Introduction of Secondary and Tertiary Amines to Chitosan: Enhancing Delivery of Nucleic Acids

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Statement of Purpose: Nucleic acids have emerged as an important tool in the field of medicine and biotechnology. Use of DNA vaccines and the breakthroughs in both antisense and siRNA gene knockdown have provided new, more effective and specific techniques to battle diseases. However, it is important to ensure appropriate and efficient delivery of the nucleic acids to their targets. Use of modified or functionalized polymers has shown improvement in delivery efficiency by reducing cytotoxicity, improving transfection, and allowing for targeting to specific cells and tissues[1]. Chitosan, a polysaccharide, has been studied as a vehicle for non-viral gene delivery due to its biocompatibility, non-toxicity, biodegradability, and cationic charge[2]. Recently, chitosan was demonstrated as a possible delivery mechanism for siRNA[3]. However, the inherent transfection efficacy of native chitosan is significantly less than other cationic polymers (e.g. PEI) or lipids. A possible strategy to enhance the efficacy of chitosan-based gene delivery carriers would be the introduction of secondary and tertiary amines to the polymer thereby exploiting the so called "proton sponge" mechanism for improved endosomal escape. Imidazole acetic acid (IAA) has been previously demonstrated to produce cationic particles that utilize the proton sponge effect[4]. In this study we demonstrate modification of chitosan with IAA and particle formation and characterization with both pDNA and siRNA.

Methods: Imidazole-4-acetic acid monohydrochloride (AlfaAesar, Ward Hill, MA) was conjugated to the primary amines of Protasan CL113 (Novamatrix, Norway) through the use of a carbodiimide chemistry using varying amounts of 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide Hydrochloride (EDC) (Pierce Biotechnology, Inc., Rockford, IL). The reaction schematic is seen in figure 1. IAA-modified chitosan were then evaluated through a ninhydrin assay to determine amount of primary amines converted. The polymers, including non-modified chitosan as a control, were then reacted with both pDNA and siRNA to produce polyplexes. Nitrogen to phosphate ratio (N/P) was varied to provide an optimal particle formation. These particles were then characterized to determine size and zeta potential through the use of Dynamic Light Scattering (ZetaPlus, Brookhaven, NY).

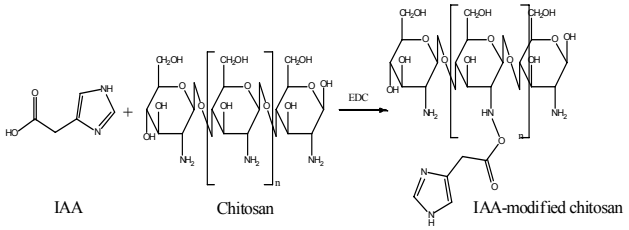

Figure 1. Schematic of IAA modification of chitosan

Results/Discussion: Through our reaction we were able to produce the IAA-modified polymer with various degrees of modification by varying the amounts of EDC used during the reaction. A quick one step reaction is necessary to avoid any self-reaction between IAA molecules. Ninhydrin assays showed an increasing degree of modification with increasing EDC content in the reaction. Degree of modification ranged from 29% to 42% of primary amine groups converted. It was necessary to dialyze the IAA-modified chitosan reaction volumes in 5mM HCl to retain polymer solubility after freeze-drying.

Particle formation resulted in IAA-Chitosan/pDNA or siRNA particles varying in size and zeta potential. Zeta potentials for the pDNA particles ranged from 20.14 mV to 22.58 mV. Mean sizes for the pDNA particles were slightly larger than expected, ranging from 355.1 nm to 580 nm. In the case of the two groups for siRNA, mean zeta potential values ranged from 24.51 mV to 25.91 mV. In the case of siRNA particles, sizes were closer to targeted dimensions with mean effective diameters of 291 nm to 285.0 nm. No aggregation was observed microscopically even after 8 hours. Cellular transfection efficiencies of these particle formulations compared to that of unmodified chitosan are now being tested. Characterization data for particles are shown in Table 1 along with values for control particles formed using plain chitosan, which showed lower zeta potentials for both pDNA and siRNA compared to experimental groups.

Samples	Degree of Modification	Molar excess of EDC	Zeta Potential (mV)	Particle Size (nm)
PDNA	0%	0	18.11 ± 0.05	306.2 ± 5.3
	29%	3.69	22.58 ± 0.48	448.0 ± 6.0
	34%	4.64	22.56 ± 0.56	580.0 ± 2.1
	38%	8.31	22.12 ± 0.50	438.0 ± 0.2
	42%	16.4	20.14 ± 0.37	355.1 ± 5.9
siRNA	0%	0	21.23 ± 0.61	317 ± 2.2
	29%	3.69	24.51 ± 0.85	291.3 ± 11
	38%	8.31	25.91 ± 1.40	285.0 ± 9.3

Table 1. Data for IAA-Chitosan/nucleic acid particles

Conclusions: We are able to successfully introduce secondary and tertiary amines to chitosan by modifying with varying degrees of IAA using carbodiimide chemistry. Efficient nanoparticle formation was demonstrated with pDNA and siRNA and dynamic light scattering characterization demonstrated stable, nanosized particles with a positive surface charge.

References:

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