Non-viral oral gene delivery for treatment of Hemophilia B.

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Statement of Purpose: Current protein replacement therapies require multiple IV injections that are expensive, painful, and inconvenient. Oral gene therapy - where exogenous genes are administered orally for therapeutic effects - can overcome these problems. Viral vectors risk random integration and limit the size of the delivered gene; non-viral gene delivery, although achieving only transient and low gene expression levels, overcomes these drawbacks. To date, oral gene delivery using chitosan (Ch, a cationic, mucoadhesive, polysaccharide) -DNA nanoparticles for treatment of hemophilia A has only improved the disease from a severe state to a mild state[1,2] therefore leaving much room for improvement. We hypothesize that adding a small amount of protamine sulfate (PS), a highly cationic protein, to the Ch-DNA nanoparticles will improve *in vivo* transfection efficiency by stabilizing the nanocomplexes extracellularly, resulting in increased therapeutic effects for patients suffering from protein deficiencies such as Hemophilia B. Therapeutic oral gene delivery has broad applications in treating protein deficiencies and providing accessible therapeutics to third world countries.

Methods: Nanoparticles were formed by bulk mixing with a ratio of 5:1:5 (w:w:w) of plasmid DNA to protamine sulfate to low molecular weight chitosan (~30kDa, ~73% deacetylated). Chitosan was dissolved in 0.2 M sodium acetate buffer (pH 5.2) at a concentration of 1 mg/mL. Plasmid DNA and protamine sulfate were dissolved in water at 0.2 µg/µL and 1 mg/mL, respectively. The plasmid DNA was mixed with protamine sulfate, chitosan was added while vortexing, and the dispersion was vortexed for 20 seconds and left at room temperature for 30 minutes. Particles were used immediately for *in vitro* experiments with Caco-2 cells and lyophilized for *in vivo* use. For *in vivo* experiments, mice were force-fed lyophilized nanoparticles re-suspended in water via oral gavage. Luciferase levels were detected by injecting luciferin into the mice and using the IVIS optical imaging system.

Results: Ternary complexes of Ch-PS-luciferase plasmid (pLuc) were determined to be 430nm±100nm with a zeta potential of 31mV±3mV. These particles were tested *in vitro* by transfecting Caco-2 cells and comparing luciferase protein levels of the Ch-PS-pLuc transfection to those of Ch-pLuc transfection. Results show that the Ch-PS-pLuc nanoparticles have significantly higher transfection efficiency than the standard Ch-pLuc nanoparticles. The Ch-PS-pLuc nanoparticles also displayed higher stability after lyophilization when compared to Ch-pLuc nanoparticles (data not shown). Transfection with lyophilized Ch-PS-pLuc nanoparticles that were stored at room temperature for 6, 7, or 8 weeks showed a decrease in transfection efficiency by ~50%, 50%, and 25%, respectively. A pilot study of mice fed with Ch-PS-pLuc nanoparticles showed luciferase expression in the stomach and the large intestine as compared to the untreated mice which exhibited no significant luciferase expression.

Conclusions: Ch-PS-pLuc nanoparticles have higher transfection efficiency than Ch-pLuc nanoparticles *in vitro*; because the Ch-pDNA system has proven to work to a small extent *in vivo*[1,2] the increased transfection efficiency of Ch-PS-pDNA is expected to increase *in vivo* efficacy and therapeutic effects. To prevent aggregation for therapeutic use, the nanoparticles are lyophilized immediately after assembly. Although there is a 50% drop of transfection efficiency after lyophilization, the transfection efficiency did not continue to decrease from day 1 to day 49, demonstrating the stability of the nanoparticles for 7 weeks when stored at room temperature. The stability of the Ch-PS-DNA nanoparticles indicates potential for use in third world countries where the product must be imported and stored at low costs. Preliminary *in vivo* results with Ch-PS-pLuc nanoparticles show luciferase production in the stomach and intestine. Currently, Ch-PS-phFIX (a human FIX plasmid that expresses the FIX protein) is being tested in a nude mouse model. Human FIX protein is being detected in mouse blood using ELISA and a functional chromogenic assay. With proof of human FIX protein production after oral delivery to the nude mouse, the nanoparticles will be used to treat hemophilic B mice and Activated Partial Thromboplastin Time (APTT) will be used to determine extent of disease correction.

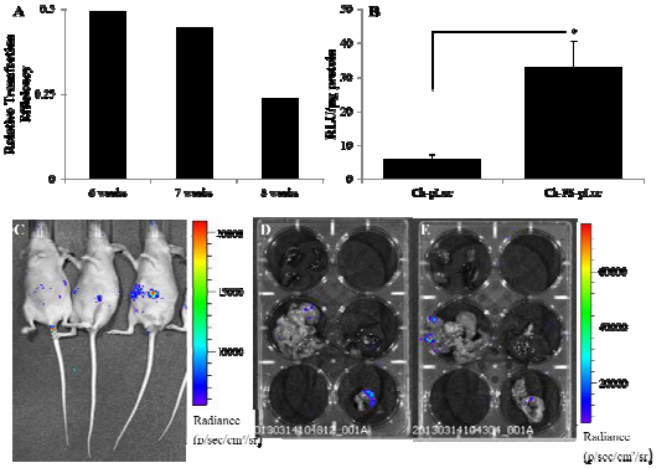

Figure 1: (A) Luciferase transfection efficiency of lyophilized Ch-PS-pLuc nanoparticles in Caco-2 cells relative to that of freshly made Ch-PS-pLuc nanoparticles; (B) Transfection efficiency of Ch-PS-pLuc and Ch-pLuc nanoparticles in Caco-2 cells; (C) IVIS images detecting luciferase expression: control 1, control 2, treated (mice from left to right); intestines from (D) control 2 and (E) treated, from left to right: kidneys and spleen, blank, intestines, liver, blank, stomach.

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References: **1**: Dhadwar, S.S. et. Al. *J. Thromb. Haemost.* (2010) 8: 2743-2750. **2**: Bowman, K. et. Al. *J. Control. Release*. (2008) 132: 252-259.