a 10 μg siRNA dose per mouse in a maximum of 20 μL. A single injection study was initially performed to determine gene knockdown GAPDH followed by a second study of multiple injections (three injections over three days). As seen in Figure 3A Chi-IAA 30 showed no significant difference in knockdown at 25%, when compared to non-modified chitosan; however it effectively matched the siPORT Amine positive control. Multiple injections demonstrated a greater knockdown at 52% for Chi-IAA 30/siRNA nanoparticle as seen in Figure 3B; however once again little to no different was seen when compared to non-modified chitosan. Results of these experiments provide the potential need for secondary modifications to further enhance the IAA-modified by providing greater mucoadhesiveness as well as further particle stability. Nanoparticles formed with negative control or scrambled siRNA (purchased from Ambion, Inc, Austin, TX) produced no gene knockdown when applied in these studies as controls demonstrating no gene knockdown effect as a result of chitosan or IAA-modified chitosans.

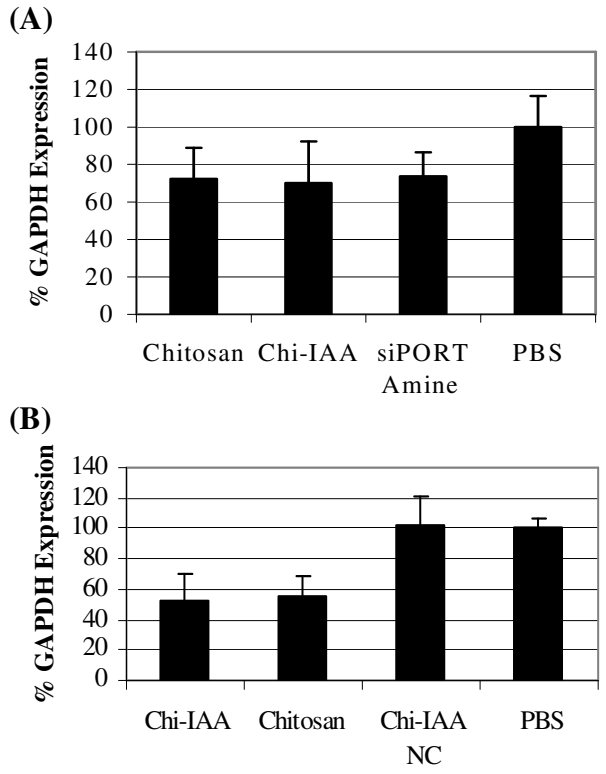

Figure 3. Chitosan and Chi-IAA 30 mediated delivery of siRNA *in vivo* via intranasal administration with A) single administration and B) multiple (3 Day) administrations at an N/P ratio of 50.

4. Discussion and Conclusion

Despite the inherent potential of therapeutic nucleic acids, effective delivery and distribution of stable and functional nucleic acids is necessary for successful and optimal function of gene-based therapies. Delivery vectors are required to overcome the inherent barriers present in cellular targeting, penetration, and distribution. In this effort, the need for biocompatible and highly effective nanocarriers has become a growing field of study. Limitations found in current potential delivery vehicles such as low transfection efficiency, cytoxicity, immunogenicity, and oncogenicity have lead researchers to place focus on tailorable and natural polymers as potential replacements. Despite lower transfection efficiencies with current non-viral delivery vectors, the modifiable nature of such polymers allows for numerous avenues to enhance multiple characteristics for specific route-specific and target-specific delivery.

We hypothesized that covalent conjugation of secondary and tertiary amines along the backbone of chitosan would provide enhanced transfection efficiency of nucleic acids through improved buffering, solubility, and dissociation kinetics, while retaining polymer biocompatibility. Here, we have reported results demonstrating the enhanced transfection of siRNA with IAA-modified chitosan with increased solubility and buffering at neutral and basic pH levels. The benefits of enhanced solubility also lend the polymer to further modifications not previously capable of being achieved. The polymer was also proven to retain biocompatibility of the chitosan post modification (data not shown).

In this study, we have demonstrated the introduction of imidazole acetic acid to chitosan. We have also shown nanoparticle formulation with siRNA using both modified and non-modified chitosans producing particles that are both cationic and below 300 nm in effective diameter. We have also demonstrated significantly enhanced gene knockdown *in vitro* with IAA-modified chitosan nearly doubling siRNA efficacy in HEK293T cells. *In vivo* studies did not, however, demonstrate a significant enhancement in comparison to non-modified chitosan. Here, potential issues may arise from reduced mucoadhesive properties as ineffectiveness via intranasal administration. It should be noted that both chitosan and IAA-modified chitosan nanoparticles matched the positive control for gene knockdown.

In conclusion, we have developed a biocompatible, polycationic polymer showing highly efficient delivery of siRNA with the potential to provide further enhancement of delivery and development of nucleic acid-based therapies.

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